THE ROLE OF THE QUATERNARY STRUCTURE OF A BIFUNCTIONAL ENZYME

by

Wendy A. Findlay

Department of Biochemistry

McGill University

Montreal, Quebec, CANADA

July, 1988

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

Formiminotransferase (E.C. 2.1.2.5) - cyclodeaminase (E.C. 4.3.1.4) catalyses two sequential folate-dependent reactions. It has much higher affinity for polyglutamate than monoglutamate folate substrates, and "channels" formiminotetrahydropteroylpolyglutamate between the active sites. The porcine enzyme is a circular octamer of identical subunits (M_r 62,000) with two types of subunit-subunit interaction. Monofunctional dimers with either transferase or deaminase activity are produced upon dissociation by urea in the presence of substrate analogues. The two dimers isolate different subunit interfaces and further dissociation leads to loss of activity. Transferase-active dimers are also produced during renaturation of formiminotransferase-cyclodeaminase from 6 M guanidine hydrochloride. They can be "trapped" by the presence of 1.5 M urea, but reassociate to form bifunctional octamer upon dialysis. Only deaminase dimers exhibit higher affinity for polyglutamate than monoglutamate folate substrates. We propose that the transferase and deaminase activities require formation of alternate subunit interfaces in the native octamer and that polyglutamate binding requires the same interface as deaminase activity.

RÉSUMÉ

La formiminotransférase (E.C. 2.1.2.5)-cyclodésaminase (E.C. 4.3.1.4) catalyse deux réactions successives qui utilisent le folate. Cette enzyme a beaucoup plus d'affinité pour les substrats folates polyglutamylés et transfère le formiminotétrahydroptéroylpolyglutamate entre les sites actifs. L'enzyme d'origine porcine est un octamère circulaire formé de sous-unités identiques (M_r 62,000) qui sont associées selon deux façons différentes. Des dimères monofonctionnels possédant soit l'activité de transférase, soit l'activité de désaminase sont produits après dissociation par l'urée en présence d'analogues des substrats. Les deux dimères présentent des interactions différentes entre les sous-unités et toute dissociation subséquente mène à la perte d'activité. Les dimères possédant l'activité de transférase peuvent aussi être produits durant la renaturation de la formiminotransférase-cyclodésaminase après un traitement au chlorhydrate de guanidine 6 M. Ils peuvent être isolés en présence d'urée 1.5 M, mais ils se réassocient en octamère bifonctionnel après dialyse. Seuls les dimères avec l'activité de désaminase présentent plus d'affinité pour les substrats folylpolyglutamates. Nous proposons que les activités de transférase et de désaminase requièrent la formation d'interfaces alternées de l'octamère d'origine et que le site liant le polyglutamate exige la même interface que l'activité de désaminase.

TABLE OF CONTENTS

FOREWORD

A part of this thesis includes the text of original papers submitted for publication. In compliance with the Faculty of Graduate Studies and Research "Guidelines Concerning Thesis Preparation", the text of section 7 entitled "Manuscripts and Authorship" is cited below:

"The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text (see below), of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interests of cohesion.

It is acceptable for theses to include as chapters authentic copies of papers already published, provided these are duplicated clearly on regulation thesis stationery and bound as a integral part of the thesis. Photographs or other materials which do not duplicate well must be included in their original form. In such instances, connecting texts are mandatory and supplementary explanatory material is almost always necessary.

The inclusion of manuscripts co-authored by the candidate and others is acceptable but the candidate is required to make an explicit statement on who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims, e.g. before the Oral Committee. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear. Candidates following this option must inform the Department before it submits the thesis for review".

This format for thesis preparation has been approved by the Department of Biochemistry. Each chapter has its own numeration of figures and tables. All references are compiled at the end of the thesis and presented in alphabetical order.

ACKNOWLEDGEMENTS

I am very much indebted to Dr. Robert E. MacKenzie, my research supervisor, for the privilege of working in his laboratory and for his excellent guidance during the course of my work. His interest, advice, and enthusiasm were much appreciated and I thank him also for proofreading and constructive criticism of this thesis. I thank the other members of his laboratory, particularly Dr. Drummond Smith, Dr. Joanne Paquin, Ethel Rios-Orlandi and Scott Taylor, for interesting scientific discussions and for contributing to a good working atmosphere. The encouragement and support provided by my parents during the course of my studies were also greatly appreciated.

I am grateful to Dr. Charles Baugh (University of South Alabama), who supplied various folylpolyglutamates, Dr. Joanne Paquin, who synthesized several of the tetrahydropteroylpolyglutamates, and Nori Churchill-Smith, who prepared enzyme for much of the preliminary work.

I thank Margaret Licorish for typing Chapters 1 and 5 and for careful preparation of the final version of this thesis, Maureen Caron for typing Chapter 2, and Josie D'Amico for typing Chapters 3 and 4. Margot Oeltzschner drew all the figures presented in this thesis, and all the photography was provided by Kathy Teng. I am also very grateful to Claire Turbide for helping with translation of the Abstract, and to Ethel Rios-Orlandi and Dean Hum for proofreading and criticism of several sections of this thesis.

The financial support provided by M.R.C., F.C.A.C., and F.C.A.R at various stages of this work was much appreciated, as was financial assistance from the Faculty of Medicine during the final months.

Publication of the Work Presented in the Thesis

Dissociation of the Octameric Bifunctional Enzyme Formiminotransferase-Cyclodeaminase in Urea. Isolation of Two Monofunctional Dimers. W.A. Findlay and R.E. MacKenzie, Biochemistry 26, 1948-1954 (1987).

Renaturation of Formiminotransferase-Cyclodeaminase from Guanidine Hydrochloride. Quaternary Structure Requirements for the Activities and Polyglutamate Specificity. W.A. Findlay and R.E. MacKenzie, Biochemistry 27, 3404-3408 (1988).

The work presented in Chapter 4 will be submitted to Biochimica et Biophysica Acta under the title "An Improved Procedure for Purifying Formiminotransferase-Cyclodeaminase from Pig Liver. Kinetics of the Transferase Activity with Tetrahydropteroylpolyglutamates". by W.A. Findlay, C.G. Zarkadas, and R.E. MacKenzie.

The contribution of Dr. Zarkadas to this work was to perform the amino acid analysis of the enzyme and transferase-active fragment. Dr. R.E. MacKenzie provided the normal supervision and advice appropriate to his role as research director.

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF TABLES

LIST OF ABBREVIATIONS

$H_4PteGlu_n$: tetrahydropteroylpolyglutamate with a total of n glutamates.

5-NHCH-$H_4PteGlu_n$: 5-formiminotetrahydropteroylpolyglutamate

5,10-CH^+-$H_4PteGlu_n$: 5,10-methenyltetrahydropteroylpolyglutamate

PteGlu: folic acid

PEG: polyethylene glycol

DTT: dithiothreitol

EDTA: ethylenediaminetetraacetic acid

TEA.Cl: triethanolamine chloride

Gdn.HCl: guanidine hydrochloride

HCl: hydrochloric acid

TCA: trichloracetic acid

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis.

CHAPTER 1

GENERAL INTRODUCTION

"The most beautiful thing we can experience is the mysterious. It is the source of all art and science".

A. Einstein

The importance of the role of enzyme organization in the overall efficiency and regulation of many metabolic pathways is now becoming apparent. Multifunctional enzymes, where two or more activities are catalysed by a single type of polypeptide chain, represent one type of enzyme organization, and several examples are found in the pathways of folate-mediated metabolism. This thesis deals with the octameric bifunctional folate-dependent enzyme formimino-transferase-cyclodeaminase, which catalyses two sequential reactions in the catabolism of histidine in mammalian liver. This enzyme exhibits much higher affinity for the naturally occurring polyglutamate derivatives of its folate substrates and "channeling" of the intermediate between the two activities is observed. Formimino-transferase-cyclodeaminase is an excellent system for studying both site-site interaction and the relationship between structure and function in a multifunctional enzyme.

A discussion of enzyme structure and organization, and the use of protein denaturation/renaturation to study the role of quaternary structure in enzyme activity, as well as a review of the properties of formiminotransferase-cyclodeaminase will be presented prior to outlining the objectives of this thesis.

1.1 STRUCTURE AND ORGANIZATION OF ENZYMES

The three dimensional native structure of a protein can be described at a hierarchy of levels. Primary structure refers to the sequence of amino acids in the polypeptide. Short range interactions between amino acids form the elements of secondary structure, which combine to form the tertiary structure of the correctly folded monomer. Quaternary structure refers to the interaction between subunits to form oligomeric enzymes or multienzyme complexes. The catalytic activity of enzymes requires formation of the native structure.

The physical association of different activities by either covalent or non-covalent interactions is a form of enzyme organization, and its role in a number of metabolic pathways is currently being elucidated (see Srere, 1987). Although the extent of enzyme organization observed to date probably represents only a fraction of that present in living cells, we can now begin to understand some of the principles involved in the function of enzymes *in vivo* as an extension of the previous observations *in vitro* using very dilute solutions of highly purified individual enzymes.

In this section I present a summary of the principles of protein structure from the primary to the quaternary level, followed by a selection of examples of various types of enzyme organization and some of their features, and finally discuss a number of consequences of enzyme organization.

PROTEIN STRUCTURE: PRIMARY TO QUATERNARY

Proteins are synthesized as linear polymers of amino acids joined by peptide bonds. There are twenty commonly occurring amino acids which can be classed according to side chain as nonpolar, neutral polar, acidic or basic (Dickerson and Geis, 1969). The primary structure of a protein is the sequence of amino acids in the polypeptide chain, and is specified by the mRNA translated by the ribosome, and ultimately coded by the DNA of the organism.

The peptide bond formed between amino acid residues is approximately planar and rotation around this bond is inhibited by resonance. The peptide bond in proteins is almost always trans. The side chain is attached to the α-carbon of the residue, and three dihedral angles for rotation are defined: Ψ around the C_α-C=O bond, ϕ around the C_α-N bond and χ around the C_α-C_β bond of the side chain. The size and structure of the side chain imposes steric limits on possible values of Ψ, ϕ and χ, as is shown by Ramachandran plots of Ψ vs ϕ for the various amino acids (Ramachandran and Sasisekharan, 1968). While the map for glycine covers a fairly large area, that for residues with a side chain β-carbon is significantly reduced. These restrictions are respected in the formation of α-helices and β-sheets, the most common elements of secondary structure (Pauling et al., 1951 and Pauling and Corey, 1951).

The right-handed α-helix has 3.6 residues per turn with hydrogen bonding between the carboxyl- and amino- groups of the peptide backbone four residues apart. The side chains point out from the helix and this

structure is compatible with most amino acids except proline since its backbone N atom is bonded to the side chain ring and therefore is not available for hydrogen bonding, and its rigid structure interferes with the helix packing.

The β-sheet is formed by extended polypeptide chains which aggregate side by side and form hydrogen bonds between the backbone carbonyl and amino groups of adjacent strands, and can be oriented either parallel or antiparallel. In general, β-sheets have a twist which is right handed when viewed along their strands due to the greater range of ϕ and Ψ values allowed by this conformation. The side groups project alternately above and below the plane of the sheet, and again most residues, apart from proline, are compatible with this structure.

Most of the rest of the polypeptide chain forms turns, which are short connecting stretches between helices and β-sheets, or is present as loops of random coil.

Although the sequence of a polypeptide ultimately specifies its three dimensional structure, the correlation between short pieces of sequence and secondary structure is not very good. Tables have been prepared showing the frequency of occurrence of the various residues in the different secondary structures (for examples see Schulz and Schirmer (1979), Chapter 6) and various programs for predicting secondary structure from sequence have been developed, but in general are only 50-60% accurate (Kabsch and Sander, 1983). Argos (1987) has analysed 229 identical and over 9,000 similar (one residue different) pairs of pentapeptides in unrelated proteins from the Brookhaven Data Bank. He found that helical structure was preserved for only about 24% of the

pairs, extended (β) structure less than 20%, and conversion between helix and β-structure occurred in 20 to 28% of the pairs. He concluded that "an oligopeptide, given the proper environment, can do just about anything on a structural level". This is due mainly to the influence of long range interactions which determine the tertiary organization of the polypeptide.

The tertiary structure of a protein describes the packing of α-helices and β-sheets usually into a globular structure. I will restrict this discussion to globular soluble proteins, which include the majority of enzymes, as opposed to structural proteins and intrinsic membrane proteins, which have somewhat different properties.

A review by Chothia (1984) summarizes the principles which determine the overall structure of proteins. In general, the atoms in proteins are extremely closely packed and the packing density approaches that of an organic crystal, about 20% higher than that of a liquid (Chothia, 1975).

The packing of α-helices usually involves the ridges of one helix (formed by residues four apart in the sequence) fitting into grooves between ridges on a second helix oriented at a -52° angle. The contacts between the helices involve the ends of side chains. There is a strong tendency for α-helices to pack against β-sheets with their axes parallel to the sheet strands, to generate a surface complementary to the twisted β-sheet. For β-sheets, two kinds of packing are observed - aligned β-sheet packing, where two essentially independent β-sheets are packed face-to-face with their strands oriented at an ~30° angle, and orthogonal β-sheet packing, where a β-sheet is folded on itself so that

the layers are packed face-to-face, but their strands are at 90° to each other and the main chain passes from one layer to the next at diagonally opposite corners. The β-sheet surfaces that form interfaces with other elements of secondary structure contain high concentrations of valine, leucine, isoleucine, phenylalanine and alanine.

Sequentially linked elements of secondary structure are often in contact in the tertiary structure forming "supersecondary structures" such as: αα, two antiparallel packed α-helices; ββ, two antiparallel β-strands; βαβ, a helix packed against two parallel β-sheet strands; and αββ or ββα, a helix against two adjacent antiparallel β-strands. These arrangements of secondary structures occur frequently and Richardson (1980, 1981) has proposed that proteins can be classified into four major categories - all-α (mainly antiparallel α-helices), all-β (mainly antiparallel β-sheet), parallel α/β (alternating α and β structures) and small disulfide-rich or ligand-rich structures (containing a relatively low proportion of secondary structure). Combination of elements of supersecondary structure results in formation of structural domains - regions of closely packed secondary structures usually linked by a single strand of polypeptide chain.

Rossman and Argos (1981) have suggested the following properties for domains - that 1) similar domain structures or amino acid sequences be repeated in the same polypeptide or be present in different proteins, 2) domains within a polypeptide are spatially separate from each other, 3) domains have a specific function i.e. binding a substrate or cofactor, and 4) the active center of a molecule is usually at the interface between domains. Schultz and Schirmer (1979) distinguish

between functional domains, which are functionally autonomous (i.e. ligand-binding or enzymatic activity) regions of the polypeptide chain, and structural domains, which are geometrically separate entities which can be classified by secondary structure and usually contain from 100 to 200 residues. Most functional domains of molecular weight greater than 20,000 possess more than one structural domain and have their active sites at the interface between structural domains.

Traut (1986) has proposed that domains can be further subdivided into "modules" of ~5000 Da molecular weight, each of which is involved in either binding a ligand (substrate or effector) or interacting with another subunit. He suggests that the average size of these units corresponds well with the amount of polypeptide required to form a stable, folded structure and with that encoded by the average exon. The size of an enzyme subunit should therefore be determined by the number of modules required to fulfill all its biological functions (catalytic, regulatory and interactive).

It has been suggested that the diversity of proteins present in organisms today arose by gene fusion of smaller genes coding for structural domains, because of the limited number of supersecondary elements present. In general protein structure is conserved to a far greater extent than amino acid sequence, for example in the NAD-binding domains of various dehydrogenases compared by Rossman et al. (1975). It is often difficult to distinguish between the results of divergence of different proteins from a common ancestor and convergence of two proteins which arose separately towards a similar stable folding pattern. Fothergill-Gilmore (1986) has reviewed similarities in the

structure, function and sequences of 15 glycolytic enzymes (for which more detailed structural data is known than for any other group of enzymes). Several enzymes in this pathway catalyse similar classes of reactions and others bind similar ligands (e.g. ATP or NAD) and their substrates are fairly similar, i.e. small negatively charged molecules. She concludes that "it seems unlikely that the glycolytic enzymes catalysing similar reactions have evolved from primordial kinases, mutases, dehydrogenases, etc. Any similarities have probably arisen by convergence towards a few stable structures on which have been arranged the amino acid chains required for ligand binding and catalysis". The only exceptions to this so far are diphosphoglycerate mutase which appears to have evolved from phosphoglycerate mutase, and glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase which may have shared a common ancestor, however detailed structural data for five of the other enzymes is not yet available. By looking at sequence homology between enzymes from different sources and between isozymes, she has determined that the approximate "rate of evolution" for the glycolytic enzymes is fairly slow.

The overall stability of a globular protein is conferred mainly by hydrogen bonding, both within the secondary structures and between polar side groups, and by the sequestering of hydrophobic large nonpolar side groups inside the protein. For some proteins there is an additional smaller contribution by disulfide crosslinks formed between cysteines, and by salt bridges formed by pairs of internalized charged residues.

Quaternary structure refers to the association of folded polypeptides to form oligomeric proteins. As shown by Darnall and Klotz (1972) for more than 300 proteins, dimers are by far the most common oligomers (~50%), followed by tetramers (~20%), and proteins with even numbers of subunits are more common than ones with odd numbers.

Chothia (1974) has shown a linear relationship between the accessible surface area of amino acids and their hydrophobicity, as determined by Nozaki and Tanford (1971). He demonstrated that for monomeric proteins, the proportion of accessible surface area buried on folding is directly proportional to the molecular mass and yields a hydrophobic contribution to the free energy of folding of 24 cal/$Å^2$, whereas the proportion of polar groups forming intramolecular hydrogen bonds is constant (Chothia, 1975). Miller et al. (1987) showed that the accessible surface area buried in oligomeric proteins is also proportional to the total molecular mass, although the proportions buried within and between subunits can vary widely for different proteins. This confirms that the hydrophobic effect, due to burying of protein surface area on formation of tertiary and quaternary structure, is an important contribution to the stability of the folded protein.

The quaternary structure can involve either identical or non-identical subunits - for example, some enzymes consist of both catalytic and regulatory subunits and others are oligomers of a single kind of subunit. The areas of contact must be complementary and association is generally very specific. Interaction between identical subunits can be

either isologous, involving the same surface of two subunits and forming a closed dimeric structure which can interact at other surfaces with other dimers, or heterologous, where nonidentical surfaces interact forming either open ended or cyclic structures (Monod et al., 1965).

Recently a number of enzymes have been reported to have their catalytic sites at subunit-subunit interfaces with active site residues contributed by both polypeptides. Aspartate aminotransferase and glutathione reductase are dimeric enzymes which must contain a single isologous subunit-subunit interaction and two active sites have been found at the interface for both enzymes (Ford et al., 1980, and Thieme et al., 1981). For enzymes with heterologous interactions between subunits, such as the catalytic trimers of aspartate carbamoyltransferase (Krause et al., 1985) and the dodecameric glutamine synthase (Almassy et al., 1986), a single active site has been found at each interface.

Another possible consequence of formation of quaternary structure is cooperation between subunits and its effect on enzymatic catalysis (Huang et al., 1982). This is particularly important for regulatory enzymes, almost all of which are oligomeric. An excellent example of this is aspartate transcarbamoylase from E. coli, which has been studied extensively (see Schachman, 1987). This enzyme consists of two catalytic trimers linked by three regulatory dimers and is allosterically inhibited by CTP, which causes it to undergo a measurable conformational change from the R-state to the T-state (Monod et al., 1965). It also shows cooperativity with respect to the substrate aspartate. Both the concerted allosteric transition and the observed

cooperativity in binding aspartate (or the substrate analogue PALA) require communication between the catalytic subunits, and between catalytic and regulatory subunits.

Most of the information about protein structure has been obtained by x-ray crystallography, which presents a rather static picture, but to understand enzyme function will also require knowledge of protein dynamics (Pain, 1983). It is now becoming possible to study protein conformation in solution using high resolution nuclear magnetic resonance (N.M.R.) spectroscopy (see Kabsch and Rösch, 1986), which can provide information about the mobility of side chains and conformational changes on binding substrates or inhibitors. The coordinates provided by x-ray crystallography or NMR can also be used as a starting point for theoretical modeling of protein dynamics as described by McCammon and Karplus (1983).

ENZYME ORGANIZATION

It is becoming apparent that cellular metabolism is ordered in both space and time, mainly as a result of compartmentalization of enzymes involved in different metabolic pathways. Physical segregation results from the enzymes being in separate organelles - for example the enzymes of the Krebs TCA cycle in the mitochondrial matrix or the enzymes of oxidative phosphorylation in the inner mitochondrial membrane (Srere, 1985). Other enzymes may undergo reversible, specific association with various subcellular structures as reviewed by Friedrich (1984), such as the binding of glycolytic enzymes to band 3 protein of erythrocytes or to the I-band of skeletal muscle fibers. Multienzyme complexes are

characterized by strong non-covalent interactions so that two or more activities can be shown to copurify but are the property of two or more polypeptide chains. If more than one activity is catalysed by a single type of polypeptide chain, it is known as a multifunctional enzyme or multienzyme conjugate (Friedrich, 1984). Welch (1977) has proposed the term "enzyme cluster" to denote any physically associated enzyme system of physiological significance.

Two well characterized examples of multienzyme complexes are tryptophan synthase from E. coli and both bacterial and mammalian pyruvate dehydrogenases. Bacterial tryptophan synthase has an $\alpha_2\beta_2$ quaternary structure and the α and β_2 subunits have been shown to catalyse sequential reactions in the formation of tryptophan from indoleglycerol phosphate and serine. Formation of the complex greatly increases the rate of the overall reaction with an ~100-fold increase in turnover number (see Miles, 1979).

Pyruvate dehydrogenase forms a much larger multienzyme complex and in E. coli consists of a core of 24 lipoate acetyltransferase (E2) subunits to which 24 pyruvate decarboxylase (E1) and 12 lipoamide dehydrogenase (E3) subunits are bound (CaJacob et al., 1985). The mammalian pyruvate dehydrogenase complex has a core of 60 E2 subunits to which non-stoichiometric amounts of the other two catalytic (E1 and E3) and two regulatory subunits are bound. Wu and Reed (1984) determined that six E_3 dimers and 9-14 E_1 tetramers bind to the E_2 core. Brandt et al. (1983) observed 7 high affinity and 13 low affinity binding sites for the E1 component per core. Binding and release of the E1 tetramers

from the low affinity binding sites would allow migration of this component between cores (Cate et al., 1980).

Weaker interactions between enzymes in solution may only occur at the high protein concentrations found inside cells or may require the presence of particular metabolites. Weber and Bernhard (1982) demonstrated formation of an enzyme-substrate-enzyme complex by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 3-phosphoglycerate kinase (PGK) to transfer 1,3-diphosphoglycerate (P_2G) and showed that it is actually the complex PGK-1,3-P_2G which acts as substrate for glyceraldehyde-3-phosphate dehydrogenase. Srivastava and Bernhard (1986) have extended this study to show pairwise interaction of ten enzymes of glycolysis for direct transfer of intermediates. They also suggest interaction of dehydrogenases to transfer NADH when the two enzymes bind NADH in different conformations (i.e. exposing opposite "faces" of the nicotinamide ring) and again an E_1-NADH complex acts as substrate for the second enzyme.

Formation of some multienzyme complexes may depend on the metabolic or developmental state of the cell. Reddy and Pardee (1980) observed cosedimentation on a sucrose gradient of 7 enzymes in the DNA synthetic pathway extracted from the nucleus of mammalian cells in S phase. In cells which had not initiated DNA synthesis, the enzymes were dispersed in the cytoplasm.

Srere (1987) has reviewed the evidence for complexes of sequential metabolic enzymes involved in DNA replication ("replitase"), RNA synthesis (various RNA polymerases), protein synthesis (the ribosome and its interaction with other protein factors) and glycogen metabolism, as

well as those involved in the metabolism of precursor molecules - purines and pyrimidines, amino acids, and lipids - and in glycolysis and the tricarboxylic acid cycle. However, the extent of enzyme organization observed to date may reflect only a fraction of that actually present in cells.

Improvements in purification techniques, the use of protease inhibitors and the development of SDS-PAGE have led to the discovery of a number of multifunctional enzymes, where two or more independent reactions are catalysed by a protein consisting of a single type of polypeptide. A number of examples of multifunctional enzymes have been compiled by Kirshner and Bisswanger (1976) and Schmincke-Ott and Bisswanger (1980). In almost all cases the functions are related to each other, often catalytic activities in the same pathway which may or may not be sequential. Multifunctional enzymes are more common in eukaryotes, particularly in metabolic pathways which occur in the cytoplasm (Coggins and Hardie, 1986), and many are composed of several functional domains which can be separated as active fragments after proteolytic cleavage. Schmincke-Ott and Bisswanger (1980) suggest that multienzyme complexes evolved by gene fusion via either gene duplication, translocation or deletion after which the added function may be conserved or modified to a different function.

The pentafunctional _arom_ enzyme which catalyses five sequential reactions in aromatic amino acid biosynthesis has been cloned and sequenced from _S. cerevisiae_ by Duncan et al. (1987). They showed that it is a mosaic of five functional domains exhibiting 15 to 38% homology with the corresponding monofunctional enzymes from _E. coli_.

Evidence for the evolution of fatty acid synthase multifunctional enzymes has been presented by Hardie and McCarthy (1986). The seven reactions involved in fatty acid synthesis are catalysed by discrete enzymes in most bacteria and in chloroplasts, which have a separate acyl carrier protein (M_r 10,000) containing a phosphopantotheine cofactor to which the elongating fatty acid is attached. In other eukaryotes the enzymes exist as conjugates. In fungi the complex is of the form $\alpha_6\beta_6$, where the α subunit catalyses two reactions and contains an acyl carrier domain, and the β subunit catalyses the remaining four reactions. Most other eukaryotes have a homodimeric (α_2) fatty acid synthase where a single polypeptide carries all seven enzyme activities and an acyl carrier domain. Although complete sequence data is not available, a number of proteolytic fragments have been obtained which either express one or more activity or can be active-site labelled to determine the order of functional domains in the polypeptides. Since this order is different for the vertebrate and yeast multifunctional enzymes, it has been proposed that they arose by independent gene fusion events (McCarthy et al., 1983). The mechanism for the action of vertebrate fatty acid synthase suggests that the two subunits are aligned "head-to-tail" and that groups are transferred between catalytic sites on different polypeptides during the elongation cycle (Stoops and Wakil, 1981). This suggests that the active sites must be close to the subunit-subunit interface.

Multifunctional enzymes which catalyse non-sequential reactions are also known, for example the bifunctional aspartokinase-homoserine

dehydrogenase from E. coli (Cohen and Dautry-Varsat, 1980), which catalyses the first reaction in the synthesis of Lys, Met, Ile and Thr from aspartate and the third reaction which is the branch point between synthesis of Lys and the other three amino acids.

Most experimental work on enzyme organization has used either multifunctional enzymes or tightly bound multienzyme complexes, because the activities remain associated in vitro, allowing the system to be studied in isolation from other cellular components.

CONSEQUENCES OF ENZYME ORGANIZATION

As summarized by Gaertner (1978) and developed in greater detail by Welch (1977) the unique catalytic properties possessed by enzyme clusters are coordinate effects, such that binding of a single effector can affect the catalytic properties of several different enzymes, and effects due to compartmentalization of intermediates. The proximity of active sites for sequential reactions decreases both the transit time - the time required for a metabolite to diffuse from one active site to the next - and the transient time - the time for the overall rate process catalysed by the multienzyme sequence. The reduction in transit time allows the overall cellular concentration of intermediates to be low while still maintaining optimal local concentrations of substrates in the vicinity of the active sites. The smaller pools of intermediates required decreases the time required for the system to change metabolic state, which may be important in metabolic regulation, and prevents interference between competing pathways.

Rate enhancement can be achieved in enzyme clusters in several ways — 1) by energy transfer if energy released in one chemical reaction can be retained in the protein conformation and used in a subsequent catalytic event, 2) by transfer of intermediates between sites in optimal steric orientations for catalysis, 3) by stabilization of enzyme-substrate complexes and 4) by restriction of individual polypeptides to a single conformation optimal for catalysis. Welch (1977) defines the "α-spectrum" as the total effects of protein-protein or protein-matrix interaction on the rate properties of an enzyme and suggests that the "basic nature" of the catalytic centre "for a given enzyme was determined very early in biological evolution" and since then natural selection has been mainly acting on the "α-spectrum" to favour protein-protein interactions which increase the speed and efficiency of overall pathways. This is an extension of the concept of enzyme evolution for increasing catalytic efficiency developed by Albery and Knowles (1976) for independent, diffusible, non-regulatory enzymes.

Strongly self-associating or covalently-linked activities require no energy expenditure to maintain structure and function, whereas transient interactions are dependent on constant energy dissipation, but allow highly coordinated, versatile, and dynamic control of metabolic processes.

Kurganov (1986) has proposed that a metabolic pathway which corresponds to a structurally ordered multienzyme complex be designated a "metabolic system". He suggests that such complexes may form by

association with "anchor proteins", for example the glycolytic enzymes bound to the I-band of muscle fibers or band 3 protein of the erythrocyte membrane, and may be influenced by chemical signals such as Ca^{++} binding.

One of the most interesting consequences of association of sequential enzyme activities is the phenomenon of channeling. In the most general sense, channeling refers to preferential transfer of the product of one reaction to the active site of the next reaction without equilibration with the medium.

The first demonstration of metabolic channeling was by Davis in 1967. Using mutant strains of Neurospora he demonstrated that there are two different forms of carbamoylphosphate synthetase associated with aspartate transcarbamylase and ornithine transcarbamylase, providing separate pools of carbamylphosphate for pyrimidine and for arginine biosynthesis, respectively. Mutants lacking one of the carbamyl phosphate synthases are unable to make the corresponding end product. Davis (1967) proposed a "surface" model and a "compartment" model of channeling. In the "surface" model, two genetically distinct enzymes catalyse formation of the same intermediate but are physically associated with enzymes of different pathways, forming two pools of intermediate bound to the surfaces of the two multifunctional units. This requires high affinity and incomplete saturation of the second catalytic site with the intermediate. The "compartment" model requires that the intermediates of a pathway are restricted to a self-assembled phase in the cytoplasm, for example in a channel formed by a multienzyme

complex or in a separate membrane-bound organelle. We now know that in Neurospora the carbamylphosphate synthase and ornithine transcarbamylase of the arginine pathway are located in the mitochondria while there is a bifunctional carbamylphosphate synthase - aspartate transcarbamylase involved in pyrimidine biosynthesis in the cytoplasm, so this example includes features of both models.

Matchett (1974) showed that the bifunctional enzyme tryptophan synthase from Neurospora channels the intermediate indole through the two activities, which are catalysed by separate active sites. This observation appears to be consistent with the "surface" model proposed by Davis (1967), since V_{max} for site II is higher than for site I, and the affinity of the second site for indole is fairly high (K_m = 0.1 mM).

Duggleby et al. (1978) proposed a molecular model for channeling of intermediate between two sites on a bifunctional enzyme and defined the degree of channeling as the partitioning of the product of the first reaction between transfer to the second site and dissociation from the enzyme. Channeling is often measured by the release of intermediate into the bulk medium, so unless the rate of the second reaction is fast, experimentally observed channeling reflects release of intermediate from both sites. Duggleby et al. (1978) simulated progress curves for the reactions of chorismate mutase-prephenate dehydrogenase from E. coli and showed that the enzyme has separate active sites for the two activities and does not exhibit channeling of the prephenate intermediate.

Storer and Cornish-Bowden (1974) have kinetically analysed coupled enzyme systems and provide a method for calculating the lag time required for the velocity of the second reaction to approach that of the first as a function of V_{max} and K_m. The reduction or elimination of this lag time can also be used as an indication of channeling in vitro.

The artificial bifunctional enzyme β-galactosidase/galactokinase created by Bülow (1987) was found to channel galactose and this effect was increased by adding polyethylene glycol to mimic intracellular conditions. In this case channeling appears to be due solely to proximity of the two active sites, and the enhancement in the presence of polyethylene glycol to an increase in viscosity.

In several cases, channeling involves a covalently bound intermediate. For example, in the multifunctional enzyme fatty acid synthase from both mammals (α_2) and yeast ($\alpha_6\beta_6$) the lengthening fatty acyl chain is transferred between active sites for successive reactions while attached to the sulfhydryl group of the phosphopantotheine prosthetic group of the acyl carrier protein domain. Similarly, in both the pyruvate and α-ketoglutarate dehydrogenase multienzyme complexes, the activated intermediate is attached to a lipoamide prosthetic group of the transacetylase core. Both of these examples provide flexible "arms" to allow the intermediate to interact with distinct active sites while remaining bound to the enzyme or complex, without requiring major conformational changes in the proteins involved. Packman et al. (1983) have shown that a single E1 (pyruvate decarboxylase) dimer can reductively acylate all 24 E2 (lipoate acetyltransferase) chains in the

enzyme core in the E. coli pyruvate dehydrogenase complex and almost all 60 E2 chains in the enzyme core of the ox heart enzyme. This provides an extreme example of active site coupling in a multienzyme complex.

Channeling of intermediate can also be observed between transiently associating enzymes. Reddy and Pardee (1982) showed rapid and specific channeling of ribonucleotide diphosphates into DNA upon formation of the "replitase" complex during S-phase of chinese hamster cells. The transfer of metabolite via enzyme-substrate-enzyme complexes observed for glycolytic enzymes by Srivastava and Bernhard (1986) is the quintessential form of metabolic channeling, where the concentrations of the various intermediates are much lower than the number of high affinity sites available to bind them.

Interaction between active sites depends on association of the corresponding activities, and is more easily studied in multifunctional enzymes or strongly associated multienzyme complexes. Dissociation and reassociation of these complex systems can provide information about the relationship between their structure and function, including active site interactions leading to channeling of intermediates.

1.2 DENATURATION AND RENATURATION OF ENZYMES

Protein synthesis *in vivo* is a very complicated process requiring transcription of DNA to yield RNA, which is translated by ribosomes into polypeptide. Although the basic mechanism of transcription and translation have been fairly well worked out, the correspondence between the primary structure of a polypeptide (a one dimensional sequence) and the three dimensional structure of the native protein is much less well understood.

Individual polypeptides undergo various processing and folding steps to form structured monomers, which can then undergo specific association to form oligomeric enzymes or multienzyme complexes. After the correct quaternary or quinary structure is formed, further conformational changes may be required before an enzyme can express catalytic activity.

Because of the complexity of the *in vivo* process, much work on the relationship between structure and function of enzymes, and on the mechanism of protein folding and subunit-subunit interaction has been performed by studying refolding and reassociation of purified proteins *in vitro*. Many enzymes have been shown to undergo reversible denaturation/renaturation under the right conditions (Jaenicke, 1982).

In this section I will summarize some of the results obtained by such studies, particularly with respect to the principles governing protein structure formation, and the relationship between activity and quaternary structure in enzymes.

PROTEIN DENATURATION

Denaturation of a protein refers to the disruption of the native three dimensional structure. It can be induced by high temperature, extremes of pH, or the presence of organic solvents such as alcohols, organic solutes such as urea or guanidine hydrochloride, detergents, or inorganic salts, and is often reversible under the right conditions (Ghélis and Yon, 1982).

The overall difference in free energy between a folded and unfolded polypeptide is only of the order of -10 kcal/mole in aqueous solution at 20°C, and depends on enthalpy and entropy changes of both the polypeptide and the surrounding solvent. Nonpolar groups in a protein make a smaller contribution than polar groups to the enthalpy changes of the polypeptide and solvent, but a much larger contribution to the change in entropy of the solvent (Schulz and Schirmer, 1979). As discussed previously in section 1.1, much of the stability of a folded protein is due to sequestering of nonpolar groups in the interior, away from the aqueous solvent.

Privalov (1979) provides a very good thermodynamic treatment of denaturation of proteins by heat, pH and chemical denaturants (urea and guanidine hydrochloride), partly based on microcalorimetry studies from his own laboratory. For small globular proteins, the denaturation profile obtained by monitoring ultraviolet absorbance, circular dichroism, optical rotation, viscosity, stability against proteolysis or rate of hydrogen exchange at increasing temperature, pH or denaturant concentration is characterized by a large sigmoidal change, corresponding to denaturation, and much smaller pre- and post-denaturational

changes. Denaturation of small globular proteins can be approximated by a two state model.

Calorimetric studies of the influence of temperature on the state of a protein show that the heat capacity per unit of protein mass is approximately the same for all globular proteins (0.32 ± 0.02 cal $K^{-1}g^{-1}$ at 25°C). It increases slowly with temperature prior to denaturation, increases rapidly during denaturation, then continues to increase slowly with temperature. The most important contribution to the heat capacity change on unfolding is due to the interactions of non-polar groups with water and the negative heat of disruption of hydrophobic contacts accounts for at least 80% of the temperature dependence of the enthalpy of unfolding (Privalov, 1979).

The enthalpy of unfolding of a protein does not depend on the manner of unfolding, and is a linear function only of the temperature at which unfolding occurs. It is independent of the pH and denaturant concentration once their effects on the solvent are taken into account. This suggests that denaturational changes are associated with a transition between two clearly defined macroscopic states of the protein and correspond to a highly cooperative process involving many bonds. A variety of measurements suggest that the unfolded state is close to a random coil.

The entropy of unfolding of a polypeptide increases monotonically with temperature, but is not linear, and is strongly affected by disulfide crosslinks between different portions of the polypeptide chain. Privalov (1979) suggests that at high temperatures, the effects of water become negligible and the observed entropy change can be

attributed to the increase in conformational freedom of the polypeptide on unfolding. The entropy of unfolding versus temperature profiles of different proteins extrapolates at high temperature to a value of 4.2 cal K^{-1} mol^{-1} per residue which corresponds to about an eight-fold increase in possible conformations.

The stability of the native state of a protein is maximal at temperatures close to physiological or lower, where the entropy of unfolding is zero and stabilization is due to the enthalpy factor. As the temperature is decreased, the entropy of unfolding becomes negative (and therefore stabilizing), however at still lower temperatures the enthalpy also changes sign and becomes a destabilizing factor. Stability of different proteins does not differ greatly and has an average value of about (12 ± 5) kcal mol^{-1} near physiological temperatures. For small proteins, disulfide cross-links are required for additional stability.

Predenaturational changes are connected with much faster processes than the major denaturational change and probably correspond to local fluctuations of the native structure. Small fluctuations of protein structure depend on microstability which is defined by the energy required for local unfolding. This brings internal groups into rapid contact with the solvent and can be observed by studying the exchange of amide hydrogens (see Delepierre et al., 1987).

In a later review, Privalov (1982) described calorimetric studies of the thermal denaturation of multidomain proteins. In general, for

such proteins unfolding cannot be considered a two state process and the specific heat capacity function with respect to temperature can be deconvoluted to give profiles corresponding to the different domains. The domains of multidomain proteins are indistinguishable thermodynamically from small globular proteins and form definite cooperative subsystems. The upper limit to the size of these cooperative units corresponds to a maximum energy of stabilization of ~60 kJ mol^{-1} at physiological temperature. To considerably exceed the energy of thermal motion, the minimum stability of a folded region must be at least 12 kJ mol^{-1}, which can only be achieved by the cooperative interaction of at least 30 residues (in the absence of disulfide crosslinks). The domain structure of proteins thus reflects thermodynamic requirements for a stable protein structure, as well as providing flexibility and multifunctionality, and allowing the relative displacement of domains for large proteins.

Much experimental work on protein unfolding/refolding has been done using the chemical denaturants urea or guanidine hydrochloride, because at high concentration (6-8 M) they completely unfold most proteins and the transition can often be reversed by either diluting or dialysing out the denaturant.

Saito and Wada (1983 a,b) used spectroscopic and chromatographic techniques to study the guanidine hydrochloride denaturation profiles of 17 globular proteins. They found an inverse correlation between the concentration of guanidine hydrochloride at the midpoint of the denaturation transition and the molecular weight of the protein, and

also classified the proteins according to the number and apparent cooperativity of the transitions observed. They suggest that the two separate cooperative transitions observed in four of the proteins correspond to breaking of β-structure and then of the remaining α-helix, and therefore that a multiphasic denaturational profile does not necessarily correspond to a multidomain structure.

During denaturation of enzymes, loss of activity usually precedes the major unfolding transition, as shown by Yao et al. (1984) for denaturation of creatine kinase by urea. Almost complete inactivation occurs at a lower urea concentration than that required for significant conformational change. At higher urea concentrations the rate of inactivation is 1000-fold faster than unfolding of the enzyme.

Strambini and Gonelli (1986) compared the effects of urea and guanidine hydrochloride on equine liver alcohol dehydrogenase by monitoring intrinsic tryptophan fluorescence and phosphorescence. They suggest that at predenaturational concentrations of guanidine hydrochloride loss of activity corresponds to a loosening of intramolecular interactions, resulting in a conformation similar to the native state but with greater fluidity of the protein interior. In contrast, with increasing concentrations of urea, loss of activity and unfolding occur in a highly cooperative two-stage process.

REFOLDING

For many enzymes conditions can be adjusted so that the effects of denaturation are reversible, and refolding of the polypeptides and reassociation of subunits can be monitored. Theories of protein refolding have been developed mainly based on results of studies with small monomeric proteins, both for simplicity and to separate refolding from reassociation since the two are usually coupled in oligomeric proteins.

The "thermodynamic hypothesis" of protein folding was proposed by Anfinsen in 1963 based on work on the refolding of ribonuclease from 8 M urea (for a review see Anfinsen, 1973). It states that "the three dimensional structure of a native protein in its normal physiological milieu... is the one in which the Gibb's free energy of the system is lowest". The main evidence for this was the observation that thermodynamic studies of unfolding and refolding of small proteins produced highly cooperative transitions which could be explained satisfactorily by a two state model. However, as pointed out by Karplus and Weaver (1976), the time taken for a random polypeptide of 100 residues to sample all possible conformations would be $\sim 10^{50}$ yr or many times the age of the universe. This led to the proposal of pathways of folding, which started the search for kinetic intermediates and suggested that the Gibb's free energy of the folded protein might be a local rather than a global minimum.

The first experimentally determined pathway for protein folding was elucidated by Creighton (1977) for bovine pancreatic trypsin inhibitor (BPTI), a small protein of 58 residues containing three disulfide bonds. By trapping intermediates with one and two disulfide bonds, he showed

that the refolding of BPTI does not occur by a simple sequence of formation of its three disulfide bonds. Three different two disulfide intermediates are formed which rearrange to a form with two "correct" disulfides in the slowest transition in the pathway, then the third disulfide required for native structure is formed rapidly. Kosen et al. (1983) used CD spectroscopy to show that the slow transition between two disulfide intermediates corresponds to a major change in the conformation of the protein, yielding a nearly native structure. More recently, Marks et al. (1987) have shown that mutants of BPTI lacking two cysteines involved in the formation of the three two-disulfide intermediates observed by Creighton are able to refold to a state approximating the native structure (lacking one disulfide bond) at 37° or 52°C (but not at 25°C) but refolding of the mutant proteins is 3-fold slower than that of native BPTI.

Complex kinetics of refolding have also been used as evidence for kinetic intermediates on a folding pathway; however, Garel and Baldwin (1973) showed that the biphasic kinetics observed for refolding of thermally denatured ribonuclease A are due to a fast and a slow folding reaction both of which yield fully active enzyme. They proposed that there are two (or more) unfolded forms which interconvert slowly but are linked to a common folding pathway to yield native enzyme.

Brandts et al. (1975) proposed that the slow phase of refolding observed for six small proteins is due to cis-trans isomerization of proline in the unfolded polypeptide. They calculated that prolines in a random coil would spend 10-30% of the time in cis configuration, although most prolines in native proteins are known to be trans. Using

dipeptides as models, they showed that the energy of activation of proline isomerization (19.8 kcal/mole) was very similar to the energy of activation of the slow phase of refolding measured for 6 small proteins (16-20 kcal/mole). Also, a form of carp parvalbumin containing one proline has an additional slow step during unfolding and refolding not present in two forms without proline (Lin and Brandts, 1978).

Kim and Baldwin (1982) reviewed the evidence for specific intermediates in the refolding of a number of small proteins and summarized the various kinetic models proposed for protein folding. The "biased random search" model suggests that the number of possible conformations of a polypeptide is drastically reduced if only sterically possible ones are allowed, with a substantial decrease in the time required to find the native conformation. According to the "nucleation-growth" model, folding cannot begin until an initial reaction (nucleation) forms an initial element of structure, after which subsequent folding occurs rapidly. The "diffusion-collision-adhesion" (or microdomain coalescence) model proposes that short segments of unfolded chain fold independently into unstable "microdomains" which diffuse, collide with each other, coalesce and become stable. The "sequential folding" model suggests that folding occurs in a unique and definite sequence of steps, and therefore that it should be possible to detect specific well-populated intermediates.

The high rate of the elementary processes of folding and the cooperative nature of the folding/unfolding transition make it difficult to distinguish between the models proposed and for comparison with experimental results two generalized working models are used. In the

"framework" model, hydrogen bonded secondary structure is formed first and rearranged to generate the final tertiary structure, whereas in the "modular assembly" or "folding by parts" model, different regions of the polypeptide fold independently at different rates, then interact to form the overall structure. Refolding of large proteins probably combines elements of both models.

Ptitsyn and Finkelstein (1980) propose that either α-helices or β-hairpins can act as initiating structures for folding, since they form quickly enough and their energy of growth is close to zero. In contrast, Scheraga (1980) has proposed that nucleation is dominated by hydrophobic interactions e.g. formation of a hydrophobic pocket by residues 106-118 of ribonuclease during refolding.

For large multidomain proteins, it is now generally thought that folding starts independently in many regions of the polypeptide chain and elements of secondary structure then interact to form local compact tertiary structures (domains) which assemble to yield the folded polypeptide (Goldberg, 1985). In a review by Wetlaufer (1981) evidence is presented for folding to native-like structure by fragments of β-galactosidase, lysozyme, serum albumin, penicillinase and tryptophan synthase. This provides support for the "folding by parts" hypothesis and he suggests that the lower size limit to form a stable compact structure is 20-40 amino acids.

Support for the "framework" model has also been obtained since an intermediate conformational state with secondary structure similar to the native protein but fluctuating tertiary structure has been observed

during denaturation of α-lactalbumin (Dolgikh et al., 1981) and in acid-denatured cytochrome C (Ohgushi and Wada, 1983). This conformation is termed the "molten globule" state and is almost as compact as the native state but with much more mobility of side chains. Dolgikh et al. (1984) have observed accumulation of a "molten globule" state during refolding of bovine carbonic anhydrase, with native-like secondary structure and compactness but no fixed tertiary structure or enzymic activity.

It appears likely that refolding of small proteins or of individual domains proceeds via formation of a "framework" of secondary structure followed by rearrangement to form tertiary structure, and that the independent folding of domains is consistent with the "folding by parts" model for larger proteins. Conformational changes can occur during folding of domains, after association of domains and after association of subunits in oligomeric proteins. These events have been well studied during renaturation of the β_2 subunit of E. coli tryptophan synthase.

Tryptophan synthase from E. coli has an $\alpha_2\beta_2$ quaternary structure which can be dissociated to active α and β_2 subunits. The β_2 subunit can be "nicked" by chymotrypsin to yield two fragments (F_1 and F_2) which can be isolated and have been shown to refold independently and to reassociate to form "nicked" β_2 (Zetina and Goldberg, 1982). Blond and Goldberg (1985) showed that the spontaneous association of folded F_1 and F_2 fragments to yield "nicked" β_2 proceeds via association of one F_1 and

one F_2, before the rapid association to dimer. A slow isomerization then occurs in the dimer to yield "native-like" protein able to bind pyridoxal phosphate. A slow isomerization step precedes the rapid dimerization observed during renaturation of uncleaved β_2; this may correspond to a rate-limiting step that takes place during the independent refolding of the isolated fragments. They propose that the pathway of folding of uncleaved β_2 is rapid folding of domains, followed by a slow conformational change in the monomer then rapid dimerization followed by another isomerization in the dimer presumably induced by interdomain interactions. There is an additional slow isomerization within the F_1 domain which occurs independently of F_2 and of dimerization, and is required for reactivation and formation of the native structure. In a later paper, Blond and Goldberg (1987) showed that two monoclonal antibodies recognizing the two domains of the native β_2 subunit of tryptophan synthase recognized structures formed at an early stage in the refolding pathway of these subunits, suggesting that partly native epitopes are already present on early intermediates.

Protein folding <u>in vivo</u> is obviously quite different from refolding <u>in vitro</u>, and may well occur during translation as has been proposed by Bergman and Kuehl (1979). They isolated nascent immunoglobulin light chains to show that the disulfide bond between cysteines 35 and 100 in the amino-terminal domain is formed before translation of the carboxy-terminal domain is complete, suggesting that sequential folding is initiated from the NH_2-terminal end in growing nascent chains prior to release from the ribosome.

Purvis et al. (1987) have proposed that efficient folding of some proteins depends on controlled rates of translation *in vivo*. They suggest that strings of "rare" codons for which there are fewer isoaccepting tRNA's may cause pauses during translation influencing the folding pathway of the polypeptide. Such strings of rare amino acids occur between the domains containing the catalytic and allosteric sites in pyruvate kinase and between several of the functional domains in the pentafunctional *arom* enzyme. A similar string occurs in the middle of the second functional domain of anthranilate synthase II - indole-3-glycerate phosphate synthase. Their hypothesis suggests that translation "pausing" prevents abnormal interactions between unfolded regions of different protein domains, or intramolecular aggregation, and may explain why some polypeptides do not refold well *in vitro*.

REASSOCIATION OF SUBUNITS

Jaenicke (1982) has reviewed work on the refolding and renaturation of many oligomeric enzymes, and has shown that usually refolding, reassociation and reactivation occur simultaneously. As he has pointed out, "the initial function of oligomeric proteins is their own self-assembly". Refolding and reassociation must be properly coordinated because formation of the correct quaternary structure requires specific recognition between complementary surfaces on "structured monomers". In most cases cited, reactivation parallels the formation of the native quaternary structure. His discussion focuses on refolding and reassociation of enzymes *in vitro* after denaturation, since it is not yet feasible to study folding and association of nascent polypeptides. The correct coupling of refolding and reassociation is critical during

renaturation of oligomeric enzymes and "structured monomers" must be formed before correct association will occur. If the relative rates of the two processes are altered, for example by increasing the protein concentration, aggregation occurs forming inactive complexes of monomeric chains with partial secondary structure. They can often be denatured again, then will renature correctly at a higher dilution.

Dissociation of oligomeric enzymes without unfolding the subunits can sometimes be achieved by mild forms of denaturation (i.e. pH changes, high salt concentration, or low concentrations of urea or guanidine hydrochloride in the presence of protective agents like Na_2SO_4), during cold inactivation or by high hydrostatic pressure (~1,000 bar). Studies of the reassociation kinetics using chemical crosslinking or fluorescence depolarization can be used to correlate reactivation with reassociation.

For many proteins, consecutive first and second order processes are confirmed as rate-limiting steps in reassociation, yielding the following general kinetic scheme (Jaenicke, 1982)

$$nM' \longrightarrow nM \longrightarrow \frac{n}{2}\ D' \longrightarrow \frac{n}{2}\ D \longrightarrow \frac{n}{4}\ T' \longrightarrow \frac{n}{4}\ T \ldots$$

where M, D and T represent monomer, dimer and tetramer respectively and the primed forms are different conformational states.

In very few cases were structured monomers found to be active in solution, and dimers formed en route to a native tetramer are usually inactive or have very low activity. The binding of ligands increases the rate of renaturation for some enzymes, and may also protect "structured monomers" from "wrong aggregation" suggesting that at least some monomers already possess ligand binding sites.

The interactions between subunits have been shown to be highly specific by renaturing enzymes in the presence of other proteins. Cook and Koshland (1969) renatured various enzymes after acid dissociation or complete denaturation by 8 M urea and observed little interference between different proteins from the same or different species, or between homologous proteins from different species. They were unable to detect cross-hybridization using gel electrophoresis, and even enzymes added to yeast cell extract before denaturation were able to renature.

Jaenicke and coworkers have extensively studied the refolding and reassociation of the tetrameric lactate dehydrogenase (4 x 35,000) from pig muscle. Rudolf and Jaenicke (1976) monitored the reassociation and reactivation of the enzyme after acid dissociation. They showed that dissociation of the tetramer to monomers and deactivation occurred in parallel with decreasing pH and that a highly cooperative transition occurred around pH 4. Neutralization led to almost complete reactivation (83%) and removal of inactive aggregates by filtration yielded a product with the specific activity, molecular weight and spectral properties characteristic of native enzyme. The rate-limiting step in reactivation was second order, which suggests that the monomer is not active. Limited proteolysis of lactate dehydrogenase by thermolysin during reactivation arrests reassociation at the level of dimers, which appeared to be enzymatically inactive, and have been internally nicked to yield fragments of about 18,000 and 12,000. The dimer to tetramer transition is the rate-limiting step in renaturation and reactivation parallels tetramer formation (Girg et al., 1981).

Hermann et al. (1981) used fast chemical crosslinking with glutaraldehyde followed by SDS-PAGE to follow the kinetics of reassociation of LDH after acid dissociation, and separated the process into a monomer-dimer equilibrium ($K = 3 \times 10^8$ L/mol) followed by a rate-limiting association of dimers to tetramers ($k = 3 \times 10^4$ L mol^{-1} s^{-1}). Reactivation was found to parallel tetramerization.

A similar study by Zettlmeissl et al. (1982) on renaturation of LDH fully denatured by Gdn.HCl showed that formation of dimers is determined by a first order reaction ($k = 8 \times 10^{-4}$ s^{-1}) which corresponds to folding of monomers, and confirmed that the monomer to dimer association is fast and that the second rate-limiting step in reactivation corresponds to formation of tetramer. Girg et al. (1983) have shown that in the presence of the stabilizing salt $(NH_4)_2SO_4$ (1.5M) the dimeric intermediates during renaturation either from 6M Gdn.HCl or after acid dissociation possess 50% of the activity of the native tetramer. They suggest that in the absence of $(NH_4)_2SO_4$ the dimers have too much flexibility to be active.

For a few oligomeric enzymes, monomers have been shown to possess catalytic activity. The monomeric and dimeric intermediates formed during renaturation of tetrameric phosphoglycerate mutase from baker's yeast exhibit ~35% of the activity of native enzyme (Hermann et al., 1985). Borchik et al. (1985) have shown that dissociation by isopropanol of the hexameric inorganic pyrophosphatase from E. coli yields monomers which retain the full catalytic activity of the native enzyme. Anderson (1986) has shown that monomers of E. coli carbamoyl

phosphate synthetase are active *in vivo*. There is a substrate-induced equilibrium between inactive and active monomers, and a concentration-dependent association of active monomers to form active oligomers (dimers and tetramers). Although the oligomers do not have different catalytic or ligand binding properties from the active monomers, association increases the concentration of active species by mass-action effects, which is important at nonsaturating levels of substrate.

The nonidentical but homologous α and β subunits of the heterodimeric bacterial luciferase are encoded by adjacent genes (lux A and lux B). Waddle et al. (1987) expressed the two genes from *Vibrio harveyi* on separate plasmids in *E. coli*. Active luciferase was present in extracts formed from *E. coli* infected with both plasmids and fairly high levels of the corresponding subunits were expressed by cells infected by the individual genes. However, mixing extract from *E. coli* carrying the lux A gene with that carrying the lux B gene yielded very low levels of active luciferase. Denaturation of the mixed extracts by urea followed by renaturation greatly increased the formation of active luciferase. This suggests that the isolated monomers do not fold to the correct conformation required for association and that their interaction must occur before folding is complete.

Luther et al. (1986) have used sedimentation velocity centrifugation to study the self-association of rabbit muscle phosphofructokinase (PFK). They suggest that the noncovalent forces governing dimerization are different from those involved in tetramerization. Formation of dimer is characterized by negative entropy and enthalpy changes with no change in heat capacity and is increased at low ionic

strength, suggesting it involves ionic or Van der Waals forces. Formation of tetramer is characterized by positive changes in enthalpy and entropy and a large change in heat capacity, and is enhanced by higher ionic strength, suggesting the involvement of hydrophobic effects.

Based on microcalorimetric studies of the assembly of E. coli aspartate transcarbamoylase from the catalytic trimers (c_3) and regulatory dimers (r_2), McCarthy and Allewell (1983) have shown that association is characterized by negative enthalpy and heat capacity changes and positive entropy changes as well as the uptake of six protons, presumably to protonate six ionizable groups. Based on their work and from the literature on other proteins, they suggest a pattern of hydrogen bonding at allosteric interfaces (such as the c-r interface in aspartate transcarbamoylase) and hydrophobic bonding at noncooperative interfaces of multisubunit proteins.

Lane et al. (1984) have studied the binding of α subunits of E. coli tryptophan synthase to the β_2 subunit using rapid mixing techniques and monitoring fluorescence changes to measure the rate of assembly. Binding of each α subunit is followed by a slow isomerization of the αβ protomer, which greatly stabilizes the $\alpha\beta_2$ and $\alpha_2\beta_2$ complexes. Negative cooperativity is observed in the binding of the second α subunit, possibly due to steric hindrance. The kinetics of binding of L-serine to the α subunit and indolepropanol phosphate (a substrate analog) to the β subunit suggest that isomerization of both the α and β subunits in the $\alpha_2\beta_2$ complex is required for full activity. It therefore appears that association of the α and β subunits induces reciprocal conformational changes.

Even the very large multienzyme complex pyruvate dehydrogenase from Bacillus stearothermophilus has been reconstituted after denaturation by low pH or by 8 M urea (Jaenicke and Perham, 1982). Here also the rate-limiting step appeared to be first order and may correspond to reshuffling of the reassembled polypeptides, possibly involving mutually induced conformational changes.

For the bifunctional enzyme aspartokinase I - homoserine dehydrogenase I from E. coli, Garel and Dautry-Varsat (1980) have shown that several intermediates on the renaturation pathway have different kinetic and allosteric properties. Their observations suggest a sequential model for renaturation of this enzyme - a first order reaction corresponds to refolding of monomers which only have kinase activity, a second order reaction produces dimers with both kinase and threonine-inhibitable dehydrogenase activity, and a final second order process yields the native tetramer in which both activities can be inhibited by threonine.

Vaucheret et al. (1987) have shown that rapid refolding of aspartokinase-homoserine dehydrogenase occurs within seconds of transfer of completely unfolded enzyme from 6 M guanidine hydrochloride to more native conditions. The rate of reappearance of kinase activity, the index of formation of "structured monomers", is significantly slower (order of minutes) and is inversely proportional to solvent viscosity. This slow step is proposed to reflect the relative movement of already folded regions to allow association of the domains required for the kinase activity, and occurs during renaturation of both the complete

enzyme (with three domains) and a kinase-active fragment (the two NH_2-terminal domains). It appears not to correspond to cis-trans isomerization of a proline. Reappearance of dehydrogenase activity follows biphasic kinetics, reflecting folding of monomers, dimerization, then minor structural rearrangements within the dimer. This reassociation is not viscosity dependent and the second-order rate constant is much smaller than for a diffusion-limited encounter. A final association of functional dimers yields the native tetramer.

In vitro denaturation and renaturation of enzymes has yielded much information on protein refolding and reassociation of subunits, which has allowed the development of a number of models to explain these processes. It is now of great interest to determine how similar these in vitro processes are to the in vivo folding and association of newly synthesized polypeptides. In vitro studies also provide much information on the relationship between quaternary structure and function of oligomeric enzymes.

1.3 FORMIMINOTRANSFERASE - CYCLODEAMINASE

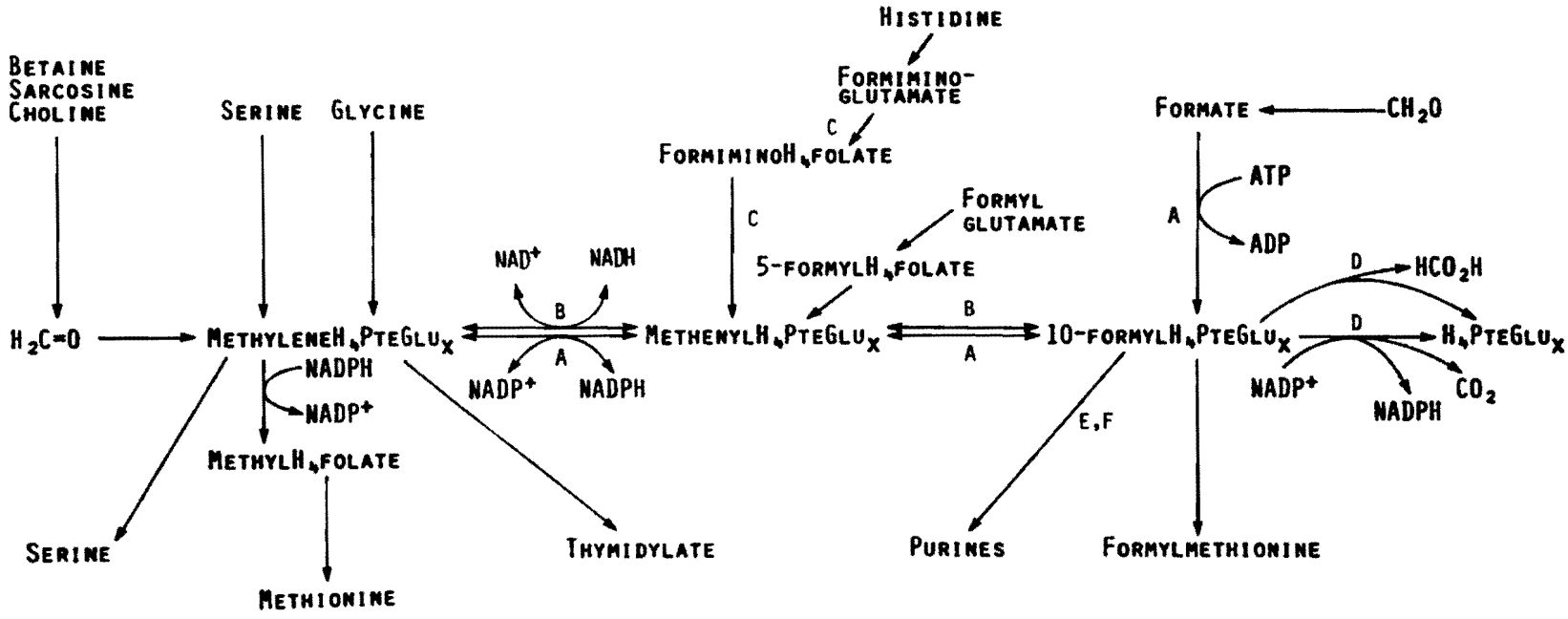
The subject of this thesis is the role of the quaternary structure of a folate-dependent bifunctional enzyme, which I have studied using chemical denaturation and renaturation. This is only one example of enzyme organization in this broad metabolic area, so I will briefly discuss the evidence for and some consequences of enzyme organization in the pathways of folate-mediated metabolism before reviewing in more detail the work to date on formiminotransferase-cyclodeaminase.

ENZYME ORGANIZATION IN FOLATE METABOLISM

Folate derivatives are involved in many biosynthetic and degradative pathways, and an outline of the various folate-dependent reactions which occur in higher vertebrates is shown in Figure 1. Many of the activities catalysing the interconversion of one carbon substituted tetrahydrofolate derivatives are properties of multifunctional enzymes, and associated activities are indicated by a single letter. The most important sources of one carbon units are serine and glycine, which enter the active one carbon pool as 5,10-CH_2-H_4folate. Serine hydroxymethyltransferase in the cytosol converts serine to glycine with addition of a methylene group to tetrahydrofolate. Methylene tetrahydrofolate is either used to synthesize thymidylate, converted to 5-methyl-H_4folate for methionine synthesis, or converted to 10-formyl-H_4folate for purine biosynthesis. In mitochondria, the glycine cleavage system and catabolism of choline and sarcosine yield methylene tetrahydrofolate which is used by serine hydroxymethyltransferase to convert glycine to serine for transport of one carbon units to the cytosol.

In eucaryotic cells the activities interconverting 5,10-methylene- and 10-formyl-H_4folate are catalysed by the NADP-dependent trifunctional enzyme 5,10-methylene-H_4folate dehydrogenase- 5,10-methenyl-H_4folate cyclohydrolase - 10-formyl-H_4folate synthetase (Enzyme A in Figure 1), which also synthesizes 10-formyl-H_4folate from formate and H_4folate in the presence of ATP. These activities have been well characterized in a number of species (reviewed by MacKenzie, 1984) and occur in prokaryotes either as separate monofunctional enzymes or as a bifunctional dehydrogenase-cyclohydrolase.

FIGURE 1: Pathways of folate-mediated one carbon metabolism in mammalian liver (adapted from MacKenzie, 1984). Multifunctional enzymes are indicated by letters, as follows: A: 5,10-methyleneH_4folate dehydrogenase - 5,10-methenylH_4folate cyclohydrolase - 10-formylH_4folate synthetase. B: NAD-dependent 5,10-methyleneH_4folate dehydrogenase - 5,10-methenylH_4folate cyclohydrolase (found in embryonic and transformed cells only). C: 5-formimino H_4folate: glutamate formiminotransferase - 5-formiminoH_4folate cyclodeaminase. D: 10-formylH_4folate dehydrogenase-10-formylH_4folate hydrolase. E,F: GAR transformylase and AICAR transformylase, each is part of a multifunctional enzyme involved in purine biosynthesis and uses 10-formylH_4folate as cofactor.


BETAINE
SARCOSINE
CHOLINE
SERINE
GLYCINE
HISTIDINE
FORMIMINO-GLUTAMATE
C
FORMIMINOH4FOLATE
FORMATE
CH2O
ATP
A
ADP
FORMYL GLUTAMATE
C
5-FORMYLH4FOLATE
D
HCO2H
NAD+
NADH
B
H2C=O
METHYLENEH4PTEGLUX
METHENYLH4PTEGLUX
10-FORMYLH4PTEGLUX
H4PTEGLUX
A
NADP+
NADPH
NADPH
NADP+
METHYLH4FOLATE
E,F
CO2
SERINE
THYMIDYLATE
PURINES
FORMYLMETHIONINE
METHIONINE

Embryonic and transformed mammalian cells also express an NAD-dependent dehydrogenase-cyclohydrolase (Mejia and MacKenzie, 1985) which is bifunctional enzyme B in figure 1. Formiminotransferase-cyclodeaminase (enzyme C) is a bifunctional enzyme found in mammalian liver and kidney which catalyses two sequential reactions in histidine catabolism to yield 5,10-methenyl-H_4folate (Tabor and Wyngarden, 1959; Drury et al., 1975).

A bifunctional enzyme catalysing non-sequential reactions (Enzyme D) breaks down 10-formyltetrahydrofolate either by NADP-dependent release of carbon dioxide or by hydrolysis to formate, both regenerating free tetrahydrofolate. Rios-Orlandi et al. (1986) have shown that the two activities occur simultaneously, apparently at separate sites.

A multifunctional protein (Enzyme E) catalyses three reactions in the pathway of de novo purine biosynthesis in chicken liver (Daubner et al., 1985) including glycinamide ribonucleotide synthetase, the folate-dependent glycinamide ribonucleotide transformylase and aminoimidazole ribonucleotide synthetase. A second folate-dependent transformylase enzyme in de novo purine synthesis, 5-amino-4-imidazole-carboxamide ribonucleotide transformylase (Enzyme F in Figure 1), is part of a bifunctional enzyme which also catalyses the inosinicase (or ring closure) reaction (Mueller and Benkovic, 1981).

Caperelli et al. (1980) observed copurification from chicken liver of glycinamide ribonucleotide transformylase with the activities of the trifunctional dehydrogenase - cyclohydrolase - synthetase and with serine transhydroxymethylase, suggesting that the enzymes involved in de novo purine biosynthesis may form an enzyme complex in vivo.

The relationship between the active sites of a multifunctional enzyme catalysing sequential folate-dependent reactions has been studied using the eucaryotic NADP-dependent dehydrogenase-cyclohydrolase-synthetase. A domain catalysing only the dehydrogenase and cyclohydrolase activities has been isolated after limited proteolysis of the porcine enzyme, and different conditions allow isolation of a synthetase-active domain (Tan and MacKenzie, 1977, 1979). The dehydrogenase-cyclohydrolase domain (M_r 33,000) comprises the aminoterminal portion of the polypeptide (M_r 100,000). Smith and MacKenzie (1983) have suggested that the dehydrogenase and cyclohydrolase activities share a common folate binding site, based on chemical modification and protection studies and on the inhibition of the cyclohydrolase by NADP, a substrate of the dehydrogenase reaction. They later used affinity labelling with tritium-labelled activated folate to show that inactivation occurred with incorporation of one mole ^{3}H-folate per mole of polypeptide chain confirming that the activities share a single folate binding site (Smith and MacKenzie, 1985).

Villar et al. (1985) have used chymotryptic digestion of the rabbit trifunctional enzyme to allow simultaneous isolation of two fragments, one containing the synthetase activity and the other the dehydrogenase and cyclohydrolase activity, confirming that the native enzyme has two independent functional domains.

Because one carbon derivatives of folate are involved in many different biosynthetic and catabolic pathways, organization of the folate-dependent enzymes may be an important means of compartmentalization, which should be manifested as preferential transfer of the product of one activity to the next active site on a pathway. This

"channeling" of intermediate has been observed in several folate-dependent enzymes.

Cohen and MacKenzie (1978) have shown preferential transfer of methenyltetrahydrofolate from the dehydrogenase to the cyclohydrolase activity in the pig liver trifunctional enzyme, corresponding to 60% channeling. A similar extent of channeling is found for the NAD-dependent bifunctional dehydrogenase-cyclohydrolase although the two activities are thought to be less closely associated in this enzyme (Rios-Orlandi and MacKenzie, 1988). Wasserman et al. (1983) have observed 85% channeling of 5,10-methenyltetrahydropteroyltriglutamate from the dehydrogenase to the cyclohydrolase activity of the chicken liver trifunctional enzyme.

Intracellular folates occur mainly as polyglutamylated derivatives with glutamates joined in peptide linkage via their γ-carboxyls, as shown in Figure 2. Cichowicz and Shane (1987) found that the hexaglutamate derivative (~55%) and pentaglutamate derivative (~22%) were the predominant products formed in vivo in hog liver from tetrahydropteroylmonoglutamate. They have purified and characterized the enzyme folylpoly-γ-glutamate synthetase from hog liver, which catalyses the MgATP-dependent addition of glutamate to tetrahydrofolates.

Many folate-dependent enzymes have much higher affinity for polyglutamate derivatives of their folate substrates and inhibitors. Baggott and Krumdieck (1979) proposed that alterations in the poly-γ-glutamate chain length may regulate pathways of one-carbon metabolism. Matthews et al. (1985) have shown that four folate-dependent enzymes from pig liver exhibit very different

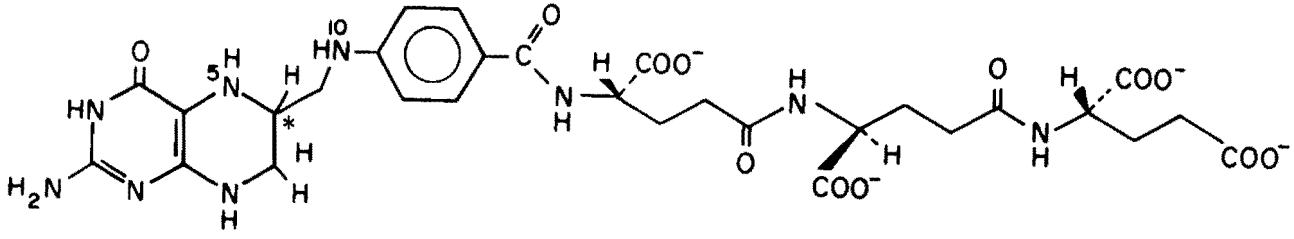


FIGURE 2: Structure of tetrahydropteroyltriglutamate. One carbon substitutions are at the N_5 and N_{10} positions and the glutamates are joined in γ-linkage.

specificity with respect to polyglutamate chain length. They propose that the number of glutamyl residues on a folate derivative may be an important determinant of flux through competing metabolic pathways.

The fundamental kinetic properties of some enzymes are altered with polyglutamate substrates. For thymidylate synthase the order of addition of substrates is different with the monoglutamate than with the pentaglutamate derivative of methylenetetrahydrofolate as substrate (Lu et al., 1984). Strong et al. (1987) have suggested a similar change in order of addition of substrates for 10-formyltetrahydrofolate synthetase from rabbit liver when tetrahydropteroylpolyglutamate is used as substrate instead of the monoglutamate form. They have also postulated a conformational change in the synthetase domain, which is part of a trifunctional enzyme, on binding tetrahydropteroylpolyglutamates.

MacKenzie and Baugh (1980) have shown that the length of polyglutamate chain greatly affects the channeling of formiminotetrahydropteroylpolyglutamate by the bifunctional enzyme formiminotransferase-cyclodeaminase. The mono- and tri-glutamate derivatives were not transfered between the activities, whereas with the pentaglutamate derivative complete channeling is obtained and the extent of channeling decreases again for the heptaglutamate. They proposed that an optimal length of polyglutamate chain is required to anchor the substrate to the enzyme while the pteroyl portion is transferred between the active sites. No increase in extent of channeling of folylpolyglutamate substrate was observed for the dehydrogenase/cyclohydrolase activities of either the NADP-dependent trifunctional enzyme (MacKenzie and Baugh, 1980) or the NAD-dependent bifunctional one (Rios-Orlandi and MacKenzie, 1988).

FOLATE-DEPENDENT HISTIDINE CATABOLISM

The role of folates in the synthesis and catabolism of histidine has been reviewed recently by Shane and Stokstad (1984). I will present here an overview of histidine degradation, focusing on the properties of the folate-dependent formiminotransferase and cyclodeaminase activities.

In both mammalian liver and microorganisms, histidine is converted to formiminoglutamic acid by three distinct enzymes. Only in liver is the catabolism of formiminoglutamate folate dependent. Formimino-transferase catalyses the transfer of the formimino group to the N_5 position of tetrahydrofolate, releasing free glutamate, and the cyclodeaminase activity cyclizes the 5-formiminotetrahydrofolate with the release of ammonia to yield 5,10-methenyltetrahydrofolate which enters the pool of activated one carbon folate derivatives.

These activities have been found in the liver and kidneys of all mammalian species tested. Formiminotransferase was not detected in insects or bacteria, but both activities have been reported in filaria. Some Clostridia possess a cyclodeaminase activity to metabolize formiminotetrahydrofolate derived from formiminoglycine arising from degradation of purines, and this activity appears to be associated with a 5,10-methenyltetrahydrofolate cyclohydrolase (Uyeda and Rabinowitz, 1967).

Tabor and Wyngarden (1959) purified three enzymes involved in the degradation of formiminoglutamic acid. Starting with an acetone powder of hog liver, formiminotransferase and cyclodeaminase copurified over 600-fold during ammonium sulfate fractionation, differential extractions

and pH precipitation, and they were only able to separate the two activities by treatment with chymotrypsin to destroy the cyclodeaminase or with NH_4OH at pH 10.5 to destroy the formiminotransferase. Ultracentrifugation of the untreated, purified enzyme gave a single peak, providing the first indication that formiminotransferase and cyclodeaminase are strongly associated in mammalian liver.

This procedure was modified by Drury et al. (1975) to purify formiminotransferase-cyclodeaminase 740-fold directly from frozen pig liver. Two polyethylene glycol precipitations were inserted before the ammonium sulfate step, and a crystallization step was added after the pH precipitation. The purified enzyme had both activities and gave a single band of M_r 62,000 on SDS-PAGE. A native molecular weight of 540,000 was determined by sedimentation equilibrium.

Beaudet and MacKenzie (1976) used isoelectric focusing and cyanogen bromide cleavage to confirm that the subunits are identical and that the enzyme consists of bifunctional polypeptides. Electron microscopy demonstrated that the porcine enzyme is composed of eight subunits arranged in a ring. Preliminary evidence suggests that the filarial enzyme may also be an octamer of identical bifunctional subunits (Jaffe et al., 1980).

FORMIMINOTRANSFERASE-CYCLODEAMINASE FROM PIG LIVER

Most of the work on this system has been performed by MacKenzie and coworkers using enzyme prepared by the method of Drury et al. (1975). This yields a homogeneous preparation of enzyme of native molecular weight 540,000 and subunit molecular weight 62,000, based on SDS-PAGE. Beaudet and MacKenzie (1976) used electron microscopy to show that both in solution and at early stages of crystallization the enzyme is a planar ring. Using rotational reinforcement of negatives of electron micrographs of single molecules of formiminotransferase-cyclodeaminase negatively stained with 1% potassium phosphotunstate, they distinguished eight subunits per circular molecule. They confirmed that the subunits were all identical based on molecular weight, isoelectric focusing and comparison of cyanogen-bromide peptides with the amino acid composition. The protein was found to contain 2.8% carbohydrate, and a second minor band observed during isoelectric focusing was proposed to be due to variations in non-protein material. The aminoterminal appeared to be blocked, and only alanine was obtained as a carboxyterminal residue.

Chymotryptic digestion of the native octamer in the presence of folic acid generates a transferase-active fragment containing polypeptides of M_r 39,000 (MacKenzie et al., 1980). Cross-linking with dithiobis(succinimidyl propionate) indicated that the active fragment is dimeric. Cross-linking of the native enzyme with difluorodinitrobenzene yielded primarily dimers and tetramers suggesting two types of subunit interaction in the circular octamer, one of which is isolated in the dimeric transferase fragment.

Formiminotransferase-cyclodeaminase catalyses the two activities shown in Figure 3, and which can be summarized as follows:

formiminoglutamate + $H_4PteGlu_n$ $\rightleftharpoons$ 5-formimino-$H_4PteGlu_n$ + glutamate

5-formimino-$H_4PteGlu_n$ $\rightleftharpoons$ 5,10-methenyl-$H_4PteGlu_n$ + NH_4^+

The kinetic mechanism of the formiminotransferase activity was worked out by Beaudet and MacKenzie (1975) and appears to be rapid equilibrium random with formation of an enzyme-tetrahydrofolate-glutamate dead end complex.

MacKenzie and Baugh (1980) demonstrated that formiminotransferase-cyclodeaminase had much higher affinity for polyglutamate than monoglutamate forms of $H_4PteGlu_n$ as substrate of the transferase activity and of $PteGlu_n$ as inhibitor of the deaminase activity, and were able to demonstrate channeling of polyglutamate substrate between the active sites. When tetrahydropteroyl mono- or triglutamate was provided as substrate there was a definite lag in the appearance of the 5,10-methenyl-$H_4PteGlu_n$ final product and production of substantial amounts of the 5-formimino-$H_4PteGlu_n$ intermediate. When the pentaglutamate substrate was supplied, no lag and no accumulation of intermediate was observed, corresponding to 100% channeling. The extent of channeling decreased significantly when the heptaglutamate substrate was used.

By mixing enzyme modified with dithionitrobenzene to inactivate only the deaminase activity (Drury and MacKenzie, 1977) with enzyme modified with diethylpyrocarbonate to inactivate the transferase, MacKenzie and Baugh (1980) showed that channeling must occur between sites on the same molecule. With tetrahydropteroylmonoglutamate the

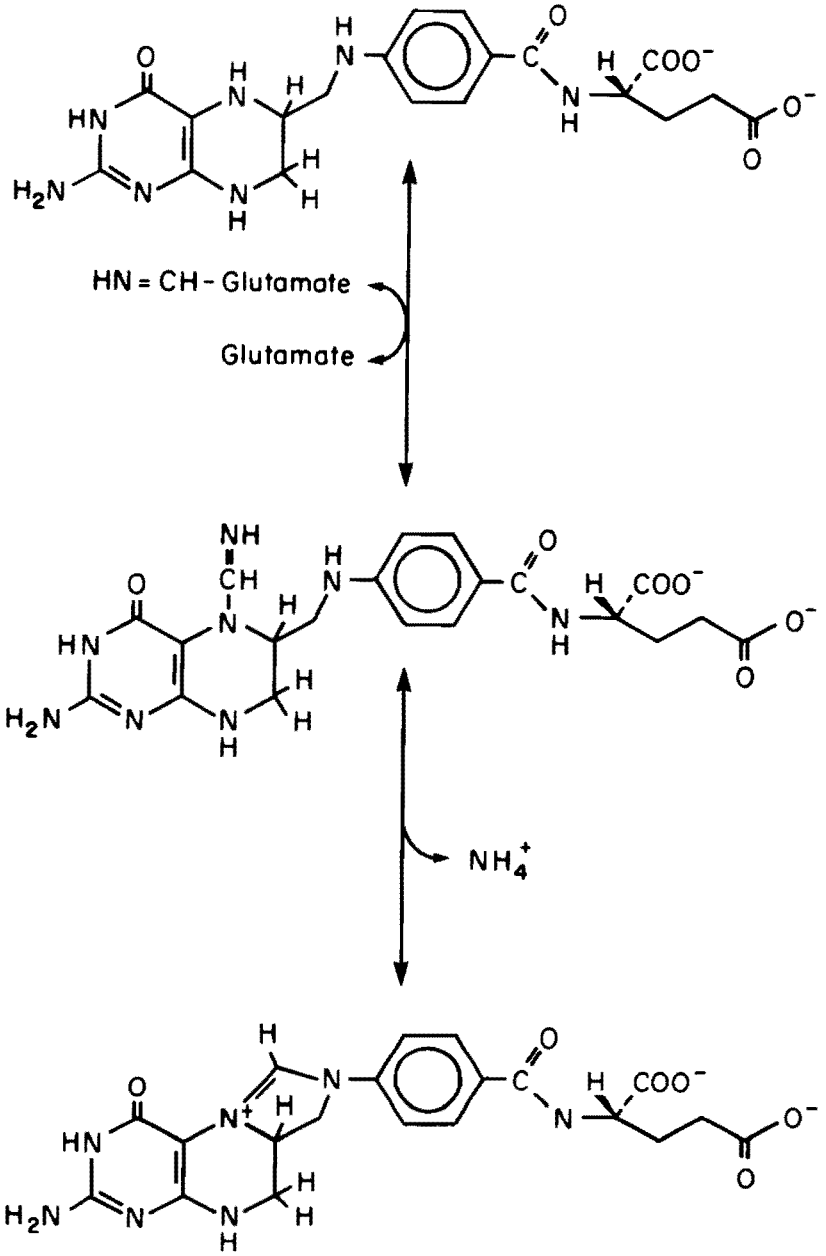


FIGURE 3: The two sequential reactions catalysed by formimino-transferase-cyclodeaminase.

same time course of appearance of products was observed with the native and the mixture of modified enzymes, but with the pentaglutamate substrate there was a definite lag in the production of methenyl-H_4PteGlu$_5$ by the mixture of modified enzymes. They proposed that the polyglutamate chain could "anchor" the intermediate to the enzyme molecule while the tetrahydropteroyl moiety is transferred between active sites, and that the pentaglutamate is the optimal chain length for this transfer.

A more complete study of the channeling of tetrahydropteroyl-polyglutamates between the active sites of formiminotransferase-cyclodeaminase was reported by Paquin et al. (1985), who used binding and kinetic studies to determine the molecular mechanism of the preferential transfer. The values of K_d obtained from Scatchard plots indicated that the enzyme has 10- to 100-fold greater affinity for tetrahydropteroylpolyglutamates with chain length from 4 to 7 than for the mono- or triglutamate substrate and tightest binding is exhibited with the hexaglutamate. They also determined kinetic parameters for the two activities. For the transferase the K_m for (6S)-H_4PteGlu$_n$ decreases 40 to 70-fold for $n \geq 4$, while V_{max} decreases only by a factor of 2. The values of K_m for (6S)-5-formimino-H_4PteGlu$_n$ also decrease 70-fold for $n \geq 4$ compared to $n = 1$, but the V_{max} decreases about 10-fold. The V_{max} for the deaminase is always at least twice that of the transferase for any value of n. The catalytic efficiency, defined as V_{max}/K_m, does not distinguish between $n = 4,5,6$ or 7, and is very similar for the two activities for these chain lengths. Complete channeling of intermediate was observed only with the pentaglutamate substrate. The extent of channeling was reduced to about 70% with

either the tetra- or hexaglutamate substrate, and to about 40% with the heptaglutamate. No channeling of the mono- or triglutamate substrate was observed. They suggest that complete transfer of intermediate between the active sites requires an optimal length of polyglutamate chain, and is not simply due to the tighter binding of polyglutamylated folate substrates. By calculating and plotting the relative free energy of binding as a function of polyglutamate chain length, the largest difference in free energy (5.7 kJ mol^{-1}) occurs on binding the fourth glutamate, which appears to be important in anchoring the substrate during transfer of the pteroyl moiety between the active sites. The distance between the α-carboxyl group of the tightly bound fourth glutamate and nitrogen-5 of the H_4PteGlu ring where the formimino group is attached was calculated to be ~20-25 Å based on measurement of a space filling model. The "anchored" substrate can thus span a distance of up to 50 Å, which is greater than the diameter of a subunit (~32 Å from electron micrographs - Beaudet and MacKenzie,1976).

There are only 4 high affinity binding sites for tetrahydropteroyl-polyglutamates on the native octamer, which appear to correspond to polyglutamate binding subsites rather than the active sites. Paquin et al. (1985) were unable to determine the number of catalytic sites directly, but used inhibition by (R)-H_4PteGlu (the inactive isomer of the substrate) to confirm that the transferase and deaminase active sites are distinct. They also showed kinetically that the two activities function independently. Using different combinations of mono- and pentaglutamate $H_4PteGlu_n$ substrate and exogenous formimino-$H_4PteGlu_n$ intermediate, they showed that only when at least

one of the substrates is the monoglutamate derivative is the enzyme able to use exogenous formimino-H_4PteGlu$_n$. This suggests that there is a single polyglutamate binding site per pair of transferase/ deaminase active sites.

STATEMENT OF THE PROBLEM

The main objective of the work presented in this thesis is to determine the relationship between the unusual quaternary structure of formiminotransferase-cyclodeaminase, the two activities, and the mechanism of channeling of tetrahydropteroylpolyglutamates between the active sites.

The results summarized in the previous section show that the native enzyme is a circular octamer of identical subunits with two types of subunit-subunit interaction, one of which can be isolated in a proteolytically-derived transferase fragment. There are only 4 high affinity sites for binding tetrahydropteroylpolyglutamates to the octamer and apparently one polyglutamate-binding site per pair of transferase/deaminase active sites. This leads to the following questions:

1) Is the dimer the basic functional unit of this enzyme, with both activities and the ability to channel polyglutamate substrates? 2) Is there evidence for site formation between subunits? and 3) What is the relationship between the two catalytic sites, and is there a separate polyglutamate binding site?

To attempt to answer these questions I have used partial denaturation of the enzyme by urea to generate active dimers whose properties could be studied, and complete renaturation of the enzyme after

unfolding by guanidine hydrochloride to correlate formation of quaternary structure (via dimers to octamer) with recovery of the two activities and channeling. I have also developed a new procedure using column chromatography to purify formiminotransferase-cyclodeaminase, which will allow isolation of the amounts of pure protein required for X-ray crystallography, the next step in determining the relationship between the structure and function of this enzyme.

CHAPTER 2

DISSOCIATION OF THE OCTAMERIC BIFUNCTIONAL ENZYME FORMIMINOTRANSFERASE-CYCLODEAMINASE IN UREA. ISOLATION OF TWO MONOFUNCTIONAL DIMERS

"At every level we find something new,
a new breathtaking vista".

A. Szent-Györgyi

ABSTRACT

Partial denaturation of the circular octameric bifunctional enzyme formiminotransferase-cyclodeaminase in increasing urea concentrations leads to sequential dissociation via dimers to inactive monomers. In potassium phosphate buffer, dissociation to dimers in 3 M urea coincides with loss of both activities and a major decrease in intensity of intrinsic tryptophan fluorescence. In the presence of folic acid, these dimers retain the deaminase activity, but with folylpolyglutamates, both activities are protected and the native octameric structure is retained. The protection profiles with polyglutamates are cooperative with a Hill coefficient greater than 2, suggesting that binding of more than one folylpolyglutamate per octamer is required to stabilize the native structure. In triethanolamine•hydrochloride buffer, transferase-active dimers which retain the intrinsic tryptophan fluorescence can be obtained in 1 M urea, and stabilized at higher urea concentration by the addition of glutamate. Deaminase-active dimers are obtained by the protection of folate in 3 M urea. Proteolysis of the two kinds of dimers by chymotrypsin leads to very different fragmentation patterns indicating that they are structurally different. We propose that the two dimers retain different subunit-subunit interfaces, one of which is required for each activity. This suggests that the native octameric structure is required for expression of both activities and therefore for "channeling" of intermediates.

INTRODUCTION

The folate-dependent bifunctional enzyme formiminoglutamate: tetrahydrofolate formiminotransferase (EC 2.1.2.5) - formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4) catalyses the following sequential reactions of the histidine degradation pathway in mammalian liver:

$$\text{formiminoglutamate} + H_4\ \text{PteGlu}_n \rightleftharpoons \text{formimino} - H_4\ \text{PteGlu}_n + \text{glutamate}$$

$$\text{formimino} - H_4\ \text{PteGlu}_n \rightleftharpoons \text{methenyl} - H_4\ \text{PteGlu}_n + NH_4^+$$

With longer polyglutamate derivatives, $H_4\ \text{PteGlu}_n$ ($n \geq 4$), direct transfer of the formimino-intermediate from the transferase to the deaminase site has been observed, with complete "channeling" of the pentaglutamate (Paquin et al., 1985). The channeling requires non-covalent attachment of the intermediate to the enzyme, possibly to a separate polyglutamate binding site which "anchors" the substrate while the pteroyl moiety is transferred from the transferase to the deaminase catalytic site (MacKenzie and Baugh, 1980). There appears to be a steric requirement for optimal channeling since the efficiency of channeling does not correlate directly with either binding affinity or kinetic efficiency (V_m/K_m) as polyglutamate chain length increases (Paquin et al., 1985).

When isolated from pig liver, the enzyme is composed of eight identical polypeptides of M_r= 62,000 arranged in a ring, as observed by electron microscopy (Beaudet and MacKenzie, 1976). Cross-linking

experiments with 1,5-difluoro- 2,4-dinitrobenzene indicated two types of subunit interaction, suggesting a tetramer of dimers structure which was confirmed by the production of a dimeric transferase-active fragment on treatment of the enzyme with chymotrypsin (MacKenzie et al., 1980). Paquin et al. (1985) have observed only four high affinity sites for binding of H_4-pteroylpolyglutamates to the native octamer, presumably one site per dimer. Kinetic experiments suggest that the catalytic sites can function independently but that there is a single polyglutamate binding site per pair of transferase/ deaminase sites. The existence of only four polyglutamate binding sites suggests that the dimer may be the basic functional unit of this enzyme and that the sites may be formed by one of the two kinds of subunit-subunit interactions. The role of the unusual quaternary structure of formiminotransferase-cyclodeaminase in its ability to channel intermediate has not been determined.

In this paper, we present results showing that partial denaturation at low urea concentrations dissociates the octameric enzyme to produce two active dimeric species. The isolation and characterization of these dimers helps to define the role of the quaternary structure in enzyme activity and provides evidence for site formation by each type of subunit-subunit association.

MATERIALS AND METHODS

The formiminotransferase-cyclodeaminase enzyme was prepared and assayed as described previously (Drury et al., 1975). Preparations routinely had a transferase specific activity of 41 μmoles $min^{-1}mg^{-1}$ and yielded a single band on SDS-PAGE. The transferase-active fragment was generated by chymotryptic cleavage of the enzyme in the presence of folate (MacKenzie et al., 1980); it had a specific activity of 29 μmoles $min^{-1}mg^{-1}$ and also yielded a single band on SDS-PAGE after purification by gel filtration.

Ultrapure urea was purchased from Canadian Scientific Products, folic acid and formimino-L-glutamic acid were from Sigma Chemical Company, and bis(sulfosuccinimidyl)suberate was from Pierce Chemical Co. Pteroylpolyglutamates were a gift from Dr. Charles Baugh, University of South Alabama. Other chemicals were reagent grade: Tween-80, triethanolamine, and EDTA from Fisher Scientific, sodium and potassium phosphate from J.T. Baker Chemical Co., 2-mercaptoethanol from Kodak and DTT from Boehringer- Mannheim. (6R,S)-H_4PteGlu was prepared chemically and purified as described previously (Drury et al., 1975).

Assays - The transferase assays were performed as reported by Drury et al. (1975), but using 0.5 ml incubation volume and measuring A_{350} on a Bausch and Lomb Spectronic 2000 spectrophotometer. Deaminase assays contained 60 μM (6S)-formimino-H_4PteGlu in 0.5 ml of 0.1 M potassium phosphate (pH 7.3) and 35 mM 2-mercaptoethanol. The appearance of (6R)-5,10-methenyl-H_4PteGlu was monitored at 355 nm (ε = 24,900) with either a Spectronic 2000 or a Beckman DU-7 spectrophotometer after addition of 10 μL (50 ng) of enzyme. The (6S)-formimino-H_4PteGlu was

prepared from 1.8 mM (6R,S)-H_4PteGlu using the transferase fragment, as described by Paquin et al. (1985). All activity measurements are the average of assays done in duplicate or triplicate.

Denaturation - Stock enzyme solution (2 mg/ml in 0.1 M potassium phosphate (pH 7.3), 40% glycerol, 35 mM 2-mercaptoethanol) was diluted 400-fold into 0.1 M potassium phosphate (pH 7.3) or 0.1 M TEA•Cl (pH 7.3) buffer containing 35 mM 2-mercaptoethanol, 0.05% Tween-80 and the required urea concentration. After 2 h at room temperature the two activities were assayed. Control experiments showed that the enzyme was stable under these conditions in the absence of urea, and time courses in the presence of urea indicated that the major decrease in activity occurs in the first two hours, followed by a slower loss thereafter. For the protection experiments, the substrate analogs and the enzyme were added to the buffer 15 min before addition of an equal volume of urea in the same buffer, then incubated 2 h at room temperature before assay. The experiments with transferase fragment were performed in the same manner except that only a 20-fold dilution was used from a stock solution containing 102 µg/ml.

Fluorescence - A Perkin-Elmer LS-5 fluorescence spectrophotometer was used with excitation at 290 nm and the emission scanned from 320-400 nm to determine the wavelength and peak height of maximum emission. Slit widths of 3 mm were used for both excitation and emission, and samples were prepared exactly as for the denaturation experiments.

Cross-linking and Electrophoresis - A 10 mg/ml solution of bis(sulfosuccinimidyl)suberate was freshly prepared in 0.05 M sodium phosphate (pH 7.4) and 30 µl aliquots added to 20 µg formimino-

transferase-cyclodeaminase or 10 µg fragment which had been incubated for 2 h in 0.5 ml of 0.1 M potassium phosphate (pH 7.3) or 0.1 M TEA•Cl (pH 7.3), 0.05% Tween - 80, 1 mM DTT, 1 mM EDTA, containing the required urea concentration and protective agents (as indicated in the Figure Legends). After 20 min, 55 µL of 20 mg/ml lysine was added and left for 10 min to react with excess cross-linker. The samples were precipitated by the method of Bensadoun and Weinstein (1976). For electrophoresis, the phosphate system of Weber and Osborn (1969) was used with samples redissolved in 50 µl dissolving buffer, loaded on 4% polyacrylamide tube gels, and electrophoresed at 6-8 mA per gel for ~ 4 h. For experiments with the transferase fragment, 5% polyacrylamide gels were used.

Limited Proteolysis - Enzyme (15 µg in 15 µL) was added to 400 µL of 0.1 M TEA•Cl (pH 7.3), 35 mM 2-mercaptoethanol, 0.05% Tween containing the required protecting agent (20 mM glutamate or 1 mM PteGlu). After 10 min, 400 µL of urea solution in the same buffer was added to give the required final urea concentration. Samples were incubated for 2 h at room temperature, then assayed for both activities. Prior to proteolysis, each sample was diluted to contain 2 M urea and both protecting agents, glutamate and PteGlu. To the final volume of 1.2 ml was added 20 µL of 40 µg/ml chymotrypsin in 1 mM HCl. The samples were left for 1 hr at room temperature, then 20 µL of 2.5 mg/ml PMSF in 30% isopropanol was added to stop proteolysis. After another 10 min at room temperature, the samples were cooled on ice and cold 100% (w/v) trichloroacetic acid was added to give a final concentration of 20% (w/v) TCA. After 15 min on ice, the samples were centrifuged at 13,000 g in a Fisher microcentrifuge for 15' and the supernatant

removed. The pellets were washed with 250 µL cold 2% (w/v) trichloroacetic acid, and the tubes centrifuged again for 15 min. The pellets were resuspended in 50 µL of sample buffer for electrophoresis using the discontinuous SDS system of Laemmli (1970) with a 12% resolving and a 3% stacking gel.

RESULTS

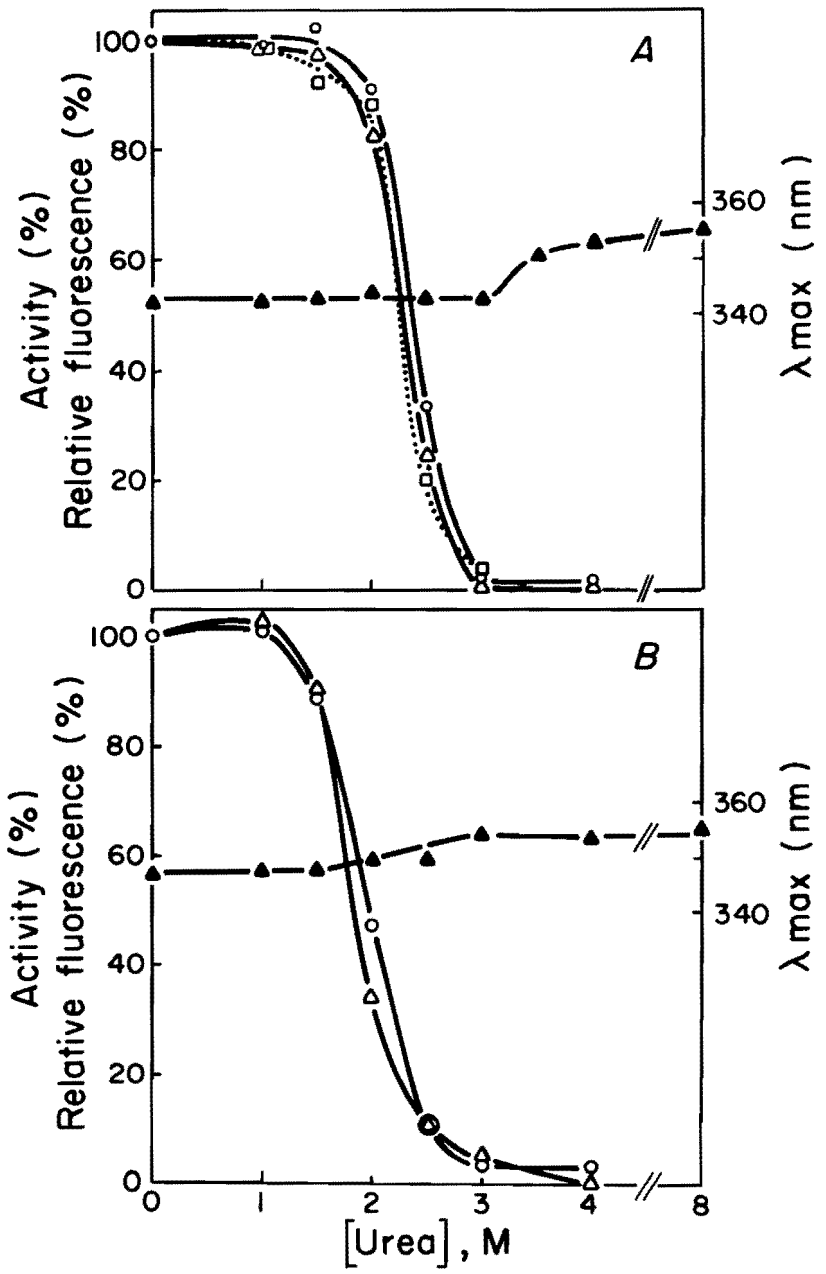
Inactivation in urea and protection by substrate analogs - In potassium phosphate buffer both activities of the bifunctional enzyme decrease simultaneously as the urea concentration is increased from 2 to 3 M (Fig. 1A). As has been found in other systems (Ghélis and Yon, 1982), the inactivation profile is highly cooperative.

In an attempt to differentiate between the two catalytic sites, addition of substrate analogs was used to protect against the effects of 3 M urea. Folic acid was found to protect only the deaminase activity (Fig. 2A) and exerted its half maximal effect at 160 µM. Although the values of K_i determined kinetically for folate against the transferase (44 µM) and the deaminase (50 µM) are similar (J. Paquin, unpublished), no protection of the transferase activity was observed with up to 500 µM folic acid. Both activities were protected by the addition of pteroylpolyglutamates. In the presence of the pentaglutamate, which is the optimal length for substrate channeling (Paquin et al., 1985), substantial fractions of both activities were retained (Fig. 2C). Half maximal protection is observed at ~ 12 µM $PteGlu_5$ and the initial portions of the two curves are similar with divergence at higher $PteGlu_5$ concentrations. Pteroyltriglutamate, whose tetrahydro-derivative is not channeled by the enzyme, also protected both activities but the two

FIGURE 1. A) Inactivation and intrinsic tryptophan fluorescence of formiminotransferase-cyclodeaminase in different urea concentrations. Final concentrations were 10 nM enzyme in 0.1 M potassium phosphate (pH 7.3), 35 mM 2-mercaptoethanol, and 0.05% Tween-80.
B) Inactivation and intrinsic tryptophan fluorescence of a proteolytically-derived dimeric transferase fragment in different urea concentrations.

Final concentrations were 65 nM dimeric fragment in 0.1 M potassium phosphate (pH 7.3), 35 mM 2-mercaptoethanol, 0.05% Tween-80 and indicated urea concentration. (o) % original transferase activity (□)% original deaminase activity, (Δ) % relative fluorescence = $(F-F_4)/(F_0-F_4)$ where F is maximum peak height, F_0 is fluorescence in the absence of urea and F_4 is fluorescence in 4 M urea, and (Δ) wavelength of maximum emission.

None of the fluorescence data were corrected for possible "inner filter effects", however the absorbance of all solutions used was much less than 0.1 at the wavelengths of excitation and emission.


A
B
Activity (%)
Relative fluorescence (%)
λmax (nm)
100
80
60
40
20
0
360
340
0
1
2
3
4
8
[Urea], M

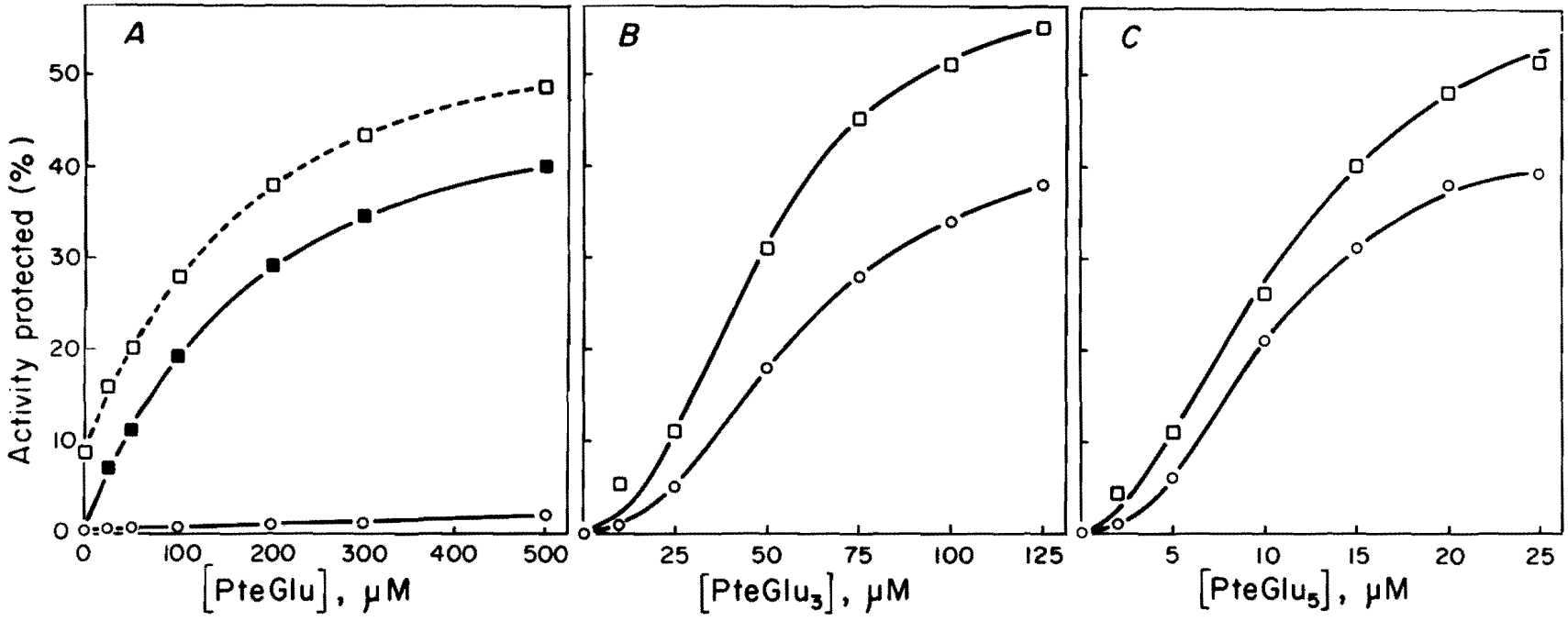


FIGURE 2. Protection of enzyme activities in 3 M urea by substrate analogs. Activities as a function of concentration of A) PteGlu, B) $PteGlu_3$, and C) Pte Glu_5, with 10 nM enzyme in 0.1 M potassium phosphate (pH 7.3), 35 mM 2-mercaptoethanol, 0.05% Tween-80 and 3 M urea. (o) transferase activity, (□) deaminase activity.

In A) (■) indicates that the residual deaminase activity in the absence of PteGlu has been subtracted from the values indicated by (□). In B) and C) only the corrected values are shown.

curves are more divergent (Fig. 2B) with less protection of the transferase activity. Half maximal protection occurred with 50-60 μM $PteGlu_3$ and the three fold decrease from the value for folic acid agrees well with the three fold decrease in dissociation constant for the triglutamate vs. monoglutamate derivative of tetrahydrofolate for this enzyme (Paquin et al., 1985).

The extent of protection as a function of the concentration of each of the polyglutamates indicates that the effect is very cooperative, and a computer fit of the data to the Hill equation using the program Kinfit (Knack and Röhm, 1981) yielded the parameters shown in Table 1. These values were used to generate the curves in Fig. 2, with the residual activities in the absence of folates (~ 10% for deaminase and ~ 3% for transferase) subtracted. The average value of the Hill coefficient was 2.2 for polyglutamates but was close to 1 for folate itself.

Changes in protein fluorescence - The intensity of the intrinsic tryptophan fluorescence was found to decrease concomitantly with the loss of activity over the same range of urea concentration (Fig. 1A), reflecting a corresponding physical change in the structure of the enzyme between 2 and 3 M urea. The coincidence between the profiles of inactivation and decrease in fluorescence intensity suggest a two-state process, possibly either subunit dissociation or a major conformational change. At higher urea concentrations (between 3 and 4 M) a red shift in λ_{max} of fluorescence emission is observed, corresponding to a second physical change in the enzyme, further exposing the tryptophan residues.

TABLE I: Parameters for Fitting Protection Profiles by Hill Equation

SUBSTRATE ANALOG	ACTIVITY	MAXIMUM PROTECTION (%)	CONCENTRATION AT HALF MAXIMUM (μM)	h
$PteGlu_5$	transferase	44 ± 2	10.4 ± 0.5	2.5 ± 0.2
	deaminase	67 ± 7	12 ± 2	1.8 ± 0.2
$PteGlu_3$	transferase	45 ± 1	60 ± 2	2.3 ± 0.1
	deaminase	62 ± 1	48 ± 1	2.3 ± 0.1
$PteGlu_1$	transferase	< 5	-	-
	deaminase	50 ± 1	154 ± 4	1.15 ± 0.02

Parameters and standard deviations were generated by computer fits of the data in Figs. 2A, B and C.

The Hill equation was used to analyse this data based on the assumption that binding of the protecting agent affected only the equilibrium between the active and inactive forms of the enzyme. If the protecting agent affects the inactivation rate constant and the system does not reach equilibrium in 2 hours, similar profiles would be observed, but the effect would not be considered "cooperative".

The transferase fragment also shows coincidence of the deactivation profile in urea and the decrease in intensity of intrinsic tryptophan fluorescence (Fig. 1B). This transition occurs at a lower urea concentration (1.5 to 2.5 M), suggesting that the dimeric fragment is less stable than the native octamer. A smaller change in λ_{max} of fluorescence emission was observed over the same range of urea concentration as the loss of activity, so the separate physical changes observed during denaturation of the enzyme occur together in the fragment.

Cross-linking - The hydrophilic bifunctional reagent bis(sulfosuccinimidyl)-suberate, which reacts primarily with lysine groups, was found to give good cross-linking of the enzyme under the experimental conditions. It resulted in production of substantial amounts of all species up to octamer as shown in Lane a of Fig. 3, and a plot of log MW (or number of subunits) vs. R_f was linear from dimer to hexamer (not shown). Two bands are observed for the octamer probably corresponding to linear and circular forms. Darker bands corresponding to dimer, tetramer, hexamer and octamer confirm the tetramer of dimers structure.

Cross-linking of the enzyme in the presence of urea indicated that the loss of activity corresponded to a change in the quaternary structure. As shown in Fig. 3, little difference in the cross-linking profile is observed in 0, 1 and 2 M urea. In 3 M urea there is a drastic reduction in the amount of species higher than dimer, and in 4 M urea a significant amount of dimer is retained, but only monomer is observed in 8 M urea. Dissociation of the octamer in 3 M urea was

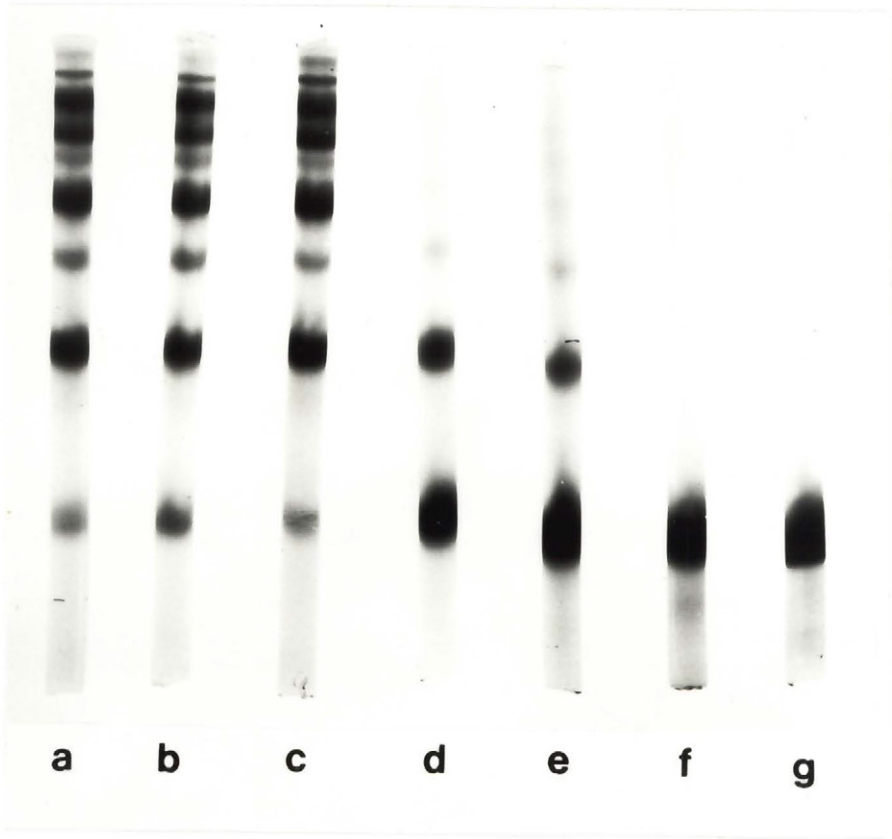


FIGURE 3. SDS-PAGE analysis (Weber and Osborn, 1969) of formiminotransferase-cyclodeaminase cross-linked in different urea concentrations. Each gel has 20 µg enzyme cross-linked with 0.55 mg/ml bis(sulfosuccinimidyl)suberate in 0.1 M potassium phosphate, 1 mM DTT, 1 mM EDTA, 0.05% Tween-80, and a) 0 M, b) 1 M, c) 2 M, d) 3 M, e) 4 M, f) 8 M urea, and g) no cross-linking.

confirmed by gel filtration on a column of LKB AcA-34 gel, in the presence and absence of 500 μM folic acid.

Bis(sulfosuccimimidyl)suberate also gives reasonably good cross-linking of the transferase fragment and, as shown in Fig. 4, loss of activity with increasing urea concentration accompanies dissociation of the dimer.

The presence of 20 μM pteroylpentaglutamate preserves both subunit-subunit interactions of the octameric enzyme in 3 M urea, as indicated by the presence of all species up to octamer after cross-linking (Fig. 5). A similar effect on quaternary structure was observed with 125 μM triglutamate (data not shown). The binding of polyglutamates thus seems to strengthen dimer-dimer interactions. However, the addition of 500 μM folic acid increased only the amount of dimer observed after cross-linking in 3 M urea, and had little effect on the amount of higher forms. It appears to protect by binding to the deaminase site and may strengthen subunit-subunit interaction within the dimer.

Production of two kinds of dimer by urea denaturation in triethanolamine hydrochloride buffer - Formiminotransferase-cyclodeaminase is less stable in TEA•Cl buffer than in potassium phosphate, and under these conditions, the deaminase is inactivated in 1 M urea, while the transferase is preferentially retained, as shown in Fig. 6. The loss of transferase activity with increasing urea concentration is less cooperative than observed in potassium phosphate, and the midpoint of the profile occurs at 1.5 M urea. The loss of intrinsic tryptophan fluorescence intensity corresponds more closely to inactivation of the

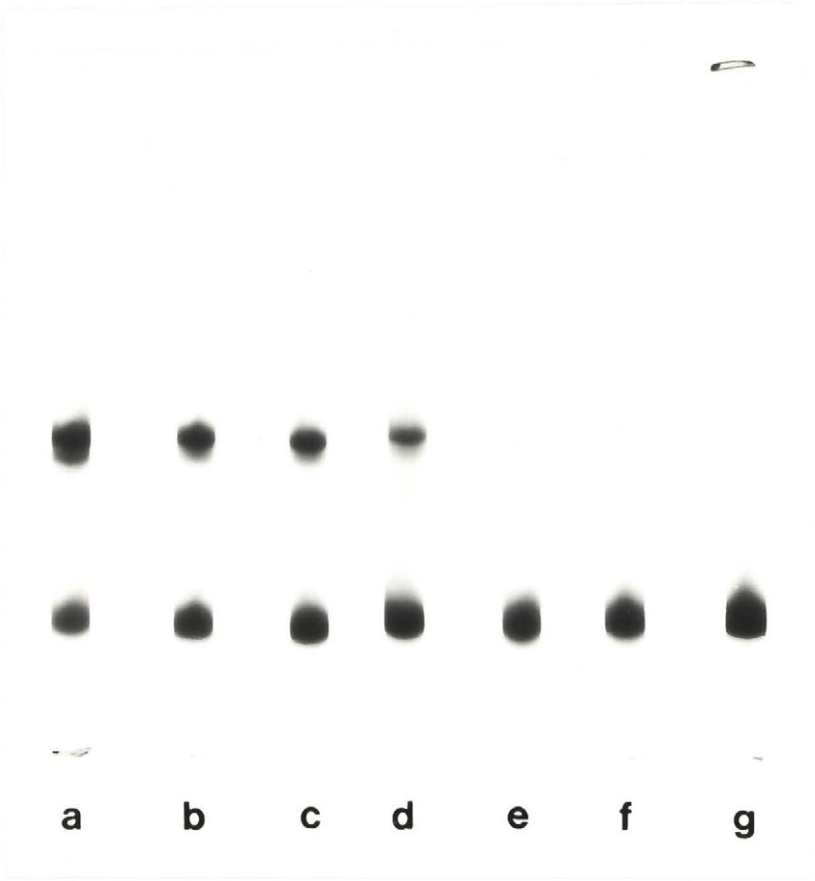


FIGURE 4. SDS-PAGE analysis of transferase fragment cross-linked in different urea concentrations. Each gel has 10 μg of fragment cross-linked with 0.55 mg/ml bis(sulfosuccinimidyl)suberate in 0.1 M potassium phosphate, 1 mM DTT, 1 mM EDTA, 0.05% Tween-80, and a) 0 M, b) 1 M, c) 1.5 M, d) 2 M, e) 3 M, and f) 4 M urea, and g) no cross-linking.

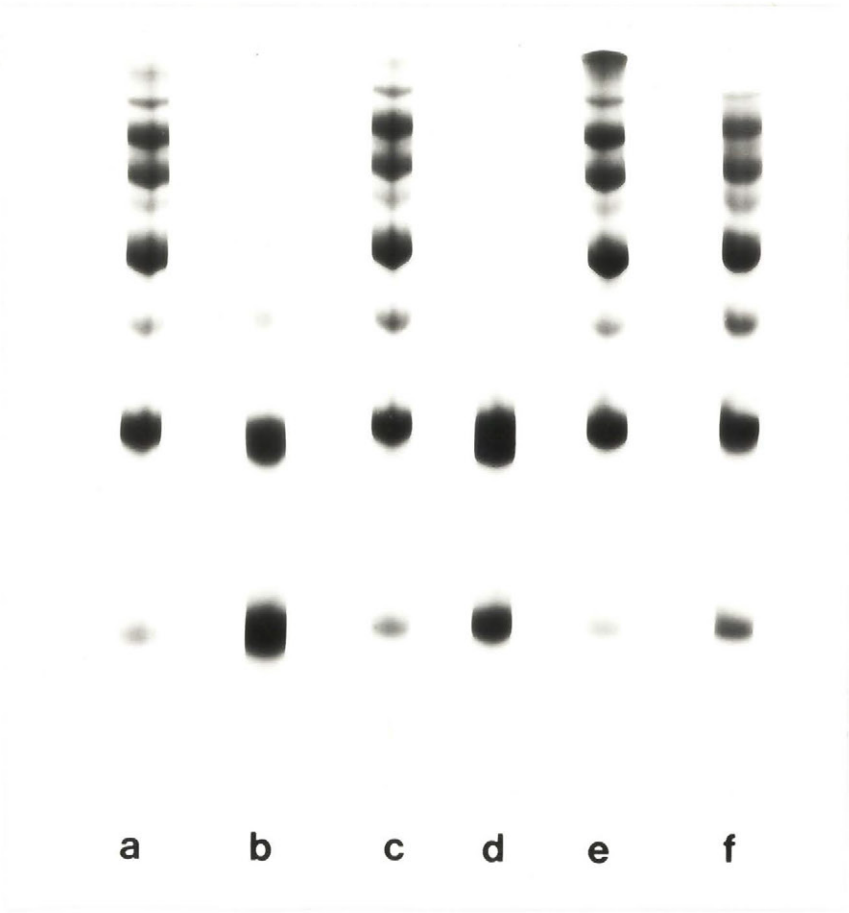


FIGURE 5. SDS-PAGE analysis of formiminotransferase-cyclodeaminase cross-linked in the presence and absence of urea and substrate analogs. Gels have 20 µg protein cross- linked by 0.55 mg/ml bis(sulfosuccinimidyl)suberate in 0.1 M potassium phosphate, 1 mM EDTA, 1 mM DTT, 0.05% Tween-80 and indicated concentration of urea and protective agent. a) no additives, b) 3 M urea, c) 500 µM PteGlu, d) 3 M urea and 500 µM PteGlu, e) 22 µM $PteGlu_5$, and f) 3 M urea and 22 µM $PteGlu_5$.

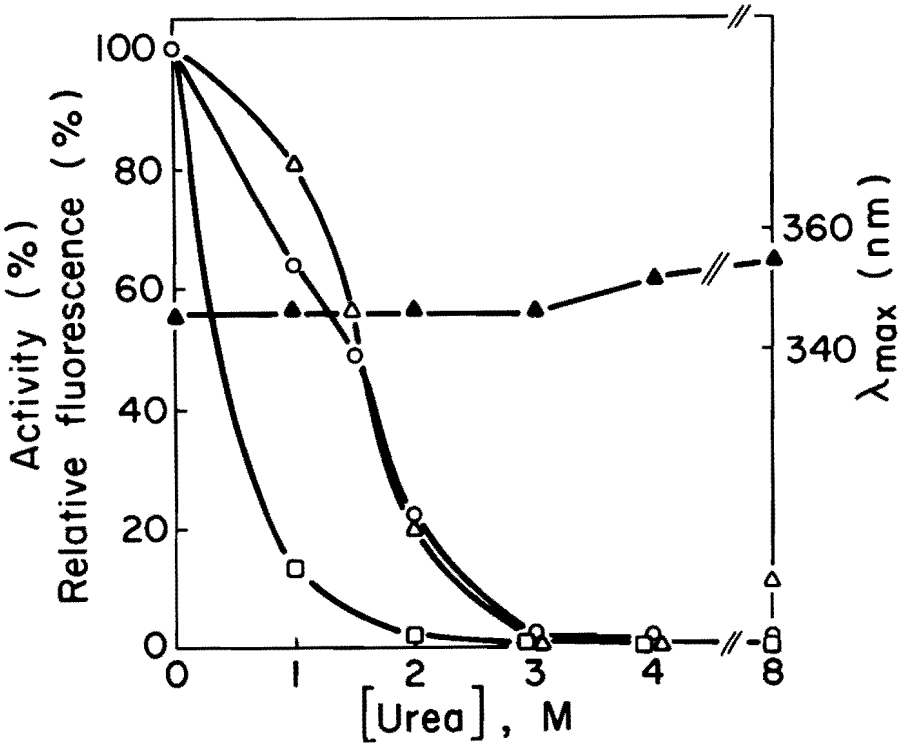


FIGURE 6. Inactivation and intrinsic tryptophan fluorescence of formiminotransferase-cyclodeaminase in TEA•Cl and increasing urea concentration. Final concentration was 10 nM octameric enzyme in 0.1 M TEA•Cl (pH 7.3), 35 mM 2-mercaptoethanol, 0.05% Tween-80 and indicated urea concentration. (o) transferase and (□) deaminase activities, (Δ) relative fluorescence intensity (see Fig. 1), (▲) wavelength of maximum emission.

transferase than the deaminase. The red shift of the wavelength of maximum fluorescence emission occurs between 3 and 4 M urea, which is the same as in potassium phosphate.

It is possible to protect the two activities separately against urea denaturation in TEA•Cl buffer. Table II shows protection by glutamate of the transferase activity and of the intrinsic tryptophan fluorescence in 2 M urea. The effect of glutamate is not cooperative and is half maximal at about 1 mM. The deaminase activity is protected by the presence of folate, even in 3 M urea, as shown in Fig. 7. The protection by folate is not cooperative ($h = 1.1 \pm 0.1$) and half maximal protection occurs at a concentration of 260 μM, which is somewhat higher than the value in potassium phosphate (154 μM). Folylpolyglutamates did not protect either activity in 3 M urea.

Cross-linking by bis(sulfosuccinimidyl)suberate in the presence of urea and TEA•Cl indicates that dissociation to dimers occurs between 0 and 1 M urea (Fig. 8A). As the urea concentration is increased, dissociation of the dimers occurs, and little dimer is left in 4 M urea. The cross-linking patterns are changed by the addition of the protecting agents, as shown in Fig. 8B. In the presence of folic acid, the band corresponding to dimer in the native enzyme is more intense and in 3 M urea a marked increase in the amount of dimer is observed. The addition of glutamate to protect the transferase activity in 2 M urea also appears to increase the amount of dimer present. In both cases, the amount of higher forms is not increased.

The production of monofunctional dimers retaining either the transferase or deaminase activity raised the possibility that different

TABLE II: Protection by Glutamate of the Transferase Activity and Intrinsic Tryptophan Fluorescence of the Enzyme in 2 M Urea

[Glutamate] mM	Transferase Activity %	Fluorescence %
0	12	47
0.5	28	56
1	35	61
2	36	69
4	41	76
8	49	83
native enzyme (+8 mM glutamate)	100	100

Addition of glutamate affected fluorescence of native enzyme by less than 5%.

No protection of deaminase activity was observed.

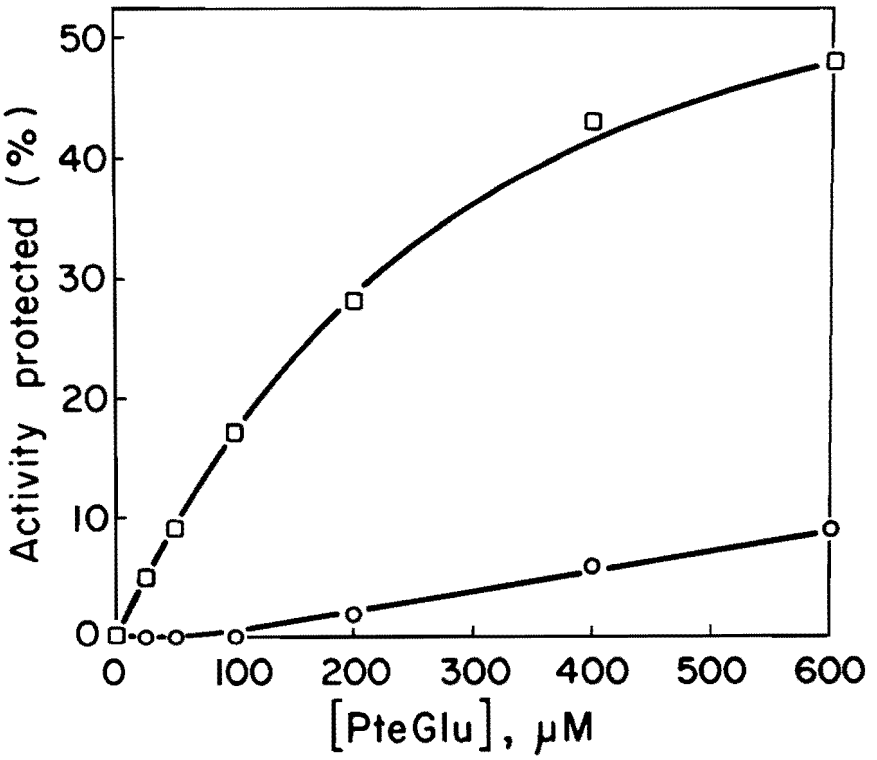


FIGURE 7. Preferential protection of deaminase activity in 3 M urea and TEA•Cl by PteGlu. Final concentration was 10 nM octameric enzyme in 0.1 M TEA•Cl (pH 7.3), 35 mM 2-mercaptoethanol, 0.05% Tween-80, 3 M urea and indicated PteGlu concentration. (□) deaminase and (o) transferase activities.

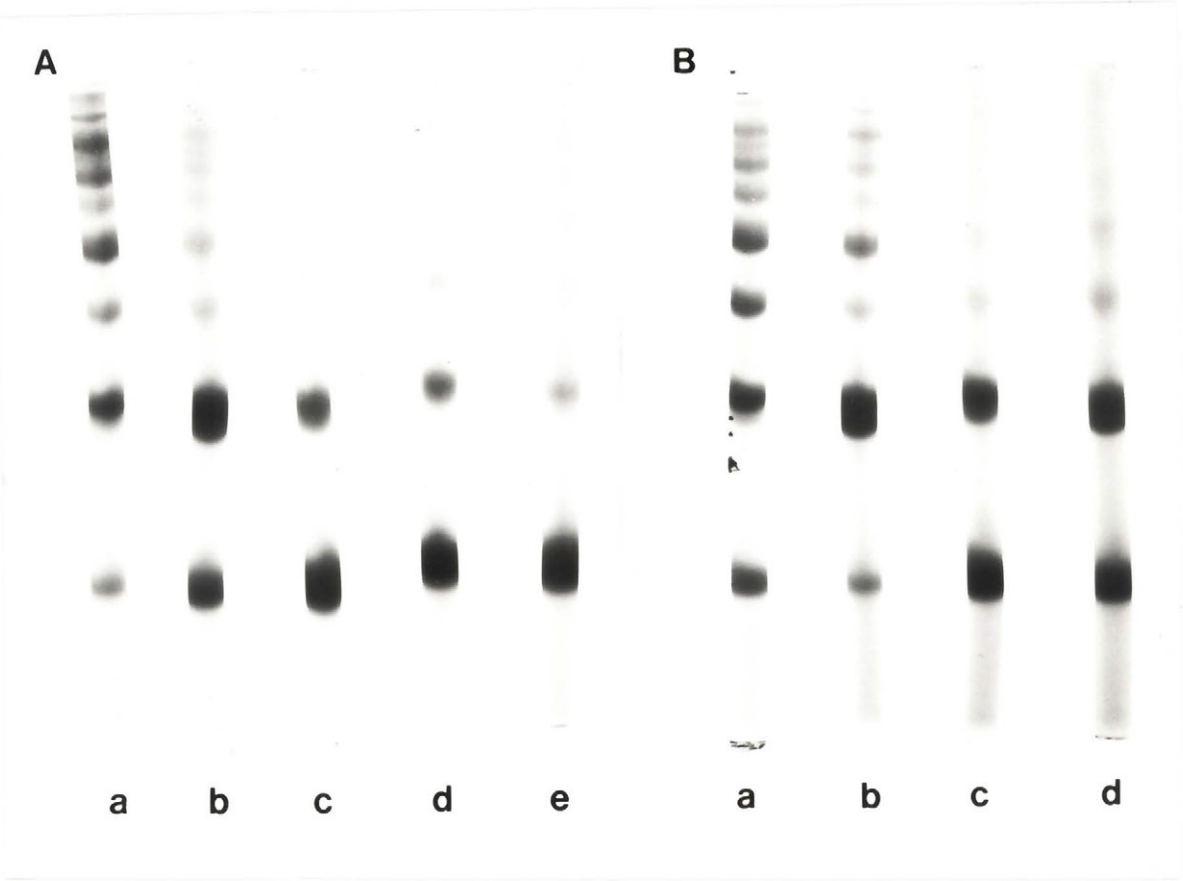


FIGURE 8. SDS-PAGE analysis of formiminotransferase-cyclodeaminase cross-linked in different urea concentrations and in the presence of substrate analogs. Each gel has 20 µg of enzyme cross-linked with 0.55 mg/ml bis(sulfosuccinimidyl)suberate in 0.1 M TEA•Cl, 1 mM DTT, 1 mM EDTA, and 0.05% Tween-80. A) Effect of increasing urea concentration, a) 0 M, b) 1 M, c) 2 M, d) 3 M, and e) 4 M urea. B) Effect of protecting agents, a) 0 M urea and 10 mM glutamate, b) 0 M urea and 500 µM PteGlu, c) 2 M urea and 10 mM glutamate, and d) 3 M urea and 500 µM PteGlu.

subunit-subunit interfaces had been isolated from the native tetramer of dimers structure. The sensitivities of the two dimers to proteolysis by chymotrypsin supported this, since quite different peptides were obtained on SDS-PAGE (Fig. 9). Digestion of the transferase-active dimer gave results similar to the native enzyme; two major bands at 39,000 M_r and 24,000 M_r. The deaminase-active dimer showed a much different peptide pattern, without the production of the 39,000 M_r species. The deaminase activity is lost during proteolysis, whereas the transferase activity is retained by both the transferase-active dimer and the native enzyme (Table III).

DISCUSSION

Most intracellular proteins occur as oligomers, with dimers and tetramers being by far the most common. If the subunits are identical, the interactions between them can be either isologous or heterologous yielding different symmetries in the quaternary structure (Ghélis and Yon, 1982). These interactions are mainly hydrophobic and serve to increase the stability of the protein, so arrangements maximizing subunit contacts are preferred. Methods of studying the folding and association of proteins have been reviewed by Jaenicke (1982) and most results show that folded monomers are inactive and that full enzymatic activity requires subunit association.

The unusual circular octameric structure of formiminotransferase-cyclodeaminase, combined with its bifunctional nature and its ability to channel non-covalently bound polyglutamate substrates between active sites, makes this enzyme a particularly suitable system for studying site-site interactions and the relationship of activity to quaternary

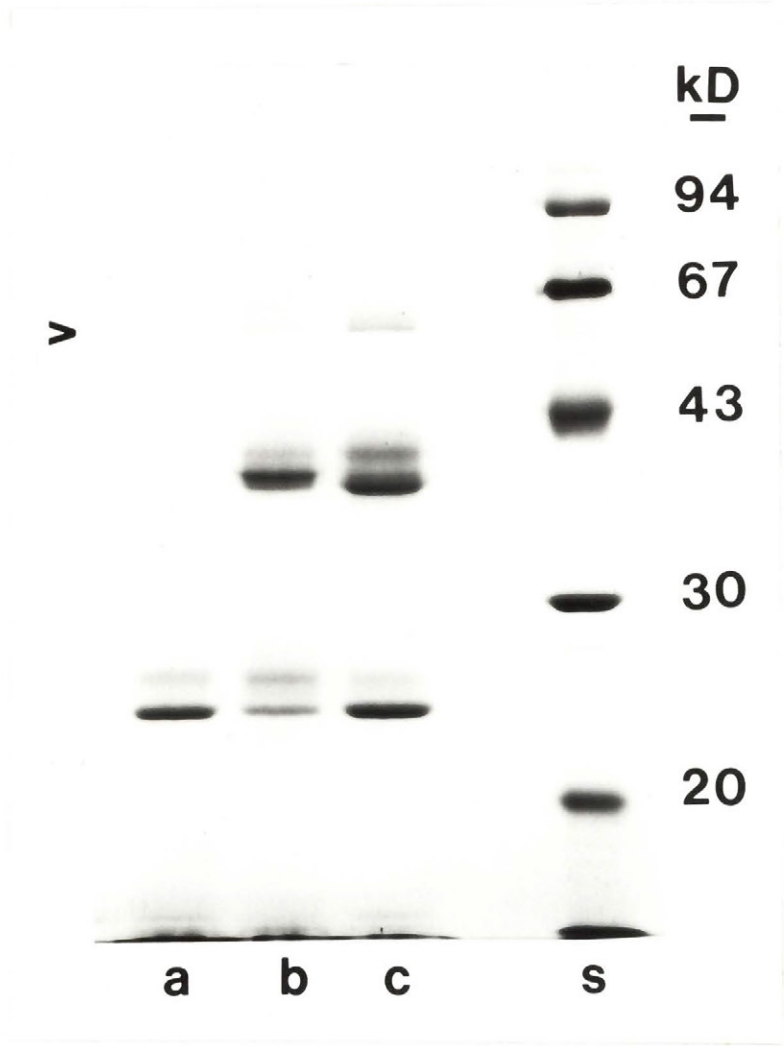


FIGURE 9. SDS-PAGE analysis (Laemmli, 1970) of products of limited proteolysis of two kinds of dimer. The first three lanes contain 15 µg of enzyme treated with 0.67 µg/ml chymotrypsin under identical conditions as described in Methods. a) deaminase dimer produced in 3 M urea + 500 µM PteGlu, b) transferase dimer produced in 2 M urea + 10 mM glutamate, and c) native enzyme incubated with 500 µM PteGlu and 10 mM glutamate. Lane 5 contains Pharmacia low molecular weight standards. The arrowhead indicates the position of uncleaved subunit.

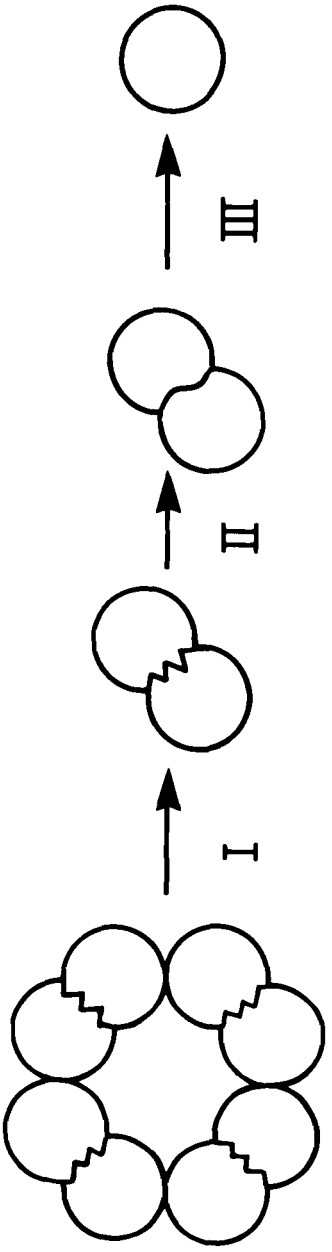
TABLE III: Activities Before and After Proteolysis of Transferase- and Deaminase-Active Dimers by Chymotrypsin

Enzyme Form	Activity Before Proteolysis (%)		Activity After Proteolysis (%)	
	Transferase	Deaminase	Transferase	Deaminase
Native Octamer	100	100	70	5
Transferase Dimer	58	4	54	< 1
Deaminase Dimer	5	62	< 1	2

structure. Binding studies and kinetic experiments suggested that the dimer may be the basic functional unit of this enzyme, with a single polyglutamate binding site per pair of transferase/ deaminase sites (Paquin et al., 1985). In the present study we have used low concentrations of urea to dissociate the bifunctional octameric enzyme. Assays for both activities, intrinsic tryptophan fluorescence, ligand induced stabilization, and cross-linking of species to determine quaternary structure were used to identify intermediates.

Dissociation by urea in phosphate buffer. Using partial denaturation by urea in potassium phosphate buffer we were able to observe three separate transitions for which we propose the scheme shown on the next page. The first transition (I) occurs as the urea concentration is increased from 2 to 3 M and is observed as a simultaneous loss of both activities (in the absence of folate) accompanied by a decrease in intensity of the intrinsic tryptophan fluorescence and dissociation of the octameric enzyme to dimers. Transition II corresponds to a physical change in these dimers between 3 and 4 M urea and is observed as a red shift in the wavelength of maximum fluorescence emission. At urea concentrations above 4 M, dissociation of the dimers to monomers constitutes the third transition (III). In the presence of folic acid, transition I generates monofunctional deaminase-active dimers.

The dimeric transferase-active fragment, which isolates one type of subunit-subunit interaction, undergoes a single transition, with the loss of activity in urea accompanied by a decrease in intensity of tryptophan fluorescence and by dissociation of the dimer to monomers. We propose that the interface isolated in the dimeric transferase



SCHEME 1

fragment is the one that is disrupted in transition I, since in both cases a substantial decrease in intensity of intrinsic tryptophan fluorescence, and complete loss of transferase activity accompany the dissociation.

The presence of substrate analogs protected the enzyme from inactivation in 3 M urea. The marked differential protection of the two activities by folate and pteroyltriglutamate confirm that the active sites are separate as previously indicated by chemical modification (Drury and MacKenzie, 1977; and MacKenzie and Baugh, 1980) and kinetic studies (Paquin et al., 1985). The protection profile for folate is not cooperative ($h \simeq 1$), suggesting that it binds directly to the deaminase catalytic site, stabilizing the portion of the polypeptide responsible for this activity and possibly strengthening subunit- subunit interaction within the dimer. Protection of the two activities by pteroylpentaglutamate may be related to the ability of the enzyme to "channel" the pentaglutamate substrate completely, if it binds to a separate polyglutamate site and the H_4 pteroyl portion can reach both active sites. This does not necessarily imply that the two active sites are close together since the distance from the N-5 position of the H_4pteroyl ring to the α-carboxyl group of the tightly binding fourth glutamate is calculated to be 20-25 Å (Paquin et al., 1985). Binding of polyglutamates appears to strengthen the dimer-dimer interactions, and the apparent cooperativity ($h>2$) may be explained if the four polyglutamate-binding sites per octamer observed by Paquin et al. (1985) are located between dimers, and if binding to two or more sites is required to stabilize the octameric structure.

Production of two types of dimer in triethanolamine•HCl. The native transferase-deaminase is stabilized or activated by both monovalent cations and polyvalent anions (Tabor and Wyngarden, 1959; Drury et al., 1975), and is clearly less stable in the absence of potassium and phosphate. Dissociation of the enzyme by urea in TEA•Cl buffer proceeds by a different sequence from that seen in potassium phosphate, with dissociation to dimers and preferential retention of the transferase activity in only 1 M urea. As the concentration of urea is increased from 1 to 3 M, dissociation to monomers, loss of transferase activity, and a decrease in the intrinsic tryptophan fluorescence of the protein all occur. The transferase dimer produced in low concentrations of urea retains the native fluorescence, which is lost upon further dissociation, and the addition of glutamate protected both the transferase activity and the intrinsic tryptophan fluorescence at higher urea concentration. As found for the studies in phosphate buffer, the deaminase activity was preferentially stabilized by folic acid and in 3 M urea dimers with only the deaminase activity were produced in the presence of folate.

The formation of the two monofunctional dimers appears to involve dissociation of different subunit-subunit interactions. This is supported by the association in all cases of fluorescence changes and transferase activity with a single interface, as well as by the very different stabilities of the two dimers to urea. Further evidence is provided by limited proteolysis of the two dimers by chymotrypsin. The domain responsible for transferase activity (M_r=39,000) is protected in both the native enzyme and the transferase dimer, but is completley

digested in the deaminase dimer, as expected if different portions of the polypeptide are protected by contact between subunits.

For several enzymes, the requirement of subunit association for activity has been shown to be due to the formation of active sites at the interfaces between subunits, with essential residues contributed by both polypeptides. Catalytic sites can be formed by either isologous (Ford et al., 1980; Theime et al., 1981) or heterologous (Krause et al., 1985; Almassy et al., 1986) interactions. The physical location of the two catalytic sites of formiminotransferase-cyclodeaminase, and their relationship to the polyglutamate binding site will only be resolved by x-ray crystallography of this enzyme.

The results presented here suggest that the transferase and deaminase activities require the integrity of alternate subunit-subunit interfaces. This indicates that dimers are not the basic functional unit of this enzyme, and that the octameric structure is required for simultaneous expression of both activities and therefore is essential for substrate channeling.

CHAPTER 3

RENATURATION OF FORMIMINOTRANSFERASE - CYCLODEAMINASE FROM GUANIDINE HYDROCHLORIDE. QUATERNARY STRUCTURE REQUIREMENTS FOR THE ACTIVITIES AND POLYGLUTAMATE SPECIFICITY.

"A process cannot be understood by stopping it.
Understanding must move with the flow of process,
must join it and flow with it".

F. Herbert

ABSTRACT

Formiminotransferase-cyclodeaminase denatured in 6 M guanidine•HCl refolds and reassembles to the native octameric structure upon dilution into buffer. Both enzymic activities are recovered to greater than 90%, and the renatured enzyme "channels" the formiminotetrahydropteroyl-pentaglutamate intermediate. Under conditions where the two activities are recovered simultaneously, the rate-limiting step in reactivation is first order with respect to protein, with $k = 1.9 \times 10^{-5}$ s^{-1} at 22°C and $\Delta E^{\ddagger} \approx 15$ kcal mol^{-1}. In the presence of 1.5 M urea, renaturation is arrested at the level of dimers having only transferase activity. Subsequent dialysis to remove the urea leads to recovery of deaminase activity and formation of octamer. Kinetic studies with mono- and pentaglutamate derivatives of the folate substrates demonstrated that native and renatured enzyme as well as deaminase-active dimers (Findlay and MacKenzie, Biochemistry 26, 1948-54) have much higher affinity for polyglutamate substrates, while the transferase-active dimers do not. These results indicate that the transferase activity is associated with one type of subunit-subunit interaction in the native tetramer of dimers, and that the polyglutamate binding site and the deaminase activity are associated with the other interface. A dimeric transferase-active fragment generated by limited proteolysis of the native enzyme can also be renatured from 6 M guanidine•HCl, confirming that it is an independently folding domain capable of reforming one type of subunit interaction.

INTRODUCTION

The bifunctional enzyme formiminotetrahydrofolate:glutamate formiminotransferase (EC 2.1.2.5) - formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4) isolated from porcine liver is composed of eight identical subunits (M_r 62,000) arranged in a ring (Beaudet and MacKenzie, 1976). This unusual octameric quaternary structure consists of two types of subunit-subunit interaction (MacKenzie et al., 1980) forming a tetramer of dimers arrangement. The enzyme catalyses two sequential reactions involved in histidine degradation in mammalian liver and has much greater affinity for the naturally occurring polyglutamate derivatives of folates as substrates.

$$\text{formiminoglutamate} + H_4\text{PteGlu}_n \rightleftharpoons \text{formimino-}H_4\text{PteGlu}_n + \text{glutamate}$$

$$\text{formimino-}H_4\text{PteGlu}_n \rightleftharpoons \text{5,10-methenyl-}H_4\text{PteGlu}_n + NH_4^+$$

With the longer polyglutamates (n>4), direct transfer of the formimino-H_4PteGlu$_n$ from the transferase to the deaminase catalytic site is observed, with complete "channeling" of the pentaglutamate (Paquin et al., 1985). A model has been proposed, with the intermediate "anchored" to the enzyme via noncovalent attachment to a polyglutamate binding site while the pteroyl moiety is transferred between the catalytic sites (MacKenzie and Baugh, 1980).

Only four high affinity sites for binding tetrahydropteroylpoly-glutamates were observed per octamer (Paquin et al., 1985) and kinetic

evidence suggested a single polyglutamate site per pair of transferase/deaminase catalytic sites. These results raised the possibility that the basic functional unit of the enzyme is a dimer, which would have both activities and the ability to channel polyglutamate substrate between the active sites. However, dissociation of formiminotransferase-cyclodeaminase by low concentrations of urea under different conditions generated monofunctional dimers with either transferase or deaminase activity, and led to the suggestion that these dimers isolate different subunit-subunit interactions (Findlay and MacKenzie, 1987). This would mean that the dimer could not be the basic functional unit of this enzyme, and that the activities require the integrity of alternate subunit interfaces in the native octamer.

In this paper we report conditions under which formiminotransferase-cyclodeaminase can be renatured from guanidine•HCl. Isolation and characterization of dimeric intermediates further defined the relationship between the enzymic activities and quaternary structure, and established the location of the polyglutamate binding site.

MATERIALS AND METHODS

The formiminotransferase-cyclodeaminase enzyme was prepared and assayed as described previously (Drury et al., 1975), and stored at -20°C as a stock solution of 2.0-2.5 mg/ml in 0.1 M potassium phosphate (pH 7.3), 40% glycerol, and 35 mM 2-mercaptoethanol. The purified enzyme had a transferase specific activity of 41 μmol $min^{-1}mg^{-1}$ and yielded a single band on SDS-PAGE. The transferase- active fragment was generated by chymotryptic cleavage of the enzyme in the presence of folate and purified by gel filtration (MacKenzie et al., 1980). It was then concentrated 6-fold by dialysis in Spectra/Por 3 membrane tubing (Fisher Scientific) against 40% polyethylene glycol 8000 (J.T. Baker Chemical Company), 0.1 M potassium phosphate (pH 7.3), 35 mM 2-mercaptoethanol. After a second dialysis into 40% glycerol, 0.1 M potassium phosphate (pH 7.3), 35 mM 2-mercaptoethanol, it was stored at -20°C as a stock solution of 0.75 mg/ml, with a specific activity of 31 μmol $min^{-1}mg^{-1}$. It also gave a single band on SDS-PAGE.

Ultra pure guanidine hydrochloride and urea were purchased from Canadian Scientific Products, folic acid and formiminoglutamate (hemibarium salt) were from Sigma Chemical Co., and bis(sulfosuccinimidyl)suberate and high purity Triton X-100 were from Pierce Chemical Co. Pteroylpentaglutamate was purchased from Dr. B. Schircks Laboratories (Switzerland). (6 R,S)-H_4PteGlu was prepared chemically (Drury et al., 1975) and (6S)-H_4PteGlu and (6S)- $H_4PteGlu_5$ were prepared enzymatically (MacKenzie and Baugh, 1980). Other chemicals were reagent grade: Tween 80 and EDTA from Fisher Scientific, sodium and potassium phosphates from J.T. Baker Chemical Co.,

2-mercaptoethanol from Eastman Kodak Company and DTT from Boehringer-Mannheim.

Renaturation - Assays for the two enzyme activities, and chemical cross-linking by bis(sulfosuccinimidyl)suberate were performed as previously described (Findlay and MacKenzie, 1987). The enzyme was denatured by a four-fold dilution from the stock solution of 2.0-2.5 mg/ml into 8 M guanidine•HCl (final concentration 6 M), in 0.1 M potassium phosphate (pH 7.3), 1 mM DTT, 1 mM EDTA. After 30 min incubation at room temperature, renaturation was accomplished by dilution of at least one hundred fold into renaturation buffer containing 0.1 M potassium phosphate (pH 7.3), 40% glycerol, 1 mM DTT, 1 mM EDTA and 0.1% Triton X-100 or 0.05% Tween 80, and the recovery of the two activities followed with time. All renaturations were carried out at a final concentration of 0.06 M Gdn•HCl. Controls showed that native enzyme under the same conditions retained almost 100% activity in the presence of Tween 80 and over 90% activity in the presence of Triton X-100 for several days at room temperature. For renaturation of the transferase-active fragment the same procedure was used starting with a stock solution of 0.75 mg/ml. To reduce sample volumes in the cross-linking experiments, only a 10-fold dilution into buffer was used, followed by dialysis against the same buffer.

The program KINFIT (Knack and Röhm, 1981) was used to determine initial velocities of reactivation under various conditions by performing either an initial velocity or a first order fit of the data for the activity versus time profiles.

Fluorescence - A Perkin-Elmer LS-5 fluorescence spectrophotometer was used with excitation at 290 nm and emission scanned from 310-400 nm. Slit widths were 3 mm for excitation and 5 mm for emission, and samples were prepared exactly as in the renaturation experiments. Duplicate scans were averaged and the background subtracted.

Gel Filtration - A 48 ml column (1.6 x 24 cm) of AcA-34 (Pharmacia) was equilibrated with 3-4 column volumes of 0.1 M potassium phosphate (pH 7.3), 40% glycerol, 1 mM EDTA, 5 mM DTT, and 0.1% Triton X-100, containing 1.5 M urea when required. Samples of 0.5 ml of native enzyme in renaturation buffer containing 1.5 M urea, enzyme renatured in the presence of 1.5 M urea, and enzyme renatured in the absence of urea were applied separately, eluted at room temperature with the same buffer as used for equilibration and 30 drop (0.47 ml) fractions were collected and assayed for transferase activity. Native enzyme and transferase fragment diluted in renaturation buffer were used as markers.

Kinetic Experiments - Transferase activity was assayed in 0.5 ml of 0.1 M potassium phosphate (pH 7.3), 5 mM formiminoglutamate, 35 mM 2-mercaptoethanol and various concentrations of (6S)-H_4PteGlu$_n$ (n=1 or 5), at 30°C. The reaction was initiated by addition of enzyme and stopped after 3 min with 25 µL of 5 N HCl, then heated in boiling water for 1 min and cooled on ice. For blanks the acid was added with the enzyme. A Beckman DU-7 spectrophotometer was used to measure the absorbance at 350 nm due to (6R)-5,10-methenyl-H_4PteGlu$_n$.

Deaminase activity was assayed in 0.5 ml of 0.1 M potassium phosphate (pH 7.3), 35 mM 2-mercaptoethanol, and various concentrations of (6S)-5-formimino-H_4PteGlu$_n$ (n=1 or 5), prepared as reported

previously (Paquin et al., 1985). After addition of enzyme, the absorbance at 355 nm was monitored using the Beckman DU-7 spectrophotometer to observe the production of methenyl-$H_4PteGlu_n$ at 23°C. For blanks, the rate was measured in the absence of enzyme.

After subtraction of the blanks, the data for both activities were fit to the Michaelis-Menten equation to determine values of K_m, using the computer program KINFIT (Knack and Röhm, 1981) with an unweighted fit.

"Channeling" was monitored by following the time course of appearance of the products of the two activities at 30°C for up to 2 min as reported previously (Paquin et al., 1985), using 0.5 ml of assay mix containing 0.1 M potassium phosphate (pH 7.3), 35 mM 2-mercaptoethanol, 5 mM formiminoglutamate and 25 μM (6S)-$H_4PteGlu_5$.

RESULTS

Preliminary experiments to establish optimal conditions for renaturation of formiminotransferase-cyclodeaminase indicated that both 40% glycerol and a low concentration (≤0.1% w/v) of non-ionic detergent (Tween 80 or Triton X-100) are required for complete renaturation of this enzyme. In the absence of detergent, less than 40% of the activities could be recovered.

Figure 1A shows the time course of recovery of the transferase activity after a hundred-fold dilution into renaturation buffer (0.1 M potassium phosphate (pH 7.3), 40% glycerol, 1 mM DTT and 1 mM EDTA) containing 0.05% Tween-80. The renatured enzyme has over 90% of both activities after 48 h, and under these conditions the two activities are recovered simultaneously as shown in the inset in Figure 1A. Figure 1B

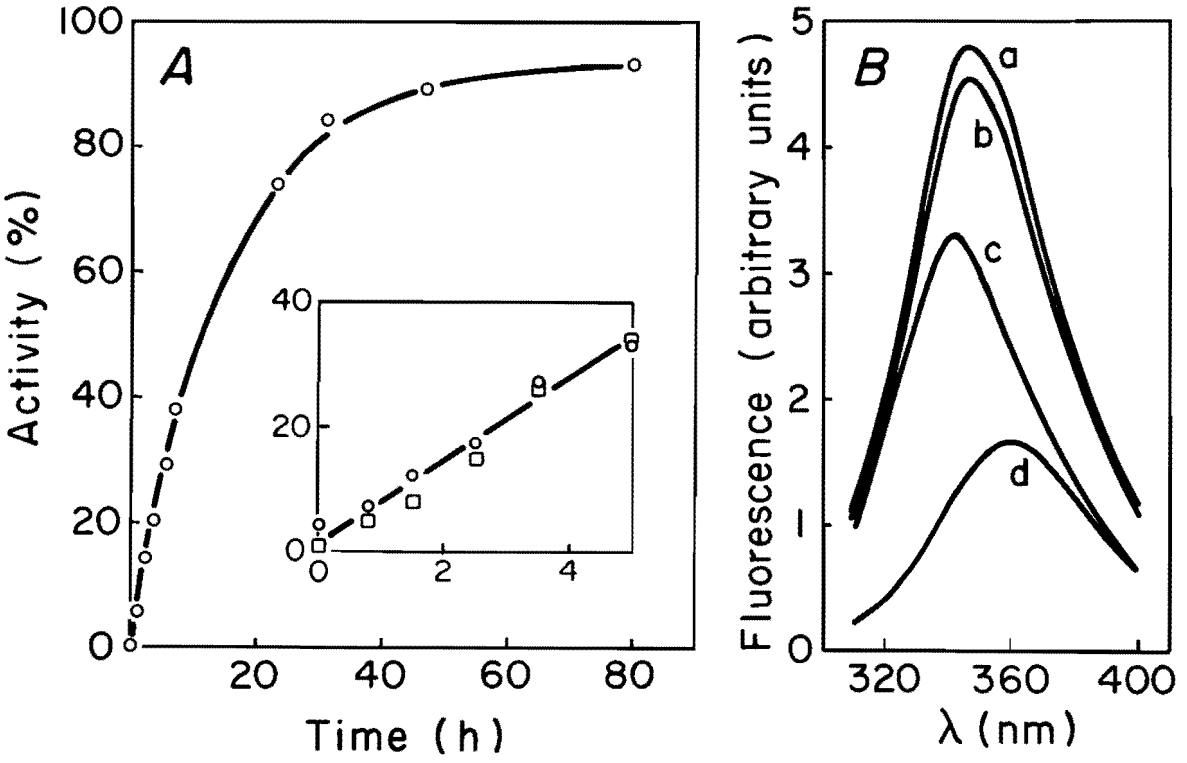


FIGURE 1. Renaturation of formiminotransferase - cyclodeaminase after dilution from 6 M Gdn•HCl. Final protein concentration was 76 nM subunit (4.7 µg/ml) in renaturation buffer (0.1 M potassium phosphate, 40% glycerol, 1 mM DTT and 1 mM EDTA) containing 0.05% Tween 80 and 0.06 M Gdn•HCl. (A) Recovery of activity with time after dilution; expanded scale for the first five hours is shown in the inset. Symbols: (o) % of original transferase activity and (□) % of original deaminase activity. (B) Spectrum of intrinsic tryptophan fluorescence of a) native enzyme, b) enzyme renatured for 48 h, c) enzyme renatured for 5 min, and d) enzyme in 6 M Gdn•HCl.

shows that renatured enzyme has ~ 95% of the intrinsic tryptophan fluorescence of native enzyme, and that denatured enzyme recovers ~ 70% of the native fluorescence in the first 5 min after dilution. The fluorescence intensity of the enzyme in 6 M Gdn•HCl is much lower and the wavelength of maximum emission is shifted from 345 nm to 360 nm, consistent with unfolding of the polypeptide chain.

The rate limiting step for reactivation under these conditions is a first order process. The initial rate of reactivation of both the transferase and deaminase activities varies linearly with enzyme concentration as shown in Figure 2A, and the rate constant for reactivation is $(1.9\pm0.2)\times10^{-5}$ s^{-1} at 22°C. Figure 2B is an Arrhenius plot of the initial rate of transferase reactivation at temperatures from 15° to 30°C. Since it is linear, a single step appears to be rate-limiting over this range of temperatures and the energy of activation is ~ 15 kcal/mol.

When Tween 80 is replaced by Triton X-100 in the renaturation buffer, the transferase and deaminase activities are recovered at different rates, as shown in Figure 3A. The transferase is reactivated somewhat more quickly than it is in the presence of Tween 80, but the deaminase is reactivated significantly more slowly. After 48 hours, over 80% of both activities has been recovered (90% of control). Since we observe two different initial rates, there appear to be two rate-limiting steps during renaturation under these conditions, and reactivation of each activity is apparently first order (not shown).

The addition of 1.5 M urea to the renaturation buffer completely prevents recovery of the deaminase activity, while further increasing

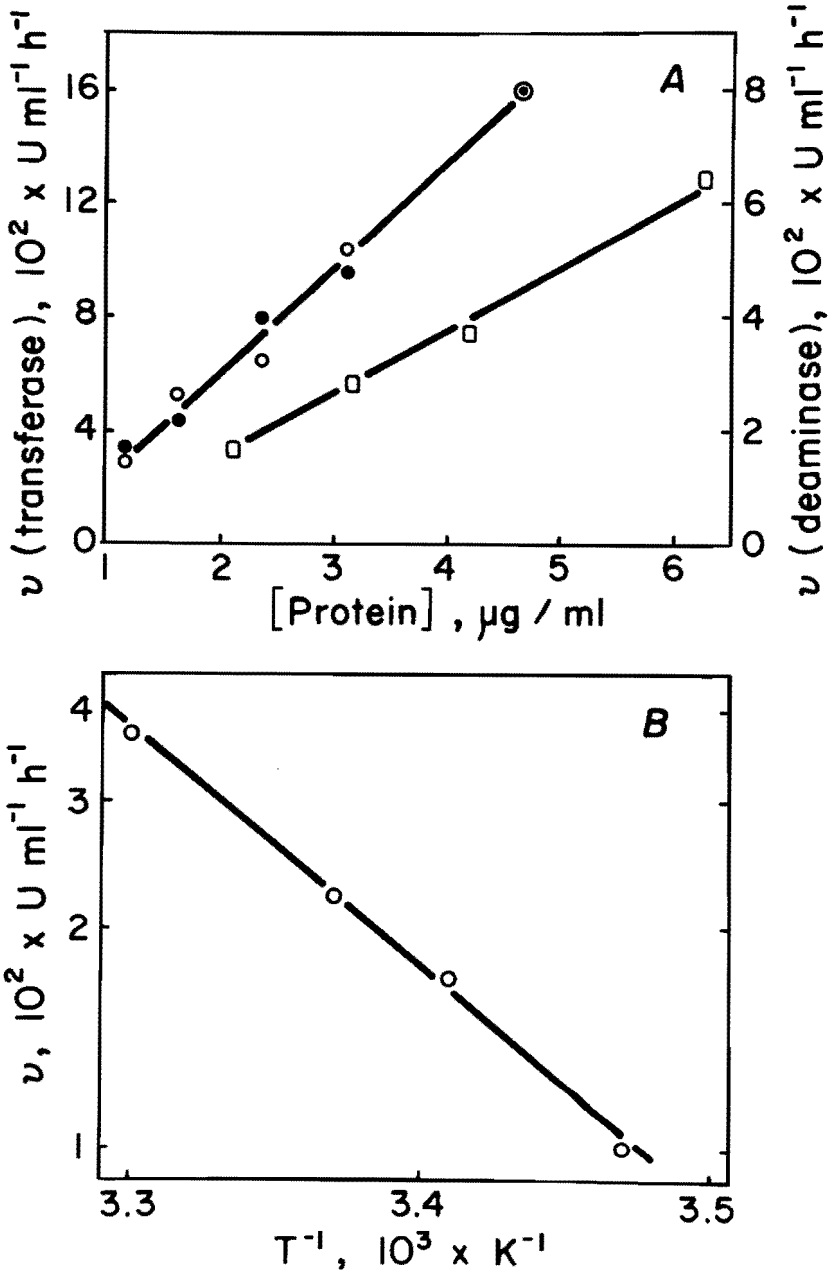


FIGURE 2. The effects of enzyme concentration and temperature on initial rates of reactivation (v) after dilution from 6 M Gdn•HCl into renaturation buffer containing 0.05% Tween 80. (A) Initial velocity values at different protein concentrations obtained by initial velocity fit of the transferase (o) and deaminase (□) reactivation profiles and by first order fits of the transferase (●) profiles using the program KINFIT (Knack and Röhm, 1981). (B) Arrhenius plot of transferase reactivation at a protein concentration of 4.7 µg/ml. Initial velocity values were obtained as in (A).

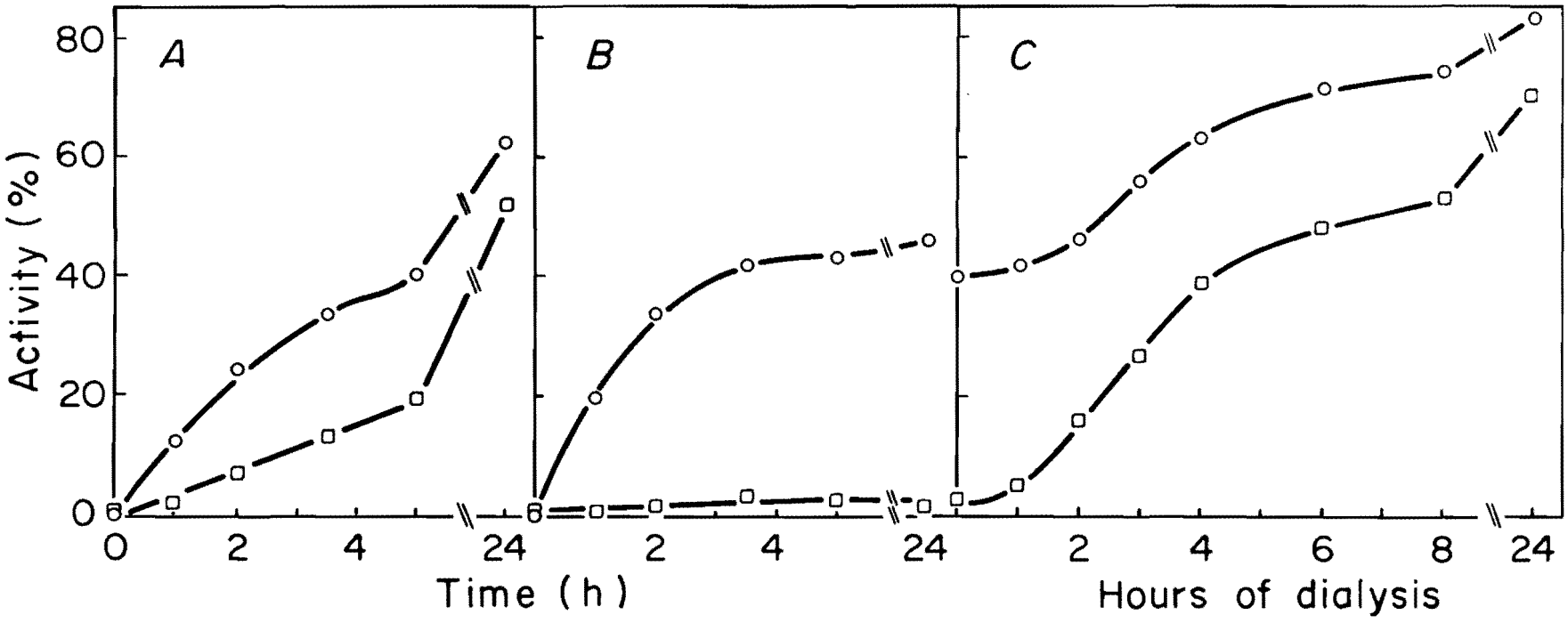


FIGURE 3. Effect of urea on reactivation of formiminotransferase - cyclodeaminase after dilution from 6 M Gdn•HCl into buffer containing Triton X-100. Final enzyme concentration was 100 nM subunit (6.25 µg/ml) in renaturation buffer containing 0.1% Triton X-100 and (A) no urea, (B) 1.5 M urea, and (C) during dialysis after 20 h renaturation in 1.5 M urea. Symbols: (o) % original transferase activity and (□) % original deaminase activity.

the initial rate of reactivation of the transferase (Figure 3B). However, the final extent of reactivation is less than 50% of the initial transferase activity, even after 24 hours. Dialysis against renaturation buffer to remove the urea leads to reactivation of the deaminase as well as an increase in transferase activity, as shown in Figure 3C. After 48 h of dialysis, over 85% of both activities is recovered (not shown).

As reported previously (MacKenzie et al., 1980), limited proteolysis of the enzyme by chymotrypsin in the presence of folic acid generates a dimeric transferase-active fragment (2 x 39 kDa). This fragment can also be completely renatured after denaturation in 6 M guanidine•HCl, under the same conditions as the native enzyme. Figure 4 shows recovery of the transferase activity with time for the dimeric fragment, and the inset illustrates the initial rate of reactivation at different protein concentrations. The rate-limiting step in renaturation is apparently first order for the fragment also, and has a rate constant $k=(2.4\pm0.3)\times10^{-4}$ s^{-1}, which is about 5-fold faster than the value obtained for the native enzyme (Figure 3A). It is also slightly faster (<2-fold) than that for reactivation of the transferase alone in 1.5 M urea (Figure 3B).

Cross-linking of the enzyme with the bifunctional reagent bis(sulfosuccinimidyl)suberate under the various conditions shows that octameric structure is regained after 48 h of renaturation in the presence of either Tween 80 or Triton X-100, and after 24 h renaturation in the presence of 1.5 M urea, followed by removal of the urea by dialysis for 48 h (Figure 5A). Cross-linking of the renatured

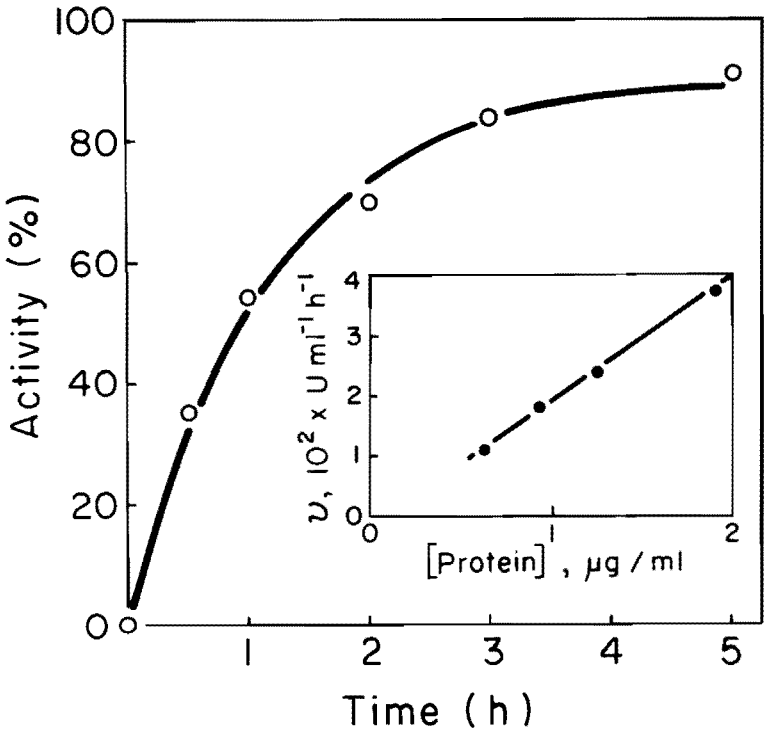


FIGURE 4. Reactivation of the proteolytically - derived transferase fragment after dilution from 6 M Gdn•HCl. Final protein concentration was 1.88 µg/ml fragment (48 nM monomer) in renaturation buffer containing 0.1% Triton X-100. Inset shows initial velocity values obtained by computer fit of reactivation profiles at different protein concentrations using the program KINFIT (Knack and Röhm, 1981).

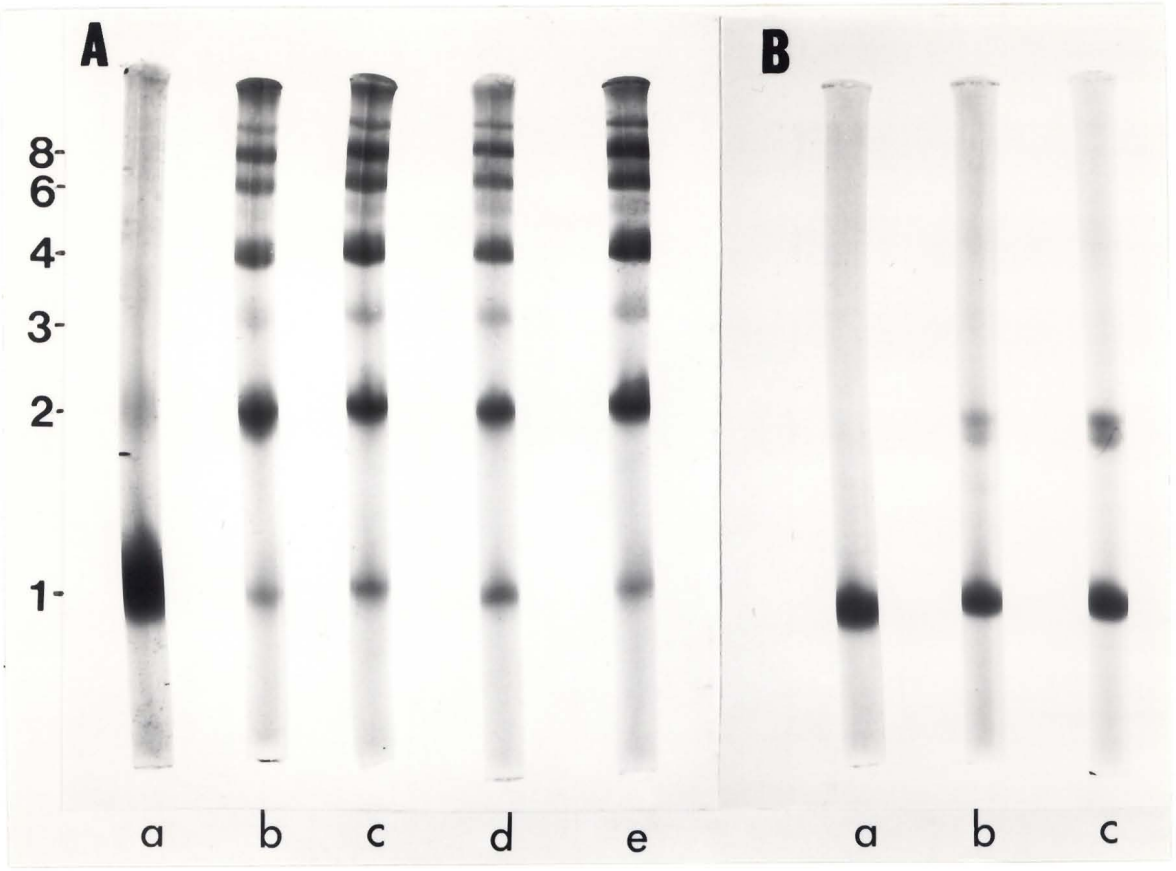


FIGURE 5. SDS-PAGE analysis (Weber and Osborn, 1969) of formiminotransferase-cyclodeaminase and the proteolytically-derived fragment cross-linked after renaturation under different conditions (as described in Materials and Methods) in the presence of 0.1% Triton X-100 unless otherwise indicated. (A) Each 4% polyacrylamide tube gel contains 20 µg of formiminotransferase-cyclodeaminase cross-linked with 0.55 mg/ml bis(sulfosuccinimidyl)suberate: a) at zero time, b) after 48 h in the presence of 0.05% of Tween 80 (no Triton X-100), c) after 48 h, d) after dialysis for 48 h following 20 h renaturation in 1.5 M urea, and e) native enzyme diluted in renaturation buffer. (B) Each 5% polyacrylamide tube gel contains 10 µg fragment cross-linked with 1.5 mg/ml bis(sulfosuccinimidyl)suberate a) at zero time, b) after 6 h and c) fragment diluted in renaturation buffer (not denatured). The number of subunits in each cross-linked species is indicated.

proteolytic fragment (Figure 5B) shows that it recovers its dimeric structure as it is reactivated.

Cross-linking of the enzyme renatured in 1.5 M urea showed some dimer and no higher forms (not shown), and gel filtration on an AcA-34 column was used to confirm the size of the active species. As shown in Figure 6, completely renatured enzyme and native enzyme in the presence and absence of 1.5 M urea elute at the same position confirming that they are all octameric. Enzyme renatured in the presence of 1.5 M urea appears to be dimeric since it elutes much later than the octamer but earlier than the transferase fragment ($M_r \cong 80,000$). It appears that the presence of 1.5 M urea during renaturation prevents formation of the second subunit-subunit association present in native enzyme, thereby generating a dimeric transferase-active species. This may be an active intermediate in the complete renaturation process, as shown in Scheme I. Refolding of the denatured polypeptides to form structured monomers is indicated by step I, followed by reassociation of the monomers to form transferase-active dimers (step II) which undergo further association (step III) to give the native octamer.

Table 1 shows K_m values determined with mono- and pentaglutamate substrates for native and renatured enzyme, and for the two monofunctional dimers. For the transferase activity, the K_m values for (6S)-H_4PteGlu are similar in the three cases, but the transferase dimers generated by renaturation in 1.5 M urea have ~ 30-fold higher K_m values for the pentaglutamate derivative than either native or renatured enzyme. Addition of 1.5 M urea to native enzyme had no effect on the K_m values (not shown). The K_m values for (6S)-5-formimino-H_4PteGlu

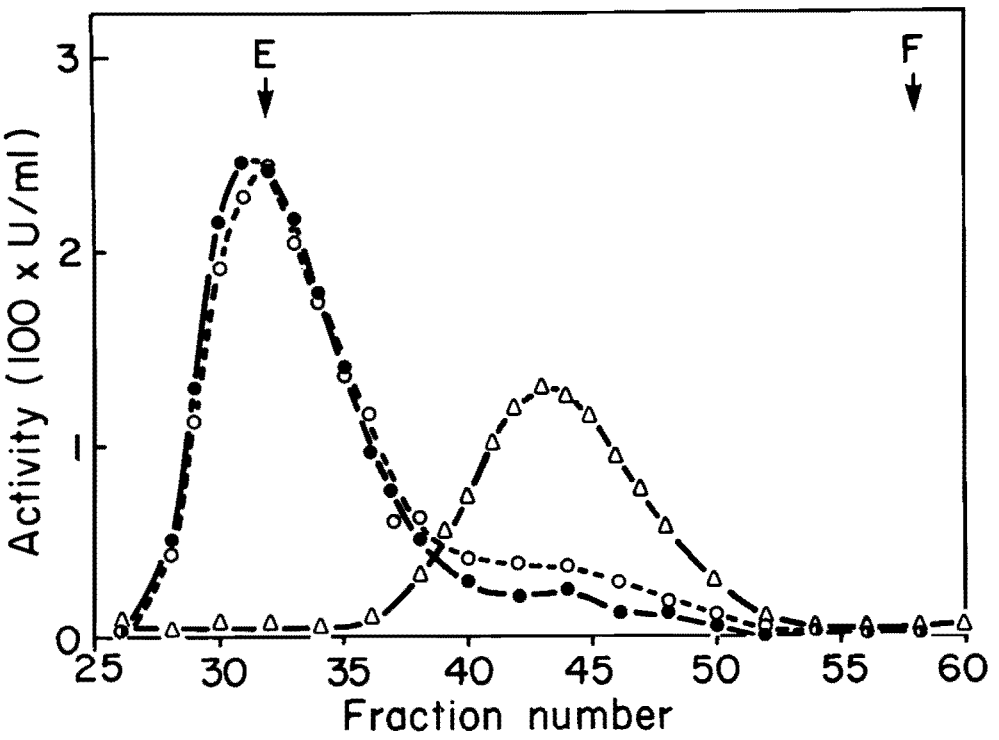
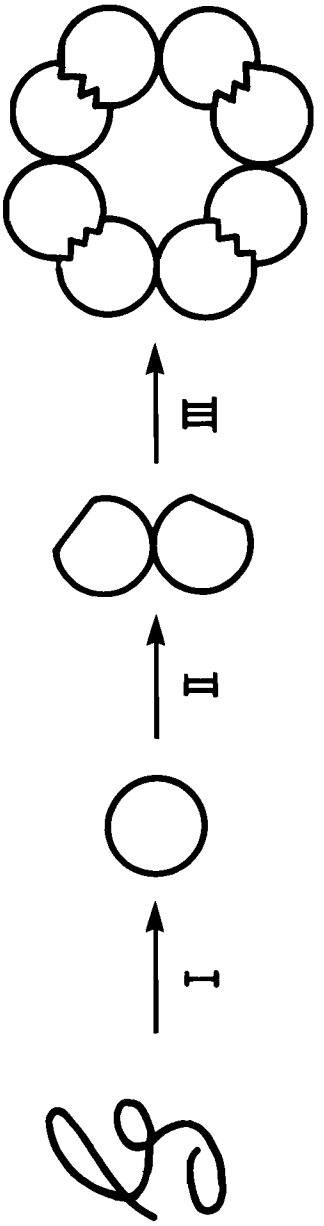


FIGURE 6. Gel filtration of formiminotransferase - cyclodeaminase renatured in the presence and absence of 1.5 M urea. After dilution from 6 M Gdn•HCl into renaturation buffer containing 0.1% Triton X-100, 0.5 ml (~ 3 µg protein) was applied to a 48 ml column of AcA-34 as described in Materials and Methods. Fractions of 0.47 ml were collected and assayed for transferase activity. Symbols: (o) native enzyme diluted in renaturation buffer containing 1.5 M urea, (●) enzyme renatured for 50 h in the absence of urea, and (Δ) enzyme renatured for 20 h in the presence of 1.5 M urea. E indicates the elution position of native enzyme (M_r ~ 500,000) in the absence of urea, and F indicates that of the proteolytically derived transferase-active fragment (M_r ~ 80,000).



SCHEME 1

Table I: Values of K_m for the folate substrates[a] of native and renatured enzyme, and of monofunctional dimers.

Substrate	Previous Value[b]	Control Enzyme	Renatured Enzyme	Dimers	
				Transferase	Deaminase
			μM		
(6S)-H_4PteGlu	48±14	43±21	21±5	27±1	-
(6S)-H_4PteGlu$_5$	0.7±0.3	2.1±1.7	0.9±0.2	70±30	-
(6S)-5-HCNH-H_4PteGlu	149±14	114±26	52±12	-	79±14
(6S)-5-HCNH-H_4PteGlu$_5$	2.0±0.7	3.4±0.5	2.0±0.6	-	17±7

[a]Values are expressed as averages ± standard deviations for 3 separate determinations.

[b]From Paquin *et al.* (1985), determined for native enzyme.

determined for native and renatured enzyme, and for deaminase dimers formed by dissociation of the enzyme in 3 M urea in the presence of folic acid (Findlay and MacKenzie, 1987) are similar for the three cases, and the K_m values for the pentaglutamate derivative are ~ 25-fold lower for both the native and renatured enzyme, and ~ 5-fold lower for the deaminase dimers. It appears that the deaminase dimers have polyglutamate specificity while the transferase dimers do not, suggesting that the polyglutamate binding site requires the same subunit-subunit interface as the deaminase activity.

Formiminotransferase-cyclodeaminase renatured for ≥48 h in renaturation buffer containing 0.1% Triton X-100 channeled 85% of the (6S)-5-formimino-H_4PteGlu$_5$ produced by the first reaction. The same extent of channeling was observed with native enzyme incubated under the same conditions, but enzyme diluted into cold renaturation buffer and assayed immediately exhibited 100% channeling (data not shown).

DISCUSSION

Formiminotransferase-cyclodeaminase is an excellent system for studying both site-site interactions involved in the "channeling" of a non-covalently bound intermediate, and the relationship between structure and function of an oligomeric enzyme with two types of subunit-subunit interaction. In this paper we have used renaturation of formiminotransferase-cyclodeaminase from 6 M Gdn•HCl to further probe the relationship of the two activities to quaternary structure and to help localize the two catalytic sites and the polyglutamate-binding site.

Jaenicke (1982) has reviewed work on denaturation and renaturation of many oligomeric enzymes, and has proposed the following general scheme for renaturation of an octameric enzyme:

$$n\ U \xrightarrow{uni} n\ M' \xrightarrow{uni} n\ M \xrightarrow{bi} \frac{n}{2}\ D' \xrightarrow{uni} \frac{n}{2}\ D \xrightarrow{bi} \frac{n}{4}\ T' \xrightarrow{uni} \frac{n}{4}\ T \xrightarrow{bi} \frac{n}{8}\ O' \xrightarrow{uni} \frac{n}{8}\ O$$

where U is unfolded polypeptide and M, D, T, and O are monomer, dimer, tetramer and octamer respectively, with the primed forms representing different conformational states. This scheme involves a series of alternating first and second order reactions, but the final symmetry of formiminotransferase- cyclodeaminase raises the possibility that four dimers may associate directly to form the circular octamer, resulting in a fourth order step.

Because reassembly requires several second (or higher) order steps, it was rather unexpected that even at very low protein concentrations (~ 1 µg/ml) all reactivation steps observed were apparently first order. In the presence of Tween 80, both activities are recovered simultaneously and to >90% in 48 h. The rate-limiting step has a rate constant $k=(1.9\pm0.2)\times10^{-5}\ s^{-1}$ at 22°C and an activation energy of ~ 15 kcal mol^{-1}. It must correspond to either a step in the initial refolding of monomers or a conformational change after one or more association steps. The intrinsic tryptophan fluorescence spectra confirm that the enzyme is unfolded in 6 M Gdn·HCl, but suggest that major refolding occurs within the first 5 min after dilution. Chemical

cross-linking indicates that fully reactivated enzyme has recovered its native octameric structure.

In the presence of Triton X-100, the initial rate of transferase reactivation is more than twice that of the deaminase, indicating that there are two rate-limiting steps under these conditions, both of which appear to be first order. In the presence of a low concentration of urea, renaturation is arrested at the level of a transferase-active dimer which we propose is also an intermediate in the complete renaturation to octamer in the presence of Triton X-100. One rate-limiting step would therefore be either refolding of monomer or a conformational change forming active dimer. The second rate-limiting step would be somewhere on the dimer to octamer pathway, since it appears that formation of octamer is required for expression of both activities. The reactivated enzyme has also recovered the ability to "channel" the formimino- $H_4PteGlu_5$ intermediate between the catalytic sites.

The proteolytically-derived transferase fragment comprises 63% of the polypeptide chain and contains one subunit interface of the native octamer. It can be completely renatured under the same conditions as the enzyme, confirming that it is an independently folding domain. The rate-limiting step here is also first order, and the initial rate is slightly ($\leq$2-fold) faster than that of the transferase dimer.

The results of these renaturation experiments provide independent confirmation of the proposal that the two activities require alternate subunit interfaces in the native octamer, which was based on the

isolation of two monofunctional dimers by dissociation of formimino-transferase-cyclodeaminase in urea (Findlay and MacKenzie, 1987).

Both activities of native formiminotransferase-cyclodeaminase have been shown to exhibit much lower K_m values (>50-fold) for polyglutamate forms of their folate substrates (Paquin et al., 1985). We have shown here that the deaminase dimers formed by dissociation in urea in the presence of folate (Findlay and MacKenzie, 1987) show much higher affinity for pentaglutamate than monoglutamate substrate. However, the transferase dimer has lower affinity for the pentaglutamate substrate, similar to results obtained previously with the proteolytically-derived transferase fragment, where a 4-fold increase in the K_m for $H_4PteGlu_5$ versus $H_4PteGlu$ was reported (MacKenzie et al., 1980). Since both the transferase dimer and fragment have no polyglutamate specificity, while the deaminase dimer has ~ 5-fold higher affinity for the pentaglutamate substrate, it appears that the 4 polyglutamate sites observed by binding studies (Paquin et al., 1985) are formed by the interfaces required for deaminase activity.

There have been a number of recent reports of enzyme active site formation at interfaces between subunits, mainly based on x-ray crystallographic studies. In dimers with a single isologous interaction such as aspartate aminotransferase and glutathione reductase, two active sites are formed at the interface (Ford et al., 1980; and Thieme et al., 1981). For enzymes with heterologous interactions between subunits, such as the catalytic trimer of aspartate carbamoyltransferase (Krause et al., 1985) and glutamine synthetase with twelve identical subunits

(Almassy et al., 1986), a single active site is formed at each interface.

Formiminotransferase-cyclodeaminase appears to be unusual in that catalytic sites for two different reactions require two kinds of subunit-subunit interfaces, but that there appears to be a single site associated with each isologous interaction. X-ray crystallography is now required to determine if the active sites of this enzyme are formed by the interfaces between subunits, which would explain why the native octameric structure is required for expression of the two activities and for "channeling" of polyglutamate intermediates.

CHAPTER 4

AN IMPROVED PROCEDURE FOR PURIFYING FORMIMINOTRANSFERASE-CYCLODEAMINASE FROM PIG LIVER. KINETICS OF THE TRANSFERASE ACTIVITY WITH TETRAHYDROPTEROYLPOLYGLUTAMATES

"The course of nature...

seems delighted with transmutations".

I. Newton

ABSTRACT

Formiminotransferase-cyclodeaminase is stabilized and activated ~ 40% in the presence of low concentrations (< 0.2%) of Triton X-100, possibly because the average hydrophobicity (1.10 kcal/residue) and the frequency of large non-polar side chains (0.34) of this protein are both somewhat higher than average. This stabilization enabled us to develop a new purification procedure for the enzyme using chromatography on Matrex™ Gel Orange A and Heparin-Sepharose columns in the presence of Triton X-100. This procedure is easier, much more reproducible, and gives slightly higher yield than the previous method (Drury et al., 1975). Further investigations on the role of tetrahydropteroylpoly-glutamates with formiminotransferase-cyclodeaminase reveal that the use of polyglutamylated folate substrates does not change the mechanism of the transferase reaction, but decreases the K_m for formiminoglutamate, the second substrate, more than ten-fold, bringing it closer to the expected physiological range.

INTRODUCTION

Formiminotransferase-cyclodeaminase catalyses the following two sequential reactions in the degradation of histidine in mammalian liver:

$$\text{NHCH-L-glutamate} + H_4\text{PteGlu}_n \rightleftharpoons 5\text{-NHCH-}H_4\text{PteGlu}_n + \text{glutamate}$$

$$5\text{-NHCH-}H_4\text{PteGlu}_n \rightleftharpoons 5,10\text{-CH}^+\text{-}H_4\text{PteGlu}_n + NH_4^+$$

Both activities were shown to have much higher affinity for polyglutamate forms of their folate substrates ($4 \leq n \leq 7$) and "channeling" of the NHCH-H_4PteGlu$_n$ intermediate was observed (Paquin et al., 1985). Beaudet and MacKenzie (1976) used cyanogen bromide cleavage, isoelectric focusing and electron microscopy to show that the enzyme consists of eight identical subunits (M_r 62,000) arranged in a ring. Since only four binding sites for H_4PteGlu$_n$ ($4 \leq n \leq 7$) were observed for the native octamer (Paquin et al., 1985), and cross-linking and generation of a dimeric transferase-active fragment by limited proteolysis had suggested a tetramer of dimers structure (MacKenzie et al., 1980), it was proposed that the basic functional unit of this enzyme is a dimer.

Partial denaturation of the enzyme by urea under various conditions yielded dimers with either transferase or deaminase activity, which appeared to isolate different subunit-subunit interfaces (Findlay and MacKenzie, 1987). Complete renaturation of the enzyme from 6 M guanidine hydrochloride in the presence of 1.5 M urea is arrested at the level of transferase-active dimers which reassociate during dialysis to

form the bifunctional octamer (Findlay and MacKenzie, 1988). The deaminase dimers have higher affinity for polyglutamate than monoglutamate substrate, but the transferase dimers have no polyglutamate specificity. We therefore propose that the polyglutamate binding site and deaminase activity require the integrity of one kind of subunit-subunit interaction and the other interaction is required for expression of the transferase activity.

The enzyme used in the previous studies was prepared by the method of Drury et al. (1975), and is a modification of the original procedure of Tabor and Wyngarden (1959), who first showed copurification of the activities from an acetone powder of hog liver. This modified method purifies formiminotransferase-cyclodeaminase 740-fold from frozen pig liver and yields ~ 15-20 mg of enzyme from 600 g of liver. It consists of a series of precipitations and differential extractions, since the properties of the enzyme did not allow column chromatography. However, this method is not very reproducible and requires adjustment for different sources of liver.

Our observation that low concentrations of non-ionic detergent stabilize formiminotransferase-cyclodeaminase has enabled us to develop a more reliable method of purifying the enzyme using column chromatography. This should allow us to obtain the amounts of pure enzyme required for X-ray crystallography, which will localize the two active sites and the polyglutamate binding site within the quaternary structure of this protein. We have also used this enzyme for kinetic studies to compare the mechanism of the transferase reaction with polyglutamate versus monoglutamate folate substrates.

MATERIALS AND METHODS

Folic acid and formimino-L-glutamic acid were purchased from Sigma Chemical Co., Matrex^{TM} Gel Orange A was from Amicon Corp., and Heparin-Sepharose CL-6B and ultrapure Triton X-100 were from Pierce Chemical Co. Pteroylpenta-γ-L-glutamic acid was purchased from Dr. B. Schircks Laboratories (Switzerland) and converted enzymatically to (6S)-H_4PteGlu$_5$ (MacKenzie and Baugh, 1980). The other tetrahydropteroylpolyglutamates were prepared by Dr. J. Paquin using the same procedure. For routine assays we used (6R,S)-H_4PteGlu prepared chemically (Drury et al., 1975). All other chemicals were reagent grade.

Amino Acid Analysis - Formiminotransferase-cyclodeaminase was purified by the method of Drury et al. (1975) and the transferase fragment prepared as described by MacKenzie et al. (1980). Duplicate samples of 5.0 μg of the two proteins were used for regular analysis and a separate methionine and cysteine determination, and 80 μg aliquots were used for determination of tryptophan. All materials, methods and data analysis are as described by Rios-Orlandi et al. (1986).

Enzyme Assays - Standard assays for transferase activity contained 0.1 M potassium phosphate (pH 7.3), 35 mM 2-mercaptoethanol, 4 mM Na-formiminoglutamate and 1.0 mM (6 R,S)-H_4PteGlu. Aliquots of 10 μL of enzyme solution were added to 0.5 mL aliquots of assay solution at 30°C, and after 5-10 min incubation, 0.5 mL of 0.36 M HCl was added. The samples were boiled for 1 min to convert 5-NHCH-H_4PteGlu to 5,10-CH^+-H_4PteGlu, cooled, and absorbance at 350 nm was measured (ε=24,900 $M^{-1}cm^{-1}$). Much lower concentrations of tetrahydropteroyl-polyglutamates were used (as indicated in figure legends) and assays

were stopped with 10 µL of 9.6 N HCl after 3 min incubation. All activity measurements are the average of duplicate or triplicate determinations.

Enzyme Purification - The purification procedure developed by Drury et al. (1975) was followed to the ammonium sulphate precipitation step, and all manipulations were performed at 0-4°C. Frozen pig liver (100 g) was homogenized for 2 min with 300 mL of 0.1 M potassium phosphate (pH 7.3) and 35 mM 2-mercaptoethanol in a Waring blender. The homogenate was centrifuged at 20,000 g for 1 h, and the supernatant poured through glass wool, then 0.14 volumes of 50% (w/v) polyethylene glycol was added slowly while stirring. After standing for 30 min, the suspension was centrifuged at 20,000 g for 20 min. A further 0.5 volumes (based on the original supernatant) of 50% polyethylene glycol was added to the supernatant, while stirring slowly. After standing for 30 min, the suspension was centrifuged at 20,000 g for 20 min, then the pellets were resuspended in 80 mL of 0.1 M potassium phosphate (pH 7.3) and 35 mM 2-mercaptoethanol. Solid ammonium sulfate (19.4 g/100 ml) was added slowly to this solution, while stirring and maintaining the pH at 7 by adding concentrated ammonium hydroxide. After standing for at least 1 h or overnight, the suspension was centrifuged for 1 h at 20,000 g. The pellet was resuspended in 4-5 ml of 0.035 M 2-mercaptoethanol by stirring for ~ 2 h, then centrifuged for 90 min at 30,000 g. This pellet was resuspended in 2.5 ml Buffer A (0.05 M potassium phosphate (pH 7.3), 35 mM 2-mercaptoethanol, 0.02% Triton X-100). After

centrifugation at 30,000 g for 45 min, the supernatant was saved and the pellet resuspended in 5 mL Buffer A and centrifuged again at 30,000 g for 30 min. The pellet was extracted a third time using 2.5 ml Buffer A. All supernatants were assayed for transferase activity, most of which (~ 55-60% of original) is usually found in the second Buffer A extraction. The enzyme was then dialysed overnight against 1 L of cold Buffer A, and applied to a 34 ml (2.5x7.0 cm) column of Matrex^{TM} Gel Orange A resin (Amicon Corp.) previously equilibrated with Buffer A. After 30 min, the column was washed with 100 ml Buffer A, and eluted with a linear gradient of 100 ml Buffer A and 100 ml of 0.5 M KCl in Buffer A, while collecting 2.7 ml fractions. The fractions containing highest transferase activity were pooled and dialysed overnight against 1 L Buffer A. The enzyme was then applied slowly to a 10 ml (1.5x6.0 cm) column of Heparin-Sepharose CL-6B (Pharmacia) equilibrated with Buffer A. After 30 min, the column was washed with 40 ml Buffer A, 25 ml 0.1 M KCl in Buffer A and eluted with 25 ml 0.25 M KCl in Buffer A, while collecting 2.7 ml fractions. Again, the fractions with highest transferase activity were pooled and dialysed overnight against Buffer A containing 40% glycerol, which concentrates the enzyme about 3-fold and allows it to be stored at -20°C without freezing. It is stable under these conditions for at least 6 months. Protein determinations were by the method of Bensadoun and Weinstein (1976), and SDS-PAGE (Laemmli, 1970) was used to monitor the purity.

Kinetic Experiments - The K_m for formiminoglutamate was determined with several tetrahydropteroylpolyglutamates ($H_4PteGlu_n$, n=1,3,4,5,7) using

concentrations of 3-5 times K_m for the folate substrates (values from Paquin et al., 1985) and measuring v at various concentrations of formiminoglutamate. All assays were done in duplicate, and the data was fitted to the Michaelis-Menten equation using the program KINFIT (Knack and Röhm, 1981). Each value of K_m reported is the average of at least 3 independent determinations. Inhibition by the product glutamate against formiminoglutamate and tetrahydropteroylpentaglutamate was determined by measuring velocity with increasing concentrations of glutamate at three concentrations of one substrate and saturating concentration ($> 10xK_m$) of the other substrate. Three independent experiments were performed under identical conditions and the results averaged.

RESULTS

Formiminotransferase-cyclodeaminase is activated by low concentrations ($\leq$ 0.2%) of non-ionic detergent, as shown in Figure 1. For enzyme incubated with increasing amounts of Triton X-100, transferase activity is enhanced by about 40%. The profile appears hyperbolic and reaches a plateau above 0.05% detergent. To determine whether this effect is due solely to the presence of detergent carried over into the assay, we repeated the experiment with 0.002% Triton added to the assay solution (equivalent to 0.1% Triton in the first experiment). This gave ~ 10-15% increase in the transferase activity of enzyme incubated with low concentrations of Triton, but had no apparent effect at higher concentrations (above 0.05%). The deaminase activity is stimulated to a slightly lesser extent (30 to 35%), and is unaffected by 0.002% Triton in the assay buffer (not shown).

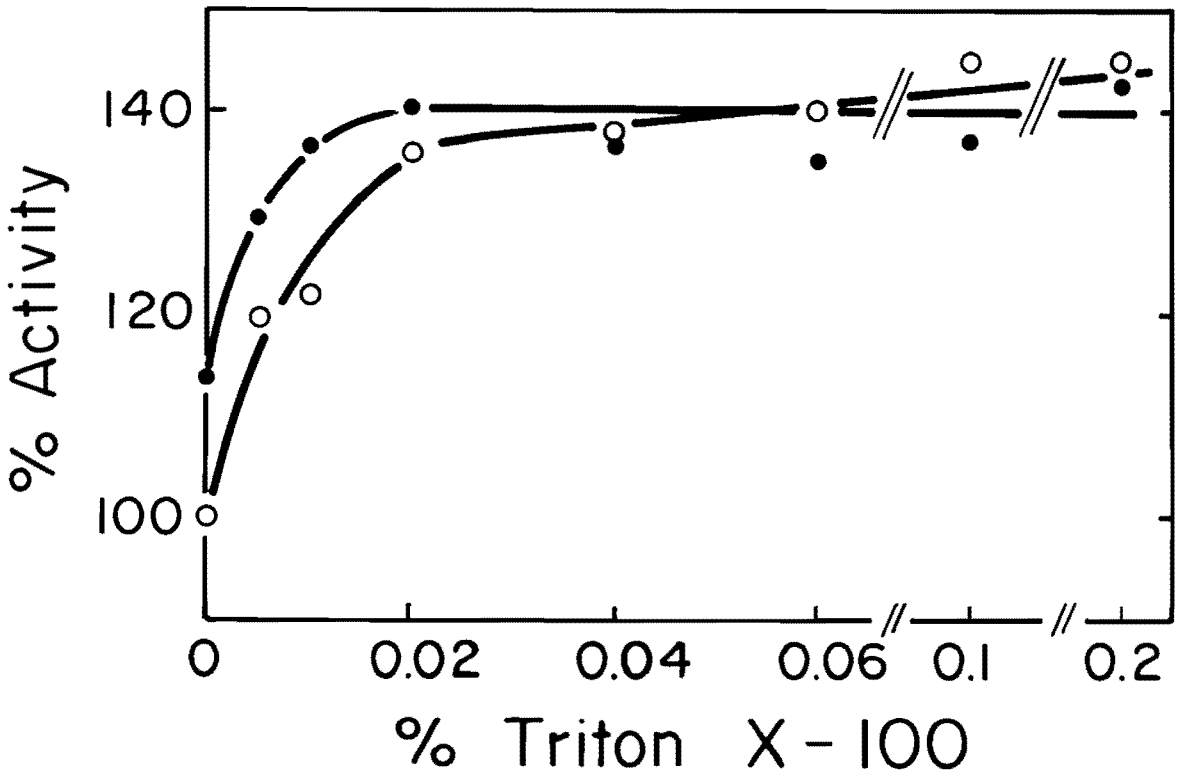


FIGURE 1: Stimulation of transferase activity upon incubation in buffer containing Triton X-100. Symbols: (o) no additional Triton in assay solution, (●) with 0.002% Triton added to assay solution.

Proteolysis of formiminotransferase-cyclodeaminase with chymotrypsin in the presence of folic acid generates two polypeptides of 39,000 and 24,000 molecular weight. The 39 kDa polypeptide forms a dimeric transferase-active fragment (MacKenzie et al., 1980) and has been shown to be an independently refolding domain after denaturation by guanidine•HCl (Findlay and MacKenzie, 1988), but the 24 kDa fragment appears to be inactive. Amino acid analysis was performed on the enzyme and the 39 kDa fragment, and the results are reported in Table I. The composition of the remaining polypeptide is predicted by the difference between that of the enzyme and the transferase fragment (39 kDa), and indicates unequal distribution of amino acids between the two domains, especially glycine, cysteine and tryptophan. The composition of formiminotransferase-cyclodeaminase is in good agreement with a previously reported analysis (Beaudet and MacKenzie, 1976).

Table II contains various parameters calculated from the amino acid analysis of the enzyme and two fragments. The calculated specific volumes are all 0.73 ml/g, somewhat lower than the experimentally measured value of 0.77 ml/g determined for the native enzyme (Drury et al., 1975). Bigelow (1967) calculated the average hydrophobicity, frequency of large nonpolar side chains (NPS) and ratio of polar to nonpolar volume (p) for 109 proteins and peptides. Our calculated values of average hydrophobicity (~ 1.10 kcal/mole) and of p (0.88-0.89) are higher and lower respectively than the average from his data. Variation between the domains can be seen from the frequency of nonpolar side chains, which is higher than average for both the enzyme and the

Table I: Amino acid compositions of formiminotransferase - cyclodeaminase and the proteolytically-derived transferase fragment.

Amino acid	Nearest Integer Ratios		
	Enzyme	39 kDa fragment	Remainder‡ of polypeptide
Asx	42	29	13
Thr	23	12	11
Ser	27	17	10
Glx	69	48	21
Pro	25	19	6
Gly	39	35	4
Ala	71	36	35
Val	48	31	17
Met	6	3	3
Ile	16	12	4
Leu	69	43	26
Tyr	14	10	4
Phe	21	14	7
Lys	31	18	13
His	14	8	6
Arg	39	23	16
Cys	21	6	15
Trp	6	5	1
TOTAL	581	369	212
MW	63,952	40,582	23,370

‡Values obtained by difference between enzyme and 39 kD fragment.

Table II: Parameters calculated from amino acid composition

Parameters	Enzyme	39 kD fragment	24 kD fragment
$\bar{v}^a$(ml/g)	0.729	0.728	0.729
$H\Phi^b_{ave}$ (kcal/mole)	1.10	1.11	1.10
NPS[c]	0.343	0.363	0.307
P[d]	0.885	0.889	0.878

[a] calculated from values of Zamyatnin (1972)

[b] as defined by Bigelow (1967)

[c] frequency of large nonpolar side-chains (Waugh, 1954)

[d] ratio of polar to nonpolar volume (Fisher, 1964)

transferase fragment, and is ~ 18% higher for the 39 kDa fragment than for the remainder of the polypeptide.

We have developed a more reliable method of purifying formiminotransferase-cyclodeaminase, which is outlined in Table III. The first few steps of the procedure of Drury et al. (1975) were retained - two polyethylene glycol precipitations and an ammonium sulfate precipitation reduce the amount of protein by 90%. Extraction of the pellet with 35 mM mercaptoethanol, then with buffer containing Triton X-100 yields enzyme which is purified 100-fold. Previous purifications relied on a pH precipitation and a later crystallization, both of which required fairly high protein concentration and specific activity to be successful. We have circumvented this requirement by dialysing the enzyme extracted from the $(NH_4)_2SO_4$ pellet and applying it to an Orange A dye column in the presence of 0.02% Triton X-100. As shown in Figure 2, most protein does not bind to this matrix and appears in the wash, whereas ~ 0.2 M KCl is required to elute formiminotransferase-cyclodeaminase, which is now ~ 70% pure. After dialysis the enzyme is applied to a Heparin-Sepharose column for a final 1.5-fold purification. The gel in Figure 3 shows the different stages of purification, and confirms that the enzyme is > 95% pure after the Heparin-Sepharose column.

The final specific activity is 48 U/mg which is higher than the previously reported value of 37 U/mg (Drury et al., 1975) due to the presence of Triton X-100 in all buffers. Yields range from 23-26%, slightly higher than the 18% yield obtained by Drury et al. (1975).

Paquin et al. (1985) reported a dramatic decrease in K_d and K_m

Table III: Purification of Formiminotransferase-Cyclodeaminase

Fraction	Volume (ml)	Protein (mg)	Transferase Activity (μmoles/min)	Specific Activity (μmoles/min/mg)	Yield
1st supernatant	310	11,300	675	0.060	100%
PEG fraction	80	4,400	560	0.13	83%
K-phosphate/Triton	5.5	59.4	372	6.3	55%
Orange A column	36	5.51	188	34	28%
Heparin-Sepharose	9.0	3.26	156	48	23%

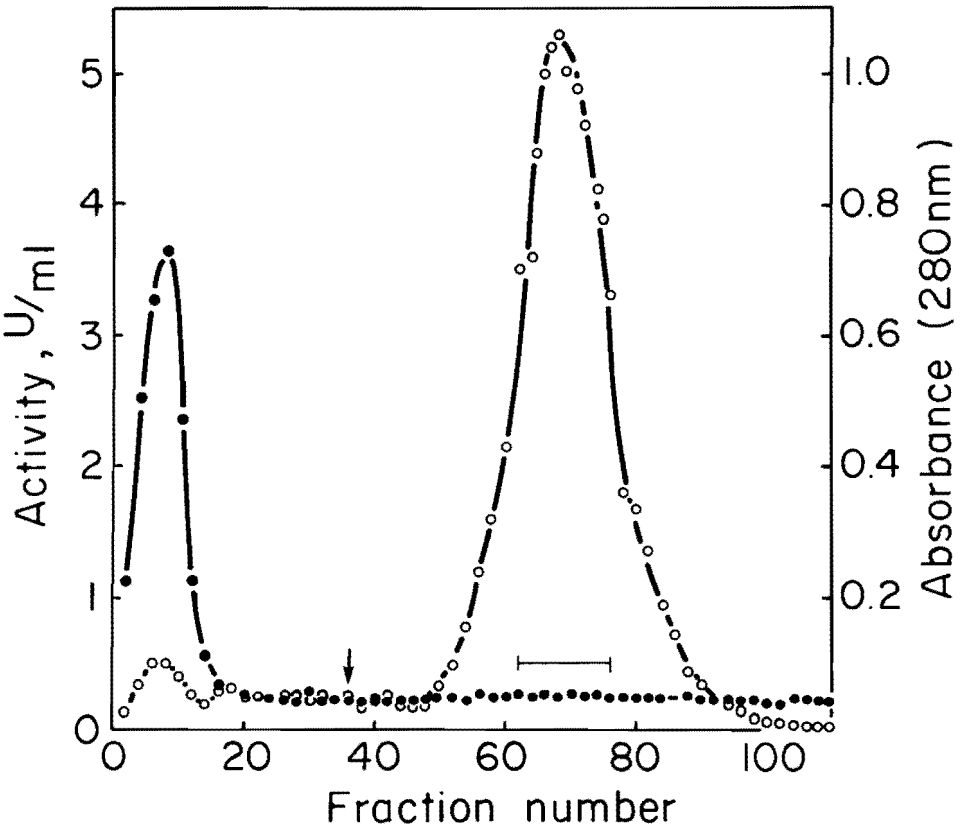


FIGURE 2: Profile of elution of formiminotransferase-cyclodeaminase from Matrex™ Gel Orange A column. Symbols: (o) transferase activity and (●) absorbance at 280 nm of a 1:10 dilution of each fraction. The arrow indicates the start of the gradient and the bar indicates the fractions pooled.

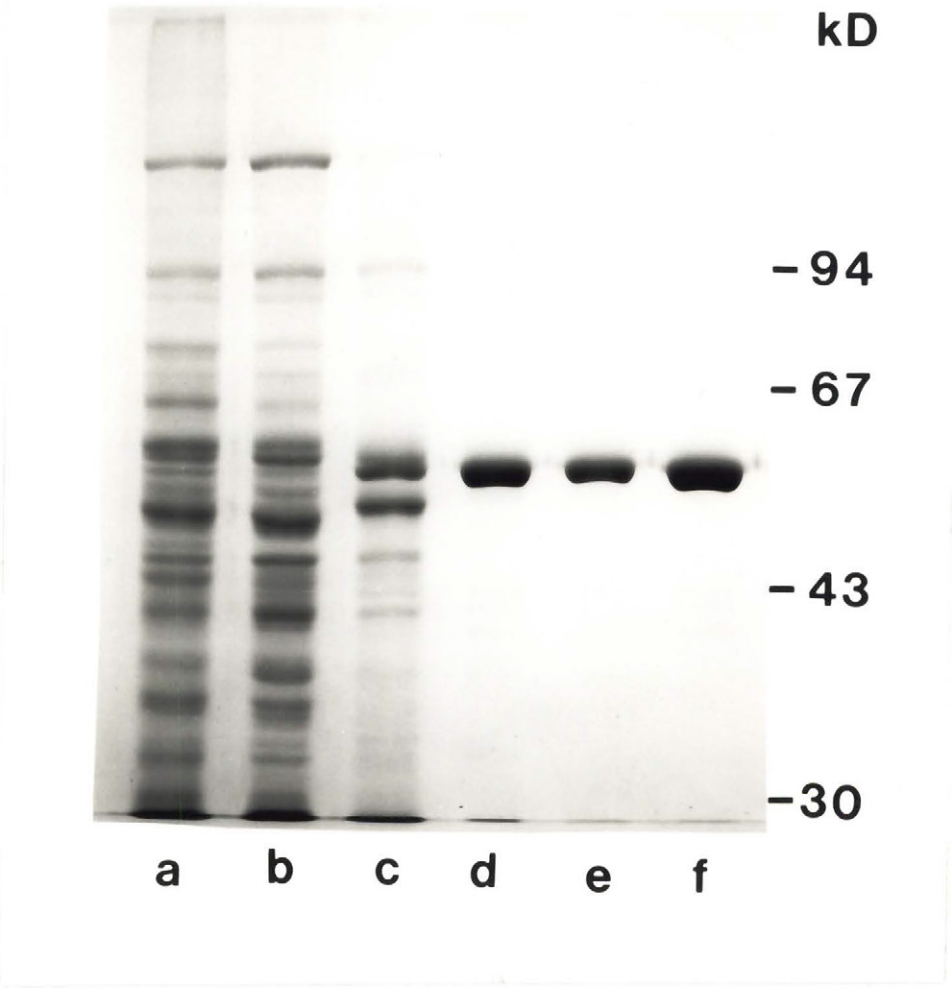


FIGURE 3: SDS-PAGE analysis of fractions from various steps in the purification of formiminotransferase-cyclodeaminase. a) extract (40 µg protein), b) polyethylene glycol fraction (40 µg), c) potassium phosphate/Triton X-100 extraction (20 µg), d) pooled fractions from Orange dye column (10 µg), and e) 5 µg and f) 10 µg protein from the Heparin-Sepharose column.

values for polyglutamate derivatives of the folate substrates of formiminotransferase- cyclodeaminase. Figure 4 shows that the use of polyglutamate substrates also significantly decreases the K_m value for formiminoglutamate, the second substrate of the transferase reaction. For the tetra-, penta- and heptaglutamate derivatives, it is more than 10-fold lower than with tetrahydropteroylmonoglutamate.

We were unable to use initial velocity studies to determine whether the kinetic mechanism was altered by using the polyglutamate substrates because the K_m values for $H_4PteGlu_n$ ($4 \leq n \leq 7$) are less than micromolar (Paquin et al., 1985), too low for accurate determination by the regular spectrophotometric assay. Instead, we were forced to rely on product inhibition by glutamate against formiminoglutamate and $H_4PteGlu_5$. The results are shown as Dixon plots in Figure 5. Both sets of lines intersect to the left of the ordinate, and replots of s/v versus I appear parallel (not shown), indicating that glutamate inhibits competitively against both substrates. This observation rules out an ordered mechanism but is consistent with the rapid equilibrium random mechanism determined by Beaudet and MacKenzie (1975) with tetrahydropteroylmonoglutamate as substrate.

DISCUSSION

Formiminotransferase-cyclodeaminase provides an excellent model for studying the relationship between structure and function of an oligomeric enzyme, and the interaction between catalytic sites of a bifunctional enzyme. We have shown previously (Findlay and MacKenzie, 1987,1988) that the two activities require the integrity of alternate subunit-subunit interactions in the circular octameric structure, and

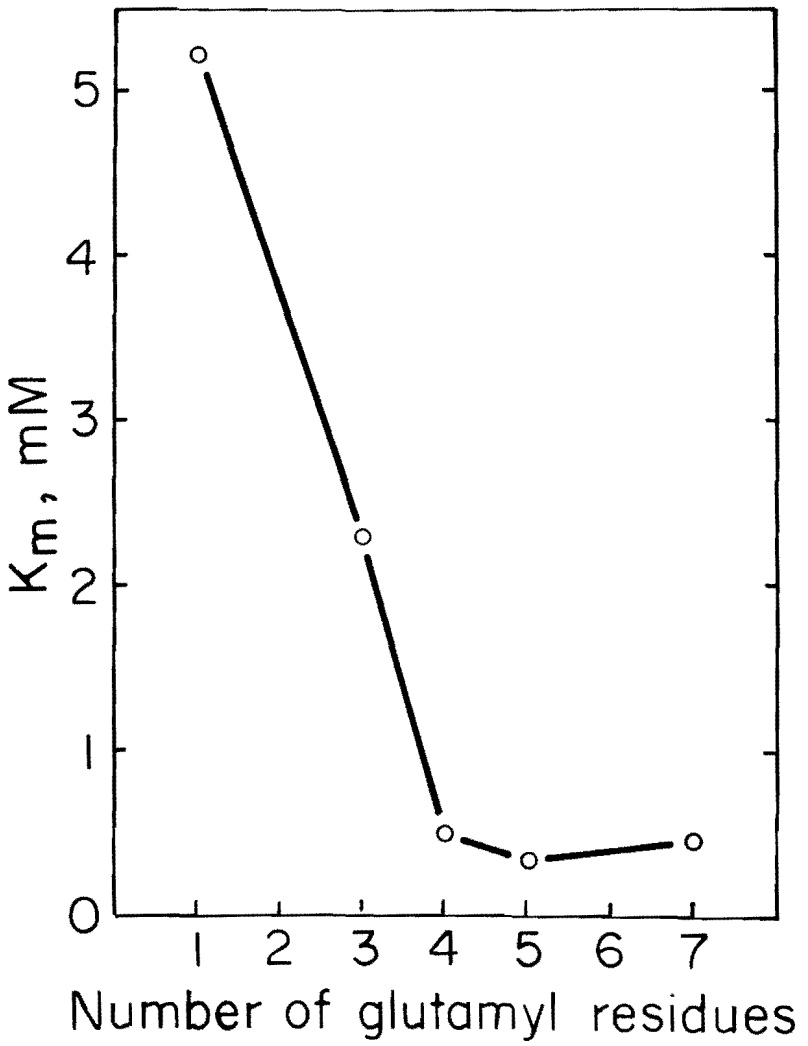


FIGURE 4: Effect of the number of glutamates in the $H_4PteGlu_n$ substrate on the K_m for formiminoglutamate.

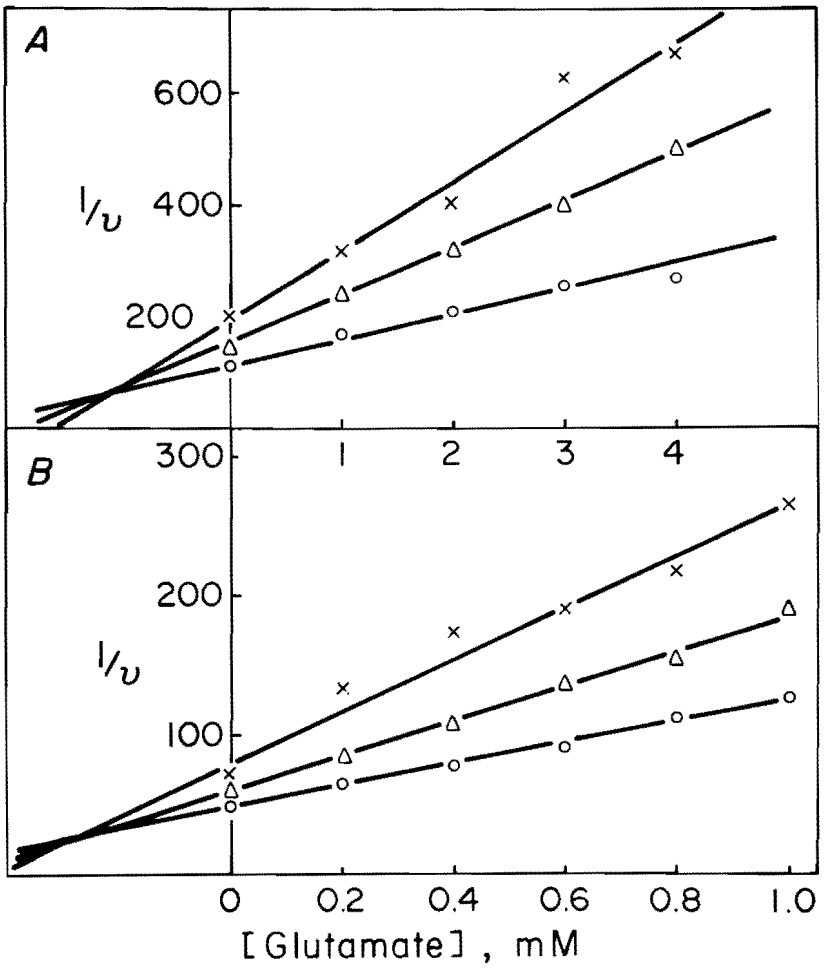


FIGURE 5: Dixon plot of product inhibition of the transferase activity by glutamate. A) Formiminoglutamate concentration was held constant at 4.5 mM and glutamate varied at three fixed concentrations of $H_4PteGlu_5$: (x) 3 μM, (Δ) 6 μM, and (o) 10 μM. B) (6S)-$H_4PteGlu_5$ concentration was held constant at 10 μM and glutamate varied at three fixed concentrations of formiminoglutamate: (x) 0.3 mM, (Δ) 0.6 mM and (o) 0.9 mM.

that the polyglutamate binding site is formed at the same interface as is required for deaminase activity. This enzyme also provides one of the best examples observed to date of "channeling" of a non-covalently bound intermediate, since up to 100% channeling of tetrahydropteroyl-polyglutamates between the two activities has been observed (Paquin et al., 1985).

The observation that the presence of low concentrations of Triton X-100 stabilizes and activates formiminotransferase-cyclodeaminase confirms previously reported effects of nonionic detergents during denaturation and renaturation of the enzyme (Findlay and MacKenzie, 1987,1988). We have also observed stabilization and activation of a proteolytically-derived dimeric transferase- active fragment (M_r=2x39,000). Both formiminotransferase-cyclodeaminase and the transferase fragment are fairly hydrophobic ($H\phi_{ave}$=1.10 and 1.11 kcal/residue, respectively) and have a higher than average frequency of large non-polar side chains, which may help to explain the interaction with detergent.

The purification procedure developed by Drury et al. (1975) yields good amounts (15-20 mg) of high purity, crystalline formimino-transferase-cyclodeaminase, but has a less than 50% success rate (unpublished observations). Each stage of the procedure requires an optimum protein concentration and specific activity to be successful, so insufficient purification during an early step cannot be overcome during the subsequent steps. Previous attempts to purify the enzyme using ion exchange chromatography led to poor recovery of active enzyme. The addition of Triton X-100 to all buffers allows us to purify

formiminotransferase-cyclodeaminase by column chromatography on Orange A dye-ligand agarose and heparin-Sepharose gels. This procedure produces milligrams of pure enzyme and can be scaled up to obtain the amounts of protein required for X-ray crystallography, the next step in determining the relationship between the structure and function of this enzyme.

We have also shown that the use of polyglutamylated folate substrates (which are the naturally occurring forms in vivo) greatly reduces the K_m value for formiminoglutamate, the second substrate of the transferase reaction, to less than millimolar which is closer to expected physiological concentrations. It was shown previously that binding of pteroylpolyglutamates protects formiminotransferase-cyclodeaminase from dissociation by 3 M urea (Findlay and MacKenzie, 1987), and prevents limited proteolysis by chymotrypsin (MacKenzie et al. 1980), suggesting that it causes a conformational change in the enzyme.

Strong et al. (1987) observed a similar decrease in the K_m of 10-formyltetrahydrofolate synthetase for Mg-ATP and formate when tetrahydropteroylpolyglutamates were used as substrates. The binding of $H_4PteGlu_n$ (n>3) in the presence of Mg-ATP and NH_4^+ appears to cause a conformational change in the synthetase domain of this trifunctional protein and preliminary evidence suggests that the order of addition of substrates is changed when the polyglutamate form is used. Lu et al. (1984) showed that the ordered mechanism of thymidylate synthase is reversed for both substrates and products when methylene-$H_4PteGlu_4$ is used instead of methylene-$H_4PteGlu_1$. However, for formiminotransferase-cyclodeaminase, product inhibition by glutamate suggests that the

mechanism of the transferase reaction does not change from rapid equilibrium random when tetrahydropteroylpentaglutamate is used as substrate.

As well as physically localizing the two active sites and the polyglutamate-binding site within the octameric quaternary structure of formiminotransferase-cyclodeaminase, it is now also of interest to look at conformational changes in the enzyme on binding folylpolyglutamates, to elucidate the mechanism of "channeling" of tetrahydropteroylpoly-glutamates between the active sites.

CHAPTER 5

GENERAL DISCUSSION

"We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time."

T.S. Eliot

The bifunctional enzyme formiminotransferase-cyclodeaminase has an unusual circular octameric quaternary structure with eight identical polypeptides forming two types of subunit-subunit association. Each interface can be isolated by dissociation of the octamer in low concentrations of urea under different conditions to yield either transferase-active or deaminase-active dimers (Chapter 2), and further dissociation to monomers causes loss of activity. The expression of the two activities appears to require the integrity of alternate subunit-subunit interfaces, suggesting that the complete octameric structure is required for "channeling" of intermediate.

Renaturation of this enzyme from 6 M guanidine hydrochloride can be arrested at the level of transferase-active dimers, which can undergo reassociation to form the bifunctional octamer (Chapter 3). A proteolytically-derived transferase fragment can also refold and dimerize, confirming that it is an independent functional domain. Both activities of the native enzyme have much higher affinity for longer polyglutamate forms of their folate substrates (Paquin et al., 1985), but the transferase-active dimer observed during renaturation of formiminotransferase-cyclodeaminase does not. The deaminase-active dimer produced by dissociation in urea retains the polyglutamate specificity, suggesting that the polyglutamate-binding site requires formation of the same subunit interaction as the deaminase activity.

Although the polyglutamate binding site seems to be more closely associated with the deaminase activity, the presence of a polyglutamate tail on the folate substrate of the transferase activity greatly decreases its own K_m as well as that of the second substrate

formiminoglutamate. Unlike some examples discussed in Chapter 4, the use of tetrahydropteroylpolyglutamates does not change the kinetic mechanism of the transferase.

The improved purification procedure described in Chapter 4 will allow isolation of sufficient quantities of formiminotransferase-cyclodeaminase for x-ray crystallography of the enzyme, to determine its tertiary structure and the location of the two catalytic sites and the polyglutamate-binding site within the tertiary and quaternary structure.

The results presented in this thesis raise some interesting aspects of the relationship between structure and function of oligomeric enzymes, the interaction between catalytic sites in multienzyme systems, and the role of evolution in enzyme organization. Some of these aspects and potential future studies using formiminotransferase-cyclodeaminase are discussed in this final chapter.

5.1 ROLE OF THE QUATERNARY STRUCTURE IN CATALYSIS

The observation of only four high affinity sites for binding tetrahydropteroylpolyglutamates to the native octamer (Paquin et al., 1985) suggests that the polyglutamate binding sites are formed at one of the subunit interfaces. This suggestion is strengthened by the observation that only the deaminase dimers have higher affinity for polyglutamate substrates (Chapter 3) which helps to localize the sites to a particular interface.

Based on the number of polyglutamate binding sites and kinetic evidence, Paquin et al. (1985) suggested that there is a single polyglutamate binding site per pair of transferase/deaminase active sites. In combination with the observation that the two activities

require the integrity of alternate subunit interfaces in the native octamer (Chapter 2), this is consistent with the formation of the active sites by subunit-subunit association. A number of enzymes have recently been shown to have intersubunit catalytic sites, including the dimeric citrate synthase (Remington et al., 1982), mitochondrial aspartate aminotransferase (Ford et al., 1980), and glutathione reductase (Thieme et al., 1981), tetrameric catalase (Fita and Rossman, 1985), dodecameric glutamine synthetase (Almassy et al., 1986) and the catalytic trimers of aspartate carbamoyltransferase (Krause et al., 1985). The occurrence of catalytic sites at subunit interfaces is an extension of site formation between domains in a subunit to formation of sites between domains on different subunits. Two identical active sites are formed by the isologous interactions present in the three dimeric enzymes and in catalase, whereas a single active site is formed at each heterologous interface in aspartate carbamoyltransferase and glutamine synthase. The predicted single active site at each isologous interface of formimino-transferase-cyclodeaminase may be due to constraints imposed by curvature of the ring.

To ascertain whether the active sites of formiminotransferase-cyclodeaminase are in fact formed by the subunit-subunit interactions will require x-ray crystallography of the enzyme in the presence and absence of substrates. Once the enzyme has been cloned and sequenced, it may be possible to delete portions from the amino- and carboxyl-terminal regions of the polypeptide and obtain fragments with either transferase or deaminase activity. This would define the minimum structure required for each activity and tell us more about the domain structure of this enzyme. Site-specific mutagenesis could then be used

to determine the amino acids essential for each catalytic activity, for polyglutamate binding and for interaction between subunits.

The complete renaturation of formiminotransferase-cyclodeaminase after treatment with 6 M guanidine hydrochloride confirms that all the information necessary for refolding and reassociation is present in the amino acid sequence. The sequence of events involved seems to be initial refolding of monomers ($t_{\frac{1}{2}} < 5$ min), formation of transferase-active dimers ($t_{\frac{1}{2}} \sim 1\frac{1}{2}$ h), and association to bifunctional octamer ($t_{\frac{1}{2}} \sim$ 10 h) under our conditions of renaturation (Chapter 3).

The analogous processes in vivo may occur in a different order and may be much faster. Syntranslational folding would be possible since the transferase fragment has been shown to be an independently folding domain and is postulated to comprise the amino terminal portion of the polypeptide chain (aminotermini of both the enzyme and the transferase fragment are blocked). The overall concentration of the enzyme in liver is about 30 times higher than that used for the renaturation studies and local concentrations could be higher still, which may increase the rate of reassociation. The two rate-limiting steps observed in vitro were both first order, presumably conformational changes involved in forming active dimer and active octamer (Chapter 3). The same steps may not be rate-limiting in vivo.

The sequence of reassociation and reactivation observed for formiminotransferase-cyclodeaminase involves inactive monomers, transferase-active dimers, then bifunctional octamer able to channel polyglutamate substrates. This is roughly analogous to that observed for aspartokinase-homoserine dehydrogenase; kinase-active monomers,

bifunctional dimers, then threonine inhibitable bifunctional tetramer (Garel & Dautry-Varsat, 1980). In both cases, different properties emerge with formation of different levels of quaternary structure. This may be an important attribute of multifunctional enzymes, in contrast to monofunctional oligomeric enzymes where intermediates formed during reassociation are usually inactive or express lower levels of the same activity as the native enzyme (Jaenicke, 1982).

Understanding the mechanisms of refolding and reassociation of oligomeric proteins is particularly important now that recombinant DNA technology allows the cloning and expression of eukaryotic proteins (many with potential medical applications) into bacteria. Over-expression can yield large quantities of proteins of reproducibly high purity, but in some cases the synthesized protein is inactive and is segregated into inclusion bodies, which can be isolated and the protein denatured and renatured in vitro to achieve its native structure (Pain, 1987).

5.2 INTERACTION BETWEEN CATALYTIC SITES

Formiminotransferase-cyclodeaminase has been shown to completely "channel" the formiminotetrahydropteroylpentaglutamate produced by the transferase reaction to the deaminase site. Paquin et al. (1985) demonstrated preferential transfer of intermediate with tetrahydro-pteroylpolyglutamates containing 4 to 7 glutamates, but 100% channeling is observed only with the pentaglutamate form, and the efficiency of transfer does not correlate directly with binding affinity or catalytic efficiency. They propose that transfer of the pteroyl moiety between the catalytic sites occurs via binding of the polyglutamate portion to a

separate polyglutamate binding site, with the pentaglutamate being the optimal length of "anchor", calculated to be ~25 Å. Electron micrographs suggest a subunit diameter of ~32 Å for formimino-transferase-cyclodeaminase (Beaudet and MacKenzie, 1976).

One interesting aspect of this proposed mechanism of channeling of pteroylpolyglutamates is its similarity to the "swinging arm" mechanism proposed for channeling by fatty acid synthase and by pyruvate dehydrogenase (as discussed in the Introduction) both of which channel a covalently bound intermediate. For formiminotransferase-cyclodeaminase, the intermediate appears to be bound non-covalently. Also, for the first two enzymes flexibility is provided by the protein itself transmitted through a prosthetic group (phosphopantotheine for fatty acid synthase and lipoamide in pyruvate dehydrogenase), but the flexibility required for channeling of tetrahydropteroylpolyglutamates appears to reside entirely in the substrate.

Binding of folylpolyglutamates induces a conformational change in the enzyme which prevents limited proteolysis by chymotrypsin (MacKenzie et al., 1980), and dissociation of the enzyme by 3 M urea (Chapter 2). Although the polyglutamate binding site is more closely associated with the deaminase site (Chapter 3), the use of tetrahydropteroyl-polyglutamate substrate decreases the K_m for formiminoglutamate, the second substrate of the transferase activity, more than 10-fold (Chapter 4). The use of the naturally occurring tetrahydropteroylpolyglutamates thus decreases the K_m for both the folate substrate and formimino-glutamate, to allow the enzyme to function efficiently with the expected

intracellular concentrations of the two substrates. Channeling of the longer polyglutamate derivatives further improves the efficiency of the pathway and prevents wasteful accumulation of formiminotetrahydrofolate, which is not used in any other metabolic reaction.

The evidence obtained to date for enzyme organization in folate metabolism has been summarized in Chapter 1, Part 3. In mammalian liver, five multifunctional enzymes are involved in interconverting one carbon derivatives of tetrahydrofolate, and evidence has been provided for further association of all the enzymes involved in de novo purine biosynthesis (Caperelli et al., 1980).

Higher levels of organization may also be present in some of the other pathways, and formiminotransferase-cyclodeaminase might be associated in liver with other enzymes of histidine catabolism and/or with the trifunctional dehydrogenase-cyclohydrolase-synthetase, which could convert the methenyl-H_4folate produced to either methylene-H_4-folate for thymidylate or methionine synthesis or to 10-formylH_4folate for purine synthesis. Now that theoretical and experimental techniques for studing weak or transient interactions between enzymes are being developed, it will become possible to determine the full extent of organization of folate-dependent enzymes in liver.

Different folate-dependent activities exhibit different specificity for the number of glutamates in their tetrahydropteroylpolyglutamate substrates (MacGuire and Coward, 1984; Matthews et al., 1985) and this has been postulated as a means of controlling flux through different pathways (Baggott and Krumdieck, 1979). If the enzymes are associated in vivo, channeling of folylpolyglutamates could enhance this effect.

The mechanism of transfer of tetrahydropteroylpolyglutamates between the active sites in formiminotransferase-cyclodeaminase may also be feasible for "channeling" between catalytic sites of different enzymes.

5.3 EVOLUTION AND ENZYME ORGANIZATION

The evolution of enzyme activity has been expected to optimize the ratio K_{cat}/K_m, but Keleti and Welch (1984) have proposed that the ratio of V_{max} to K_m, the "kinetic power", is the real measure of the efficiency of an enzyme *in situ*. Evolution of independent enzymes would tend to maximize this quantity, up to the limit of diffusion control, but in regulatory enzymes, the requirements for regulation may prevent the maximization of "kinetic power". In organized multienzyme sequences, the efficiency of metabolic compartmentation depends on the "kinetic power" of the individual enzymes, which in turn is affected by the microenvironment formed by the associated enzymes, i.e. by the increased local concentration of both enzymes and subtrates. The optimization of cell metabolism would require evolutionary improvement of complete multienzyme sequences.

Genetic events which alter the structure of a protein are of two types (MacLachlan, 1980): 1) minor steps - single point mutations or small insertions or deletions usually in loops between elements of secondary structure - may change the specificity of an enzyme or the interaction between subunits but leaves the overall structure of a monomer unaltered and 2) major steps - massive deletions or insertions, frame shifts, termination codon changes, misaligned chromosome crossing over, gene fusion or tandem gene duplication - allow new combinations of existing structure elements. The former generates new members of the

same family of proteins while the latter generates a new protein which may express altered and/or additional properties compared to the original protein. Many multidomain proteins appear to have evolved through genetic rearrangements which combined the structural units from existing simpler proteins, and it has been proposed that multifuctional enzymes evolved in this manner (Schmincke-Ott and Bisswanger, 1980) since the component activities are often catalysed by discrete functional domains. For example, aspartokinase I - homoserine dehydrogenase I from E. coli is a bifunctional enzyme consisting of three domains (Fazel et al., 1983). Analysis of fragments produced by limited proteolysis suggests that one domain is associated with each activity and the third domain is involved in subunit-subunit interactions.

Multifunctional enzymes are more common in eukaryotes and evidence for homology between domains of a eukaryotic multifunctional enzyme and the corresponding prokaryotic monofunctional enzymes has been demonstrated for the arom pentafunctional enzyme from S. cerevisiae (Duncan et al., 1987) and the mammalian and yeast multifunctional fatty acid synthases (Hardie et al., 1986; Schweizer, 1986).

Zalkin and Yanofsky (1982) have shown homology between the α and β chains of the E. coli tryptophan synthase ($\alpha_2\beta_2$) and two regions of the bifunctional yeast tryptophan synthase. They propose that the yeast enzyme consists of fused α and β domains; however, they are in the reverse order from the structural genes for the α and β chains in the E. coli operon. Yanofsky et al. (1987) fused the trp B and trp A genes of E. coli and recovered a fusion protein of the correct size with about

one third the activity of the native enzyme, but with the same substrate affinities as the wild type. The order of the domains in this fusion protein was the opposite of that in the yeast bifunctional tryptophan synthase. Crawford et al. (1987) expressed the yeast bifunctional tryptophan synthase in E. coli and varied the interdomain connecting region. They suggest that the length of the "connector" may be more important than its sequence.

Formiminotransferase-cyclodeaminase may be the result of gene fusion, but because the activities are not found in most prokaryotes, it will be difficult to determine the precursor proteins. The enzyme is expressed at fairly high levels in the livers and kidneys of mammals (Tabor and Wyngarden, 1959), but is not detectable in most other tissues, so its expression must be highly regulated, although the mechanism of this regulation is not yet known. The enzyme appears to have evolved to use the naturally occurring tetrahydropteroylpoly-glutamates found in mammalian liver, and both activities have at least 10-fold greater catalytic efficiency with folylpolyglutamate substrates with four to seven glutamates than with the monoglutamate substrates.

In conclusion, the next step will be to use x-ray crystallography to physically localize the catalytic sites and the polyglutamate binding site within the quaternary structure of formiminotransferase-cyclodeaminase. Once the enzyme has been cloned, site specific mutagenesis can be used to determine the role of the amino acids involved in catalysis, polyglutamate binding and in interactions between subunits. By deleting portions of the polypeptide, it may be possible

possible to define the minimum structure required for expression of each activity (i.e. to produce transferase-active or deaminase-active fragments). To determine if formiminotransferase-cyclodeaminase is part of a higher level of organization of enzymes in liver cells, it will also be of interest to look for association between it and the enzymes of histidine degradation and/or the trifunctional methyleneH_4folate dehydrogenase-methenylH_4folate cyclohydrolase-formylH_4folate synthetase.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

I have investigated the role of the unusual circular octameric quaternary structure of formiminotransferase-cyclodeaminase and its relationship to the two catalytic sites and the polyglutamate binding site, using two independent approaches as summarized below. Previous observations of two types of subunit-subunit interaction suggesting a tetramer of dimers arrangement, and only four high affinity sites for binding folylpolyglutamates to the native octamer raised the possibility that the dimer might be the basic functional unit of this enzyme. The work presented in this thesis, however, leads to the conclusion that the complete octameric structure is required for simultaneous expression of both activities and therefore for "channeling" of folylpolyglutamate substrate between the active sites.

1. Denaturation by urea

i) Octameric formiminotransferase-cyclodeaminase is dissociated by low concentrations of urea to yield either transferase-active or deaminase-active dimers depending on the substrate analogue present. The two dimers appear to isolate different subunit-subunit interactions and in both cases further dissociation leads to loss of activity.

ii) Binding of pteroylpolyglutamate protects both activities and prevents dissociation of the enzyme by 3 M urea. The protection profiles are cooperative with Hill coefficients of about 2. Dissociation of the enzyme by 3 M urea in the presence of folic acid (pteroylmonoglutamate) protects only the deaminase activity, noncooperatively ($h \simeq 1$), and strengthens the subunit-subunit interaction within the dimer.

2. Renaturation from Guanidine hydrochloride

i) Formiminotransferase-cyclodeaminase can be completely renatured by dilution from 6 M guanidine hydrochloride. The rate-limiting step is apparently first order with an activation energy of - 15 kcal/mole and a rate constant of 1.9×10^{-5} s^{-1} at 22°C. A dimeric transferase-active fragment produced by limited proteolysis of the native enzyme by chymotrypsin can also be completely renatured under the same conditions.

ii) In the presence of 1.5 M urea, renaturation is arrested at the level of transferase-active dimers, which reassociate upon dialysis to form the bifunctional octamer. These dimers do not exhibit the much higher affinity for polyglutamate than monoglutamate substrates observed for the native and fully renatured enzyme, however the deaminase dimers produced by dissociation of the enzyme by urea retain the polyglutamate specificity. This suggests that the polyglutamate binding site requires the integrity of the same subunit interface as does the deaminase activity.

To obtain the quantities of pure protein necessary to attempt x-ray crystallography, which is now required to physically localize the active sites and polyglutamate binding site within the tertiary and quaternary structure of this enzyme and to determine whether the sites are in fact formed by the subunit interfaces, I have developed an easier and more reliable method of purifying formiminotransferase-cyclodeaminase from pig liver. This procedure uses chromatography on Matrex ™ Gel Orange A and Heparin-Sepharose columns in the presence of low concentrations of non-ionic detergent (0.1% Triton X-100), which stabilizes the enzyme.

REFERENCES

Albery, W.J. and Knowles, J.R. (1976) Biochemistry 15, 5631-5640.

Almassy, R.J., Janson, C.A., Hamlin, R., Xuong, N.-H and Eisenberg, D. (1986) Nature 323, 304-309.

Anderson, P.M. (1986) Biochemistry 25, 5576-5582.

Anfinsen, C.B. (1973) Science 181, 223-230.

Argos, P. (1987) J. Mol. Biol. 197, 331-348.

Baggott, J.E. and Krumdieck, C.L. (1979) Biochemistry 18, 1036-1041.

Beaudet, R. and MacKenzie, R. (1975) Biochim. Biophys. Acta 410, 252-261.

Beaudet, R. and MacKenzie, R.E. (1976) Biochim. Biophys. Acta 453, 151-161.

Bensadoun, A. and Weinstein, D. (1976) Analyt. Biochem. 70, 241-250.

Bergman, L.W. and Kuehl, W.M. (1979) J. Biol. Chem. 254, 8869-8876.

Bigelow, C.C. (1967) J. Theoret. Biol. 16, 187-211.

Blond, S. and Goldberg, M.E. (1985) J. Mol. Biol. 182, 597-606.

Blond, S. and Goldberg, M. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 1147-1151.

Borschik, I.B., Pestova, T.V., Sklyankina, V.A. and Avaeva, S.M. (1985) FEBS Letters 184, 65-67.

Brandt, D.R., Roche, T.E. and Pratt, M.L. (1983) Biochemistry 22, 2958-2965.

Brandts, J.F., Halvorson, H.R. and Brennan, M. (1975) Biochemistry 14, 4953-4963.

Bülow, L. (1987) Eur. J. Biochem. 163, 443-448.

CaJacob, C.A., Frey, P.A., Hainefeld, J.F., Wall, J.S., and Yang, H. (1985) Biochemistry 24, 2425-2431.

Caperelli, C.A., Benkovic, P.A., Chettur, G. and Benkovic, S.J. (1980) J. Biol. Chem. 255, 1885-1890.

Cate, R.L., Roche, T.E. and Davis, L.C. (1980) J. Biol. Chem. 255, 7556-7562.

Chothia, C. (1974) Nature 248, 338-339.

Chothia, C. (1975) Nature 254, 304-308.

Chothia, C. (1984) Ann. Rev. Biochem. 53, 537-572.

Cichowicz, D.J. and Shane, B. (1987) Biochemistry 26, 504-512.

Coggins, J.R. and Hardie, D.G. (1986) in Multidomain Proteins- Structure and Evolution (eds. D.G. Hardie and J.R. Coggins), Elsevier Science Publishers B.V., The Netherlands, pp. 1-12.

Cohen, G.N. and Dautry-Varsat, A. (1980) in Multifunctional Proteins (eds. H. Bisswanger and E. Schmincke-Ott) John Wiley & Sons, U.S.A., pp. 49-121.

Cohen, L. and MacKenzie, R.E. (1978) Biochim. Biophys. Acta 522, 311-317.

Cook, R.A. and Koshland, D.E. (1969) Proc. Natl. Acad. Sci. 64, 247-254.

Crawford, I.P., Clarke, M., van Cleemput, M. and Yanofsky, C. (1987) J. Biol. Chem. 262, 239-244.

Creighton, T.E. (1977) J. Mol. Biol. 113, 275-293.

Darnall, D.W. and Klotz, I.M. (1972) Arch. Biochem. Biophys. 149, 1-14.

Daubner, S.C., Schrimsher, J.L., Schendel, F.J., Young, M., Henikoff, S., Patterson, D., Stubbe, J. and Benkovic, S.J. (1985) Biochemistry 24, 7059-7062.

Davis, R.H. (1967) in Organizational Biosynthesis (eds. H.J. Vogel, J.O. Lampen and V. Bryson) Academic Press, U.S.A., pp. 303-325.

Delepierre, M., Dobson, C.M., Karplus, M., Poulsen, F.M., States, D.J. and Wedin, R.E. (1987) J. Mol. Biol. 197, 111-130.

Dickerson, R.E. and Geis, I. (1969) The Structure and Action of Proteins, W.A. Benjamin, Inc., U.S.A., pp. 17-21.

Dolgikh, D.A., Gilmanshin, R.I., Brazhnikov, E.V., Bychkova, V.E., Semisotnov, G.V., Venyaminov, S. Yu. and Ptitsyn, O.B. (1981) FEBS Letters 136, 311-315.

Dolgikh, D.A., Kolomiets, A.P., Bolotina, I.A. and Ptitsyn, O.B. (1984) FEBS Letters 165, 88-92.

Drury, E.J. and MacKenzie, R.E. (1977) Can. J. Biochem. 55, 919-923.

Drury, E.J., Bazar, L.S. and MacKenzie, R.E. (1975) Arch. Biochem. Biophys. 169, 662-668.

Duggleby, R.G., Sneddon, M.K. and Morrison, J.F. (1978) Biochemistry 17, 1548-1554.

Duncan, K., Edwards, R.M. and Coggins, J.R. (1987) Biochem. J. 246, 375-386.

Fazel, A., Müller, K., LeBras, G., Garel, J.-R., Véron, M. and Cohen, G.N. (1983) Biochemistry 22, 158-165.

Findlay, W.A. and MacKenzie, R.E. (1987) Biochemistry 26, 1948-1954.

Findlay, W.A. and MacKenzie, R.E. (1988) Biochemistry 27, 3404-3408.

Fisher, H.F. (1964) Proc. Natl. Acad. Sci. 51, 1285-1291.

Fita, I. and Rossmann, M.G. (1985) J. Mol. Biol. 185, 21-37.

Ford, G.C., Eichele, G. and Jansonius, J.N. (1980) Proc. Natl. Acad. Sci. USA 77, 2559-2563.

Fothergill-Gilmore, L.A. (1986) in Multidomain Proteins-Structure and Evolution (eds. D.G. Hardie and J.R. Coggins) Elsevier Science Publishers B.V., The Netherlands, pp. 85-174.

Friedrich, P. (1984) Supramolecular Enzyme Organization Pergamon Press, Oxford, England.

Gaertner, F.H. (1978) T.I.B.S. 3, 63-65.

Garel, J.-R. and Baldwin, R.L. (1973) Proc. Natl. Acad. Sci. USA 70, 3347-3351.

Garel, J.-R. and Dautry-Varsat, A. (1980) Proc. Natl. Acad. Sci. USA 77, 3379-3383.

Ghélis, C. and Yon, J. (1982) Protein Folding, Academic Press, U.S.A. 223-296.

Girg, R., Rudolph, R. and Jaenicke, R. (1981) Eur. J. Biochem. 119, 301-305.

Girg, R., Rudolph, R. and Jaenicke, R. (1983) FEBS Letters 163, 132-135.

Goldberg, M.E. (1985) T.I.B.S. 10, 388-391.

Hardie, D.G. and McCarthy, A.D. (1986) in Multidomain Proteins - Structure and Evolution (eds. D.G. Hardie and J.R. Coggins), Elsevier Science Publishers B.V., The Netherlands, pp. 229-258.

Hardie, D.G., McCarthy, A.D. and Braddock, M. (1986) Biochem. Soc. Trans. 14, 568-570.

Hermann, R., Jaenicke, R. and Rudolph, R. (1981) Biochemistry 20, 5195-5201.

Hermann, R., Jaenicke, R. and Price, N.C. (1985) Biochemistry 24, 1817-1821.

Huang, C.Y., Rhee, S.G. and Chock, P.B. (1982) Ann. Rev. Biochem. 51, 935-971.

Jaenicke, R. (1982) Biophys. Struct. Mech. 8, 231-256.

Jaenicke, R. and Perham, R.N. (1982) Biochemistry 21, 3378-3385.

Jaffe, J.J., Chrin, L.R. and Smith, R.B. (1980) J. Parasitology 66, 428-433.

Kabsch, W. and Rosch, P. (1986) Nature 321, 469-470.

Kabsch, W. and Sander, C. (1983) FEBS Letters 155, 179-182.

Karplus, M. and Weaver, D.L. (1976) Nature 260, 404-406.

Keleti, T. and Welch, G.R. (1984) Biochem. J. 223, 299-303.

Kim, P.S. and Baldwin, R.L. (1982) Ann. Rev. Biochem. 51, 459-489.

Kirschner, K. and Bisswanger, H. (1976) Ann. Rev. Biochem. 45, 143-166.

Knack, I. and Röhm, K.-H. (1981) Hoppe-Seyler's Z. Physiol. Chem. 362, 1119-1130.

Kosen, P.A., Creighton, T.E. and Blout, E.R. (1983) Biochemistry 22, 2433-2440.

Krause, K.L., Volz, K.W., and Lipscomb, W.N. (1985) Proc. Natl. Acad. Sci. USA 82, 1643-1647.

Kurganov, B.I. (1986) J. Theor. Biol. 119, 445-455.

Laemmli, U.K. (1970) Nature 227, 680-685.

Lane, A.N., Paul, C.H. and Kirschner, K. (1984) The EMBO Journal 3, 279-287.

Lin, L.-N. and Brandts, J.F. (1978) Biochemistry 17, 4102-4110.

Lu, Y.-Z, Aiello, P.D. and Matthews, R.G. (1984) Biochemistry 23, 6870-6876.

Luther, M.A., Cai, G.-Z. and Lee, J.C. (1986) Biochemistry 25, 7931-7937.

MacGuire, J.J. and Coward, J.K. (1984) in Folates and Pterins Volume 1 - Chemistry and Biochemistry of Folates. (eds. R.L. Blakley and S.J. Benkovic), John Wiley and Sons, U.S.A. pp. 135-190.

MacKenzie, R.E. (1984) in Folates and Pterins: Volume 1-Chemistry and Biochemistry of Folates (eds. R.L. Blakley and S.J. Benkovic) John Wiley & Sons, U.S.A., pp. 255-306.

MacKenzie, R.E. and Baugh, C.M. (1980) Biochim. Biophys. Acta 611, 187-195.

MacKenzie, R.E., Aldridge, M. and Paquin, J. (1980) J. Biol. Chem. 255, 9474-9478.

Marks, C.B., Naderi, H., Kosen, P.A., Kuntz, I.D. and Anderson, S. (1987) Science 235, 1370-1373.

Matchett, W.H. (1974) J. Biol. Chem. 249, 4041-4049.

Matthews, R.G., Lu, Y.-Z., Green, J.M. and MacKenzie, R.E. (1985) in Proceedings of the Second Workshop on Folyl and Antifolyl Polyglutamates (ed. I.D. Goldman) Praeger Publishers, U.S.A., pp. 65-75.

McCammon, J.A. and Karplus, M. (1983) Accounts of Chemical Research 16, 187-193.

McCarthy, M.P. and Allewell, N.M. (1983) Proc. Natl. Acad. Sci. USA 80, 6824-6828.

McCarthy, A.D., Goldring, J.P.D. and Hardie, D.G. (1983) FEBS Letters 162, 300-304.

McLachlan, A.D. (1980) in Protein Folding (ed. R. Jaenicke) Elsevier/North Holland Biomedical Press, The Netherlands, pp. 79-99.

Mejia, N.R. and MacKenzie, R.E. (1985) J. Biol. Chem. 260, 14616-14620.

Miles, E.W. (1979) Adv. Enzymol. Relat. Areas Mol. Biol. 49, 127-186.

Miller, S., Lesk, A.M., Janin, J. and Chothia, C. (1987) Nature 328, 834-836.

Monod, J., Wyman, J. and Changeux, J.-P. (1965) J. Mol. Biol. 12, 88-118.

Mueller, W.T. and Benkovic, S.J. (1981) Biochemistry 20, 337-344.

Nozaki, Y. and Tanford, C. (1971) J. Biol. Chem. 246, 2211-2217.

Ohgushi, M. and Wada, A. (1983) FEBS Letters 164, 21-24.

Packman, L.C., Stanley, C.J. and Perham, R.N. (1983) Biochem. J. 213, 331-338.

Pain, R.H. (1983) Nature 305, 581-582.

Pain, R. (1987) T.I.B.S. 12, 309-312.

Paquin, J., Baugh, C.M. and MacKenzie, R.E. (1985) J. Biol. Chem. 260, 14925-14931.

Pauling, L., Corey, R.B. and Branson, H.R. (1951) Proc. Natl. Acad. Sci. 37, 205-211.

Pauling, L. and Corey, R.B. (1951) Proc. Natl. Acad. Sci. 37, 251-256.

Privalov, P.L. (1979) Adv. Prot. Chem. 33, 167-241.

Privalov, P.L. (1982) Adv. Prot. Chem. 35, 1-104.

Ptitsyn, O.B. and Finkelstein, A.V. (1980) in Protein Folding (ed. R. Jaenicke) Elsevier/North-Holland Biomedical Press, The Netherlands, pp. 101-115.

Purvis, I.J., Bettany, A.J.E., Santiago, T.C., Coggins, J.R., Duncan, K., Eason, R. and Brown, A.J.P. (1987) J. Mol. Biol. 193, 413-417.

Ramachandran, G.N and Sasisekharan, V. (1968) Adv. Prot. Chem. 23, 283-437.

Reddy, G.P.V. and Pardee, A.B. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3312-3316.

Reddy, G.P.V. and Pardee, A.B. (1982) J. Biol. Chem. 257, 12526-12531.

Remington, S., Wiegand, G. and Huber, R. (1982) J. Mol. Biol. 158, 111-152.

Richardson, J.S. (1980) in Protein Folding (ed. R. Jaenicke) Elsevier/North-Holland Biomedical Press, The Netherlands, pp. 41-52.

Richardson, J.S. (1981) Adv. Prot. Chem. 34, 167-339.

Rios-Orlandi, E.M. and MacKenzie, R.E. (1988) J. Biol. Chem. 263, 4662-4667.

Rios-Orlandi, E.M., Zarkadas, C.G. and MacKenzie, R.E. (1986) Biochim. Biophys. Acta 871, 24-35.

Rudolph, R. and Jaenicke, R. (1976) Eur. J. Biochem. 63, 409-417.

Rossman, M.G. and Argos, P. (1981) Ann. Rev. Biochem. 50, 497-532.

Rossman, M.G., Liljas, A., Brändén, C.-I. and Banaszak, L.J. (1975) in The Enzymes (ed. P.D. Boyer) Vol. 11, Academic Press, U.S.A., pp. 61-102.

Saito, Y. and Wada, A. (1983a) Biopolymers 22, 2105-2122.

Saito, Y. and Wada, A. (1983b) Biopolymers 22, 2123-2132.

Schachman, H.K. (1987) Biochem. Soc. Trans. 15, 772-775.

Scheraga, H.A. (1980) in Protein Folding (ed. R. Jaenicke) Elsevier/ North-Holland Biomedical Press, The Netherlands, 261-288.

Schmincke-Ott, E. and Bisswanger, H. (1980) in Multifunctional Proteins (eds. H. Bisswanger and E. Schmincke-Ott) John Wiley & Sons, U.S.A., pp. 1-29.

Schulz, G.E. and Schirmer, R.H. (1979) Principles of Protein Structure Springer-Verlag New York Inc., U.S.A.

Schweizer, M. (1986) in Multidomain Proteins- Structure and Evolution (eds. D.G. Hardie and J.R. Coggins) Elsevier Science Publishers, The Netherlands, 195-227.

Shane, B. and Stokstad, E.L.R. (1984) in Folates and Pterins. Vol. 1- Chemistry and Biochemistry of Folates (eds. R.L. Blakley and S.J. Benkovic) John Wiley and Sons Inc., U.S.A. pp. 433-455.

Smith, D.D.S. and MacKenzie, R.E. (1983) Can. J. Biochem. Cell Biol. 61, 1166-1171.

Smith, D.D.S. and MacKenzie, R.E. (1985) Biochem. Biophys. Res. Commun. 128, 148-154.

Srere, P.A. (1985) in Organized Multienzyme Systems (ed. G.R. Welch) Academic Press Inc., U.S.A. pp. 1-61.

Srere, P.A (1987) Ann. Rev. Biochem. 56, 89-124.

Srivastava, D.K. and Bernhard, S.A. (1986) Science 234, 1081-1086.

Stoops, J.K. and Wakil, S.J. (1981) J. Biol. Chem. 256, 5128-5133.

Storer, A.C. and Cornish-Bowden, A. (1974) Biochem. J. 141, 205-209.

Strambini, G.B. and Gonnelli, M. (1986) Biochemistry 25, 2471-2476.

Strong, W., Joshi, G., Lura, R., Muthukumaraswamy, N. and Schirch, V. (1987) J. Biol. Chem. 262, 12519-12525.

Tabor, H. and Wyngarden, L. (1959) J. Biol. Chem. 234, 1830-1846.

Tan, L.U.L. and MacKenzie, R.E. (1977) Biochim. Biophys. Acta. 485, 52-59.

Tan, L.U.L. and MacKenzie, R.E. (1979) Can. J. Biochem. 57, 806-812.

Thieme, R., Pai, E.F., Schirmer, R.H. and Schulz, G.E. (1981) J. Mol. Biol. 152, 763-782.

Traut, T.W. (1986) Mol. Cell. Biochem. 70, 3-10.

Uyeda, K. and Rabinowitz, J.C. (1967) J. Biol. Chem. 242, 24-31.

Vaucheret, H., Signon, L., Le Bras, G. and Garel, J.-R. (1987) Biochemistry 26, 2785-2790.

Villar, E., Schuster, B., Peterson, D. and Schirch, V. (1985) J. Biol. Chem. 260, 2245-2252.

Waddle, J.J., Johnston, T.C. and Baldwin, T.O. (1987) Biochemistry 26, 4917-4921.

Wasserman, G.F., Benkovic, P.A., Young, M. and Benkovic, S.J. (1983) Biochemistry 22, 1005-1013.

Waugh, D.F. (1954) Adv. Prot. Chem. Vol. IX, 326-437.

Weber, J.P. and Bernhard, S.A. (1982) Biochemistry 21, 4189-4194.

Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.

Welch, G.R. (1977) Prog. Biophys. Mol. Biol. 32, 103-191.

Wetlaufer, D.B. (1981) Adv. Prot. Chem. 34, 61-92.

Wu, T.-L. and Reed, L.J. (1984) Biochemistry 23, 221-226.

Yanofsky, C., Paluh, J.L., van Cleemput, M. and Horn, V. (1987) J. Biol. Chem. 262, 11584-11590.

Yao, Q-Z., Tian, M. and Tsou, C.-L. (1984) Biochemistry 23, 2740-2744.

Zalkin, H. and Yanofsky, C. (1982) J. Biol. Chem. 257, 1491-1500.

Zamyatnin, A.A. (1972) Prog. Biophys. Mol. Biol. 24. 107-123.

Zetina, C.R. and Goldberg, M.E. (1982) J. Mol. Biol. 157, 133-148.

Zettlmeissl, G., Rudolph, R. and Jaenicke, R. (1982) Biochemistry 21, 3946-3950.