INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Molecular Basis of Biotin-Responsive Multiple Carboxylase Deficiency

By

Lucie Dupuis

Department of Biology

McGill University, Montreal

August 1996

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Master of Sciences

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and in French, an introduction which clearly stated the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.**

TABLE OF CONTENTS

ABSTRACT

Multiple carboxylase deficiency (MCD) results from a decreased activity of holocarboxylase synthetase (HCS) which is responsible for the biotinylation of the four biotin-dependent carboxylases found in humans. The disease can be treated with pharmacologic doses of oral biotin (biotin-responsiveness). The cDNA for HCS contains a biotin-binding domain deduced by analogy with the sequence and crystal structure of the *E. coli* BirA biotin ligase. *E. coli birA*$^-$ mutations causing biotin-auxotrophy all localize to this region. Of six point mutations I have identified in MCD patients, four localize to the biotin-binding region. In order to assess the HCS activity associated with patient mutations, I used an assay based on the expression of mutant HCS in *E. coli*. The method is based on the ability of mutant HCS to biotinylate the biotin carboxyl carrier protein (BCCP) of acetyl-CoA carboxylase in a temperature-sensitive *birA*$^-$ *E. coli* strain using 3H-biotin as tracer. I have shown that all of the mutations cause a severe decrease in HCS activity. In addition, I have shown that five of the mutant HCS are biotin-responsive. These findings are a major contribution to the understanding of the mechanism of biotin-responsiveness.

RÉSUMÉ

La déficience multiple des carboxylases (DMC) est le résultat d'une diminution de l'activité de l'enzyme holocarboxylase synthétase (HCS). Cette enzyme est responsable de la liaison covalente de la biotine à chacune des quatres carboxylases qui se trouvent chez l'humain. Cette maladie peut cependant être traitée avec des doses élevées de biotine. L'ADNc codant pour HCS contient une région de liaison de la biotine déduite par analogie avec la séquence et la structure tridimensionnelle de la biotine ligase (BirA) de *E. coli*. Des mutations auxotrophes pour la biotine chez *E. coli* (*birA*$^-$) sont toutes localisées dans cette région. J'ai identifie six mutations chez des patients qui souffrent de DCM, dont quatre se trouvent dans la région de la liaison de la biotine. Afin de mesurer l'activité HCS associée avec ces mutations, j'ai développé un assai basé sur l'expression d'HCS mutée et exprimée chez *E. coli*. Cette méthode est basée sur la capacité d'HCS mutantes à biotinyler la sous-unité BCCP de l'acétyl CoA carboxylase dans la souche themosensible de *E. coli birA*$^-$ en utilisant la ^{3}H-biotine comme marqueur. J'ai démontré que toutes les mutations causent une baisse sévère de l'activité HCS. De plus, j'ai démontré que cinq des six HCS mutées répondent a la biotine. Ces résultats représentent une contribution majeure dans la compréhension du mécanisme de réponse à la biotine.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor, Dr. Roy A. Gravel, for giving me the opportunity to work in his extraordinary laboratory. Although the research that led to this thesis did not always go as smoothly as anticipated, he was a constant source of ideas and enthusiasm.

I would like to thank in particular Dr. Alfonso Leon-Del-Rio for introducing me to the world of HSC and for his friendship. I would also like to thank Dr. Daniel Leclerc for helping throughout these two years with technical support and ideas.

My special thanks to Jing-Qi Huang for making my stay in the lab so enjoyable. I would like to acknowledge the help given to me by other members of the lab: Eric Campeau, Farzad Saberi, Beverly Akerman and Marlene Aardse.

My special thanks to Jose Guilmette for his patience, understanding and love.

I dedicate this thesis to my parents Dr. Gilles and Patricia Dupuis who have been a constant source of love, support and encouragement: You are the wind beneath my wings.

LIST OF FIGURES

CHAPTER I

CHAPTER II

CHAPTER III

LIST OF TABLES

CHAPTER I

CHAPTER II

CHAPTER III

ABBREVIATIONS

MCD: multiple carboxylase deficiency

HCS: holocarboxylase synthetase

PCC: propionyl CoA carboxylase

MCC: ß-methylcrotonyl CoA carboxylase

PC: pyruvate carboxylase

ACC: acetyl CoA carboxylase

bio operon: biotin operon

BirA: biotin ligase protein

*Bir*A: biotin ligase gene

*bir*A: biotin ligase mutant

THESIS OUTLINE

Holocarboxylase synthetase (HCS) catalyzes the biotinylation of all four biotin-dependent carboxylases in humans. An inherited deficiency in HCS activity results in the neonatal form of multiple carboxylase deficiency (MCD), a rare autosomal recessive disorder. The disease is characterized by metabolic acidosis, organic aciduria and varying degrees of hyperammonemia. Clinical manifestations include skin rashes, seizures and developmental delay followed by coma and death. This potentially fatal disease can be cured in virtually all cases with pharmacological doses of oral biotin.

My project was to determine the molecular basis of the biotin-responsiveness observed in patients with neonatal MCD. To fulfil this goal, I proposed to identify disease-causing mutations in the HCS gene, to express them in an *in vivo* system and to determine the relative affinity for biotin of normal and mutant HCS.

In chapter II, I describe the identification of mutations in HCS deficient patients. This was achieved by performing SSCP (single stranded length conformational polymorphism) analysis on cDNA obtained by RT-PCR of HCS mRNA and direct or automated sequencing of PCR products which displayed band shifts on SSCP gels. The identified mutations were then confirmed by restriction enzyme digests of PCR amplified gDNA.

In chapter III, I have determined the mechanism of biotin-responsiveness by expressing the mutations in a biotin ligase-deficient *E. coli* strain and determined the kinetics of BCCP (biotin carboxyl carrier protein) activation *in vivo*.

The results of this study contribute to an increased understanding of the mechanism of HCS action and the basis of biotin-responsiveness in patients.

CHAPTER I

LITERATURE REVIEW

DISCOVERY OF BIOTIN AND ITS ROLE

It was observed in 1916 that a diet rich in dried raw eggs was highly toxic to rats (1). When raw egg whites were the only source of protein, the animals developed neuromuscular disorders, severe dermatitis and hair loss (2). However, the condition was preventable by heating the egg whites or by feeding the rats foods such as yeast, liver or a component from egg yolk that promoted the growth of yeast (3). In 1934, the protective component was identified as biotin or vitamin H (4). It was determined that avidin, a compound with a high affinity for biotin ($K_d = 10^{-15}M$) was the cause of the toxicity (5).

Biotin is a water soluble vitamin of the B complex group. Structurally, it is composed of a heterocyclic ureido ring substituted with an aliphatic carboxylic acid group (Fig. 1). Biotin can be found in low amounts in most foods (6). About 30 types of prokaryotes are able to synthesize biotin (7). In humans, only the intestinal microflora can synthesize biotin but its relative contribution remains obscure. It is estimated that the daily requirement for biotin is 150-300 μg of biotin for adults and 35 μg for children (8).

Two causes of biotin deficiency are known in humans: genetic deficiencies, in which there are inherited defects of biotin utilization or recycling of protein-bound biotin, and nutritional deficiencies due to biotin deficient diets or ingesting foods with high avidin content. This thesis is concerned with the disease caused by an inherited defect of biotin utilization known as multiple carboxylase deficiency (MCD).

CARBOXYLASES

Biotin is essential for all organisms because it is the cofactor for enzymes involved in carboxylation reactions (9-12). In humans, the biotin-dependent carboxylases participate

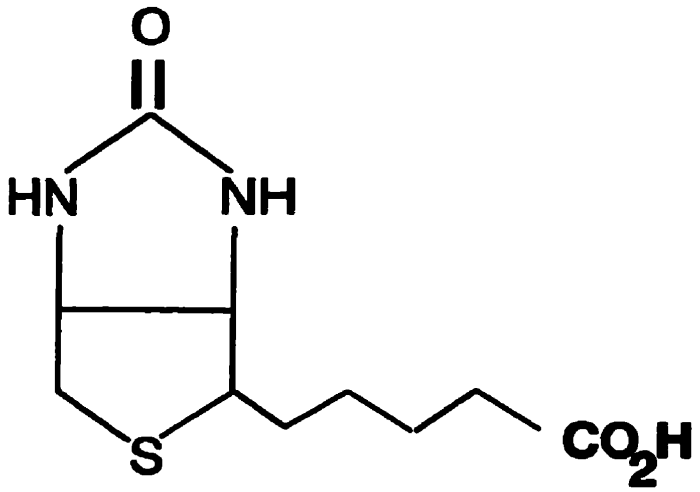

Figure 1. Structure of biotin.

in fatty acid synthesis, gluconeogenesis, and the catabolism of metabolites such as branched chain amino acids, fatty acids with odd number of carbon atoms and the amino acid leucine. The carboxylase enzymes are synthesized in the apo form and become activated only through the covalent attachment of the biotin cofactor. Biotin serves as a carrier of CO_2 by attachment of a carboxyl substituent at its ureido ring. The biotin ring system is at the end of a 14Å long flexible arm which permits the carboxylase to transfer bicarbonate between donor and acceptor sites (13).

Classes of carboxylases

The biotin-dependent enzymes are involved in three classes of carboxylation reactions and are believed to proceed by similar two-step mechanisms (14,15). They are distinguished by their source of carboxyl group. Class I enzymes utilize bicarbonate as a source of CO_2 and the reaction is driven by ATP (Fig. 2). All eukaryotic carboxylases and most bacterial carboxylases belong to this class. The carboxyl acceptor can be acyl CoA's (e.g. propionyl CoA) or carboxylic acids (e.g. pyruvate) (9,10). Class II proteins are involved in the production of energy and are found in anaerobic bacteria. They are sodium transport decarboxylases (Fig. 2). The carboxyl donors are ß-keto acids or their CoA derivatives (e.g. methylmalonyl CoA) (16). Class III includes a single enzyme *P. Shermani* transcarboxylase (Fig. 2). It transfers a carboxyl group between a pyruvate and methylmalonylCoA (9). It does not require ATP or CO_2 (bicarbonate) in the reaction. Humans have only class I carboxylase enzymes.

Role of carboxylases

Humans possess four biotin-dependent enzymes: PC (pyruvate carboxylase; EC 6.4.1.1), PCC (propionyl CoA carboxylase, EC 6.4.1.3), MCC (ß-methylcrotonyl CoA carboxylase, EC 6.4.1.4) and ACC (acetyl CoA carboxylase; EC 6.4.1.2) (Fig. 3 and 4). The first three are mitochondrial and the last one is cytosolic. PC is involved in gluconeogenesis since it catalyses the conversion of pyruvate to oxaloacetate which is an intermediate in the biosynthesis of phosphoenolpyruvate and eventually glucose. ACC is involved in the biosynthesis of fatty acids. It catalyses the first step in the synthesis

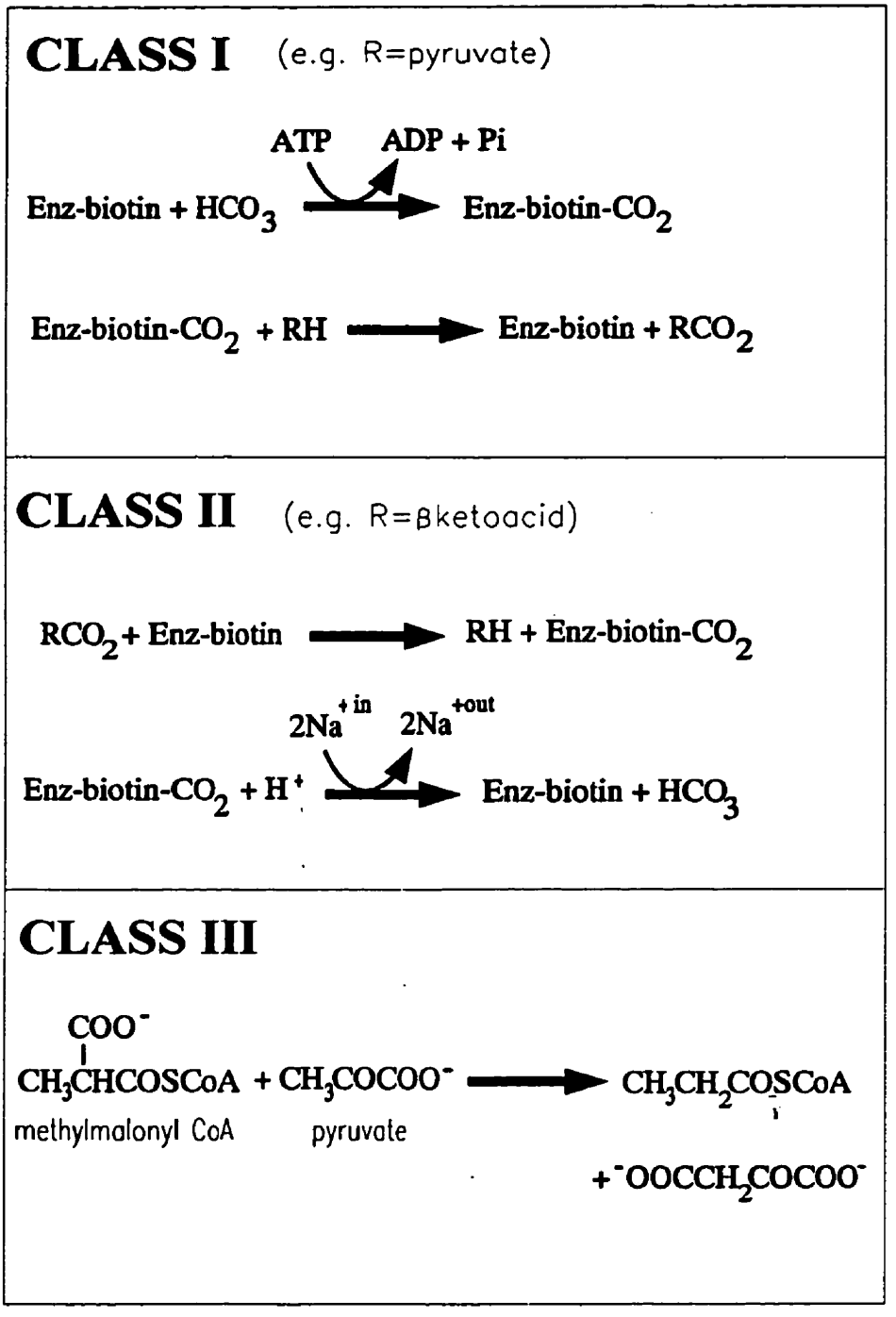

Figure 2. Classes of metabolic processes involving carboxylases.

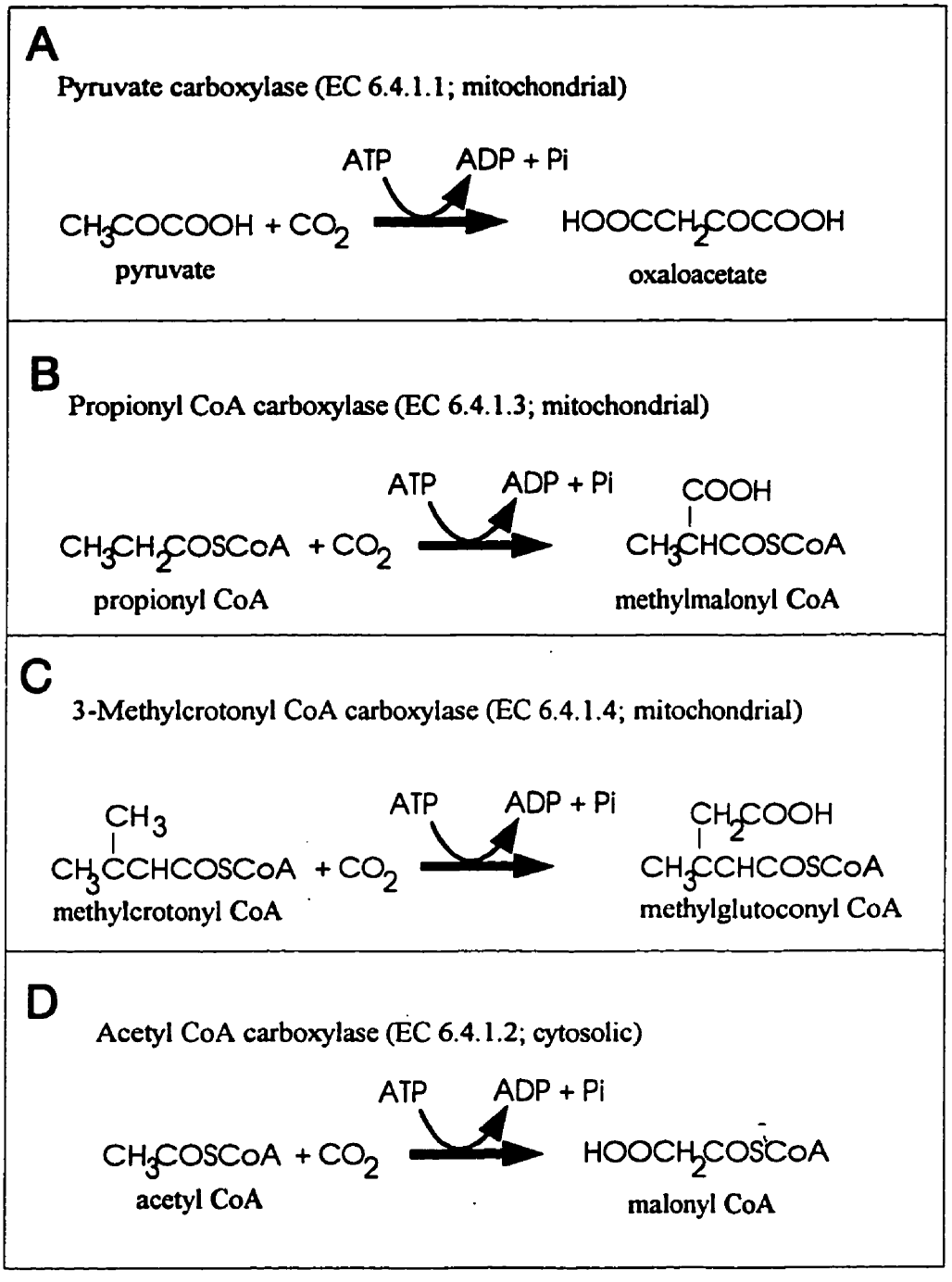

Figure 3. Reactions catalyzed by human biotin-dependent enzymes.

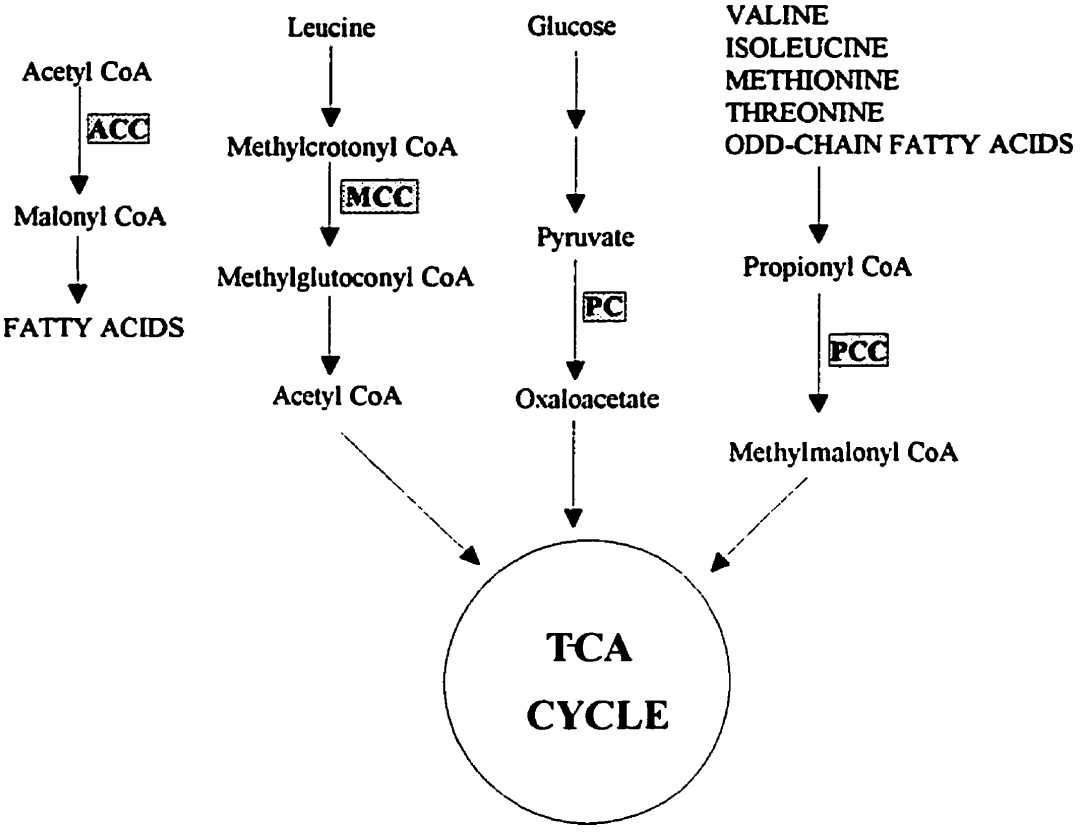

Figure 4. Role of biotin-dependent carboxylases in metabolism.

of fatty acids by converting acetyl CoA to malonyl CoA. PCC converts propionyl CoA to methylmalonyl CoA which can enter the tricarboxylic acid cycle. It is involved in the catabolism of branched chain amino acids, as well as fatty acids with odd numbered carbon length. MCC plays a role in leucine catabolism. It catalyses the conversion of ß-methylcrotonyl CoA to ß-methylglutaconyl CoA. In *E. coli*, there is a single biotin-dependent enzyme, acetyl CoA carboxylase (ACC) which carries out all CO_2-dependent reactions.

Structural categories of carboxylases

Carboxylases can be categorized into three different groups based on number of independent subunits comprising the enzymes (13). Group 1 enzymes contain three functionally distinct subunits. An example is ACC in *E. coli* (Fig. 5A and B) (17-20). The BCCP subunit (biotin carboxyl carrier protein) carries the biotin cofactor. The BC subunit (biotin carboxylase) carries out biotin carboxylation. The CT subunit (carboxyl transferase) transfers the carboxyl group from biotin onto the appropriate acceptor. Group 2 proteins are made up of two different polypeptides. An example is human PCC (Fig. 5B). In this case, the α-subunit carries the biotin cofactor and possesses biotin carboxylase activity. The ß-subunit contains the carboxyl transferase activity. Human MCC also belongs to this group. Group 3 proteins are made up of a single polypeptide which carries the biotin cofactor and possesses the carboxylase and carboxyl transferase activities. Examples are human ACC and PC (Fig. 5B).

Activation of apocarboxylases

Carboxylases are initially synthesized in the apo form. In order to carry out their respective functions, they must be activated by the addition of a biotin molecule. HCS is the enzyme responsible for the biotinylation of apocarboxylases in eukaryotes and biotin ligase (BirA) in the case of prokaryotes. Biotinylation of apocarboxylases in prokaryotes and eukaryotes has been extensively studied (21-33). The results of these studies indicate that biotin ligase and HCS proceed via a common enzymatic mechanism. However, these two enzymes are different both structurally and functionally as discussed

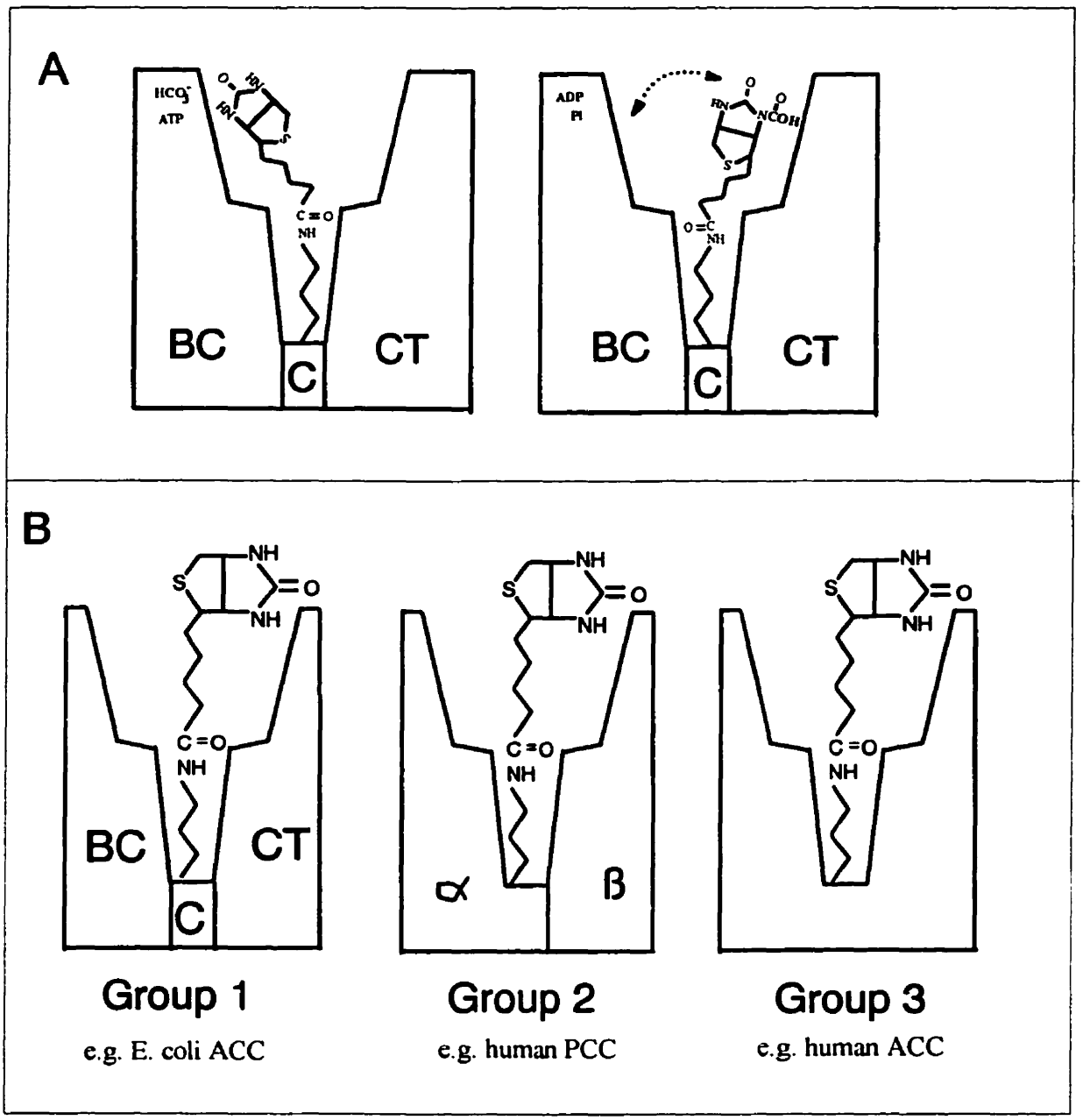

Figure 5. Structural categories of carboxylases. In A) schematic representation of the carboxyl translocation by biotin in ACC of *E. coli*. In B) group 1 (e.g. *E. coli* ACC), group 2 (e.g. human PCC) and group 3 (e.g. human ACC).

in a later section.

Prokaryotes. In a study of the partially purified biotin ligase from *P. shermanii*, it was shown that the conversion of apotranscarboxylase to active holotranscarboxylase occurred in two steps (31)(Fig. 6A). The first step involves the activation of biotin by ATP with the formation of biotinyl-5'-AMP and the release of pyrophosphate. The carbonyl group on the biotin molecule becomes activated so that it is more susceptible to nucleophilic attack by the ϵ amino group of a lysine residue on the carboxylase. In the second step, the biotinyl group is covalently linked to the lysine residue with the concomitant release of AMP.

Eukaryotes. The existence of HCS was first demonstrated by developing an assay which utilized soluble apoPCC isolated from biotin-depleted rat liver (27,34,35). ATP and a divalent ion were found to be necessary for the biotinylation of PCC. Through the isolation of biocytin (ϵ-N-biotinyl-L-lysine) from yeast extract, the site of attachment of biotin was located to a lysine residue (36). Similar results to those found with *P. shermanii* for the proposal of a two-step reaction were obtained using apoPCC isolated from rabbit liver with the corresponding HCS (23).

UTILIZATION OF BIOTIN

In mammals.

A major source of biotin in mammals is previously-formed protein-bound biotin. Its metabolic recycling and reutilization is known as the biotin cycle. It begins with the intake of biotin from the diet that is used for the activation of the apocarboxylases by HCS. Through the natural turnover of cellular proteins, the activated carboxylases are ultimately degraded proteolytically to release biotin as biocytin (i.e. biotinyl-lysine). At this point, the second major enzyme of the cycle, biotinidase, cleaves the biotin-lysine amide bond so that released biotin becomes available for reutilization (Fig. 6B and 7). In humans, it has been suggested that two-thirds of the biotin necessary for biotinylation comes from metabolic recycling and the rest from the diet (37).

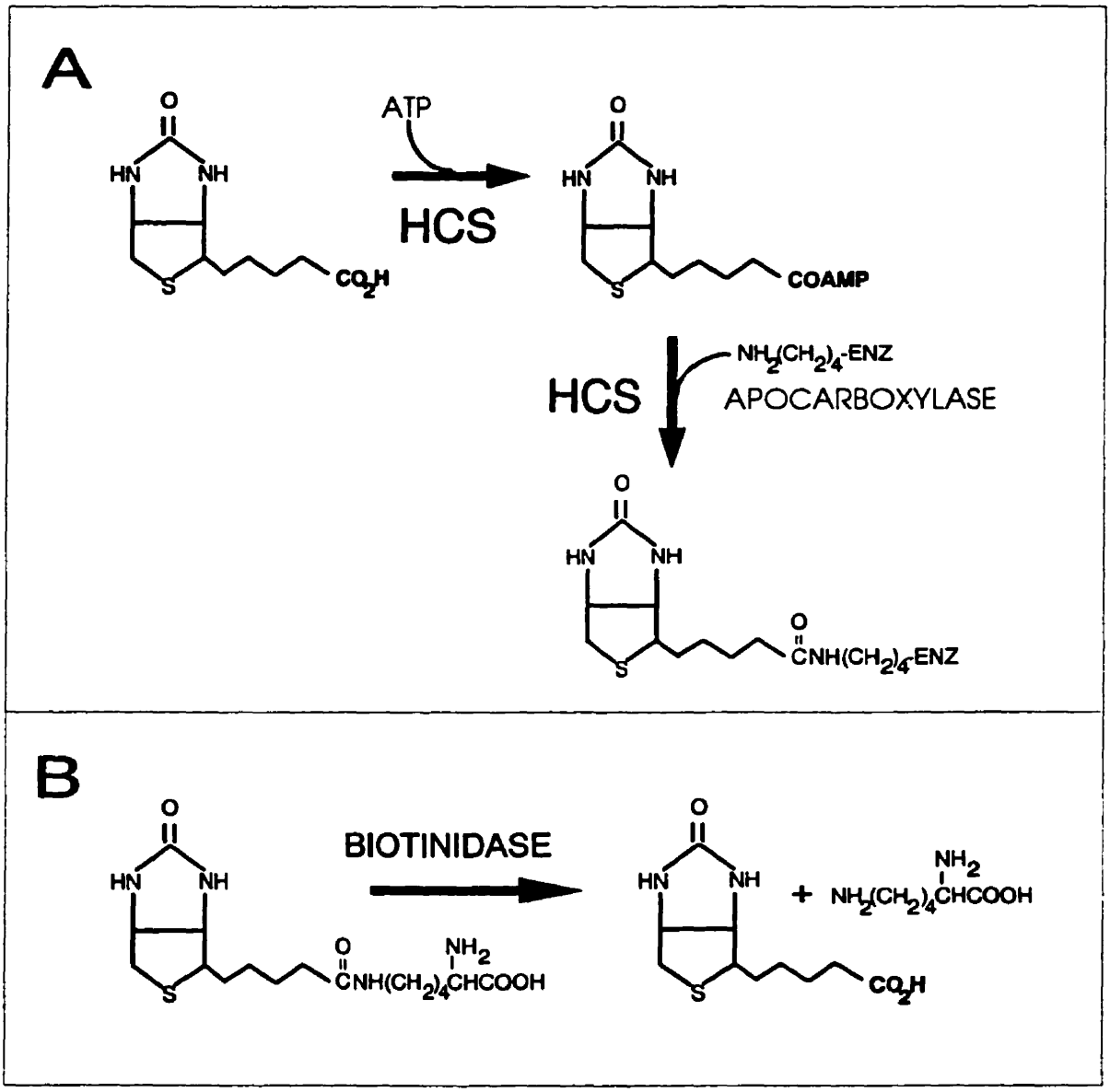

Figure 6. Reactions catalysed by HCS or biotinidase. In A) activation of apocarboxylases occurs in a two-step reacton, each half-reaction is catalyzed by HCS. In B) recycling of biotin catalyzed by biotinidase.

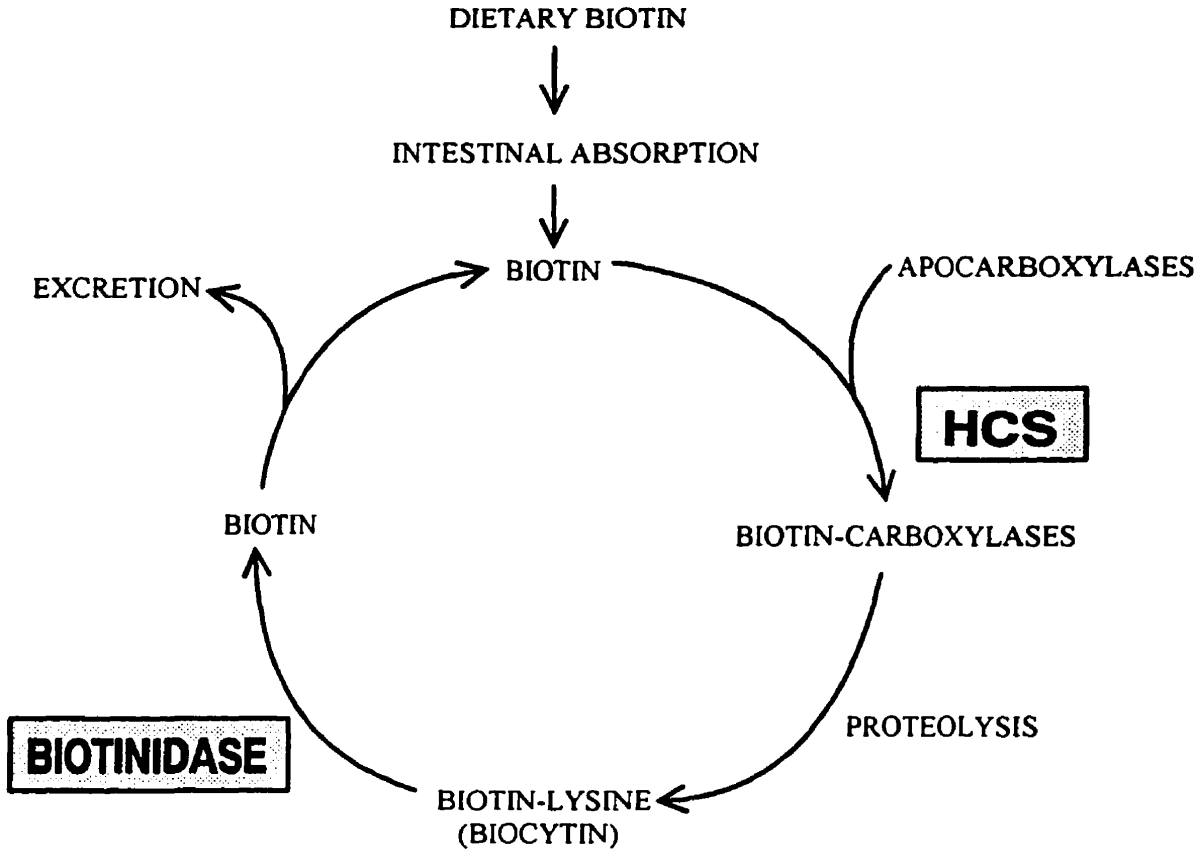

Figure 7. The biotin cycle.

In *E. coli*.

E. coli derives biotin through exogenous uptake or *de novo* synthesis. It regulates the relationship between these two sources through the biotin operon (bio operon). The principal protein associated with biotin utilization is the 34 kDa BirA protein which is 321 amino acids in length. It serves as corepressor of the bio operon and contains the biotin ligase function. Biotin is synthesized from pimeloyl CoA and alanine by a set of enzymes encoded by a cluster of genes (with one exception) that make up the bio operon (38,39). The transcription of these genes is from two "face to face" overlapping promoters. One transcript is from the bioBCDEF genes and the second is from the bioA gene with an ORF of unknown function (38).

The uptake of exogenous biotin in *E. coli* occurs through an active transport system studied by Prakash and Eisenberg (40). They demonstrated that biotin uptake depends on energy, temperature, pH and is inhibited by iodoacetate. They showed that biotin uptake was controlled by the amount of biotin in the external medium. They calculated an apparent K_m and V_{max} for uptake of biotin of 140 nM and 6.6 pmol biotin/mg dry weight of cells/min, respectively. They also measured free and bound biotin in biotin-auxotrophs and showed that most of the biotin taken up is in the bound form. It is believed that uptake occurs by passive diffusion at concentration greater than 4 μM (41).

Regulation of the bio operon. The bio operon is regulated by BirA in an unusual yet highly effective manner. To inhibit biotin synthesis in the presence of high exogenous biotin, the repressor (BirA) and corepressor (biotinyl-5'-AMP, which is obtained in the first half of the biotinylation reaction) are necessary. Biotin is manufactured by the cell only if its supply of biotin is limiting (biotin starvation). When biotinyl-5'-AMP is produced it will immediately be used for biotinylation of apoBCCP. Therefore, there will be no net excess of biotinyl-5'-AMP. If the level of biotin is high, i.e. all apoBCCP is biotinylated, then there will be excess biotinyl-5'-AMP made and the biotin ligase biotinyl-5'-AMP complex will accumulate. This complex is the active repressor of the

bio operon. It will bind to the operator, inhibiting further transcription of the proteins needed to synthesize biotin. Therefore, transcription of the bio operon depends not only on the level of free biotin in the cell but also the level of apoBCCP (41). Synthesis has been shown to be almost completely repressed by exogenous biotin concentrations greater than 40 nM (42,43,44).

BirA crystal structure and identification of mutations. The three-dimensional structure of BirA was defined by Wilson *et al.* (45) (Fig. 8). BirA contains three domains. One defined by the N-terminal region contains a helix-turn-helix DNA-binding motif and is loosely connected to the rest of the molecule. The central domain containing the active site is made up of both ß-sheets and α-helices and is exposed to the solvent. It contains the biotin-binding site and a sequence GRGRRG previously identified as a consensus sequence for ATP binding. The C-terminal end is made up of ß-sheets and is of undefined function. Significant to this thesis, several *bir*A mutations leading to an increased requirement for biotin in the growth medium (biotin-auxotrophy) have been localized to the biotin-binding region (see Chapter III, Fig. 8).

HUMAN HCS

In eukaryotes, HCS catalyses the biotinylation of apocarboxylase. The human HCS cDNA was recently cloned by Suzuki *et al.* (46) and by Leon-del-Rio *et al.* (47) in our laboratory. The former was achieved based on peptide sequence data from the purified bovine HCS and the latter used functional complementation of a *bir*A mutant of *E. coli* defective in the bacterial biotin ligase activity. Human HCS is a 64 KDa protein of 726 amino acids. The HCS mRNA (5.8 Kb) is present in highest amounts in skeletal muscle, kidney and pancreas (47).

Localization of HCS. HCS can biotinylate individual subunits of apocarboxylases located in the cytoplasm (prior to assembly) or in the mitochondria (after assembly) (48-50). HCS appears to biotinylate both mitochondrial and cytosolic apocarboxylases. The

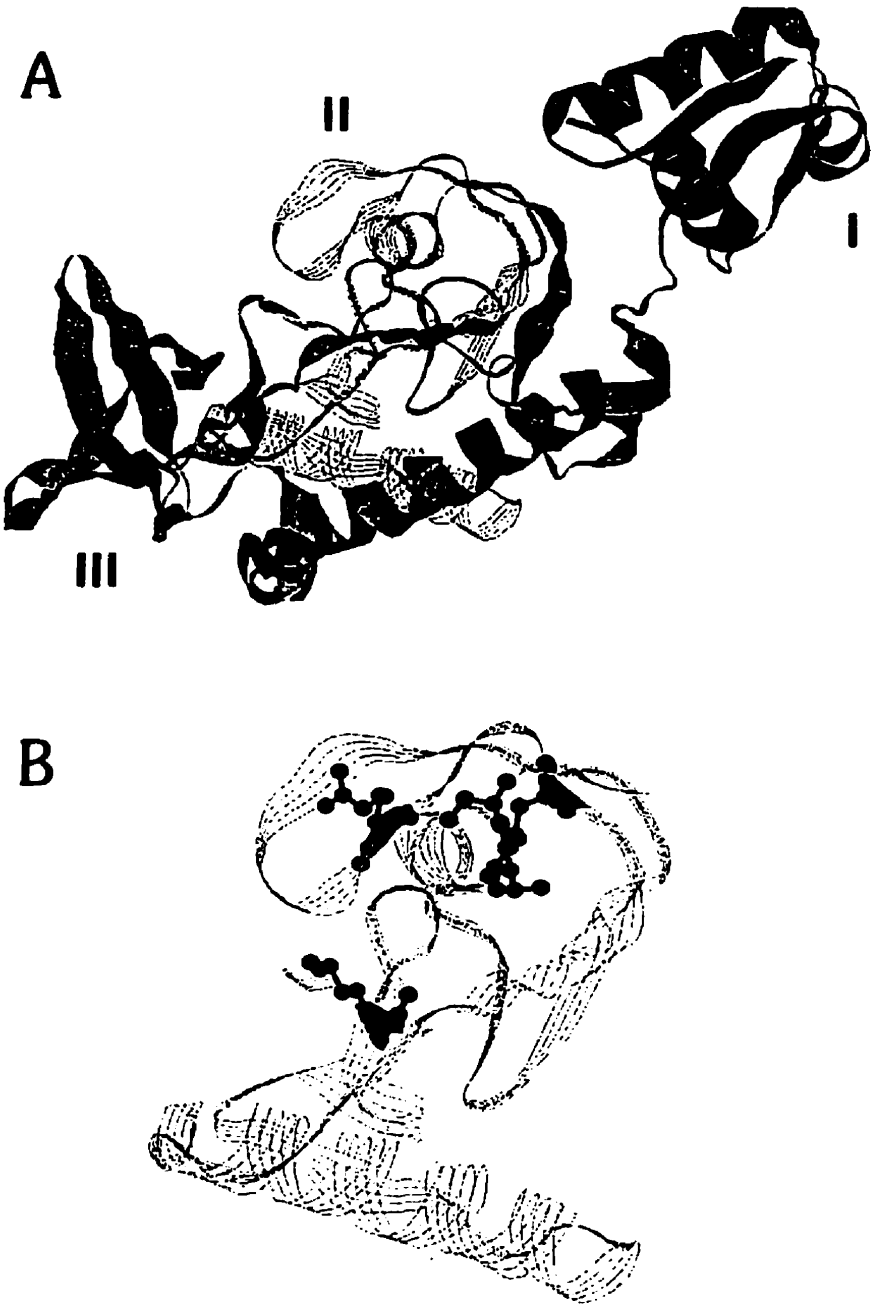

Figure 8. Three-dimensional structure of BirA in *E. coli*. In A) the ribbon structure of the BirA protein with the biotin-binding region highlighted. I represents the N-terminal region contains, II represents the central domain containing the biotin-binding site and III represents the C-terminal region. In B) localization of the contact points for biotin (45).

mechanism for targeting of HCS to the mitochondria versus the cytosol may be alternative splicing to generate different N-terminal sequences, though this has not been completely resolved (47). Support for a single gene encoding an HCS which is directed to both compartments comes initially from patients who were shown to have a decrease in activity of both mitochondrial and cytosolic carboxylases (37).

SPECIFICITY OF BIOTIN LIGASES

Biotin ligases need an energy source, a cofactor (biotin) and an apocarboxylase to carry out their function. HCS and BirA are highly specific for biotin but the specificity for high energy phosphate donor or the apoenzyme acceptor is not as high (23,28,30,31,53). Even human HCS must be able to recognize four different apocarboxylase substrates. The biotin-binding site is located near the C-terminus in PC, PCC, MCC and near the N-terminus in ACC. This suggests the presence of recognition sites for biotinylation in biotin-dependent enzymes. There is strong homology in the biotin-binding site of these enzymes. The biotinylated lysine is located within the conserved sequence Met-Lys-Met in all carboxylases, the only exception is ACC from Anabaena sp (Met-Lys-Leu) (54). Leon-del-Rio *et al.* (55), in our laboratory demonstrated that a conserved Pro-X-Pro sequence, as well as several conserved Gly residues are also essential as part of a recognition domain in the C-terminal sequence of PCC-α. Substitutions made in the amino acids flanking the critical lysine residue or elimination of the last three amino acids in PCC-α were found to have little or no effect on biotinylation despite their high conservation in biotin dependent carboxylases. The minimum sequence of PCC-α necessary for biotinylation was found to be the last 67 amino acids. It has also been shown that the C-terminal 87 residues of *E. coli* BCCP could act as an independent domain for biotinylation (56). In addition, several studies have shown that HCS from one species is able to biotinylate apocarboxylases from another (47,57). For example, in studies by Leon-del Rio *et al.* (47), we have shown that human HCS is able to biotinylate the BCCP subunit of ACC in *E. coli*.

HUMAN BIOTINIDASE

Biotinidase (EC 3.5.1.12) cleaves biocytin or biotinyl peptides to biotin and lysine (37). It may also play a role in release of biotin from dietary proteins. The human cDNA encoding biotinidase was recently isolated and characterized (58). It encodes a 543 amino acid protein with a 41 amino acid potential signal peptide. The gene is located on chromosome 3p29 (59). It is a glycoprotein composed of a single polypeptide of 67-76 kDa (60-62). Northern blot analysis showed the presence of biotinidase mRNA in several tissues including human liver, kidney, pancreas, lung skeletal muscle, heart, brain and placenta.

ISOLATED CARBOXYLASE DEFICIENCY

Deficiencies of isolated carboxylases will not be discussed in great detail in this thesis. They are mentioned only to distinguish them from the disorders that affect the attachment or removal of biotin from the carboxylases.

Isolated deficiencies of each of the mitochondrial carboxylases have been well described in humans (37). A single case of deficiency in the cytosolic carboxylase, ACC, has been reported (63). Each deficiency is due to mutations in the gene encoding the carboxylase protein that affect its structure and/or its function. Treatment involves a protein restricted diet to minimize accumulation of metabolites. Treatment with oral biotin has been proven ineffective.

COMBINED CARBOXYLASE DEFICIENCY

The disorder that affects the activity of the four carboxylases is termed multiple carboxylase deficiency (MCD). The symptoms are a summary of the isolated carboxylase deficiencies. There exists two types: the early onset (or neonatal) and the late onset (or juvenile) MCD. In general, individuals with the early onset form suffer from HCS deficiency and those with late onset from biotinidase deficiency (37). However, the age of onset overlaps in the two forms. In both diseases, patients respond to biotin therapy with complete reversal of clinical and biochemical manifestations (37). HCS deficiency

can be fatal if left untreated. Both diseases are inherited in an autosomal recessive fashion.

HCS deficiency

The first patient ultimately shown to have HCS deficiency was described in 1971 with symptoms of ß-methylcrotonyl glycinuria and clinical responsiveness to biotin therapy (64). A few years later, it was demonstrated that PCC and MCC activities from cultured fibroblasts from this patient were undetectable at low concentrations of biotin but their activity was restored at high concentrations (65). Another group obtained similar results and suggested a defect in HCS activity (66). Our laboratory also showed a defect in the third mitochondrial carboxylase, PC (67). In 1981, Burri *et al.* provided evidence that neonatal MCD arose from a defect in HCS (21).

The age of onset of neonatal MCD occurs from a few hours to several months after birth (37). The common symptoms include ketolactic acidosis, organic aciduria, and hyperammonemia. A summary of the symptoms is found in Table 1. If left untreated, this disease leads to coma and death.

There is no direct assay for HCS. Saunders *et al* (68) evaluated HCS in a coupled assay in which patient fibroblast cell extracts were combined with a carboxylase-free extract from normal cells. It was shown that there was a block in the activation of apocarboxylases in MCD patients. Burri *et al* (69) went on to use partially-purified liver cell extracts from biotin-starved rats as a source of apocarboxylase for the assay of HCS and determined a K_M for biotin of 15 nM also by assay of activated carboxylase as a measure of product formation. The K_M for biotin of mutant HCS varied from 50-1050 nM in different patient fibroblast lines. These results were further substantiated by Bartlett's group who measured a normal K_M for biotin of 100 nM and a greatly increased K_M of 554 nM for a MCD patient. This group used mitochondria isolated from cells grown in a medium containing biotin depleted fetal calf serum, which were incubated with ATP, biotin and propionyl-CoA. They measured the incorporation of radioactive

CLINICAL AND BIOCHEMICAL FEATURES OF BIOTINIDASE DEFICIENCY	
% affected	symptom
>50	alopecia, developmental delay, hypotonia, ketolactic acidosis, organic aciduria, seizures, skin rash/infection
25-50	ataxia, conjunctivitis, hearing loss, lethargy, mild hyperammonemia, visual and hearing problems
10-25	coma, feeding problems, fungal infections
<10	hepatomegaly, speech problems, splenomegaly
CLINICAL AND BIOCHEMICAL FEATURES OF HCS DEFICIENCY	
% affected	symptom
100	ketolactic acidosis, organic aciduria
75-100	hyperammonemia, breathing problems
50-75	skin rash
25-50	lethargy, irritability, feeding problems, hypotonia/hypertonia, developmental delay, coma
<10	ataxia, tremor, alopecia

Table 1. Summary of clinical and biochemical features of HCS and biotinidase deficiency

bicarbonate into methylmalonyl-CoA. The two studies showed that mutant HCS in MCD patients has a lower affinity for biotin. The difference between the K_M values measured in these two studies may lie in the source of apoenzyme. Both studies demonstrated dramatic increases in the K_m for biotin in patient cells but left unclear the affinity of biotin for the native enzyme.

Biotinidase deficiency

The first patient diagnosed with biotinidase deficiency was treated with pharmacological doses of oral biotin (70). All the clinical and biochemical abnormalities were reversed including severe rash, alopecia, ketosis and ataxia. A summary of the manifestations of biotinidase deficiency is illustrated in Table 1. Measurement of the activities of the three mitochondrial carboxylases showed an increase in activity in response to the biotin treatment (70). Other groups studied patients with late onset MCD and found that carboxylase activities were normal (71-73) which led to the hypothesis that the defect in late-onset MCD lay in the transport of biotin. However, it was later demonstrated that the defect was due to biotinidase deficiency (74). A reduction in biotinidase activity results in lower amounts of available free biotin thus producing an effect similar to the effect of HCS deficiency. The age of onset is from a few weeks to several years. The manifestations of this disease are variable even among affected siblings (37).

There exists a simple procedure for detecting biotinidase deficiency that is used routinely for newborn screening (75,76). It involves measuring the ability of biotinidase to release p-aminobenzoate (PAB) from a synthetic substrate N-biotinyl-p-aminobenzoate (B-PAB) in a coupled reaction with a naphthol derivative. A purple coloration results which can then be compared to that of known biotinidase activity.

Treatment for MCD

Fortunately, all patients with either form of MCD can be treated with pharmacological doses of oral biotin (37). All the clinical and biochemical abnormalities are reversed within weeks of beginning treatment (37). As well, biotin supplement can be given to

mothers of affected fetuses to allow normal development. This has been documented to be very effective (77,78). At birth, the children are normal and remain symptom-free on biotin treatment.

REFERENCES

1. Bateman, W.G. (1916): The digestibility and utilization of egg protein. *J. Biol. Chem.* 26:263

2. Parsons, H.T., Lease, J.G., and Kelly, E. (1937): LIX. The interrelationship between dietary egg white and requirement for a protective factor in the cure of the nutritive disorder due to egg white. *Biochem. J.* 31:424

3. Gyorgy, P. (1939): The curative factor (vitamin H) for egg white injury, with particular reference to its presence in different foodstuffs and in yeast. *J. Biol. Chem.* 131:733

4. Du Vignaud, V., Hoffmann, K., and Melville, D.B. (1942): On the structure of biotin. *J. Am. Chem. Soc.* 64:188

5. Eakin, R.E., Snell, E.E., and Williams, R.J. (1940): Concentration and assay of avidin, injury producing agents in raw egg white. *J. Biol. Chem.* 136:801

6. Harding, M.G. and Crooks, H. (1961): Lesser known vitamins in foods. *J. Am. Diet. Assoc.* 38:204

7. Gyorgy, P. and Lager, B.W. (1967): The Vitamin VII-Chemistry, Physiology, Pathology, Methods. In: *Biotin.* edited by W.H.J. Sebrell, et al, pp. 303, Academic, New York.

8. National Research Council. (1980): Recommended Dietary Allowances. 8th ed.

9. Wood, H.G. and Barden, R.E. (1977): Biotin enzymes. *Annu. Rev. Biochem.* 46:385-413.

10. Dakshinamurti, K., Chalifour, L., and Bhullar, R.P. (1985): Requirement for biotin and the function of biotin in cells in culture. *Ann. NY Acad. Sci.*, 447:38-55.

11. Moss, J. and Lane, M.D. (1971): The biotin-dependent enzymes. [Review]. *Adv. Enz. Rel. Areas Mol. Biol.*, 35:321-442.

12. Alberts, A.W. and Vagelos, P.R. (1972): *The Enzymes.* Academic Press, Orlando, FL.

13. Lynen, F. (1979): New experiments of biotin enzymes. *CRC Critical Rev. Biochem.*, 7:103-119.

14. Samols, D., Thornton, C.G., Murtif, V.L., Kumar, G.K., Haase, F.C., and Wood, H.G. (1988): Evolutionary conservation among biotin enzymes. *J. Biol. Chem.* 263:6461-6464.

15. Knowles, J.R. (1989): The mechanism of biotin-dependent enzymes. *Annu. Rev. Biochem.* 58:195-221.

16. Dimroth, P. (1987): Sodium ion transport decarboxylases and other aspects of sodium ion cycling in bacteria. [Review]. *Microbiol. Rev.*, 51:320-340.

17. Li, S.J. and Cronan, J.E., Jr. (1992): The gene encoding the biotin carboxylase subunit of Escherichia coli acetyl-CoA carboxylase. *J. Biol. Chem.* 267:855-863.

18. Li, S.J. and Cronan, J.E.J. (1992): The genes encoding the two carboxyltransferase subunits of Escherichia coli acetyl-CoA carboxylase. *J. Biol. Chem.* 267:16841-16847.

19. Alberts, A.W., Nervi, A.M., and Vagelos, P.R. (1969): Acetyl CoA carboxylase, II. Deomonstration of biotin-protein and biotin carboxylase subunits. *Proc. Natl Acad. Sci. USA*, 63:1319-1326.

20. Guchhait, R.B., Polakis, S.E., Dimroth, P., Stoll, E., Moss, J., and Lane, M.D. (1974): Acetyl coenzyme A carboxylase system of Escherichia coli. Purification and properties of the biotin carboxylase, carboxyltransferase, and carboxyl carrier protein components. *J. Biol. Chem.*, 249:6633-6645.

21. Burri, B.J., Sweetman, L., and Nyhan, W.L. (1981): Mutant holocarboxylase synthetase: Evidence for the enzyme defect in early infantile biotin-responsive multiple carboxylase deficiency. *J. Clin. Invest.* 68:1491-1495.

22. Barker, D.F. and Campbell, A.M. (1981): Genetic and biochemical characterization of the birA gene and its product: Evidence for a direct role of biotin holoenzyme synthetase in repression of the biotin operon in Escherichia coli. *J. Mol. Biol.* 146:469-492.

23. McAllister, H.C. and Coon, M.J. (1966): Further studies on the properties of liver propionyl coenzyme A holocarboxylase synthetase and the specificity of holocarboxylase formation. *J. Biol. Chem.* 241:2855-2861.

24. Cazzulo, J.J., Sundaram, T.K., Dilks, S.N., and Kornberg, H.L. (1971): Synthesis of pyruvate carboxylase from its apoenzyme and (+)-biotin in Bacillus stearothermophilus. Purification and properties of the apoenzyme and the holoenzyme synthetase. *Biochem. J.*, 122:653-661.

25. Wood, H.G., Harmon, F.R., Wuhr, B., Hubner, K., and Lynen, F. (1980): Comparison of the biotination of apotranscarboxylase and its aposubunits. Is assembly

essential for biotination? *J. Biol. Chem.*, 255:7397-7409.

26. Lane, M.D., Young, D.L., and Lynen, F. (1964): The enzymatic synthesis of holotranscarboxylase from apotranscarboxylase and (+)- biotin. I. Purification of apoenzyme and synthetase; characteristics of the reaction. *J. Biol. Chem.* 239:2858

27. Kosow, D.P. and Lane, M.D. (1961): Restoration of biotin-deficiency-induced depression of propionyl CoA carboxylase activity in vivo and in vitro. *Biochem. Biophys. Res. Commun.* 4:92

28. Kosow, D.P., Huang, S.C., and Lane, M.D. (1962): Propionyl holocarboxylase synthesis. *J. Biol. Chem.* 12:3633

29. Foote, J.L., Cristner, J.E., and Coon, M.J. (1963): Biotin and adenosine triphosphate-dependent activation of propionyl apocarboxylase. *Biochim. Biophys. Acta*, 67:676

30. Seigel, L., Foote, J.L., Christner, J.E., and Coon, M.J. (1963): Propionyl CoA holocarboxylase synthesis from biotinyl adenylate and the apocarboxylase in the presence of an activating enzyme. *Biochem. Biophys. Res. Commun.* 3:307

31. Lane, M.D., Rominger, K.L., Young, D.L., and Lynen, F. (1964): The enzymatic synthesis of holotranscarboxylase from apotranscarboxylase and (+)-biotin. II. Investigation of the reaction mechanism. *J. Biol. Chem.* 239:2865

32. Christner, J.E., Schlesinger, M.J., and Coon, M.J. (1964): Enzymatic activation of biotin: Biotinyl adenylate formation. *J. Biol. Chem.* 239:3997

33. Seigel, L., Foote, J.L., and Coon, M.J. (1965): The enzymatic synthesis of

propionyl coenzyme A holocarboxylase from d-biotinyl 5-adenylate and the apocarboxylase. *J. Biol. Chem.* 240:1025

34. Kosow, D.P. and Lane, M.D. (1961): Propionyl apocarboxylase activation catalyzed by cell-free enzyme extracts. *Biochem. Biophys. Res. Commun.* 5:191-195.

35. Kosow, D.P. and Lane, M.D. (1962): Biotin-dependent activation of propionyl apocarboxylase. *Fed. Proc.* 21:236

36. Wright, L.D., Cresson, E.L., Skegs, H.R., Wood, T.R., Peck, R.L., Wolf, D.E., and Folkers, K.J. (1952): Isolation of crystalline biocytin from yeast extract. *J. Amer. Chem. Soc.* 74:1996-1999.

37. Wolf, B. (1995): Disorders of Biotin Metabolism. In: *The Metabolic and Molecular Bases of Inherited Disease*, edited by C.R. Scriver, et al, pp. 3151-3177. McGraw-Hill, New York.

38. Otsuka, A.J., Buoncristiani, M.R., Howard, P.K., Flamm, J., Johnson, C., Yamamoto, R., Uchida, K., Cook, C., Ruppert, J., and Matsuzaki, J. (1988): The Escherichia coli biotin biosynthetic enzyme sequences predicted from the nucleotide sequence of the bio operon. *J. Biol. Chem.* 263:19577-19585.

39. Cronan, J.E.Jr. (1989): The E. coli bio operon: Transcriptional repression by an essential protein modification enzyme. *Cell*, 58:427-429.

40. Prakash, O. and Eisenberg, M.A. (1974): Active transport of biotin in Escherichia coli K-12. *J. Bacteriol.*, 120:785-791.

41. Cronan, J.E., Jr. (1988): Expression of the biotin biosynthetic operon of Escherichia

coli is regulated by the rate of protein biotination. *J. Biol. Chem.*, 263:10332-10336.

42. Pai, C.H. and Lichstein H.C. (1962): *Bichem. Biophys. Acta* 65:1559-160.

43. Eisenberg, M.A. (1973): Biotin: biogenesis, transport, and their regulation. *Adv. Enz. Rel. Areas Mol. Biol.*, 38:317-372.

44. Eisenberg, M.A. (1985): Regulation of the biotin operon in E. coli. *Ann. NY Acad. Sci*, 447:335-349.

45. Wilson, K.P., Shewchuk, L.M., Brennan, R.G., Otsuka, A.J., and Matthews, B.W. (1992): Escherichia coli biotin holoenzyme synthetase/bio repressor crystal structure delineates the biotin- and DNA-binding domains. *Proc. Natl Acad. Sci. USA*, 89:9257-9261.

46. Suzuki, Y., Aoki, Y., Ishida, Y., Chiba, Y., Iwamatsu, A., Kishino, T., Niikawa, N., Matsubara, Y., and Narisawa, K. (1994): Isolation and characterization of mutations in the human holocarboxylase synthetase cDNA. *Nat. Genet.* 8:122-128.

47. Leon-Del-Rio, A., Leclerc, D., Akerman, B., Wakamatsu, N., and Gravel, R.A. (1995): Isolation of a cDNA encoding human holocarboxylase synthetase by functional complementation of a biotin auxotroph of Escherichia coli. *Pro. Natl Acad. Sci. USA*, 92:4626-4630.

48. Ahmad, P.M. and Ahmad, F. (1991): Mammalian pyruvate carboxylase: effect of biotin on the synthesis and translocation of apo-enzyme into 3T3-L adipocyte mitochondria. *FASEB J.* 5:2482-2485.

49. Landman, A.D. (1976): Activation of biotin-enzymes: a possible biochemical rationale. *Life Sci.*, 19:1377-1381.

50. Taroni, F. and Rosenberg L.E. (1991): The presursor of the biotin-binding subunit of mammalian propionyl CoA carboxylase can be translocated into mitochondria as apo- or holoprotein. *J. Biol. Chem.*, 266:13267-13271.

51. Barker, D.F., Kuhn, J. and Campbell, A.M. (1981): Sequence and properties of operator mutations in the bio operon of *Escherichia coli, Gene*, 13:89-102.

52. Dupuis, L., Leon-Del-Rio, A., Leclerc, D., Campeau, E., Sweetman, L., Saudubray, J.M., Herman, G., Gibson, K.M., and Gravel, R.A. (1996): Clustering of mutations in the biotin-binding region of holocarboxylase synthetase in biotin-responsive multiple carboxylase deficiency. *Hum. Mol. Gen.* 5:1011-1016.

53. Achuta Murthy, P.N. and Mistry, S.P. (1964): Synthesis of biotin-dependent carboxylases from their apoproteins and biotin. *Biochem. Rev.* 239:2858

54. Gornicki, P., Scappino, L.A., and Haselkorn, R. (1993): Genes for two subunits of acetyl coenzyme A carboxylase of Anabaena sp. strain PCC 7120: biotin carboxylase and biotin carboxyl carrier protein. *J. Bacteriol.*, 175:5268-5272.

55. Leon-Del-Rio, A. and Gravel, R.A. (1994): Sequence requirements for the biotinylation of carboxyl-terminal fragments of human propionyl-CoA carboxylase alpha subunit expressed in Escherichia coli. *J. Biol. Chem.*, 269:22964-22968.

56. Chapman-Smith, A., Turner, D.L., Cronan, J.E., Jr., Morris, T.W., and Wallace,

J.C. (1994): Expression, biotinylation and purification of a biotin-domain peptide from the biotin carboxy carrier protein of Escherichia coli acetyl-CoA carboxylase. *Biochem. J.*, 302:881-887.

57. Burri, B.J., Sweetman, L., and Nyhan, W.L. (1985): Heterogeneity of holocarboxylase synthetase in patients with biotin-responsive multiple carboxylase deficiency. *Am. J. Hum. Genet.* 37:326-337.

58. Cole, H., Reynolds, T.R., Lockyer, J.M., Buck, G.A., Denson, T., Spence, J.E., Hymes, J., and Wolf, B. (1994): Human serum biotinidase. cDNA cloning, sequence, and characterization. *J. Biol. Chem.* 269:6566-6570.

59. Cole, H., Weremowicz, S., Morton, C.C., and Wolf, B. (1994): Localization of serum biotinidase (BTD) to human chromosome 3 in band p25. *Genomics*, 22:662-663.

60. Craft, D.V., Goss, N.H., Chandramouli, N., and Wood, H.G. (1985): Purification of biotinidase from human plasma and its activity on biotinyl peptides. *Biochemistry*, 24:2471-2476.

61. Chauhan, J. and Dakshinamurti, K. (1986): Purification and characterization of human serum biotinidase. *J. Biol. Chem.*, 261:4268-4275.

62. Wolf, B., Miller, J.B., Hymes, J., Secor McVoy, J., Ishikawa, Y., and Shapira, E. (1987): Immunological comparison of biotinidase in serum from normal and biotinidase-deficient individuals. *Clin. Chim. Acta*, 164:27-32.

63. Blom, W. and Scholte, H.R. (1981): Acetyl-CoA carboxylase deficiency: An inborn error of de novo fatty acid synthesis. *N. Engl. J. Med.* 305:465

64. Gompertz, D., Draffan, G.H., Watts, J.L., and Hull, D. (1971): Biotin-responsive B-methylcrotonylglycinuria. *Lancet*, 22-24.

65. Bartlett, K. and Gompertz, D. (1976): Combined carboxylase defect: biotin-responsiveness in cultured fibroblasts. *Lancet*, 804

66. Weyler, B., Sweetman, L., Maggio, D.C., and Nyhan, W.L. (1977): Deficiency of propionyl-CoA carboxylase in a patient with methylcrotonylglycinuria. *Clin. Chim. Acta*, 76:321-328.

67. Saunders, M., Sweetman, L., Robinson, B., Roth, R., Cohn, R., and Gravel, R.A. (1979): Biotin-response organicaciduria: Multiple carboxylase defects and complementation studies with propionicacidemia in cultured fibroblasts. *J. Clin. Invest*, 64:1695-1702.

68. Saunders, M.E., Sherwood, W.G., Duthe, M., Surh, L., and Gravel, R.A. (1982): Evidence for a defect ofholocarboxylase synthetase activity in cultured lymphoblasts from a patient with biotin-responsive multiple carboxylase deficiency. *Am. J. Hum. Genet.* 34:590-601.

69. Burri, B.J., Sweetman, L., and Nyhan, W.L. (1985): Heterogeneity of holocarboxylase synthetase in patients with biotin-responsive multiple carboxylase deficiency. *Am. J. Hum. Genet.* 37:326

70. Cowan, M.J., Wara, D.W., Packman, S., Ammann, A.J., Yoshino, M., Sweetman, L., and Nyhan, W. (1979): Multiple biotin-dependent carboxylase deficiencies associated with defects in T-cell and B-cell immunity. *Lancet*, 2:115-118.

71. Charles, B.M., Hosking, G., Green, A., Pollitt, R., Bartlett, K., and Taitz, L.S.

(1979): Biotin-responsive alopecia and developmental regression. *Lancet*, 2:118-120.

72. Thoene, J., Baker, H., Yoshino, M., and Sweetman, L. (1981): Biotin-responsive carboxylase deficiency associated with subnormal plasma and urinary biotin. *N. Engl. J. Med.*, 304:817-820.

73. Thoene, J.G., Lemons, R., and Baker, H. (1983): Impaired intestinal absorption of biotin in juvenile multiple carboxylase deficiency. *N. Engl. J. Med.*, 308:639-642.

74. Wolf, B., Grier, R.E., Allen, R.J., Goodman, S.I., and Kien, C.L. (1983): Biotinidase deficiency: the enzymatic defect in late-onset multiple carboxylase deficiency. *Clin. Chim. Acta*, 131:273-281.

75. Wolf, B., Heard, G.S., Jefferson, L.G., Proud, V.K., Nance, W.E., and Weissbecker, K.A. (1985): Clinical findings in four children with biotinidase deficiency detected through a statewide neonatal screening program. *N. Engl. J. Med.*, 313:16-19.

76. Heard, G.S., Wolf, B., Jefferson, L.G., Weissbecker, K.A., Nance, W.E., McVoy, J.R., Napolitano, A., Mitchell, P.L., Lambert, F.W., and Linyear, A.S. (1986): Neonatal screening for biotinidase deficiency: results of a 1-year pilot study. *J. Ped.* 108:40-46.

77. Roth, K.S., Yang, W., Allan, L., Saunders, M., Gravel, R.A., and Dakshinamurti, K. (1982): Prenatal administration of biotin in biotin responsive multiple carboxylase deficiency. *Ped. Res.*, 16:126-129.

78. Packman, S., Cowan, M.J., Golbus, M.S., Caswell, N.M., Sweetman, L., Burri, B.J., Nyhan, W.L., and Baker, H. (1982): Prenatal treatment of biotin responsive multiple carboxylase deficiency. *Lancet*, 1:1435-1438.

CHAPTER II

Preface to chapter II

The cloning of the human HCS cDNA in our laboratory permitted me to begin mutation analysis on cell lines from patients with multiple carboxylase deficiency. The work involved performing SSCP analysis (single stranded length conformational polymorphism) analysis on cDNA obtained by RT-PCR of HCS mRNA and direct or automated sequencing of PCR products which displayed band shifts on SSCP gels. The identified mutations were then confirmed by restriction enzyme digests of PCR amplified gDNA.

This work has been published in Human Molecular Genetics, 5(7), 1011-1016, 1996, under the title: Clustering of mutations in the biotin-binding region of holocarboxylase synthetase in biotin-responsive multiple carboxylase deficiency. The authors are: Lucie Dupuis, Alfonso Leon-Del-Rio, Daniel Leclerc, Eric Campeau, Lawrence Sweetman, Jean-Marie Saudubray, Gail Herman, K. Michael Gibson and Roy A. Gravel. LS, JMS, GH and KMG provided the clinical and biochemical characterization of patients and fibroblast cell lines used in this study. EC identified the uncharacterized splice mutation in the patient CP. ALDR and DL provided indispensable suggestions and participated in crucial discussions. The paper is presented exactly as it was published except that the abstract has been omitted

I have demonstrated that the identified mutations cluster in the biotin binding region of HCS. This localization may account for the lower affinity for biotin in mutant HCS from MCD patients and hence the biotin responsiveness observed in all patients. In addition, I have identified two mutations outside this region suggesting a second domain involved in biotin responsiveness.

Clustering of mutations in the biotin-binding region of holocarboxylase synthetase in biotin-responsive multiple carboxylase deficiency

INTRODUCTION

Holocarboxylase synthetase (HCS; EC 6.3.4.10) catalyzes the biotinylation of the four biotin-dependent carboxylases found in humans: the mitochondrial propionyl-CoA carboxylase (PCC), pyruvate carboxylase (PC) and ß-methylcrotonyl-CoA carboxylase (MCC) and the cytosolic acetyl-CoA carboxylase (ACC) (1). An inherited deficiency of HCS activity results in the neonatal form of multiple carboxylase deficiency (MCD), a rare autosomal recessive disorder. The disease is characterized by a decrease in activity of all four biotin-dependent carboxylases so as to impair gluconeogenesis, fatty acid metabolism and amino acid catabolism (1). Affected individuals show metabolic acidosis, organic aciduria and varying degrees of hyperammonemia when they first become symptomatic, usually in the first months of life. Clinical manifestations include skin rashes, seizures and developmental delay followed by coma and death if left untreated (1). Both the clinical and biochemical symptoms are dramatically resolved with pharmacological doses of oral biotin (1). A milder form of the disease (late onset MCD) has also been described (2). Most cases are caused by a defect in biotinidase, the enzyme responsible for the recycling of biotin, although some appear to be mild cases of HCS deficiency (3). This additional form of MCD can also be treated with oral biotin.

HCS catalyzes the ATP-dependent covalent attachment of biotin to the apocarboxylases (1). While the four mammalian carboxylases differ substantially in their structure, they share a highly homologous biotin-binding site located near the C-terminus in three of them and near the N-terminus in ACC (4). HCS activity is found in both the mitochondria and cytosol (5,6), which allows for the biotinylation of apocarboxylases in both compartments.

Recently, we (Leon-Del-Rio *et al.* (7)) and Suzuki *et al.* (8) cloned the cDNA encoding

human HCS, the former by functional complementation of a *bir*A mutant of *E. coli* defective in the bacterial "biotin ligase" activity and the latter based on peptide sequence data from the purified bovine HCS. Analysis of the predicted amino acid sequence of human HCS (726 amino acids) showed that it is homologous with bacterial biotin ligases in a segment previously identified as the biotin-binding domain of the *E. coli* BirA protein by X-ray crystallography (9). The BirA protein (321 amino acids) serves as co-repressor of the biotin biosynthetic operon as well as carrying the biotin ligase function. Significantly, mutations in *bir*A mutants, showing an increased requirement for biotin in the growth medium (biotin-auxotrophy) have been localized to the biotin-binding region.

In MCD, mutant HCS has been shown to be associated with a reduced affinity for biotin. Burri *et al.* (10), used partially-purified extracts from biotin-starved rats as a source of apocarboxylase for the measurement of HCS activity and determined a K_M for biotin by assay of activated carboxylase as a measure of product formation. They obtained increases of up to 350 fold in the K_M for biotin in MCD patients. Ghneim *et al.* (11), also reported an increased K_M in a neonatal MCD patient by assaying the activation of carboxylases in mitochondrial homogenates of biotin-starved fibroblasts. Recently, Suzuki *et al.* (8), identified two mutations, a single base deletion and a point mutation which may be common in Japanese patients with neonatal MCD. However, neither mutation was located in the biotin-binding region.

In the present report, we have screened more than 95% of the HCS coding sequence of six patients with neonatal MCD to reveal several mutations which cluster in the biotin-binding region.

RESULTS

The HCS cDNA was divided into six overlapping segments (Table 1) in order to permit SSCP analysis of relatively small fragments. Two PCR products were obtained for fragment I because the 5' end of HCS mRNA is alternatively spliced (7). Fragments V

SEGMENT (position[a])	PRIMERS SEQUENCE[b]	SIZE[c] (bp)	RESTRICTION ENZYME[d] & FRAGMENT SIZES[d]
I (-289 to 79)	TGAGAATTTACAGAGATCATCCTC/ TCAGAGTGGAGTCCTGCAAGTGCA	369 & 223	*Alu*I 213, 94, 62 and 94, 67, 62
II (-75 to 465)	GCTTGCAGACCTGGGGATCCTTAT/ ACTCTCCTCCCTTCTCTTTCG	541	*Mbo*II 241, 207 &93
III (347 to 869)	ACCATTGAGTCAGTCAAGTTTGCG/ CTGCAGCCACTGCTCAAGACGCT	523	*Fok*I 254, 140 & 129
IV (724 to 1218)	GAAGGTGTTGGGCCTGTCTTCATC/ ATGTTTCCCAAGCCACTGCATAAG	495	*Nla*III 232, 176 & 87
V (1123 to 1669)	CTGTGACATGAAACAAGTTCCTGC/ GAATGGACCTCACTGCTTCCACGA	547	*Hae*III 171, 165, 125 & 86
VI (1527 to 2181)	GAGGGAATGTGTGGCTGAGCCCTG/ TTACCGCCGTTTGGGGAGGATGAG	655	*Hae*III 261, 178, 118 & 98

Table 1. Primers for Amplification of HCS cDNA. The a indicates numbering as in Leon-Del-Rio *et al.* (7), the b sequences of primer pairs given 5' to 3', for sense and antisense respectively and the c expected size of amplified fragments. For segment I, two products sizes are given due to alternative splicing (7). The d represents restriction enzymes for digestion of SSCP samples for analysis with resulting calculated sizes of fragments.

and VI contain the putative biotin-binding region. Sequencing the PCR products that showed band shifts on the SSCP gels resulted in the identification of six mutations, of which four were localized to the biotin-binding region (Table 2).

In patients MC, CP and YL, a G_{1648}→A mutation (Val550Met) was identified which created an *Nla*III restriction site. A 206 bp PCR fragment was amplified from genomic DNA using intronic sense and antisense primers (Table 3). When the PCR fragment was digested with *Nla*III, a 206 bp fragment was obtained for the normal allele, while fragments of 151 and 55 bp were obtained as a result of the base substitution (Fig. 1A). MC and CP proved to be heterozygous for the mutation and YL appeared homozygous, although partial or complete gene deletion in one allele cannot be excluded in the latter since the parents were unrelated.

A G_{1553}→A mutation (Gly518Glu) was identified in one allele of HB which did not alter a restriction site. Therefore, an oligonucleotide was designed to create a *Taq*I restriction site in the presence of the mutation (Table 3). The altered sense primer contained only exonic sequence while the antisense primer contained intronic sequence. When genomic DNA was amplified by PCR with these primers, a band of 177 bp in length was obtained. Upon digestion of the PCR fragment with *Taq*I, the normal sequence was not cut whereas the mutation resulted in fragments of 155 and 22 bp (Fig. 1B). HB was confirmed as heterozygous for the mutation.

A T_{647}→G mutation (Leu216Arg) was found in patient VE. The mutation was detected using a modified sense primer to create a single *Hpa*II restriction site in the PCR fragment amplified from genomic DNA (Table 3). Two exonic primers were used to amplify a 245 bp fragment. When digested with *Hpa*II, fragments of 224 and 21 bp in length were obtained when the mutation was present. The patient was found to be heterozygous for the mutation (Fig. 1C).

Patient Initials	Ref.	Mutations outside the biotin-binding region		Mutations within the biotin-binding region				Unidentified second mutation
		$T_{647}\rightarrow G$ (L216R)	$T_{1088}\rightarrow A$ (V363D)	$C_{1522}\rightarrow T$ (R508W)	$G_{1553}\rightarrow A$ (G518E)	$G_{1648}\rightarrow A$ (V550M)	$G_{1711}\rightarrow A$ (D571N)	
VE		X	X					
MMC			X	X				
PD				X			X	
JRi	(23-27)			X				X
MK				XX				
HB	a				X			X
MC	(28)					X		X
CP						X		X[b]
YL	(29)					XX		

Table 2. Summary of mutations found in patients with HCS deficiency. The a indicates it was presented at the Vth Nordic Meeting of Medical Genetics in Laugarvatn, Iceland, August 27-28, 1988 (abstract 220). The b indicates an uncharacterized splice mutation (not analyzed at level of genomic DNA).

Mutation	Primers	Expected size (bp)	Enzyme restriction site created by:		Expected size after digestion (bp):	
			mutation	mutation & primer	normal	mutant
T_{467}→G	CCGTGGACGGACAACTGTCTCC/ CTGCAGCCACTGCTCAAGACGCT	245		HpaII	245	224 & 21
T_{1008}→A	GCTCAAGTCAAGCAATTTTAGCAG/ acCAAGGGTTGTCAGAATCTCTC	61	BbsI		61	36 & 25
C_{1322}→T	cactgtggcctgtgtccagCA/ tagaaggagactgaactgtaccta	206		NlaIII	206	182 & 24
$^{*}C_{1322}$→T	CGGCAGACCGAGGGCAAAGCA/ TTACCGCCGTTTGGGGAGGATGAG	681		NlaIII	378, 234, 36 & 33	378, 211, 36, 33 & 23
G_{1353}→A	GGAATGTGTGGCTGAGCCCTGTCG/ tagaaggagactgaactgtaccta	177		TaqI	177	155 & 22
G_{1668}→A	cactgtggcctgtgtccagCA/ tagaaggagactgaactgtaccta	206	NlaIII		206	151 & 55
G_{1771}→A	cttaattaatgtgcagattcccct/ tagaaggagactgaactgtaccta	242	SapI		242	181 & 61

Table 3. Stategy for PCR-based diagnostics of mutations identified in the human HCS gene. The (a) indicates RT-PCR was used to amplify the region with the mutation. The lower case letters indicate intronic sequence and the upper case letters coding sequence. The double underline letters indicate the nucleotide was altered to create a new restriction site. The single underline letters indicate the nucleotide was previously altered for a different diagnostic.

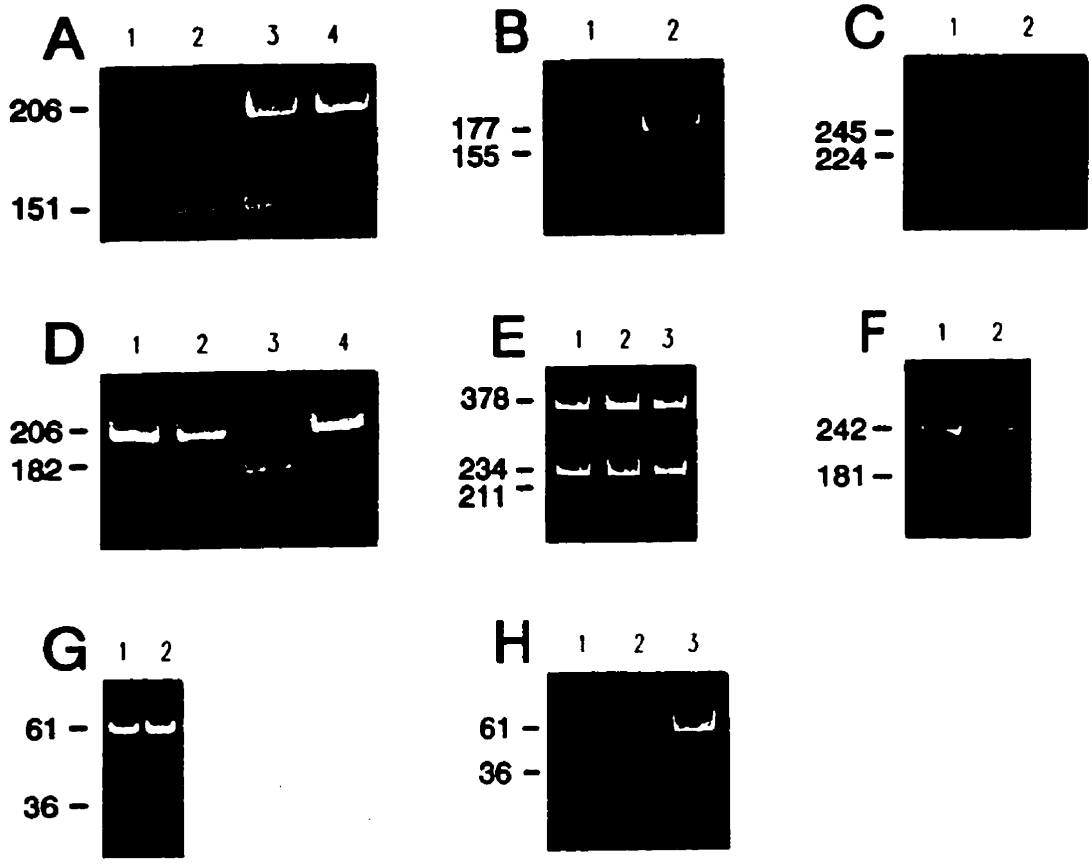

Figure 1. DNA diagnostic tests for mutations in the *HCS* gene. Details are given in Table 3. **A.** G_{1648}→A (in genomic DNA): lane 1, CP; lane 2, YL; lane 3, MC; lane 4, normal. **B.** G_{1553}→A (in genomic DNA): lane 1, HB; lane 2, normal. **C.** T_{647}→G: lane 1, normal; lane 2, VE. **D.** C_{1522}→T (in genomic DNA): lane 1, JRi; lane 2, PD; lane 3, MK; lane 4, normal. **E.** C_{1522}→T (in reverse transcribe mRNA): lane 1, normal; lane 2, MMC; lane 3, PD. **F.** G_{1711}→A (in genomic DNA): lane 1, normal; lane 2, PD. **G.** T_{1088}→A (in genomic DNA): lane 1, VE; lane 2, Normal. **H.** T_{1088}→A (in reversed transcribed mRNA): lane 1, VE; lane 2, MMC; lane 3, Normal.

In patients MMC, PD, JRi and MK, a C_{1522}→T transition was identified (Arg508Trp). An oligonucleotide was synthesized that generated an *Nla*III restriction site in the presence of the mutation (Table 3). PCR products for JRi, MK and PD were generated from genomic DNA templates using an altered sense primer which was partially intronic and an antisense primer containing intronic sequence only. For the normal allele, a fragment of 206 bp in length was obtained when the PCR product was digested with *Nla*III compared to fragments of 182 and 24 bp when the mutation was present (Fig. 1D). PD and JRi were heterozygous for the mutation. MK appeared homozygous for the mutation, however, a partial or complete gene deletion in one allele can not be excluded due to nonconsanguinity. The mutation was also demonstrated in one allele of MMC using an RT-PCR based diagnostic test since genomic DNA was not available. The two cDNA primers, including an altered sense primer, generated a fragment of 681 bp in length (Table 3). When the normal PCR product was cut with the enzyme *Nla*III, fragments of 378, 234, 36 and 33 bp were generated (only the two largest fragments could be discerned on the gel). In the presence of the mutation, the 234 bp fragment was cut into fragments of 211 and 23 bp (Fig. 1E).

A G_{1711}→A mutation (Asp571Asn) was identified in PD which created a single *Ssp*I restriction site. Two intronic primers were used to generate the 242 bp fragment by PCR using genomic DNA as the template (Table 3). The 242 bp fragment was cut into segments of 181 and 61 bp in length in the presence of the mutation (Fig. 1F).

A T_{1088}→A base substitution (Val363Asp) was identified in VE and MMC which created a *Bbs*I restriction site (Fig. 1G). The PCR primers were almost fully exonic and generated a product 61 bp in length (Table 3). Cleavage of the PCR product generated fragments of 36 and 25 bp in the presence of the mutation. In the case of MMC, the PCR products were generated from reversed transcribed mRNA since genomic DNA was not available (Fig. 1H).

For each of the above mutations, 20 control samples were analyzed for each diagnostic test of a mutation to insure the mutations were not polymorphisms. In none of them was the mutation identified in a normal individual.

DISCUSSION

Our results support the prediction originally made by Sweetman's group, that biotin-responsiveness in neonatal MCD arises from mutations affecting the affinity of HCS for biotin (12). We observed a cluster of four mutations in the predicted biotin-binding region, of which two are candidates for common mutations. The C_{1522}→T (Arg508Trp) mutation accounted for 5/24 of the alleles examined (with one patient homozygous for the mutation), while G_{1648}→A (Val550Met) accounted for 4/24 of the alleles (with one patient homozygous for the mutation). Furthermore, we have identified a patient with two mutations outside the biotin-binding region. Although we have not expressed these mutations in an *in vitro* system, the clustering of the mutations, the occurrence of two of them as common alleles, and the absence of all of them in a sampling of normal individuals provide compelling evidence that they are responsible for the disease.

We anticipate that the four mutations in the biotin-binding region of HCS, will account for the high K_M for biotin measured in patients with neonatal MCD (10,11). For example, two of the patient fibroblast lines we studied, JRi and MC, had a reported K_M of 346 and 48 nM, respectively, compared to 15 nM for the normal enzyme (10). JRi was found to have an Arg508Trp mutation and MC was found to have a Val550Met mutation (in each case, the second mutation has yet to be identified). While it is premature to conclude that these mutations are causative of the elevated K_M, their location in the binding biotin region and the fact that of three of the four mutations affect residues conserved among human, *P. denitrificans, E. coli, B. subtilis, S. typhimurium*, mouse (data not shown) and yeast biotin ligases (Fig. 2) is consistent with this notion. A stronger case can be made for Arg508Trp, which has a major impact on both the structure and charge of the side chain, than for Val550Met, which represents a conservative change at a site which is variable in the above species.

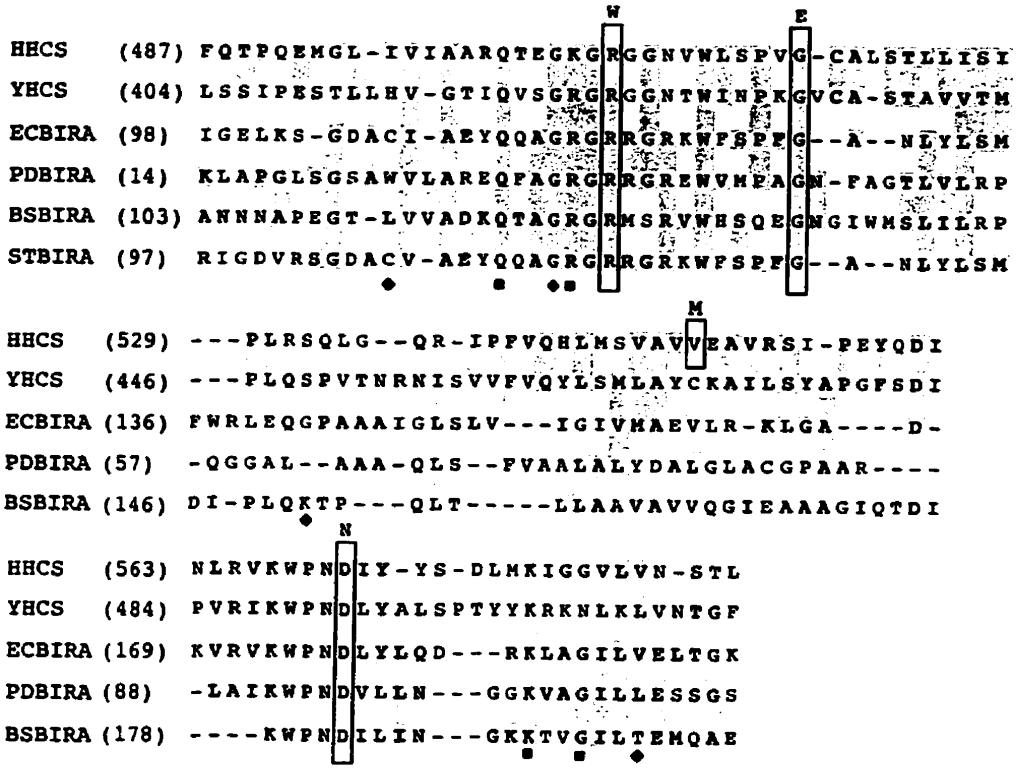

Figure 2. Comparison of the protein sequence of a fragment of human HCS (HHCS (7)) with related sequences of yeast HCS (YHCS (18)) and the BirA proteins of *E. coli* (ECBirA (19)), *Paracoccus denitrificans* (PDBirA (20)), *Bacillus subtilis* (BS1BirA and BS2BirA (21)) and *Salmonella typhimurium* (STBirA, partial sequence (22)). BSBirA has two forms which differ in a single amino acid (A169 or E169). Conserved residues and similar residues (A,G; S,T; D,E; N,Q; R,K; V,L,I,M; F,W,Y) are located in the shaded areas. For each protein, the position of the initial residue in each line is shown on the left of each row. The symbol (■) shows residues that are in contact with biotin in the BirA protein of *E. coli* (9). Positions identified by (◆) refer to mutations affecting the activity of the BirA protein of *E. coli* (*birA*815, *birA*A1, *birA*71, *birA*215 and *birA*104) and which are within 10 Å of the crystallographically determined biotin-binding site (9). The boxes denote mutations identified in MCD patients and above the boxes is the amino acid change produced by the mutation. The alignment at V550M is uncertain given the gaps surrounding the site.

The Arg508Trp change is located two amino acids from a demonstrated contact point for biotin in the corresponding *E. coli* enzyme. However, the mutation is within the site G_{505}-K-G-R-G-G, which is also compatible with a putative ATP binding site [G-X-G-X-X-G (9)]. The role of this site in relation to the participation of ATP and biotin in the biotin-activation step has not been established.

In one biotin-responsive patient, VE, two mutations were found outside the biotin-binding region and indeed beyond the limits corresponding to the BirA protein, at Leu216Arg and Val363Asp. The Leu216Arg mutation was localized to a region where Suzuki *et al.* (8), also reported a point mutation in a biotin-responsive Japanese patient, Leu237Pro. The second mutation in their patient was a deletion of G_{838} that created a stop codon at position 280. Since the frameshift presumably causes a complete loss of HCS activity, the allele containing the point mutation must be involved in biotin-responsiveness. VE's second mutation, Val363Asp, was also found in patient MMC. MMC's second mutation is located in the biotin-binding region and is most likely the one involved in biotin-responsiveness, suggesting that Val363Asp need not be necessary for biotin binding. We therefore predict that the area which contains the mutations Leu216Arg and Leu237Pro is also involved in the biotin-responsiveness of patients. It may act as a second domain involved in biotin binding or it may act more indirectly in the stabilization of the enzyme as a result of biotin binding. We have screened the cDNA of 12 MCD patients for the two mutations reported by Suzuki's group and have found them not to be present in any of our patients.

Patients with HCS deficiency who are not biotin-responsive have yet to be identified, although one appears to be only partially responsive (13). Presumably, null expression of the HCS gene would be associated with an unresponsive phenotype. Such patients would likely be homozygous or heteroallelic for mutations such as chain terminating frameshift or point mutations, deletions or additions, or splice mutations. Thus far, we have not found two functionally null HCS alleles in a patient, although the 5' end of the cDNA was not fully analyzed. Since it is subject to extensive alternate splicing, further

mutation analysis will need to be done in genomic DNA as the required sequences are determined. It is also possible that null genotypes have not been seen because they are lethal *in utero*.

MATERIALS AND METHODS

Cell lines

Fibroblast lines MMC, VE, MC, EG and HB, originating from HCS-deficient patients, were from the cell bank of the Children's Hospital, Los Angeles, CA. Cell line PD was from the Groupe Hospitalier Necker-Enfants Malades, Paris. Additional cell lines surveyed for the identified mutations were from the Baylor Research Institute, Dallas, TX (YL), the Baylor College of Medicine, Houston, TX (MK) and the cell bank of the Children's Hospital, Los Angeles, CA (JRi and CP). The fibroblast lines were grown in α-MEM medium containing 20% FCS, added biotin (20 mM) and antibiotics/antimycotics (Gibco-BRL). Control fibroblasts were grown in the same medium (without added biotin) in the presence of antibiotics and 10% FCS.

Materials

Taq polymerase was purchased from Perkin-Elmer. The T/A cloning kit used for subcloning of PCR products was from Invitrogen. The Gene Clean Kit was obtained from Bio 101 inc. and the Wizard Mini-Preps used for plasmid purification were obtained from Promega. Plasmid sequencing was done using the Sequenase kit from United States Biochemicals. The α-[^{35}S]dATP (12.5 mCi/mmol) was purchased from Dupont. The oligonucleotide primers were synthesized by R. Clarizio of the Montreal Children's Hospital Research Institute Oligonucleotide Synthesis Facility.

Reverse transcription of total RNA and cDNA amplification

Total RNA isolated from fibroblasts was reverse transcribed and the HCS cDNA was amplified in six overlapping segments of 450 to 650 bp in length by PCR according to the methods described earlier (14), except that 0.5 μg of oligonucleotide primers were used and the annealing temperature ranged from 60°C to 66°C, depending on the

segment being amplified. For PCR prior to SSCP analysis, the concentration of dATP was reduced to 6.25 nM, 12.5 μCi of α-[^{35}S]dATP was added and the concentration of dTTP, dGTP and dCTP was changed to 12.5 nM.

SSCP analysis and DNA sequencing

SSCP analysis was performed according to Orita *et al.* (15), as modified by Triggs-Raine *et al.* (14), except that the α-^{35}S-labelled PCR products were digested with various restriction enzymes to generate smaller fragments in order to enhance SSCP sensitivity. The fragments were subjected to electrophoresis in a gel containing 6% acrylamide and 10% glycerol in 1X TBE. The digested and non-digested samples were denatured (100°C, 3 min.) prior to loading. As well, an aliquot of each sample was run without prior heating to identify the duplex product. The gels were run at 6 watts for 16 hours. Fragments that displayed band shifts were sequenced directly. PCR products were sequenced either manually (16) or were done at the Sequencing Core Facility of the Canadian Genetic Diseases Network (Ottawa, ON) using an Applied Biosystems automated sequencer.

Inverse PCR and identification of exon boundaries

In order to confirm some of the mutations in genomic DNA, the sequences of the flanking exons were determined by inverse PCR to allow the design of primers for the PCR-based diagnostic tests (17). Genomic DNA was digested with different enzymes (*Alu*I, *Rsa*I, *Taq*I, *Mse*I, *Msp*I or *Hae*II), ligated with T4 DNA ligase (Gibco), and amplified by PCR using adjacent oligonucleotides priming in opposite direction. The PCR products were purified with Gene Clean and were subcloned in the pCRII vector and transformed into *E. coli* as per the supplier's protocol (TA Cloning Kit, Invitrogen). The candidate clones were then sequenced.

Confirmation of mutations

The identified mutations were confirmed directly in PCR amplification products from genomic DNA or reversed transcribed mRNA. The latter was used if DNA was not

available. If the mutation created or destroyed a restriction enzyme site, this site was used to confirm the presence of the mutation. A 15 μl sample of the PCR product was digested with the appropriate restriction enzyme and analyzed by electrophoresis on an 8% acrylamide gel. If the base substitution did not change a restriction site, one was created through the diagnostic strategy. An oligonucleotide primer terminating one nucleotide from the mutation was altered one or two nucleotide from its 3' end so as to generate (or eliminate) a restriction site in combination with the mutation. Cleavage of the PCR product with the corresponding restriction enzyme would reveal the presence or absence of the mutation (14). For each mutation, a control digestion was included to insure the PCR products were completely digested.

ACKNOWLEDGEMENTS

We thank Stephen Baird (Sequencing Core Facility of the Canadian Genetic diseases Network, Ottawa) for invaluable sequencing assistance and Beverly Akerman (Montreal Children's Hospital Research Institute) for growing the cell cultures. These studies were supported by a grant from the Medical Research Council of Canada. LD is a recipient of a Scholarship from the Montreal Children's Hospital Research Institute. EC is a recipient of a Scholarship from the Fonds de la Recherche en Santé du Québec. We are grateful to the physicians who provided the cell lines used in this study.

ABBREVIATIONS

HCS: holocarboxylase synthetase

MCD: multiple carboxylase deficiency

PCC: propionyl-CoA carboxylase

REFERENCES

1. Wolf, B. (1995) The Metabolic and Molecular Basis of Inherited Diseases, C.R. Scriver, A.L. Beaudet, W.S. Sly and Valle, D. (Eds.), McGraw-Hill, New York, Vol.7, pp 3151-3177.

2. Wolf, B., Grier, R.E., Allen, R.J., Goodman, S.I., and Kien, C.L. (1983) Biotinidase deficiency: the enzymatic defect in late-onset multiple carboxylase deficiency. *Clin. Chim. Acta.*, **131**, 273-281.

3. Sherwood, W.G., Saunders, M., Robinson, B.H., Brewster, T., and Gravel, R.A. (1982) Lactic acidosis in biotin responsive multiple carboxylase deficiency caused by holocarboxylase synthetase deficiency of early and late onset. *J. Pediatr.*, **101**, 546-550.

4. Lamhonwah, A.M., Quan, F., and Gravel, R.A. (1987) Sequence homology around the biotin-binding site of human propionyl-CoA carboxylase and pyruvate carboxylase. *Arch. Biochem. Biophys.*, **254**, 631-636.

5. Chang, H.I. and Cohen, N.D. (1983) Regulation and intracellular localization of the biotin holocarboxylase synthetase of 3T3-L1 cells. *Arch. Biochem. Biophys.*, **225**, 237-247.

6. Chiba, Y., Suzuki, Y., Aoki, Y., Ishida, Y., and Narisawa, K. (1994) Purification and properties of bovine liver holocarboxylase synthetase. *Arch. Biochem. Biophys.*, **313**, 8-14.

7. Leon-Del-Rio, A., Leclerc, D., Akerman, B., Wakamatsu, N., and Gravel, R.A. (1995) Isolation of a cDNA encoding human holocarboxylase synthetase by functional complementation of a biotin auxotroph of Escherichia coli. *Proc. Natl Acad. Sci. U.S.A.*, **92**, 4626-4630.

8. Suzuki, Y., Aoki, Y., Ishida, Y., Chiba, Y., Iwamatsu, A., Kishino, T., Niikawa, N., Matsubara, Y., and Narisawa, K. (1994) Isolation and characterization of mutations in the human holocarboxylase synthetase cDNA. *Nat. Genet.*, **8**, 122-128.

9. Wilson, K.P., Shewchuk, L.M., Brennan, R.G., Otsuka, A.J., and Matthews, B.W. (1992) Escherichia coli biotin holoenzyme synthetase/bio repressor crystal structure delineates the biotin- and DNA-binding domains. *Proc. Natl Acad. Sci. U.S.A.*, **89**, 9257-9261.

10. Burri, B.J., Sweetman, L., and Nyhan, W.L. (1985) Heterogeneity of holocarboxylase synthetase in patients with biotin-responsive multiple carboxylase deficiency. *Am. J. Hum. Genet.*, **37**, 326-337.

11. Ghneim, H.K. and Bartlett, K. (1982) Mechanism of biotin-responsive combined carboxylase deficiency [letter]. *Lancet*, **1**, 1187-1188.

12. Burri, B.J., Sweetman, L., and Nyhan, W.L. (1981) Mutant holocarboxylase synthetase: Evidence for the enzyme defect in early infantile biotin-responsive multiple carboxylase deficiency. *J. Clin. Invest.*, **68**, 1491-1495.

13. Wolf, B., Hsia, Y.E., Sweetman, L., Feldman, G., Boychuk, R.B., Bart, R.D., Crowell, D.H., DiMauro, R.M., and Nyhan, W.L. (1981) Multiple carboxylase deficiency: clinical and biochemical improvement following neonatal biotin treatment. *Pediatrics*, **68**, 113-118.

14. Triggs Raine, B.L., Akerman, B.R., Clarke, J.T., and Gravel, R.A. (1991) Sequence of DNA flanking the exons of the HEXA gene, and identification of mutations in Tay-Sachs disease. *Am. J. Hum. Genet.*, **49**, 1041-1054.

15. Orita, M., Suzuki, Y., Sekiya, T., and Hayashi, K. (1989) Rapid and sensitive detection of point mutations and DNA polmorphisms using the polymerase chain reaction. *Genomics*, **5**, 874-879.

16. Winship, P.R. (1989) An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. *Nucl. Acids Res.*, **17**, 1266.

17. Ochman, H., Medhora, M.M., Garza, D., and Hartl, D.L. (1990) Amplification of Flanking Sequences by Inverse PCR. In: PCR Protocols A guide to Methods and Applications, M.A. Innes, D.H. Gelfand J.J. Sninsky and T.J. White (Eds), Academic Press, San Diego, pp. 219-227.

18. Cronan, J.E., Jr. and Wallace, J.C. (1995) The gene encoding the biotin-apoprotein ligase of Saccharomyces cerevisiae. *FEMS Microb. Lett.*, **130**, 221-229.

19. Howard, P.K., Shaw, J., and Otsuka, A.J. (1985) Nucleotide sequence of the BirA gene encoding the biotin operon repressor and biotin holoenzyme synthetase functions of Escherichia coli. *Gene*, **35**, 321-331.

20. Xu, X., Matsuno Yagi, A., and Yagi, T. (1993) DNA sequencing of the seven remaining structural genes of the gene cluster encoding the energy-transducing NADH-quinone oxidoreductase of Paracoccus denitrificans. *Biochemistry*, **32**, 968-981.

21. Bower, S., Perkins, J., Yocum, R.R., Serror, P., Sorokin, A., Rahaim, P., Howitt, CL, Prasad, N., Ehrlich, S.D., and Pero, J. (1995) Cloning and characterization of the Bacillus subtilis BirA gene encoding a repressor of the biotin operon. *J. Bacteriol.*, **177**, 2572-2575.

22. Dombrosky, P.M., Schimd, M.B., and Young, K.D. (1994) GenBank accession number P37416.

23. Gompertz, D., Draffan, G.H., Watts, J.L., and Hull, D. (1971) Biotin-responsive B-methylcrotonylglycinuria. *Lancet*, 22-24.

24. Sweetman, L., Bates, S.P., Hull, D., and Nyhan, W.L. (1977) Propionyl-CoA carboxylase deficiency in a patient with biotin-responsive 3-methyl-crotonylglycinuria. *Pediatr. Res.*, **11**, 1144-1147.

25. Saunders, M., Sweetman, L., Robinson, B., Roth, K., Cohn, R., and Gravel, R.A. (1979) Multiple carboxylase defects and complementation studies with propionicacidemia in cultured fibroblasts. *J. Clin. Invest.*, **64**, 1695-1702.

26. Bartlett, K. and Gompertz, D. (1976) Combined carboxylase defect: biotin-responsiveness in cultured fibroblasts. *Lancet*, 804.

27. Weyler, B., Sweetman, L., Maggio, D.C., and Nyhan, W.L. (1977) Deficiency of propionyl-CoA carboxylase in a patient with methylcrotonylglycinuria. *Clin. Chim. Acta*, **76**, 321-328.

28. Packman, S., Caswell, N., Gonzalez Rios, M.C., Kadlecek, T., Cann, H., Rassin, D., and McKay, C. (1984) Acetyl CoA carboxylase in cultured fibroblasts: differential biotin dependence in the two types of biotin-responsive multiple carboxylase deficiency. *Am. J. Hum. Genet.*, **36**, 80-92.

29. Livne, M., Gibson, K.M., Amir, N., Eshel, G., and Elpeleg, O.N. (1994) Holocarboxylase synthetase deficiency: a treatable metabolic disorder masquerading as cerebral palsy. *J. Child Neurology*, **9**, 170-172.

CHAPTER III

Preface to chapter III

The preceding described how I identified six mutations in patients suffering from neonatal MCD. To assess which mutations were responsible for the biotin-responsiveness observed in patients, I improved the assay used to examine the ability of HCS to biotinylate a subunit of the *E. coli* carboxylase, BCCP.

This assay was initially developed in our lab by Alfonso Leon-Del-Rio during the cloning of human HCS to prove the identity of HCS clones. Two key modifications have been made. First, the radioactive substrate for HCS was changed from ^{14}C-biotin to ^{3}H-biotin. This allowed the use of lower concentrations of biotin in the assay because of its higher specific activity. Second, the system used to detect radioactivity was also improved. Instead of running proteins samples on an SDS-PAGE, transferring onto a membrane then quantitating the radioactivity incorporated into BCCP with a phosphorimage analyzer, I precipitated the proteins with TCA. The precipitate was then filtered and counted in a liquid scintillation counter. This procedure increased the number of samples that could be analyzed at once and reduced the amount of time.

These improvements allowed me to characterize mutant HCS expressed in *E. coli* and showed that all the identified mutations are associated with biotin-responsiveness except for the L216R mutation located outside the biotin-binding region. In addition, this system allowed estimation of K_m and V_{max} for biotin of wild type HCS.

All the data in this chapter were generated by me. In our lab, Daniel Leclerc provided me with technical support. Studies will be completed by another graduate student in our lab prior to submitting this data for publication.

Expression of mutations in holocarboxylase synthetase from patients with biotin-responsive multiple carboxylase deficiency.

INTRODUCTION

Biotin-responsive multiple carboxylase deficiency (MCD) is an inherited disorder of intermediary metabolism that is characterized by deficient activity of acetyl CoA carboxylase (ACC; EC 6.4.1.2), pyruvate carboxylase (PC; EC 6.4.1.1), propionyl CoA carboxylase (PCC; EC 6.4.1.3) and ß-methylcrotonyl CoA carboxylase (MCC; EC 6.4.1.4). MCD is caused by a deficiency of holocarboxylase synthetase (HCS; EC 6.3.4.10) or biotinidase (EC 3.5.1.12) activity (1). A defect in HCS results in the severe neonatal form of MCD whereas a defect in biotinidase results in the milder juvenile form. In both diseases, patients present similar clinical and biochemical phenotypes including alopecia, skin rashes and metabolic acidosis (1). Fortunately, the biochemical abnormalities and clinical symptoms improve dramatically after treatment with pharmacological doses of oral biotin. If left untreated the disease can lead to coma and death.

HCS and biotinidase are the major enzymes of biotin metabolism in humans. HCS is responsible for the activation of apocarboxylases through the covalent attachment of a biotin molecule to a specific lysine residue. The reaction proceeds in two half-reactions, the first involves the activation of biotin with ATP to form biotin-5'-AMP, which is then transferred to the ϵ amino group of the lysine residue on the carboxylase in the second half-reaction, with release of AMP. Biotinidase functions in the recovery of protein-bound biotin for reutilization during the natural turnover of cellular proteins. It cleaves the amide bond between biotin and the lysine residue of biocytin (biotinyl-lysine).

Whereas there exists a simple and rapid method for the diagnosis of biotinidase deficiency (2), the assay of HCS activity is complicated by the requirement for apocarboxylases as one of the substrates. Circumstantial evidence of HCS deficiency can

be obtained by finding low activity of the carboxylases in cultured fibroblasts grown in the presence of a low concentration of biotin in the medium with restoration of carboxylase activities in high biotin medium (3-5). HCS activity can also be measured by assaying the incorporation of radioactive biotin into apocarboxylases (6) or, indirectly, by measuring the activity of the resulting holocarboxylases (3,4). The apocarboxylase substrate necessary for these assays must be purified from biotin-starved animals (3,6) or by culturing cells in a biotin-depleted medium (4), making it difficult to use these methods as routine assays. Using the activation of apo-PCC as an indicator of HCS activity, Burri *et al.* (13) estimated a K_m for biotin for wild type HCS of 15 nM. They obtained increases of up to 350 fold in the K_m for biotin in MCD patients. Ghneim *et al.* (14) also reported an increased K_m in a neonatal MCD patient by assaying the activation of carboxylases in mitochondrial homogenates of biotin-starved fibroblasts. However, their estimate of K_m for biotin of the wild type enzyme was much higher (100 nM).

The predicted amino acid sequence (726 amino acids) of human HCS cDNA showed that it is homologous with bacterial biotin ligases (BirA protein) in a region previously identified as the biotin binding domain of the *E. coli* BirA protein by X-ray crystallography (7). In *E. coli,* the BirA protein (321 amino acids) has the dual function of serving as the repressor of the biotin biosynthetic operon and as the biotin ligase for the biotin-dependent enzyme acetyl CoA carboxylase (ACC). In the *Bir*A gene, mutations leading to an increased requirement for biotin in the growth medium (biotin auxotrophy) have been localized to the biotin binding region (8-10). In previous studies, we suggested that the mutations in the HCS gene that account for the biotin responsiveness observed in patients may cluster in the biotin binding region as they do in the case of *bir*A mutants (17). Subsequently, we identified four mutations in the biotin binding region (R508W, G518E, V550M and D571N)(11). Four other mutations were located outside the region, two by our group (L216R and V363D)(11) and two by Suzuki *et al.* (L237P and delG1067) (12). The delG1067 mutation creates a stop codon a few amino acids downstream and should therefore result in inactive HCS. Suzuki *et al.* (12) have shown

that the L237P mutation also results in a decrease in HCS activity. They determined HCS activity indirectly by measuring PCC activity through the incorporation of ^{14}C-bicarbonate into apoPCC after transient expression of the enzyme in cultured fibroblasts from a patient.

To assess the biotin-responsiveness of individual mutations, we developed an assay to measure HCS activity based on biotinylation of BCCP in *E. coli* strain (*BirA*$^-$) transformed with an expression vector carrying mutant HCS sequences. We demonstrate that the mutations identified in the MCD patients are indeed responsible for the reduced HCS activity. Further, we show that HCS containing the mutations R508W, G518E, V550M, D571N, and V363D are responsible for the biotin-responsiveness observed in patients.

MATERIALS AND METHODS

Materials

Taq polymerase was purchased from Perkin-Elmer. The T/A cloning kit used for subcloning of PCR products was from Invitrogen. The Gene Clean Kit was obtained from Bio 101 Inc. and the Wizard Mini-Preps used for plasmid purification were obtained from Promega. The calf intestinal alkaline phosphatase (CIAP) and T4 DNA ligase were purchased from Life Technologies. ^{3}H-biotin (45 mCi/mmol) was purchased from Dupont and the ^{14}C-biotin (57 mCi/mmol) from Amersham. The CytoScint ES scintillation fluid was obtained from ICN and the glass microfibre filters 934-AH from Whatman. The oligonucleotide primers were synthesized by R. Clarizio of the Montreal Children's Hospital Research Institute Oligonucleotide Synthesis Facility. *E. coli birA*104 was kindly provided by Dr. A.M. Campbell (Stanford University) (15,16). It is a strain which contains a temperature sensitive mutation in BirA that makes it auxotrophic for biotin.

Subcloning of mutations in the vector pSE936 and/or BlueScript

A. Mutations in the biotin-binding region:

Mutations in the biotin-binding region were PCR amplified from cDNA of fibroblasts of

patients known to carry the mutation. The PCR was done as described (11) except that the annealing temperature was 63°C for the primers 5'-CTGTGACATGAAACAAGTTCCTGCand5'-TTACCGCCGTTTGGGGAGGATGAG. The previously published mutations D571N and R508W were amplified from cDNA from PD, G518E from HB and V550M from YL (11). The PCR products (1060 bp) were subcloned in the pCRII vector. The candidate clones were tested for the presence of the mutation since most patients were heteroallelic for two mutations. Mutations were confirmed by diagnostic tests detailed in Dupuis *et al.* (11) except that the DNA template was 30 ng of the Mini-Prep and the primers used for PCR amplification were complementary to cDNA sequences rather than genomic sequences (Table 1). Once the clones containing the mutations had been identified, 10 μl of the Mini-Prep was digested with BstEII at 60°C for one hour and then with HpaI at 37°C for one hour. The digested DNA was separated on a 1% agarose and the 442 bp fragment was cut out and purified using the GeneClean procedure. A Mini-Prep of the pSE936 vector containing the HCS clone BL11 or BlueScript with HCS clone BL04 (17) was also digested with the same enzymes and treated with CIAP thirty minutes before separating the DNA on a 1% agarose gel and purifying the band using the Gene Clean procedure.

Eight μl of the 422 bp insert containing the mutation and 8 μl of dephosphorylated vector were incubated with T4 DNA ligase (10 U) overnight at 14°C. Ten μl of the ligation mixture was used to transform *bir*A104- and Top10F'-competent cells. The transformation was plated on LB-agar containing ampicillin (100 μM) and biotin (5 μM). Colonies were picked and screened for the correct size of the insert and the presence of the mutation (as described above).

B. Mutations outside the biotin binding region:

The mutations outside the biotin binding region were amplified from patient cDNA using the following primers and an annealing temperature of 60°C: 5'-GCTTGCAGACCTGGGGATCCTTATand5'-GAATGGACCTCACTGTCTCCACGA. The cDNA used to amplify the L216R and V363D mutations were from VE. The PCR

Mutation	Primers	Expected size (bp)	Enzyme restriction site created by:		Expected size after digestion (bp):	
			mutation	mutation & primer	normal	mutant
T_{647}→G	CCGTGGACGGACAACTGTCTCC/ CTGCAGCCACTGCTCAAGACGCT	245		HpaII	245	224 & 21
T_{1088}→A	GCTCAAGTCAAGCAATTTTAGCAG/ ACCAAGGGTTGTCAGAATCTCTC	61	BbsI		61	36 & 25
C_{1522}→T	CGGCAGACCGAGGGCAAAGCA/ AACCAGAACTCCGCCGATCTTCAT	155		NlaIII	135 & 20	114, 21 & 20
G_{1553}→A	GGAATGTGTGGCTGAGCCCTGTCG/ AACCAGAACTCGCCGATCTTCAT	127		TaqI	127	103 & 24
G_{1648}→A	CGGCAGACCGAGGGCAAAGCA/ AACCAGAACTCCGCCGATCTTCAT	155	NlaIII		135 & 20	86, 49 & 20
G_{1711}→A	CGGCAGACCGAGGGCAAAGCA/ AACCAGAACTCCGCCGATCTTCAT	155	SspI		155	112 & 43

Table 1. Strategy for PCR-based diagnostics of mutations identified in the human HCS gene. The double underline letters indicate the nucleotide was altered to create a new restriction site. The single underline letters indicate the nucleotide was previously altered for a different diagnostic.

product (1746 bp) was subcloned into the T/A vector and screened for the correct size of the insert and the presence of the mutation (Table 1) as above. Ten μl of the Mini-Prep containing the mutation was digested with BstEII one hour at 60°C. The DNA was separated on a 1% agarose gel. The linearized vector was cut out and purified by the GeneClean procedure. The DNA was digested with a second enzyme, PshAI, at 37°C for one hour, separated on a 1% agarose gel and the 690 bp fragment purified as before. BlueScript-BL04 was cut with the same enzymes (BstEII/PshAI) and was dephosphorylated by treatment with CIAP for 30 minutes at 37°C prior to separating the DNA on a 1% agarose gel and purifying. Ligation and transformation were done as described above.

Biotinylation assay using ^{14}C-biotin

*E. coli birA*104 cells containing mutant or wild type pSE936-BL11 vector were grown overnight in 5 ml LB medium containing ampicillin (100 μg/ml) and biotin (5 μM) at 30°C. Prior to experiments, a 1:100 dilution was made in the same LB medium and the culture was grown for 3 hours at 30°C (until an OD_{600} of about 0.4). About $15x10^8$ cells were centrifuged, washed twice with cold PBS and resuspended in three ml of MEM-A (18). The cells were incubated at either 30°C or 42°C for 15 minutes. Three μl of ^{14}C-biotin were added to the culture and incubated with vigorous shaking for 2-4 hours at 30°C or 42°C. The cells were centrifuged, washed with cold PBS and resuspended in 50 μl of resuspension buffer (50 mM Tris, 1 mM EDTA, 0.1 mM DTT, 5% glycerol and 2 mM PMSF). Protein determinations were made using the Bio-Rad kit. Fifty μg of protein were separated on a 12% SDS-polyacrylamide gel. Biotinylation of BCCP was quantitated with a Phosphor Image analyzer (Fujix BAS 2000; Fuji, Japan) after the proteins had been transferred to nitrocellulose filters (18). The filters were also exposed to an X-ray film for two weeks.

Biotinylation assay using ^{3}H-biotin

*E. coli birA*104 cells transformed with Bluescript-BL04 containing mutations, were grown overnight in 5 ml LB containing ampicillin (100 μg/ml) and biotin (5 μM) at

30°C. A 1:20 dilution was made in MEM-A (18). The culture was grown for 3.5 hours at 30°C. The cells were centrifuged and resuspended in fresh MEM-A to a concentration of is $5x10^7$ cells/ml of culture. The intact cells were kept on ice until use. Seven ml samples were preincubated in a 42°C bath for 10 min. Radioactive biotin was added to start the reaction and the tube was placed in a shaker at 42°C. The culture contained 20-4000 nM radioactive biotin (1 μCi/ml for 20 nM biotin, 2 μCi/ml up to 400 nM biotin, 3 μCi/ml up to 2400 nM biotin and 4 μCi/ml for 4000 nM biotin). Duplicate one ml samples were taken at various time intervals and added to a tube containing 5 ml of cold 10% TCA containing 500 μM biotin to precipitate the proteins. The samples were filtered through a glass fibre filter pre-soaked in 500 μM biotin. The filter was washed three times with 3 ml of 5% TCA containing 500 μM biotin and rinsed with 99% ethanol. Once the filters were dry, 5 ml of scintillation fluid was added and the samples were counted in a Rack Beta Liquid Scintillation Counter (LBK/Wallace).

RESULTS

Biotinylation assay (^{14}C-biotin)

Initially, we confirmed the ability of human HCS in pSE936 to biotinylate BCCP in the mutant *E. coli* strain *bir*A104 that has biotin ligase (BirA) function at 30°C (Fig. 1A, lane 3) but not at 42°C (Fig. 1B, lane 3), in agreement with the findings of Barker and Campbell (8). When the cells were transformed with the HCS cDNA clone, biotinylation of BCCP was detected at 42°C (Fig. 1B, lane 1) and at 30°C (Fig. 1B, lane 1). These results were similar to those obtained previously in our lab (17), validating the use of *bir*A104 for the assay of HCS. Furthermore, the data also show that the only significant incorporation of the ^{14}C-label is in BCCP.

When patient mutations were introduced into HCS and expressed in *bir*A104 cells, biotinylation of BCCP was only observed at 30°C due to active BirA (Fig. 1A, lanes 4-8). Virtually no biotinylated product could be detected at 42°C (Fig. 1B, lanes 4-8). These results demonstrate that the mutations D571N, R5082W, G518E and V550M, are deleterious and are responsible for the decreased HCS activity in MCD

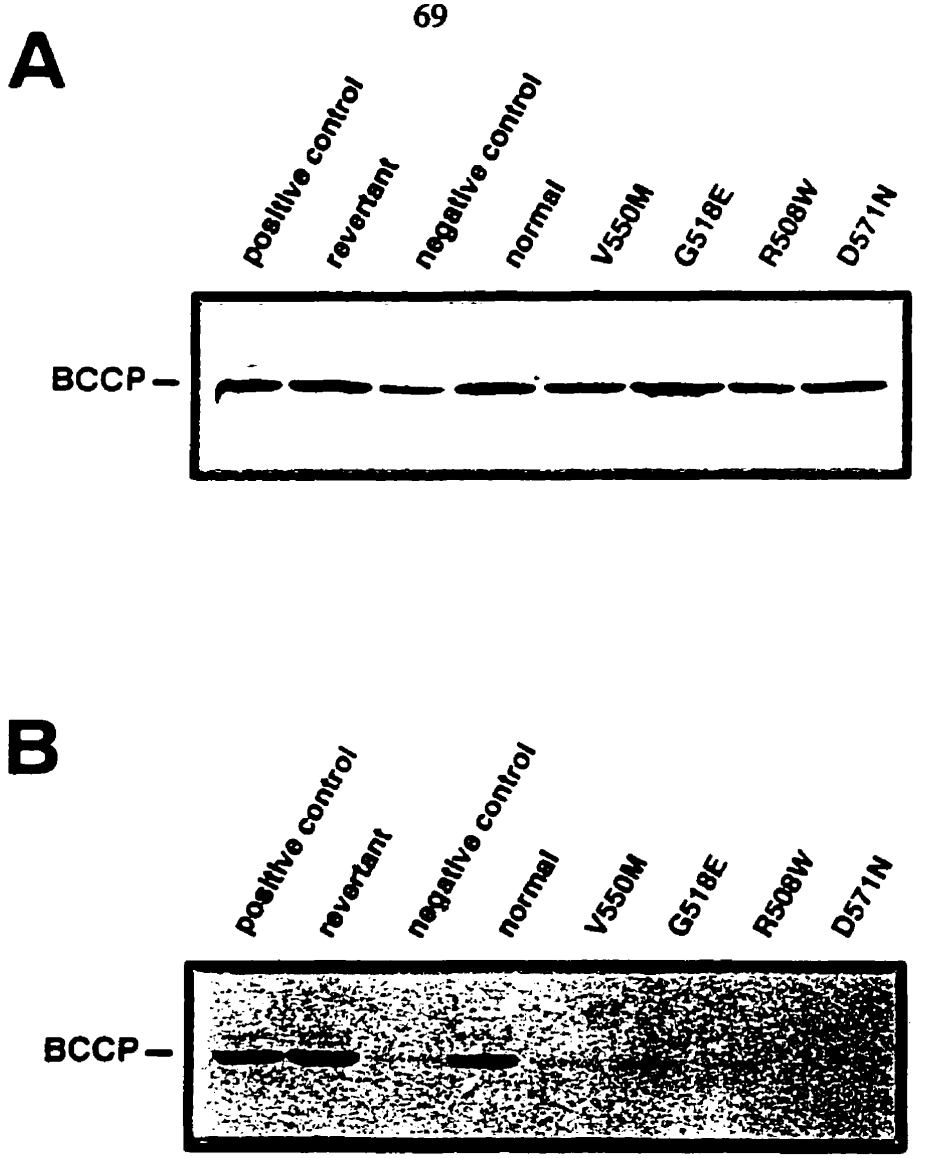

Figure 1. Biotinylation of BCCP by normal or mutant HCS using ^{14}C-biotin as a tracer. In A) at 30°C, BCCP is biotinylated in all cases. In B) at 42°C, only the positive control, revertant and normal show significant biotinylation of BCCP. The four mutations analyzed are found in the biotin-binding region, the normal represent the wild type sequence inserted back into the pSE936-BL11 vector. The positive control is the pSE936-BL11 without manipulations. The negative control is *bir*A104 without the vector. The *bir*A104 revertant was obtained during the cloning of human HCS in our lab (17).

patients.

We next examined whether HCS containing mutations were associated with biotin-responsiveness using the same biotin incorporation assay. Expression of HCS containing the G518E mutation was examined by incorporation of ^{14}C-biotin label into BCCP at three different concentrations of biotin in the culture medium. The results showed an increase in the biotinylated product correlating with the increased concentration of biotin in the medium (Fig. 2, lanes D, E and F). In contrast, the wild type HCS showed no increase in biotinylated product suggesting that the wild type enzyme was under saturating conditions even at the lowest concentration of biotin used (Fig. 2, lanes A, B and C). We conclude that the biotin responsiveness observed patients is an inherent property of mutant HCS. These experiments show that the biotin incorporation assay could be used effectively for the determination of biotin-responsiveness. However, the concentration of biotin used ranged from 0.88-2.6 μM, several logs higher than the concentration of circulating biotin in humans. We therefore elected to use ^{3}H-biotin for subsequent experiments because of its higher specific activity in order to obtain a wider range of biotin concentrations for the incorporation experiments. We also modified the experiment to detect TCA-precipitable radioactivity as a measure of biotinylated BCCP since this was the only molecule showing incorporation of label.

Biotinylation assay (^{3}H-biotin)

Because of its high biotinylating activity, the rate at which wild type HCS biotinylates BCCP was examined over a very short time period (2.5 minutes). Figure 3A shows the response of velocity as a function of biotin concentration in the medium. Although biotinylation of BCCP depends on biotin transport as well as HCS activity, it was possible to estimate the apparent K_m and V_{max} for biotin for the overall reaction. In separate experiments, the K_m was found to be 87 and 129 nM as determined from a Lineweaver-Burk plot (Fig. 3B). The measurements of V_{max} were found to be highly variable (52 and 232 pmol of biotin/mg protein/min) which may be related to variation in the steady state level of HCS in different cultures of *E. coli* despite their derivation

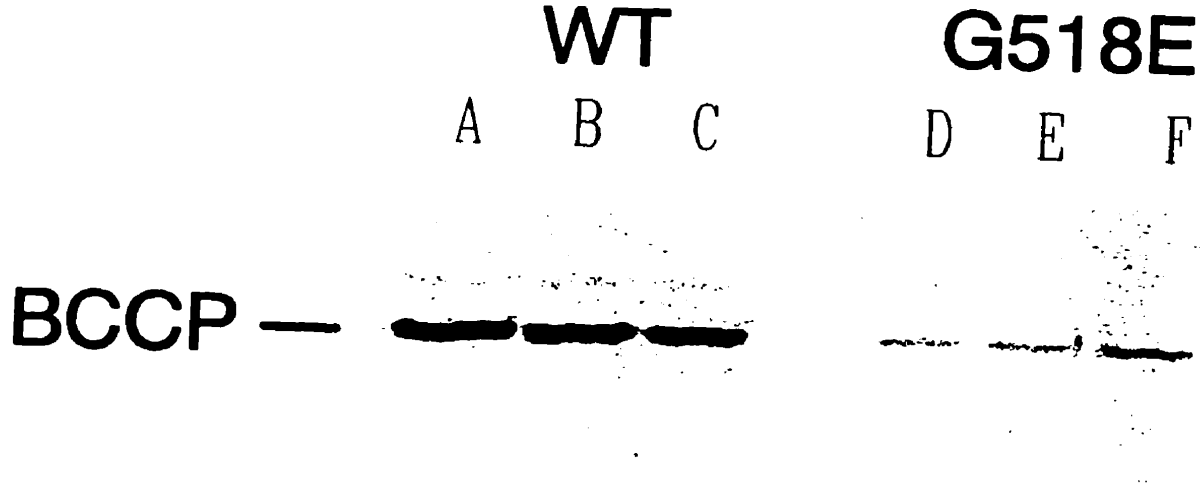

Figure 2. Biotinylation of BCCP by wild type HCS and mutant HCS with increasing concentrations of ^{14}C-biotin at 42°C. In wild type (A, B and C), no significant increase in biotinylated product was observed. However, a dramatic increase in biotinylated product was found for HCS containing the G518E mutation. The concentration of biotin used was 0.88 μM in A and D, 1.76 μM in B and E and 2.64 μM in C and F.

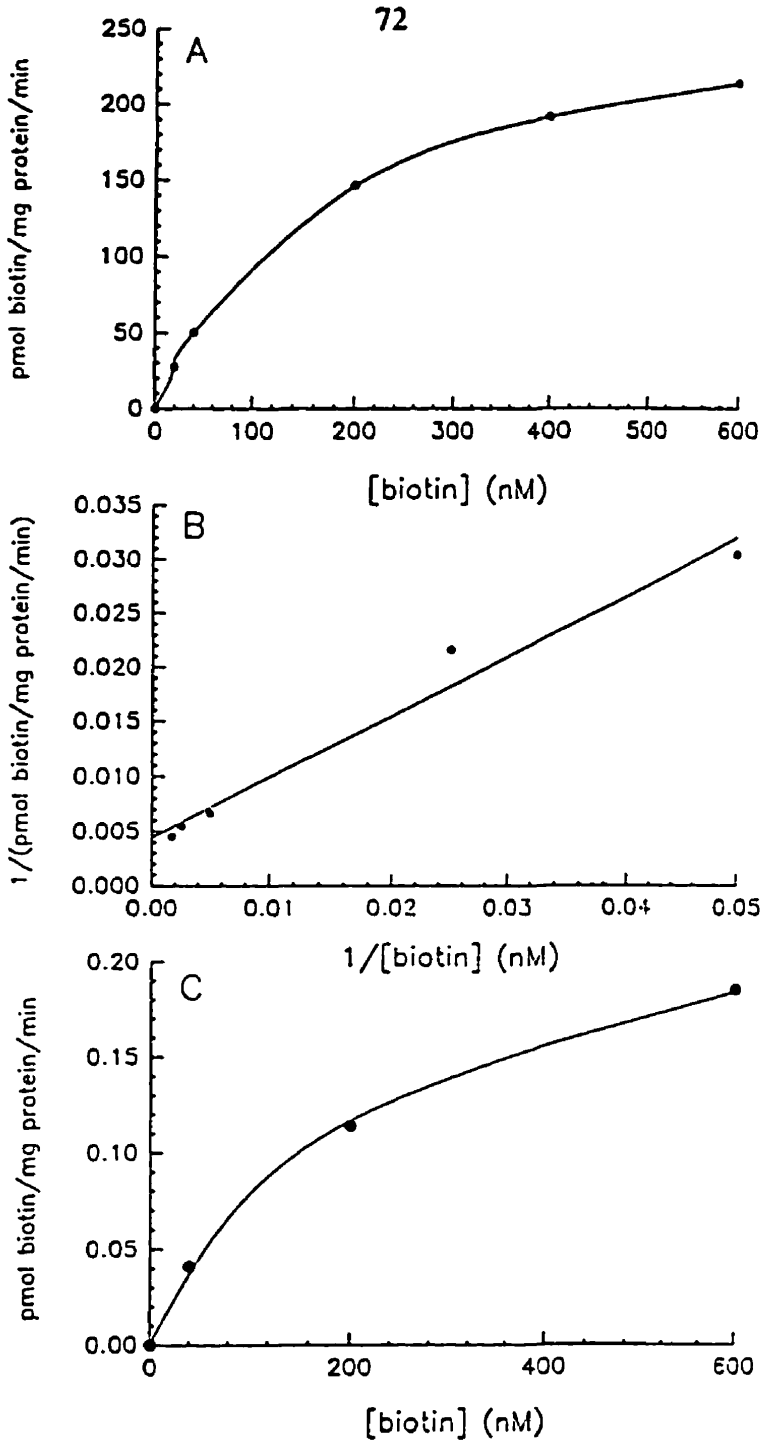

Figure 3. Incorporation of ^{3}H-biotin into BCCP as a function of concentration of biotin and determination of kinetic parameters for biotin for wild type HCS at 42°C. An increase in biotinylating activity was observed with increasing concentration of biotin for wild type HCS (in A) and mutant HCS containing the V363D mutation (in C) (with extrapolation to zero in both cases). In B) a Lineweaver-Burk plot was generated from the data for wild type HCS (in A). In this case, the calculated K_m for biotin for HCS was found to be 129 nM and the V_{max} was found to be 232 pmol of biotin/mg protein/min.

from the same clone. The transfection efficiency may also play a role in the variation in V_{max}.

Using ^{3}H-biotin incorporation into BCCP, we observed the same dramatically reduced biotinylation activity at 42°C for mutant HCS as we did using the ^{14}C-biotin biotinylation assay (Fig. 4). HCS containing each mutation was surveyed at different medium biotin concentrations to determine if the lesion was associated with biotin-responsiveness. The assay for mutant HCS was extended for up to 160 minutes except for the experiments with R508W which were assayed for up to only 60 minutes. Despite the high variability due to low enzyme activity in mutants, most of the mutations were found to be associated with biotin-responsiveness. The HCS containing five of the six mutations were biotin responsive, four of them significantly so. The R508W HCS was the most responsive of all the mutants (Fig. 4C). This is illustrated at 2400 nM biotin. At this very high biotin concentration, the mutant HCS behaves essentially as wild type, reaching a maximal activity of about 400 pmol biotin/mg protein. HCS containing the G518E mutation also demonstrated high biotin responsiveness (Fig. 4D). HCS containing V550M and V363D are moderately biotin-responsive (Fig. 4E and 4G, respectively) whereas D571N HCS (Fig. 4F) is only slightly responsive.

In order to compare the relative severity of each mutation, biotinylating activities are presented at a single biotin concentration of 40 nM (Fig. 5). Although below the apparent K_m for biotin of the wild type HCS, this concentration far exceeds the concentration of biotin (free and bound) circulating in human plasma which is estimated to be between 0.9 and 4 nM (19-23). L216R showed essentially no enzyme activity and V550M, V363D and D571N gave very low activity (0.14 to 0.40% of wild type) at this concentration. G518E showed moderate HCS activity (5.1% of wild type) and R508W displayed the highest activity (25% of wild type) of all the mutants. In the order of decreasing activity, the residual HCS activity associated with different mutations at 40 nM external biotin is: R508W > G518E > V550M > D571N = V363D > L216R. The data are from a single experiment to accommodate the variability in V_{max} obtained for wild type HCS

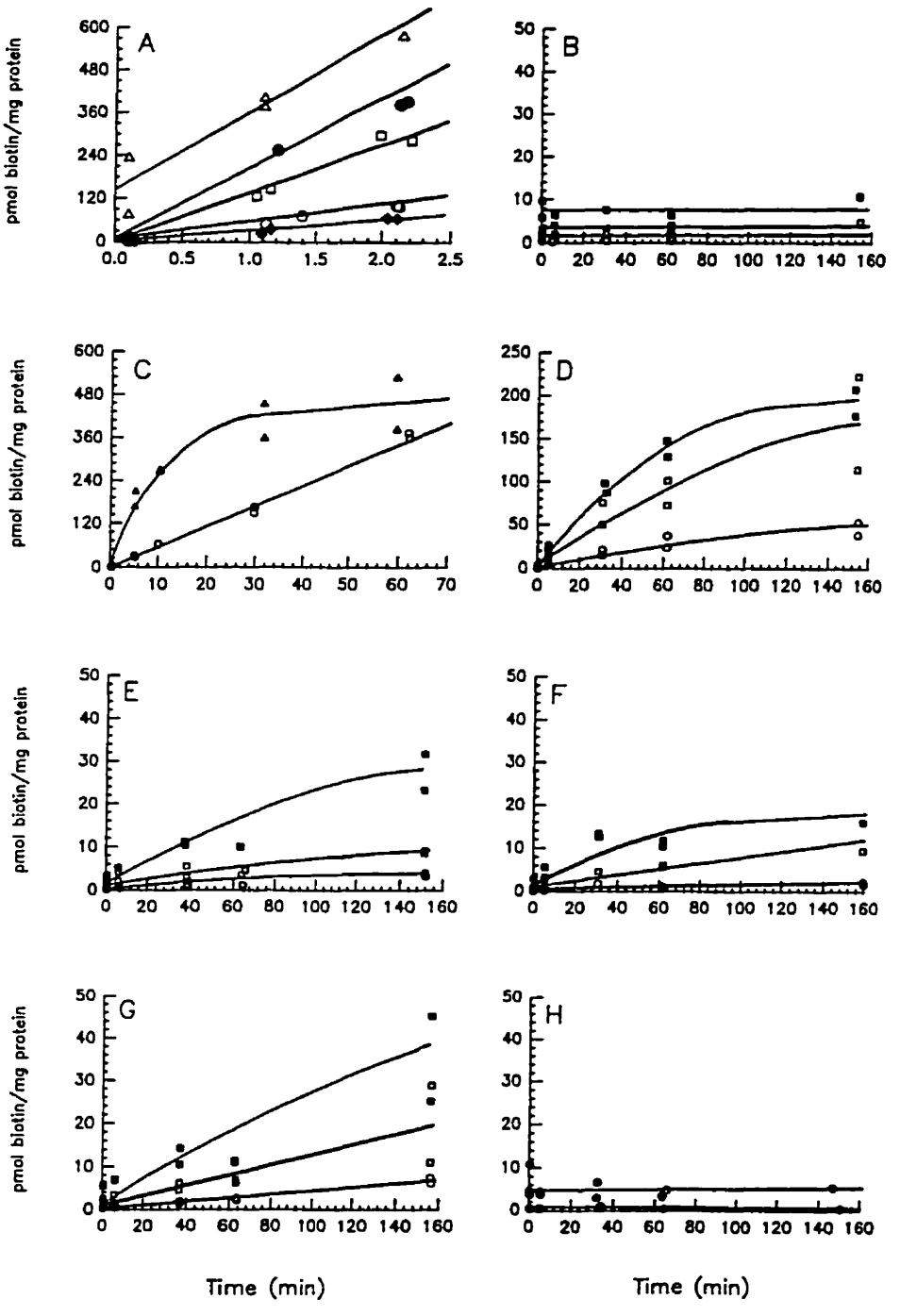

Figure 4. Biotinylating activity as a function of time with increasing concentrations of biotin at 42°C. A) wild type HCS, B) *bir*A104 containing the BlueScript vector without HCS, C) HCS containing the R508W mutation, D) HCS containing the G518E mutation, in E) HCS containing the V550M mutation, F) HCS containing the D571N mutation, G) HCS containing the V363D mutation and H) HCS containing the L216R mutation. The concentrations of biotin used were: 20 nM (♦), 40 nM (○), 200 nM (□), 400 nM (●), 600 nM (■), 2400 nM (▲) and 4000 nM (△).

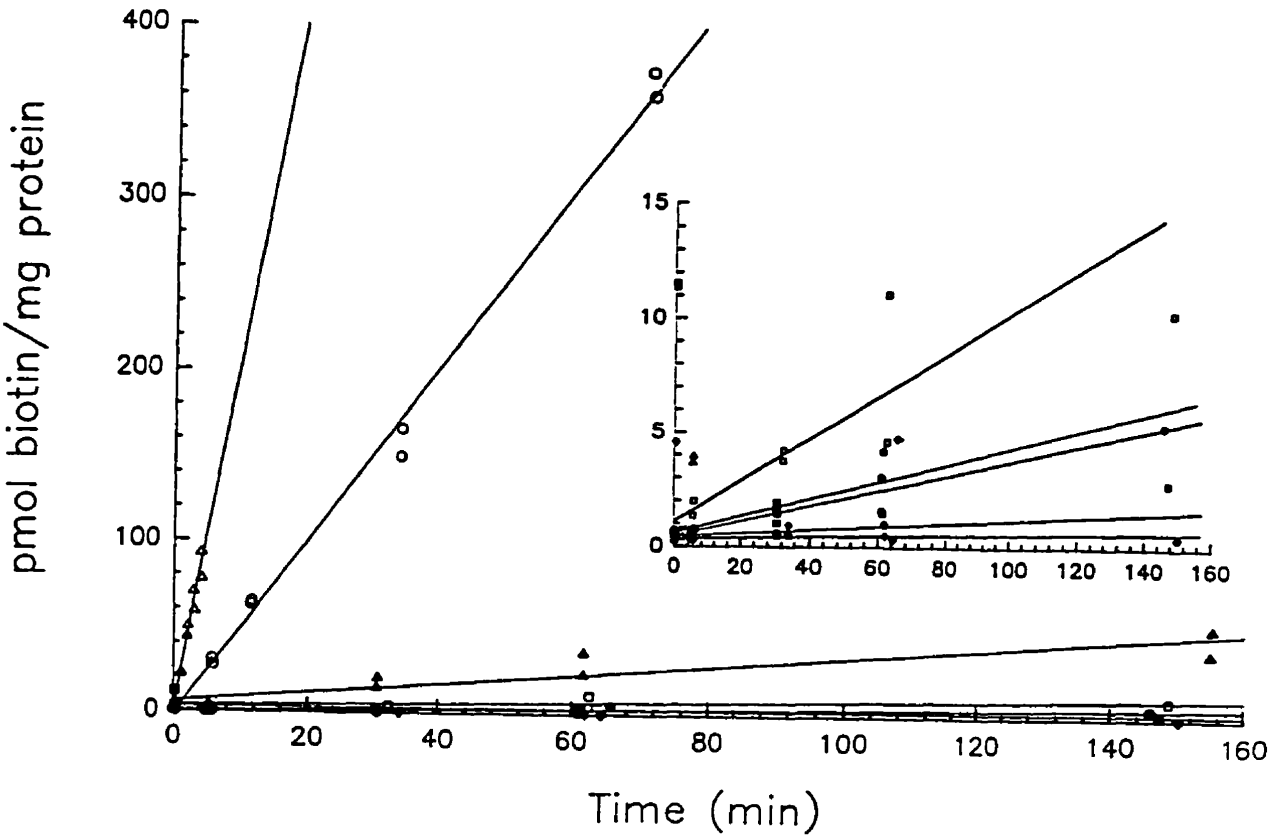

Figure 5. Comparison of biotinylation activity as a function of time at 42°C and 40 nM biotin. Wild type HCS is represented by △, HCS containing the R508W mutation by ○, HCS containing the G518E mutation by ▲, HCS containing the V550M mutation by □, HCS containing the D571N mutation by ●, HCS containing the V363D mutation by ■, HCS containing the L216R mutation by ◆ and *bir*A104 containing the BlueScript vector without an HCS insert by ◇.

between experiments. Similar ranking were obtained in different experiments.

DISCUSSION

Virtually all patients with MCD respond to biotin therapy, indicating that at least one of the two HCS alleles must be biotin responsive. The assay we have developed measures the effect of individual mutations on HCS expressed in bacteria in response to increasing concentrations of biotin. Of the six mutations we previously identified, all were found to be deleterious and five of them were found to show varying degrees of biotin-responsiveness when expressed in *E. coli*. Two mutant HCS's showed significant biotinylating activity and were biotin responsive (R508W and G518E). Two others with low HCS activity (V550M and V363D) were also biotin-responsive and one mutant HCS with very low HCS activity (D571N) still showed some degree of biotin-responsiveness. Only one mutant HCS, with background activity (L216R), did not seem to be biotin-responsive at the concentrations examined. Because the assay is conducted at 42°C, mutations may also be temperature sensitive. In essence, any mutant HCS with detectable enzyme activity was biotin-responsive.

Two mutations, V550M and R508W, previously detected at high frequency among patients we analyzed (11), proved to be biotin-responsive. Both were present in homozygous fashion in patients, YL in the case of V550M and MK in the case of R508W. In the latter, the mutation was confirmed in each parent (data not shown). These patients confirm the link between biotin-responsiveness *in vivo* and allele expression in *E. coli* (Table 2). CP and MC also carry the biotin responsive V550M mutation. In each case, the second mutation has yet to be identified although it need not necessarily be biotin-responsive. JRi, PD and MMC also carry the biotin responsive R508W mutation. JRi has a second unidentified mutation. PD's second mutation, D571N, is more severe than R508W so that R508W may provide most of the observed biotin-responsiveness. MMC's second mutation, V363D, seems only slightly biotin responsive. VE also possesses the V363D mutation and her second mutation, L216R, has biotinylating activity

Patient	Ref	Mutation*				Dose of biotin (mg/ day)	Clinical Biotin Responsiveness **
		Allele 1	Biotin Responsiveness	Allele 2	Biotin Responsiveness		
VE	26	L216R	0	V363D	++	10-40	Good
MMC	26	V363D	++	R508W	+++++	?	complete
PD	27	R508W	+++++	D571N	+	5	complete
JRi	28-32	R508W	+++++	unknown	?	10	complete
MK	33	R508W	+++++	R508W	+++++	10	complete
HB	34	G518E	+++	unknown	?	10	complete
MC	35	V550M	++	unknown	?	10	complete
CP	26	V550M	++	unknown	?	10	complete
YL	36	V550M	++	V550M	++	10	complete

* Allele frequency are L216R (1/24 alleles examined), V363D (2/24), R508W (5/24), G518E (1/24), V550M (4/24) and D571N (1/24) as previously determined (11).

** Good refers to full clinical responsiveness although some metabolites continue to be detected in urine; complete refers to full responsiveness and normal metabolite levels.

Table 2. Summary of patient mutations and biotin-responsiveness.

at background level at the concentrations examined. Therefore, the allele carrying the V363D mutation appears to be responsible for the biotin-responsiveness despite the low activity of the resulting HCS. HB carries a G518E mutation also shown to be biotin responsive and a second unidentified mutation.

The predicted amino acid sequence of human HCS cDNA is homologous to that of BirA of *E. coli* in a region previously identified as the biotin-binding domain by X-ray crystallography (7) (Fig. 6A). Three of the four mutations we have identified in the biotin binding region (Fig. 6D, R508W, G518E, and D571N) are close to contact points of biotin with BirA (Fig. 6B) or to bacterial mutations in the *Bir*A gene (Fig. 6C) that lead to an increased requirement for biotin in the growth medium. The fourth mutation identified in HCS (V550M) is located in the α-helix at the base of the structure (α-helix F (7)) whose role remains to be defined.

Using our cellular assay, we determined the apparent K_m for biotin of 108 nM (87 and 129 nM) based on two independent series of experiments. Biotin transport, an essential component of this assay, and BirA activity have been shown to be tightly coupled (8,9,25). We corroborated this finding confirming that there was no transport of biotin or biotinylation of BCCP in the absence of active BirA or wild type HCS (data not shown). We conclude therefore, that the apparent K_m for biotin represents a de facto measure of the affinity of biotin for HCS despite the use of whole bacterial cells for the assay. Significantly, Prakash and Eisenberg (24) determined a K_m for transport of biotin of 140 nM in *E. coli*. We suspect they were actually measuring the K_m for biotin of wild type BirA.

Although it has been proposed that there is active transport of biotin (24), the absence of transport by cells deficient in BirA activity led Barker and Campbell (9) to conclude that biotin uptake was by facilitated diffusion. By this mechanism, uptake is determined by the removal of internalized biotin by the action of BirA (or as we have shown, HCS) to generate 5'biotin-AMP and then BCCP-biotin.

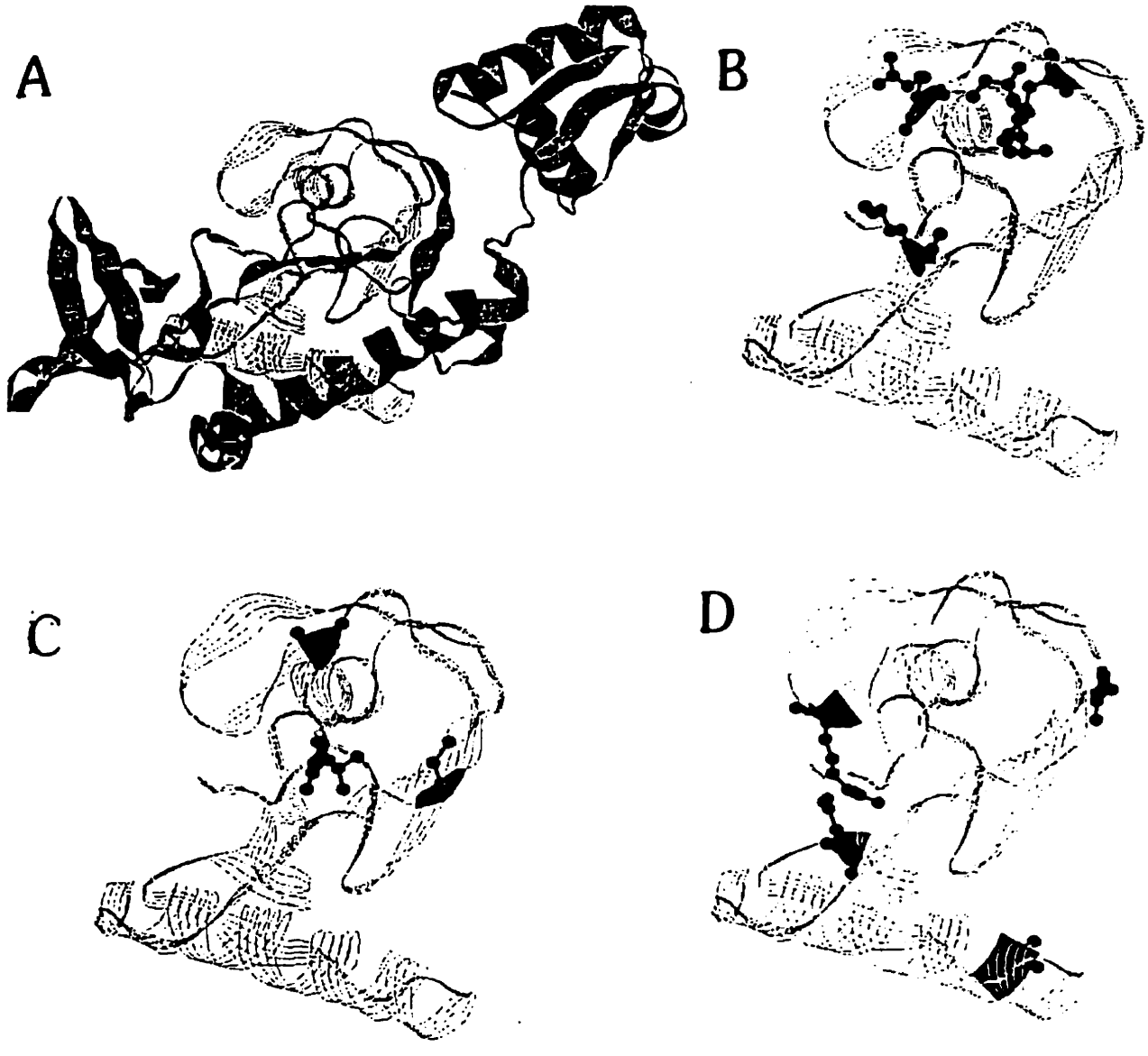

Figure 6. Three-dimensional structure of BirA in *E. coli*. In A) ribbon structure of the BirA protein with the biotin-binding region highlighted. In B) localization of the contact points for biotin (7), in C) localization of mutations in BirA leading to biotin-auxotrophy (8,9) and in D) localization of mutations in human HCS in the corresponding amino acid of BirA (11).

We also attempted to determine the apparent K_m for biotin for mutant HCS. Unfortunately, it could not calculated due to the scatter in the data with some of the values being barely above the background activity of *bir*A104. Nevertheless, the data do show a biotin concentration-dependent increase in activity.

The bacterial assay illustrates the impact of biotin supplementation on increasing the biotinylating activity of mutant HCS. This is shown by the effect of biotin concentration on the activity of V363D HCS (Fig. 3C). The circulating level of free biotin in human plasma is approximatively 1.6 nM, as determined by Mock *et. al* (19), about 70 times below the estimated K_m for biotin of wild type HCS. If this concentration is extrapolated to the bacterial cells, the specific activity of wild type HCS would be approximatively 2.6 pmol of biotin/mg protein/min. To achieve this same activity by G518E HCS, an external medium containing over 1800 nM biotin would be required. Whether or not a direct comparison can be made between bacterial and human cells, it is evident that the effective biotin concentration that reaches HCS is likely far below the K_m of HCS for biotin. It follows, as it has been observed, that all patients should be biotin-responsive if they possess residual HCS activity. An example is the patient VE. She has an unresponsive L216R mutation and a slightly responsive V363D mutation. Yet, she has a good clinical response to biotin, although some of the characteristic metabolites in her urine remain slightly elevated even on biotin doses ranging from 10-40 mg/day (26). These results also suggest that individuals with null mutations should exist who are unresponsive to biotin treatment. Thus far, none have been documented. It is possible that biotin-unresponsive MCD is lethal *in utero*.

Our finding of biotin-responsiveness associated with different mutations extends the early experiments of Burri *et al.* (13) and Ghneim *et al.* (14), who showed that the mutant HCS of patient cell extracts was responsible for biotin-responsiveness. We suggest that the bacterial expression system for HCS that we developed provides a valid test of the biotin-responsiveness associated with specific mutations.

REFERENCES

1. Wolf, B. (1995): Disorders of Biotin Metabolism. In: *The Metabolic and Molecular Bases of Inherited Disease*, edited by C.R. Scriver, et al, pp. 3151-3177. McGraw-Hill, New York.

2. Yamaguchi, A., Fukushi, M., Arai, O., Mizushima, Y., Sato, Y., Shimizu, Y., Tomidokoro, K., and Takasugi, N. (1987): A simple method for quantification of biotinidase activity in dried blood spot and its application to screening of biotinidase deficiency. *Tohoku J. Exp. Med.*, 152:339-346.

3. Burri, B.J., Sweetman, L., and Nyhan, W.L. (1985): Heterogeneity of holocarboxylase synthetase in patients with biotin-responsive multiple carboxylase deficiency. *Am. J. Hum. Genet.* 37:326

4. Bartlett, K., Ghneim, H.K., Stirk, H.J., and Wastell, H. (1985): Enzyme studies in biotin-responsive disorders. *J. Inherit. Metab. Dis.* 8 Suppl 1:46-52.

5. Saunders, M.E., Sherwood, W.G., Duthe, M., Surh, L., and Gravel, R.A. (1982): Evidence for a defect ofholocarboxylase synthetase activity in cultured lymphoblasts from a patient with biotin-responsive multiple carboxylase deficiency. *Am. J. Hum. Genet.* 34:590-601.

6. Chang, H.I. and Cohen, N.D. (1983): Regulation and intracellular localization of the biotin holocarboxylase synthetase of 3T3-L1 cells. *Arch. Biochem. Biophys.* 225:237-247.

7. Wilson, K.P., Shewchuk, L.M., Brennan, R.G., Otsuka, A.J., and Matthews, B.W. (1992): Escherichia coli biotin holoenzyme synthetase/bio repressor crystal structure

delineates the biotin- and DNA-binding domains. *Proc. Natl Acad. Sci. USA.* 89:9257-9261.

8. Barker, D.F. and Campbell, A.M. (1981): The BirA gene of Escherichia coli encodes a biotin holoenzyme synthetase. *J. Mol. Biol.* 146:451-467.

9. Barker, D.F. and Campbell, A.M. (1981): Genetic and biochemical characterization of the BirA gene and its product: Evidence for a direct role of biotin holoenzyme synthetase in repression of the biotin operon in Escherichia coli. *J. Mol. Biol.* 146:469-492.

10. Buoncristiani, M.R., Howard, P.K., and Otsuka, A.J. (1986): DNA-binding and enzymatic domains of the bifunctional biotin operon repressor (BirA) of Escherichia coli. *Gene*, 44:255-261.

11. Dupuis, L., Leon-Del-Rio, A., Leclerc, D., Campeau, E., Sweetman, L., Saudubray, J.M., Herman, G., Gibson, K.M., and Gravel, R.A. (1996): Clustering of mutations in the biotin-binding region of holocarboxylase synthetase in biotin-responsive multiple carboxylase deficiency. *Hum. Mol. Gen.* 5:1011-1016.

12. Suzuki, Y., Aoki, Y., Ishida, Y., Chiba, Y., Iwamatsu, A., Kishino, T., Niikawa, N., Matsubara, Y., and Narisawa, K. (1994): Isolation and characterization of mutations in the human holocarboxylase synthetase cDNA. *Nat. Genet.* 8:122-128.

13. Burri, B.J., Sweetman, L., and Nyhan, W.L. (1985): Heterogeneity of holocarboxylase synthetase in patients with biotin-responsive multiple carboxylase deficiency. *Am. J. Hum. Genet.* 37:326-337.

14. Ghneim, H.K. and Bartlett, K. (1982): Mechanism of biotin-responsive combined

carboxylase deficiency [letter]. *Lancet*, 1:1187-1188.

15. Barker, D.F. and Campbell, A.M. (1980): Use of bio-lac fusion strains to study regulation of biotin biosynthesis in Escherichia coli. *J. Bacteriol.* 143:789-800.

16. Campbell, A., del Campillo-Campbell, A., and Chang, R. (1972): A mutant of Escherichia coli that requires high concentrations of biotin. *Proc. Natl Acad. Sci. USA*, 69:2219-2223.

17. Leon-Del-Rio, A., Leclerc, D., Akerman, B., Wakamatsu, N., and Gravel, R.A. (1995): Isolation of a cDNA encoding human holocarboxylase synthetase by functional complementation of a biotin auxotroph of Escherichia coli. *Proc. Natl Acad. Sci. USA*, 92:4626-4630.

18. Leon-Del-Rio, A. and Gravel, R.A. (1994): Sequence requirements for the biotinylation of carboxyl-terminal fragments of human propionyl-CoA carboxylase alpha subunit expressed in Escherichia coli. *J. Biol. Chem.*, 269:22964-22968.

19. Mock, D.M. and Malik, M.I. (1992): Distribution of biotin in human plasma: most of the biotin is not bound to protein. *Am. J. Clin. Nut.*, 56:427-432.

20. Mock, D.M., deLorimer, A.A., Liebman, W.M., Sweetman, L., and Baker, H. (1981): Biotin deficiency: an unusual complication of parenteral alimentation. *N. Engl. J. Med.*, 304:820-823.

21. Mock, D.M., Baswell, D.L., Baker, H., Holman, R.T., and Sweetman, L. (1985): Biotin deficiency complicating parenteral alimentation: diagnosis, metabolic repercussions, and treatment. *J. Pediatr.* 106:762-769.

22. Suormala, T., Wick, H., Bonjour, J.P., and Baumgartner, E.R. (1985): Rapid differential diagnosis of carboxylase deficiencies and evaluation for biotin-responsiveness in a single blood sample. *Clin. Chim. Acta*, 145:151-162.

23. Velazquez, A., Teran, M., Baez, A., Gutierrez, J., and Rodriguez, R. (1995): Biotin supplementation affects lymphocyte carboxylases and plasma biotin in severe protein-energy malnutrition. *Am. J. Clin. Nutr.*, 61:385-391.

24. Prakash, O. and Eisenberg, M.A. (1974): Active transport of biotin in Escherichia coli K-12. *J. Bacteriol.*, 120:785-791.

25. Cronan, J.E., Jr. (1988): Expression of the biotin biosynthetic operon of Escherichia coli is regulated by the rate of protein biotination. *J. Biol. Chem.*, 263:10332-10336.

26. Sweetman, L. (1996): Data on MCD patients. (personal communication)

27. Saudubray, J.-M. (1996): Data on MCD patient (PD). (personal communication)

28. Gompertz, D., Draffan, G.H., Watts, J.L., and Hull, D. (1971) Biotin-responsive B-methylcrotonylglycinuria. *Lancet*, 22-24.

29. Sweetman, L., Bates, S.P., Hull, D., and Nyhan, W.L. (1977) Propionyl-CoA carboxylase deficiency in a patient with biotin-responsive 3-methyl-crotonylglycinuria. *Pediatr. Res.*, **11**, 1144-1147.

30. Saunders, M., Sweetman, L., Robinson, B., Roth, K., Cohn, R., and Gravel, R.A. (1979) Multiple carboxylase defects and complementation studies with propionicacidemia in cultured fibroblasts. *J. Clin. Invest.*, **64**, 1695-1702.

31. Bartlett, K. and Gompertz, D. (1976) Combined carboxylase defect: biotin-responsiveness in cultured fibroblasts. *Lancet*, 804.

32. Weyler, B., Sweetman, L., Maggio, D.C., and Nyhan, W.L. (1977) Deficiency of propionyl-CoA carboxylase in a patient with methylcrotonylglycinuria. *Clin. Chim. Acta*, **76**, 321-328.

33. Herman, G. (1996): Data on MCD patient (MK). (personal communication)

34. Abstract presented at the Vth Nordic Meeting of Medical Genetics in Laugarvatn, Iceland, August 27-28, 1988 (abstract 220)

35. Packman, S., Caswell, N., Gonzalez Rios, M.C., Kadlecek, T., Cann, H., Rassin, D., and McKay, C. (1984) Acetyl CoA carboxylase in cultured fibroblasts: differential biotin dependence in the two types of biotin-responsive multiple carboxylase deficiency. *Am. J. Hum. Genet.*, **36**, 80-92.

36. Livne, M., Gibson, K.M., Amir, N., Eshel, G., and Elpeleg, O.N. (1994) Holocarboxylase synthetase deficiency: a treatable metabolic disorder masquerading as cerebral palsy. *J. Child Neurology*, **9**, 170-172.

CHAPTER IV

GENERAL DISCUSSION

Patients suffering from neonatal MCD have a defect in holocarboxylase synthetase. Fortunately, these patients respond to treatment with pharmacological doses of oral biotin. All the biochemical and clinical manifestations are reversed within weeks of treatment. If the disease is left untreated, it can lead to coma and death. It is therefore crucial to detect the disease early.

The work included in this thesis has advanced our knowledge of the cause of MCD and the basis of biotin-responsiveness in patients. In chapter II, I described six mutations in the gene from HCS-deficient patients. It has long been thought that mutations responsible for the disease would localize to the region of the HCS protein involved in biotin binding. I have identified four mutations in the biotin-binding region of HCS (1). This region was determined through homology to various biotin ligases including BirA from *E. coli* whose three-dimensional structure has been defined (2). I have also found two mutations outside this region, suggesting a second domain involved in biotin binding or in establishing the proper conformation of the protein as a result of biotin binding. The only requirement is that the mutant HCS possess residual activity. All of the identified mutations lie in functional domains and is the reason for the clustering. Presumably, mutations outside these domains are benign and therefore are not detected. Further studies of normal individuals should reveal their identity. Patients with two functionally null alleles have not been seen since it may be lethal *in utero*.

In chapter III, I described the effect of each of the mutations on the binding of biotin by expressing them in *E. coli birA*$^-$ cells. I examined their effect on biotinylation of BCCP under different concentrations of biotin. I showed that three mutations (R508W, G518E and V550M) are associated with high to moderate HCS activity and were biotin

responsive, two mutations (D571N and V363D) showed low to very low HCS activity and were biotin responsive and the last mutation (L216R) showed background activity and was not biotin responsive under the conditions used in the assay.

Apparent K_m and V_{max} of HCS for biotin were determined for wild type HCS. Since the assay was done in whole cells, the kinetic constants represent a combination of biotin transport and HCS activity. However, previous studies suggested that biotin uptake and biotinylation are highly coupled (4-6). My data corroborates these findings, confirming that biotin uptake was by facilitated diffusion and that the apparent K_m was a reflection of the K_m for biotin of HCS. Unexpectedly, the calculated V_{max} varied considerably in different experiments. Perhaps the variation was due to different amounts of human HCS being produced by the bacteria (i.e. not well controlled), despite identity of the vector and growth conditions. A solution to this problem would be to normalize the HCS activity to HCS protein (i.e. Western blot) when an antibody becomes available or to co-expressed ß-galactosidase activity.

The kinetic parameters could not be determined for HCS containing the various mutations because of dispersion of the data, especially mutants that showed only slight activity. However, it would be possible to obtain these values for at least two of the six mutant HCS's (R508W and G518E) with a more extensive evaluation.

Determining the kinetic parameters for biotin (K_m and V_{max}) of HCS carrying the more common mutations would have important implications for the treatment of neonatal MCD. Once the affinity of mutant HCS for biotin has been determined, it would be possible to treat MCD patients more efficiently by determining more accurate doses of biotin needed for treatment instead of the conventional 10 mg of biotin per day or a trial and error method.

A more sensitive and perhaps more reliable assay to determine these kinetic parameters would be to use an *in vitro* assay that makes use of a carboxylase substrate (p-67) for

HCS developed in our lab by Alfonso Leon-Del-Rio (7). It contains the last 67 amino acids of human PCC-α and is the minimum sequence required for recognition by human HCS for biotinylation. It could be used much the same way as the *in vivo* ^{3}H-biotin assay. ^{3}H-biotin incorporation and precipitation of protein with TCA would be used to determine the amount of radioactive biotin bound to p-67. It could also be used as the substrate for cell extracts to determine patient HCS activity. For the analysis of individual mutations, the source of HCS would be an extract of an *E. coli* strain defective in the synthesis of biotin transformed with mutant HCS. This approach would solve several problems including the control of the concentration and purity of the apocarboxylase substrate. In addition, endogenous biotin synthesis would no longer be a factor.

There are many questions concerning MCD and the mechanism of HCS action that remain unanswered. Future work could include determining which form of HCS is targeted to the mitochondria or to the cytosol. Constructs of HCS cDNA containing the different N-terminal sequences generated by alternative splicing (7) could be fused to the sequence of the "green fluorescent protein" (GFP) from *Aequorea Victoria* at the C-terminus. The fluorescent moiety would be used as a marker to facilitate the identification of the mitochondrial versus the cytoplasmic fate of the fusion protein using fluorescence microscopy for detection. One could also determine if the cytoplasmic version of HCS can biotinylate mitochondrial apocarboxylases while in transit to the organelle.

All patients with MCD are biotin responsive although some respond less well than others. To date, no truly unresponsive patients have been identified. We previously suggested that this could be due to lethality *in utero* or, alternatively, to a second enzyme with a low affinity for biotin that may be able to substitute for HCS in this case (8). My results suggest yet another explanation. Because the estimated K_m for biotin is far below the concentration of biotin found in human plasma, increasing the biotin concentration in normal cells will increase the activity of HCS. Therefore, all MCD patients should be

biotin-responsive if they possess any residual HCS activity.

An interesting question to answer would be to define the affinity that HCS has for each of the carboxylases. If the minimum sequence of the carboxylase needed for biotinylation could be found for PC, MCC and ACC as is was done for PCC, one could examine relative affinities. Kinetic constants could be determined directly for the acceptor peptides. In addition, competition assays could be done with pairs or multiples of the apocarboxylase substrates using radioactive biotin as a tracer and running the proteins on SDS-PAGE then quantifying. Also, one could determine the minimum sequence of human HCS needed to carry out biotinylation given that only a small domain of 129 amino acids is conserved between the bacterial and human enzymes. This could be done by expressing fragments of HCS in *E. coli birA*104 cells and determining if biotinylation of BCCP occurs at 42°C. The results would give insight on the mechanism of biotinylation of apocarboxylases.

In summary, I have identified six disease-causing mutations in MCD patients. I have demonstrated the level of biotin-responsiveness of HCS containing each of these mutations. Furthermore, I have provided an explanation for the near universal biotin responsiveness observed in MCD patients.

REFERENCES

1. Dupuis, L., Leon-Del-Rio, A., Leclerc, D., Campeau, E., Sweetman, L., Saudubray, J.M., Herman, G., Gibson, K.M., and Gravel, R.A. (1996): Clustering of mutations in the biotin-binding region of holocarboxylase synthetase in biotin-responsive multiple carboxylase deficiency. *Hum. Mol. Gen.* 5:1011-1016.

2. Wilson, K.P., Shewchuk, L.M., Brennan, R.G., Otsuka, A.J., and Matthews, B.W. (1992): Escherichia coli biotin holoenzyme synthetase/bio repressor crystal structure delineates the biotin- and DNA-binding domains. *Proc. Natl Acad. Sci USA* 89:9257-9261.

3. Prakash, O. and Eisenberg, M.A. (1974): Active transport of biotin in Escherichia coli K-12. *J. Bacteriol.*, 120:785-791.

4. Barker, D.F. and Campbell, A.M. (1981): The birA gene of Escherichia coli encodes a biotin holoenzyme synthetase. *J. Mol. Biol.* 146:451-467.

5. Barker, D.F. and Campbell, A.M. (1981): Genetic and biochemical characterization of the birA gene and its product: Evidence for a direct role of biotin holoenzyme synthetase in repression of the biotin operon in Escherichia coli. *J. Mol. Biol.* 146:469-492.

6. Cronan, J.E., Jr. (1988): Expression of the biotin biosynthetic operon of Escherichia coli is regulated by the rate of protein biotination. *J. Biol. Chem.*, 263:10332-10336.

7. Leon-Del-Rio, A. and Gravel, R.A. (1994): Sequence requirements for the biotinylation of carboxyl-terminal fragments of human propionyl-CoA carboxylase alpha subunit expressed in Escherichia coli. *J. Biol. Chem.*, 269:22964-22968.

8. Leon-Del-Rio, A., Leclerc, D., Akerman, B., Wakamatsu, N., and Gravel, R.A. (1995): Isolation of a cDNA encoding human holocarboxylase synthetase by functional complementation of a biotin auxotroph of Escherichia coli. *Proc. Natl Acad. Sci. USA*, 92:4626-4630.

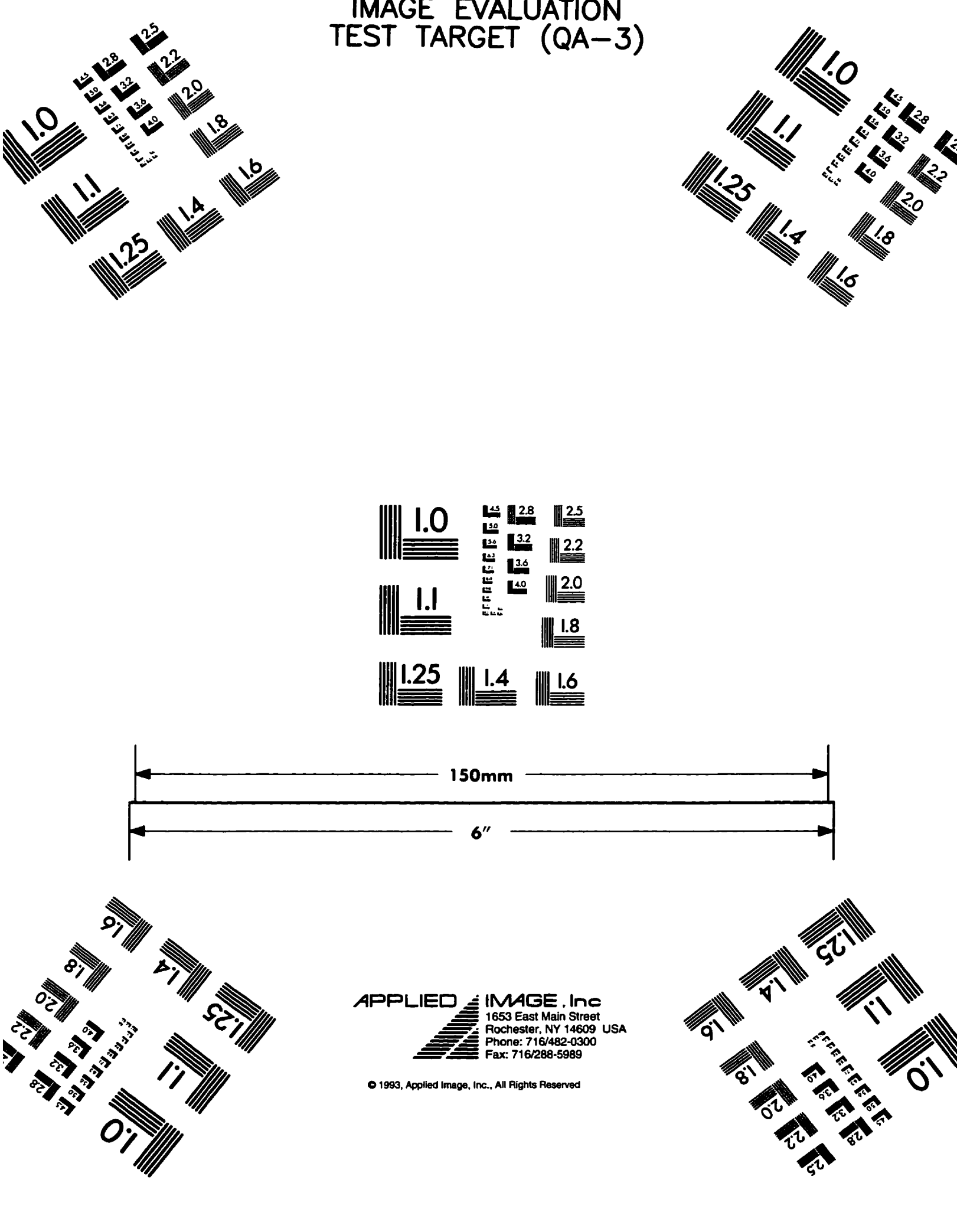
IMAGE EVALUATION
TEST TARGET (QA-3)
1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
150mm
6"
APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989
© 1993, Applied Image, Inc., All Rights Reserved