Investigations of the biocatalytic activity of human P450 2D6

Jin Zhao

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

Department of Chemistry
McGill University
Montreal, Quebec, Canada
H3A 2K6
August, 2007

Your file Votre référence
ISBN: 978-0-494-51359-0
Our file Notre référence
ISBN: 978-0-494-51359-0

Abstract

The cytochrome P450 enzymes (CYPs) are very attractive biocatalysts because of their ability to regio- and stereo-selectively catalyze the insertion of a single atom of molecular oxygen into inactivated C-H bonds. There are many drawbacks, however, limiting the use of these enzymes in organic synthesis, including the need for expensive cofactors, low stability, and low tolerance to organic solvents. The goal of this thesis was to overcome some of these drawbacks for human CYP2D6. This isoform was selected because of its broad substrate promiscuity and high importance in drug metabolism.

We have tested inexpensive chemicals to replace the natural cofactors of CYP2D6, NADPH and cytochrome P450 reductase (CPR). The results showed that cumene hydroperoxide and *tert*-butyl hydroperoxide can successfully substitute CPR and NAD(P)H with retained regio- and stereo-selectivity. Moreover, with these surrogates, product formation and initial rates are increased by as much as two fold compared to the use of the natural cofactors.

It is widely accepted that even small proportions of organic solvents in the buffer can deactivate most enzymes including P450s. Our studies on the biocatalysis of CYP2D6 in organic solvent/buffer emulsions showed that under the optimized conditions, as much as 76% of the enzyme activity was retained. Product formation in biphasic solvent systems is comparable whether the natural redox partner and cofactor are used, or a surrogate. In addition, a correlation was observed between the log P and the suitability of a solvent for enzymatic activity, with higher log P resulting in higher enzymatic activity. These results were obtained with dextromethorphan (DXM), a water soluble substrate. A very hydrophobic substrate, 7-benzyloxy-4-*N,N*-diethylaminomethyl-coumarin (BDAC), was also tested successfully to demonstrate the utility of this method.

Lyophilization is usually required to remove water before using enzymes in nearly anhydrous solvents. This physical process is harmful to P450 enzyme activity.

We therefore tested numerous sugars as lyoprotectant during lyophilization. Addition of trehalose or sucrose before lyophilization allowed the retention of 80% of the CYP2D6 activity, compared to 40% remaining activity in its absence. CYP2D6 co-lyophilized with trehalose was next tested in selected hydrophobic organic solvents in the absence of water. The enzymatic activity was found to strongly depend on the hydrophobicity of the solvent. Interestingly, the enzyme showed higher catalytic ability in *n*-decane or *n*-dodecane than in the standard buffer. This was unexpected considering that the activity of most enzymes was reported to decrease to 10% or less in nearly anhydrous organic solvents.

The last objective of this thesis was to improve the stability and/or activity of CYP2D6. Use of DNA self-assemblies to encapsulate P450 enzymes was envisaged to potentially increase their stability. Indeed, DNA assemblies have many advantages compared to traditional solid supports reported for enzymes. Our preliminary results showed that CYP2D6 templated the formation of cyclic DNA dimeric and tetrameric over polymeric self-assemblies. Characterization of the CYP2D6 activity in the presence of the DNA self-assemblies revealed no loss of activity or stability.

Résumé

Les enzymes cytochromes P450 (CYPs) ont des propriétés attrayantes comme biocatalyseurs à cause de leur habileté à catalyser l'insertion d'un seul atome d'oxygène dans une liaison C-H inactivé de manière régio- et stéréo-selective. Par contre, plusieurs inconvénients limitent l'utilisation des ces enzymes en synthèse organique tels que l'exigence de cofacteurs dispendieux pour leur activité enzymatique, leur manque de stabilité ainsi que leur manque de tolérance envers les solvant organique. Le but de cette thèse était de surmonter quelques uns de ces inconvénients pour l'enzyme CYP2D6 d'origine humaine.

Nous avons examiné plusieurs produits chimiques peu coûteux en vue de remplacer les cofacteurs indigènes de CYP2D6 dont NADPH et la réductase des cytochromes P450. Les résultats démontrent que l'hydroperoxyde de cumène et l'hydroperoxyde tert-butylique peuvent remplacer les cofacteurs indigènes tout en retenant les régio- et stéréo-sélectivités de l'enzyme. De plus, en utilisant ces substituants, la formation de produit et les vitesses réactionelles initiales sont augmenteés jusqu'à deux fois comparé aux réactions avec les cofacteurs naturels.

Il est reconnu que même des quantités minimes de solvant organique dans une solution tampon peuvent désactiver la plupart des enzymes incluant les P450s. Nos études démontrent que dans les conditions idéales, jusqu'à 76% de l'activité de CYP2D6 est maintenue dans des émulsions de solvant organique et de solution tampon. Dans ce genre de solvant biphasique, il y a peut de différence entre la formation de produits par CYP2D6 avec les cofacteurs naturels ou des substituants. De plus, on observe une corrélation entre le logP et l'aptitude d'un solvant pour l'activité enzymatique tel qu'un logP plus élevé engendre une activité enzymatique supérieure. Ces résultats on été obtenues avec dextrométhorphane (DXM), un substrat hydrophile. Un autre substrat très hydrophobe, 7-benzyloxy-4-*N*,*N*-diethylaminomethyl-coumarine (BDAC), a également été testé pour démontrer l'utilité de cette méthode.

La lyophilisation d'enzymes est habituellement requise pour enlever l'eau avant

leur utilisation dans des solvants anhydres. Ce processus physique est cependant néfaste à l'activité des P450s. Nous avons donc examiné plusieurs sucres pour leur effet lyoprotecteur durant la lyophilisation. L'addition du trehalose ou du sucrose avant la lyophilization permet la rétention de 80% de l'activité de CYP2D6 comparé à une rétention de 40% en son absence. Ensuite, l'enzyme CYP2D6 co-lyophilizée avec le trehalose a été testée dans des solvants hydrophobes en absence d'eau. L'activité de CYP2D6 dans ce milieu dépend fortement sur l'hydrophobicité du solvant. En effet, l'habileté catalytique de l'enzyme est plus élevée dans les solvants n-decane et n-dodecante que dans une solution tampon standard. Ceci a largement dépassé toute attente puisque l'activité de la plupart des enzymes diminue généralement de 90% ou plus dans les solvants organiques anhydres.

Le dernier objectif de cette thèse était d'améliorer la stabilité et/ou l'activité de CYP2D6. Nous avons envisagé l'utilisation d'ADN auto-assemblées pour encapsuler les P450s et augmenter leur stabilité. Nos résultats préliminaires suggèrent que CYP2D6 favorise la formation de dimères et de tetramères cyclique d'ADN au depend des polymères. L'activité et la stabilité de CYP2D6 en présence des capsules d'ADN auto-assemblées est inchangée.

Acknowledgments

I would like to express my gratitude to all those who help me through this journey, because this degree means so much to me. I am so lucky to fulfill my dream in McGill University.

I especially thank Professor Karine Auclair for giving me the opportunity to focusing on this interesting project and keeping supervising me throughout all my studies at McGill University. Lots of knowledge and skills learned from her will benefit me for lifetime.

I thank all professors in the department who taught me not only chemistry but also the passion for research.

I thank Dr. Saade Nadim for his help with Mass Spectroscopy, Chantal Marotte and other administrative stuffs for their support.

I also thank my lab mates and fellows for making so many good memories and joys. Especially, Amandine helped me to start my experiments; Faisal explained to me how DNA-assemblies work and supplied excellent graphs and schemes used in chapter 6; Amelie helped me to translate my abstract into French. To my brotherhood, Olivier, Alain, Alan, Luke, and Frank, you guys rock!

Last, but not the least, I would like to thank my grandparents and parents for their constant support and encouragement. I would not do anything without their love.

Contributions of co-authors

This thesis comprises one introduction, three publications (**chapter 2**, **3**, and **4**), two drifts (**chapter 5** and **6**), and one conclusion (**chapter 7**). **Chapter 5** will be submitted for publication shortly and **chapter 6** will be continually studied by others in Dr. Auclair's and Dr. Sleiman's research groups. All the work described in these manuscripts was performed as part of my study and research for Master degree.

As my supervisor, Professor Karine Auclair supervised each sub-project presented in this thesis and a co-author for each manuscript. All other co-authors are described below.

Chapter 2: I accomplished the experiments and collected data related to CYP2D6. Amandine Chefson was responsible for the study related to CYP3A4.

Chapter 3: I accomplished the behavior of CYP2D6 in different organic-buffer biphasic systems. Elaine Tan carried out the experiments related to CD. Julian Ferras synthesized and characterized the substrate BDAC.

Chapter 4: I accomplished the experiments and data related to CYP2D6. Amandine Chefson is responsible for the parts related to CYP3A4.

Chapter 5: I performed all the experiments reported.

Chapter 6: I carried out the expression, purification, and characterization of CYP2D6. I also performed high ionic buffer tolerance experiments and enzyme stability studies. Faisal Aldaye synthesized the DNA building blocks and performed the polyacrylamide gel electrophoresis (PAGE) analyses of the DNA self-assemblies.

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Abbreviation

2' 5' ADP – adenosine 2' 5' diphosphate

δ-ALA – 5-Aminolevulinic Acid

AdR – Aadrenodoxin reductase

Adx – adrenodoxin

AHMC – 3-[2-(*N*,*N*-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin

AMMC – 3-[2-(*N*,*N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin

BHP – *tert*-butyl hydroperoxide

[bmim]BF_6 – 1-butyl -3-methylimidazolium hexafluoroborate

CHAPS – 3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid

CHP – cumene hydroperoxide

CO – carbon monoxide

CPR – cytochrome P450 reductase

CYP2D6 – cytochrome P450 2D6

DEAE – diethylaminoethyl

DMF – dimethyl formamide

DMSO – dimethyl sulfoxide

DTT – dithiothreitol

EDTA – ethylenediaminetetraacetic acid

FAD – flavin adenine dinucleotide

FdR – ferredoxin reductase

Fdx – ferredoxin

FMN – flavin mononucleotide

HLADH – horse liver alcohol dehydrogenase

HPβCD – hydropropyl-β-cyclodextrin

HPLC – high performance liquid chromatography

HRP – horseradish peroxidase

IL – ionic liquid

IPTG – Isopropyl β-D-1-thiogalactopyranoside

Kpi buffer – potassium phosphate buffer

LB media – Luria-Bertani media

MβCD – methyl-β-cyclodextrin

MeOH – methanol

MnP – manganese peroxidase

MOPS – 3-*N*-morpholino propanesulfonic acid

MXM – 3-methoxymorphinan

NAPH – nicotinamide adenine dinucleotide, reduced form

NADPH – nicotinamide adenine dinucleotide phosphate, reduced form

PAHs – polycyclic aromatic hydrocarbons

PCMC – protein-coated microcrystals

PMSF – phenylmethanesulfonyl fluoride

SDS PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SPB – sodium perborate

SPC – sodium percabonate

TFA – trifluoroacetic acid

THF – tetrahydrofuran

TTN – total turnover number

Tris – trishydroxymethylaminomethane

UHP – urea hydrogen peroxide

Chapter One

Literature review: P450 enzymes as biocatalysts

1.1 Enzymes

Proteins, derived from the translation of mRNA, are essential components for life. They play versatile roles ranging from structural components to signaling. Among them, proteins which catalyze reactions are referred to as enzymes. Typically composed of a linear combination of the 20 amino acids, the structures of enzymes are dynamic and flexible. As with chemical catalysts, most enzymes speed reactions up by lowering the energy of activation. Enzymes have evolved to fulfill a variety of needs in adjusting the rates of reactions essential to living cells. As of June 26, 2007, 4037 types of enzymes were identified (http://www.expasy.ch/enzyme/), ribozymes excluded. Researchers have learned lessons from these natural catalysts in their search for man-made catalysts. To date, however, artificial catalysts cannot compete with biocatalysts' efficiency. Carbonic anhydrase (CA), an enzyme able to catalyze up to 10^6 reactions per sec [1], is the most proficient enzyme reported so far. More interestingly, some enzymes are known to catalyze reactions yet unachievable chemically. One example is hydroxylation at specific inactivated C-H bonds, catalyzed by cytochrome P450 enzymes amongst others [2, 3]. This thesis work has focused on improving the stability and activity of one P450 isoform, human CYP2D6, under typical conditions of biocatalysis.

1.2 Historical perspective of cytochrome P450 Enzymes

The first cytochrome P450 enzyme (CYP) was identified more than 50 years ago in a search for pigments [4]. In 1963, a group of pigments were ultimately characterized as cytochromes and named from the spectroscopic absorption maxima at 450 nm observed after reduction and ligation with carbon monoxide [5]. This absorption is caused by the cysteine ligated heme prosthetic group found at the core of these enzymes. This ligand distinguishes P450 enzymes from other heme proteins such as hemoglobin which contains a histidine heme iron ligand. There are only two other enzyme families with a proximal heme iron cysteine ligand, chloroperoxidase (CPO) and nitric oxide synthase

(NOS). The cysteine ligand is believed to facilitate the activation of molecular oxygen by the heme group [6-9].

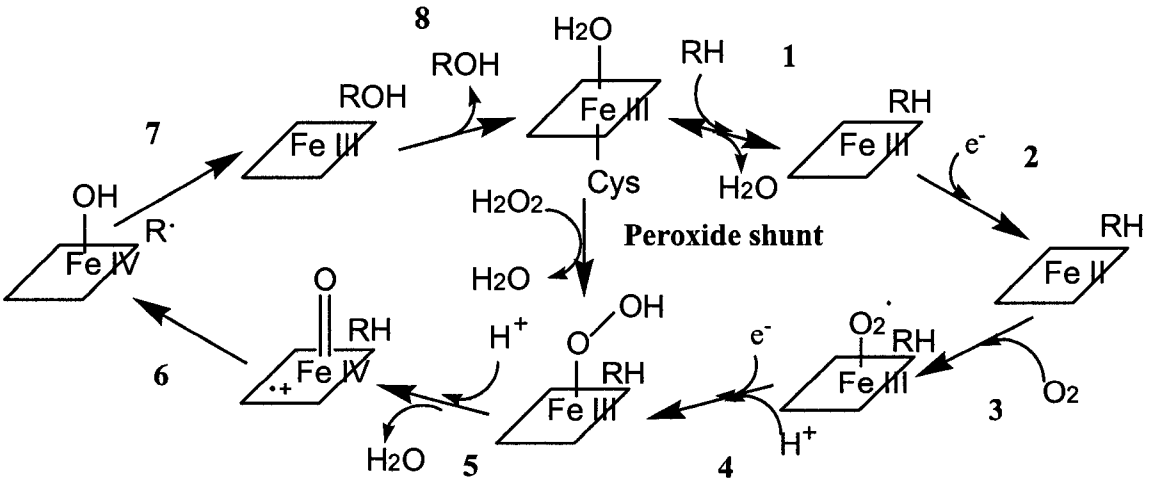
Almost all species of the kingdom produce and utilize P450 enzymes to accomplish at least one of the following functions, i) food uptake; ii) endogenous compounds syntheses; iii) exogenous compounds (including drugs and pollutants) biodegradation; and iv) defensive compounds syntheses. Plants for example, develop resistance to herbicides and pesticides via expression of P450 enzymes which break down the "harmful" molecules [10]. Human utilize P450 enzymes not only in the metabolism of xenobiotics, but also in the biosynthesis of vitamins, steroids and hormones [11]. Insect P450 enzymes execute syntheses and biodegradation of ecdysteroids and juvenile hormones, and detoxify insecticides [12].

The nomenclature of P450s is based on amino acid identity, phylogeneticity and gene organization. Typically, a root symbol *CYP* is followed by a number indicating a family sharing more than 40% amino acids sequence similarity. Then a letter identifies the subfamily, with members showing at least 55% similarity. A subsequent number designates the gene (http://drnelson.utmem.edu/CytochromeP450.html). As of March 09, 2007, 787 isoforms of P450 enzymes had been reported (http://www.icgeb.org/~p450srv/new/p450.html).

1.3 P450 enzymes reactions and mechanism

P450 enzymes have attracted the interests of biochemists, medicinal chemists, toxicologists and pharmacologists for decades because of their importance in drug metabolism. Synthetic chemists have joined this area of research because of the exceptional ability of these enzymes to catalyze many difficult reactions [2, 13-17], including hydrocarbon hydroxylation, heteroatom oxidation and dealkylation, epoxidation, aromatic ring formation, carbon-carbon bond formation or cleavage, dehydrogenation, olefin or aromatic ring oxidation, dehalogenation, and many more. The most sought-after reaction among these, and also the most prevalent in P450 catalysis, is hydroxylation at inactivated carbon-hydrogen bonds. Not only does this reaction proceed with high regioselectivity and stereoselectivity, but the enzyme also performs it under very mild conditions [18].

A general mechanism for P450 enzymes including multiple intermediates was proposed more than two decades ago. Evidences for these intermediates have been reported in the last decade through versatile spectroscopic techniques [19-22], synthetic metalloporphyrins [23, 24], computational chemistry [25, 26], and novel diagnostic substrates [27-29]. The widely accepted mechanism includes (**Scheme 1.1**): 1) in the resting state the heme iron is 6-coordinate low spin; then, binding of a substrate triggers a state change to low spin 5-coordinate with release of the water molecule from the heme distal site; 2) one electron is next transferred from nicotinamide adenine dinucleotide phosphate (reduced) (NAD(P)H) to the P450 heme iron via one or more electron transfer proteins (redox partners such as CPR) thus reducing the ferric heme to ferrous; 3) ferrous heme binds molecular oxygen to form a P450-dioxygen complex; 4) reduction of this system with a second electron and protonation of the distal oxygen atom yields a ferric hydroperoxy intermediate; 5) further protonation of the distal oxygen and water release generates the highly reactive iron-oxo (ferryl, compound I) intermediate; 6) hydrogen abstraction on the substrate produces a radical and a ferryl-hydroxy intermediate; 7) rebound of the hydroxyl group onto the substrate radical leads to hydroxylation of the substrates; 8) dissociation of the product regenerates the enzyme resting state. At step 4 or 5 the release of superoxide or hydrogen peroxide may occur. This so-called uncoupling mechanism is common, and results in the suppression of hydroxylation with consumption of NAD(P)H.



Scheme 1.1 The catalytic cycle of P450 enzymes illustrating the proposed intermediates. The rhombus represents protopophyrin IX. The proximal Cys ligand remains attached to the heme iron but is shown here only once for clarity. See test for a detailed description.

1.4 P450 cofactors and oxygen surrogates

As indicated in **Section 1.3**, P450 enzymes must be reduced for catalysis. Two electrons must be transferred separately to the P450 heme group and therefore cannot be donated directly by NAD(P)H (hydride donors; donation of two electrons at a time). P450 enzymes must work with other proteins, referred to as redox partners, which accept electrons from NAD(P)H and transfer them to the P450 one at a time.

The P450 enzymes are generally grouped into 3 classes based on the electron transfer system that they use [30, 31]. **Figure 1.1** illustrates the most common modes of electron transfer systems observed. Class I family comprises bacterial and mitochondrial P450s, to which electrons are transferred from a sulfur-iron ferredoxin (Fdx, bacteria) or adrenodoxin (AdX, mitochondria) proteins. FdX or AdX must first be reduced by ferredoxin reductase (FdR) or adrenodoxin reductase (AdR) respectively, using electrons from NAD(P)H. Class II P450s systems, use a cytochrome P450 reductase (CPR) containing flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) cofactors which transfer electrons from NAD(P)H to the P450 enzyme. Most class II systems have a separate electron transfer protein (CPR) and a separate P450 protein, which unite for catalysis by sliding along membranes, anchored at their *N*-terminals. Some bacterial P450 enzymes of class II, however, have evolved into a single protein containing both the heme domain and the redox partners. Class III P450 enzymes contain FMN, iron-sulfur clusters, and heme binding domains, all in one protein. NAD(P)H reduces FMN which transfer the electron to the iron-sulfur cluster before transferring it to the P450 heme. A prototypical example is P450-PFOR fusion enzymes such as CYP116 [32].

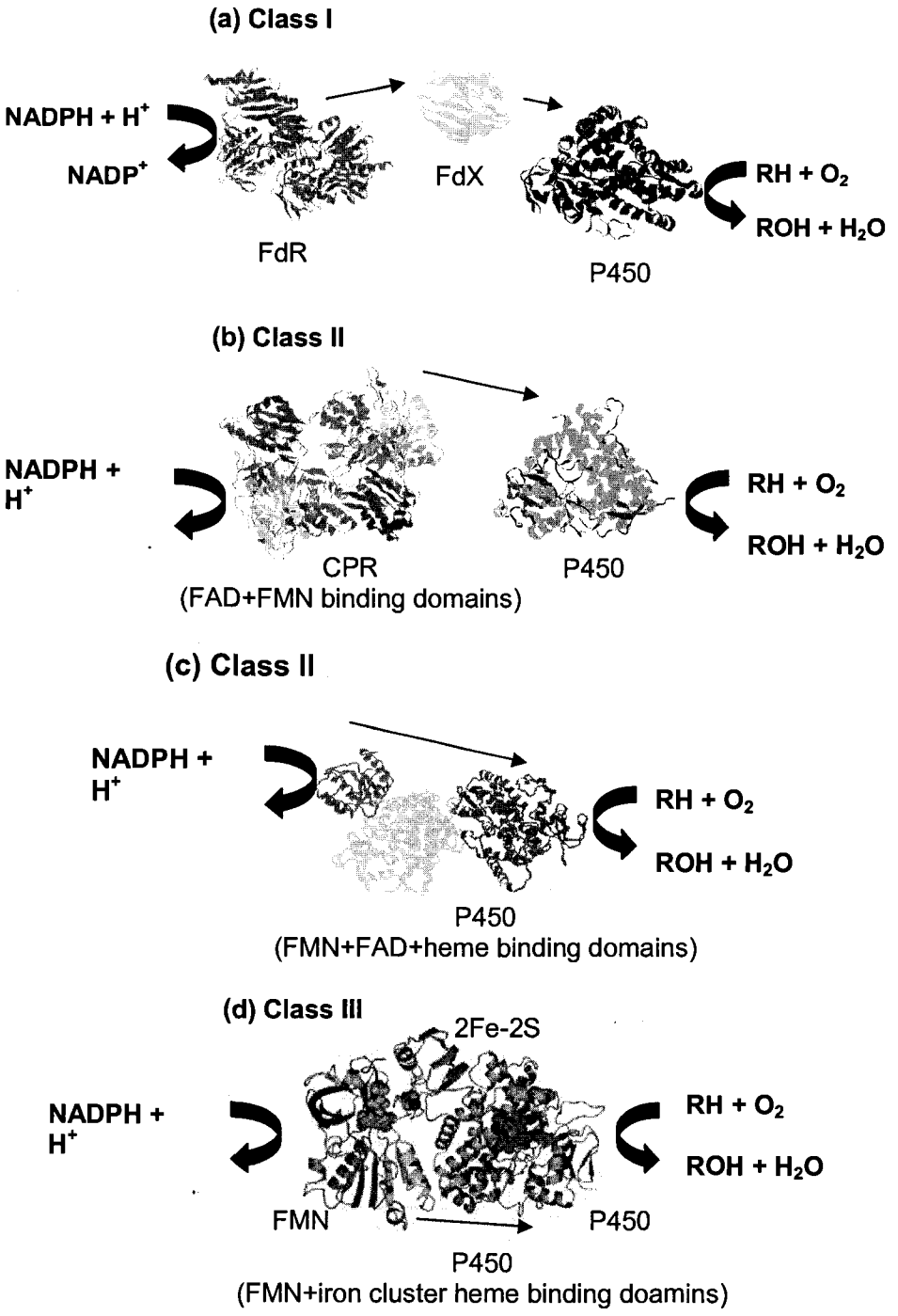


Figure 1.1 schematic illustrations of the 3 classes of P450 enzymes based on electron transfer systems. See text for detailed explanation. The linear arrows highlight the approximate direction of electron transfer.

For many years, researchers have known that these complex electron transfer systems can sometimes be replaced with hydrogen peroxide (peroxide shunt pathway),

although with low efficiency (**Scheme 1.1**) [33]. The cofactor surrogates reported include hydrogen peroxide, *tert*-butyl hydroperoxide (BHP), cumene hydroperoxide (CHP), peracids, periodate, chlorite and iodosobenzene [34-37]. A number of studies aiming to increase the peroxide shunt efficiency by mutagenesis have been reported in the last decades [38-42]. Another alternative to replace the P450 redox partner(s) and NAD(P)H involves the use of electrode [43, 44]. Vilker and coworkers observed a reaction rate of 36 nmol of 5-*exo*-hydroxycamphor per nmol of P450cam per min using tin oxide electrode compared to 2300 nmol with the natural system [45]. This method is promising for the assembly of biosensors. Shumyantseva *et al.* discovered that the use of "reversed" electrodes (immobilized substrate on the electrode) is effective for P450scc detection [46].

Recently, a bacterial P450 isoform, $P450_{BS\beta}$, has attracted interest because it merely utilizes hydrogen peroxide as a source of electrons and oxygen atoms [47-49]. This enzyme shows comparable catalytic activity to $P450_{BM3}$.

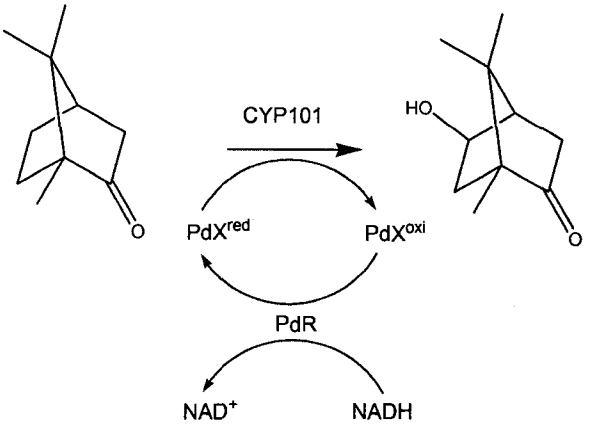
Chapter 2 of this thesis reports our success in replacing the natural cofactors of human P450 3A4 and 2D6 with cheap chemical surrogates. Under optimal conditions, the use of cumene hydroperoxide with CYP2D6 generates twice as much product compared to the use of the natural cofactors (CPR / NADPH) [50].

1.5 CYP101 (P450cam) and CYP102 ($P450_{BM-3}$)

The most studied P450 enzymes are by far CYP101 (also known as P450cam) from class I and CYP102 (also known as $P450_{BM-3}$) from class II. CYP101 is expressed by *Pseudomonas putida* to allow the use of camphor as a carbon source. Most of the current mechanistic knowledge of P450 enzymes comes from studies with CYP101 [2]. The first P450 crystal structure was also obtained with this isoform [51]. CYP101 catalyzes the 5-exo-hydroxylation of camphor using putidaredoxin (PdX), putidaredoxin reductase (PdR), and NADH (see **Scheme 1.2**).

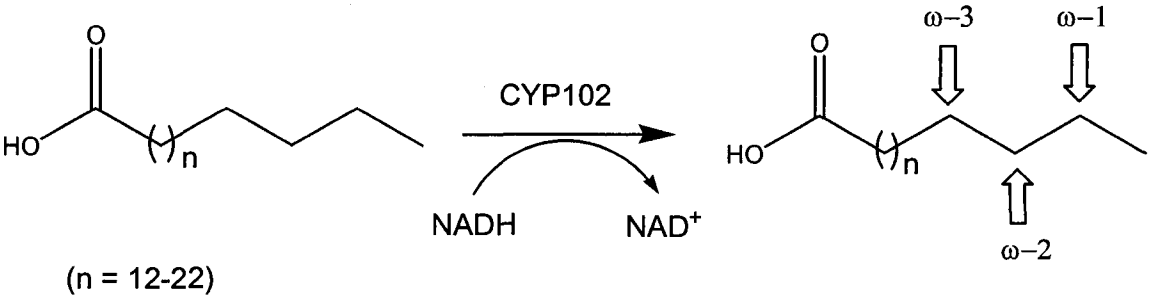
The availability of numerous crystal structures of CYP101 makes this enzyme an excellent candidate for mutagenic studies. A significant amount of work was devoted to modification of CYP101 for industrial applications [52-57]. The high specificity of CYP101 for the substrate camphor, however, is a serious limitation to its use as a biocatalyst [2]. A triple mutant designed based on the crystal structure shed light on the

possibility of trimming the active site for accommodating substrates other than camphor [53]. CYP101 variants able to convert propane or ethane, to propanol [54] or ethanol [55] was obtained after mutating five to nine amino acids including residues located far from active site. Mutation at F87 and Y96 enhanced the ability of the enzyme to modify polycyclic aromatic hydrocarbons such as phenanthrene, fluoranthene, pyrene and benzo[*a*]pyrene [56]. Another disadvantage associated with the use of CYP101 is that the coupling/uncoupling ratio of CYP101 with substrates other than camphor is low. A double mutant (Y96F–V247L) was designed to address this problem and showed 60% coupling efficiency compared to < 2% for the wild type [57].



Scheme 1.2 Transformation of camphor to 5-exo-hydroxycamphor by CYP101 (P450cam). Abbreviations: PdX^{red}: putidoredoxin in the reduced form; PdX^{oxi}: putidoredoxin in the oxidized form.

Since its discovery, CYP102 from *Bacillus megaterium* has attracted much more attention than any other isoforms for fine chemical conversion because of its natural fusion to the redox partner and experimentally high catalytic activity. The natural substrates and reactions catalyzed by CYP102 are illustrated in **Scheme 1.3**.



Scheme 1.3 Typical substrates of CYP102. Hydroxylation is catalyzed at ω-1, ω-2, and/or ω-3 positions (as indicated by the arrows).

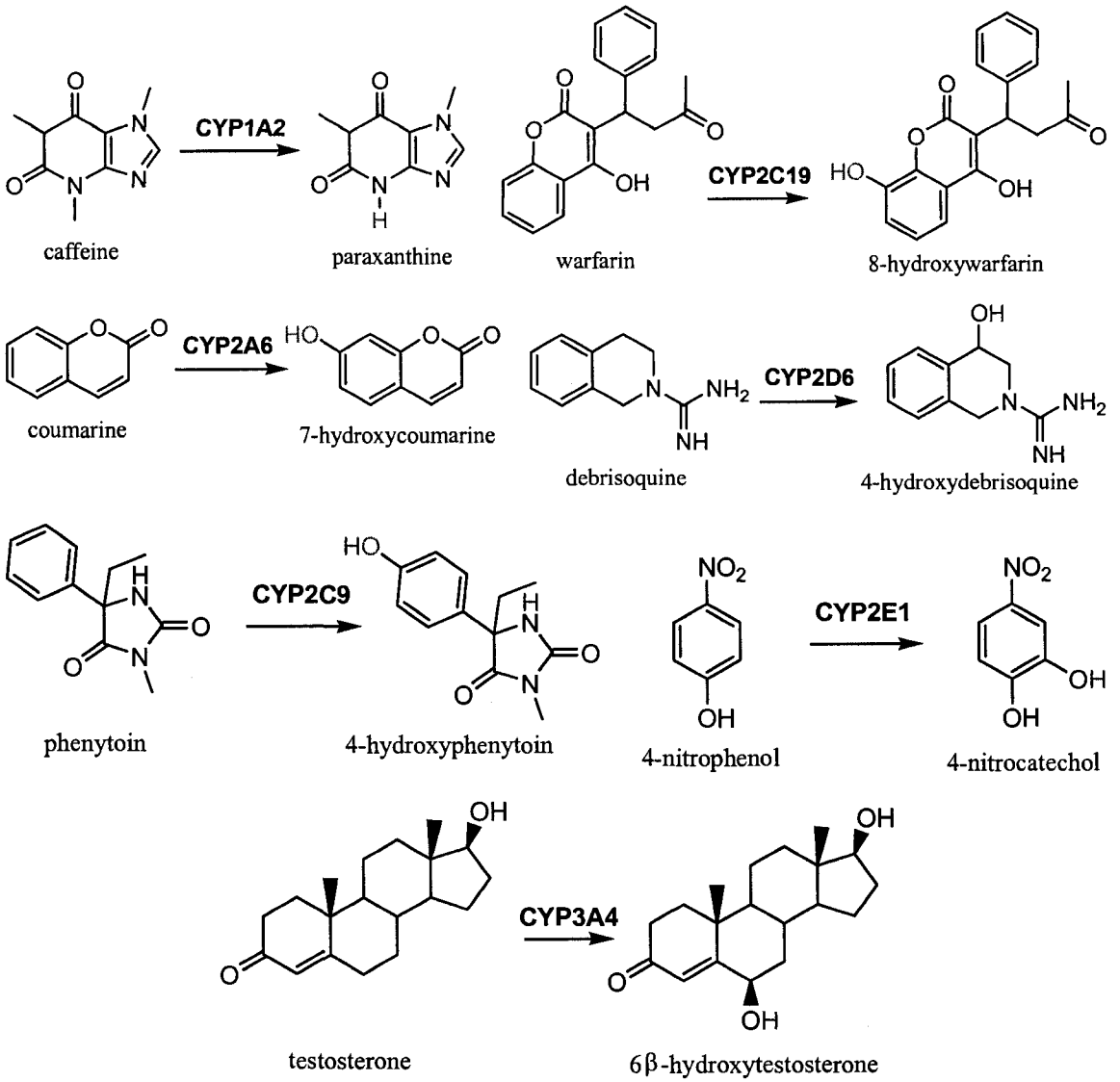
It was recently reported that wild-type CYP102 can modify certain pharmaceuticals in the same way that human P450s enzymes do [58]. The substrate specificity of wild-type CYP102 is, however, much narrower than for its mammalian counterparts and the yields of drug metabolites are generally low and highly variable. Mutagenesis has, however, modified the substrate specificity of CYP102. For example, several research groups have created variants showing high turnover numbers and high regioselectivity in the epoxidation of terminal alkenes such as 1-hexene [59]. High activity towards branched fatty acids has also been reported for several mutants [60, 61]. Other reactions that have been accessed after CYP102 random mutagenesis include the transformation of propranolol to 4'-hydroxylpropranolol and 5'-hydroxylpropranolol [62], the conversion of short length alkanes including gaseous ethane to the corresponding alcohols [63]. CYP102 mutants with high activity for the oxidation of benzene, styrene, cyclohexane, 1-hexene, and 1-propylene have also been engineered [64], as well as increased thermostability and peroxide resistance [65]. Finally, CYP102 was also used as a template to make artificial family of P450 enzymes with recombination of subunits made by cutting the chain at 7 crossover locations [66]. Several recent reviews about the potential of CYP102 as a biocatalyst summarize the field [67, 68].

1.6 Mammalian cytochrome P450 enzymes

In mammals, P450 enzymes are responsible for the synthesis of endogeneous regulators such as hormones, steroids, as well as the phase I metabolism of exogeneous compounds such as drugs and pollutants [69]. Like bacterial P450 enzymes, they oxidize substrates by reducing molecular oxygen to water. Bacterial P450 enzymes are generally soluble proteins whereas mammalian P450 enzymes and CPR are membrane-bound proteins. Two-dimensional movements of the mammalian enzymes allow side-by-side interactions between them.

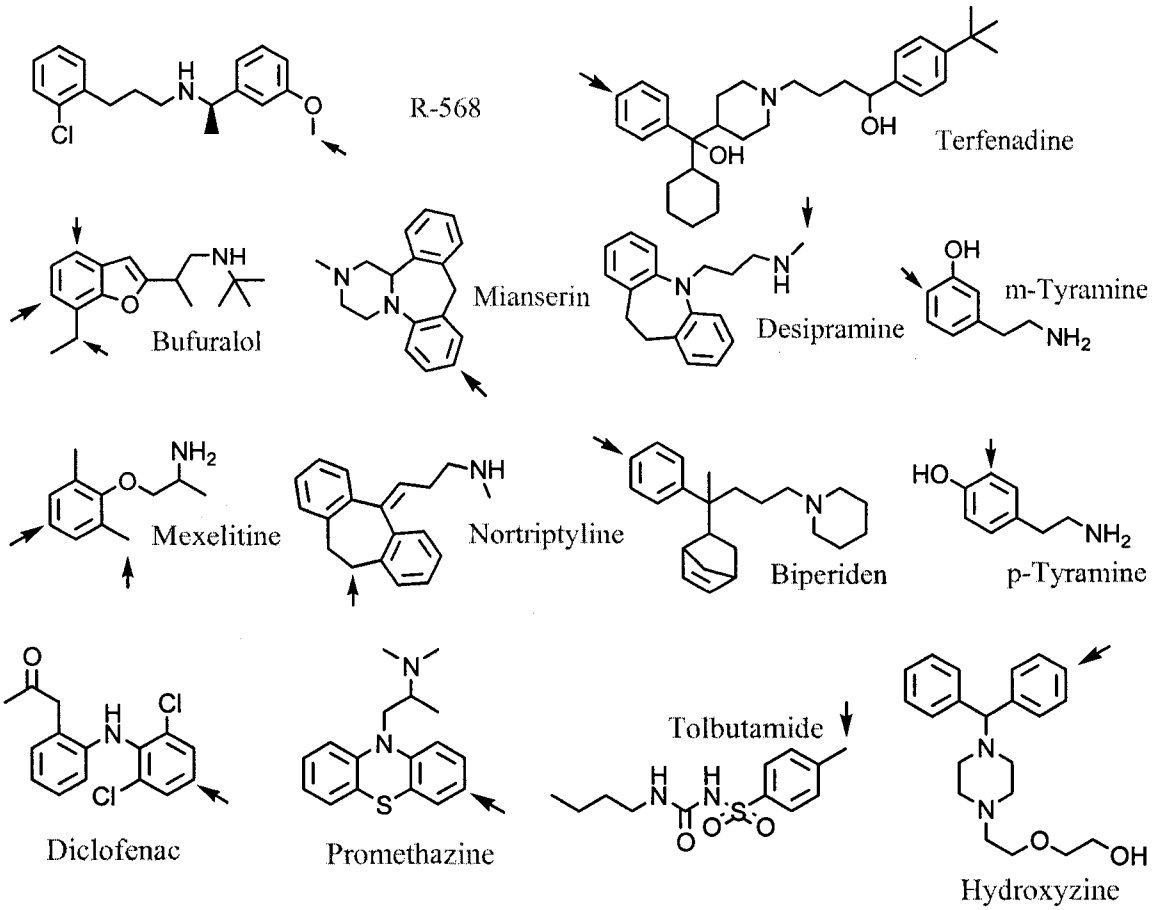
The human genome reveals 57 P450 isoforms, with the seven major isoforms involved in drug metabolism being CYP3A4, CYP2C9, CYP2D6, CYP2C19, CYP1A2,

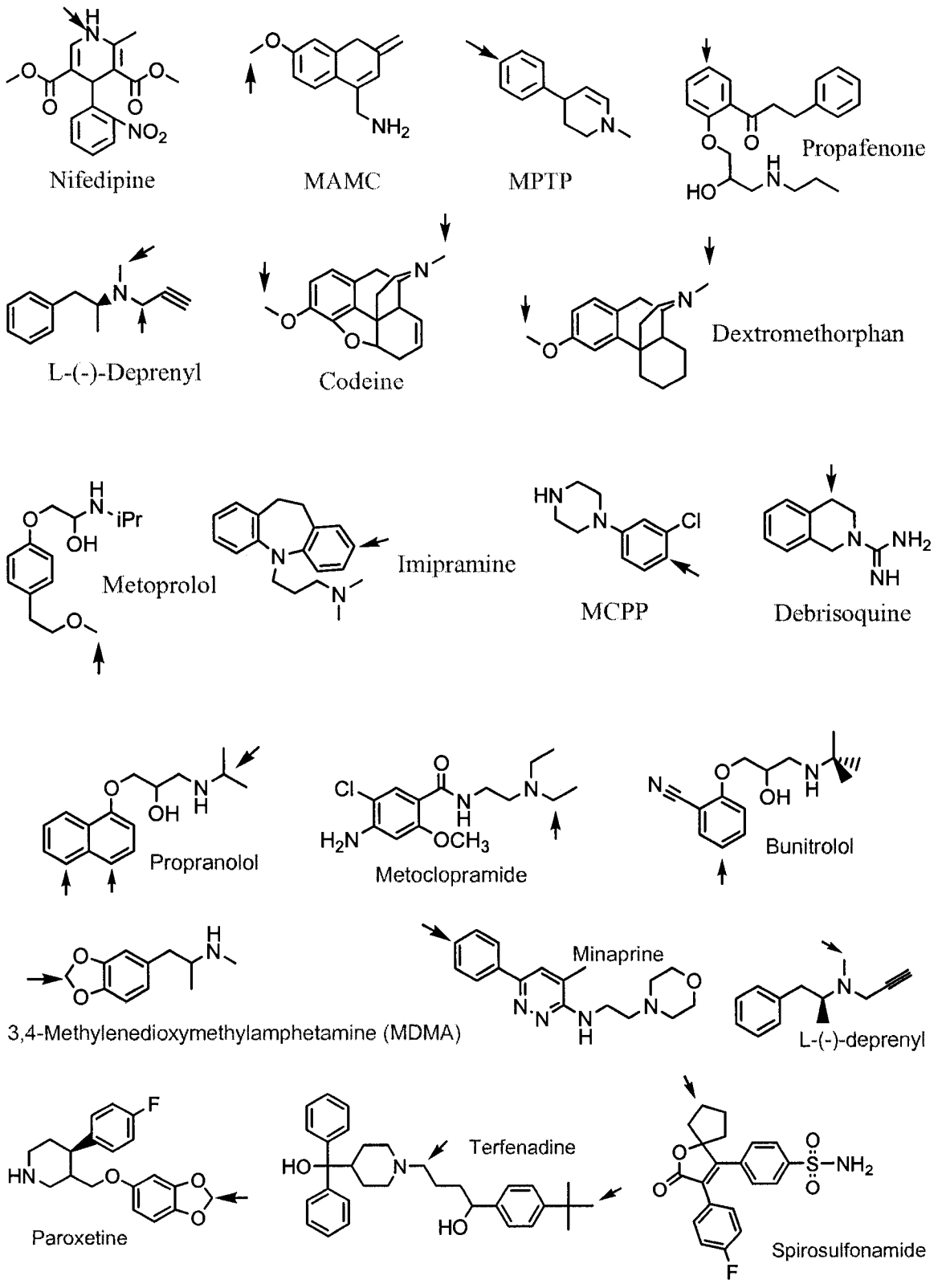
CYP2E1 and CYP2A6. Three of them (CYP3A4, CYP2D6, and CYP2C9) are involved in the metabolism of ~75% of all current pharmaceuticals. For the purpose of metabolism studies, a series of marker substrates, each specific to one isoform, have been identified and are shown on **Scheme 1.4** along with the major product formed. Also, novel fluorescent probes have recently been developed as marker substrates to facilitate screening [70, 71].



Scheme 1.4 Standard transformations used as marker for the seven human P450 enzymes, CYP1A2 [72], CYP2A6 [73], CYP2C9 [74], CYP2C19 [75], CYP2D6 [76], CYP2E1 [77], and CYP3A4 [78].

Human cytochrome P450 enzymes have been studied more for their pharmacological involvement than for fine chemicals biotransformation. Their very high substrate promiscuity, however, makes them especially attractive biocatalysts among P450 enzymes [2, 69]. For example, **Scheme 1.5** shows selected CYP2D6 substrates and the positions of transformation. Drawbacks that limit applications of human P450s as biocatalysts include low turnover numbers, limited tolerance to organic solvents, and the need for expensive cofactors. Some research groups have reported studies to overcome these disadvantages; however, a lot more work is needed [42, 79-82]. The ability of P450s to catalyze hydroxylation at inactivated carbon-hydrogen bond in a regio- and stereo-selective manner is a strong incentive to this endeavour.





Scheme 1.5 Diversity of CYP2D6 substrates. The arrows show the main positions where catalysis occurs.

In conclusion, mammalian P450 enzymes have a great potential in a variety of useful applications.

1.7 Enzymes in organic solvents

Most enzymes work naturally under aqueous conditions. Organic solvents, however, are preferred by chemists for a number of reasons including substrate solubility, moisture sensitivity of some substrates, reagents or products, shift of the reaction equilibrium by water, and for the ease of harvesting products [83]. Organic solvents molecules, nevertheless, were initially believed to deactivate enzymes because of enzyme denaturation. It was found later that organic media can keep enzymes active by rigidifying enzymes in active conformation. Media engineering has been successfully applied for enzymatic esterification, transesterification, and enantioselective resolution [84-87] with commercial or chemically modified lipases. Surprisingly, the change of reaction media from aqueous to selected organic solvents can affect not only the yield and the transformation rate but also the stereoselectivity of the enzyme [84, 88]. For example, Hirose *et al.* reported that *Pseudomonas* sp. lipase shows opposite yet outstanding stereoselectivity in transforming dihydropyridine dicarboxylates to (R)-monoesters in water saturated cyclohexane or to (S)-monoesters in water saturated diisopropyl ether [89].

Interestingly, some enzymes are more stable in *anhydrous* hydrophobic solvents than in buffers [90, 91]. This may be explained by a more rigid conformation adopted in the absence of bulk water molecules. A very small amount of water molecules are nevertheless considered essential as absolute *anhydrous* conditions to support enzymatic activity [92]. Hydrophilic organic solvents are more detrimental than hydrophobic organic solvents for they can stripe essential water molecules. The exact amount of water needed is enzyme-dependent, and thus, water activity is normally an important parameter to standardize enzyme activity. Water activity (a_w) is the relative availability of water in a substance. It is defined as the vapor pressure of water divided by that of pure water at the same temperature. Many studies show that the water activity of the reaction medium strongly correlates to the enzyme activity [93]. An interesting correlation has been

observed in galactooligosaccharides synthesis by the commercial enzyme β-glycosidase. CloneZyme Gly-001-02 can be optimized to catalyze different length of oligosaccharide formation by varying water activity and substrate concentration [94]. The widespread use of lipase-catalyzed reactions in synthesis is easily explained by the high stability of these enzymes, and their lack of cofactorial needs.

Oxido-reductases are much more complex systems, including oxidants and/or reductants. A change of medium may detrimentally affect not only the protein fold and pH but also electron shuttling, essential to redox reactions. It has been reported that the catalytic properties of some oxido-reductases are retained in certain organic solvents [95-97]. Horse liver alcohol dehydrogenase works very well (*ee* of 95 to 100%) in isopropyl ether on a 1 to 10 mmol scale [98]. Chloroperoxidase can catalyze chlorination reactions in the presence of organic solvents with log *P* values less than 0 [99]. Manganese peroxidase shows significant activity towards polycyclic aromatic hydrocarbons when up to 36 % (v/v) of acetone is added to the medium [100].

Physical or chemical modifications are, however, often necessary to overcome the effect of solvents on enzymes. These may include, for example, adsorption on a solid support [101, 102], encapsulation in sol-gel or polymers [103, 104], entrapment in reverse micelles [105], and cross-linking or covalent addition of stabilizing groups [106]. For instance, maleic anhydride or 2,4-bis(*o*-methyoxypolyethylene glycol)-6-chloro-*s*-triazine can dramatically increase stability of horseradish peroxidase in organic solvent such as benzene [107, 108].

Some achievements have been accomplished to physically or chemically modify P450 enzymes. Enzyme immobilization on stable supports has been used to facilitate applications as in biosensors. For examples, P450 1A2 was immobilized using Langmiur-Blodgett films as a solid support [109]. This system allowed detection of many organic substrates. Immobilized CYP102 in sol-gel was found to transform β-ionone, octane and naphthalene using a NADH recycling system with formate dehydrogenase [110].

Molecular dynamics (MD) has been used to study loss of P450 activity in presence of organic medium [111, 112]. It suggests that the activity loss results from organic solvent molecules pertubing the heme coordination and conformational change near active site access tunnel. Most of the engineering work to increase the tolerance of

P450 enzymes towards organic solvent has involved random mutagenesis (directed revolution) [42, 81-82]. For instance, both a single and double mutants of CYP102 were found with 10 fold increased specific activity in 2% (v/v) THF and 6-fold in 25% (v/v) DMSO compared to the wild type. The tolerance to other organic solvents was also improved [113]. Kumar and coworkers have screened >3000 colonies derived from random mutagenesis and found a P450 2B1 mutant, V183L/F202L/L209A/S334P or QM, possessing enhanced hydrogen peroxide mediated catalysis. After 3 extra mutations (QM/K236I/D257N/L295H) the variant was found to exhibits more than 2-fold higher activity than QM in nearly pure DMSO [114].

1.8 Enzyme stability

A major factor limiting enzyme applications in industry is their low stability, especially under harsh operation conditions and/or after numerous operation cycles. As described in **Section 1.7**, enzyme immobilization on inert solid supports has been effective at increasing their kinetic stability. Few such examples have been reported for oxido-reductases, and a handful for P450 enzymes.

A limited number of thermopilic enzymes have been identified [115]. By definition, these enzymes show high activity and stability at relatively high temperature. To date, three thermophilic P450 isoforms have been identified and purified, including CYP119 from *Sulfolobus solfataricus* [116], P450st from *Sulfolobus tokodaii* [117], and CYP175A1 from *Thermus thermophilus* HB27 [118]. The factors contributing to higher enzyme stability may include a higher abundance of salt bridges, more extended aromatic clusters, and minimal alanine/isoalanine interactions in the interior of the proteins leading to better side-chain packing [115]. Although thermophilic enzymes are very attractive for industrial applications, the very narrow substrate range of these 3 P450s is a significant obstacle.

In summary, P450 enzymes represent powerful biocatalysts because of their ability to catalyze regio- and stereo-specific hydroxylations at inactivated C-H bonds. The human CYP2D6 and CYP3A4 are of special interest in synthesis because of their high substrate promiscuity. There are, however, many drawbacks that have restricted the applications of P450 enzymes including expensive cofactor requirements, low organic

solvents tolerance, and low stability. In this thesis, the first two issues have been addressed and effective solutions to overcome these limitations are described. Some preliminary studies also suggest the possible stabilization of P450 enzymes by DNA self-assembly.

1.9 Objective of this thesis

Although P450 enzymes represent powerful biocatalysts because of their ability to catalyze region- and stereo-specific hydroxylations at inactivated C-H bonds, the use of P450 enzymes as biocatalysts is limited by a number of technical factors. The goal of this thesis is to overcome the major drawbacks associated with handling P450 2D6. More specific objectives include: 1) replacement of the expensive cofactors, 2) develop conditions for use of CYP2D6 in organic solvents, 3) increase 2D6 stability.

Chapter 2 describes our efforts at replacing the expensive natural cofactors of CYP2D6 and CYP3A4 with inexpensive chemical oxidants.

In chapter 3, the behaviours of CYP2D6 in different biphasic solvent systems were evaluated. The purpose of introducing biphasic systems is to enlarge the scope of application of the enzyme as most of CYP2D6 substrates are insoluble in water.

Next, we hope to study the activity of CYP2D6 in nearly anhydrous solvents. This required enzyme lyophilisation which is known to dramatically reduce enzyme activity. Various lyoprotectants were investigated to protect CYP2D6 and CYP3A4 against the harmful effects of lyophilisation.

Chapter 5 is an extension of chapter 3. In order to enlarge further the scope of application of CYP2D6 to water sensitive reactions, the activity of this enzyme was tested in a variety of selected nearly anhydrous organic solvents.

Due to great features of DNA self-assembly such as programmability and the rigidity of the double helices, encapsulation of CYP2D6 within DNA self-assemblies was investigated as method to stabilize the enzyme. In chapter 6, preliminary studies of P450 enzymes with DNA self-assemblies are reported.

Overall, each chapter aims at conquering one major drawback limiting the applications of P450 enzymes and each new chapter builds on the previous ones.

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Chapter Two

P450 enzymes attract a lot of attentions to chemists due to their unique and powerful catalysis of doing hydroxylation at inactivated C-H bond in regiospecific and stereospecific manners. The use of such enzymes in organic syntheses will largely reduce synthetic processes and increase efficiencies. As two major human isoforms, CYP3A4 and CYP2D6 are especially attractive owing to high promiscuity (A list of substrates of CYP2D6 is recorded in **chapter one**.). The catalyses of human P450 enzymes, however, are restricted by the requirement of coenzyme CPR (details in **chapter one**) and electron donor NADPH. The achievement of utilizing two enzymes together in large scale remains difficulties. In addition, there is economically unflavoured to use NADPH as electron donor due to its very high price. Therefore, finding cheap surrogates to replace both coenzyme CPR and NADPH is the first step towards using human P450 enzymes in large scale production.

Contributions of co-authors

This chapter is a copy of a published communication and is reproduced with permission from the journal ChemBioChem. It is cited as Amandine Chefson, Jin Zhao, and Karine Auclair, "Replacement of the natural cofactors by selected hydrogen peroxide donors or organic peroxides results in improved activity for CYP3A4 and CYP2D6" *ChemBioChem* **2006**, 7, 916-919. I accomplished the experiments and collected data related to CYP2D6. Amandine Chefson was responsible for the study related to CYP3A4.

Replacement of the natural cofactors by selected hydrogen peroxide donors or organic peroxides results in improved activity for CYP3A4 and CYP2D6

The cytochrome P450 enzymes (P450s or CYPs) form a ubiquitous family of heme proteins able to catalyze the monooxygenation of a wide range of substrates. P450s are of considerable interest in synthetic organic chemistry because of their impressive ability to catalyze the insertion of oxygen into non-activated C-H bonds. This useful reaction in organic chemistry has received much attention over several decades, but still remains a significant challenge. Some metal catalysts have been successfully used,[1] and biomimetic nonheme iron catalysts have been developed [2] but the regio- and/or stereo-selectivity usually remains poor. Biocatalysts such as P450 enzymes represent a promising alternative.[3] One limitation to the use of P450s in synthesis is the need for a complex system of cofactors including NADPH and a redox partner such as cytochrome P450 reductase (CPR) or a ferrodoxin/ferredoxin reductase system. A number of groups have attempted to overcome this drawback. Electrochemical methods,[4] cobaltocene,[5] and cobalt(III)sepulchrate,[6] have all been used to replace the cofactors albeit with limited success or applicability. Although many P450 enzymes are also known to accept peroxides or aqueous hydrogen peroxide as a source of oxygen (shunt pathway),[7] this pathway is generally not efficient. Some mutants of $P450_{BM-3}$ and $P450_{cam}$ have been engineered by directed evolution to efficiently use hydrogen peroxide in the absence of cofactors.[8] A heme domain mutant of $P450_{BM-3}$ has been engineered to catalyze regio- and stereo-selective oxidations in the presence of hydrogen peroxide instead of its natural cofactors.[9] The initial reaction rates were however significantly lower than those observed with the wild type enzyme under natural conditions (NADPH). Moreover, this enzyme is

naturally very specific for fatty acids and must be mutated to accept any new substrates.[10]

We selected the use the two human P450 isoforms CYP2D6 and CYP3A4 because of their high substrate promiscuity-a significant advantage for use in synthesis. Neither of these enzymes showed significant activity in the presence of aqueous hydrogen peroxide. We show here that without mutagenesis, the two studied isoforms can use various hydrogen peroxide donors or organic peroxides such as sodium percarbonate (SPC), cumene hydroperoxide (CHP) and *tert*-butylhydroperoxide (tBHP), to catalyze reactions in aqueous medium. Interestingly, product formation and initial rates are increased by as much as *two fold compared to the use of the natural cofactors*. Moreover, the regio- and stereo-selectivities are maintained.

The activity of CYP2D6 was monitored using the standard reaction of dextromethorphan demethylation to dextrorphan. All the reactions performed with hydrogen peroxide donors or organic peroxides were compared to the control reaction performed with the natural cofactors (NADPH/CPR) standardized to 100% activity (**Figure 2.1**). HPLC traces show a significant amount of unreacted substrate because a very large excess was used. The shoulder on the substrate peak in **B** is assigned to cumene alcohol. The results obtained with cofactor surrogates are summarized in **Table 2.1**. The best cofactor replacement was CHP, which yielded after one hour up to 210% of the amount of product obtained under the natural conditions. Very low concentrations of CHP were optimal. tBHP also showed good activity (80%) compared to the solid sources of hydrogen peroxide which yielded at best 53% activity (30 mM SPC, calculated in peroxide equivalents). Spectrometric analysis (LC-MS) was used to confirm the identity of the product. Combined with HPLC analysis of *N*-demethylated and *O*-demethylated standards, the data allowed us to confirm that the major product was dextrorphan in all cases (*O*-demethylation).

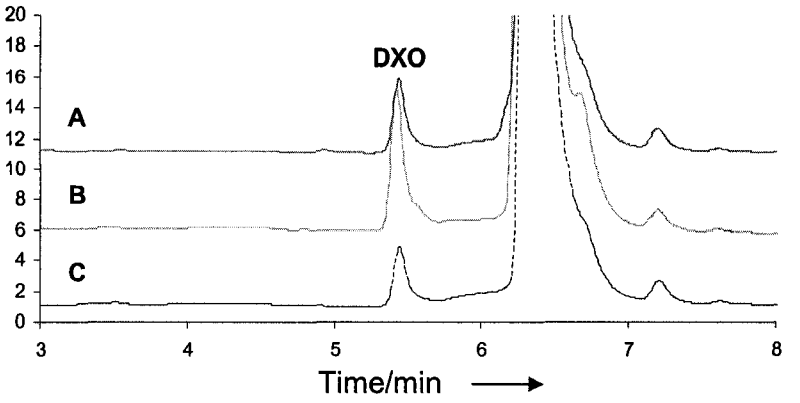

Figure 2.1: HPLC chromatograms with fluorescence detection (λ_{exc} = 280 nm, λ_{em} = 310 nm) after incubation at 37 °C for 1 hr of CYP2D6 (0.20 μM) and dextromethorphan (100 μM) in 0.1 M potassium phosphate at pH 7.4. **A:** reaction with CPR (0.83 μM) and NADPH (1.67 mM). **B:** reaction with cumene hydroperoxide (0.1 mM). **C:** reaction with *tert*-butylhydroperoxide (2 mM). The retention times for the substrate dextromethorphan (DXM) and the product dextrorphan (DXO) were 6.2 and 5.4 min respectively.

Some product formation was also observed in the control reactions without enzyme, generally representing $\leq$5% of the product formed in the presence of the enzyme. The only exception was with 100 mM SPB (sodium perborate) which yielded more of the product in the absence than in the presence of enzyme (depicted as negative values in **Table 2.1**). Aqueous hydrogen peroxide on the other hand did not yield detectable products under the conditions tested.

Table 2.1: CYP2D6-catalyzed formation of dextrorphan in the presence of various hydrogen peroxide donors or organic peroxides expressed as a percentage of activity compared to the reaction with the natural cofactors (CPR and NADPH).

Peroxide equiv. (mM)	SPC[a,b]	SPB[a,c]	UHP[a,d]	CHP[a,e]	tBHP[a,f]	H_2O_{2aq}[a,g]
0.1	-	-	-	**210 ± 4**	16 ± 2	N.D.[h]
0.2	-	-	-	197 ± 7	28 ± 7	N.D.
0.5	-	-	-	168 ± 13	50 ± 3	N.D.
1	-	-	-	124 ± 2	69 ± 2	N.D.
2	-	-	-	83 ± 2	**80 ± 4**	N.D.
10	14 ± 4	8 ± 4	17 ± 2	-	-	N.D.
20	48 ± 8	-	-	-	-	N.D.
30	**53** ± 12	**27 ± 11**	28 ± 4	-	-	N.D.
50	9 ± 14	1 ± 15	**29 ± 3**	-	-	N.D.
100	-17 ± 6	-57 ± 37	16 ± 4	-	-	N.D.

[a]Dextrorphan was quantified by HPLC after 1 hr of reaction at 37 °C for CHP and tBHP and 4 hrs for SPC, SPB and UHP. The mixtures (300 μL) contained CYP2D6 (0.2 μM, all from the same batch), dextromethorphan (166 μM for SPB, SPC and UHP, or 100 μM for CHP and tBHP) in 0.1 M potassium phosphate at pH 7.4, to which was added various concentrations of hydrogen peroxide donors or organic peroxides. The control reaction was performed with the natural cofactors CPR (0.83 μM) and NADPH (1.67 μM) and standardized to 100% activity. [b]SPC = sodium percarbonate; [c]SPB = sodium perborate; [d]UHP = urea-hydrogen peroxide; [e]CHP = cumene hydroperoxide; [f]tBHP = *tert*-butylhydroperoxide; [g]H_2O_2aq = aqueous hydrogen peroxide. [h]N.D: below the detection limit of 0.1 μM (3.5%).

The activity of CYP3A4 was monitored using the standard reaction of testosterone 6β-hydroxylation. Potassium phosphate buffer was used at a concentration of 100 mM, except with SPC where the buffer was optimum at 1 M to offset the expected increase of pH induced by the release of carbonates in the reaction mixture. Again, five hydrogen peroxide donors or organic peroxides were tested (**Figure 2.2, Table 2.2**). Similar to CYP2D6, the best activity was obtained with CHP and reached 132% of the activity promoted by the natural cofactors (CPR and NADPH). Relatively low concentrations of CHP were sufficient. SPC was also found to efficiently support the reaction, initially yielding 77% of the natural activity (see supporting information).

The reaction with SPC was further optimized by varying the buffer concentration and pH, and reached 119% of the activity with CPR/NADPH when 1 M of potassium phosphate at pH 7.0 instead of 7.4 was used. Thus after optimization, CHP and SPC yield comparable activities, although CHP is effective at lower total concentrations. The other hydrogen peroxide donors and organic peroxides produced less than 50% of the natural activity. The optimal concentration for SPC was 500 mM (calculated in peroxide equivalent), which is about 10 times higher than for CYP2D6, hence the need to increase the buffer strength. In all cases no product formation was detected in the control reactions without CYP3A4. Finally, aqueous hydrogen peroxide did not lead to the formation of any detectable products.

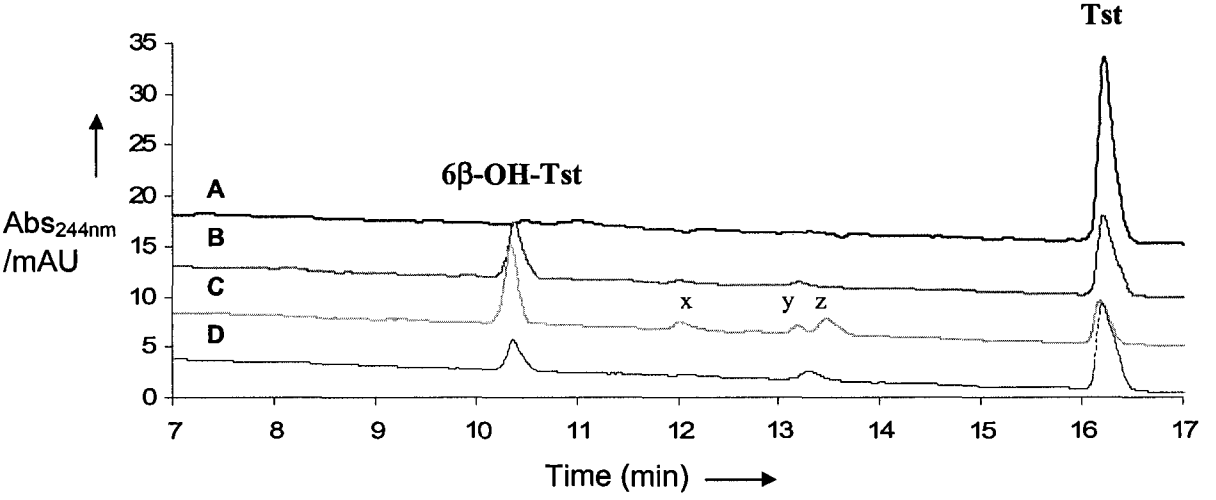

Figure 2.2: HPLC chromatograms with detection at 244 nm following incubation at 37°C for 4 hrs of CYP3A4 (0.9 μM) and testosterone (5 μM) in 0.1 M potassium phosphate at pH 7.4, unless otherwise stated. The internal standard cortexolone (14.2 min) was subtracted from the graphs for clarity. **A**: control reaction with no cofactor. **B:** reaction initiated with CPR (3.6 μM) and NADPH (1 mM + 1 mM every 30 min) **C:** reaction with cumene hydroperoxide (1 mM). **D**: reaction with sodium percarbonate (equiv. to 500 mM H_2O_2) in 1 M potassium phosphate buffer at pH 7.0.The retention times for the product 6β-hydroxytestosterone (6β-OH-Tst), and the substrate testosterone (Tst) were 10.4 and 16.2 min respectively. The peaks x, y and z are other metabolites formed during the reaction, unidentified but very likely to be the 1β, 15β and 2β hydroxylated products according to Guengerich and coworkers.[11]

Table 2.2: Formation of 6β-hydroxytestosterone by CYP3A4 with hydrogen peroxide donors or organic peroxides expressed as a percentage of the reaction with the natural cofactors (CPR and NADPH).

Peroxide equiv. (mM)	**SPC**[a,b]	**SPB**[a,c]	**UHP**[a,d]	**CHP**[a,e]	**TBHP**[a,f]	**H_2O_{2aq}**[a,g]
0.1	-	-	-	83 ± 8	6 ± 2	N.D.[h]
0.5	-	-	-	114 ± 12	13 ± 4	N.D.
1	-	-	-	**132 ± 17**	21 ± 2	N.D.
2	-	-	-	92 ± 10	**35 ± 3**	N.D.
5	-	-	-	61 ± 6	16 ± 1	N.D.
50	28 ± 3	2 ± 2	2 ± 1	-	-	N.D.
100	47 ± 5	4 ± 3	3 ± 3	-	-	N.D.
500	**119[h] ± 12**	**10 ± 3**	**7 ± 4**	-	-	N.D.
1000	28 ± 4	6 ± 2	4 ± 3	-	-	N.D.

[a]Analysis of 6β-hydroxytestosterone (6β-OHT) formation by HPLC after 1 hr incubation at 37°C of CYP3A4 (0.9 μM) and testosterone (115 μM), initiated with various concentrations of hydrogen peroxide donors or organic peroxides. The buffer was 0.1 M potassium phosphate at pH 7.4, and 1 M potassium phosphate at pH 7.4 for the reaction with SPC. The control reaction was with the natural cofactors CPR (2.4 μM) and NADPH (1 mM); [b]SPC = sodium percarbonate; [c]SPB = sodium perborate; [d]UHP = urea-hydrogen peroxide adduct; [e]CHP = cumene hydroperoxide; [f]tBHP = *tert*-butylhydroperoxide; [g]H_2O_2aq = aqueous hydrogen peroxide; [h]Several potassium phosphate buffer concentrations and pHs were tested with 500 mM SPC, 1 M potassium phosphate at pH 7.4 gave 77% activity, while the highest activity of 119% was obtained at pH 7.0. [h]N.D.: below the detection limit of 0.1 μM (1%).

SPC, SPB and UHP are powders with an active oxygen content equivalent to 27.5%, 32% and 35% H_2O_2 respectively. These hydrogen peroxide donors present the advantage of releasing H_2O_2 slowly and locally on application, creating milder oxidation conditions around the enzyme than aqueous hydrogen peroxide. This was verified by comparing the activity of CYP3A4 with SPC when the reaction mixture

was shaken after addition, which led to complete dissolution of the salt, or when the dissolution occurred slowly overtime without shaking. In the former case no product formation was detected, confirming the need for a slow release of hydrogen peroxide in the reaction mixture.

Interestingly, for both enzymes, the increased product formation observed in the presence of CHP or SPC (**Tables 2.1** and **2.2**) can be explained by a proportional increase in the initial rates of product formation (**Table 2.3**). Thus the calculated initial rate for the reaction of CYP2D6 in the presence of 0.1 mM CHP is twice that of the reaction promoted by CPR/NADPH. Similarly, for reactions of CYP3A4, the initial rates are ~30% higher with optimal amounts of SPC or CHP than with CPR/NADPH.

Table 2.3: Initial rates of product formation and maximum turnover numbers for the CYP2D6-catalyzed formation of dextrorphan and the CYP3A4-catalyzed 6β-hydroxylation of testosterone in the presence of CPR/NADPH or peroxide equivalents.

	CYP3A4[a]			**CYP2D6[b]**		
Cofactor	CPR/ NADPH	CHP[c]	SPC[d]	CPR/ NADPH	CHP	tBHP[e]
Initial rate ($\mu mol\ \mu mol^{-1}\ min^{-1}$)	5.9 ± 0.9	9.1 ± 1.5	7.1 ± 1.2	2.4 ± 0.3	4.7 ± 0.3	2.2 ± 0.2
Turnover	46 ± 4	42 ± 5 50 ± 4*	26 ± 2	107 ± 3	92 ± 2	41 ± 2

[a]6β-hydroxytestosterone (6β-OHT) formation as measured by HPLC after incubation at 37°C for different times of CYP3A4 (0.9 μM) and testosterone (115 μM). The turnover number was calculated after a 4 hr reaction. The buffer was 0.1 M potassium phosphate at pH 7.4, and 1 M potassium phosphate at pH 7.0 for the reaction with SPC. The cofactors tested were either CPR (3.6 μM) and NADPH (1 mM + 1 mM added every 30 min), CHP (1 mM + 1 mM added every 30 min, or *0.1 mM + 0.1 mM added every 30 min) or SPC (500 mM); [b]Dextrorphan (DXO) formation as measured by HPLC after incubation at 37°C of CYP2D6 (0.18 μM) and dextromethorphan (100μM). The

turnover number was calculated after a 4 hr reaction. The buffer was 0.1 M potassium phosphate at pH 7.4. The cofactors tested were either CPR (0.53 μM) and NADPH (1.67 mM + 1.67 mM added every 30 min), CHP (0.1 mM) or tBHP (2 mM); [c]CHP = cumene hydroperoxide; [d]SPC = sodium percarbonate, [e]tBHP = *tert*-butyl hydroperoxide.

Because peroxides have been reported to react with the heme moiety of P450s and lead to enzyme inactivation,[12] we have compared the stability of CYP2D6 and CYP3A4 in the presence of surrogate and natural cofactors. UV-based measurements typically used to measure P450 stability were not convenient here because of interfering absorption by cofactors. Instead, a large excess of substrate and cofactor(s) were used (fresh cofactor was added every 30 min) and product formation was monitored over time. The enzyme was considered inactive when product formation reached a plateau. Under these conditions, CYP2D6 lost activity after ~1 hour in the presence of CHP or tBHP but after ~1.7 hours when the natural cofactors were used (**Figure 2.3**). This difference in stability is much smaller than expected based on previous literature,[12] most likely because of the very small amount of organic peroxides used. Although CYP2D6 displays lower stability in the presence of CHP compared to CPR/NADPH, its higher initial rate with CHP results in a comparable maximum turnover numbers when compared to CPR/NADPH (**Table 2.3**). CYP3A4 on the other hand shows comparable stability whether CPR/NADPH or CHP are used (~3 hours, **Figure 2.4**). This was not expected based on previous literature,[12] and is again most likely explained by the small amount of CHP used. When the quantity of CHP added is further reduced (multiple additions of 0.1 mM instead of 1 mM, see **Table 2.3** and **Figure 2.4**) the initial rate decreases; however, the enzyme is active for ~4 hours and the maximum turnover number rises (still comparable to CPR/NADPH).

CYP3A4 and CYP2D6 represent ideal biocatalysts because of their high substrate promiscuity and their ability to catalyze chemically challenging

hydroxylations at inactivated C-H bonds. In summary, we report an improvement of up to two fold in reaction rates when the natural cofactors of CYP2D6 or CYP3A4 are replaced with SPC or CHP. This is the first time that such an *improvement compared to the use of natural cofactors* is reported for P450 enzymes. This is accomplished with amounts of cofactor surrogates much lower than previously reported, which likely allowed minimization of side reactions. These results suggest that the need for expensive cofactors can be circumvented without significant decreases in yields by replacement with very cheap commercial compounds, directly with the wild type enzymes. We anticipate that this alternative approach may apply to other P450 enzymes and will be of considerable use for future applications of P450s in synthesis.

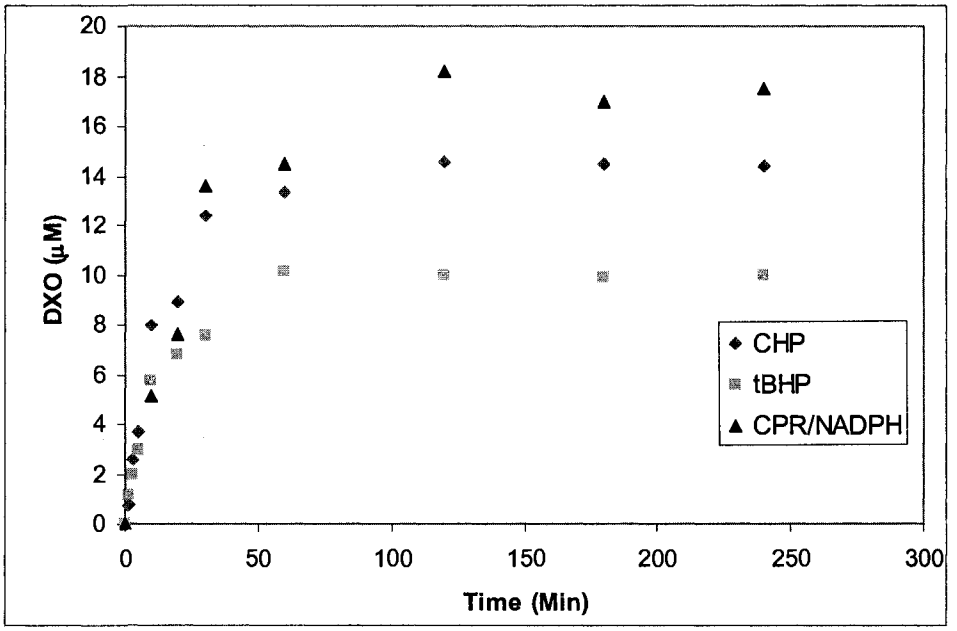

Figure 2.3: Dextrorphan (DXO) formation as measured by HPLC after incubation at 37°C of CYP2D6 (0.18 μM) and dextromethorphan (100 μM) in 0.1 M potassium phosphate buffer at pH 7.4 (total volume 300 μL). Each point on the plot represents an average of two measurements. The reaction was initiated with 0.1 mM CHP (♦), 2 mM tBHP (■), or CPR (0.5 μM) and NADPH (1.67 mM + 1.67 mM every 30 min) (▲).

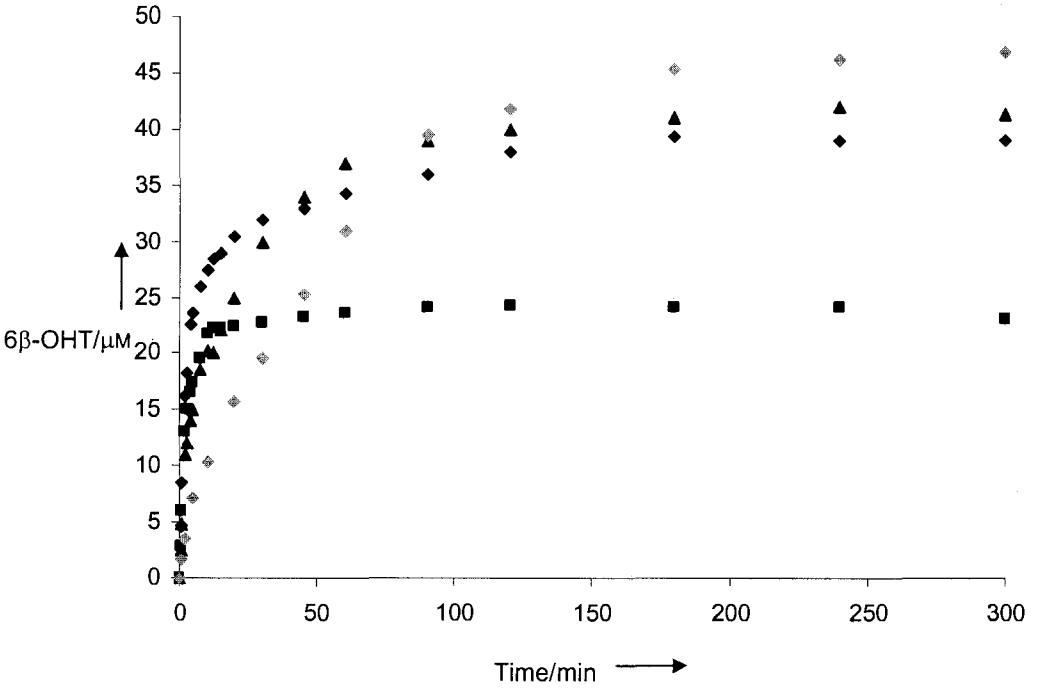

Figure 2.4: 6β-hydroxytestosterone (6β-OHT) formation as measured by HPLC (244 nm) after incubation at 37°C for different times of CYP3A4 (0.9 μM) and testosterone (115 μM) in 0.1 M potassium phosphate buffer at pH 7.4, or 1 M at pH 7.0 with SPC. Each point on the plot represents an average of two measurements, and for each, a total reaction volume of 300 μL was used. The reaction was initiated with CHP (1 mM + 1 mM added every 30 min (♦), 0.1 mM + 0.1 mM added every 30 min (◈)), 500 mM SPC (■), or CPR (3.6 μM) and NADPH (1 mM + 1 mM after 15 min then every 30 min) (▲).

Acknowledgements

The authors wish to acknowledge Prof. J. R. Halpert from the University of Arizona, Prof. F. P. Guengerich from Vanderbilt University and Prof. Charles B. Kasper from the University of Wisconsin-Madison for their kind gift of the CYP3A4, CYP2D6 and CPR expression plasmids, respectively. This work was supported by the Natural Science and Engineering Council of Canada (NSERC), Astra Zeneca Canada Inc., Boehringer Ingelheim Canada Ltd., and Merck Frosst Canada & Co.

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Chapter Two – Appendix

Experimental section (published as supporting information)

2A.1 Chemicals

The CYP3A4 pSE3A4His expression plasmid was a gift from Dr. J. R. Halpert from the University of Arizona, the CYP2D6 expression plasmid (DB6 with His_5 and M374V) was obtained from Dr. F. P. Guengerich from Vanderbilt University, and the cytochrome P450 reductase OR263 plasmid was kindly donated to us by Dr. Charles B. Kasper from the University of Wisconsin. The culture media ingredients yeast extract, tryptone, peptone, as well as the CYP2D6 substrate AMMC (3-[2-(*N,N*-diethyl-*N*-methylammonium)ethyl]-7-methoxy-4-methylcoumarin) and the product AHMC (3-[2-(diethylamino)ethyl]-7-hydroxy-4-methylcoumarin hydrochloride) were purchased from BD Biosciences (San Jose, CA). DH5α supercompetent cells and agarose were from Invitrogen (Carlsbad, CA). The 2',5'-ADP Sepharose 4B and DEAE Sepharose resins were from Amersham Bioscience (Baie d'Urfé, QC, Canada). The His-Select™ Nickel Affinity Gel was purchased from Sigma (St. Louis, US). The CYP3A4 substrate testosterone (4-androsten-17β-ol-3-one) was a king gift from Dr. Eisenberg in our department and the metabolites 6β-hydroxytestosterone (4-androsten-6β,17β-diol-3-one) and 6α-hydroxytestosterone (4-androsten-6α,17β-diol-3-one) were purchased from Steraloids (Newport, RI). All solvents were purchased from Fisher and were of HPLC grade. All chemicals were used without further purification. Water was obtained from a Milli-Q Synthesis (Millipore, San Jose, CA) filtration system. All other chemicals were purchased from Sigma or Aldrich.

2A.2 Instruments

UV absorption spectra were recorded on a Cary 5000 UV spectrophotometer (Varian, Mississauga, ON, Canada). Fluorescence measurements were obtained on a

Spectramax GeminiXS (Molecular Devices Corp., Sunnyvale, CA) using 96-well flat bottom assay plate (Corning Incorporated Life Sciences, New York, NY). Analytical HPLC analyses were performed on an Agilent 1100 modular system consisting of an auto-sampler, a quaternary pump system, a photodiode-array detector, a fluorescence detector, and a thermostated column compartment. The Agilent Chemstation software version A.10.02 was used to control the operation and data acquisition. Analysis of dextromethorphan *O*-demethylation by CYP2D6 was carried out using a Synergi 4 μm Hydro-RP 80 Å column with mobile phase A (0.05% TFA in water) and B (100% acetonitrile) at a flow rate of 1 mL/min. The elution gradient was from 20% to 90% B over 6 min, then held for 2 min. Fluorescence (λ_{ex} = 280 nm, λ_{em} = 310 nm) was used to monitor the elution. The retention times for dextromethorphan and dextrorphan were 5.4 and 6.3 min respectively. Analysis of testosterone 6β-hydroxylation by CYP3A4 used a 150 X 4.6 mm Zorbax Eclipse XDB-C8 5μm column from Agilent protected by an analytical guard column. The elution consisted of a first isocratic step at 15% acetonitrile in water for 4 min, before a linear gradient to 50% acetonitrile over 12 min. The flow rate was 1.5 mL/min, and the column temperature was set to 30°C. Detection was at 244 nm. Under these conditions, the retention times of testosterone, 6β-hydroxytestosterone, 6α-hydroxytestosterone and cortexolone were 16.3, 9.9, 10.5 and 14.2 min respectively.

2A.3 Expression and purification of His-tagged CYP2D6

The CYP2D6 plasmid was transformed into *E. coli* DH5α supercompetent cells and grown overnight at 37°C on Luria-Bertani (LB) agar plates with ampicillin (100 mg/L). LB media consisted of: 10 g tryptone, 5 g yeast extract and 10 g NaCl per liter, autoclaved for 20 min at 121°C. One separate colony was added to Terrific Broth (TB) medium (5 mL prepared from: 12 g tryptone, 24 g yeast extract, 4 mL glycerol, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4 per liter, autoclaved) containing ampicillin (100 mg/L). After incubation (300 rpm, 37°C, 17 hrs), a portion of this culture (2 mL) was diluted in TB (100 mL) containing ampicillin (100 mg/L), potassium dihydrogen phosphate (0.23

g) and potassium hydrogen phosphate (1.25 g). After a second incubation (180 rpm, 33°C, 5 hrs), this mixture was further diluted in TB (10 mL in 500 mL) containing ampicillin (100 mg/L), trace elements solution (150 μL), potassium dihydrogen phosphate (1.1 g), and potassium hydrogen phosphate (5.23 g). The trace elements solution consisted of 100 mM $FeCl_3$, 10 mM $ZnCl_3$, 10 mM Na_2MoO_4, 14.3 mM $CaCl_2$, 7.4 mM $CuCl_2$, 10 mM H_3BO_3 in 1 mL of concentrated HCl + 9 mL of MilliQ water. The culture was fermented for 30 min at 33°C and 250 rpm. Next, after addition of the heme precursor δ-aminolevulinic acid (δ–ALA, 8.4 mg/L), thiamine (0.68 g/L) and quinidine (0.03 mM), the culture was incubated at 37°C and 180 rpm until OD_{600} 0.7-0.8 (~2.5 hrs). Protein expression was induced with β-D-thiogalactoside (IPTG, 0.5 mM) and the culture was further grown at 25°C and 150 rpm for 48 hrs.

The cells were harvested by centrifugation (4000 × g, 25 min), and the harvested cells (100 g) were suspended in TES buffer (12 mL/gram cells of: 500 mM Tris acetate at pH 7.5, 250 mM sucrose, 0.25 mM EDTA), followed by addition of lysozyme (3 mg/g cells). The same volume of pre-cooled water was added and gently stirred for 30 min at 4°C before centrifugation (3393 × g, 4°C, 15 min). The pellets were resuspended in sonication buffer (30 mL of: 0.1 M potassium phosphate buffer at pH 7.4 containing 20% glycerol (v/v) and 6 mM magnesium acetate). The cells were further lyzed by sonication (7 times at 60% duty cycle and power 8 for 20 sec each time) after addition of leupeptin (1 μg/mL), aprotinin (0.04 μg/mL), bestatin (1 μM) and β-mercaptoethanol (20 mM). A salt-ice bath was applied to keep the mixture cool. Following centrifugation (10,000 × g for 20 min and 75,000 × g for 60 min at 4°C), the supernatant was loaded onto the nickel affinity column (1 mL) equilibrated with buffer C (20 mM potassium phosphate buffer at pH 7.4 containing 20% glycerol, 2 g/L 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 0.5 M potassium chloride), and buffer D (buffer C containing 10 mM β-mercaptoethanol). The column was washed with buffer D (10 mL) and buffer E (10 mL of: 20 mM potassium phosphate buffer at pH 7.4 containing 20% glycerol (v/v), 0.5 M potassium chloride, and 10 mM β-mercaptoethanol). The protein was eluted with buffer E without

β-mercaptoethanol but containing imidazole (200 mM). The colored fractions were pooled and dialyzed for 4 hrs against buffer F (2 × 4 L of 10 mM potassium phosphate buffer at pH 7.4 containing 0.13 mM EDTA and 0.1 mM DTT). The fractions containing 50 kDa proteins were identified by SDS-PAGE (Homogeneous 12.5%). The overall yield was calculated to be 0.563 mg/L.

2A.4 Expression and purification of His-tagged CYP3A4

CYP3A4 was expressed using a modification of the procedure used by Domanski *et al.* The plasmid was transformed into *Escherichia coli* DH5α supercompetent cells, and the cells grown overnight on LB agar plates with ampicillin (50 μg/mL). Culture tubes containing LB media supplemented with ampicillin (50 μg/mL) were inoculated next. After 24 hrs of growth at 37°C with shaking at 250 rpm, 10 flasks of 750 mL TB medium containing ampicillin (50 μg/mL) were inoculated each with 3 intermediate culture tubes. The flasks were placed at 37°C with shaking at 250 rpm for 2 hrs (i.e. until OD_{600} = 0.6), after which protein expression was induced with IPTG (1 mM) followed by addition of δ-ALA (80 mg/mL). The incubation temperature was reduced to 30°C, with shaking set to 190 rpm for 48 hrs. The cells were collected by centrifugation at 4000 × g and 4°C for 15 min. The pellets were resuspended in buffer A (50 mL of: 100 mM 3-[*N*-morpholino]propanesulfonic acid] (MOPS) at pH 8 containing 10% glycerol, 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride (PMSF)). All subsequent procedures were performed at 4°C. Lysozyme (10 mg/g of cells) was added, and the suspension was stirred slowly for 15 min, after which the suspension was supplemented with protease inhibitors (1.6 μg/mL leupeptin, 1 μg/mL aprotinin, 0.8 μg/mL bestatin, and 0.7 μg/mL pepstatin A). The suspension was then sonicated on an ice-salt bath at 60% and power 8. After 2 sonication cycles of 20 sec each, the suspension was centrifuged at 100,000 × g for 60 min. The supernatant was removed and the pellets resuspended in buffer B (10 mL of: 100 mM MOPS at pH 7.4, containing 10% glycerol and 2 mM PMSF). CHAPS (0.5%) and potassium chloride (0.5 M) were then added. Cells were homogenized and gently stirred for 2 hrs

before centrifugation (1 hr at 100,000 × g). This CHAPS-solubilized P450 preparation was then stored at -80°C until the next step.

The His-tagged protein was purified by affinity column chromatography using the Ni-NTA metal affinity resin (1.5 mL), pre-equilibrated with 5 bed volumes of EQ-buffer (buffer B containing 0.5% CHAPS, 0.5 M KCl, and 5 mM imidazole) at a flow rate of 0.5 mL/min. The P450 preparation was then loaded onto the column at the same flow rate. The column was washed with 10 bed volumes of EQ-buffer, then with 10 bed volumes of 100 mM MOPS buffer at pH 7.4 containing 10% glycerol and 10 mM imidazole. The P450 was eluted in EL-buffer (100 mM MOPS at pH 7.4 containing 10% glycerol and 200 mM imidazole). The orange-colored fractions were compared spectroscopically for protein content and by SDS-PAGE (12.5% homogeneous gels) for purity assessment. The fractions containing the CYP3A4 protein were pooled and dialyzed (twice 4 L of: 100 mM MOPS buffer at pH 7.4 containing 10% glycerol, 0.2 mM dithiothreitol and 1 mM EDTA) at 4°C. The sample was aliquoted and stored at -80°C. The overall yield was 0.6 mg per liter of culture.

2A.5 Quantification of P450 enzymes

The P450 content was measured by reduced carbon monoxide difference spectra following the method described by Omura and Sato [2]. Thus heme was reduced by addition of a few solid grains of sodium dithionite ($Na_2S_2O_4$) in quartz cuvettes containing the buffered enzyme solution, and formation of the enzyme-carbon monoxide complex was achieved by slow bubbling of CO gas into the reduced enzyme solution for about 20 sec. The UV absorption spectra was recorded, and the concentration of P450 was determined using the extinction coefficient $\varepsilon_{450} = 91\ mM^{-1}\ cm^{-1}$.

2A.6 Cytochrome P450 reductase (CPR) expression and purification

E. coli DH5α supercompetent cells were transformed with the cytochrome P450 reductase OR263 plasmid and spread onto LB medium agar plate containing ampicillin (100 mg/L) and incubated overnight at 37°C. Four separate colonies were added to LB medium (6 mL) containing ampicilin (100 mg/L). The culture was incubated overnight (225 rpm, 37°C, 17 hrs) before dilution in TB (3 mL in 1 L) supplemented with ampicillin (100 mg/L) and riboflavin (1 mg/L). CPR expression was induced with IPTG (0.5 M) at OD_{600} ~ 0.8 (~3 h at 37°C and 225 rpm). The cultures were further grown at 37°C for 20 hrs. Unless mentioned otherwise, all the steps in the protein purification were carried at 4°C and storage was at -80°C. Cells were collected by centrifugation (4614 ×g, 25 min) and the harvested cells (46.8 g) were resuspended in TSE buffer (60 mL). Lysozyme (1.2 mg) was added and the mixture was incubated for 20 min. After centrifugation (3000 × g, 30 min), the pellet was lysed for 20 min in lysis buffer (60 mL, containing 50 mM Tris base at pH 8.0, 0.5 M EDTA, 10 mg/L aprotinin, and 1 mM PMSF). The cells were further sonicated 7 times at 60% duty cycle and power 8 for 30 sec each time with salt-ice bath cooling. Centrifugation (12,000 × g, 10 min, 4°C) was used to remove the supernatant and the pellet was sonicated again in the same matter. After the second centrifugation, the two batches of supernatant were combined and suspended in affinity buffer (120 mL of: 50 mM Tris base at pH 8.0 containing 10% glycerol (v/v), 0.1% Triton X100 (v/v), 0.1 mM EDTA, and 0.05 mM DTT). After another centrifugation (41,000 × g, 45 min), PMSF (1 mM) was added. A homogenous solution was obtained after 3 hrs of gentle stirring. The protein solution was applied to a 2',5' ADP Sepharose 4B column (16 mL) equilibrated with affinity buffer (60 mL). The column was first washed with buffer A (40 mL of affinity buffer containing 0.5 mM adenosine), followed by elution of the protein with buffer B (affinity buffer containing 2 mM adenosine 2'-monophosphate (2'-AMP)). The fractions containing the 70 kDa protein were pooled. The sample was loaded onto a fast flow DEAE Sepharose column (8 mL) equilibrated with DEAE-EQ buffer (100 mL of: 0.2 M potassium phosphate buffer at pH 7.4 containing 20% glycerol (v/v) and 1 g/L CHAPS), and washed with buffer C (16 mL of: DEAE-EQ buffer containing 10 mM β-mercaptoethanol). The column was washed with buffer C (40 mL) and eluted with a

linear gradient increasing from 0.1 to 0.5 M potassium chloride in buffer C. The fractions containing the 70 kDa protein were identified by SDS-PAGE (homogeneous 12.5%) and dialyzed against buffer G (1 L of 0.1 M potassium phosphate buffer at pH 7.4 containing 20% glycerol (v/v). The overall yield was calculated to be 0.643 mg/L.

2A.7 CPR concentration and activity assay

The concentration of CPR was determined by oxidation with potassium ferricyanide. An aliquot of CPR (130 μL) was diluted in 100 mM potassium phosphate buffer at pH 7.6 (266 μL) before addition of potassium ferricyanide (7 μM). The concentration was calculated using an extinction coefficient of 21.2 mM^{-1} at 455 nm for the oxidized form of CPR. The activity of CPR was evaluated by cytochrome c reduction in the presence of NADPH. The assay mixture contained CPR (0.5 μM), cytochrome c (50 μM), and NADPH (100 μM) in 300 mM phosphate buffer at pH 7.6. The blank did not contain NADPH. Cytochrome c reduction by CPR is revealed by absorption peaks at 520 and 550 nm.

2A.8 AMMC demethylation by CYP2D6

AMMC was dissolved in acetonitrile. For the reference reaction, a mixture (300 μL) containing AMMC (200 μM), CPR (0.7 μM), CYP2D6 (0.12 μM) in potassium phosphate buffer (0.1 M at pH 7.4) was incubated at 37°C for 5 min. The reaction was initiated with the addition of NADPH (0.83 mM) and monitored by fluorescence (λ_{ex} = 390 nm, λ_{em} = 460 nm) for 15-20 min. Blank reactions were performed in parallel without CYP2D6.

2A.9 Dextromethorphan demethylation by CYP2D6

Dextromethorphan (DXM) was demethylated to dextrorphan (DXO) by CYP2D6 in the presence of cofactors CPR and NADPH or hydrogen peroxide donors.

In the reference reaction, the mixture (300 μL) containing CYP2D6 (200 nM), CPR (825 nM), and DXM (167 μM) in potassium phosphate buffer (0.1 M, pH 7.4) was incubated for 5 min at 37°C. The reaction was initiated with the addition of NADPH (3 mM) and incubated for 1 or 4 hrs at 250 rpm and 37°C. The reaction was quenched with 23% (v/v) perchloric acid (100 μL). For the reactions with the hydrogen peroxide donors, CYP2D6 (200 nM) and DXM (167 μM with SPB, SPC, or UHP, and 100 μM with CHP or tBHP) and the mixture did not contain CPR/NADPH. The reactions were initiated by one of different hydrogen peroxide donors (10 mM to 100 mM) or organic peroxides (0.1 mM to 2.5 mM). The reaction was quenched with 23% (v/v) perchloric acid (100 μL) after incubated for 2 or 4 hrs at 250 rpm and 37°C. The range of concentrations used for hydrogen peroxide, cumene hydroperoxide and *tert*-butyl hydroperoxide was 0.25 mM to 2.5 mM. The quenched mixtures were centrifuged (16000 × g, 10 min). The supernatants were filtered through 0.2 μM Teflon filter units before HPLC analysis. Blank reactions without CYP2D6 were carried in parallel.

2A.10 Quantification of 6β-hydroxytestosterone

Quantification of the 6β-hydroxytestosterone formed during the enzymatic reaction was achieved with a calibration curve obtained by spiking the incubation mixture (no CYP) with 6β-hydroxytestosterone and cortexolone as the internal standard. Concentrations ranging from 0.5 to 5 nmol/mL were used for the calibration curve which was generated by plotting the area ratio (6β-hydroxytestosterone/cortexolone) against the concentration of 6β-hydroxytestosterone. A linear regression plot of peak-area ratio versus concentration was constructed and the concentration of 6β-hydroxytestosterone determined from the peak-area ratio relative to the calibration graph.

2A.11 Testosterone hydroxylation assays for CYP3A4

The reaction mixtures (300 μL) were prepared by mixing CYP3A4 (580 pmol, 50 μL of a 3.5 μM enzyme solution) and testosterone (115 μM) in potassium phosphate buffer (100 mM, pH 7.4). The reference reaction contained the same components plus CPR (1.5 nmol). The molar ratio of CYP3A4:CPR of 1:4 was selected with regard to near optimal activity as shown by the study of Wang *et. Al.* After 5 min of preincubation at 37°C, the reaction was initiated using various concentrations of SPC, SPB, UHP, CuOOH, *t*BuOOH, or aqueous H_2O_2, and NADPH (1 mM) for the reference reaction. After addition of the solid SPC, SPB or UHP, the reaction mixture was not immediately shaken, to allow the slow release of the hydrogen peroxide as the dissolution occurred. After 15 min the mixture was homogenized by gentle shaking. The reaction was terminated after 1 h of incubation at 37°C by addition of methylene chloride (500 μL total) immediately followed by introduction of the internal standard cortexolone (15 μL of a 200 μL solution in methanol). The sample was vigorously vortexed, the layers separated by centrifugation (1500 × g for 2 min), and the organic layer was transferred to a dry vial. The extraction was repeated twice with CH_2Cl_2 (500 μL), and the pooled organic extracts were evaporated to dryness using a roto-evaporator. The residue was redissolved in HPLC-grade methanol (150 μL), vigorously vortexed, sonicated and filtered through a polypropylene syringe filters (0.2 μm pore size, National Scientific Company) before injection on the HPLC. The identity of the products was established by co-elution with authentic standards and by mass spectroscopy. The blank incubation assays were lacking either CYP and NADPH, or the peroxide surrogates, and did not show any detectable products. The extraction efficiency, or recovery, of this procedure was estimated by comparing the peak-area of extracted standards to those of non-extracted standards, and was superior to 96% for both 6β-hydroxytestosterone and testosterone at the concentrations used.

Effect of the buffer in reactions with sodium percarbonate: In order to evaluate the importance of the buffer composition for the reaction with SPC, the experiment was repeated using the conditions described above but in 12 different buffers: 3 potassium

phosphate concentrations (0.5 M, 0.75 M and 1 M), and 4 pHs for each (6.0, 6.5, 7.0 and 7.5).

Table 2A.1: Effect of the buffer in CYP3A4 reactions with sodium percarbonate.

Potassium phosphate concentration	pH	% of the activity with CPR/NADPH
500 mM	6.0	86 ± 2.2
	6.5	78 ± 4.3
	7.0	86 ± 2.8
	7.5	15 ± 1.5
750 mM	6.0	61 ± 6.6
	6.5	82 ± 8.1
	7.0	99 ± 4.7
	7.5	63 ± 3.7
1 M	6.0	54 ± 4.1
	6.5	81 ± 5.8
	7.0	**119** ± 3.4
	7.5	77 ± 2.4

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Chapter Three

In the last chapter, our results have demonstrated that the expensive and unmanageable natural coenzyme CPR and electron donor NADPH can be replaced successfully with inexpensive chemicals. The catalysis condition is, then, significantly simplified. As other enzymes, cytochrome P450s are being evolved to work in aqueous. This nature, however, restricts of use the enzymes in catalyzing water-insoluble substrates. In order to enlarge the scope of application, we have optimized a biphasic solvent system for CYP2D6. Many enzymes have been shown to be applicable in biphasic system [1-2]. The activity of P450 enzymes, however, had not been studied under such conditions. This chapter describes the effect of a series of emulsion on CYP2D6.

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Contributions of co-authors

This chapter is a copy of a published communication and is reproduced with permission from the journal Biotechnology & Bioengineering. It is cited as Jin Zhao, Elaine Tan, Julian Ferras and Karine Auclair, "Activity of human P450 2D6 in biphasic solvent systems" *Biotechnol.Bioeng.* **2007**, 98, 508-513. I studied the behavior of CYP2D6 in different organic-buffer biphasic systems. Elaine Tan carried out the experiments related to CD. Julian Ferras synthesized and characterized the substrate BDAC.

Activity of human P450 2D6 in biphasic solvent systems

3.1 Abstract

Several limitations have restricted the use of P450 enzymes in synthesis, including the narrow substrate specificity of some P450 isoforms, the need for a redox partner and an expensive cofactor, incompatibility with organic solvents, and poor stability. We previously demonstrated that the natural redox partner and cofactor of the promiscuous P450s 3A4 and 2D6 can be efficiently substituted with some cheap hydrogen peroxide donors or organic peroxides. We report here that P450 2D6 maintains as much as 76% of its activity when used in buffer/organic emulsions. Product formation in biphasic solvent systems is comparable whether the natural redox partner and cofactor are used, or a surrogate. As reported for other enzymes, a correlation is observed between the logP and the suitability of a solvent for enzymatic activity. Moreover, the utility of our system was established by demonstrating the transformation of a novel hydrophobic substrate, not modified by P450 2D6 in the absence of organic solvent.

3.2 Introduction

P450 enzymes (P450s, CYPs) have attracted the interest of chemists in part because of their impressive ability to catalyze the insertion of oxygen into inactivated C-H bonds [1]. Very few chemical catalysts are known that directly hydroxylate aliphatic or aromatic C-H bonds, and most are not selective or of limited scope [2-4]. Biocatalysts such as P450s represent a promising alternative; however, a number of limitations have restricted their use in synthesis. These include the narrow substrate specificity of some P450 isoforms, the need for one or more redox partners and NAD(P)H, incompatibility with organic solvents, low activity and poor stability. Unlike lipases and esterases for which significant progresses have been achieved to overcome the drawbacks associated with their handling [5, 6], little advancements have been made with oxidoreductases such as P450s [7-10]. Most of the work with purified P450s has focused on bacterial enzymes and was aimed at modulating substrate specificity and improving stability [11, 12]. A few investigations concerning the activity of P450s in organic solvents have been reported.

Carbon tetrachloride [13] and halothane [14] were shown to inactivate human liver microsomal P450s by irreversibly modifying the heme cofactor. A similar inactivation process was observed with *N,N*-dimethylformamide and *N,N*-dimethylacetamide [15]. Small percentages of commonly used water soluble organic solvents such as acetonitrile and methanol produce a significant inhibition of the activity of human P450s [16-18]. Directed evolution has been used to engineer bacterial P450-BM3 for improved tolerance to organic solvents [19]. The activity of the mutant enzyme increased 10-fold in the presence of THF (2% v/v) and 6-fold when DMSO (25% v/v) was added, compared to the wild type enzyme. Molecular dynamics simulations of P450 BM-3 in DMSO/water mixtures suggest a conformational shift blocking the substrate access channel [20]. Mutants of P450 102A1 were prepared that catalyze the hydroxylation of cyclohexane, octane and myristic acid in biphasic solvent systems with cofactor recycling [21]. Finally, P450 2B1 was the first mammalian P450 laboratory-evolved to generate a mutant with enhanced catalytic tolerance to DMSO [22]. Two mechanisms have been proposed to explain the deactivation of enzymes by organic solvents, protein denaturation and displacement of enzyme-bound water molecules [23-27].

Substrate promiscuity and the ability to show activity in the presence of organic solvents and in the absence of biological cofactors are great assets for biocatalysts. We have selected the very promiscuous human P450 2D6 to overcome the substrate specificity issue. We recently reported that the cheap reagents cumene hydroperoxide and *tert*-butylhydroperoxide can be used with P450 2D6 to efficiently replace its natural redox partner cytochrome P450 reductase (CPR), and cofactor nicotinamide adenine dinucleotide phosphate (NADPH) [28]. We report here that P450 2D6 shows very good activity with CPR/NADPH or cumene hydroperoxide in selected biphasic solvent systems.

3.3 Results and discussion

Choice of substrates

Most of the commercial substrates of mammalian P450 2D6 were discovered through drug metabolism studies and are therefore water soluble [29]. The majority of

synthetic projects however, involve molecules insoluble in water. It was envisaged to use both water soluble and insoluble substrates to investigate the behavior of P450 2D6 in biphasic solvent systems. Based on previous studies [30, 31], we designed the P450 2D6 substrate 7-benzyloxy-4-*N,N*-diethylaminomethyl-coumarin (BDAC). The P450 2D6 substrate BDAC was prepared using modified literature procedures [32], (see supporting information for more details). BDAC is not soluble in water but highly soluble in chloroform and acetonitrile, and slightly soluble in hexane and isooctane. This contrasts well with the known P450 2D6 substrate dextromethorphan which is soluble in water but poorly soluble in non-halogenated hydrophobic organic solvents.

Transformation of dextromethorphan and BDAC by P450 2D6 in buffer

HPLC analysis confirmed that the *O*-demethylation of dextromethorphan to dextrorphan by P450 2D6 proceeds well in buffer in the presence of either CPR/NADPH or cumene hydroperoxide. With BDAC however, no product was detected in the absence of organic solvent, likely due to a lack of solubility of this substrate in water. When acetonitrile (5%) was added to the buffer; however, BDAC was *N*-deethylated to 7-benzyloxy-4-*N*-ethylaminomethyl-coumarin (BEAC) by P450 2D6 (**Figure 3.1**). The yield was comparable whether the natural redox partner and cofactor, or the surrogate cumene hydroperoxide were used. The expected product of 7-*O*-debenzylation (4-*N,N*-diethylaminomethyl-coumarin, DAC) was not detected. Besides BEAC, the only other product was minor (<5%, retention time of 13.1 min, too small to be visible on Figure 1B). Although the reaction of P450 2D6 with these two substrates result in overall dealkylations, the major catalytic role of the enzyme in these transformations is to hydroxylate the carbon adjacent to the heteroatom and yield an unstable hemiacetal (dextromethorphan) or hemiaminal (BDAC). These functional groups are known to be unstable and spontaneously break down to the corresponding aldehyde, and alcohol or amine. Therefore, even though these assays do not allow the direct assessment of the enzyme stereoselectivity, they are a valid example of P450 hydroxylations and provide a rapid means for measuring activity.

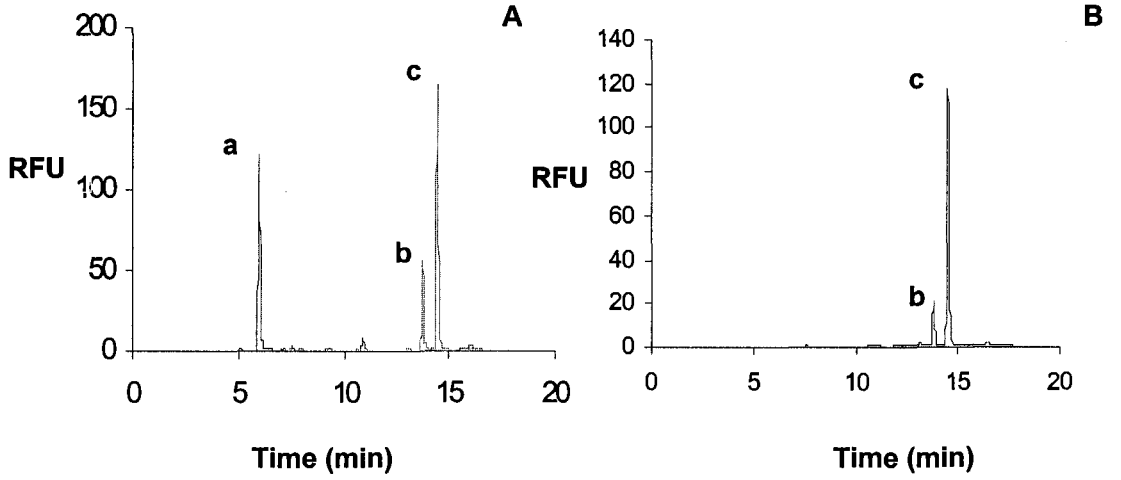

Figure 3.1 (A) HPLC trace of all standards: (a) possible product DAC at 6.1 min; (b) possible product BEAC at 13.7 min; and (c) substrate BDAC at 14.4 min; (B) Chromatogram recorded for the reaction mixture of P450 2D6 (0.2 μM) with BDAC (100 μM) and cumene hydroperoxide (0.1 mM) in buffer (0.1 M potassium phosphate, pH 7.4). Detection was achieved with λ_{ex} = 350 nm, λ_{em} = 420 nm. See experimental section for elution details.

Optimization of the amount of CHP used to support P450 2D6 activity in buffer/organic solvent emulsions

Preliminary investigations of the P450 2D6-catalyzed transformations of dextromethorphan or BDAC in buffer/hexane or buffer/isooctane emulsions revealed minimal losses in activity. Buffer/isooctane 2/1 emulsions were next used to optimize the concentration of cumene hydroperoxide. The cumene hydroperoxide-supported activity of P450 2D6 is susceptible to enzyme deactivation by the peroxide. In biphasic solvent systems, partitioning of the peroxide between the two phases and enzyme denaturation by the solvent are also key factors. For dextromethorphan, a substrate soluble in buffer, 0.1 mM cumene hydroperoxide was optimal in the presence of organic solvents, which is comparable to the optimal amount needed in buffer alone [28], (**Figure 3.2A**). For the hydrophobic substrate BDAC however, cumene hydroperoxide was optimized at 5 mM

(**Figure 3.2B**). We suspect that this difference may be the result of higher uncoupling rates with BDAC leading to increased consumption of CHP. This is expected based on the fact that BDAC is a poorer substrate compared to DXM and/or present near the enzyme at lower local concentration due to its lower solubility in buffer. Interestingly, the activity was less sensitive to the amount of cumene hydroperoxide in the presence of organic solvents than in its absence. The ability of excess cumene hydroperoxide to partition into the isooctane phase contributes to increased stability of the enzyme in the presence of the peroxide. This is consistent with our previous observation that a slow release of hydrogen peroxide from sodium percarbonate favors P450 catalysis over inactivation by the peroxide [28].

Comparison of the P450 2D6 activity at different isooctane/buffer volume ratios, with the natural cofactor and redox partner, or with a surrogate

As mentioned above, we recently reported that the cheap reagents cumene hydroperoxide, *tert*-butyl hydroperoxide and sodium percarbonate can be used to replace the redox partner and the expensive cofactor of human P450s 3A4 and 2D6. Moreover the P450 2D6 activity observed in the presence of small amounts of cumene hydroperoxide is two fold superior to that in the presence of CPR and NADPH [28]. To verify that this also applies in biphasic solvent systems, we compared the P450 2D6 activity in buffer/isooctane with cumene hydroperoxide or CPR/NADPH. As shown on **Figure 3.2C**, both the natural and the chemical cofactors supported P450 2D6-catalyzed dextromethorphan *O*-demethylation. This suggests that both P450 2D6 and CPR tolerate isooctane. Interestingly, reactions with cumene hydroperoxide were slightly more sensitive to the amount of organic solvent added than those with CPR/NADPH (higher slope). This may be explained by the partitioning of cumene hydroperoxide in isooctane (away from the enzyme). A steady yet slow access to cumene hydroperoxide is important to maximize enzymatic activity and minimize degradation.

When the isooctane/buffer volume ratio was varied, the amount of product formed decreased as the percentage of isooctane was increased with the water soluble substrate

dextromethorphan (**Figure 3.2C**). This is likely the effect of enzyme denaturation by the solvent. In contrast, with the hydrophobic solvent BDAC the addition of isooctane was beneficial until it reached 50% (**Figure 3.2D**). The different behavior observed with these two substrates can be explained by the difference in solubility.

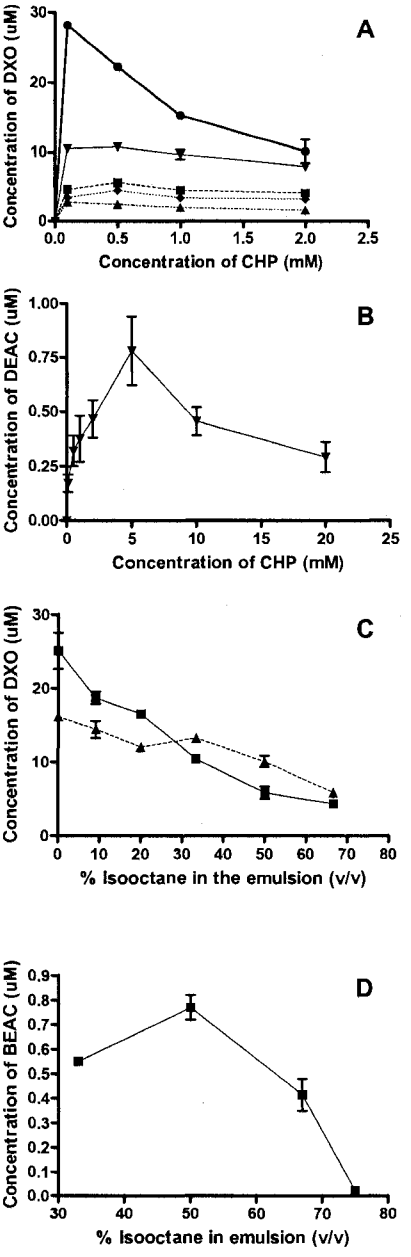

Figure 3.2 (A) Effect of the concentration of cumene hydroperoxide (CHP) on the production of dextrorphan (DXO) from dextromethorphan (DXM, 100 μM) by P450 2D6

(0.2 μM) in different biphasic solvent systems (1/2 v/v ratios) after 1 h at 37°C and 200 rpm. Key: (●) buffer alone; (▼) isooctane/buffer; (■) hexane/buffer; (◆) pentane/buffer; and (▲) cyclohexane/buffer. (B) Effect of the concentration of CHP on the production of 7-benzyloxy-4-*N*-ethylaminomethyl-coumarin (BEAC) from 7-benzyloxy-4-*N,N*-diethylaminomethyl-coumarin (BDAC) by P450 2D6 (0.2 μM) in isooctane/buffer (1/2 v/v ratio) after 2 h at 37°C and 200 rpm. (C) Effect of different isooctane/buffer volume ratios on the activity of P450 2D6 (0.2 μM) with DXM (100 μM) supported by (▲) CPR/NADPH (0.6 μM/1.6 mM) or (■) CHP (0.1 mM), after 1 h at 37°C and 200 rpm. (D) Effect of different isooctane/buffer volume ratios on the transformation of BDAC to BEAC by P450 2D6 (0.2 μM) supported by CHP (2.0 mM), and incubated for 2 h 37°C and 200 rpm. The buffer used in all cases was potassium phosphate (200 μL, 0.1 M, pH 7.4). The experiments were carried out in duplicate.

Effect of the choice of organic solvent for P450 2D6 activity in biphasic reaction systems

The activity of P450 2D6 in different biphasic solvent systems was evaluated after one hour of reaction and compared to the activity in buffer alone. The results with the substrate dextromethorphan (**Figure 3.3A**) suggest rapid inactivation of P450 2D6 in the presence of aromatic or halogenated solvents. The enzyme precipitated within minutes of exposure and negligible amounts of product were detected. Halogenated solvents have been reported to form reactive radical intermediates in the presence of P450s, and deactivate the enzyme by transforming the heme moiety [13]. Alkanes and ethers on the other hand did not eliminate enzymatic activity. After one hour of reaction in isooctane/buffer emulsions, the amount of product (6.6 μM) was a little over half that of the reaction in buffer alone (13.0 μM). The regioselectivity of the reaction was not affected noticeably.

In the absence of organic solvent, debenzylation of the synthetic substrate BDAC by P450 2D6 was below the detection limit. The solvent leading to the best product yield was isooctane with 0.13 μM of BEAC. Here again, isooctane, hexane and cyclohexane

were the most promising solvents, whereas dichloromethane inhibited enzymatic activity (**Figure 3.3B**).

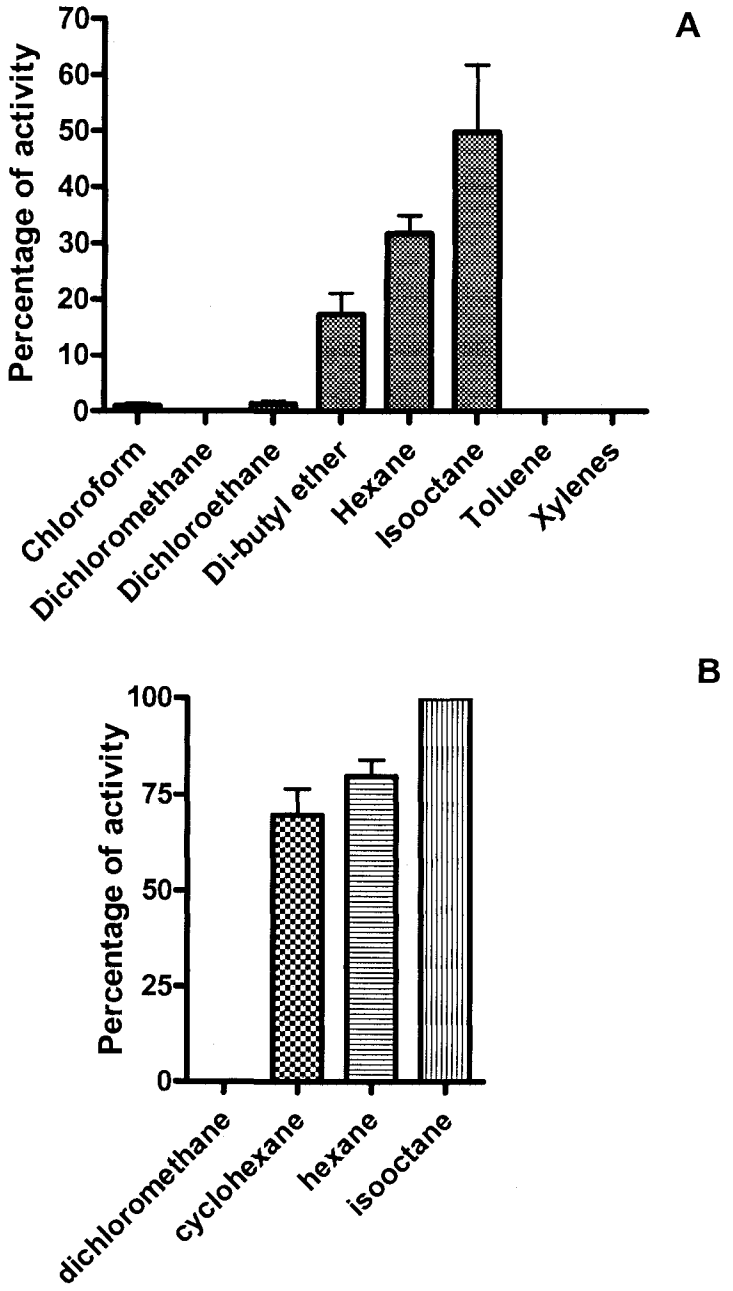

Figure 3.3 Concentration of product formed by P450 2D6 in the presence of different biphasic solvent systems. (A) The formation of the product dextrorphan (DXO) is

monitored from a reaction consisting of P450 2D6 (0.2 μM), dextromethorphan (100 μM) and cumene hydroperoxide (0.1 mM) in potassium phosphate buffer (200 μL, 0.1 M, pH 7.4) and organic solvent (100 μL). The emulsions were incubated at 37°C and 200 rpm for 1 hr. (B) The formation of the product 7-benzyloxy-4-*N*-ethylaminomethyl-coumarin (BEAC) is monitored from a reaction consisting of P450 2D6 (0.2 μM) and cumene hydroperoxide (0.1 mM) in potassium phosphate buffer (200 μL, 0.1 M, pH 7.4) to which BDAC (100 μM) was added as a solution in the organic solvent (100 μL). The emulsion was incubated at 37°C and 200 rpm for 2 h. The results are averages from triplicates.

A number of groups have reported a correlation between solvent hydrophobicity and enzyme activity [33-35] or cell viability [36] in biphasic solvent systems. The enzymatic activities reported here were plotted as a function of different physical properties of the organic solvents (**Figure 3.4** shows a few selected properties). For both substrates tested, our results reveal a correlation between the enzyme activity and the logP of the solvent used in the biphasic reaction systems.

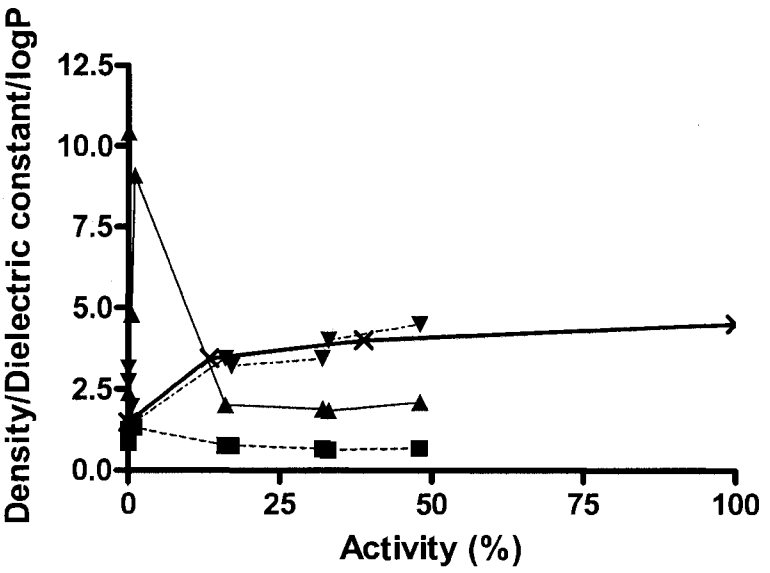

Figure 3.4 Activity of P450 2D6 (0.2 μM) in buffer/solvent emulsions (2/1, total of 300 μL), correlated to different properties of the organic solvents: (■) density in g/mL, (▲)

dielectric constant, and (▼) logP with the substrate dextromethorphan (100 μM), and (✕) logP with the substrate BDAC (100 μM). The reaction conditions were the same as for Figure 3.3.

Initial rates and maximum turnover numbers

The kinetics of P450 2D6-catalyzed dextromethorphan dealkylation in 2/1 buffer/isooctane emulsions were investigated in the presence of cumene hydroperoxide (**Table 3.1**). As expected, the total turnover number (TTN, defined here as the number of mole of substrate that a mole of catalyst can convert before becoming inactivated, with units of μmol of product per μmol of enzyme) for the reaction in the biphasic system (70 ± 5 μmol μmol^{-1}) was slightly lower than that in buffer (92 ± 2 μmol μmol^{-1}), likely due to enzyme denaturation by the organic solvent. This represents 76% remaining activity. In terms of enzyme stability, the cumene hydroperoxide-supported activity of P450 2D6 decreased below 20% after ~90 min in pure buffer, but after only 60 min in a buffer/isooctane emulsion. In other words, the addition of isooctane results in a reduction of the enzyme stability by ~30 min. On the other hand, the initial rate measured with cumene hydroperoxide was higher in the buffer/isooctane biphasic system (6.4 ± 0.2 $\mu mol\ \mu mol^{-1}\ min^{-1}$) than in pure buffer (4.7 ± 0.3 $\mu mol\ \mu mol^{-1}\ min^{-1}$), suggesting that partitioning of cumene hydroperoxide between the two layers may ensure a steady source of oxidant to the enzyme, yet slow enough to minimize reaction with the heme group. As proposed above, the organic solvent may moderate the effects of cumene hydroperoxide on the enzyme.

Table 3.1 Initial rates and total turnover numbers (TTNs) for the formation of dextromethorphan catalyzed by P450 2D6 with the surrogate cumene hydroperoxide in either buffer alone or isooctane/buffer biphasic system.

	In buffer[c]	In isooctane/buffer[d]
Initial rate[a] (min^{-1})	4.7 ± 0.3	6.4 ± 0.2
TTN[b] ($\mu mol\ \mu mol^{-1}$)	92 ± 2	70 ± 5

[a]The initial rate is the number of μmoles of product formed per μmol of enzyme per minute. [b]The TTN is defined here as the number of mole of substrate that a mole of catalyst can convert before becoming inactivated, with units of μmol of product per μmol of enzyme. [c]These results were published earlier by us (Chefson et al., 2006b). [d]P450 2D6 (0.2 μM), the substrate dextromethorphan (100 μM), and cumene hydroperoxide (0.1 mM) in potassium phosphate buffer (10 mM, pH 7.4, 200 μL) and isooctane (100 μL) were incubated at 37°C and 200 rpm for up to 2 hours.

CD results

We hypothesized that the decreased activity observed could correlate with changes in the protein folding and be reflected in the secondary structure elements. Circular dichroism was used to monitor the overall percentages of helices, beta-sheets and turns, and random coils. The effect of shaking P450 2D6 in biphasic solvent systems containing either isooctane or dichloromethane was investigated over time. For the control reaction in buffer the percentage of each of the secondary structures remained constant over 2 hours and enzyme precipitation was not observed during that time. Dichloromethane (33%), a solvent that inhibits P450 2D6 activity, precipitated the enzyme within minutes, which prohibited CD analysis. In the presence of 33% isooctane, no effect could be

detected on the secondary structure; however, the enzyme started to precipitate after 60 minutes. The inability to detect changes in the secondary structure of P450 2D6 in the presence of solvents may be explained by a rapid aggregation and precipitation following enzyme unfolding.

P450 enzymes have an enormous potential as biocatalysts; however, their use has been limited because of the narrow substrate specificity of some isoforms, need for a redox partner and a cofactor, incompatibility with organic solvents and low stability. The results presented here combined with our earlier studies [28] overcome three of these drawbacks. By selecting the promiscuous P450 2D6 we expect that numerous organic molecules will be substrates of this enzyme. We previously reported that cumene hydroperoxide is a highly efficient replacement for the natural redox partner and the cofactor of P450 2D6 in buffer. Here we demonstrate that this applies in the presence of organic solvents as well. We also report that in 2/1 buffer/isooctane emulsions, this enzyme catalyzes the transformation of the water soluble substrate dextromethorphan or the hydrophobic substrate BDAC, with comparable activity whether the natural redox partner and cofactor, or the surrogate cumene hydroperoxide are used. This establishes the utility of our system since BDAC is not transformed significantly in the absence of organic solvent. Under optimized biphasic conditions, the TTN was >75% of that of the natural system (with CPR/NADPH and in buffer). Although the use of purified P450 2D6 enzymes in industry remains impractical, our results represent one important step towards this goal.

Acknowledgements

The authors wish to acknowledge Prof. F. Peter Guengerich from Vanderbilt University and Prof. Charles B. Kasper from the University of Wisconsin-Madison for their kind gift of the P450 2D6 and CPR expression plasmids, respectively. We also thank Dr. Nadim Saade for his assistance with LC-MS. Finally, this work was supported by the

Natural Science and Engineering Council of Canada (NSERC), Astra Zeneca Canada Inc., Boehringer Ingelheim Canada Ltd., and Merck Frosst Canada & Co.

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Chapter Three – Appendix

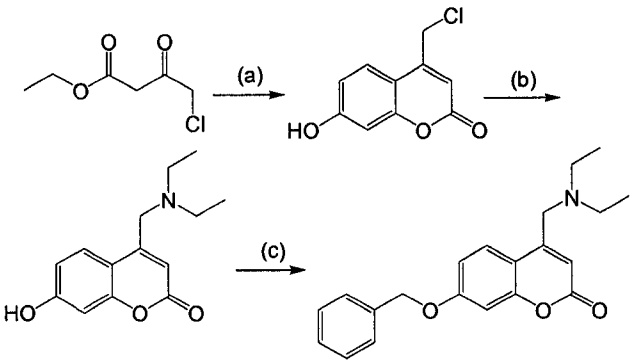
Experimental section (published as supporting information)

3A.1 Chemicals and instruments

Dextromethorphan, dextrorphan, 3-methoxymorphinan, NADPH, trifluoroacetic acid (TFA), cumene hydroperoxide (CHP), L-α-phosphatidylcholine, 1,2-di[cis-9-octadecenoyl]-sn-glycero-3-phosphocholine, and 2,2,4-trimethylpentane (isooctane) were purchased from Sigma-Aldrich (Montreal, QC, Canada). Chloroform, 1,2-dichloroethane, xylenes, toluene, hexane, pentane and HPLC grade acetonitrile were from Fisher (Nepean, ON, Canada). Dichloromethane was purchased from EMD Chemicals Inc. (Cincinnati, OH). Water was obtained from a Milli-Q Synthesis filtration system (Millipore, San Jose, CA). All chemicals were used without further purification.

An Agilent 1100 modular system equipped with an auto-sampler, a quaternary pump system, a photodiode-array detector, a fluorescence detector, and a thermostated column compartment was operated for HPLC analyses. UV absorption spectra were recorded using a Cary 5000 UV spectrophotometer (Varian, Mississauga, ON, Canada). LC-MS analyses were performed on a Finnigan LCQDUO mass spectrometer from Thermo Separation Products including LC pump P4000 and UV spectrophotometer UV2000. Circular Dichroism spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco Inc., Easton, MD) in rectangular 1 mm quartz cuvettes (Starna Cell, Inc., Atascadero, CA). ^{1}H and ^{13}C NMR spectra were recorded on Varian Mercury 200 or 300 spectrometers. The peak patterns are included as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublet; m, multiplet, etc.

3A.2 Synthesis of the substrate 7-benzyloxy-4-*N*,*N*-diethylaminomethyl-coumarin (BDAC).



Scheme 3A.1 (a) resorcinol, $ZrCl_4$ (8%, in mol), 70°C, 3 hrs, 21%. (b) diethylamine, 60°C, 48 hrs, 70%. (c) THF, NaH, 0°C, 1 hr; benzyl bromide and tetrabutylammonium iodide, 70°C, 3 hrs, 45%. Details for specific steps are given below.

3A.3 7-Hydroxy-4-chloromethyl-coumarin

A mixture of ethyl 4-chloro-3-oxobutanoate (10 mmol) and resorcinol (10 mmol) was heated at 70°C in the presence of zirconium (IV) chloride (184 mg, 8% in mol), for 3 hrs. The mixture was then cooled to room temperature and ice cold water was added to afford a yellowish precipitate. The solid was collected by filtration, washed with ice cold water, dried and purified by flash chromatography (silica gel, 2:3 ethyl acetate:hexane) to obtain white crystals of the known 7-hydroxy-4-chloromethyl-coumarin [2] in 21% yield (442 mg). ^{1}H NMR (d_6-DMSO) δ 10.65 (1H, s), 7.65 (1H, d, J = 9.0 Hz), 6.81 (1H, dd, J = 9.0, 2.2 Hz), 6.73 (1H, d, J = 2.2 Hz), 6.4 (1H, s), 4.93 (2H, s); ^{13}C NMR (d_6-DMSO) δ 162.20, 160.83, 155.98, 151.64, 127.16, 113.78, 111.71, 110.02, 103.22, 42.05; ESMS Calc ($C_{10}H_7ClO_3$, M^+), 211.2, Obs 210.0.

3A.4 7-Hydroxy-4-*N*,*N*-diethylaminomethyl-coumarin

7-Hydroxy-4-chloromethyl-coumarin (10 mmol) was added to diethylamine (5 mL). The resulting yellow solution was stirred under nitrogen for 48 hrs at 60°C. The mixture was evaporated in vacuo to afford a brown oil which was purified by flash chromatography (silica gel, chloroform followed by 1:1 ethyl acetate:hexane) to obtain the desired product as yellowish crystals. Yield 1.7 g, 70%. ^{1}H NMR ($CDCl_3$) δ 7.79 (1H, d, J = 8.8 Hz), 6.79 (1H, dd, J = 8.8, 2.2 Hz), 6.71 (1H, d, J = 2.2 Hz), 6.39 (1H, s), 3,74 (1H, s), 2.62 (4H, q, J = 7.2 Hz), 1,08 (6H, t, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 163.26, 160.51, 155.71, 155.47,

126.07, 113.60, 112.40, 110.81, 103.57, 54.80, 47.86, 12.04; HRMS Calc ($C_{14}H_{17}NO_3$, M^+), 247.12084, Obs 247.12049.

3A.5 7-Benzyloxy-4-*N,N*-diethylaminomethyl-coumarin (BDAC) To a solution of 7-hydroxy-4-*N,N*-diethylaminomethyl-coumarin (10 mmol) in anhydrous THF at 0°C, sodium hydride (10 mmol) was slowly added. The mixture was stirred for 1 hr at room temperature. A homogeneous greenish-yellow solution was obtained. Benzyl bromide (10 mmol) and tetrabutylammonium iodide (1 mmol) were added and the resulting mixture was stirred for 3 hrs at 70°C. THF was evaporated in vacuo and the residue was redissolved in a minimum of ethyl acetate. The organic phase was washed with saturated aqueous $NaHCO_3$, and with saturated aqueous NH_4Cl. It was dried over magnesium sulfate and concentred in vacuo. Flash chromatography (silica gel, 1:8 ethyl acetate:hexane followed by 1:2) afforded the desired compound in 45% yield (1.5 g, purity >99%.). ^1H NMR ($CDCl_3$) δ 7.76 (1H, d, J = 9.0 Hz,), 7.39 (5H, m), 6.91 (2H, m), 6.44 (1H, s), 5.11 (2H, s), 3.63 (2H, s), 2.57 (4H, q, J = 6.6 Hz), 1.04 (6H, t, J = 6.6 Hz). ^{13}C NMR ($CDCl_3$) δ 161.93, 161.69, 155.7, 154.41, 136.13, 128.96, 128.55, 127.72, 125.93, 113.02, 112.95, 111.90, 101.11, 70.64, 54.89, 47.76, 12.08; MS Calc ($C_{21}H_{23}NO_3$, M^+), 337.2, Obs 338.2.

3A.6 Enzyme expression and purification

P450 2D6 and cytochrome P450 reductase (CPR) were expressed and purified as previously described [1]. The use of lipids such as L-α-phosphatidylcholine and/or 1,2-di[cis-9-octadecenoyl]-sn-glycero-3-phosphocholine to reconstitute P450 2D6 was not necessary. The enzymatic activity was unchanged by the addition of lipids for reactions in pure buffer or in buffer/organic emulsions.

3A.7 Enzymatic assays

The dextromethorphan stock solution (10 mM) was prepared in potassium phosphate buffer (0.1 M, pH 7.4). The BDAC stock solution was prepared in the same organic solvent as used for the enzymatic reactions in biphasic solvent systems. P450 2D6 (0.2 μM) and the desired substrate (dextromethorphan or BDAC, 100 μM) were mixed in

potassium phosphate buffer (0.1 M, pH 7.4). Different volumes of organic solvents (20-400 µL) were added before initiating the reaction with cofactors (0.6 µM CPR and 1.67 mM NADPH, or 1 mM CHP). The biphasic reaction mixture (aqueous layer of 200 µL) was incubated for 60 min with dextromethorphan or 120 min with BDAC, at 37°C and 200 rpm. The reaction vessels were positioned horizontally to maximize the turbulence. For total reaction volumes of 200-300 µL, vials of 650 µL were used, whereas vials of 1.5 mL were more appropriate for mixtures of 400-600 µL. Perchloric acid (23% v/v, 10 µL) was added to precipitate the enzyme after reactions with dextromethorphan. The mixture was vortexed and left on ice for 5 min. A gentle stream of air was applied to remove the organic solvent. For assays with BDAC, the organic solvent was evaporated under a gentle stream of air, followed by addition of acetonitrile (200 µL) and vortexing to precipitate the enzyme. The samples were centrifuged at 16,000 × g for 5 min before injection (5 µL) in the HPLC system.

3A.8 HPLC analysis to monitor the *O*-dealkylation of dextromethorphan by P450 2D6

DXM DXO

Separation was achieved using a 250 × 4.60 mm Synergi 4 µ Hydro-RP 80 Å column with mobile phase A (0.1% TFA in water) and B (100% acetonitrile) at a flow rate of 0.2 mL/min, kept at 30°C. The initial mobile phase consisted of 50:50 A:B (v:v) and was linearly changed to 80:20 A:B over 20 min. The excitation and emission wavelengths of the fluorescence detector were set at 280 and 310 nm respectively. The retention times of dextrorphan, 3-methoxymorphinan and dextromethorphan were 13.4, 17.2, and 18.0 min, respectively. For product quantification, a calibration curve was prepared using dextrorphan samples that underwent the same treatment as the assay samples, and of concentrations ranging from 2.5 µM to 50 µM.

3A.9 HPLC analysis to monitor the *N*-deethylation of DBAC by P450 2D6

BDAC BEAC

An Agilent 300 Extend-C_{18}, 4.6 × 250 mm, 5μ column was used with mobile phase B (see above) and mobile phase C (0.05% TFA in water). Elution was performed with a linear gradient from 10:90 B:C to 40:60 over 10 min, kept at 40:60 for 2 min, and brought linearly to 10:90 over 3 min. The flow rate was 0.8 mL/min and the column was kept at 22°C. Elution was monitored either by UV at 325 nm or by fluorescence with excitation at 350 nm and emission at 420 nm. The major product BEAC and the substrate BDAC eluted at 13.7 and 14.4 min, respectively. For product quantification, a calibration curve was prepared using dextrorphan samples that underwent the same treatment as the assay samples, and of concentrations ranging from 9 μM to 225 μM.

3A.10 Measurement of initial rates and TTNs

A set of mixtures of P450 2D6 (0.2 μM) and dextromethorphan (100 μM) in potassium phosphate buffer (10 mM, pH 7.4, 200 μL) and isooctane (100 μL) were pre-warmed at 37°C for 3 min before initiation of the reaction with cumene hydroperoxide (0.1 mM). The mixtures were allowed to react at 37°C and 200 rpm for up to 2 hrs. Reactions were terminated with the addition of perchloric acid (23% v/v, 10 μL). The same workup was performed as with other enzymatic assays (see above). Product formation was calculated after 25 sec, 40 sec, 1 min, 2 min, 5 min, 10 min, 30 min and 1 hr. (each in duplicate). A kinetic plot was drawn from the concentration of product formed as a function of time. The initial rate was determined from the slope of the first 5 points of this plot. The total turnover number (TTN) is defined here as "the number of mole of substrate that a mole of catalyst can convert before becoming inactivated", with units of μmol of product per μmol of enzyme. It is calculated when the kinetic plot reaches a plateau.

3A.11 CD analyses

The samples (300 μL) were prepared by diluting P450 2D6 (0.275 μM) in potassium phosphate buffer (10 mM, pH 7.4), with or without the substrate dextromethorphan (100 μM). The samples to be shaken for 5 min or less were first brought to 37°C before addition of isooctane (100 μL). Isooctane (100 μL) was directly added to the other samples. After addition of the organic solvent, the biphasic mixtures were left at 37°C and 200 rpm. The control samples did not contain isooctane. Each sample and control was prepared and analyzed in triplicate. After incubation, isooctane was evaporated under a gentle stream of air. The aqueous mixture was transferred to a 0.1cm pathlength CD cuvette and incubated for 5 min at 37°C before CD analysis. Spectra were obtained by averaging 3 scans measured at a scan rate of 20 nm min^{-1}, a time constant of 1 sec, and a bandwidth of 1 nm for both far and near UV, at 37°C. Blank spectra of the buffer (10 mM potassium phosphate at pH 7.4) were used to correct the experimental spectra. The spectra were converted into molar ellipticity units. Data points ranging from 190 to 240 nm at 0.5 nm intervals were analyzed using the software CDPro (available at http://lamar.colostate.edu/~sreeram/CDPro/main.html) to obtain the percentage of each secondary structure. For simplicity, fractions of regular and distorted α-helices were combined together. The same applies to regular and distorted β-strands, and turns.

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Chapter Four

In last two chapters, we demonstrated that the catalytic conditions of CYP2D6 can be simplified by replacing natural coenzyme CPR and electron donor NADPH to peroxides and extended in organic solvent / buffer biphasic systems. The utilization of the enzyme in such systems is restricted due to the short lifetime in two-phase emulsion condition. The stabilization of enzyme may be increased in nearly hydrophobic organic solvents due to rigidifying enzyme conformation [1, 2]. Therefore, the study of CYP2D6 in anhydrous conditions is needed. Lyophilization is the most common pretreatment to remove water from enzyme. This physical process, however, is easy to deactivate enzymes. Lyoprotection with neutral chemicals is an effective manner to reserve enzymes activity. In this chapter, the use of lyoprotectants on CYP2D6 is discussed.

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Contributions of co-authors

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Sugar-mediated lyoprotection of purified human CYP3A4 and CYP2D6

4.1 Abstract

P450 enzymes are of great interest for drug metabolism and as potential biocatalysts. Like most P450s, purified CYP3A4 is normally handled and stored in solution because lyophilization greatly reduces its activity. We show here that co-lyophilization of this enzyme with sucrose or trehalose, but not mannitol, crown ethers or cyclodextrins, allow recovery of full enzymatic activity after rehydration. Sorbitol was almost as efficient, with 85% retention of the original activity. We also show that similar protection is observed through co-lyophilization of CYP2D6 with trehalose. This procedure should greatly facilitate handling, storage, or use of these enzymes in anhydrous media.

4.2 Introduction

P450 enzymes (P450s, CYPs) have gained considerable attention amongst chemists for their unequalled catalytic properties. Indeed, P450s have the impressive ability to catalyze the regio- and stereo-selective hydroxylation of inactivated C-H bonds. P450s are not only studied as potential novel biocatalyst but also because of their importance in drug metabolism and interactions. The pharmaceutical industry uses them in the prediction and production of drug metabolites. For practical purposes, these studies are most often carried out using hepatocytes, recombinant *E. coli* whole cells, or microsomes, instead of pure enzymes. A few companies, like BioCatalytics Inc. or BD Gentest,^{TM} commercialize kits for metabolites production. These kits contain one or more P450s plus the necessary components (reductase, cytochrome b5, etc.) as a lyophilized formulation, or as supersome or microsome solutions. Purified enzymes are often desirable for biochemical, structural, mechanistic or synthetic studies. Invitrogen has recently added a few P450 enzymes to their product list.

The past decades have seen great progress towards the easier use of purified P450 enzymes [1-3] yet poor storage and operational stability is one of the drawbacks limiting applications with these enzymes. Lyophilization of enzymes is useful not only for handling and storage but also before use in non-aqueous environments. Over the last few years it has been realized that the low activity of lyophilized enzymes in organic solvents is largely due to protein inactivation during freeze-drying [4]. Dehydration is believed to induce some conformational changes not recovered upon rehydration and potentially detrimental to biological activity [5, 6]. The use of additives during lyophilization has sometimes been found to protect enzymes from damaging changes [7-9]. Sugars are the most widely used lyo- and cryo-protectants for enzymes [10-14]. Carbohydrates and cyclodextrins are believed to compensate for the losses in the hydration shell of the enzyme, allowing preservation of the native structure in the dehydrated state [5, 6, 8, 15-23]. Interestingly, it has been demonstrated that the weight ratio sugar/protein directly correlates with the protection of catalase during freeze-drying, but the bulk concentration of lyoprotectant does not [24]. Crown-ethers have also had some success as lyoprotectants [25-28], and are believed to protect the enzymes by forming non-covalent macrocyclic complexes with lysine ammonium groups [29, 30].

We selected to study the human P450 isoform CYP3A4 for its wide substrate promiscuity, a significant advantage for possible applications in synthesis. This isoform is also responsible for the metabolism of more than half of the known drugs and is highly studied. We evaluated the consequences of freeze-drying on its activity, and optimized the conditions to minimize the harmful effects of the process.

4.3 Results and Discussion

We have previously demonstrated that the P450 enzyme CYP3A4 can efficiently use cumene hydroperoxide (CHP) as a substitute for the natural cofactors NADPH and cytochrome P450 reductase [31]. In this study, the CHP-supported

6β-hydroxylation of testosterone by CYP3A4 was used to monitor enzymatic activity, yet applicability was also demonstrated with CPR and NADPH. Product quantification was achieved by HPLC, with cortexolone as the internal standard (see supporting information for more details). Following purification, CYP3A4 is usually kept in buffer containing 10% glycerol as a cryoprotectant for subsequent storage at -80°C. The presence of glycerol during lyophilization tends to produce a sticky solid or a viscous oil because glycerol does not evaporate. The importance of glycerol for long term storage at -80°C was therefore evaluated. After two weeks, the activity decreased by only 3% and 5% with or without glycerol respectively, and after 4 months, the activity decreased by 7% and 13% respectively. This suggests that cryoprotection is not necessary for the storage of CYP3A4. All subsequent enzyme batches were stored without addition of glycerol in the dialysis buffer.

Our preliminary experiments revealed that freeze-dried CYP3A4 resolubilized in buffer had lost ~50% of its activity towards testosterone.While the total turnover number (TTN, defined here as the number of mole of substrate that a mole of catalyst can convert before becoming inactivated, in μmol μmol^{-1}) reached 40 ± 8 for non-lyophilized enzyme, it dropped to 23 ± 7 upon lyophilization. On the other hand, no significant loss in activity was observed after freezing and thawing the enzyme solution once or twice (38 ± 9 and 39 ± 11 respectively). This indicates that among the two stress factors involved in the freeze-drying process, freezing and dehydration [32, 33], dehydration is the most harmful to the CYP3A4 activity. A variety of compounds have been used as *cryoprotectants* with other enzymes, such as glycerol, polyethyleneglycol, sugars, glycine, proline, but only sugars were effective as *lyoprotectants* [5, 16, 18, 34-36]. This led us to evaluate the effect of various additives during lyophilization of CYP3A4, including the disaccharides trehalose and sucrose, the polyols sorbitol and mannitol, as well as two cyclodextrins, methyl-β-cyclodextrin (MβCD) and hydroxypropyl-β-cyclodextrin (HPβCD), and two crown ethers, 18-crown-6 (18C6) and 15-crown-5 (15C5).

As shown on **Figures 4.1** and **4.2**, the co-lyophilization of CYP3A4 with

cyclodextrins or crown ethers did not significantly help protecting the enzyme. Only low concentrations of 18C6 and 15C5 (molar ratio to enzyme of ~50) increased the activity and the effect was very modest (from 52% without to 60% with the additive). High concentrations of crown ethers (> 100 molar ratio to CYP) were even detrimental to the P450 activity.

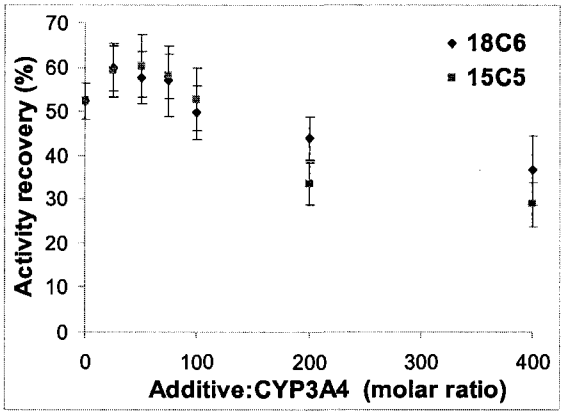

Figure 4.1: Effect of co-lyophilization (24 h) with different amounts of 18C6 and 15C5 on the 6β-hydroxylation of testosterone (115 μM) catalyzed by CYP3A4 (0.4 μM, 14.5 μg) upon rehydration in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 700 μl). The reaction was initiated with cumene hydroperoxide (1 mM) and incubated for 1 h at 37°C.

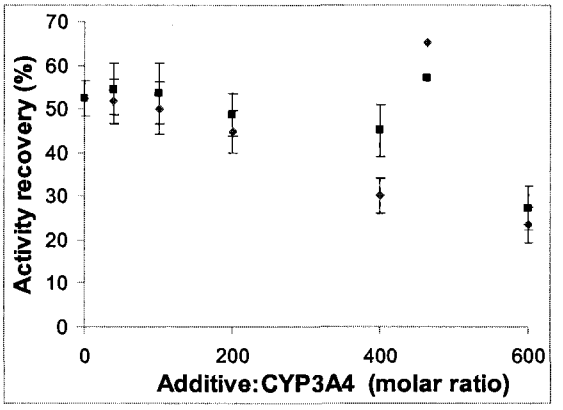

Figure 4.2: Effect of co-lyophilization (24 h) with different amounts of methyl-β-cyclodextrin (MβCD) and hydropropyl-β-cyclodextrin (HPβCD) on the 6β-hydroxylation of testosterone (115 μM) catalyzed by CYP3A4 (0.4 μM, 14.5 μg) upon rehydration in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 700 μl).

The reaction was initiated with cumene hydroperoxide (1 mM) and incubated for 1 h at 37°C.

The addition of sugars, reduced or not, before lyophilization was much more effective at maintaining the CYP3A4 enzymatic activity (**Figure 4.3**). Both sucrose and trehalose confered a similar level of lyoprotection. The recovered activity, measured after initiation with cumene hydroperoxide, was dependent on the amount used. Very high ratios sugar/enzyme w/w (5000-10000) allowed the retention of > 90% of the activity, compared to 52% for the enzyme freeze-dried alone. Sorbitol was almost as effective at lower concentrations (1000-2000 w/w), helping retain about 85% of the activity. Its beneficial effect quickly dropped when the amount of sorbitol increased further, and no protection remained at ratios of 10000 and higher. At proportions ≤1000 w/w, sorbitol, sucrose and trehalose had similar effects. Mannitol was the least effective, barely increasing the residual activity to 60% at weight ratios of 2000-5000. In all cases, no improvement of the activity was observed in the controls containing the additives but not lyophilized, suggesting that the sugars protect against the harmful effects of dehydration, as opposed to directly activating the enzyme.

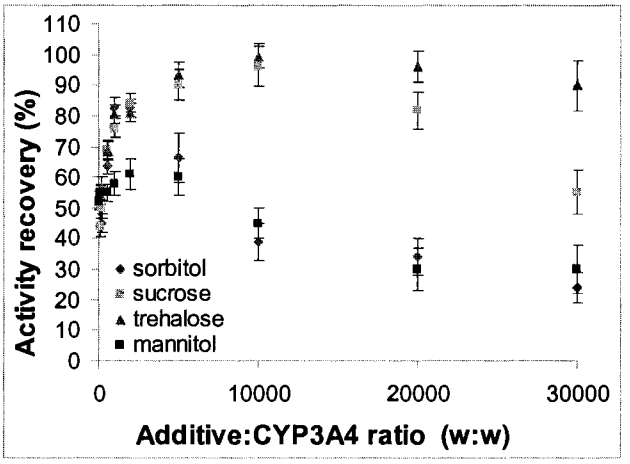

Figure 4.3: Effect of co-lyophilization (24 h) with different amounts of sorbitol, sucrose, trehalose and mannitol on the 6β-hydroxylation of testosterone (115 μM) catalyzed by CYP3A4 (0.4 μM, 14.5 μg) upon rehydration in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 700 μl). The reaction was initiated with cumene hydroperoxide (1 mM) and incubated for 1 h at 37°C.

After lyophilization in the presence of the optimized amount of sucrose or trehalose, the activity of CYP3A4 was also tested with the natural cofactors (CPR and NADPH). The lyoprotection effect observed was very similar (data not shown) to that described above for the CHP-supported reactions. Without lyophilization, the TTN was 40 with CHP and 44 with CPR/NADPH. After lyophilization *without* additive, the TTN dropped to 19 with CHP (47% remaining activity), and to 23 with CPR/NADPH (52% remaining activity). With the addition of sucrose (10000 w/w) before lyophilization, the TTN reached 36 with CHP and 37 with CPR/NADPH, corresponding to 90 and 84% remaining activity. With trehalose (10000 w/w), the remaining activity was 87.5% with CHP and 88.5% with CPR/NADPH (TTNs of 35 and 39 respectively).

To investigate the scope of this method, the activity of human CYP2D6 was investigated after lyophilization in the absence or in the presence of sorbitol, sucrose, mannitol or trehalose. Lyophilization of CYP2D6 in the absence of lyoprotectant led to the loss of approximately 60% of the enzymatic activity, and was greatly affected by the duration and conditions of the process. Co-lyophilization of CYP2D6 with sorbitol yielded inconsistent results (data not shown). The effect of mannitol on the activity was minimal and dropped rapidly as the mannitol/enzyme ratio increased (**Figure 4.4**). On the other hand, the addition of either sucrose (at a sucrose:CYP2D6 ratio of 20,000) or trehalose (at a trehalose:CYP2D6 ratio of 5,000) allowed recovery of about 80% of the activity. These results are very similar to those obtained with CYP3A4, therefore suggesting applicability to other P450 isoforms.

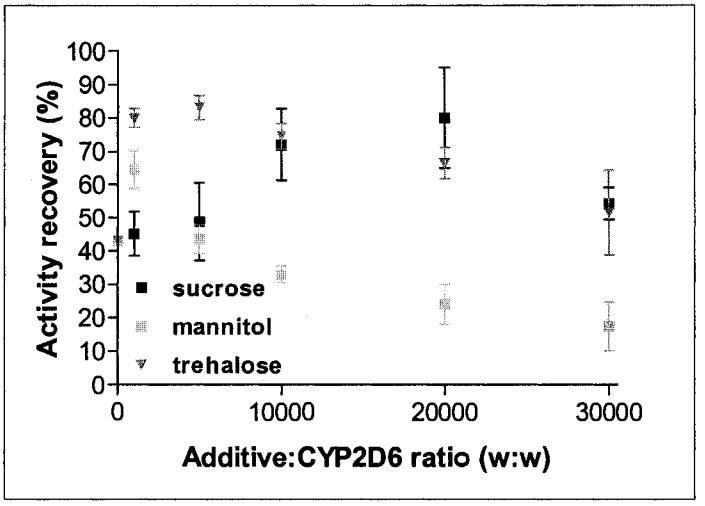

Figure 4.4: Effect of co-lyophilization (22 h) with different amounts of sucrose, trehalose or mannitol on the demethylation of dextromathorphan (100 μM) catalyzed by CYP2D6 (0.14 μM) upon rehydration in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 800 μl). The reaction was initiated with cumene hydroperoxide (0.1 mM) and incubated for 1 h at 37 °C.

CYP3A4 and CYP2D6 are some of the most important enzymes in drug metabolism. Alone, CYP3A4 transforms more than half of all known pharmaceuticals. CYP3A4 and CYP2D6 are versatile and unequalled biocatalysts because of their high substrate promiscuity and their ability to catalyze the regio- and stereo-selective oxidation of inactivated C-H bonds. Lyophilization facilitates the use of enzymes, but without additives, it greatly reduces the activity of CYP3A4 and CYP2D6. We demonstrate here that the addition of sugars before lyophilization can help preserve most of the enzymatic activity upon rehydration. Saccharides were the most efficient lyoprotectants, especially sucrose and trehalose which lead to complete retention of activity at high concentrations. The possibility to lyophilize CYP3A4 or CYP2D6 without activity loss greatly eases handling, storage and use of this enzyme in anhydrous environments. This is a considerable asset for general applications of purified P450s, especially in synthesis.

Acknowledgements

The authors wish to acknowledge Prof. J. R. Halpert from the University of Arizona for his kind gift of the CYP3A4 expression plasmid. The authors are also grateful to Prof. F. Peter Guengerich from Vanderbilt University and Prof. Charles B. Kasper from the University of Wisconsin-Madison for their kind gift of the P450 2D6 and CPR expression plasmids, respectively. This work was supported by the Natural Science and Engineering Council of Canada (NSERC), Astra Zeneca Canada Inc., Boehringer Ingelheim Canada Ltd., and Merck Frosst Canada & Co.

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Chapter Four – Appendix

Experimental section (published as supporting information)

4A.1 Chemicals

The CYP3A4 pSE3A4His expression plasmid was a gift from Dr. J. R. Halpert from the University of Arizona. The culture media ingredients yeast extract, tryptone and peptone were purchased from BD Biosciences (San Jose, CA). DH5α supercompetent cells and agarose were from Invitrogen (Carlsbad, CA). The His-Select™ Nickel Affinity Gel was purchased from Sigma (St. Louis, US). The CYP2D6 substrate dextromethorphan and its metabolite dextrorphan were purchased from Sigma (Oakville, Canada). The CYP3A4 substrate testosterone (4-androsten-17β-ol-3-one) was a kind gift from Dr. Eisenberg in our department and the metabolite 6β-hydroxytestosterone (4-androsten-6β,17β-diol-3-one) was purchased from Steraloids (Newport, RI). All solvents were purchased from Fisher and were of HPLC grade. All chemicals were used without further purification. Water was obtained from a Milli-Q Synthesis (Millipore, San Jose, CA) filtration system. All other chemicals were purchased from Sigma or Aldrich.

4A.2 Instruments

Lyophilization was performed on a Labconco freeze dry system Freezone ® 4.5 at a condenser temperature of -52°C and a pressure $\leq 70 \times 10^{-3}$ mbars. Analytical HPLC analyses were performed on an Agilent 1100 modular system consisting of an auto-sampler, a quaternary pump system, a photodiode-array detector, a fluorescence detector, and a thermostated column compartment. The Agilent Chemstation software version A.10.02 was used to control the operation and data acquisition. Analysis of testosterone 6β-hydroxylation by CYP3A4 used a 150 X 4.6 mm Zorbax Eclipse XDB-C8 5μm column from Agilent protected by an analytical guard column. The

elution consisted of a first isocratic step at 15% acetonitrile in water for 4 min, before a linear gradient to 50% acetonitrile over 12 min. The flow rate was 1.5 ml/min, and the column temperature was set to 30°C. Detection was at 244 nm. Under these conditions, the retention times of testosterone, 6β-hydroxytestosterone, 6α-hydroxytestosterone and cortexolone were 16.3, 9.9, 10.5 and 14.2 min respectively. A Synergi 4 μm Hydro-RP 80 Å column with a matching guard column were used to analyze the formation of dextrorphan from dextromethorphan. A combination of 50% water containing 0.1% TFA and 50% acetonitrile was linearly changed to 80% acidic water and 20% acetonitrile over 20 min. This eluent was maintained for 4 min, before returning to 50:50 over 2 min. Under these conditions, the retention times of dextrorphan and dextromethorphan were 13.4 min and 18.0 min respectively.

4A.3 Expression and purification of enzymes

The expression and purification of CYP2D6 was performed as described earlier [2]. CYP3A4 was expressed using a modification of the procedure used by Domanski *et al* [1]. The plasmid was transformed into *Escherichia coli* DH5α supercompetent cells, and the cells grown overnight on LB agar plates with ampicillin (50 μg/ml). Culture tubes containing LB media supplemented with ampicillin (50 μg/ml) were inoculated next. After 24 h of growth at 37°C and 250 rpm, 10 flasks of TB medium (750 ml each) containing ampicillin (50 μg/ml) were inoculated each with 3 intermediate culture tubes. The flasks were incubated at 37°C and 250 rpm for 2 h (i.e. until OD_{600} = 0.6), after which protein expression was induced with IPTG (1 mM) followed by addition of δ-ALA (80 mg/ml). The incubation temperature was reduced to 30°C, and shaking was set to 190 rpm for 48 h. The cells were collected by centrifugation at 4000 × g and 4°C for 15 min. The pellets were resuspended in buffer A (50 ml of: 100 mM 3-[*N*-morpholino]propanesulfonic acid] (MOPS) at pH 8 containing 10% glycerol, 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride (PMSF)). All subsequent procedures were performed at 4°C. Lysozyme (10 mg/g of cells) was added, and the suspension was stirred slowly for 15 min, after which the suspension

was supplemented with protease inhibitors (1.6 μg/ml leupeptin, 1 μg/ml aprotinin, 0.8 μg/ml bestatin, and 0.7 μg/ml pepstatin A). The suspension was then sonicated on an ice-salt bath at 60% and power 8. After 2 sonication cycles of 20 s each, the suspension was centrifuged at 100,000 × g for 60 min. The supernatant was removed and the pellets resuspended in buffer B (10 ml of: 100 mM MOPS at pH 7.4, containing 10% glycerol and 2 mM PMSF). 3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid (CHAPS, 0.5%) and potassium chloride (0.5 M) were then added. Cells were homogenized and gently stirred for 2 h before centrifugation (1 h at 100,000 × g). This CHAPS-solubilized P450 preparation was then stored at -80°C until the next step.

The His-tagged protein was purified by affinity column chromatography using the Ni-NTA metal affinity resin (1.5 ml), pre-equilibrated with 5 bed volumes of EQ-buffer (buffer B containing 0.5% CHAPS, 0.5 M KCl, and 5 mM imidazole) at a flow rate of 0.5 ml/min. The P450 preparation was then loaded onto the column at the same flow rate. The column was washed with 10 bed volumes of EQ-buffer, then with 10 bed volumes of 100 mM MOPS buffer at pH 7.4 containing 10% glycerol and 10 mM imidazole. The P450 was eluted in EL-buffer (100 mM MOPS at pH 7.4 containing 10% glycerol and 200 mM imidazole). The orange-colored fractions were compared spectroscopically for protein content and by SDS-PAGE (12.5% homogeneous gels) for purity assessment. The fractions containing the CYP3A4 protein were pooled and dialyzed (twice 4 l of: 100 mM MOPS buffer at pH 7.4 containing 0.2 mM dithiothreitol and 1 mM EDTA) at 4°C. The sample was aliquoted and stored at -80°C. The overall yield was 0.6 mg per liter of culture.

4A.4 Quantification of P450 enzymes

The P450 content was measured by reduced carbon monoxide difference spectra following the method described by Omura and Sato [3]. Thus heme was reduced by addition of a few solid grains of sodium dithionite ($Na_2S_2O_4$) in quartz cuvettes containing the buffered enzyme solution, and formation of the enzyme-carbon

monoxide complex was achieved by slow bubbling of CO gas into the reduced enzyme solution for about 20 s. The UV absorption spectra was recorded immediately, and the concentration of P450 was determined using the extinction coefficient ε_{450} = 91 mM^{-1} cm^{-1}.

4A.5 Effect of freezing and lyophilization on the enzyme activity

Samples containing glycerol-free CYP3A4 (100 µl of a 11.5 µM solution) in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 300 µl) were frozen at -80°C and lyophilized for 24 h. Three holes were made in the Eppendorfs' cap using a red-hot needle, and the pierced caps were replaced with new ones after freeze-drying. The dried samples were redissolved in 0.1 M potassium phosphate buffer at pH 7.4 (287 µl), and the substrate testosterone (115 µM, 10 µl of a 1 mg/ml solution in methanol) was added (final CYP3A4 concentration of 1.3 µM). Control samples were prepared using the same amount of CYP3A4 (100 µl of a 11.5 µM solution) and the substrate testosterone (115 µM, 10 µl of a 1 mg/ml solution in methanol) in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 300 µl). To test the effect of freezing alone, some samples containing a CYP3A4 solution were frozen at -80°C and thawed on ice once or twice. After 5 min of pre-incubation at 37°C, the reaction was initiated with the addition of cumene hydroperoxide (1 mM, 7 µl of a 0.1 M stock solution in water:methanol 90:10), and the samples were shaken at 37°C and 250 rpm for 1 h. After incubation, the samples were spiked with the internal standard cortexolone (15 µl from a 200 µM solution in methanol) and extracted with CH_2Cl_2 (3 × 500 µl). The combined organic layers were evaporated in vacuo. The residue was redissolved in MeOH (150 µl) and analyzed by HPLC.

4A.6 Effects of additives during lyophilization

Samples containing glycerol-free CYP3A4 enzyme (24 µl of a 11.5 µM solution) and different amounts of additives (18-crown-6, 15-crown-5,

methyl-β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin, sucrose, sorbitol, mannitol or trehalose) in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 700 µl) were lyophilized for 24 h. For sucrose, sorbitol, mannitol and trehalose the amounts added are reported as w/w ratio additive/enzyme, whereas molar ratios are used for all other additives tested. The dried samples were redissolved in 0.1 M potassium phosphate buffer at pH 7.4 (687 µl), and the substrate testosterone (50 µM, 10 µl of a 1 mg/ml solution in methanol) was added (final CYP3A4 concentration of 0.4 µM). After 5 min of pre-incubation at 37°C, the reaction was initiated by the addition of cumene hydroperoxide (1 mM, 7 µl of a 0.1 M stock solution in water:methanol 90:10), and the samples were shaken at 37°C and 250 rpm for 1 h. A first set of controls was prepared for each additive at every concentration with non-lyophilized enzyme. Another control reaction was also prepared with lyophilized enzyme and no additives. After incubation, the samples were spiked with the internal standard cortexolone (15 µl from a 200 µM solution in methanol) and extracted with CH_2Cl_2 (3 × 500 µl). The combined organic layers were evaporated in vacuo. The residue was redissolved in MeOH (150 µl) and analyzed by HPLC.

The activity of CYP3A4 lyophilized with the optimized amounts of sucrose or trehalose was also tested with cytochrome P450 reductase (CPR) and NADPH. Samples containing glycerol-free CYP3A4 enzyme (0.4 µM, 25 µl of a 11.2 µM solution) and sucrose (145 mg, 10000 w/w) or trehalose (145 mg, 10000 w/w) in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 700 µl) were lyophilized for 24 h. The dried samples were redissolved in 0.1 M potassium phosphate buffer at pH 7.4 (632 µl), and CPR was added (1.6 µM, 45 µl of a 25 µM solution). The substrate testosterone (50 µM, 10 µl of a 1 mg/ml solution in methanol) was added, and after 5 min of pre-incubation at 37°C, the reaction was initiated with the addition of NADPH (1 mM, 28 µl of a 25 µM solution in buffer). The samples were shaken at 37°C and 250 rpm for 1 h. Reactions were also carried out after initiation with CHP under the same conditions. Control reactions with non-lyophilized enzyme (initiation with CHP or CPR/NADPH) and enzyme lyophilized without additives (initiation with CHP or

CPR/NADPH) were also analyzed. After incubation, the samples were spiked with the internal standard cortexolone (15 µl from a 200 µM solution in methanol) and extracted with CH_2Cl_2 (3 × 500 µl). The combined organic layers were evaporated in vacuo. The residue was redissolved in MeOH (150 µl) and analyzed by HPLC.

Samples containing glycerol-free CYP2D6 enzyme (8 µl of a 13.85 µM solution) and different amounts of sugar additives (sorbitol, sucrose, mannitol, or trehalose; ratios in w/w) in 0.1 M potassium phosphate buffer at pH 7.4 (final volume of 1000 µl) were lyophilized for 22 hrs. The lyophilized enzyme (final concentration of 0.14 µM), with or without additive, was redissolved in 0.1 M potassium phosphate buffer at pH 7.4, and the substrate dextromethorphan (100 µM) was added. The reaction was initiated with the addition of the cofactor surrogate cumene hydroperoxide (0.1 mM). The samples were shaken at 37°C and 200 rpm for 1 h. The reaction was terminated with the addition of perchloric acid (23%, 40 µl). After cooling to 4°C for 30 min, the samples were centrifuged at 10000 rpm for 5 min. The resulting supernatant was directly injected in the HPLC.

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Chapter Five

In chapter 3 of this thesis, studies of CYP2D6 showed very good activity for this enzyme in organic/aqueous biphasic solvent systems. Although this methodology allows for the first time transformation of hydrophobic substrates, it is not appropriate for moisture sensitive substrates or products, for water-soluble products and for reactions where water shifts the equilibrium towards substrates. Enzyme studies in anhydrous organic solvents require the elimination of water by lyophilization, a process harmful to P450 enzymes. To pave the way for further studies of CYP2D6 in organic solvents, different additives were tested as lyoprotectants for this enzyme in chapter 4. Trehalose was found to effectively protect the enzyme from being deactivated during lyophilization. In this chapter, the behaviour of CYP2D6 in nearly anhydrous organic solvents is investigated after lyophilization with trehalose and in the presence of the surrogate CHP.

Contributions of co-authors

This chapter is reproduced from a manuscript that will be submitted for publication shortly. I performed all the experiments described in this chapter myself, under the supervision of Professor Karine Auclair.

The activity of human CYP2D6 in low water organic solvents

5.1 Abstract

P450 enzymes are of high interest for synthetic applications due to their ability to catalyze hydroxylation reactions at inactivated C-H bonds. The low solubility of many substrates in buffer, however, is limiting the applications of P450s. Our recent demonstration that P450 2D6 can function very well in biphasic solvent systems is one step towards overcoming this drawback, but is not practical when substrates or products are unstable in water or with water soluble products. An alternative strategy which also facilitates enzyme recycling is to directly resuspend lyophilized enzyme into nearly anhydrous organic solvents. Interestingly, we report here that CYP2D6 co-lyophilized with trehalose and suspended in *n*-decane shows higher activity than in aqueous buffer. This study demonstrates the unexpected high tolerance of wild type CYP2D6 to organic solvents and provides an alternative strategy to facilitate the use of this enzyme in synthesis.

5.2 Introduction

Although most enzymes have evolved to catalyze reactions under aqueous conditions, many of them have been reported to show activity in the presence of organic solvents [1, 2]. Tolerance to organic media expands the biocatalytic potential of enzymes. Indeed, the use of non-aqueous solvents can overcome problems such as substrate insolubility in water, moisture sensitivity of substrates, reagents or products, or product harvesting [3]. P450 enzymes are highly versatile catalysts involved in xenobiotic metabolism, biosynthesis of steroids, lipids, vitamins, and natural products [4]. Hydroxylation at inactivated C-H bonds is one of the most challenging reactions in organic synthesis, yet it is catalyzed under mild conditions by P450 enzymes with high stereo- and regio-selectivity [4]. As biocatalysts, mammalian P450s have an advantage over their bacterial counterparts due to their high substrate promiscuity [5, 6]. Numerous studies, however, have shown a detrimental effect of organic solvents on the activity of P450s [7-23]. In general, even small amounts of water-miscible organic solvents strongly inhibit P450 enzymes. These solvents likely strip essential water molecules off the

enzyme [24]. On the other hand, biphasic media containing hydrophobic organic solvents have proven more promising media for many enzymes [1, 2, 25]. Ichinose *et al.* reported significant activity of the bacterial P450cam embedded in reverse micelles with the cofactors NADH, putidaredoxin and putidaredoxin reductase [26]. A few groups have also attempted to increase the tolerance of bacterial P450s to organic solvents using directed evolution [27]. For instance, one P450 BM-3 variant reported by Arnold and coworkers shows an improvement in activity of 10-fold in 2% (v/v) tetrahydrofuran (THF) and 6-fold in 25% (v/v) dimethylsulfoxide (DMSO) compared to the wild-type enzyme [28]. Molecular dynamics simulations suggest P450 BM-3 remains folded in the presence of DMSO [29], but solvent molecules may replace the water molecules linked to the heme iron of this enzyme [30]. Crystal structures of P450 BM-3 obtained in different ratios of DMSO/water confirm these studies and provide a detailed structural explanation for the deactivating effect of this solvent [31]. Urlacher and coworkers generated a CYP102 mutant able to catalyze hydroxylation of cyclohexane, octane or myristic acid in biphasic solvent systems [32]. Two mutants of the mammalian CYP2B1 reported by Kumar *et al.* exhibited enhanced tolerance towards 2.5–15% DMSO [33]. A third mutant created by combination of the first two variants was > 2 folds more tolerant to DMSO [33]. Our group has recently reported a detailed survey of the activity of wild type human CYP3A4 in the presence of organic solvents and ionic liquids (ILs) [22]. The CYP3A4 enzyme activity was found to decrease significantly with the addition of solvents or ILs, except with biphasic solvent systems, which allowed retention of ~85% of the activity [22]. We have also demonstrated that human CYP2D6 can retain most of its activity in selected emulsions [34]. Nearly anhydrous solvents have also had significant success with many enzymes [1, 2], but have not been explored with P450s.

We have reported a highly efficient method to replace the expensive cofactor and redox partner of CYP2D6 and CYP3A4 (NADPH and cytochrome P450 reductase (CPR)) using cheap chemicals such as cumene hydroperoxide (CHP) or sodium percarbonate (SPC) [35]. Although we demonstrated very good CHP- or NADHPH/CPR-supported activity of CYP2D6 in selected biphasic solvent systems [34], this method may not be suitable with moisture sensitive substrates or products, or with water soluble products. An alternative strategy which also facilitates enzyme recycling is to directly resuspend

lyophilized enzymes into nearly anhydrous organic solvents. Unfortunately, removal of the water is often harmful to enzymes. A recent method published by our group allows lyophilization of CYP3A4 and CYP2D6 with minimal loss of activity [36]. We now report a detailed evaluation of the activity of human CYP2D6 in nearly anhydrous organic media. Surprisingly, our results show higher activity of lyophilized CYP2D6 in selected water-saturated hydrophobic organic solvents than in buffer.

5.3 Results and discussion

In these studies, CHP was used instead of the natural cofactor NADPH and redox partner CPR. Indeed, the goal was to investigate the effect of organic solvents on CYP2D6 and not on its redox partner the CPR enzyme. Moreover, as reported by others [37] and by our group [35], the activity of human CYP2D6 is higher with CHP than with the natural system NADPH/CPR. We previously reported that the optimal CHP concentration for supporting CYP2D6 activity in buffer is 0.1 mM [35]. We suspected that in the absence of water, the concentration of CHP would have to be optimized again. To this end, the activity of CYP2D6 in water-saturated isooctane or *n*-decane was tested at various concentrations of CHP. As illustrated in **Figure 5.1**, the optimal concentration of CHP is 0.2 mM in water-saturated isooctane and 2 mM in water-saturated *n*-decane, compared to 0.1 mM in buffer. This difference may be the result of a combination of factors, including the presence of trehalose or differences in solubility and in diffusion rates. Control reactions without enzyme do not show any product formation (data not shown). The concentration of CHP was fixed at 0.2 mM and 2 mM for all further experiments.

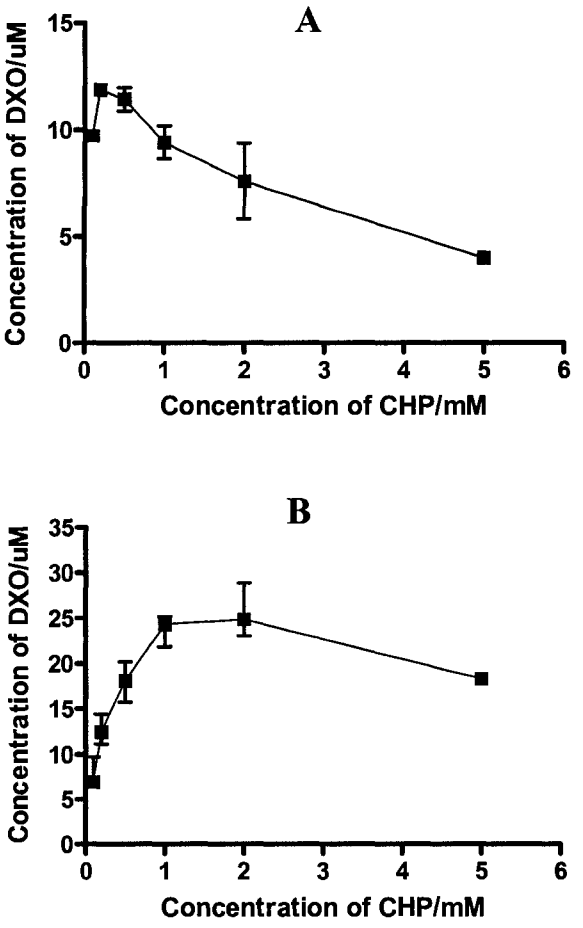

Figure 5.1 Effect of concentration of CHP on the production of dextrorphan (DXO) from dextromethorphan (DXM) by CYP2D6 (co-lyophilized with trehalose) (a) in water-saturated isooctane; (b) in water-saturated *n*-decane. The expression and purification of CYP2D6 was performed as described earlier [34]. CYP2D6 was mixed with trehalose (1:1000 w/w), frozen at -80°C, and lyophilized for 24 h using a Labconco freeze-dry system Freezone® 4.5 at a condenser temperature of -51°C and a pressure $\leq 70 \times 10^{-3}$ mbars. Lyophilized CYP2D6 (0.25 μM) and the substrate DXM (0.05 mg, < 0.46 mM, not fully soluble) were added to the reaction vial as solids. Water-saturated organic solvent was added, followed by CHP (0.1-5.0 mM, diluted in (a) isooctane or (b) *n*-decane). The final mixture (400 μl) was incubated for 2 h at 37°C and 300 rpm. Water (2 × 200 μl) was used to extract both substrate and product. The aqueous mixtures were centrifuged at 16,000 × *g* for 5 min and filtered. The filtrates were analyzed by HPLC as published earlier [34]. An alternative analytical method used the 250 × 4.60 mm Synergi 4 μ Hydro-

RP 80 Å column with mobile phase A (0.1% TFA in water) and B (100% acetonitrile) at a flow rate of 0.15 mL/min. The initial mobile phase consisted of 50:50 A:B (v:v) and was linearly changed to 80:20 A:B over 25 min. After 3 min constant 80:20 A:B, it was back to 50:50 in 2 min. UV detection was set at 230 nm.

Previous reports have suggested that water-miscible solvents may deactivate enzymes by stripping off essential water molecules [24]. Consequently, eight different non-halogenated hydrophobic organic solvents were evaluated as media for CYP2D6 activity. They were tested as anhydrous or water-saturated solvents. The high hydrophobicity of the selected solvents (especially for the best ones) limited the range of easily accessible water activities (a_w). When strictly anhydrous solvents were used, the remaining activity of CYP2D6 was below 10% (data not shown). The results with water-saturated solvents are shown in **Figure 5.2**. As reported for other enzymes [38], the activity of CYP2D6 in low water organic solvents improved with increasing hydrophobicity of the solvent. Control reactions without enzyme did not show any product formation (data not shown). As previously reported [36], the enzyme lost about ~22% activity following lyophilization in the presence of trehalose and more than 50% in the absence of trehalose (data not shown). The addition of trehalose to the reaction mixture however (enzyme not lyophilized), did not affect the activity of CYP2D6 in buffer. At CHP concentration of 0.2 mM (**Figure 5.2a**), the activity of lyophilized CYP2D6 in isooctane, *n*-decane, or *n*-dodecane was about 60% of the activity of lyophilized CYP2D6 in buffer. To our surprise, at 2 mM CHP (optimum concentration for *n*-decane), the enzymatic activity of lyophilized CYP2D6 was higher in *n*-decane (125%) than in buffer, and similar in *n*-dodecane or in buffer (**Figure 5.2b**). This was unexpected based on previous studies of enzymes in low water organic solvents where ~10% remaining activity in solvent is common [1]. For example the activity of CYP3A4 dropped to <5% under the same conditions as reported here for CYP2D6 [22]. It should be noted that the CYP2D6 activity in low water solvents is highly sensitive to the lyophilization process. The addition of trehalose is essential (a few other sugars help too, but with a less effective protection; [36]) and the optimal duration of lyophilization is 24 h (see figure captions for more details).

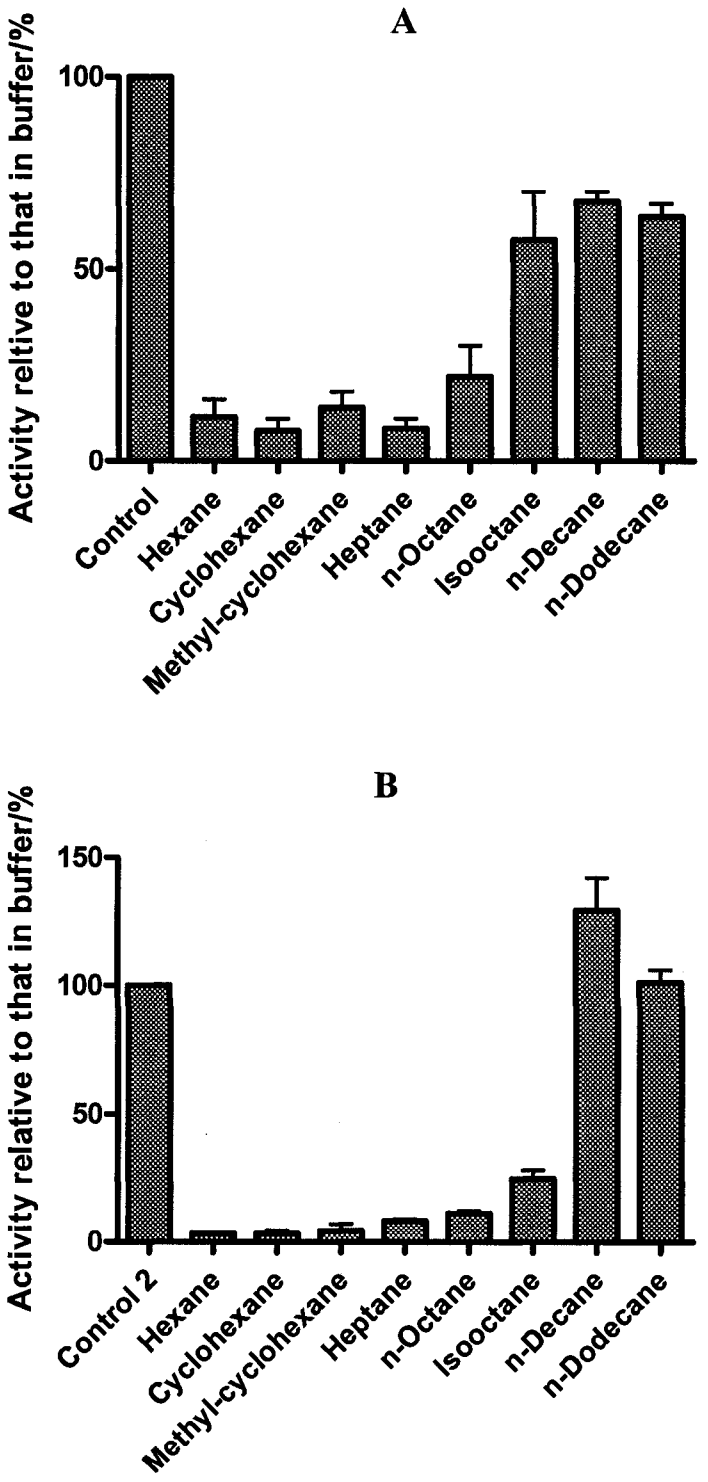

Figure 5.2 CYP2D6 enzymatic activities various low water organic solvents relative to the activity of lyophilized enzyme in buffer (Control) at two different CHP concentrations. Control: Reaction (total volume of 400 μl) of CYP2D6 (0.25 μM, lyophilized), DXM

(0.05 mg, < 0.46 mM, not fully soluble), CHP (a: 0.2 mM, b: 2 mM), trehalose (45 mM) in potassium phosphate buffer (pH 7.4, 0.1 M). Organic solvents: same as in Control but the lyophilized enzyme was resuspended in the desired water-saturated solvent instead of buffer. The reactions were incubated for 2 h at 37°C and 300 rpm before work up and analysis [34]. All organic solvents used as reaction media with suspended enzyme were equilibrated with Milli-Q water for more than a week before use. Each entry is the average of two separate experiments.

Due to the low solubility of the substrate DXM in *n*-decane and *n*-dodecane (we could not find a known CYP2D6 with high solubility in these solvents), we suspected that the high activity of CYP2D6 in these solvents may be a result of increased local concentration of DXM near the insoluble enzyme. To eliminate this possibility, the ratio of dissolved DXM over the total amount of DXM added was measured for all solvents and plotted against the enzymatic activity in the corresponding solvent, at two different CHP concentrations (**Figure 5.3**). A local concentration effect was expected to show a linear relationship between these two parameters but no correlation was observed. This lack of correlation suggests that a local concentration effect near CYP2D6 may not be a determinant factor explaining the dramatic solvent effect on enzyme activity. On the other hand, the activity of CYP2D6 in low water organic solvents improved with increasing hydrophobicity of the solvent. This is in agreement with the suggestion by others that hydrophobic solvents are less likely to strip essential water molecules off the enzyme [24].

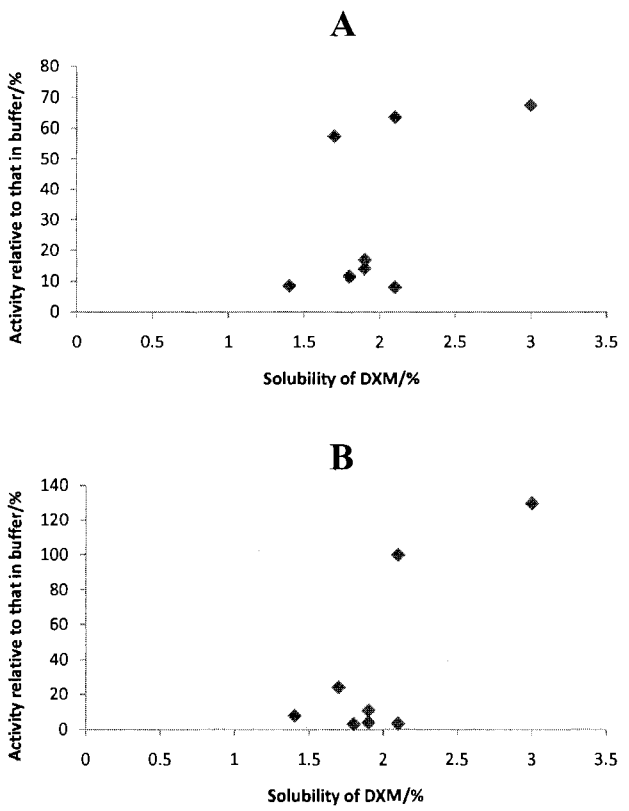

Figure 5.3 DXM solubility in each organic solvent plotted as a function of the CYP2D6 enzymatic activity in the same solvent and measured at two different CHP concentrations: (a) 0.2 mM and (b) 2 mM. DXM solubility (% w/w) was defined as the percentage of DXM dissolved in the organic solvent over the total amount of DXM added. The enzymatic activity is from **Figure 5.2**.

P450 enzymes are powerful biocatalysts. Applications of purified P450 enzymes are largely unexploited because of a number of limitations including the need for expensive cofactors and redox partners, incompatibility with organic solvents, and low stability and activity. We have previously circumvented the need for NADPH and CPR with human CYP2D6 and CYP3A4 [35]. We have also showed that CYP3A4 [22] and CYP2D6 [34] can be used in biphasic solvent systems without significant losses in activity. We report here a survey of the CYP2D6 activity in various low water organic solvents. Interestingly, after lyophilization in the presence of trehalose as previously reported [36], CYP2D6 shows ~25% more activity in *n*-decane than in buffer. This is in contrast to the ~90% loss in activity reported for CYP3A4 in low water organic solvents

[22]. To the best of our knowledge, this is the first time that human CYP2D6 was reported to show activity in nearly anhydrous organic solvents and one rare example [2, 24] where a wild type enzyme is more active in organic solvent than in buffer. This study provides an alternative strategy to facilitate the use of CYP2D6 in synthesis.

Acknowledgments

The authors wish to acknowledge Prof. F. Peter Guengerich from Vanderbilt University and Prof. Charles B. Kasper from the University of Wisconsin-Madison for their kind gift of the P450 2D6 and CPR expression plasmids, respectively. This work was supported by the Natural Science and Engineering Council of Canada (NSERC), Astra Zeneca Canada Inc., Boehringer Ingelheim Canada Ltd., and Merck Frosst Canada & Co.

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Chapter Six

In the previous chapters, we have developed new methodologies to facilitate applications of P450 enzymes. Thus, we have overcome limitations such as the need for expensive cofactors and the incompatibility with organic solvents. Another limitation associated with the use of CYP2D6 as a biocatalyst relies on its low thermodynamic and kinetic stability. In order to improve the stability of CYP2D6, we envisaged a novel strategy taking advantage of DNA self-assemblies. As reported for other enzymes [1], encapsulation (e.g. in nanogels) may rigidify the enzyme conformation, and increase the catalytic turnover. This chapter reports preliminary studies that demonstrate the templating effect of CYP2D6 in the formation of specific DNA self-assemblies.

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Contributions of co-authors

I carried out the expression, purification, and characterization of CYP2D6. I also performed high ionic buffer tolerance experiments and enzyme stability studies. Faisal Aldaye synthesized the DNA building blocks and performed the polyacrylamide gel electrophoresis (PAGE) analyses of the DNA self-assemblies. All descriptive schemes about DNA formation are from Faisal Aldaye.

CYP2D6-templated formation of cyclic DNA self-assemblies

6.1 Abstract

It was previously reported that two complementary DNA building blocks, each containing two identical DNA arms linked by a vertex, can self-assemble into a small dynamic library, including dimers, tetramers, hexagons, and oligomers. The distribution of the resulting library depends on the conditions. Upon reequilibration in the presence of $Ru(bpy)_3^{2+}$, the library proceeds mainly to tetramer formation. This discovery provides an easy synthetic route to complex DNA systems, and even an autocorrection mean for the library. DNA cages are self-assembled with building blocks whose length and flexibility can be pre-designed. It was envisaged that design of the self-assembly of DNA templated by CYP2D6 may stabilize the enzyme by encapsulating it in a way similar to sol-gels [1]. Analysis by polyacrylamide gel electrophoresis (PAGE) shows that the addition of CYP2D6 to selected DNA building blocks favours the formation of DNA dimers and tetramers over other assemblies. The activity and stability of the resulting DNA-CYP2D6 complexes are comparable to those of the enzyme alone.

6.2 Introduction

Enzymes provide "greener" alternatives to traditional synthetic chemistry, especially for high value-added fine chemical production [2, 3]. Hydroxylation at inactivated C-H bonds by P450 enzymes has attracted a lot of attention because this type of reaction is hardly accomplished regio- and stereo-selectively with traditional synthetic methods [4]. Mammalian P450s may have more potential useful applications than their bacterial counterparts due to their exceptional substrates promiscuity [5-6]. Nevertheless, many handicaps have restricted their use in synthesis, including the need for expensive cofactors, low tolerance to organic solvents and low stability. Some attempts have, at least partially, resolved these drawbacks [7-9]. Low stability of P450s, however, has remained problematic. Numerous techniques have been used to increase

enzyme stability including encapsulation of enzymes into relatively rigid frameworks [10, 11]. This strategy also provides a means to easily recycle enzymes and separate them from substrates and/or products, a very important step in industrial processes. Typical encapsulation of enzymes uses inorganic sol-gel or polymers. The disadvantages, however, relate to biocompatibility, porosity, mechanical robustness, and long term stability, thus preventing these materials from satisfying industrial requirements.

Recently, Turberfield and coworkers demonstrated the encapsulation of cytochrome c in DNA polyhedra built up from 20 base-pair double helices [12]. Their study, however, did not include stability and enzyme activity assays. The use of DNA to encapsulate enzymes has many advantages including the relative rigidity of the double helical form, ease of insertion of flexible linkers, easily tailored size, convenient self-assembly or disassembly, sequence programmability, and binding selectivity [13]. These benefits attracted us to envisage DNA as a scaffold to improve the stability and activity of P450 enzymes.

Sleiman and coworkers have reported that specific DNA building blocks can be arranged into a discrete, well-defined, two-dimensional cyclic scaffold (see **Figure 6.1**) [14, 15]. The building blocks used consisted of two DNA single strands attached either on a rigid vertex (1,3,5-tris(4-hydroxyphenyl)benzene) or a flexible vertex (alkane phosphate derivative of 1,3,5-tris(4-hydroxyphenyl)benzene). The designed complementary of the two building blocks favors self-assembly into linear or cyclic shapes. Because of their well-defined structures, cyclic self-assemblies have a high potential of application in nanotechnology [16]. **Figure 6.2** displays a 3D rendering of selected cyclic DNA self-assemblies. The empty interior space can potentially be utilized for guest molecule encapsulation. Moreover this suggests potential templating effects by guests. The empty volume can be accurately modulated by tailoring the double strands and/or number of the strands. For example, 17-base-pairs strands in cyclic tetramers are in principle of a suitable size for surrounding CYP2D6.

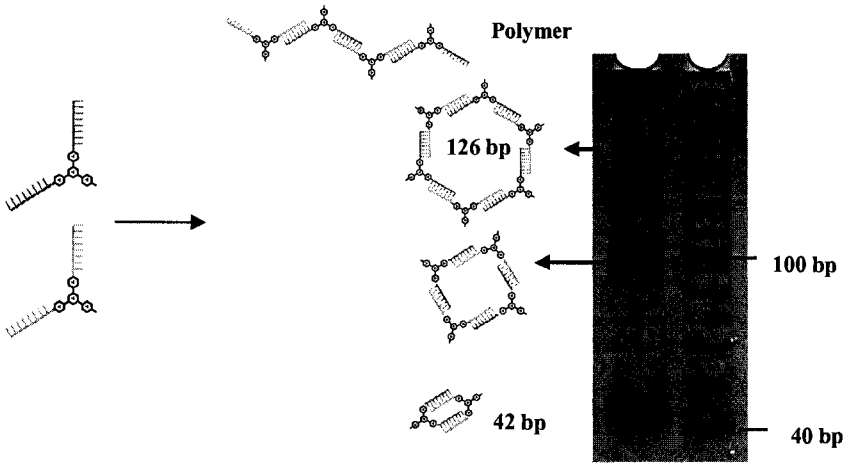


Figure 6.1 Formation of discrete DNA self-assemblies characterized by PAGE. Combs drawn on the tris-phenyl represent DNA single strands. Color differences imply different sequences and the two sequences selected here must be complementary to one another.

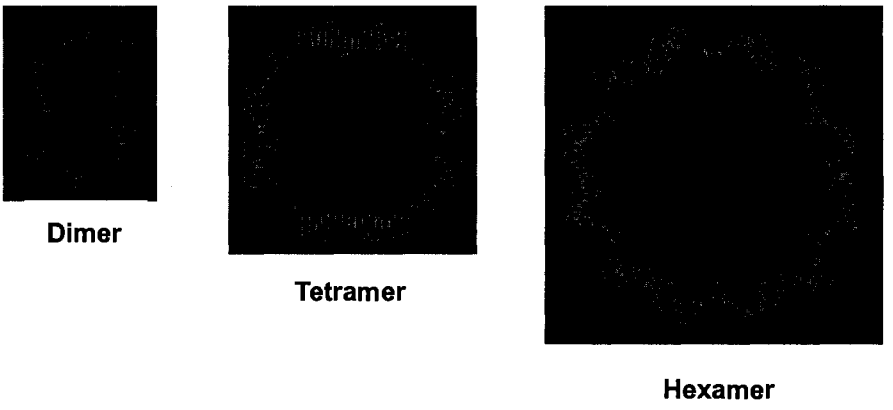


Figure 6.2 Tridimentional rendering of dimeric, tetrameric and hexameric DNA self-assemblies.

The generation of uniform size assemblies is a key step not only for decreasing production costs but also increasing biocompatibility. The simple mixing of the building blocks, however, creates a library consisting of both desired and undesired self-assemblies (**Figure 6.1**) [17]. Sleiman and coworkers have recently reported that $Ru(bpy)_3^{2+}$ favours formation of tetrameric DNA self-assemblies via the re-equilibrium of a self-assembly mixture to a single form [18] (**Figure 6.3**).

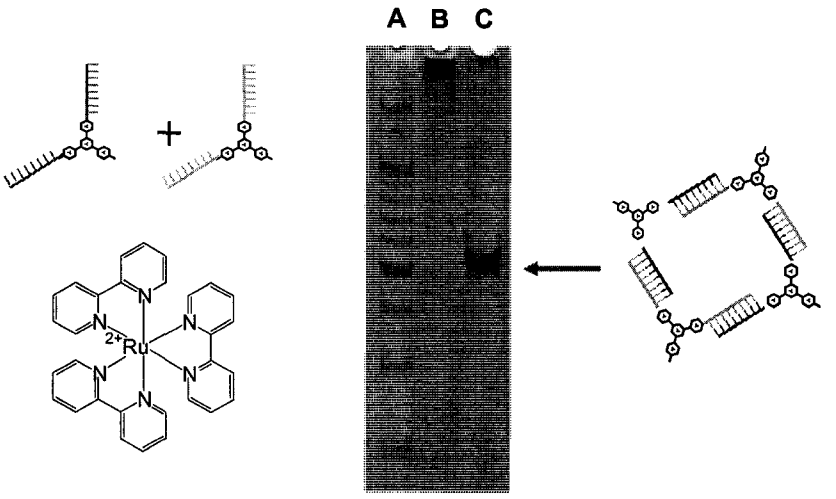


Figure 6.3 Effect of $Ru(bpy)_3^{2+}$ on the formation of DNA self-assemblies. The PAGE analysis of a mixture of two complementary building blocks (lane B); and of the same two building blocks in the presence of $Ru(bpy)_3^{2+}$ is shown in lane C. Lane A is a 20 base pair linear molecular weight DNA ladder.

The $Ru(bpy)_3^{2+}$ guest-mediated experiment suggested the possibility of enzyme-templated formation of specific cyclic assemblies, and perhaps increased stability of enzymes trapped in uniform structures. We report preliminary results demonstrating formation of discrete CYP2D6-DNA assemblies.

6.3 Materials and methods

Chemicals and instruments

All chemicals and instruments used for the preparation of DNA assemblies are published [18]. All chemicals used for CYP2D6 expression and purification, as well as the enzyme activity assay, can be found in previous chapters and in the scientific literature [19].

Design of the DNA self-assembly system

Synthesis of **6.2a**, **6.2b**, **6.3a**, and **6.3b** building blocks was previous reported by Sleiman and coworkers [18]. Briefly, it involves attaching two well-defined single

stranded DNA sequences on a 1, 3, 5-tris(4-hydroxyphenyl)benzene molecule. The DNA sequence used in each stand of **6.2a** is 5' GTGGCATTGG 3', and for its complementary **6.2b** sequence is used 5' CCAATGCCAC 3'. The DNA sequence in each strand of **6.3a** is 5' CGATCTTGTGGCATTGG 3', and its complementary sequence 5' CCAATGCCACAAGATCG 3' is used for **6.3b**..

Activity of CYP2D6 in high ionic strength buffers

To a mixture of CYP2D6 (0.2 μM), DXM (0.1 mM) and magnesium sulfate solution (0 to 50 mM) or sodium sulfate solution (0 to 120 mM) in potassium phosphate buffer (pH 7.4, 0.1 M) was added cumene hydroperoxide (0.1 mM) to initiate the enzymatic reaction. The final volume of the mixture was 200 μL. The reaction was quenched with 23% (v/v) perchloric acid (100 μL) after incubation for 1 h at 200 rpm and 37°C. The quenched reaction mixtures were centrifuged (16,000 × *g*, 5 min). The supernatants were filtered using 0.2 μM teflon filters before HPLC analysis as reported previously [19].

Generation of CYP2D6-templated formation of discrete DNA self-assemblies

One building block (**6.2a**, **6.3a**, or **6.4a**, 0.4 μM) was added to CYP2D6 (0.23 μM). After gentle mixing, the complementary building block (**6.2b**, **6.3b**, and **6.4b**, 10 μM) was introduced (final volume of 30 μL) at room temperature. DXM (0.1 mM) and potassium phosphate buffer (pH 7.4, 0.1 M) were added to monitor the enzymatic activity. CHP (0.1 mM) was added next to initiate the reaction (final volume to 300 μL). After incubation at 37°C and 300 rpm for 1.5 h, the mixture was quenched with perchloric acid (23% v/v, 10 μL), centrifuged (16,000 × *g*, 5 min), and filtered (0.2 μM pore size). The filtrate was analyzed by HPLC as previously reported [19].

Stability of the CYP2D6/DNA assemblies

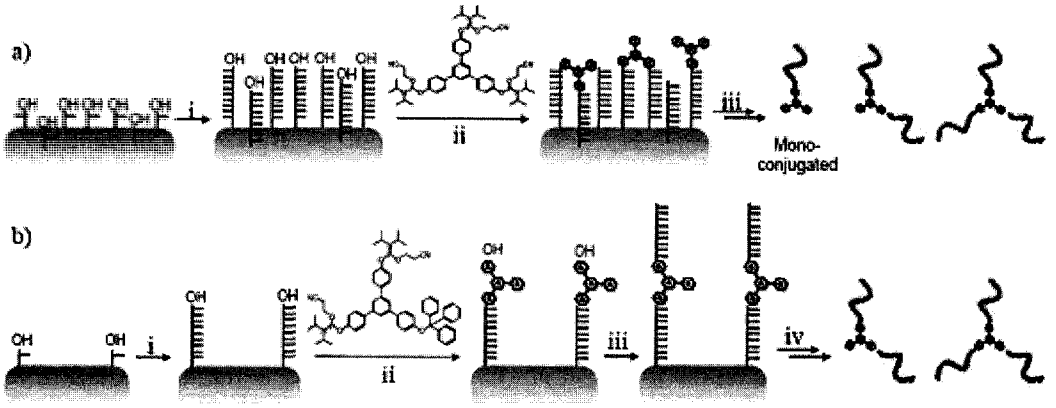
Five samples each of CYP2D6 (6.85 μM, 20 μL) or CYP2D6 (6.85 μM, 20 μL)/DNA assemblies (10 μM, 10 μL) were incubated at 25°C for various amounts of time. The enzyme activity was tested as mentioned above.

6.4 Results and discussion

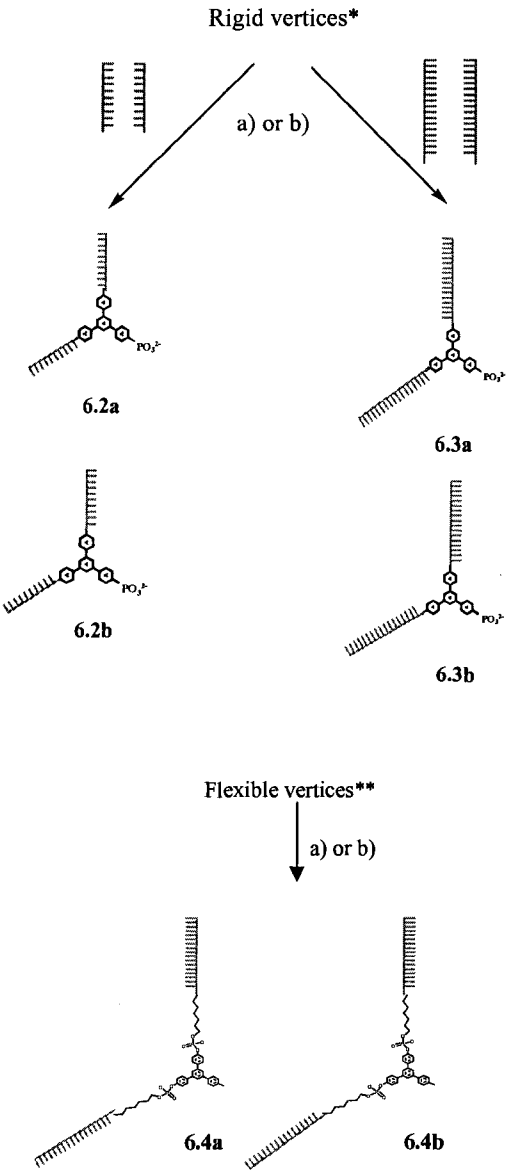
Synthesis of DNA building blocks

Detailed information about the synthesis and characterization of the different DNA building blocks with rigid vertices was previously reported by Sleiman and coworkers [18]. Synthesis of the building block with flexible vertices by Sleiman and coworkers is unpublished. A summary of the procedures is illustrated in **Schemes 6.1** (see figure caption for details).

Structures of the DNA building blocks in this study are shown on **Scheme 6.2**. Hybridization is typically accomplished by mixing two complimentary building blocks in TAEmg$_{2+}$ buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA·2Na·2H_2O, 12.5 mM $MgCl_2.6H_2O$).



Scheme 6.1 Synthetic approaches to generate branched DNA building blocks. a) DNA of the desired sequence is synthesized on 500 Å functionalized CPG at high loading density (85 μmol/g), followed by conjugation of the vertex to yield mono-, bis- and tris-branched DNA building blocks. b) DNA is synthesized on 2000 Å functionalized CPG at low loading density (5 μmol/g), followed by conjugation of the bis-branched mono-trityl protected derivative of tris-branched vertex, and the synthesis of a second DNA arm off of the vertex in a 5' to 3' direction to yield bis- and tris-branched DNA building blocks. Electrophoresis gel is used to separate the building block mixtures in both methods.



Scheme 6.2 Structures of the different building blocks used in this study. Combs facing left and right indicate sequences complementary to each other. Red comb represents a 10-base DNA helix. Green comb represents 17-base DNA helix. a) or b) indicates the building block generation procedure demonstrated in **Scheme 6.1**. * Syntheses were previously reported [18]. ** Unpublished structures (Aldaye and Sleiman).

Tolerance of human cytochrome P450 enzymes to high ionic strength buffers

DNA self-assembly is a thermodynamically favoured process, facilitated by high ionic strength buffers containing magnesium or sodium ions (e.g. TAEmg$_{2+}$ buffer).

Such conditions may have a significant impact on the behavior of enzymatic catalysis [20, 21]. Before attempting the P450-templated formation of cyclic DNA assemblies, the activity of CYP2D6 under conditions typically used for DNA self-assembly was investigated.

The results shown on **Figure 6.4a** indicate that magnesium ion concentrations up to at least 20 mM are well tolerated by CYP2D6. Enzymatic activity was even less affected by sodium ions, and addition of 50 mM Na^+ still yielded > 90% activity (**Figure 6.4b**). These results suggest that CYP2D6 may tolerate the conditions required for DNA self-assembly.

CYP3A4, on the other hand, was more sensitive to the addition of Mg^{2+} or Na^+, yet still compatible with the concentrations needed to generate the self-assemblies (data not shown).

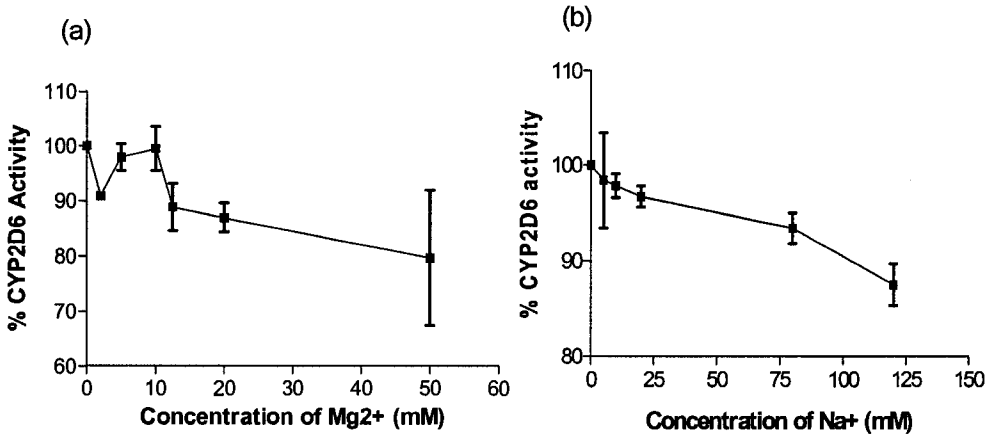


Figure 6.4 Effect of magnesium ions or sodium ions on the activity of human CYP2D6. CYP2D6 (0.2 μM) and the substrate dextromethorphan (DXM, 100 μM) were dissolved in potassium phosphate buffer (0.1 M, pH 7.4). Magnesium sulphate (a) or sodium sulphate (b) was added from a concentrated solution (0.5 M) to reach the desired concentrations. The cofactor surrogate cumene hydroperoxide (CHP, 0.1 mM) was added immediately to initiate the reaction (the total volume of 200 μL). After 1 h at 37°C and 200 rpm, the reactions were quenched with perchloric acid (23% v/v, 10 μL) and cooled to 4°C for 30 min before HPLC analysis. Each entry is the average of duplicates.

Investigation of the potential of CYP2D6 and CYP3A4 as templates in the formation of discrete DNA self-assemblies species

To study the effect of P450 enzymes on the self-assembly of DNA building blocks **6.2a** with **6.2b**, **6.3a** with **6.3b**, or **6.4a** with **6.4b**, the desired building block was added to the enzyme solution followed by its complementary building block. Polyacrylamide gel eletrophoresis (PAGE) was used to analyze the different DNA self-assemblies formed. As expected because of their shorter length, building blocks **6.2a** and **6.2b** yielded only polymeric species in the absence (**Figure 6.5**, lane A) or in the presence of CYP2D6 (lane B) or CYP3A4 (lane C). For building blocks **6.3** and **6.4**, in the absence of P450 (lanes D and G), mostly polymeric DNA self-assemblies were observed, and a significant amont of dimers was formed. The distribution of species was unaffected by the addition of CYP3A4 (lanes F, I) for both **6.3** and **6.4**. In the presence of CYP2D6, however, mostly dimeric and tetrameric DNA species were observed for **6.3** (lane E) and dimeric with **6.4** (lane H), suggesting a CYP2D6-templated annealing of DNA (lanes E, H). This effect is similar to that of $Ru(bpy)_3^{2+}$ [18].

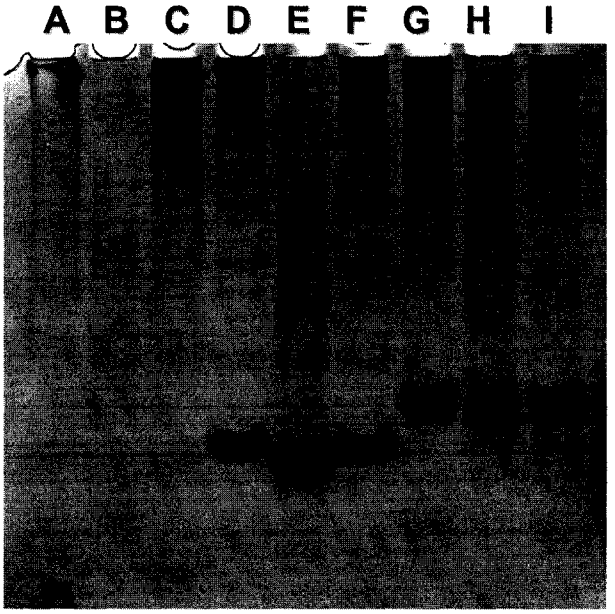


Figure 6.5 Polyacrylamide gel electrophoresis (PAGE) of the DNA self-assemblies

formed in the absence or in the presence of P450 enzymes. (A) building blocks **6.2a** and **6.2b** alone; (B) **6.2a** and **6.2b** in the presence of CYP2D6; (C) **6.2a** and **6.2b** in the presence of CYP3A4; (D) building blocks **6.3a** and **6.3b** alone; (E) **6.3a** and **6.3b** in the presence of CYP2D6; (F) **6.3a** and **6.3b** in the presence of CYP3A4; (G) building blocks **6.4a** and **6.4b** alone; (H) **6.4a** and **6.4b** in the presence of CYP2D6; (I) **6.4a** and **6.4b** in the presence of CYP3A4.

Activity of CYP2D6 in the presence of the DNA assemblies (kinetic stability)

Even though our studies show that CYP2D6 can template the formation of DNA self-assemblies, the arrangement of the DNA around CYP2D6 is unclear. This will be investigated by cross-linking studies later on. Interestingly, the resulting CYP2D6/DNA complexes, do not show the same enzymatic activity as CYP2D6 alone (**Figure 6.6**). Of note is the fact that the addition of only one building block (**6.2a**) in blank C inhibits the enzyme to about 60% activity, however double stranded DNA (e.g. **6.4a** and **6.4b**) does not significantly inhibit the enzyme, suggesting inhibition via hydrophobic effect. The activity is slightly increased in the presence of assemblies of **6.4a** and **6.4b** but not with **6.2a** and **6.2b** or **6.3a** and **6.3b**. This indicates that the interaction of the enzyme with **6.4a** and **6.4b** may facilitate catalysis, likely due to the higher flexibility of these building blocks.

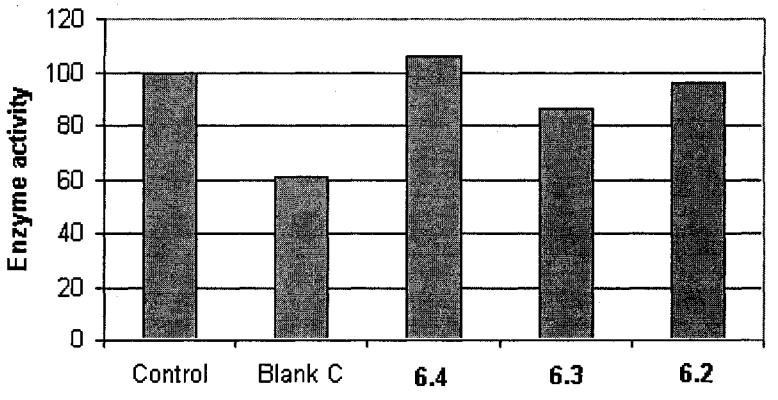


Figure 6.6 Impact of DNA assemblies on the CYP2D6 activity. CYP2D6 (0.23 μM) with or without DNA assemblies (0.4 μM), DXM (0.1 mM), and CHP (0.1 mM) in potassium phosphate buffer (pH 7.4, 0.1 M) were incubated at 25°C and 300 rpm (side

shaking), for 1.5 hrs. The control contains no DNA but was handled the dame way as the other samples. Blank C contains only building block **6.2a**. Sample **6.4** refers to the enzyme activity of CYP2D6 in self-assemblies of **6.4a** and **6.4b** used. Sample **6.3** refers to the enzyme activity of CYP2D6 in self-assemblies of **6.3a** and **6.3b** used. Sample **6.2** refers to the enzyme activity of CYP2D6 in self-assemblies of **6.2a** and **6.2b.**

Stability of CYP2D6 in the presence of DNA self-assemblies (thermodynamic stability)

The stability of CYP2D6 enzymes in the absence and in the presence of DNA self-assemblies was compared. The enzyme activity was measured using the typical dextromethorphan assay followed by HPLC analysis of the products [19]. After storing the solutions at 25°C for 10 days, the activity of the enzyme with DNA assemblies was the same as that of enzyme alone stored at 25°C. Both had gradually decreased to 30% during that time (data not shown).

In conclusion, although the stability assay did not show an improvement of the enzyme's lifetime in the presence of DNA self-assemblies, the enzymatic activity was improved slightly in the presence of **6.4a** and **6.4b**. Whenever 17-base pair long DNA self-assemblies (**6.3a** and **6.3b** or **6.4a** and **6.4b**) were used, CYP2D6 affected the library distribution to form discrete cyclic oligomers including mainly dimers and tetramers. These preliminary results demonstrate possible use of P450s in nano-technology. More studies are needed to verify whether CYP2D6 is encapsulated into the DNA assemblies. More experiments are also in progress to design assemblies able to encapsulate the enzyme and potentially stabilize it.

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Chapter Seven

Summary and conclusions

Applying biocatalysts in industrial manufacturing and processing is of increasing interest because of recent environmental concerns. P450 enzymes are especially attractive biocatalysts because of their ability to regio- and stereo-selectively catalyze the incorporation of a single atom of molecular oxygen into inactivated C-H bonds.

To date, most of the investigations to facilitate the industrial applications of P450 enzymes have focused on bacterial P450s because of their solubility, high catalytic activity, high expression yield, and fusion of electron transfer coenzymes for some bacterial species (such as P450BM-3). Substrate specificity and promiscuity, however, limit the applicability of bacterial P450s. Their mammalian counterparts, on the other hand, have exceptionally broad substrate specificity. Like for all P450s, however, their applications are limited by a number of factors including the need for expensive cofactors, low stability, and low tolerance to organic solvents. Work of this thesis has focused on the human P450 2D6 to overcome the handicaps mentioned above.

The use of the cofactor NADPH and cytochrome P450 reductase (CPR) is not economically efficient for large scale production. We have tested cheap chemical peroxides to replace these expensive cofactors. The results indicate that cumene hydroperoxide and *tert*-butyl hydroperoxide can successfully substitute CPR and NAD(P)H with retained regio- and stereo-selectivity. Moreover, with these surrogates, the product formation and initial rate are increased by as much as two fold compared to the use of the natural cofactors.

Many reports have shown that even small proportions of organic solvents in the buffer can deactivate P450 enzymes. We studied the activity of CYP2D6 in organic solvent/buffer biphasic systems. Our results demonstrate that under optimal conditions, maintains as much as 76% of the activity of CYP2D6 is retained when used in buffer/organic emulsions. Product formation in biphasic solvent systems is comparable whether the natural redox partner and cofactor are used, or a surrogate. In addition, a

correlation is observed between the logP and the suitability of a solvent for enzymatic activity, with higher logP resulting in better enzymatic activity. A very hydrophobic substrate, 7-benzyloxy-4-*N,N*-diethylaminomethyl-coumarin (BDAC), was also synthesized to demonstrate the utility of this method.

Some enzymes have also shown significant activity in nearly anhydrous organic solvents. Enzyme lyophilization is usually required to remove water before use in nearly anhydrous solvents. After noticing that this physical process was harmful to P450 activity, we introduced sugars as lyoprotectant during lyophilization. The results show that addition of trehalose or sucrose before lyophilization allows the retention of 80% activity of CYP2D6. CYP2D6 co-lyophilized with trehalose was next tested in selected non-halogenated hydrophobic organic solvents. The enzymatic activity was found to strongly depend on the hydrophobicity of the solvent. Interestingly, the enzyme shows higher catalytic ability in decane or dodecane compared to in the standard buffer.

Finally, we envisaged to use DNA self-assemblies to encapsulate P450s and potentially increase their stability. Indeed, DNA assemblies have many advantages compared to traditional solid supports. The preliminary results show that CYP2D6 favours the formation of cyclic DNA dimers and tetramers over polymers. Characterization of the DNA-bound CYP2D6 activity reveals a minimal loss of activity.

Future work

Although we have overcome many drawbacks limiting the use of human P450 enzymes in organic synthesis, there are more problems to solve before envisaging industrial applications with purified P450s. Some obvious experiments to complement this thesis are listed below:

I. CYP2D6 shows catalytic activity in nearly anhydrous organic solvents. Water activity (a_w), is an important parameter for maximal enzyme activity in organic solvent and was not varied in this thesis. Optimization of a_w may improve the activity further.

II. The preliminary studies of CYP2D6 templated DNA assemblies are interesting. More studies are needed to investigate whether the DNA assemblies are circling the enzyme or sticking on the surface. The use of different DNA building blocks including "tris-branched" ones may unveil a combination able to stabilize P450s.

III. The results showed in this thesis were all obtained with wild-type CYP2D6. Random mutagenesis or directed evolution may provide an alternative strategy to generate mutants with useful properties.

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Surname: Zhao
Street: 801 Sherbrooke West
Zip code: H3A 2K6
City: Montreal
Country: Canada
Fax:
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Journal Title: ChemBioChem
Journal Month: June
Journal Year: 2006
Journal Volume: 7(6)
Media Type: journal
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Appendix II

Reprint of published articles

DOI: 10.1002/cbic.200600006

Replacement of Natural Cofactors by Selected Hydrogen Peroxide Donors or Organic Peroxides Results in Improved Activity for CYP3A4 and CYP2D6

Amandine Chefson, Jin Zhao, and Karine Auclair*[a]

The cytochrome P450 enzymes (P450s or CYPs) form a ubiquitous family of heme proteins able to catalyze the monooxygenation of a wide range of substrates. P450s are of considerable interest in synthetic organic chemistry because of their impressive ability to catalyze the insertion of oxygen into nonactivated C–H bonds. This useful reaction in organic chemistry has received much attention over several decades, but still remains a significant challenge. Some metal catalysts have been successfully used[1] and biomimetic non-heme iron catalysts have been developed,[2] but the regio- and/or stereoselectivity usually remains poor. Biocatalysts such as P450 enzymes represent a promising alternative.[3] One limitation to the use of P450s in synthesis is the need for a complex system of cofactors including NADPH and a redox partner such as cytochrome P450 reductase (CPR) or a ferrodoxin/ferredoxin reductase system. A number of groups have attempted to overcome this drawback. Electrochemical methods,[4] cobaltocene,[5] and cobalt(III) sepulchrate,[6] have all been used to replace the cofactors, albeit with limited success or applicability. Although many P450 enzymes are also known to accept peroxides or aqueous hydrogen peroxide as a source of oxygen (shunt pathway),[7] this pathway is generally not efficient. Some mutants of $P450_{BM-3}$ have been engineered by directed evolution to efficiently use hydrogen peroxide in the absence of cofactors.[8] A heme-domain mutant of $P450_{BM-3}$ has been engineered to catalyze regio- and stereoselective oxidations in the presence of hydrogen peroxide instead of its natural cofactors.[9] The initial reaction rates were, however, significantly lower than those observed with the wild-type enzyme under natural conditions (NADPH). Moreover, this enzyme is naturally very specific for fatty acids and must be mutated to accept any new substrates.[10]

We elected to use the two human P450 isoforms CYP2D6 and CYP3A4 because of their high substrate promiscuity—a significant advantage for use in synthesis. Neither of these enzymes showed significant activity in the presence of aqueous hydrogen peroxide. We show here that without mutagenesis, the two studied isoforms can use various hydrogen peroxide donors or organic peroxides such as sodium percarbonate (SPC), cumene hydroperoxide (CHP), and *tert*-butylhydroperoxide (tBHP) to catalyze reactions in aqueous media. Interestingly, product formation and initial rates are increased by as much as twofold compared to the use of the natural cofactors. Moreover, the regio- and stereoselectivities are maintained.

The activity of CYP2D6 was monitored by using the standard reaction of dextromethorphan demethylation to dextrorphan. All the reactions performed with hydrogen peroxide donors or organic peroxides were compared to the control reaction performed with the natural cofactors (NADPH/CPR) standardized to 100% activity. HPLC traces show a significant amount of unreacted substrate because a very large excess was used (Figure 1). The shoulder on the substrate peak in (B) is assigned to cumene alcohol. The results obtained with cofactor surrogates are summarized in Table 1. The best cofactor replacement was CHP, which, after one hour, yielded up to 210% of the amount of product obtained under the natural conditions. Very low concentrations of CHP were optimal. tBHP also showed good activity (80%) compared to the solid sources of hydrogen peroxide, which yielded at best 53% activity (30 mM SPC, calculated in peroxide equivalents). Mass spectrometric analysis (LC-MS) was used to confirm the identity of the product. Combined with HPLC analysis of *N*- and *O*-demethylated standards, the data allowed us to confirm that the major product was dextrorphan in all cases (*O*-demethylation).

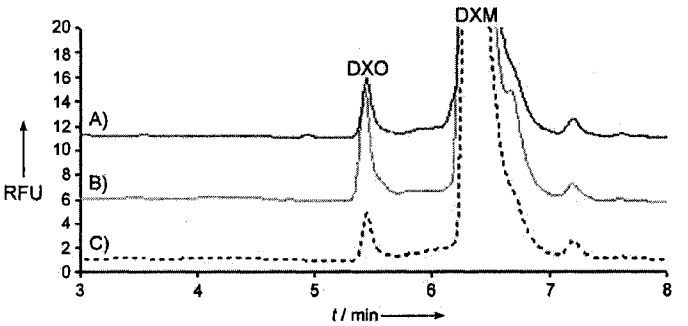

Figure 1. HPLC chromatograms with fluorescence detection (λ_{ex}=280 nm, λ_{em}=310 nm) after incubation of CYP2D6 (0.20 μM) and dextromethorphan (100 μM) in potassium phosphate (0.1 M) at pH 7.4 and 37 °C for 1 h. Reactions with A) CPR (0.83 μM) and NADPH (1.67 mM); B) cumene hydroperoxide (0.1 mM); C) *tert*-butylhydroperoxide (2 mM). The retention times for the substrate dextromethorphan (DXM) and the product dextrorphan (DXO) were 6.2 and 5.4 min, respectively.

Some product formation was also observed in the control reactions without enzyme, generally representing ≤5% of the product formed in the presence of the enzyme. The only exception was with 100 mM sodium perborate (SPB), which yielded more of the product in the absence than in the presence of enzyme (depicted as negative values in Table 1). Aqueous hydrogen peroxide, on the other hand, did not yield detectable products under the conditions tested.

[a] *A. Chefson, J. Zhao, Prof. Dr. K. Auclair*
Department of Chemistry, McGill University
801 Sherbrooke Street West, Montréal, Québec, H3A 2K6 (Canada)
Fax: (+1) 514-398-3797
E-mail: karine.auclair@mcgill.ca

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Table 1. CYP2D6-catalyzed formation of dextrorphan in the presence of various hydrogen peroxide donors or organic peroxides expressed as a percentage of the activity of the reaction with the natural cofactors (CPR and NADPH).

Peroxide equiv [mM]	SPC[a,b]	SPB[a,c]	UHP[a,d]	CHP[a,e]	tBHP[a,f]	H_2O_2(aq)[a,g]
0.1	–	–	–	**210±4**	16±2	n.d.[h]
0.2	–	–	–	197±7	28±7	n.d.
0.5	–	–	–	168±13	50±3	n.d.
1	–	–	–	124±2	69±2	n.d.
2	–	–	–	83±2	**80±4**	n.d.
10	14±4	8±4	17±2	–	–	n.d.
20	48±8	–	–	–	–	n.d.
30	**53**±12	**27±11**	28±4	–	–	n.d.
50	9±14	1±15	**29±3**	–	–	n.d.
100	−17±6	−57±37	16±4	–	–	n.d.

[a] Dextrorphan was quantified by HPLC after 1 h of reaction at 37 °C for CHP and tBHP and 4 h for SPC, SPB, and UHP. The mixtures (300 µL) contained CYP2D6 (0.2 µM, all from the same batch), dextromethorphan (166 µM for SPB, SPC, and UHP or 100 µM for CHP and tBHP) in 0.1 M potassium phosphate at pH 7.4, to which were added various concentrations of hydrogen peroxide donors or organic peroxides. The control reaction was performed with the natural cofactors CPR (0.83 µM) and NADPH (1.67 µM) and standardized to 100% activity. [b] SPC = sodium percarbonate. [c] SPB = sodium perborate. [d] UHP = urea–hydrogen peroxide; [e] CHP = cumene hydroperoxide. [f] tBHP = *tert*-butylhydroperoxide. [g] H_2O_2(aq) = aqueous hydrogen peroxide. [h] n.d. below the detection limit of 0.1 µM (3.5%).

reaction, initially yielding 77% of the natural activity (see Supporting Information). The reaction with SPC was further optimized by varying the buffer concentration and pH, and reached 119% of the activity with CPR/NADPH when 1 M of potassium phosphate at pH 7.0 instead of 7.4 was used. Thus, after optimization, CHP and SPC yield comparable activities, although CHP is effective at lower total concentrations. The other hydrogen peroxide donors and organic peroxides produced less than 50% of the natural activity. The optimal concentration for SPC was 500 mM (calculated in peroxide equivalent), which is about 10 times higher than for CYP2D6, hence the need to increase the buffer strength. In all cases, no product formation was detected in the control reactions without CYP3A4. Finally, aqueous hydrogen peroxide did not lead to the formation of any detectable products.

The activity of CYP3A4 was monitored by using the standard reaction of testosterone 6β-hydroxylation. Potassium phosphate buffer was used at a concentration of 100 mM, except with SPC for which the buffer was optimum at 1 M to offset the expected increase of pH induced by the release of carbonates in the reaction mixture. Again, five hydrogen peroxide donors or organic peroxides were tested (Figure 2, Table 2).

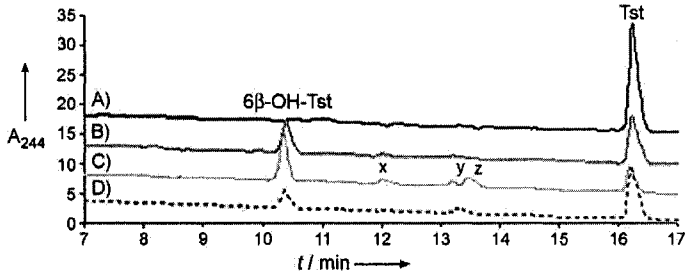

Figure 2. HPLC chromatograms with detection at 244 nm following incubation of CYP3A4 (0.9 µM) and testosterone (5 µM) in potassium phosphate (0.1 M) at pH 7.4 and 37 °C for 4 h, unless otherwise stated. The internal standard cortexolone (14.2 min) was subtracted from the graphs for clarity. A) Control reaction with no cofactor; B) reaction initiated with CPR (3.6 µM) and NADPH (1 mM + 1 mM every 30 min); C) reaction with cumene hydroperoxide (1 mM); D) reaction with sodium percarbonate (equivalent to 500 mM H_2O_2) in potassium phosphate buffer (1 M) at pH 7.0. The retention times for the product 6β-hydroxytestosterone (6b-OH-Tst), and the substrate testosterone (Tst) were 10.4 and 16.2 min respectively. The peaks x, y, and z represent other metabolites formed during the reaction, unidentified but very likely to be the 1β, 15β, and 2β hydroxylated products according to Guengerich and co-workers.[11]

SPC, SPB, and UHP are powders with an active oxygen content equivalent to 27.5%, 32%, and 35% H_2O_2 respectively. These hydrogen peroxide donors present the advantage of releasing H_2O_2 slowly and locally on application, creating milder oxidation conditions around the enzyme than aqueous hydrogen peroxide. This was verified by comparing the activity of CYP3A4 with SPC when the reaction mixture was shaken after addition, which led to complete dissolution of the salt, or when the dissolution occurred slowly over time without shaking. No product formation was detected in the former case; this confirms the need for a slow release of hydrogen peroxide in the reaction mixture.

Interestingly, for both enzymes, the increased product formation observed in the presence of CHP or SPC (Tables 1 and 2) can be explained by a proportional increase in the initial rates of product formation (Table 3). Thus the calculated initial rate for the reaction of CYP2D6 in the presence of 0.1 mM CHP is twice that of the reaction promoted by CPR/NADPH. Similarly, for reactions of CYP3A4, the initial rates are ~30% higher with optimal amounts of SPC or CHP than with CPR/NADPH.

Because peroxides have been reported to react with the heme moiety of P450s and lead to enzyme inactivation,[12] we compared the stability of CYP2D6 and CYP3A4 in the presence of surrogate and natural cofactors. The UV-based measurements typically used to measure P450 stability were not convenient here because of interfering absorption by cofactors. Instead, a large excess of substrate and cofactor(s) was used (fresh cofactor was added

Similarly to CYP2D6, the best activity was obtained with CHP and reached 132% of the activity promoted by the natural cofactors (CPR and NADPH). Relatively low concentrations of CHP were sufficient. SPC was also found to efficiently support the

Table 2. Formation of 6β-hydroxytestosterone by CYP3A4 with hydrogen peroxide donors or organic peroxides expressed as a percentage of the activity of the reaction with the natural cofactors (CPR and NADPH).

Peroxide equiv [mM]	SPC[a,b]	SPB[a,c]	UHP[a,d]	CHP[a,e]	tBHP[a,f]	H_2O_2(aq)[a,g]
0.1	–	–	–	83±8	6±2	n.d.[h]
0.5	–	–	–	114±12	13±4	n.d.
1	–	–	–	**132±17**	21±2	n.d.
2	–	–	–	92±10	**35±3**	n.d.
5	–	–	–	61±6	16±1	n.d.
50	28±3	2±2	2±1	–	–	n.d.
100	47±5	4±3	3±3	–	–	n.d.
500	**119[h]±12**	**10±3**	**7±4**	–	–	n.d.
1000	28±4	6±2	4±3	–	–	n.d.

[a] Analysis of 6β-hydroxytestosterone formation by HPLC after 1 h of incubation of CYP3A4 (0.9 μM) and testosterone (115 μM) at 37 °C, initiated with various concentrations of hydrogen peroxide donors or organic peroxides. The buffer was 0.1 M potassium phosphate at pH 7.4, and 1 M potassium phosphate at pH 7.4 for the reaction with SPC. The control reaction was performed with the natural cofactors CPR (2.4 μM) and NADPH (1 mM). [b] SPC = sodium percarbonate. [c] SPB = sodium perborate. [d] UHP = urea-hydrogen peroxide adduct. [e] CHP = cumene hydroperoxide. [f] tBHP = *tert*-butylhydroperoxide. [g] H_2O_2(aq) = aqueous hydrogen peroxide. [h] Several potassium phosphate buffer concentrations and pHs were tested with 500 mM SPC, 1 M potassium phosphate at pH 7.4 gave 77% activity, while the highest activity of 119% was obtained at pH 7.0. [i] n.d.: below the detection limit of 0.1 μM (1%).

Table 3. Initial rates of product formation and maximum turnover numbers for the CYP3A4-catalyzed 6β-hydroxylation of testosterone and CYP2D6-catalyzed formation of dextrorphan in the presence of CPR/NADPH or peroxide equivalents.

Cofactor	CYP3A4[a] CPR/NADPH	CHP[c]	SPC[d]	CYP2D6[b] CPR/NADPH	CHP	tBHP[e]
initial rate [μmol μmol^{-1} min^{-1}]	5.9±0.9	9.1±1.5	7.1±1.2	2.4±0.3	4.7±0.3	2.2±0.2
turnover	46±4	42±5 50±4*	26±2	107±3	92±2	41±2

[a] 6β-Hydroxytestosterone formation as measured by HPLC after incubation of CYP3A4 (0.9 μM) and testosterone (115 μM) at 37 °C for different times. The turnover number was calculated after 4 h of reaction. The buffer was 0.1 M potassium phosphate at pH 7.4, and 1 M potassium phosphate at pH 7.0 for the reaction with SPC. The cofactors tested were either CPR (3.6 μM) and NADPH (1 mM + 1 mM added every 30 min), CHP (1 mM + 1 mM added every 30 min, or *0.1 mM + 0.1 mM added every 30 min) or SPC (500 mM). [b] Dextrorphan formation as measured by HPLC after incubation of CYP2D6 (0.18 μM) and dextromethorphan (100 μM) at 37 °C. The turnover number was calculated after 4 h of reaction. The buffer was 0.1 M potassium phosphate at pH 7.4. The cofactors tested were either CPR (0.53 μM) and NADPH (1.67 mM + 1.67 mM added every 30 min), CHP (0.1 mM + 0.1 mM added every 30 min) or tBHP (2 mM). [c] CHP = cumene hydroperoxide. [d] SPC = sodium percarbonate, [e] tBHP = *tert*-butyl hydroperoxide.

every 15 or 30 min), and product formation was monitored over time. The enzyme was considered inactive when product formation reached a plateau. Under these conditions, CYP2D6 lost activity after ~1 h in the presence of CHP or tBHP, but only after ~1.7 h when the natural cofactors were used (Figure 3). This difference in stability is much smaller than expected based on previous literature,[12] most likely because of the very small amount of organic peroxides used. It should be noted that the results of Table 1 can only be compared to Figure 3 for the first 30 min. Indeed, Figure 3 was obtained after multiple additions of CPR/NADPH or CHP, whereas the results of Table 1 were collected after optimization with a single addition of the cofactors. Although CYP2D6 displays lower stability in the presence of CHP compared to CPR/NADPH, its higher initial rate with CHP results in comparable maximum turnover numbers when compared to CPR/NADPH (Table 3). CYP3A4, on the other hand, shows comparable stability whether CPR/NADPH or CHP are used (~3 h, Figure 4). This was not expected based on previous literature,[12] and is again most likely explained by the small amount of CHP used. When the quantity of CHP added is further reduced (multiple additions of 0.1 mM instead of 1 mM, see Table 3 and Figure 4) the initial rate decreases; however, the enzyme is active for ~4 h, and the maximum turnover number rises (still comparable to CPR/NADPH). Here again, the results of Table 2 can only be compared to Figure 4 for the first 15 min (time of the second addition of NADPH).

In summary, CYP3A4 and CYP2D6 represent ideal biocatalysts because of their high substrate promiscuity and their ability to catalyze chemically challenging hydroxylations at inactivated C–H bonds. We report an improvement of up to twofold in reaction rates when the natural cofactors of CYP2D6 or CYP3A4 are replaced with SPC or CHP. Although preliminary studies with the CYP2D6 substrate AMMC (3-[2-(*N*,*N*-diethyl-*N*-methylammonium)ethyl]-7-methoxy-4-methylcoumarin) suggested a similar trend (data not shown), rates and rate increases are likely to vary from one substrate to another. This is nonetheless the first time that such an improvement compared to the use of natural cofactors is reported for P450 enzymes. This was accomplished with much lower amounts of cofactor surrogates than previously reported, which likely allowed minimization of side reactions. These results suggest that the need for expensive cofactors can be circumvented without significant decreases in yields by replacing them with very cheap commercial compounds, directly with the wild-type enzymes. We anticipate that this alternative approach will apply to other P450 enzymes and will be of considerable use for future applications of P450s in synthesis.

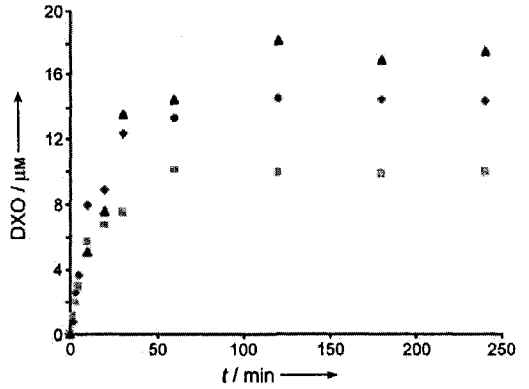

Figure 3. Dextrorphan (DXO) formation as measured by HPLC after incubation of CYP2D6 (0.18 μM) and dextromethorphan (100 μM) in potassium phosphate buffer (0.1 mM) at pH 7.4 and 37 °C (total volume 300 μL). Each point on the plot represents an average of two measurements. The reaction was initiated with CHP (0.1 mM + 0.1 mM every 30 min; ♦), tBHP (2 mM; ■), or CPR (0.5 μM) and NADPH (1.67 mM + 1.67 mM every 30 min; ▲).

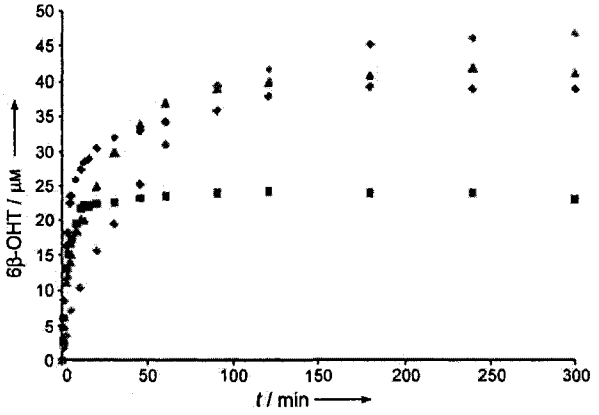

Figure 4. 6β-Hydroxytestosterone formation as measured by HPLC (244 nm) after incubation of CYP3A4 (0.9 μM) and testosterone (115 μM) in 0.1 M potassium phosphate buffer at pH 7.4, or 1 M buffer at pH 7.0 with SPC at 37 °C for different times. Each point on the plot represents an average of two measurements; a total reaction volume of 300 μL was used for each. The reaction was initiated with CHP (1 mM + 1 mM added every 30 min (♦), 0.1 mM + 0.1 mM added every 30 min (◆)), SPC (500 mM; ■), or CPR (3.6 μM) and NADPH (1 mM + 1 mM after 15 min then every 30 min; ▲).

Acknowledgements

The authors wish to acknowledge Profs. J. R. Halpert from the University of Arizona, F. P. Guengerich from Vanderbilt University, and Charles B. Kasper from the University of Wisconsin–Madison for their kind gift of the CYP3A4, CYP2D6, and CPR expression plasmids, respectively. This work was supported by the Natural Science and Engineering Council of Canada (NSERC), Astra Zeneca Canada Inc., Boehringer Ingelheim Canada Ltd., and Merck Frosst Canada & Co.

Keywords: biotechnology · cofactors · cytochrome P450 · hydrogen peroxide · hydroxylation

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Received: January 9, 2006
Published online on May 3, 2006

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Replacement of the Natural Cofactors by Selected Hydrogen Peroxide Donors or Organic Peroxides Results in Improved Activity for CYP3A4 and CYP2D6

Amandine Chefson, Jin Zhao, Karine Auclair*

Chemicals

The CYP3A4 pSE3A4His expression plasmid was a gift from Dr. J. R. Halpert from the University of Arizona, the CYP2D6 expression plasmid (DB6 with His_5 and M374V) was obtained from Dr. F. P. Guengerich from Vanderbilt University, and the cytochrome P450 reductase OR263 plasmid was kindly donated to us by Dr. Charles B. Kasper from the University of Wisconsin. The culture media ingredients yeast extract, tryptone, peptone, as well as the CYP2D6 substrate AMMC (3-[2-(*N,N*-diethyl-*N*-methylammonium)-ethyl]-7-methoxy-4-methylcoumarin) and the product AHMC (3-[2-(diethylamino)ethyl]-7-hydroxy-4-methylcoumarin hydrochloride) were purchased from BD Biosciences (San Jose, CA). DH5a supercompetent cells and agarose were from Invitrogen (Carlsbad, CA). The 2',5'-ADP Sepharose 4B and DEAE Sepharose resins were from Amersham Bioscience (Baie d'Urfé, QC, Canada). The His-Select™ Nickel Affinity Gel was purchased from Sigma (St. Louis, US). The CYP3A4 substrate testosterone (4-androsten-17β-ol-3-one) was a king gift from Dr. Eisenberg in our department and the metabolites 6β-hydroxytestosterone (4-androsten-6β,17β-diol-3-one) and 6α-hydroxytestosterone (4-

androsten-6α,17β-diol-3-one) were purchased from Steraloids (Newport, RI). All solvents were purchased from Fisher and were of HPLC grade. All chemicals were used without further purification. Water was obtained from a Milli-Q Synthesis (Millipore, San Jose, CA) filtration system. All other chemicals were purchased from Sigma or Aldrich.

Instruments

UV absorption spectra were recorded on a Cary 5000 UV spectrophotometer (Varian, Mississauga, ON, Canada). Fluorescence measurements were obtained on a Spectramax GeminiXS (Molecular Devices Corp., Sunnyvale, CA) using 96-well flat bottom assay plate (Corning Incorporated Life Sciences, New York, NY). Analytical HPLC analyses were performed on an Agilent 1100 modular system consisting of an auto-sampler, a quaternary pump system, a photodiode-array detector, a fluorescence detector, and a thermostated column compartment. The Agilent Chemstation software version A.10.02 was used to control the operation and data acquisition. Analysis of dextromethorphan *O*-demethylation by CYP2D6 was carried out using a Synergi 4 µm Hydro-RP 80 Å column with mobile phase A (0.05% TFA in water) and B (100% acetonitrile) at a flow rate of 1 mL/min. The elution gradient was from 20% to 90% B over 6 min, then held for 2 min. Fluorescence ($?_{ex}$ = 280 nm, $?_{em}$ = 310 nm) was used to monitor the elution. The retention times for dextromethorphan and dextrorphan were 5.4 and 6.3 min respectively. Analysis of testosterone 6β-hydroxylation by CYP3A4 used a 150 x 4.6 mm Zorbax Eclipse XDB-C8 5µm column from Agilent protected by an analytical guard column. The elution consisted of a first isocratic step at 15% acetonitrile in water for 4 min, before a linear gradient to 50% acetonitrile over 12 min. The flow rate was 1.5 mL/min, and the column temperature was set to 30°C. Detection was at 244 nm. Under these conditions, the retention times of testosterone, 6β-hydroxytestosterone, 6α-hydroxytestosterone and cortexolone were 16.3, 9.9, 10.5 and 14.2 min respectively.

Expression and purification of His-tagged CYP2D6

The CYP2D6 plasmid was transformed into *E. coli* DH5a supercompetent cells and grown overnight at 37°C on Luria-Bertani (LB) agar plates with ampicillin (100 mg/L). LB media consisted of: 10 g tryptone, 5 g yeast extract and 10 g NaCl per liter, autoclaved for 20 min at 121°C. One separate colony was added to Terrific Broth (TB) medium (5 mL prepared from: 12 g tryptone, 24 g yeast extract, 4 mL glycerol, 2.31 g KH_2PO_4,

12.54 g K_2HPO_4 per liter, autoclaved) containing ampicillin (100 mg/L). After incubation (300 rpm, 37°C, 17 h), a portion of this culture (2 mL) was diluted in TB (100 mL) containing ampicillin (100 mg/L), potassium dihydrogen phosphate (0.23 g) and potassium hydrogen phosphate (1.25 g). After a second incubation (180 rpm, 33°C, 5 h), this mixture was further diluted in TB (10 mL in 500 mL) containing ampicillin (100 mg/L), trace elements solution (150 µL), potassium dihydrogen phosphate (1.1 g), and potassium hydrogen phosphate (5.23 g). The trace elements solution consisted of 100 mM $FeCl_3$, 10 mM $ZnCl_3$, 10 mM Na_2MoO_4, 14.3 mM $CaCl_2$, 7.4 mM $CuCl_2$, 10 mM H_3BO_3 in 1 mL of concentrated HCl + 9 mL of MilliQ water. The culture was fermented for 30 min at 33°C and 250 rpm. Next, after addition of the heme precursor δ-aminolevulinic acid (δ–ALA, 8.4 mg/L), thiamine (0.68 g/L) and quinidine (0.03 mM), the culture was incubated at 37°C and 180 rpm until OD_{600} 0.7-0.8 (~2.5 h). Protein expression was induced with β-D-thiogalactoside (IPTG, 0.5 mM) and the culture was further grown at 25°C and 150 rpm for 48 h.

The cells were harvested by centrifugation (4000 *g*, 25 min), and the harvested cells (100 g) were suspended in TES buffer (12 mL/gram cells of: 500 mM Tris acetate at pH 7.5, 250 mM sucrose, 0.25 mM EDTA), followed by addition of lysozyme (3 mg/g cells). The same volume of pre-cooled water was added and gently stirred for 30 min at 4°C before centrifugation (3393 *g*, 4°C, 15 min). The pellets were resuspended in sonication buffer (30 mL of: 0.1 M potassium phosphate buffer at pH 7.4 containing 20% glycerol (*v/v*) and 6 mM magnesium acetate). The cells were further lyzed by sonication (7 times at 60% duty cycle and power 8 for 20 s each time) after addition of leupeptin (1 µg/mL), aprotinin (0.04 µg/mL), bestatin (1 µM) and ß-mercaptoethanol (20 mM). A salt-ice bath was applied to keep the mixture cool. Following centrifugation (10 000 *g* for 20 min and 75 000 *g* for 60 min at 4°C), the supernatant was loaded onto the nickel affinity column (1 mL) equilibrated with buffer C (20 mM potassium phosphate buffer at pH 7.4 containing 20% glycerol, 2 g/L 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 0.5 M potassium chloride), and buffer D (buffer C containing 10 mM ß-mercaptoethanol). The column was washed with buffer D (10 mL) and buffer E (10 mL of: 20 mM potassium phosphate buffer at pH 7.4 containing 20% glycerol (*v/v*), 0.5 M potassium chloride, and 10 mM ß-mercaptoethanol). The protein was eluted

with buffer E without ß-mercaptoethanol but containing imidazole (200 mM). The colored fractions were pooled and dialyzed for 4 h against buffer F (2 × 4 L of 10 mM potassium phosphate buffer at pH 7.4 containing 0.13 mM EDTA and 0.1 mM DTT). The fractions containing 50 kDa proteins were identified by SDS-PAGE (Homogeneous 12.5%). The overall yield was calculated to be 0.563 mg/L.

Expression and purification of His-tagged CYP3A4

CYP3A4 was expressed using a modification of the procedure used by Domanski *et al.*[1] The plasmid was transformed into *Escherichia coli* DH5α supercompetent cells, and the cells grown overnight on LB agar plates with ampicillin (50 μg/mL). Culture tubes containing LB media supplemented with ampicillin (50 μg/mL) were inoculated next. After 24 h of growth at 37°C with shaking at 250 rpm, 10 flasks of 750 mL TB medium containing ampicillin (50 μg/mL) were inoculated each with 3 intermediate culture tubes. The flasks were placed at 37°C with shaking at 250 rpm for 2 h (i.e. until OD_{600} = 0.6), after which protein expression was induced with IPTG (1 mM) followed by addition of δ-ALA (80 mg/mL). The incubation temperature was reduced to 30°C, with shaking set to 190 rpm for 48 h. The cells were collected by centrifugation at 4000 *g* and 4°C for 15 min. The pellets were resuspended in buffer A (50 mL of: 100 mM 3-[*N*-morpholino]propanesulfonic acid] (MOPS) at pH 8 containing 10% glycerol, 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride (PMSF)). All subsequent procedures were performed at 4°C. Lysozyme (10 mg/g of cells) was added, and the suspension was stirred slowly for 15 min, after which the suspension was supplemented with protease inhibitors (1.6 μg/mL leupeptin, 1 μg/mL aprotinin, 0.8 μg/mL bestatin, and 0.7 μg/mL pepstatin A). The suspension was then sonicated on an ice-salt bath at 60% and power 8. After 2 sonication cycles of 20 s each, the suspension was centrifuged at 100 000 *g* for 60 min. The supernatant was removed and the pellets resuspended in buffer B (10 mL of: 100 mM MOPS at pH 7.4, containing 10% glycerol and 2 mM PMSF). CHAPS (0.5%) and potassium chloride (0.5 M) were then added. Cells were homogenized and gently stirred for 2 h before centrifugation (1 h at 100 000 *g*). This CHAPS-solubilized P450 preparation was then stored at -80°C until the next step.

The His-tagged protein was purified by affinity column chromatography using the Ni-NTA metal affinity resin (1.5 mL), pre-equilibrated with 5 bed volumes of EQ-buffer

(buffer B containing 0.5% CHAPS, 0.5 M KCl, and 5 mM imidazole) at a flow rate of 0.5 mL/min. The P450 preparation was then loaded onto the column at the same flow rate. The column was washed with 10 bed volumes of EQ-buffer, then with 10 bed volumes of 100 mM MOPS buffer at pH 7.4 containing 10% glycerol and 10 mM imidazole. The P450 was eluted in EL-buffer (100 mM MOPS at pH 7.4 containing 10% glycerol and 200 mM imidazole). The orange-colored fractions were compared spectroscopically for protein content and by SDS-PAGE (12.5% homogeneous gels) for purity assessment. The fractions containing the CYP3A4 protein were pooled and dialyzed (twice 4 L of: 100 mM MOPS buffer at pH 7.4 containing 10% glycerol, 0.2 mM dithiothreitol and 1 mM EDTA) at 4°C. The sample was aliquoted and stored at -80°C. The overall yield was 0.6 mg per liter of culture.

Quantification of P450 enzymes

The P450 content was measured by reduced carbon monoxide difference spectra following the method described by Omura and Sato[2]. Thus heme was reduced by addition of a few solid grains of sodium dithionite ($Na_2S_2O_4$) in quartz cuvettes containing the buffered enzyme solution, and formation of the enzyme-carbon monoxide complex was achieved by slow bubbling of CO gas into the reduced enzyme solution for about 20 s. The UV absorption spectra was recorded, and the concentration of P450 was determined using the extinction coefficient $\varepsilon_{450} = 91\ mM^{-1}\ cm^{-1}$.

Cytochrome P450 reductase (CPR) expression and purification

E. coli DH5a supercompetent cells were transformed with the cytochrome P450 reductase OR263 plasmid and spread onto LB medium agar plate containing ampicillin (100 mg/L) and incubated overnight at 37°C. Four separate colonies were added to LB medium (6 mL) containing ampicilin (100 mg/L). The culture was incubated overnight (225 rpm, 37°C, 17 h) before dilution in TB (3 mL in 1 L) supplemented with ampicillin (100 mg/L) and riboflavin (1 mg/L). CPR expression was induced with IPTG (0.5 M) at OD_{600} ~ 0.8 (~3 h at 37°C and 225 rpm). The cultures were further grown at 37°C for 20 h. Unless mentioned otherwise, all the steps in the protein purification were carried at 4°C and storage was at -80°C. Cells were collected by centrifugation (4614 *g*, 25 min) and the harvested cells (46.8 g) were resuspended in TSE buffer (60 mL). Lysozyme

(1.2 mg) was added and the mixture was incubated for 20 min. After centrifugation (3000 *g*, 30 min), the pellet was lysed for 20 min in lysis buffer (60 mL, containing 50 mM Tris base at pH 8.0, 0.5 M EDTA, 10 mg/L aprotinin, and 1 mM PMSF). The cells were further sonicated 7 times at 60% duty cycle and power 8 for 30 s each time with salt-ice bath cooling. Centrifugation (12 000 *g*, 10 min, 4°C) was used to remove the supernatant and the pellet was sonicated again in the same matter. After the second centrifugation, the two batches of supernatant were combined and suspended in affinity buffer (120 mL of: 50 mM Tris base at pH 8.0 containing 10% glycerol (*v/v*), 0.1% Triton X100 (*v/v*), 0.1 mM EDTA, and 0.05 mM DTT). After another centrifugation (41 000 *g*, 45 min), PMSF (1 mM) was added. A homogenous solution was obtained after 3 h of gentle stirring. The protein solution was applied to a 2',5' ADP Sepharose 4B column (16 mL) equilibrated with affinity buffer (60 mL). The column was first washed with buffer A (40 mL of affinity buffer containing 0.5 mM adenosine), followed by elution of the protein with buffer B (affinity buffer containing 2 mM adenosine 2'-monophosphate (2'-AMP)). The fractions containing the 70 kDa protein were pooled. The sample was loaded onto a fast flow DEAE Sepharose column (8 mL) equilibrated with DEAE-EQ buffer (100 mL of: 0.2 M potassium phosphate buffer at pH 7.4 containing 20% glycerol (*v/v*) and 1 g/L CHAPS), and washed with buffer C (16 mL of: DEAE-EQ buffer containing 10 mM ß-mercaptoethanol). The column was washed with buffer C (40 mL) and eluted with a linear gradient increasing from 0.1 to 0.5 M potassium chloride in buffer C. The fractions containing the 70 kDa protein were identified by SDS-PAGE (homogeneous 12.5%) and dialyzed against buffer G (1 L of 0.1 M potassium phosphate buffer at pH 7.4 containing 20% glycerol (*v/v*). The overall yield was calculated to be 0.643 mg/L.

CPR concentration and activity assay

The concentration of CPR was determined by oxidation with potassium ferricyanide. An aliquot of CPR (130 µL) was diluted in 100 mM potassium phosphate buffer at pH 7.6 (266 µL) before addition of potassium ferricyanide (7 µM). The concentration was calculated using an extinction coefficient of 21.2 mM^{-1} at 455 nm for the oxidized form of CPR. The activity of CPR was evaluated by cytochrome c reduction in the presence of NADPH. The assay mixture contained CPR (0.5 µM), cytochrome c (50 µM), and NADPH

(100 μM) in 300 mM phosphate buffer at pH 7.6. The blank did not contain NADPH. Cytochrome c reduction by CPR is revealed by absorption peaks at 520 and 550 nm.

AMMC demethylation by CYP2D6

AMMC was dissolved in acetonitrile. For the reference reaction, a mixture (300 μL) containing AMMC (200 μM), CPR (0.7 μM), CYP2D6 (0.12 μM) in potassium phosphate buffer (0.1 M at pH 7.4) was incubated at 37°C for 5 min. The reaction was initiated with the addition of NADPH (0.83 mM) and monitored by fluorescence (λ_{ex} = 390 nm, λ_{em} = 460 nm) for 15-20 min. Blank reactions were performed in parallel without CYP2D6.

Dextromethorphan demethylation by CYP2D6

Dextromethorphan (DXM) was demethylated to dextrorphan (DXO) by CYP2D6 in the presence of cofactors CPR and NADPH or hydrogen peroxide donors. In the reference reaction, the mixture (300 μL) containing CYP2D6 (200 nM), CPR (825 nM), and DXM (167 μM) in potassium phosphate buffer (0.1 M, pH 7.4) was incubated for 5 min at 37°C. The reaction was initiated with the addition of NADPH (3 mM) and incubated for 1 or 4 h at 250 rpm and 37°C. The reaction was quenched with 23% (*v/v*) perchloric acid (100 μL). For the reactions with the hydrogen peroxide donors, CYP2D6 (200 nM) and DXM (167 μM with SPB, SPC, or UHP, and 100 μM with CHP or tBHP) and the mixture did not contain CPR/NADPH. The reactions were initiated by one of different hydrogen peroxide donors (10 mM to 100 mM) or organic peroxides (0.1 mM to 2.5 mM). The reaction was quenched with 23% (*v/v*) perchloric acid (100 μL) after incubated for 2 or 4 h at 250 rpm and 37°C. The range of concentrations used for hydrogen peroxide, cumene hydroperoxide and *tert*-butyl hydroperoxide was 0.25 mM to 2.5 mM. The quenched mixtures were centrifuged (16000 *g*, 10 min). The supernatants were filtered through 0.2 μM Teflon filter units before HPLC analysis. Blank reactions without CYP2D6 were carried in parallel.

Quantification of 6β-hydroxytestosterone

Quantification of the 6β-hydroxytestosterone formed during the enzymatic reaction was achieved with a calibration curve obtained by spiking the incubation mixture (no CYP) with 6β-hydroxytestosterone and cortexolone as the internal standard. Concentra-

tions ranging from 0.5 to 5 nmol/mL were used for the calibration curve which was generated by plotting the area ratio (6β-hydroxytestosterone/cortexolone) against the concentration of 6β-hydroxytestosterone. A linear regression plot of peak-area ratio versus concentration was constructed and the concentration of 6β-hydroxytestosterone determined from the peak-area ratio relative to the calibration graph.

Testosterone hydroxylation assays for CYP3A4

The reaction mixtures (300 μL) were prepared by mixing CYP3A4 (580 pmol, 50 μL of a 3.5 μM enzyme solution) and testosterone (115 μM) in potassium phosphate buffer (100 mM, pH 7.4). The reference reaction contained the same components plus CPR (1.5 nmol). The molar ratio of CYP3A4:CPR of 1:4 was selected with regard to near optimal activity as shown by the study of Wang *et. Al.*[3] After 5 min of preincubation at 37°C, the reaction was initiated using various concentrations of SPC, SPB, UHP, CuOOH, *t*BuOOH, or aqueous H_2O_2, and NADPH (1 mM) for the reference reaction. After addition of the solid SPC, SPB or UHP, the reaction mixture was not immediately shaken, to allow the slow release of the hydrogen peroxide as the dissolution occurred. After 15 min the mixture was homogenized by gentle shaking. The reaction was terminated after 1 h of incubation at 37°C by addition of methylene chloride (500 μL total) immediately followed by introduction of the internal standard cortexolone (15 μL of a 200 μL solution in methanol). The sample was vigorously vortexed, the layers separated by centrifugation (1500 *g* for 2 min), and the organic layer was transferred to a dry vial. The extraction was repeated twice with CH_2Cl_2 (500 μL), and the pooled organic extracts were evaporated to dryness using a roto-evaporator. The residue was redissolved in HPLC-grade methanol (150 μL), vigorously vortexed, sonicated and filtered through a polypropylene syringe filters (0.2 μm pore size, National Scientific Company) before injection on the HPLC. The identity of the products was established by co-elution with authentic standards and by mass spectroscopy. The blank incubation assays were lacking either CYP and NADPH, or the peroxide surrogates, and did not show any detectable products. The extraction efficiency, or recovery, of this procedure was estimated by comparing the peak-area of extracted standards to those of non-extracted standards, and was superior to 96% for both 6β-hydroxytestosterone and testosterone at the concentrations used.

Effect of the buffer in reactions with sodium percarbonate: In order to evaluate the importance of the buffer composition for the reaction with SPC, the experiment was repeated using the conditions described above but in 12 different buffers: 3 potassium phosphate concentrations (0.5 M, 0.75 M and 1 M), and 4 pHs for each (6.0, 6.5, 7.0 and 7.5).

Table S1: Effect of the buffer in CYP3A4 reactions with sodium percarbonate.

Potassium phosphate concentration	pH	% of the activity with CPR/NADPH
500 mM	6.0	86 ± 2.2
	6.5	78 ± 4.3
	7.0	86 ± 2.8
	7.5	15 ± 1.5
750 mM	6.0	61 ± 6.6
	6.5	82 ± 8.1
	7.0	99 ± 4.7
	7.5	63 ± 3.7
1 M	6.0	54 ± 4.1
	6.5	81 ± 5.8
	7.0	**119** ± 3.4
	7.5	77 ± 2.4

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COMMUNICATION TO THE EDITOR

BIOTECHNOLOGY and BIOENGINEERING

Activity of Human P450 2D6 in Biphasic Solvent Systems

Jin Zhao, Elaine Tan, Julian Ferras, Karine Auclair

Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montréal, Québec, Canada H3A 2K6; telephone: (+1)514-398-2822; fax: (+1)514-398-3797; e-mail: karine.auclair@mcgill.ca

Received 7 February 2007; accepted 26 March 2007

Published online 26 April 2007 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21449

ABSTRACT: Several limitations have restricted the use of P450 enzymes in synthesis, including the narrow substrate specificity of some P450 isoforms, the need for a redox partner and an expensive cofactor, incompatibility with organic solvents, and poor stability. We previously demonstrated that the natural redox partner and cofactor of the promiscuous P450s 3A4 and 2D6 can be efficiently substituted with some cheap hydrogen peroxide donors or organic peroxides. We report here that P450 2D6 maintains as much as 76% of its activity when used in buffer/organic emulsions. Product formation in biphasic solvent systems is comparable whether the natural redox partner and cofactor are used, or a surrogate. As reported for other enzymes, a correlation is observed between the logP and the suitability of a solvent for enzymatic activity. Moreover, the utility of our system was established by demonstrating the transformation of a novel hydrophobic substrate, not modified by P450 2D6 in the absence of organic solvent.

Biotechnol. Bioeng. 2007;98: 508–515.

KEYWORDS: biocatalysis; biphasic; CYP; hydroxylation; organic; solvent

Introduction

P450 enzymes (P450s, CYPs) have attracted the interest of chemists in part because of their impressive ability to catalyze the insertion of oxygen into inactivated C–H bonds (van Beilen and Funhoff, 2005). Very few chemical catalysts are known that directly hydroxylate aliphatic or aromatic C–H bonds, and most are not selective or of limited scope (Crabtree, 2004; Das et al., 2006; Li and Chang, 2004). Biocatalysts such as P450s represent a promising alternative; however, a number of limitations have restricted their use in synthesis. These include the narrow substrate specificity of some P450 isoforms, the need for one or more redox partners and NAD(P)H, incompatibility with organic solvents, low activity, and poor stability. Unlike lipases and esterases for which significant progresses have been achieved to overcome the drawbacks associated with their handling (Gotor-Fernández et al., 2006; Straathof et al., 2002), little advancements have been made with oxidoreductases such as P450s (Bühler and Schmid, 2004; Chefson and Auclair, 2006; Gillam, 2005; Urlacher and Eiben, 2006). Most of the work with purified P450s has focused on bacterial enzymes and was aimed at modulating substrate specificity and improving stability (Bernhardt, 2006; Eiben et al., 2006). A few investigations concerning the activity of P450s in organic solvents have been reported. Carbon tetrachloride (Manno et al., 1988) and halothane (Manno et al., 1991) were shown to inactivate human liver microsomal P450s by irreversibly modifying the heme cofactor. A similar inactivation process was observed with *N,N*-dimethylformamide and *N,N*-dimethylacetamide (Tolando et al., 2001). Small percentages of commonly used water soluble organic solvents such as acetonitrile and methanol produce a significant inhibition of the activity of human P450s (Busby et al., 1999; Easterbrook et al., 2001; Hickman et al., 1998). Directed evolution has been used to engineer bacterial P450-BM3 for improved tolerance to organic solvents (Wong et al. 2004). The activity of the mutant enzyme increased 10-fold in the presence of THF (2% v/v) and 6-fold when DMSO (25% v/v) was added, compared to the wild-type enzyme. Molecular dynamics simulations of P450 BM-3 in DMSO/water mixtures suggest a conformational shift blocking the substrate access channel (Roccatano et al., 2005). Mutants of P450 102A1 were

Correspondence to: K. Auclair

Contract grant sponsor: Natural Science and Engineering Research Council of Canada (NSERC)

Contract grant sponsor: Astra Zeneca Canada, Inc.

Contract grant sponsor: Boehringer Ingelheim Canada Ltd.

Contract grant sponsor: Merck Frosst Canada & Co

This article contains Supplementary Material available via the Internet at http://www.interscience.wiley.com/jpages/0006-3592/suppmat.

prepared that catalyze the hydroxylation of cyclohexane, octane and myristic acid in biphasic solvent systems with cofactor recycling (Maurer et al., 2005). Finally, P450 2B1 was the first mammalian P450 laboratory-evolved to generate a mutant with enhanced catalytic tolerance to DMSO (Kumar et al., 2006). Two mechanisms have been proposed to explain the deactivation of enzymes by organic solvents, protein denaturation and displacement of enzyme-bound water molecules (Burke et al., 1992; Gorman and Dordick, 1992; Schulze and Klibanov, 1991; Zaks and Klibanov, 1988a,b).

Substrate promiscuity and the ability to show activity in the presence of organic solvents and in the absence of biological cofactors are great assets for biocatalysts. We have selected the very promiscuous human P450 2D6 to overcome the substrate specificity issue. We recently reported that the cheap reagents cumene hydroperoxide and *tert*-butylhydroperoxide can be used with P450 2D6 to efficiently replace its natural redox partner cytochrome P450 reductase (CPR), and cofactor nicotinamide adenine dinucleotide phosphate (NADPH) (Chefson et al., 2006). We report here that P450 2D6 shows very good activity with CPR/NADPH or cumene hydroperoxide in selected biphasic solvent systems.

Results and Discussion

Choice of Substrates

Most of the commercial substrates of mammalian P450 2D6 were discovered through drug metabolism studies and are therefore water soluble (Rendic, 2002). The majority of synthetic projects however, involve molecules insoluble in water. It was envisaged to use both water soluble and insoluble substrates to investigate the behavior of P450 2D6 in biphasic solvent systems. Based on previous studies (Chauret et al., 2001; Onderwater et al., 1999), we designed the P450 2D6 substrate 7-benzyloxy-4-*N*,*N*-diethylamino-methyl-coumarin (BDAC). The P450 2D6 substrate BDAC was prepared using modified literature procedures (Smitha and Reddy, 2004), (see supporting information for more details). BDAC is not soluble in water but highly soluble in chloroform and acetonitrile, and slightly soluble in hexane and isooctane. This contrasts well with the known P450 2D6 substrate dextromethorphan which is soluble in water but poorly soluble in non-halogenated hydrophobic organic solvents.

Transformation of Dextromethorphan and BDAC by P450 2D6 in Buffer

HPLC analysis confirmed that the *O*-demethylation of dextromethorphan to dextrorphan by P450 2D6 proceeds well in buffer in the presence of either CPR/NADPH or cumene hydroperoxide. With BDAC however, no product was detected in the absence of organic solvent, likely due to a lack of solubility of this substrate in water. When acetonitrile (5%) was added to the buffer; however, BDAC was *N*-deethylated to 7-benzyloxy-4-*N*-ethylaminomethyl-coumarin (BEAC) by P450 2D6 (Fig. 1). The yield was comparable whether the natural redox partner and cofactor, or the surrogate cumene hydroperoxide were used. The expected product of 7-*O*-debenzylation (4-*N*,*N*-diethylaminomethyl-coumarin, DAC) was not detected. Besides BEAC, the only other product was minor (<5%, retention time of 13.1 min, too small to be visible on Fig. 1B). Although the reaction of P450 2D6 with these two substrates result in overall dealkylations, the major catalytic role of the enzyme in these transformations is to hydroxylate the carbon adjacent to the heteroatom and yield an unstable hemiacetal (dextromethorphan) or hemiaminal (BDAC). These functional groups are known to be unstable and spontaneously break down to the corresponding aldehyde, and alcohol or amine. Therefore, even though these assays do not allow the direct assessment of the enzyme

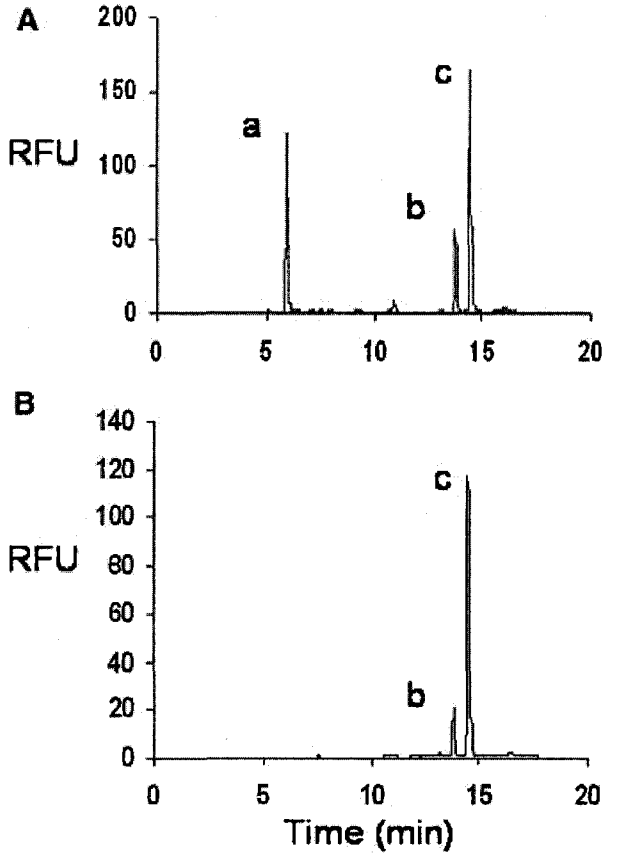

Figure 1. (A) HPLC trace of all standards: (a) possible product DAC at 6.1 min; (b) possible product BEAC at 13.7 min; and (c) substrate BDAC at 14.4 min; (B) Chromatogram recorded for the reaction mixture of P450 2D6 (0.2 μM) with BDAC (100 μM) and cumene hydroperoxide (0.1 mM) in buffer (0.1 M potassium phosphate, pH 7.4). Detection was achieved with $\lambda_{ex} = 350$ nm, $\lambda_{em} = 420$ nm. See Experimental section for elution details.

stereoselectivity, they are a valid example of P450 hydroxylations and provide a rapid means for measuring activity.

Optimization of the Amount of CHP Used to Support P450 2D6 Activity in Buffer/Organic Solvent Emulsions

Preliminary investigations of the P450 2D6-catalyzed transformations of dextromethorphan or BDAC in buffer/hexane or buffer/isooctane emulsions revealed minimal losses in activity. Buffer/isooctane 2/1 emulsions were next used to optimize the concentration of cumene hydroperoxide. The cumene hydroperoxide-supported activity of P450 2D6 is susceptible to enzyme deactivation by the peroxide. In biphasic solvent systems, partitioning of the peroxide between the two phases and enzyme denaturation by the solvent are also key factors. For dextromethorphan, a substrate soluble in buffer, 0.1 mM cumene hydroperoxide was optimal in the presence of organic solvents, which is comparable to the optimal amount needed in buffer alone (Chefson et al., 2006), (Fig. 2A). For the hydrophobic substrate BDAC however, cumene hydroperoxide was optimized at 5 mM (Fig. 2B). We suspect that this difference may be the result of higher uncoupling rates with BDAC leading to increased consumption of CHP. This is expected based on the fact that BDAC is a poorer substrate compared to DXM and/or present near the enzyme at lower local concentration due to its lower solubility in buffer. Interestingly, the activity was less sensitive to the amount of cumene hydroperoxide in the presence of organic solvents than in its absence. The ability of excess cumene hydroperoxide to partition into the isooctane phase contributes to increased stability of the enzyme in the presence of the peroxide. This is consistent with our previous observation that a slow release of hydrogen peroxide from sodium percarbonate favors P450 catalysis over inactivation by the peroxide (Chefson et al., 2006).

Comparison of the P450 2D6 Activity at Different Isooctane/Buffer Volume Ratios, With the Natural Cofactor and Redox Partner, or With a Surrogate

As mentioned above, we recently reported that the cheap reagents cumene hydroperoxide, *tert*-butyl hydroperoxide and sodium percarbonate can be used to replace the redox partner and the expensive cofactor of human P450 2D6. Moreover the P450 2D6 activity observed in the presence of small amounts of cumene hydroperoxide is two fold superior to that in the presence of CPR and NADPH (Chefson et al., 2006). To verify that this also applies in biphasic solvent systems, we compared the P450 2D6 activity in buffer/isooctane with cumene hydroperoxide or CPR/

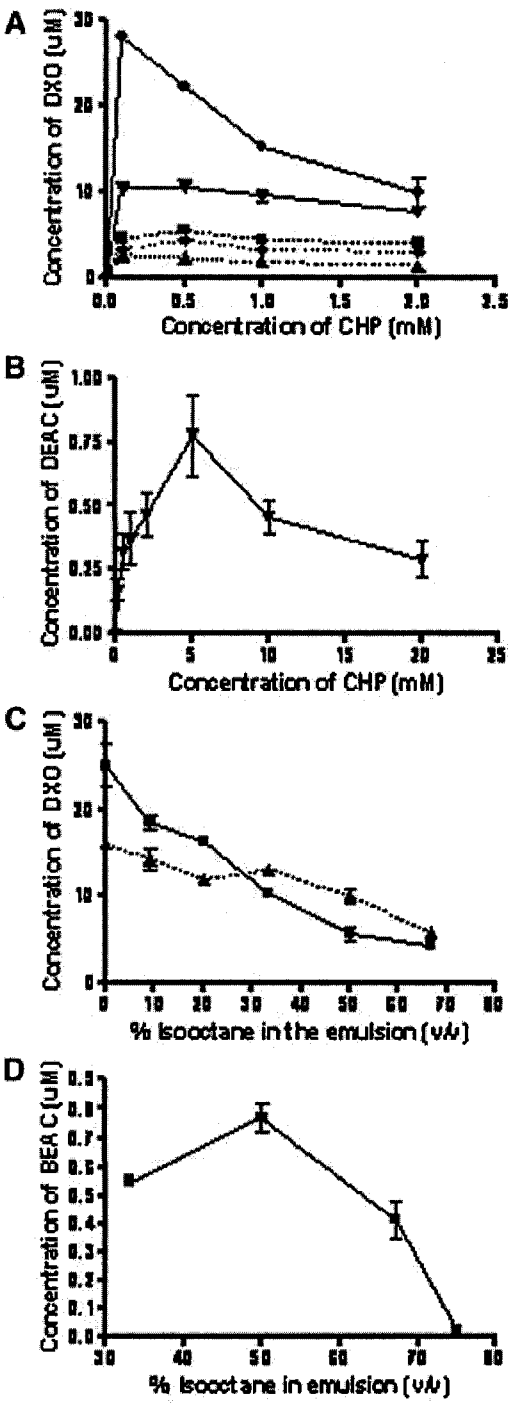

Figure 2. A: Effect of the concentration of cumene hydroperoxide (CHP) on the production of dextrorphan (DXO) from dextromethorphan (DXM, 100 μM) by P450 2D6 (0.2 μM) in different biphasic solvent systems (1/2 v/v ratios) after 1 h at 37°C and 200 rpm. Key: (●) buffer alone; (▼) isooctane/buffer; (■) hexane/buffer; (◆) pentane/buffer; and (▲) cyclohexane/buffer. B: Effect of the concentration of CHP on the production of 7-benzyloxy-4-*N*-ethylaminomethyl-coumarin (BEAC) from 7-benzyloxy-4-*N*,*N*-diethylaminomethyl-coumarin (BDAC) by P450 2D6 (0.2 μM) in isooctane/buffer (1/2 v/v ratio) after 2 h at 37°C and 200 rpm. C: Effect of different isooctane/buffer volume ratios on the activity of P450 2D6 (0.2 μM) with DXM (100 μM) supported by (▲) CPR/NADPH (0.6 μM/1.6 mM) or (■) CHP (0.1 mM), after 1 h at 37°C and 200 rpm. D: Effect of different isooctane/buffer volume ratios on the transformation of BDAC to BEAC by P450 2D6 (0.2 μM) supported by CHP (2.0 mM), and incubated for 2 h at 37°C and 200 rpm. The buffer used in ell cases was potassium phosphate (200 μL, 0.1 M, pH 7.4). The experiments were carried out in duplicate.

DOI 10.1002/bit

NADPH. As shown on Figure 2C, both the natural and the chemical cofactors supported P450 2D6-catalyzed dextromethorphan *O*-demethylation. This suggests that both P450 2D6 and CPR tolerate isooctane. Interestingly, reactions with cumene hydroperoxide were slightly more sensitive to the amount of organic solvent added than those with CPR/NADPH (higher slope). This may be explained by the partitioning of cumene hydroperoxide in isooctane (away from the enzyme). A steady yet slow access to cumene hydroperoxide is important to maximize enzymatic activity and minimize degradation.

When the isooctane/buffer volume ratio was varied, the amount of product formed decreased as the percentage of isooctane was increased with the water soluble substrate dextromethorphan (Fig. 2C). This is likely the effect of enzyme denaturation by the solvent. In contrast, with the hydrophobic solvent BDAC the addition of isooctane was beneficial until it reached 50% (Fig. 2D). The different behavior observed with these two substrates can be explained by the difference in solubility.

Effect of the Choice of Organic Solvent for P450 2D6 Activity in Biphasic Reaction Systems

The activity of P450 2D6 in different biphasic solvent systems was evaluated after 1 h of reaction and compared to the activity in buffer alone. The results with the substrate dextromethorphan (Fig. 3A) suggest rapid inactivation of P450 2D6 in the presence of aromatic or halogenated solvents. The enzyme precipitated within minutes of exposure and negligible amounts of product were detected. Halogenated solvents have been reported to form reactive radical intermediates in the presence of P450s, and deactivate the enzyme by transforming the heme moiety (Manno et al., 1988). Alkanes and ethers on the other hand did not eliminate enzymatic activity. After 1 h of reaction in isooctane/buffer emulsions, the amount of product (6.6 μM) was a little over half that of the reaction in buffer alone (13.0 μM). The regioselectivity of the reaction was not affected noticeably.

In the absence of organic solvent, debenzylation of the synthetic substrate BDAC by P450 2D6 was below the detection limit. The solvent leading to the best product yield was isooctane with 0.13 μM of BEAC. Here again, isooctane, hexane, and cyclohexane were the most promising solvents, whereas dichloromethane inhibited enzymatic activity (Fig. 3B).

A number of groups have reported a correlation between solvent hydrophobicity and enzyme activity (Carrea and Riva, 2000; Filho et al., 2003; Pace et al., 2004) or cell viability (Laane et al., 1985) in biphasic solvent systems. The enzymatic activities reported here were plotted as a function of different physical properties of the organic solvents (Fig. 4 shows a few selected properties). For both substrates tested, our results reveal a correlation between the enzyme activity and the log*P* of the solvent used in the biphasic reaction systems.

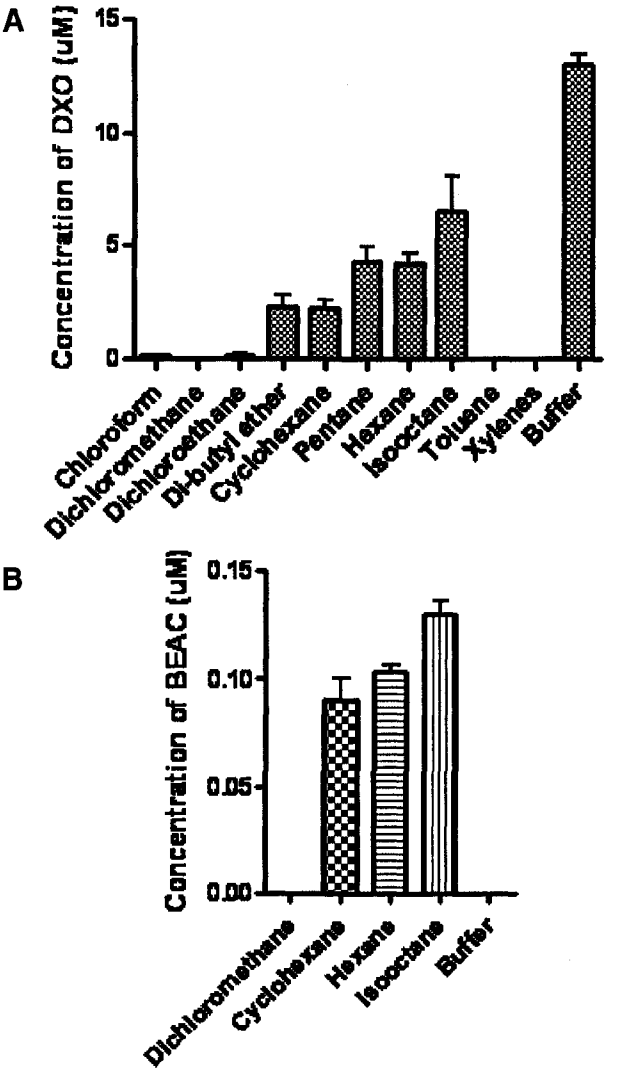

Figure 3. Concentration of product formed by P450 2D6 in the presence of different biphasic solvent systems. **A:** The formation of the product dextrorphan (DXO) is monitored from a reaction consisting of P450 2D6 (0.2 μM), dextromethorphan (100 μM) and cumene hydroperoxide (0.1 mM) in potassium phosphate buffer (200 μL, 0.1 M, pH 7.4) and organic solvent (100 μL). The emulsions were incubated at 37°C and 200 rpm for 1 h. **B:** The formation of the product 7-benzyloxy-4-*N*-ethylaminomethyl-coumarin (BEAC) is monitored from a reaction consisting of P450 2D6 (0.2 μM) and cumene hydroperoxide (0.1 mM) in potassium phosphate buffer (200 μL, 0.1 M, pH 7.4) to which BDAC (100 μM) was added as a solution in the organic solvent (100 μL). The emulsion was incubated at 37°C and 200 rpm for 2 h. The results are averages from triplicates.

Initial Rates and Maximum Turnover Numbers

The kinetics of P450 2D6-catalyzed dextromethorphan dealkylation in 2/1 buffer/isooctane emulsions were investigated in the presence of cumene hydroperoxide

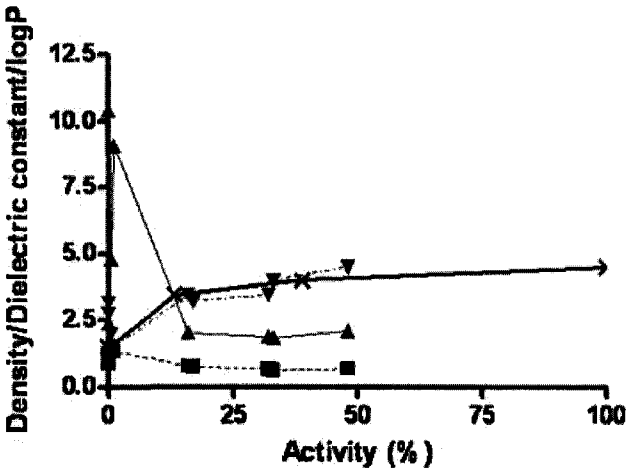

Figure 4. Activity of P450 2D6 (0.2 μM) in buffer/solvent emulsions (2/1, total of 300 μL), correlated to different properties of the organic solvents: (■) density in g/mL, (▲) dielectric constant, and (▼ log*P* with the substrate dextromethorphan (100 μM), and (✕) log*P* with the substrate BDAC (100 μM). The reaction conditions were the same as for Figure 3.

(Table I). As expected, the total turnover number (TTN, defined here as the number of mole of substrate that a mole of catalyst can convert before becoming inactivated, with units of μmol of product per μmol of enzyme) for the reaction in the biphasic system (70 ± 5 μmol μmol^{-1}) was slightly lower than that in buffer (92 ± 2 μmol μmol^{-1}), likely due to enzyme denaturation by the organic solvent. This represents 76% remaining activity. In terms of enzyme stability, the cumene hydroperoxide-supported activity of P450 2D6 decreased below 20% after ~90 min in pure buffer, but after only 60 min in a buffer/isooctane emulsion. In other words, the addition of isooctane results in a reduction of the enzyme stability by ~30 min. On the other hand, the initial rate measured with cumene hydroperoxide was higher in the buffer/isooctane biphasic system (6.4 ± 0.2 μmol μmol^{-1} min^{-1}) than in pure buffer (4.7 ± 0.3 μmol μmol^{-1} min^{-1}), suggesting that partitioning of cumene hydroperoxide between the two layers may ensure a steady source of oxidant to the enzyme, yet slow enough to minimize reaction with the heme group. As proposed above, the organic solvent may moderate the effects of cumene hydroperoxide on the enzyme.

Table I. Initial rates and total turnover numbers (TTNs) for the formation of dextromethorphan catalyzed by P450 2D6 with the surrogate cumene hydroperoxide in either buffer alone or isooctane/buffer biphasic system.

	In buffer[c]	In isooctane/buffer[d]
Initial rate (min^{-1})[a]	4.7 ± 0.3	6.4 ± 0.2
TTN (μmol μmol^{-1})[b]	92 ± 2	70 ± 5

[a]The initial rate is the number of μmol of product formed per μmol of enzyme per minute.

[b]The TTN is defined here as the number of mole of substrate that a mole of catalyst can convert before becoming inactivated, with units of μmol of product per μmol of enzyme.

[c]These results were published earlier by Chefson et al. (2006).

[d]P450 2D6 (0.2 μM), the substrate dextromethorphan (100 μM), and cumene hydroperoxide (0.1 mM) in potassium phosphate buffer (10 mM, pH 7.4, 200 μL) and isooctane (100 μL) were incubated at 37°C and 200 rpm for up to 2 h.

CD Results

We hypothesized that the decreased activity observed could correlate with changes in the protein folding and be reflected in the secondary structure elements. Circular dichroism was used to monitor the overall percentages of helices, beta-sheets and turns, and random coils. The effect of shaking P450 2D6 in biphasic solvent systems containing either isooctane or dichloromethane was investigated over time. For the control reaction in buffer the percentage of each of the secondary structures remained constant over 2 h and enzyme precipitation was not observed during that time. Dichloromethane (33%), a solvent that inhibits P450 2D6 activity, precipitated the enzyme within minutes, which prohibited CD analysis. In the presence of 33% isooctane, no effect could be detected on the secondary structure; however, the enzyme started to precipitate after 60 min. The inability to detect changes in the secondary structure of P450 2D6 in the presence of solvents may be explained by a rapid aggregation and precipitation following enzyme unfolding.

P450 enzymes have an enormous potential as biocatalysts; however, their use has been limited because of the narrow substrate specificity of some isoforms, need for a redox partner and a cofactor, incompatibility with organic solvents and low stability. The results presented here combined with our earlier studies (Chefson et al., 2006) overcome three of these drawbacks. By selecting the promiscuous P450 2D6 we expect that numerous organic molecules will be substrates of this enzyme. We previously reported that cumene hydroperoxide is a highly efficient replacement for the natural redox partner and the cofactor of P450 2D6 in buffer. Here we demonstrate that this applies in the presence of organic solvents as well. We also report that in 2/1 buffer/isooctane emulsions, this enzyme catalyzes the transformation of the water soluble substrate dextromethorphan or the hydrophobic substrate BDAC, with comparable activity whether the natural redox partner and cofactor, or the surrogate cumene hydroperoxide are used. This establishes the utility of our system since BDAC is not transformed significantly in the absence of organic solvent. Under optimized biphasic conditions, the TTN was >75% of that of the natural system (with CPR/NADPH and in buffer). Although the use of purified P450 2D6 enzymes in industry remains impractical, our results represent one important step towards this goal.

The authors wish to acknowledge Prof. F. Peter Guengerich from Vanderbilt University and Prof. Charles B. Kasper from the University of Wisconsin-Madison for their kind gift of the P450 2D6 and CPR expression plasmids, respectively. We also thank Dr. Nadim Saade for his assistance with LC-MS. Finally, this work was supported by the

DOI 10.1002/bit

Natural Science and Engineering Research Council of Canada (NSERC), Astra Zeneca Canada, Inc., Boehringer Ingelheim Canada Ltd, and Merck Frosst Canada & Co.

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Supporting Information

Activity of Human P450 2D6 in Biphasic Solvent Systems

Jin Zhao, Elaine Tan, Julian Ferras, Karine Auclair*

Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montréal, Québec, Canada H3A 2K6; Phone: (+1)514-398-2822; Fax: (+1)514-398-3797; e-mail: karine.auclair@mcgill.ca

*Corresponding author

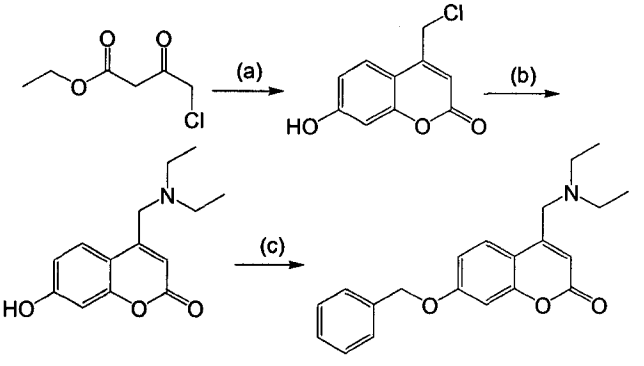
Chemicals and instruments

Dextromethorphan, dextrorphan, 3-methoxymorphinan, NADPH, trifluoroacetic acid (TFA), cumene hydroperoxide (CHP), L-α-phosphatidylcholine, 1,2-di[cis-9-octadecenoyl]-sn-glycero-3-phosphocholine, and 2,2,4-trimethylpentane (isooctane) were purchased from Sigma-Aldrich (Montreal, QC, Canada). Chloroform, 1,2-dichloroethane, xylenes, toluene, hexane, pentane and HPLC grade acetonitrile were from Fisher (Nepean, ON, Canada). Dichloromethane was purchased from EMD Chemicals Inc. (Cincinnati, OH). Water was obtained from a Milli-Q Synthesis filtration system (Millipore, San Jose, CA). All chemicals were used without further purification.

An Agilent 1100 modular system equipped with an auto-sampler, a quaternary pump system, a photodiode-array detector, a fluorescence detector, and a thermostated

column compartment was operated for HPLC analyses. UV absorption spectra were recorded using a Cary 5000 UV spectrophotometer (Varian, Mississauga, ON, Canada). LC-MS analyses were performed on a Finnigan LCQDUO mass spectrometer from Thermo Separation Products including LC pump P4000 and UV spectrophotometer UV2000. Circular Dichroism spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco Inc., Easton, MD) in rectangular 1 mm quartz cuvettes (Starna Cell, Inc., Atascadero, CA). ^{1}H and ^{13}C NMR spectra were recorded on Varian Mercury 200 or 300 spectrometers. The peak patterns are included as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublet; m, multiplet, etc.

Synthesis of the substrate 7-benzyloxy-4-*N*,*N*-diethylaminomethyl-coumarin (BDAC).



Scheme S1. (a) resorcinol, $ZrCl_4$ (8%, in mol), 70°C, 3 hrs, 21%. (b) diethylamine, 60°C, 48 hrs, 70%. (c) THF, NaH, 0°C, 1 hr; benzyl bromide and tetrabutylammonium iodide, 70°C, 3 hrs, 45%. Details for specific steps are given below.

7-Hydroxy-4-chloromethyl-coumarin

A mixture of ethyl 4-chloro-3-oxobutanoate (10 mmol) and resorcinol (10 mmol) was heated at 70°C in the presence of zirconium (IV) chloride (184 mg, 8% in mol), for 3 hrs. The mixture was then cooled to room temperature and ice cold water was added to afford a yellowish precipitate. The solid was collected by filtration, washed with ice cold water, dried and purified by flash chromatography (silica gel, 2:3 ethyl acetate:hexane) to obtain white crystals of the known 7-hydroxy-4-chloromethyl-coumarin (Smitha et al., 2004) in 21% yield (442 mg). ^{1}H NMR (d_6-DMSO) δ 10.65 (1H, s), 7.65 (1H, d, J = 9.0 Hz), 6.81 (1H, dd, J = 9.0, 2.2 Hz), 6.73 (1H, d, J = 2.2 Hz), 6.4 (1H, s), 4.93 (2H, s); ^{13}C NMR (d_6-DMSO) δ 162.20, 160.83, 155.98, 151.64, 127.16, 113.78, 111.71, 110.02, 103.22, 42.05; ESMS Calc ($C_{10}H_7ClO_3$, M^+), 211.2, Obs 210.0.

7-Hydroxy-4-*N,N*-diethylaminomethyl-coumarin

7-Hydroxy-4-chloromethyl-coumarin (10 mmol) was added to diethylamine (5 mL). The resulting yellow solution was stirred under nitrogen for 48 hrs at 60°C. The mixture was evaporated in vacuo to afford a brown oil which was purified by flash chromatography (silica gel, chloroform followed by 1:1 ethyl acetate:hexane) to obtain the desired product as yellowish crystals. Yield 1.7 g, 70%. ^{1}H NMR ($CDCl_3$) δ 7.79 (1H, d, J = 8.8 Hz), 6.79 (1H, dd, J = 8.8, 2.2 Hz), 6.71 (1H, d, J = 2.2 Hz), 6.39 (1H, s), 3,74 (1H, s), 2.62 (4H, q, J = 7.2 Hz), 1,08 (6H, t, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 163.26, 160.51, 155.71, 155.47, 126.07, 113.60, 112.40, 110.81, 103.57, 54.80, 47.86, 12.04; HRMS Calc ($C_{14}H_{17}NO_3$, M^+), 247.12084, Obs 247.12049.

7-Benzyloxy-4-*N,N*-diethylaminomethyl-coumarin (BDAC) To a solution of 7-hydroxy-4-*N,N*-diethylaminomethyl-coumarin (10 mmol) in anhydrous THF at 0°C,

sodium hydride (10 mmol) was slowly added. The mixture was stirred for 1 hr at room temperature. A homogeneous greenish-yellow solution was obtained. Benzyl bromide (10 mmol) and tetrabutylammonium iodide (1 mmol) were added and the resulting mixture was stirred for 3 hrs at 70°C. THF was evaporated in vacuo and the residue was redissolved in a minimum of ethyl acetate. The organic phase was washed with saturated aqueous $NaHCO_3$, and with saturated aqueous NH_4Cl. It was dried over magnesium sulfate and concentred in vacuo. Flash chromatography (silica gel, 1:8 ethyl acetate:hexane followed by 1:2) afforded the desired compound in 45% yield (1.5 g, purity >99%.). ^1H NMR ($CDCl_3$) δ 7.76 (1H, d, J = 9.0 Hz,), 7.39 (5H, m), 6.91 (2H, m), 6.44 (1H, s), 5.11 (2H, s), 3.63 (2H, s), 2.57 (4H, q, J = 6.6 Hz), 1.04 (6H, t, J = 6.6 Hz). ^{13}C NMR ($CDCl_3$) δ 161.93, 161.69, 155.7, 154.41, 136.13, 128.96, 128.55, 127.72, 125.93, 113.02, 112.95, 111.90, 101.11, 70.64, 54.89, 47.76, 12.08; MS Calc ($C_{21}H_{23}NO_3$, M^+), 337.2, Obs 338.2.

Enzyme expression and purification

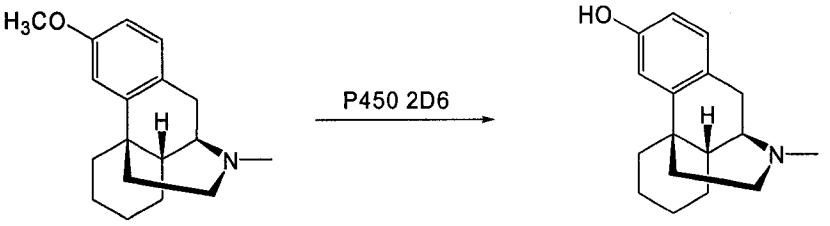
P450 2D6 and cytochrome P450 reductase (CPR) were expressed and purified as previously described (Chefson et al., 2006). The use of lipids such as L-α-phosphatidylcholine and/or 1,2-di[cis-9-octadecenoyl]-sn-glycero-3-phosphocholine to reconstitute P450 2D6 was not necessary. The enzymatic activity was unchanged by the addition of lipids for reactions in pure buffer or in buffer/organic emulsions.

Enzymatic assays

The dextromethorphan stock solution (10 mM) was prepared in potassium phosphate buffer (0.1 M, pH 7.4). The BDAC stock solution was prepared in the same organic

solvent as used for the enzymatic reactions in biphasic solvent systems. P450 2D6 (0.2 μM) and the desired substrate (dextromethorphan or BDAC, 100 μM) were mixed in potassium phosphate buffer (0.1 M, pH 7.4). Different volumes of organic solvents (20-400 μL) were added before initiating the reaction with cofactors (0.6 μM CPR and 1.67 mM NADPH, or 1 mM CHP). The biphasic reaction mixture (aqueous layer of 200 μL) was incubated for 60 min with dextromethorphan or 120 min with BDAC, at 37°C and 200 rpm. The reaction vessels were positioned horizontally to maximize the turbulence. For total reaction volumes of 200-300 μL, vials of 650 μL were used, whereas vials of 1.5 mL were more appropriate for mixtures of 400-600 μL. Perchloric acid (23% v/v, 10 μL) was added to precipitate the enzyme after reactions with dextromethorphan. The mixture was vortexed and left on ice for 5 min. A gentle stream of air was applied to remove the organic solvent. For assays with BDAC, the organic solvent was evaporated under a gentle stream of air, followed by addition of acetonitrile (200 μL) and vortexing to precipitate the enzyme. The samples were centrifuged at 10,000 rpm for 5 min before injection (5 μL) in the HPLC system.

HPLC analysis to monitor the *O*-dealkylation of dextromethorphan by P450 2D6



DXM DXO

Separation was achieved using a 250 × 4.60 mm Synergi 4 μ Hydro-RP 80 Å column with mobile phase A (0.1% TFA in water) and B (100% acetonitrile) at a flow rate of 0.2

mL/min, kept at 30°C. The initial mobile phase consisted of 50:50 A:B (v:v) and was linearly changed to 80:20 A:B over 20 min. The excitation and emission wavelengths of the fluorescence detector were set at 280 and 310 nm respectively. The retention times of dextrorphan, 3-methoxymorphinan and dextromethorphan were 13.4, 17.2, and 18.0 min, respectively. For product quantification, a calibration curve was prepared using dextrorphan samples that underwent the same treatment as the assay samples, and of concentrations ranging from 2.5 μM to 50 μM.

HPLC analysis to monitor the *N*-deethylation of DBAC by P450 2D6

BDAC BEAC

An Agilent 300 Extend-C_{18}, 4.6 × 250 mm, 5μ column was used with mobile phase B (see above) and mobile phase C (0.05% TFA in water). Elution was performed with a linear gradient from 10:90 B:C to 40:60 over 10 min, kept at 40:60 for 2 min, and brought linearly to 10:90 over 3 min. The flow rate was 0.8 mL/min and the column was kept at 22°C. Elution was monitored either by UV at 325 nm or by fluorescence with excitation at 350 nm and emission at 420 nm. The major product BEAC and the substrate BDAC eluted at 13.7 and 14.4 min, respectively. For product quantification, a calibration curve was prepared using dextrorphan samples that underwent the same treatment as the assay samples, and of concentrations ranging from 9 μM to 225 μM.

Measurement of initial rates and TTNs

A set of mixtures of P450 2D6 (0.2 μM) and dextromethorphan (100 μM) in potassium phosphate buffer (10 mM, pH 7.4, 200 μL) and isooctane (100 μL) were pre-warmed at 37°C for 3 min before initiation of the reaction with cumene hydroperoxide (0.1 mM). The mixtures were allowed to react at 37°C and 200 rpm for up to 2 hrs. Reactions were terminated with the addition of perchloric acid (23% v/v, 10 μL). The same workup was performed as with other enzymatic assays (see above). Product formation was calculated after 25 sec, 40 sec, 1 min, 2 min, 5 min, 10 min, 30 min and 1 hr. (each in duplicate). A kinetic plot was drawn from the concentration of product formed as a function of time. The initial rate was determined from the slope of the first 5 points of this plot. The total turnover number (TTN) is defined here as "the number of mole of substrate that a mole of catalyst can convert before becoming inactivated", with units of μmol of product per μmol of enzyme. It is calculated when the kinetic plot reaches a plateau.

CD analyses

The samples (300 μL) were prepared by diluting P450 2D6 (0.275 μM) in potassium phosphate buffer (10 mM, pH 7.4), with or without the substrate dextromethorphan (100 μM). The samples to be shaken for 5 min or less were first brought to 37°C before addition of isooctane (100 μL). Isooctane (100 μL) was directly added to the other samples. After addition of the organic solvent, the biphasic mixtures were left at 37°C and 200 rpm. The control samples did not contain isooctane. Each sample and control was prepared and analyzed in triplicate. After incubation, isooctane was evaporated under a gentle stream of air. The aqueous mixture was transferred to a 0.1cm pathlength CD cuvette and incubated for 5 min at 37°C before CD analysis. Spectra were obtained by

averaging 3 scans measured at a scan rate of 20 nm min^{-1}, a time constant of 1 sec, and a bandwidth of 1 nm for both far and near UV, at 37°C. Blank spectra of the buffer (10 mM potassium phosphate at pH 7.4) were used to correct the experimental spectra. The spectra were converted into molar ellipticity units. Data points ranging from 190 to 240 nm at 0.5 nm intervals were analyzed using the software CDPro (available at http://lamar.colostate.edu/~sreeram/CDPro/main.html) to obtain the percentage of each secondary structure. For simplicity, fractions of regular and distorted α-helices were combined together. The same applies to regular and distorted β-strands, and turns.

Reference:

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Journal of Biotechnology 130 (2007) 436–440
Journal of BIOTECHNOLOGY
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Short communication

Sugar-mediated lyoprotection of purified human CYP3A4 and CYP2D6

Amandine Chefson, Jin Zhao, Karine Auclair *

Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montréal, Québec H3A 2K6, Canada

Received 26 January 2007; received in revised form 25 April 2007; accepted 11 May 2007

Abstract

P450 enzymes are of great interest for drug metabolism and as potential biocatalysts. Like most P450s, purified CYP3A4 is normally handled and stored in solution because lyophilization greatly reduces its activity. We show here that colyophilization of this enzyme with sucrose or trehalose, but not mannitol, crown ethers or cyclodextrins, allow recovery of full enzymatic activity after rehydration. Sorbitol was almost as efficient, with 85% retention of the original activity. We also show that similar protection is observed through colyophilization of CYP2D6 with trehalose. This procedure should greatly facilitate handling, storage, or use of these enzymes in anhydrous media.

Keywords: Biocatalyst; Cryoprotection; Freeze-drying; Lyophilization; P450; Saccharide

1. Introduction

P450 enzymes (P450s, CYPs) have gained considerable attention amongst chemists for their unequalled catalytic properties. Indeed, P450s have the impressive ability to catalyze the regio- and stereo-selective hydroxylation of inactivated C–H bonds. P450s are not only studied as potential novel biocatalyst but also because of their importance in drug metabolism and interactions. The pharmaceutical industry uses them in the prediction and production of drug metabolites. For practical purposes, these studies are most often carried out using hepatocytes, recombinant *E. coli* whole cells, or microsomes, instead of pure enzymes. A few companies, like BioCatalytics Inc. or BD Gentest,™ commercialize kits for metabolites production. These kits contain one or more P450s plus the necessary components (reductase, cytochrome b5, etc.) as a lyophilized formulation, or as supersome or microsome solutions. Purified enzymes are often desirable for biochemical, structural, mechanistic or synthetic studies. Invitrogen has recently added a few P450 enzymes to their product list.

The past decades have seen great progress towards the easier use of purified P450 enzymes (Chefson and Auclair, 2006a; Bernhardt, 2006; Urlacher and Eiben, 2006) yet poor storage and operational stability is one of the drawbacks limiting applications with these enzymes. Lyophilization of enzymes is useful not only for handling and storage but also before use in non-aqueous environments. Over the last few years it has been realized that the low activity of lyophilized enzymes in organic solvents is largely due to protein inactivation during freeze-drying (Roy and Gupta, 2004). Dehydration is believed to induce some conformational changes not recovered upon rehydration and potentially detrimental to biological activity (Prestrelski et al., 1993a, 1993b). The use of additives during lyophilization has sometimes been found to protect enzymes from damaging changes (Izutsu and Yoshioka, 1995; Carpenter et al., 1997; Arakawa et al., 2001). Sugars are the most widely used lyo- and cryo-protectants for enzymes (Sampedro et al., 1998; Carpenter et al., 1987; Townsend and DeLuca, 2002; D'Andrea et al., 1996; Ward et al., 1999). Carbohydrates and cyclodextrins are believed to compensate for the losses in the hydration shell of the enzyme, allowing preservation of the native structure in the dehydrated state (Prestrelski et al., 1993a, 1993b; Carpenter and Crowe, 1988, 1989; Lippert and Galinski, 1992; Allison et al., 1999; Liao et al., 2002a, 2002b; DePaz et al., 2002; Santos et al., 1999; Griebenow et al., 1999). Interestingly, it has been demonstrated that the weight ratio sugar/protein directly correlates with the protection of catalase during freeze-drying, but the bulk concentration of lyoprotectant does not (Tanaka et al.,

* Corresponding author. Fax: +1 514 398 3797.
E-mail address: karine.auclair@mcgill.ca (K. Auclair).

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doi:10.1016/j.jbiotec.2007.05.009

1991). Crown-ethers have also had some success as lyoprotectants (Broos et al., 1992; Santos et al., 2001; Mine et al., 2003; Tremblay et al., 2005), and are believed to protect the enzymes by forming noncovalent macrocyclic complexes with lysine ammonium groups (van Unen et al., 2002; Lee and Dordick, 2002).

We elected to study the human P450 isoform CYP3A4 for its wide substrate promiscuity, a significant advantage for possible applications in synthesis. This isoform is also responsible for the metabolism of more than half of the known drugs and is highly studied. We evaluated the consequences of freeze-drying on its activity, and optimized the conditions to minimize the harmful effects of the process.

2. Material and methods

2.1. Chemicals and instruments

The CYP3A4 pSE3A4His expression plasmid was a gift from Dr J. R. Halpert from the University of Arizona. The culture media ingredients yeast extract, tryptone and peptone were purchased from BD Biosciences (San Jose, CA). DH5α supercompetent cells and agarose were from Invitrogen (Carlsbad, CA). The His-Select™ Nickel Affinity Gel was purchased from Sigma (St. Louis, US). The CYP2D6 substrate dextromethorphan and its metabolite dextrorphan were purchased from Sigma (Oakville, Canada). The CYP3A4 substrate testosterone (4-androsten-17β-ol-3-one) was a kind gift from Dr. Eisenberg in our department and the metabolite 6β-hydroxytestosterone (4-androsten-6β,17β-diol-3-one) was purchased from Steraloids (Newport, RI). All solvents were purchased from Fisher and were of HPLC grade. Water was obtained from a Milli-Q Synthesis (Millipore, San Jose, CA) filtration system. All other chemicals were purchased from Sigma or Aldrich. The enzymes CYP3A4, CYP2D6 and CPR were expressed and purified as previously described elsewhere (Chefson et al., 2006b). The P450 content was measured by reduced carbon monoxide difference spectra following the method described by Omura and Sato[3]. Lyophilization was performed on a Labconco freeze-dry system Freezone® 4.5. Analytical HPLC analyses were performed on an Agilent 1100 series. Analysis of testosterone 6β-hydroxylation by CYP3A4 used a 150 mm × 4.6 mm Zorbax Eclipse XDB-C8 5 μm column from Agilent, with an elution consisting of a first isocratic step at 15% acetonitrile in water for 4 min, before a linear gradient to 50% acetonitrile over 12 min. The flow rate was 1.5 ml/min, and the column temperature was set to 30 °C. Detection was at 244 nm. Under these conditions, the retention times of testosterone, 6β-hydroxytestosterone, 6α-hydroxytestosterone and cortexolone were 16.3, 9.9, 10.5 and 14.2 min, respectively. A Synergi 4 μm Hydro-RP 80 Å column was used to analyze the formation of dextrorphan from dextromethorphan. A combination of 50% water containing 0.1% TFA and 50% acetonitrile was linearly changed to 80% acidic water and 20% acetonitrile over 20 min. This eluent was maintained for 4 min, before returning to 50:50 over 2 min. Under these conditions, the retention times of dextrorphan and dextromethorphan were 13.4 and 18.0 min, respectively.

2.2. Effect of freezing and lyophilization on CYP3A4 activity

Samples containing glycerol-free CYP3A4 (100 μl of a 11.5 μM solution) in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 300 μl) were frozen at −80 °C and lyophilized for 24 h. Three holes were made in the Eppendorfs' cap using a red-hot needle, and the pierced caps were replaced with new ones after freeze-drying. The dried samples were redissolved in 0.1 M potassium phosphate buffer at pH 7.4 (287 μl), and the substrate testosterone (115 μM, 10 μl of a 1 mg/ml solution in methanol) was added (final CYP3A4 concentration of 1.3 μM). Control samples were prepared using the same amount of CYP3A4 (100 μl of a 11.5 μM solution) and the substrate testosterone (115 μM, 10 μl of a 1 mg/ml solution in methanol) in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 300 μl). To test the effect of freezing alone, some samples containing a CYP3A4 solution were frozen at −80 °C and thawed on ice once or twice. After 5 min of preincubation at 37 °C, the reaction was initiated with the addition of cumene hydroperoxide (1 mM, 7 μl of a 0.1 M stock solution in water:methanol 90:10), and the samples were shaken at 37 °C and 250 rpm for 1 h. After incubation, the samples were spiked with the internal standard cortexolone (15 μl from a 200 μM solution in methanol) and extracted with CH_2Cl_2 (3 × 500 μl). The combined organic layers were evaporated in vacuo. The residue was redissolved in MeOH (150 μl) and analyzed by HPLC.

2.3. Effects of additives during lyophilization of CYP3A4 and CYP2D6

Samples containing glycerol-free CYP3A4 enzyme (24 μl of a 11.5 μM solution) and different amounts of additives (18-crown-6, 15-crown-5, methyl-β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin, sucrose, sorbitol, mannitol or trehalose) in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 700 μl) were lyophilized for 24 h. For sucrose, sorbitol, mannitol and trehalose the amounts added are reported as w/w ratio additive/enzyme, whereas molar ratios are used for all other additives tested. The dried samples were redissolved in 0.1 M potassium phosphate buffer at pH 7.4 (687 μl), and the substrate testosterone (50 μM, 10 μl of a 1 mg/ml solution in methanol) was added (final CYP3A4 concentration of 0.4 μM). After 5 min of preincubation at 37 °C, the reaction was initiated by the addition of cumene hydroperoxide (1 mM, 7 μl of a 0.1 M stock solution in water:methanol 90:10), and the samples were shaken at 37 °C and 250 rpm for 1 h. A first set of controls was prepared for each additive at every concentration with non-lyophilized enzyme. Another control reaction was also prepared with lyophilized enzyme and no additives. After incubation, the samples were spiked with the internal standard cortexolone (15 μl from a 200 μM solution in methanol) and extracted with CH_2Cl_2 (3 × 500 μl). The combined organic layers were evaporated in vacuo. The residue was redissolved in MeOH (150 μl) and analyzed by HPLC.

The activity of CYP3A4 lyophilized with the optimized amounts of sucrose and trehalose was also tested with the nat-

ural cofactors cytochrome P450 reductase (CPR) and NADPH. Samples containing glycerol-free CYP3A4 enzyme (0.4 μM, 25 μl of a 11.2 μM solution) and sucrose (145 mg, 10000 w/w) or trehalose (145 mg, 10000 w/w) in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 700 μl) were lyophilized for 24 h. The dried samples were redissolved in 0.1 M potassium phosphate buffer at pH 7.4 (632 μl), and CPR was added (1.6 μM, 45 μl of a 25 μM solution). The substrate testosterone (50 μM, 10 μl of a 1 mg/ml solution in methanol) was added, and after 5 min of preincubation at 37 °C, the reaction was initiated by addition of NADPH (1 mM, 28 μl of a 25 μM solution in buffer), and the samples were shaken at 37 °C and 250 rpm for 1 h. Reactions were also carried out upon initiation with CHP (after lyophilization in the same conditions with sucrose or trehalose). Control reactions with nonlyophilized enzyme (initiation with CHP or CPR/NADPH) and enzyme lyophilized without additives (initiation with CHP or CPR/NADPH) were also carried out. After incubation, the samples were spiked with the internal standard cortexolone (15 μl from a 200 μM solution in methanol) and extracted with CH_2Cl_2 (3 × 500 μl). The combined organic layers were evaporated in vacuo. The residue was redissolved in MeOH (150 μl) and analyzed by HPLC.

Samples containing glycerol-free CYP2D6 enzyme (8 μl of a 13.85 μM solution) and different amounts of sugar additives (sorbitol, sucrose, mannitol, or trehalose; ratios in w/w) in 0.1 M potassium phosphate buffer at pH 7.4 (final volume of 1000 μl) were lyophilized for 22 h. The lyophilized enzyme (final concentration of 0.14 μM), with or without additive, was redissolved in 0.1 M potassium phosphate buffer at pH 7.4, and the substrate dextromethorphan (100 μM) was added. The reaction was initiated with the addition of the cofactor surrogate cumene hydroperoxide (0.1 mM). The samples were shaken at 37 °C and 200 rpm for 1 h. The reaction was terminated with the addition of perchloric acid (23%, 40 μl). After cooling to 4 °C for 30 min, the samples were centrifuged at 10,000 rpm for 5 min. The resulting supernatant was directly injected in the HPLC.

3. Results and discussion

We have previously demonstrated that the P450 enzyme CYP3A4 can efficiently use cumene hydroperoxide (CHP) as a substitute for the natural cofactors NADPH and cytochrome P450 reductase (Chefson et al., 2006b). In this study, the CHP-supported 6β-hydroxylation of testosterone by CYP3A4 was used to monitor enzymatic activity, yet applicability was also demonstrated with CPR and NADPH. Product quantification was achieved by HPLC, with cortexolone as the internal standard. Following purification, CYP3A4 is usually kept in buffer containing 10% glycerol as a cryoprotectant for subsequent storage at −80 °C. The presence of glycerol during lyophilization tends to produce a sticky solid or a viscuous oil because glycerol does not evaporate. The importance of glycerol for long term storage at −80 °C was therefore evaluated. After two weeks, the activity decreased by only 3 and 5% with or without glycerol, respectively, and after four months, the activity decreased by 7 and 13%, respectively. This suggests that cryoprotection is not necessary for the storage of CYP3A4. All subsequent enzyme batches were stored without addition of glycerol in the dialysis buffer.

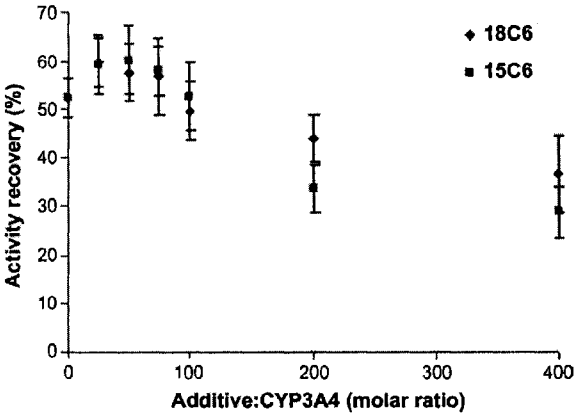

Fig. 1. Effect of colyophilization (24 h) with different amounts of 18C6 and 15C5 on the 6β-hydroxylation of testosterone (115 μM) catalyzed by CYP3A4 (0.4 μM, 14.5 μg) upon rehydration in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 700 μl). The reaction was initiated with cumene hydroperoxide (1 mM) and incubated for 1 h at 37 °C.

Our preliminary experiments revealed that freeze-dried CYP3A4 redissolved in buffer had lost ~50% of its activity towards testosterone.While the total turnover number (TTN, defined here as the number of mole of substrate that a mole of catalyst can convert before becoming inactivated, in μmol μmol^{-1}) reached 40 ± 8 for nonlyophilized enzyme, it dropped to 23 ± 7 upon lyophilization. On the other hand, no significant loss in activity was observed after freezing and thawing the enzyme solution once or twice (38 ± 9 and 39 ± 11, respectively). This indicates that among the two stress factors involved in the freeze-drying process, freezing and dehydration (Crowe et al., 1990; Carpenter et al., 1993), dehydration is the most harmful to the CYP3A4 activity. A variety of compounds have been used as cryoprotectants with other enzymes, such as glycerol, polyethyleneglycol, sugars, glycine, proline, but only sugars were effective as lyoprotectants (Prestrelski et al., 1993a; Carpenter and Crowe, 1989; Allison et al., 1999; Souillac et al., 2002a, 2002b; Kreilgaard et al., 1998). This led us to evaluate the effect of various additives during lyophilization of CYP3A4, including the disaccharides trehalose and sucrose, the polyols sorbitol and mannitol, as well as two cyclodextrins, methyl-β-cyclodextrin (MβCD) and hydroxypropyl-β-cyclodextrin (HPβCD), and two crown ethers, 18-crown-6 (18C6) and 15-crown-5 (15C5).

As shown on Figs. 1 and 2, the colyophilization of CYP3A4 with cyclodextrins or crown ethers did not significantly help protecting the enzyme. Only low concentrations of 18C6 and 15C5 (molar ratio to enzyme of ~50) increased the activity and the effect was very modest (from 52% without to 60% with the additive). High concentrations of crown ethers (>100 molar ratio to CYP) were even detrimental to the P450 activity.

The addition of sugars, reduced or not, before lyophilization was much more effective at maintaining the CYP3A4 enzymatic activity (Fig. 3). Both sucrose and trehalose confered a similar level of lyoprotection. The recovered activity, measured after

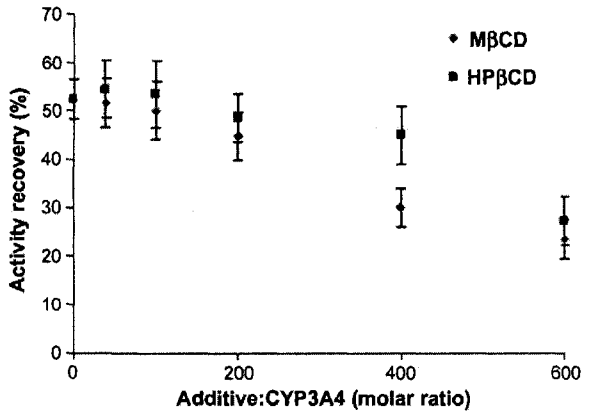

Fig. 2. Effect of colyophilization (24 h) with different amounts of methyl-β-cyclodextrin (MβCD) and hydropropyl-β-cyclodextrin (HPβCD) on the 6β-hydroxylation of testosterone (115 μM) catalyzed by CYP3A4 (0.4 μM, 14.5 μg) upon rehydration in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 700 μl). The reaction was initiated with cumene hydroperoxide (1 mM) and incubated for 1 h at 37 °C.

initiation with cumene hydroperoxide, was dependent on the amount used. Very high ratios sugar/enzyme w/w (5000–10,000) allowed the retention of >90% of the activity, compared to 52% for the enzyme freeze-dried alone. Sorbitol was almost as effective at lower concentrations (1000–2000 w/w), helping retain about 85% of the activity. Its beneficial effect quickly dropped when the amount of sorbitol increased further, and no protection remained at ratios of 10,000 and higher. At proportions ≤1000 w/w, sorbitol, sucrose and trehalose had similar effects. Mannitol was the least effective, barely increasing the residual activity to 60% at weight ratios of 2000–5000. In all cases, no improvement of the activity was observed in the controls containing the additives but not lyophilized, suggesting that the sugars protect against the harmful effects of dehydration, as opposed to directly activating the enzyme.

After lyophilization in the presence of the optimized amount of sucrose or trehalose, the activity of CYP3A4 was also tested with the natural cofactors (CPR and NADPH). The lyoprotection effect observed was very similar (data not shown) to that described above for the CHP-supported reactions. Without lyophilization, the TTN was 40 with CHP and 44 with CPR/NADPH. After lyophilization *without* additive, the TTN dropped to 19 with CHP (47% remaining activity), and to 23 with CPR/NADPH (52% remaining activity). With the addition of sucrose (10000 w/w) before lyophilization, the TTN reached 36 with CHP and 37 with CPR/NADPH, corresponding to 90 and 84% remaining activity. With trehalose (10000 w/w), the remaining activity was 87.5% with CHP and 88.5% with CPR/NADPH (TTNs of 35 and 39, respectively).

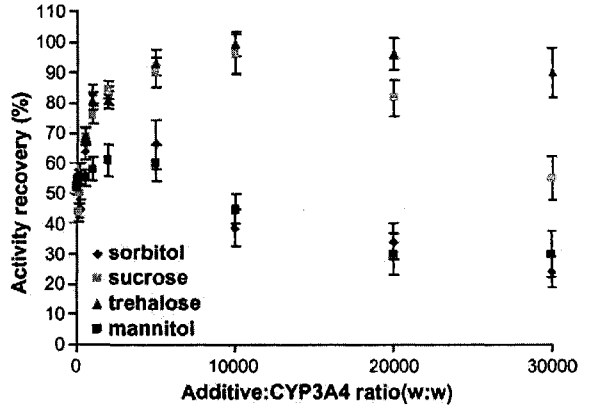

Fig. 3. Effect of colyophilization (24 h) with different amounts of sorbitol, sucrose, trehalose and mannitol on the 6β-hydroxylation of testosterone (115 μM) catalyzed by CYP3A4 (0.4 μM, 14.5 μg) upon rehydration in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 700 μl). The reaction was initiated with cumene hydroperoxide (1 mM) and incubated for 1 h at 37 °C.

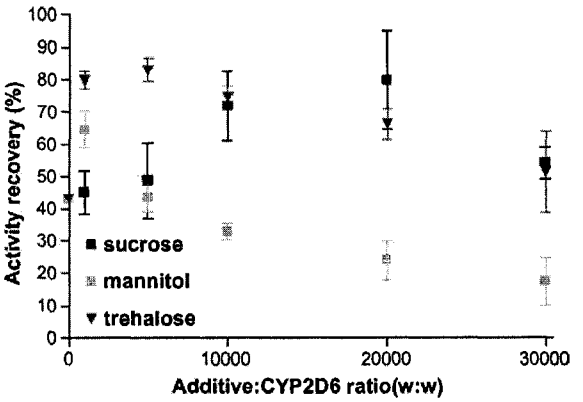

Fig. 4. Effect of colyophilization (22 h) with different amounts of sucrose, trehalose or mannitol on the demethylation of dextromathorphan (100 μM) catalyzed by CYP2D6 (0.14 μM) upon rehydration in 0.1 M potassium phosphate buffer at pH 7.4 (final volume 800 μl). The reaction was initiated with cumene hydroperoxide (0.1 mM) and incubated for 1 h at 37 °C.

To investigate the scope of this method, the activity of human CYP2D6 was investigated after lyophilization in the absence or in the presence of sorbitol, sucrose, mannitol or trehalose. Lyophilization of CYP2D6 in the absence of lyoprotectant led to the loss of approximately 60% of the enzymatic activity, and was greatly affected by the duration and conditions of the process. Colyophilization of CYP2D6 with sorbitol yielded inconsistent results (data not shown). The effect of mannitol on the activity was minimal and dropped rapidly as the mannitol/enzyme ratio increased (Fig. 4). On the other hand, the addition of either sucrose (at a sucrose:CYP2D6 ratio of 20,000) or trehalose (at a trehalose:CYP2D6 ratio of 5000) allowed recovery of about 80% of the activity. These results are very similar to those obtained with CYP3A4, therefore suggesting applicability to other P450 isoforms.

CYP3A4 and CYP2D6 are some of the most important enzymes in drug metabolism. Alone, CYP3A4 transforms more than half of all known pharmaceuticals. CYP3A4 and CYP2D6 are versatile and unequalled biocatalysts because of their high substrate promiscuity and their ability to catalyze the regio- and stereo-selective oxidation of inactivated C–H bonds. Lyophilization facilitates the use of enzymes, but without additives, it greatly reduces the activity of CYP3A4 and CYP2D6. We demonstrate here that the addition of sugars before lyophilization can help preserve most of the enzymatic activity upon rehydration. Saccharides were the most efficient lyoprotectants, especially sucrose and trehalose which lead to complete

retention of activity at high concentrations. The possibility to lyophilize CYP3A4 or CYP2D6 without activity loss greatly eases handling, storage and use of this enzyme in anhydrous environments. This is a considerable asset for general applications of purified P450s, especially in synthesis.

Acknowledgements

The authors wish to acknowledge Prof. J. R. Halpert from the University of Arizona for his kind gift of the CYP3A4 expression plasmid. The authors are also grateful to Prof. F. Peter Guengerich from Vanderbilt University and Prof. Charles B. Kasper from the University of Wisconsin-Madison for their kind gift of the P450 2D6 and CPR expression plasmids, respectively. This work was supported by the Natural Science and Engineering Council of Canada (NSERC), Astra Zeneca Canada Inc., Boehringer Ingelheim Canada Ltd., and Merck Frosst Canada and Co.

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