THE FUNCTION OF TETRAHYDROPTEROYLPOLYGLUTAMATES WITH FORMIMINOTRANSFERASE-CYCLODEAMINASE

by

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ABSTRACT

Formiminotransferase (EC 2.1.2.5)-cyclodeaminase (EC 4.3.1.4) from pig liver catalyzes two sequential tetrahydrofolate-dependent reactions. With derivatives of the tetrahydrofolate substrate having 4,5, or 6 glutamyl residues, the formiminotetrahydropteroylpolyglutamate formed by the transferase activity is preferentially transferred (channeled) to the deaminase site rather than released into the solution. This channeling is essentially complete with the pentaglutamate derivative. The enzyme has highest affinity for the hexaglutamate as measured by K_d and K_m, but does not show specificity for a given polyglutamate as measured by V_m/K_m. The results indicate that steric length of the polyglutamate chain, not simply affinity, is critical for optimal channeling. Binding studies demonstrate four sites for the binding of tetrahydropteroylpolyglutamates to the native octamer, suggesting the formation of sites between subunits. The transferase and deaminase sites are kinetically independent, but share a common polyglutamate subsite. The results support the concept that the polyglutamate chain anchors the tetrahydrofolate molecule during its transfer between active sites.

RESUME

La formiminotransférase (EC 2.1.2.5)-cyclodésaminase (EC 4.3.1.4) isolée du foie de porc catalyse deux réactions séquentielles qui utilisent le tétrahydrofolate. Avec les dérivés du substrat tétrahydrofolate ayant 4,5, ou 6 résidus glutamyles, le formiminotétrahydroptéroylpolyglutamate formé par l'activité transférase est préférentiellement transféré au site désaminase plutôt que libéré dans la solution. Ce transfert est essentiellement complété avec le dérivé pentaglutamate. L'affinité de l'enzyme, telle que mesurée par les valeurs de K_d et de K_m, est maximale avec l'hexaglutamate. Par contre, les valeurs du rapport V_m/K_m n'indiquent pas de préférence pour un polyglutamate particulier. Les résultats indiquent que la longueur même de la chaîne polyglutamate; et non la simple affinité, est une facteur déterminant de l'efficacité du transfert. Les résultats d'études de liaison montrent l'existence de quatre sites liant les tétrahydroptéroylpolyglutamates à l'octamère d'origine, suggérant la formation de sites entre les sous-unités. Les sites transférase et désaminase sont cinétiquement indépendants mais partagent un site commun pour la liaison de la chaîne polyglutamate. Les résultats s'accordent bien avec la notion d'une chaîne polyglutamate servant d'ancre à la molécule tétrahydrofolate lors de son transfert entre les sites actifs.

DEDICATION

A ma mère Madeleine Ferragne, pour son courage joyeux.

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FORWARD

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:

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This format for thesis preparation has been approved by the Department of Biochemistry. Each chapter has its own numeration of references, figures and tables. The references cited in the General Introduction (Chapter 1) and General Discussion (Chapter 5) are compiled at the end of the thesis.

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PUBLICATION OF THE WORK PRESENTED IN THIS THESIS

Preparation of tritium labeled tetrahydropteroylpolyglutamates of high specific radioactivity. J. Paquin, C.M. Baugh and R.E. MacKenzie (1985) Anal. Biochem. 145, in press.

Tetrahydrofolate polyglutamates and the function of formiminotransferase-cyclodeaminase. J. Paquin and R.E. MacKenzie (1985) in Folyl- and Antifolyl Polyglutamates (Goldman, I.D., ed.) Praeger, in press.

Formiminotransferase-cyclodeaminase from pig liver. 1. Binding of tetrahydropteroylpolyglutamates to the native octamer. Joanne Paquin, Charles M. Baugh and Robert E. MacKenzie. Submitted to J. Biol. Chem.

Formiminotransferase-cyclodeamianse from pig liver. 2. Channeling between the active sites. Joanne Paquin, Charles M. Baugh and Robert E. MacKenzie. Submitted to J. Biol. Chem.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

A. DEVELOPMENT OF TECHNIQUES

1. H_4Pteroylpolyglutamates were prepared using a malic enzyme dependent NADPH-generating system. This system enabled us to obtain the radiolabeled polyglutamate derivatives of high specific radioactivity required in binding studies. The addition of a chromatographic step involving the use of immobilized boronate permitted the removal of NADPH from labeled H_4folates. This procedure could also be used to purify unlabeled derivatives.

2. FormiminoH_4folate free from other folate derivatives was prepared using a method that was also applicable to the polyglutamate derivatives. The use of pure and stable substrate permitted the accurate measurement of the cyclodeaminase activity, and was also necessary to study the kinetic behavior of mixtures of mono- and pentaglutamate derivatives in the transferase-deaminase system.

3. The K_m values of H_4pteroylpolyglutamates with the transferase activity were very low and could not be determined with the spectrophotometric assay. The fluorescent properties of 5,10-methenylH_4-folate were used to develop a much more sensitive assay of the transferase reaction. The components of the fluorometric assay were carefully controlled to obtain reliable measurements of the fluorescence of the methenyl product.

B. FUNCTION OF TRANSFERASE-DEAMINASE

1. The results of inhibition and kinetic studies confirm the existence of separate catalytic sites on the bifunctional polypeptide for the transferase and deaminase activities. (6R)-H_4Folate is a strong inhibitor of deaminase but inhibits poorly the transferase activity.

It is possible to increase the rate of production of final methenyl-H_4folate by adding formiminoH$_4$folate intermediate to the system saturated with substrates of the transferase. This increase is apparent only with H_4folate/formiminoH$_4$folate combinations in which at least one of the folate derivatives is a monoglutamate.

2. Each of the H_4pteroylpolyglutamates binds to four sites on the native octamer. These sites are likely formed at one type of subunit interface. Their high affinity for the polyglutamate derivatives of H_4folate suggests that these sites bind the polyglutamate structure of the folate ligands. The number of these sites and the results of kinetic studies using H_4pteroylpentaglutamate/formiminoH$_4$pteroylpentaglutamate pairs support the existence of a single polyglutamate subsite per pair of transferase-deaminase activities. The large decrease in relative free energy on binding the fourth glutamate of H_4pteroylpolyglutamates indicates that this residue is particularly important in anchoring the folate molecule on the enzyme through interaction with a polyglutamate site. The calculated length of a H_4pteroylpolyglutamate molecule from the α-carboxyl group of its fourth glutamate residue to position 5 of the pteridine ring suggests a distance of 20-25Å between a polyglutamate binding subsite and an active site. The distance between transferase and deaminase catalytic sites could be considerable within this radius.

3. The affinity of the enzyme for H_4folate ligands increases with the number of added glutamyl residues to a total of six; addition of a seventh residue results in a slight decrease in affinity. Preference for the hexaglutamate derivative is shown by comparing the

values of K_d obtained from the binding of H_4pteroyl(glutamate)$_n$ (n = 1,3,4,5,6,7) ligands to the enzyme, and the values of K_m exhibited by each activity for their respective H_4pteroyl-(glutamate)$_n$ (n = 1,3,4,5,6,7) substrates. V_m of deaminase is minimal with formiminoH_4pteroyl(glutamate)$_6$ substrate suggesting that the release of methenylH_4pteroyl(glutamate)$_6$ product is slower than that of other polyglutamate derivatives. The catalytic efficiency of both activities (V_m/K_m) is much greater with substrates containing four or more glutamates, but this property does not differentiate between 4,5,6 and 7 glutamates.

4. Channeling of the formiminoH_4pteroylglutamate intermediate between the transferase and deaminase catalytic sites occurs when H_4pteroyl-(glutamate)$_n$ having n = 4,5,6 or 7 is used as substrate. Since only these ligands show high affinity for the polyglutamate binding site, the results support the hypothesis that the polyglutamate chain acts as an anchor for the transfer of the pteroyl moiety between sites. The specificity for channeling among the folylpoly-glutamates ($5 > 4 \simeq 6 > 7$) does not correlate with affinity ($6 > 7 \simeq 5 > 4$) nor with catalytic efficiency ($4 \simeq 5 \simeq 6 \simeq 7$). This suggests that the steric length, not simply affinity, of the polyglutamate chain is critical for complete channeling.

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LIST OF ABBREVIATIONS

GAR:	glycinamide ribotide
AICAR:	aminoimidazole carboxamide ribotide
H_2folate:	7,8-dihydrofolate
H_4folate:	5,6,7,8-tetrahydrofolate
PteGlu:	pteroylglutamate; pteroylmonoglutamate; folate
$PteGlu_n$:	pteroyl(glutamate)$_n$; pteroylpolyglutamate
$H_4PteGlu_n$:	tetrahydropteroyl(glutamate)$_n$
5-HCNH-$H_4PteGlu_n$:	5-formimino-tetrahydropteroyl(glutamate)$_n$
AADP:	3-aminopyridine adenine dinucleotide phosphate

CHAPTER 1

GENERAL INTRODUCTION

The potential for the organization of the enzyme activities of various metabolic pathways is greater than has actually been demonstrated to date. Organization has been observed for several of the enzymes found in folate-mediated metabolism. This thesis deals with the properties of one example of such organization. Formiminotransferase-cyclodeaminase is a bifunctional enzyme catalyzing two sequential folate-mediated reactions in mammals. The covalent linkage between the two activities represents one type of enzyme-enzyme association encountered in cells. Although the advantages resulting from the different associations of enzymic activities in this metabolism are just beginning to be investigated, it is thought that the naturally-occurring polyglutamate forms of folate coenzymes may have a special significance with these enzyme associations.

The topics of enzyme organization and of folate metabolism, including the roles of the naturally-occurring polyglutamate derivatives, as well as a review of the properties of the bifunctional transferase-deaminase will be presented to provide the context in which the objectives of this thesis can be outlined.

1.1 ENZYME ORGANIZATION

Living cells certainly are no longer considered as sacs containing dispersed enzymes catalyzing reactions using freely-diffusing metabolites. Evidence is accumulating which suggests that even the cytoplasm of cells is highly organized with their various enzyme activities associated into different types of multienzyme systems. Welch (1) has extensively reviewed the historical development of this concept of an organized

cellular metabolism through collection of experimental findings and establishment of coherent hypotheses. These hypotheses have considered the potential advantages of enzyme organization in terms of thermodynamic, kinetic and evolutionary criteria. The relation between these criteria has been further developed in more recent publications (2,3). The types and advantages of enzyme organization presented in this section have been taken from Welch's treatise (1) and from the very concise summary written by Gaertner (4). Recently, Wombacher (5) has reviewed current studies on molecular compartmentation delineated by the formation of multienzyme systems. The examples cited in this section are not intended to exhaustively cover the literature on the topic of enzyme organization but rather to illustrate different features. Examples were chosen from the reviews mentioned or from more recent publications in the field.

1.1.1 Types of enzyme organization

Three main types of physical interaction between enzyme activities have been reported. Multifunctional proteins result from the covalent linkage between autonomous enzyme activities (1,4). A multifunctional protein is thus formed by a single type of polypeptide capable of catalyzing more than one enzyme reaction. Examples and properties of this type of association have been reviewed by Bisswanger and his co-workers (6,7). The mammalian fatty acid synthase is composed of two identical polypeptide chains, each containing the seven catalytic activities required for palmitate synthesis as well as an acyl carrier site (8,9). Five enzyme activities catalyzing sequential steps in the biosynthesis of the aromatic amino acids in Neurospora are on a single polypeptide (10,11). The native protein exists as an homodimer. A very

simple multifunctional protein is the monomeric bifunctional phosphoribosylanthranilate isomerase-indoleglycerolphosphate synthase from E. coli (12). The multifunctional enzymes mentioned catalyze consecutive metabolic steps, but covalent association between non-sequential activities has also been reported. Aspartokinase I-homoserine dehydrogenase I constitutes the classical example of this type (6). Three non-sequential steps in the histidine biosynthetic pathway are catalyzed by a trifunctional protein (13).

The association of different enzyme activities by non-covalent forces results in the formation of multienzyme complexes (1,4). The activities which are found on different polypeptides can usually copurify if the interactions between subunits are strong. This is the case for the bacterial (14) and mammalian (14,15) pyruvate dehydrogenase complexes which are formed by the aggregation of several copies of three catalytic protein components in non-stoichiometric amounts. The mammalian complex contains also two regulatory components (14,16). The pyruvate dehydrogenase component of the mammalian complex was found to bind to sites of two different affinities on the transacetylase core: K_d values of 10^{-11} and 10^{-8}M are reported for the high- and low-affinity sites respectively (17). The E. coli tryptophan synthase complex catalyzes two activities which reside on separate polypeptides. It has an $\alpha_2\beta_2$ quaternary structure, and the upper limit for the dissociation constant of this complex has been estimated to be 10^{-8}M (18). Copurification of weakly associated enzyme activities is more difficult to achieve. Such interactions have been reported to exist between glycolytic enzymes (19,20), and among the Krebs' cycle enzymes in mitochondria (21,22). The strength of non-covalent interactions may be affected by the type of ligand,

either coenzyme or effector, bound to the complex (23,24), the protein concentration (23), ionic strength and pH (25,26). The dissociation constant of complex formed *in vitro* by the pig muscle glyceraldehyde-3-phosphate dehydrogenase and aldolase has been determined, and a value of 10^{-6}M is reported (25). Non-covalent aggregation of different enzyme activities can also occur transiently during the cell cycle. A clear example is the association into a multienzyme complex of several activities responsible for the synthesis of DNA in mammalian cells during the S-phase (27) of the cell cycle. It is possible that some specific enzyme-enzyme interactions that occur *in vivo* may not be isolatable as such *in vitro*. Demonstration of physiologically relevant interactions of this type is currently very difficult to achieve.

Enzymes can also be associated with structural elements in the cell (1,4,5). Certain proteins are structurally integrated in the membrane (e.g. electron transport system of the respiratory chain), but other soluble enzymes can possibly form a reversible, specific association with the membrane. The binding of several enzymes of the citric acid cycle to the inner mitochondrial membrane has been investigated (28-30). In addition, some enzymes of fatty acid β-oxidation were also found to interact with this membrane (31). Srere and his co-workers suggested that, in mitochondria, the enzymes of the Krebs' cycle, the activities of the β-oxidation of fatty acids, and the components of the electron carrier system can form large multienzyme systems *in vivo* (29,32). Their hypothesis was based on the results of kinetic and binding studies obtained in their laboratory as well as the results of electron microscopy studies reported by other investigators. Association of certain glycolytic enzymes with the erythrocyte membrane has been

reported (5,33,34), as well as with structural proteins in muscle (35-37).

The sequence "separate proteins→multienzyme complexes→multifunctional proteins" was first postulated to explain the appearance of multifunctional polypeptides in the course of evolution (6). The finding of more examples of these types of multienzyme systems combined with the characterization of these systems, and some genetic considerations suggest that separate activities can evolve either into multienzyme complexes or into multifunctional proteins in response to various biological requirements (7,38). It is also proposed that enzyme organization is an intermediate step in the process of evolution from uniform cytoplasm with separate enzyme activities to a system that is physically and functionally structured by the presence of membrane-delineated organelles (1,5). The mechanisms resulting in the formation of multifunctional proteins comprise gene duplication, gene translocation, gene fusion, and direct covalent linkage of polypeptides (6,7). Mutational modification of substrate or effector binding sites has also been proposed as a possible mechanism for the acquisition of additional activities by bacterial enzymes (39). When the components of a multifunctional protein are found in autonomously folded domains of the polypeptide chain, and when the molecular weight of the multifunctional polypeptide is consistent with the conservation of the individual molecular weight of the separate components during evolution, it is generally thought that multifunctionality arose by gene fusion.

1.1.2 Advantages of enzyme organization

The physical association of enzyme activities may confer two main functional advantages to the multienzyme systems: the compartmentation of

metabolic intermediates and the possibility of coordinate effects (1,4).

Compartmentation or metabolic channeling describes the phenomenon by which the product of the first reaction in a metabolic sequence does not equilibrate in the medium but is preferentially transferred to the next active site. The molecular mechanisms responsible for the confinement of intermediates in the vicinity of active sites are not understood in most instances. Some examples for which a mechanism has been proposed will be reported in the following section. Such a compartmentation which maintains high local concentration of intermediates at the active sites reduces the concentration of free metabolites in the cell (1,4). This feature preserves the solvent capacity of the cell and prevents wasteful accumulation of intermediates. It protects labile intermediates and prevents interference with coexisting and competing pathways (1,4).

Compartmentation may increase the catalytic efficiency of a given pathway through a reduction of the transit and/or the transient times (1,4). The transit time is the time required for the diffusion of a metabolite from one active site to the next. Although the diffusion of intermediates is not considered as a potential rate-limiting step in the function of a metabolic pathway, it is suggested that the transit time could be reduced by decreasing the distance between active sites (1,4). Whether a smaller distance facilitates the diffusion process has been recently questioned (40). However, in some membrane-bound systems, compartmentation has the potential to reduce the transit time (1,4). For example, the long chain acyl-CoA intermediates of the fatty acid β-oxidation pathway would hardly diffuse in the concentrated protein solution of the mitochondrial matrix (31). If the intermediate substrates are confined to associated enzyme activities in a given pathway,

they accumulate within smaller pools. This may reduce the transient time (1,4), that is the time required by the system to readjust to a new steady state. The Neurospora arom complex has been shown to display this property in vitro (41).

Compartmentation of intermediates has been shown to occur in several systems (1,4,6,7) using both in vivo (27,42) and in vitro studies (41,43-47). An elegant in vivo study has been done by Davis in 1967 (42) using mutant strains of Neurospora. He demonstrated that the carbamyl phosphate intermediates produced by two forms of carbamyl phosphate synthetase, one which participates in pyrimidine synthesis, and the other in the arginine synthetic pathway, do not mix as a common pool. They are channeled within their respective pathway through association of the carbamyl phosphate synthetase$_{pyr}$ with aspartate transcarbamylase in the pyrimidine pathway, and through association of the carbamyl phosphate synthetase$_{arg}$ with ornithine transcarbamylase in the arginine pathway (42). His work is often cited as a classical example of channeling, and also historically as the first important contribution to the development of the concept of channeling. In vitro, evidence for channeling can be obtained by comparing the time courses of appearance of products in the coupled assays to the time courses expected for non-interacting proteins (41,44-49). Channeling can also be examined by the approach involving exchange between labelled and unlabelled intermediates (50,51). These approaches have been used to demonstrate channeling of indole in the multifunctional tryptophan synthase of Neurospora (43).

Channeling has been demonstrated not to occur within the monomeric bifunctional phosphoribosylanthranilate isomerase-indoleglycerolphosphate

synthase from E. coli (52), or within the dimeric bifunctional chorismate mutase-prephenate dehydratase from the same organism (53). The bifunctional aspartokinase I-homoserine dehydrogenase I which catalyzes two non-sequential reactions also does not channel intermediates, and the advantage of this association is explained rather by coordinate regulation (1,4,54).

The association of functionally-related enzyme activities within a multienzyme system offers an efficient means to coordinate conformational and regulatory effects (1,4). Each activity of the system has the potential to be activated or inhibited by a single effector-ligand binding at a single site. Threonine is a common feedback inhibitor of aspartokinase I-homoserine dehydrogenase I (1,4). Four of the five activities of the arom complex are activated by binding of the first substrate (4). The binding of a single effector-ligand can also influence the conformation and/or the state of aggregation of the protein components of a multienzyme system [arom complex (4); tryptophan synthase (1); replitase multienzyme complex (55)]. The protein-protein interaction per se can also modify the intrinsic catalytic efficiency of some individual components of the multienzyme system (1,4). For example, the interaction between the α-ketoglutarate dehydrogenase complex and succinate thiokinase decreases the K_m of each activity for one of their substrates (22). Changes in K_m and V_m values of associated enzyme activities of a given pathway can decrease the transient time of the overall sequence, and can effect a compartmentalization of intermediates because they are efficiently used at lower concentrations (44).

Other advantages are postulated for enzyme-enzyme associations according to the type of association involved. The regulation of

synthesis/turnover and of aggregation is simplified with multifunctional proteins because a smaller number of separate polypeptides is handled (1,6,7). Covalent and strong non-covalent interactions could result in optimal orientation of the active sites for catalytic efficiency (1,6,7). Weaker or transient associations offer the means for a versatile and dynamic control of metabolic processes (1,21,23,56,57).

1.1.3 Examples of channeling mechanisms

Davis (42) has proposed two models to explain channeling of intermediates in multienzyme systems. The "surface model" in which an intermediate is confined or adsorbed at the surface of the enzyme system by some means requires specific kinetic conditions for efficient channeling of intermediates between the first and the second active sites: the second site must exhibit high affinity for the intermediate and must not be saturated with it (42). This model was suggested to be applicable to enzyme systems in which intermediates are tightly or covalently bound to the protein (42). When the capacity of the active sites cannot accommodate all molecules of intermediate produced, the intermediate can still be trapped within the multienzyme system depending upon the architecture of the assembled protein components. This alternate possibility was described as the "compartment model". The two possibilities can appear indistinguishable and may be grouped as "compartment" models (42). Later, Duggleby et al. (53) conceptualized a general model to describe which kinetic features in a pair of coupled enzyme reactions will influence the partitioning of the intermediate between its release in the medium or its direct transfer (channeling) to the second site.

Experimentally, channeling has been observed with several different types of enzyme systems. The clearest mechanisms of channeling are those

where covalent linkage of intermediates is involved. The transfer of covalently-linked intermediates have been highly investigated with fatty acid synthase, and the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase multienzyme systems. The animal fatty acid synthase is an homodimer and each subunit contains seven active sites and an acyl carrier site. One cycle through 6 active centers catalyzes the condensation of acetyl-CoA and malonyl-CoA; subsequent malonyl-CoA molecules are added sequentially for each additional cycle until the acyl chain has an appropriate length. The seventh activity, a thioesterase, is responsible for the termination of the growing chain and for the release of the fatty acid. The acyl intermediates formed during the synthetic process are covalently attached to an acyl carrier arm which is responsible for their transfer to the different catalytic sites. This arm is a phosphopantetheine group attached itself by a phophodiester linkage to a specific amino acid of each polypeptide. The dimeric structure is the active form for the complete process since the activity of the β-ketoacyl synthase component requires the juxtapositioning of thiol groups from each subunit (58). Moreover, the two subunits are arranged in a "head-to-tail" fashion, and one fatty acid synthesizing unit is actually formed by the interaction of one-half of a subunit with the complementary half of the other subunit (59). Details of the proposed mechanism of this enzyme are given by Wakil and his co-workers (58-60), and by Hammes' group (61,62), and some aspects have been reviewed by Kumar (63). A similar mechanism is proposed for the yeast fatty acid synthase which possesses an $\alpha_6\beta_6$ structure (64). Similarly to the acyl carrier arm mentioned above, a biotin-"arm" would serve as a carboxyl-carrier in reactions catalyzed by carboxylases (65). The lipoyl moieties of the pyruvate dehydrogenase

multienzyme system also offer sites for covalent attachment of intermediates and act as mobile arms for the transfer of intermediates between sites. Some interesting features are exhibited by this system. The three activities of the system are on separate polypeptides and the resulting aggregated structure may provide a less rigid arrangement of the active sites than the covalent linkage between the catalytic centers of the fatty acid synthase (7). However, the organization of the lipoyl groups in this system has presumably been developed to ensure efficient coupling between active sites (7). The sites of attachment of the lipoyl moieties are on mobile protruding regions of the acetyltransferase subunits (66,67) which form the core of the complex. This mobility of the lipoyl groups and the capability of a given lipoyl group to interact with adjacent lipoyl moieties (68-70) suggested that the lipoyl moieties form a network which serves as a "relay system" for the transfer of intermediates from one type of active site to another (68,70). Such a network of lipoyl groups is also proposed for the mechanism of α-ketoglutarate dehydrogenase (68,70,71). However, the mobility of protein segments which attach the lipoyl groups appear more restricted (71).

For many years the channeling of indole in the tryptophan synthase catalyzed reactions has been known to occur, as indole has not been detected as a free intermediate (72). Indole is a tightly enzyme bound intermediate but is exchangeable with exogenous compound (50). Tryptophan synthase from E. coli is an $\alpha_2\beta_2$ bienzyme complex catalyzing the formation of indole from indoleglycerolphosphate (by subunit α), and the subsequent condensation of serine and indole (by subunit β_2) to yield tryptophan. The assembly of the $\alpha_2\beta_2$ structure is complex as revealed by X-ray scattering (73) and by fluorescence and hydrodynamic studies (74).

The results of fluorescence studies suggested (74) that the active site producing indole and the active site utilizing it form two separate active sites. Cross-linking studies also support this view of two separate indole binding sites (75). It has been suggested (74) that the subunits form an internal cavity through which indole migrates from one active site to the other. The α and β domains are located on a single polypeptide chain in Neurospora (43). It is proposed that indole is also channeled between two distinct indole binding sites in this system (43,50,72).

The formation of a dimer by the mammalian bifunctional UMP synthase appeared to be required for activation of the decarboxylase reaction and for the channeling of OMP intermediate between the two active sites (76). To account for this observation and for the results of sedimentation studies, Traut has suggested a role for the quaternary structure of the protein (76). According to the model he proposed, the dimeric protein sequesters the OMP-intermediate within a cleft formed by the juxtaposition of the first active site of one subunit and the second active site of the other subunit (76).

Association of physically separate enzymes can exist potentially under certain conditions, and result in metabolic advantages. The structural change in phosphoglycerate kinase caused by the binding of the intermediate 1,3-diphosphoglycerate is suggested to be involved in the binding of kinase to glyceraldehyde-3-P dehydrogenase (77). The formation of a "heterologous dimer" permits the transfer of the intermediate between the two activities via an enzyme-substrate-enzyme complex (77). The mechanism of this transfer is still hypothetical. The binding of 3-phosphoglycerate, the substrate of the kinase activity, increases the

transfer of the intermediate to the dehydrogenase site by possible conformational change of the protein(s) (77).

The channeling models presented above involve the transfer of intermediate between separate active sites. Examples of two reactions at a single site are known. The prephenate intermediate is partially channeled in the bifunctional protein chorismate mutase-prephenate dehydrogenase from Aerobacter aerogenes (78). The results of kinetic and inactivation studies were consistent with the occurrence of both reactions at a single active site (78). The existence of a common active site thus allows the second reaction to process the intermediate as it is produced by the first reaction. A common active site model was proposed for the mutase-dehydrogenase enzyme on the basis of the experimental data and theoretical considerations for the structures of the transition states (79). In the transition states, the substrates interact with the enzyme through two ionic bonds involving their carboxyl groups, and with the nucleotide coenzyme through a hydrophobic bond. In the ground state, the hydrophobic bond and only one ionic bond are maintained. In this model, the transitional structures could thus be stabilized by means of an additional bond (79). Some degree of overlapping between active sites was also observed with the bifunctional enzyme from E. coli (80). A similar overlap has been postulated for two folate-dependent activities of a mammalian trifunctional protein (section 1.2.2).

In summary, enzyme systems can thus provide different molecular strategies for the compartmentation of intermediates. In some instances, the channeling of an intermediate has been suggested to have a special physiological or metabolic significance (section 5.2).

1.2 FOLATE METABOLISM

1.2.1 Overview of folate-mediated reactions

Folate and its derivatives are essential coenzymes in several metabolic areas: for example, biosynthesis of purine nucleotides, methionine, and thymidylate; degradation of serine, glycine, histidine, and purines. It is not within the scope of this presentation to undertake a detailed analysis of the folate-dependent pathways, but a very general overview will be given. Folate metabolism is complex and the reader is referred to (81) for an excellent and up-to-date review of different aspects of this metabolism.

Many of the folate-dependent reactions are common to most cells, but some of these reactions are species and/or tissue specific. Figure 1 presents a composite picture of folate-mediated reactions that occur in procaryotes and eucaryotes. No single species carries all these reactions. For example, only higher species utilize folate derivatives in the degradation of histidine; certain bacteria but not mammals can generate one-carbon units from the degradation of purines. The central theme of the diagram is the interconversion of a pool of one-carbon tetrahydrofolate derivatives between different oxidation states of the one-carbon substituent. These interconversions occur in all cells. The degradative pathways which provide the one-carbon units are drawn as feeding downward into this pool. In general, serine and glycine are the most important sources of one-carbon units (82). The biosynthetic routes utilizing one-carbon units are indicated from below the central pool.

The active carrier of the one-carbon units is tetrahydrofolate, the structure of which is shown on Figure 2. Hydrogenation of folate at

FIGURE 1

Pathways of folate-mediated one-carbon transfer reactions. This figure has been kindly provided by R. E. MacKenzie and taken from his recent review of the generation and interconversion of substituted tetrahydrofolates (82). The letters refer to the following enzyme activities: (A) 5,10-methylenetetrahydrofolate dehydrogenase; (B) 5,10-methenyltetrahydrofolate cyclohydrolase; (C) 10-formyltetrahydrofolate synthetase; (D) formiminoglutamate:tetrahydrofolate 5-formiminotransferase; (E) 5-formiminotetrahydrofolate cyclodeaminase; (F) 10-formyltetrahydrofolate dehydrogenase ($NADP^+$-dependent); (G) 10-formyltetrahydrofolate hydrolase; (H) 5,10-methylenetetrahydrofolate reductase; (I) serine hydroxymethyltransferase; (J + K) GAR transformylase and AICAR transformylase, two enzyme activities of the purine biosynthetic pathway which require 10-formylH_4folate as cofactor. "$H_4PteGlu_n$" represents the polyglutamate forms of tetrahydrofolate coenzyme ($H_4PteGlu$).

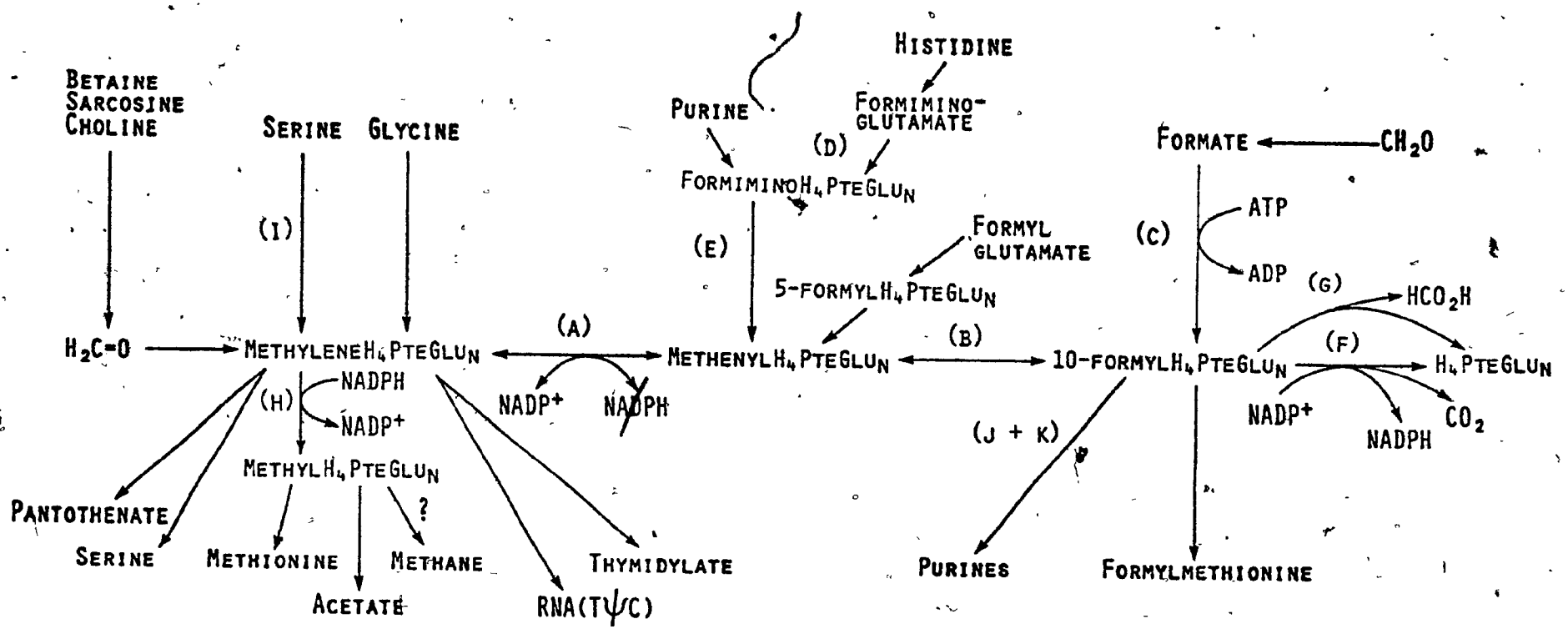
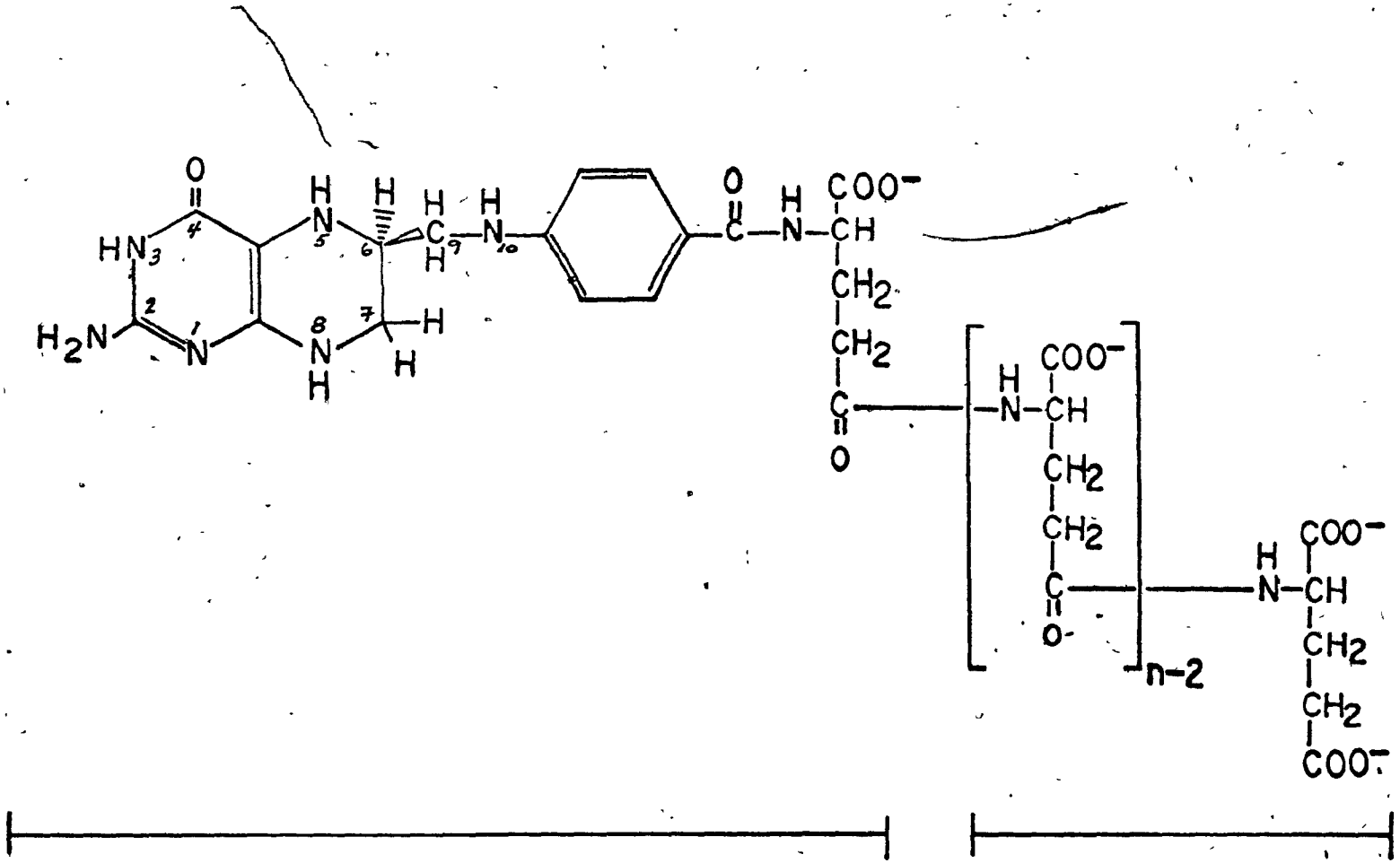

BETAINE
SARCOSINE
CHOLINE
SERINE
GLYCINE
PURINE
HISTIDINE
FORMIMINO-GLUTAMATE
(D)
FORMIMINOH4PTEGLUN
FORMATE
CH2O
(I)
(E)
FORMYL GLUTAMATE
(C)
ATP
ADP
5-FORMYLH4PTEGLUN
(G)
HCO2H
H2C=O
METHYLENEH4PTEGLUN
(A)
METHENYLH4PTEGLUN
(B)
10-FORMYLH4PTEGLUN
(F)
H4PTEGLUN
NADPH
NADP+
NADP+
NADPH
NADP+
NADPH
CO2
(H)
NADPH
NADP+
METHYLH4PTEGLUN
(J + K)
PANTOTHENATE
SERINE
METHIONINE
METHANE
?
ACETATE
THYMIDYLATE
RNA(TψC)
PURINES
FORMYLMETHIONINE

FIGURE 2

Structure of tetrahydrofolate. The natural one-carbon substituents are: 5-methyl(-CH_3); 5,10-methylene(-CH_2-); 5,10-methenyl(=CH-); 5-formyl(-CHO); 10-formyl(-CHO); and 5-formimino(-CH=NH). The polyglutamate forms are also represented.



(6S)-5,6,7,8-H_4PTEROYLGLUTAMATE

γ-LINKED GLUTAMYL RESIDUES

positions 5,6,7 and 8 generates an asymmetric center at carbon 6. The ℓ-isomer is the utilized coenzyme which corresponds to the S-configuration for unsubstituted tetrahydrofolate. The one-carbon substituents are on nitrogen atoms 5 or 10, or form a bridge between the two nitrogens. The polyglutamate derivatives of tetrahydrofolates are the naturally-occurring forms of the coenzymes in cells, and will be discussed further (section 1.2.3).

Which mechanisms govern the production and utilization of one-carbon units is not well understood, especially in mammals. Silber and Mansouri (83) have presented a general scheme for induction/repression and feedback inhibition of folate enzymes in bacteria. A model has been proposed by Krebs and his co-workers for the mammalian system (84,85). According to this model, methionine would be the modulating factor because it could activate directly or indirectly the 10-formylH_4folate dehydrogenase activity, and its S-adenosyl derivative is a potent inhibitor of mammalian methyleneH_4folate reductase. The possible activation of the 10-formyltetrahydrofolate dehydrogenase by methionine arose from the observation that addition of methionine in isolated hepatocytes increased the rate of production of CO_2 from formate and histidine (85). However, the activity of the purified enzyme is not affected by methionine and S-adenosyl methionine (82,85), thus excluding the possibility that these metabolites could have a direct effect on the enzyme activity. This enzyme presumably regenerates H_4folate when production of the one-carbon H_4folate derivatives exceeds biosynthetic needs (82,85). In this context, inhibition of the 10-formyldehydrogenase by its H_4folate product (86) could play a role in the regulation of the enzyme activity in vivo (82). It also appears that most of the folate dependent enzyme activ-

ities are susceptible to inhibition by most folate compounds (87). Two other important aspects of folate metabolism have retained the attention of the investigators in that field: the isolation of several folate-dependent enzymes in multifunctional proteins and the emergence of the polyglutamate forms of folates as the physiologically functional units in one-carbon metabolism. Both the association of enzyme activities and the natural occurrence of folylpolyglutamates are postulated to be of significance in the regulation of folate metabolism, and are the subjects of the next sections.

1.2.2 Enzyme organization in folate metabolism

Several of the folate-mediated reactions in cells are carried out by multifunctional proteins. The methyleneH_4folate dehydrogenase-methenylH_4folate cyclohydrolase-formylH_4folate synthetase activities (reactions A,B and C, Fig. 1) have been shown to form a trifunctional enzyme in yeast (88), as well as in the liver of pig (89,90), sheep (91), rabbit (92), and chicken (93). The lability of the three activities from beef liver could explain the observation of their separation in extracts of that tissue (87). Biochemical and genetic characterization of Saccharomyces cerevisiae mutated at the ade-3 locus support a physical association between the three activities (82,94,95).

Proteolysis studies (88,96,97) and analyses of terminal amino acid residues (97) suggested that the dehydrogenase-cyclohydrolase and the synthetase activities are comprised in two discrete domains, which are respectively located at the amino-terminal region and near the carboxyl-terminal portion of the polypeptide chain. A dehydrogenase-cyclohydrolase fragment has been isolated from a tryptic digest of the pig liver enzyme (96), while chymotryptic cleavage of the same enzyme in the

presence of ATP yielded a synthetase fragment (97). Similarly, a monofunctional synthetase fragment can be obtained by tryptic cleavage of the yeast trifunctional protein in vitro, or when the enzyme is purified without use of protease inhibitors (88). The dehydrogenase-cyclohydrolase activities could possibly occur at a single active site based on the relatively small polypeptide size (M_r = 33,000) of the dehydrogenase-cyclohydrolase fragment (96), the parallel loss of both activities by proteolysis (88,97), and their coordinate inactivation by chemical modification (98,99). The binding of $NADP^+$, a substrate of the dehydrogenase, affects the cyclohydrolase activity, indicating that the activities cannot act independently (99). Kinetic studies with AADP suggested that this analog of $NADP^+$ inhibits both activities by binding to the nucleotide site of the dehydrogenase (99). The degree of overlap of the dehydrogenase and cyclohydrolase sites is not yet definitively established but they could possibly share a common folate binding subsite. Folate offered identical protection to both activities against chemical inactivation, and this protection was potentiated by the presence of $NADP^+$ in each case (99). At all concentrations of $NADP^+$, both activities had a similar K_d for the folate ligand (99). The rabbit liver activities have shown similar values of K_i with various H_4folate derivatives used as inhibitors (92).

The dehydrogenase and cyclohydrolase activities appear to form part of a single protein in some bacteria, but no association of the synthetase either with the dehydrogenase-cyclohydrolase or with other folate-dependent activities has been reported in these microorganisms (82).

In mammals, a functional advantage has been demonstrated as the result of the physical association between the dehydrogenase and cyclo-

hydrolase activities. Rather than equilibrating in the solution, the methenyl-H_4folate intermediate is readily converted to formylH_4folate by the cyclohydrolase (48,92,100). The channeling of the intermediate through the second site is highly preferential in pig liver but not complete [60% (48)]. The use of the polyglutamate forms of the H_4folate substrate did not increase the efficiency of channeling as would be expected from the observed higher affinity of the dehydrogenase and cyclohydrolase for these derivatives (49). The enzyme from chicken liver (100) shows more efficient channeling using the triglutamate substrate (85%) than using the monoglutamate derivative (46%).

The two last steps in histidine catabolism in mammals are catalyzed by folate-dependent activities which have been shown to copurify from pig liver (101-104). The formiminotransferase-cyclodeaminase activities (reactions D and E, Fig. 1) are the properties of a single type of polypeptide (M_r = 62,000) (103). Chemical modification with diethylpyrocarbonate (49) and DTNB (49), as well as ammonia treatment (102) selectively inactivate a single activity, suggesting that the transferase and deaminase are separate sites. The isolation of a chymotryptic fragment with only the transferase activity also supports the hypothesis of two distinct catalytic sites (105). The two activities are found together only in higher eucaryotes (102). However, cyclodeaminase has been purified to apparent homogeneity from purine-fermenting Clostridia (106). The purified protein (M_r = 38,000) also possessed methenyl-H_4-folate cyclohydrolase activity, and separation of these activities could not be achieved (106). No significance was given for this association.

With the sequential transferase-deamihase activities from pig liver, it has been demonstrated that the product of the transferase does not

accumulate in the medium but is preferentially transferred to the deaminase site on the same enzyme molecule when tetrahydropteroylpentaglutamate but not when the monoglutamate is used as the substrate (49). In contrast to the dehydrogenase-cyclohydrolase sequence from the same tissue, the efficiency of channeling of the intermediate between the transferase and deaminase sites appears to be a function of the length of the polyglutamate chain. The process of channeling was essentially complete with the pentaglutamate and only partial with the heptaglutamate; it did not occur at all with the mono- and the triglutamate derivatives (49). The polyglutamate tail has been postulated to anchor the H_4folate molecule through association with a binding subsite during the movement of the pteroyl moiety between catalytic sites (49).

Following are more examples of multifunctional enzymes in folate metabolism but a clear advantage for their multifunctional character has not yet been demonstrated. The $NADP^+$-dependent dehydrogenase activity which regenerates H_4folate from 10-formylH_4folate (reaction F, Fig. 1) has been isolated from mammalian liver (86,107,108). The preparations showed the presence of an $NADP^+$-independent hydrolase (G) in variable ratios. Recently, Rios and MacKenzie have demonstrated that the $NADP^+$-dependent (F) and the $NADP^+$-independent (G) deformylation are catalyzed by a bifunctional enzyme (unpublished data). Up to now, these activities have been reported only in higher species and their presence in microorganisms is uncertain (82).

The folylpolyglutamate synthetase, an enzyme which catalyzes the addition of glutamyl residues to the H_4folates, has been shown to utilize H_2folate in Corynebacterium (109). This observation suggested to the authors that synthetase shares the H_2folate binding site with dihydro-

folate reductase, and the two activities can likely be found on a bifunctional protein in this organism (109). In Crithidia fasciculata (110) and in a large number of protozoans (111), dihydrofolate reductase was shown to copurify with thymidylate synthase, and both activities are apparently the properties of a single type of polypeptide. Overlap between genes encoding for both activities is reported in bacteriophage T_4 (112,113). Association of these activities has not been observed in mammals (113,114).

The partial copurification of some folate-dependent activities suggests that folate metabolism is also organized into multienzyme complexes. GAR Transformylase (reaction J, Fig. 1), one of the two enzymes which utilize the 10-formylH_4folate cofactor in purine biosynthesis, copurified with the trifunctional dehydrogenase-cyclohydrolase-synthetase from chicken liver (93). Two additional folate-requiring activities, AICAR transformylase (reaction K) and serine hydroxymethyltransferase (reaction I) can also be copurified with the activities mentioned above throughout a purification procedure involving a synthetase-specific MgATP elution from hydroxylapatite, and either a GAR affinity column (115) or an AICAR affinity column (116). The enzymes can be further separated without apparent effect on their respective activities (115-119). The possible physical association between the GAR transformylase activity and the trifunctional protein is more strongly demonstrated by cross-linking studies which detected interactions between the two enzyme species (116).

The results of pulse-chase techniques first suggested that formate could be channeled from the trifunctional enzyme to the transformylase site by way of the multienzyme complex in vitro (116). This interpre-

tation was based on the observation that formation of formylGAR was four-fold more efficient when assayed with formate and $H_4PteGlu_3$ than with methenyl$H_4PteGlu_3$, the postulated cofactor of GAR transformylase (116). The results must be reinterpreted by the discovery that 10-formylH_4folate and not methenylH_4folate is the actual cofactor (118,120). However, the association of either one or both transformylase activities with the trifunctional enzyme may have advantages with respect to overall flux and regulation of the de novo purine biosynthetic pathway (118). Whether the trifunctional protein and the folate-dependent associated enzymes constitute part of a much larger multienzyme complex involved in this pathway has not been demonstrated (121-123).

Some folate-dependent enzyme activities were also found to associate either covalently or non-covalently with activities that do not utilize these cofactors. For example, avian liver AICAR transformylase and IMP cyclohydrolase most likely reside on the same polypeptide, due to their copurification during the isolation procedure (117), in agreement with the findings of Buchanan and his co-workers (124). The reactions catalyzing the cleavage of glycine to generate methyleneH_4folate are the properties of the glycine multienzyme complex (125). During T4 phage reproduction (114,126,127) or during the active DNA-synthesizing phase of mammalian cells (27,126,128) enzymes involved in DNA synthesis, including thymidylate synthase, are structurally organized as a multienzyme complex for which the name "replitase" has been proposed (27). This association occurs with the apparent purpose of channeling the incorporation of ribonucleoside diphosphates into DNA (27,126-128).

As has been reported for the arom pathway [(1) p. 120-122; (6) p.161], the enzymes of the folate-dependent pathways show different

organizations of their activities from one organism to another. These associations of enzyme activities could result in advantages to biosynthesis, regulation, or catalytic efficiency. Of these potential advantages, channeling of the intermediate has been demonstrated with two multifunctional proteins of mammalian folate metabolism. For one of them, it has been shown that the polyglutamate tail of folylpolyglutamates can assist the transfer of the pteroyl portion of the coenzyme between active sites. Whether channeling or compartmentalization of intermediates could also be promoted by other types of enzyme organization and whether folylpolyglutamates can help in this process, remains to be demonstrated. In addition to their postulated role in channeling, the folylpolyglutamates have been assigned other important functions which will be discussed in the next section.

1.2.3 Folylpolyglutamates

Numerous investigations (129-135) have established that intracellular folates contain additional glutamates attached to the folate molecule by a peptide linkage between the α-amino and γ-carboxyl groups as shown in Fig. 2. In general, there is a distribution of lengths with one species predominating, the length of which varies with the organism and with the metabolic state of a given organism. For example, many bacteria contain predominantly $PteGlu_3$ or $PteGlu_4$ but significant amounts of longer derivatives are found (136). Mammalian cells often contain penta- or hexaglutamate as the predominant folate (136-140). Yeast and Neurospora have longer chains [$PteGlu_6$ and $PteGlu_7$ predominate (137)]. Only Clostridia has been found not to follow a pattern of different lengths: it contains only triglutamates (141). Shifts to shorter or longer glutamate chains have been observed by modification of the

metabolic conditions (109,136,142-144). No simple mechanism can explain the different distributions. Studies of the synthesis and utilization of folylpolyglutamates would ultimately help to understand their function in the cell.

Improvements in the methods of assaying folylpolyglutamates according to their oxidation level, the nature of their one-carbon substituent, as well as to the length of their polyglutamate chain (138,140,-145-149) led to important developments in characterizing the synthesis of folylpolyglutamates by cells (109,143,150-155). It was known that folates are transported and enter most cells as monoglutamates (135). Inside the cells, glutamyl residues are added one at a time to the folates by the enzyme folylpolyglutamate synthetase (109). The characterization of this enzyme activity purified or partially purified from bacterial and mammalian sources has been recently reported by Shane and his co-workers in a general review of the biosynthesis of folylpolyglutamates (109). According to the results of their studies, they suggested that in vivo distribution of folylpolyglutamates in both types of cell apparently reflects the ability of folylpolyglutamates to act as substrates for their respective folylpolyglutamate synthetase (109). Although the bacterial and mammalian enzymes differ in their folate substrate specificity, both showed decreasing activity as the number of glutamates increased in the folylpolyglutamate substrates. As a result, a predominant chain length in one species is built up because it is a poorer substrate for the synthetase (109). These findings were also made by McGuire et al. (153) using partially purified rat liver enzyme. These authors observed also that H_4pteroylpentaglutamate which exhibited very low activity with the enzyme, was a good inhibitor of the polyglutamy-

lation of shorter $H_4PteGlu_n$ (153). They suggested that folylpolyglutamates could regulate their own synthesis by end product inhibition of the synthetase (153). The low effectiveness of the pentaglutamate as a substrate with this activity did not reflect its high binding affinity as an inhibitor; it is possible that V_m of the reaction is reduced with this compound (109). More kinetic studies are needed to clarify the role of folylpolyglutamates in the regulation of the synthetase activity. An interesting hypothesis was made by McGuire and Bertino (156) while they were reviewing the transport of folates across cells. They suggested that the predominence of a given length(s) in a type of organism is a result of the compromise made by the cells during evolution between the shortest lengths that would not cross the membrane and the energetic cost of synthesizing long polyglutamates.

Because of their highly charged polyglutamate tail, folylpolyglutamates cannot pass through a lipid barrier and were thus thought to be merely the storage forms of folate coenzymes (135). This "inert" role had not been questioned for a while because only the monoglutamates supported bacterial growth, and the reduced folate monoglutamates were active coenzymes in all known folate-dependent pathways (135,137). In addition, sufficient quantities of pure polyglutamates were not available. It is now well established that folylpolyglutamates are the functional units of the one-carbon metabolism, and these findings are indebted to the development of methods for the synthesis of pure pteroylpolyglutamates (158-161), especially the solid-phase procedure as adapted from Merrifield by Krumdieck and Baugh (158). Indeed, the polyglutamates of folates are the actual cofactors <u>in vivo</u> for folate-dependent enzymes, in general having lower values of K_m than their monoglutamate counter-

parts by one to three orders of magnitude. In a few cases, the specificity of a purified enzyme activity for its folylpolyglutamate coenzymes has been tested from various sources: for example, the 10-formylH_4folate synthetase activity has been assayed from bacteria (156,162), yeast (161) and mammalian tissue (49,156); the activity of methyleneH_4folate dehydrogenase was measured from yeast (161) and pig liver (49,163); the specificities of thymidylate synthase from human cells (164) and from bacteria (156,165-167) were also determined. In general, polyglutamate specificty studies have been carried out in a disperse manner, such that a complete picture of the specificities of different enzymes in a given source, or the specificities of a given enzyme from different sources, is not available. Recent investigations reported the specificities of formiminotransferase (49) dihydrofolate reductase (168), H_4PteGlu methyltransferase (169), methyleneH_4folate reductase (170), and serine hydroxymethyltransferase (171) with enzyme preparations from mammalian tissue. The specificites of the avian liver AICAR transformylase (172) and GAR transformylase (173) activities, and of the yeast methenylH_4folate cyclohydrolase (161) activity were also added to this list. Most of the examples mentioned have been summarized recently (135,136,156,157), and other partial studies are also reported in these reviews. The values of K_m for the polyglutamate coenzymes are generally in the sub-μM range, and thus only the polyglutamates will function at the low folate concentrations encountered in the cells [the total concentration of folate is approximately 20 μM in chicken liver (172) and less than 20 μg/g in rat liver (174)]. The values of V_m vary to a lesser extent than the values of K_m, or remain unchanged, so that the polyglutamates exhibit greater catalytic efficiency (V_m/K_m) than the monoglutamates. The folylpoly-

glutamates are also more potent inhibitors of enzyme activities than their monoglutamate counterparts (49,156,157,162-165,169-171,173,175).

There is often a trend in the affinity of the enzymes for their folate substrates or inhibitors having different numbers of glutamyl residues, and there is a preferred length of the polyglutamate chain for optimal binding or catalytic efficiency. These observations have led Krumdieck (172) to propose that folylpolyglutamates can regulate the flux in the one carbon folate-dependent metabolism through affinities of the enzymes for the number of glutamates in the folates. Different specificities for the length of the chain have been reported by many investigators as summarized in the reviews mentioned. But, only recently could we compare the specificities of different folate-dependent enzymes from a single source (161,163,170,171,175).

In yeast, the methyleneH_4folate dehydrogenase and the methenylH_4 folate cyclohydrolase activities of the trifunctional protein do not show a preference for a given chain length while the 10-formylH_4folate synthetase activity exhibits a 10-fold increment in affinity by increasing the number of glutamates in 10-formylH_4folate up to four (161). However, the studies have not been carried out with the predominant lengths of folylpolyglutamates in this organism which were found to contain 6-8 glutamyl residues (176). More extensive studies were done with the pig liver activities using $PteGlu_n$ derivatives having n = 1 to 7. MethyleneH_4folate reductase preferred n = 6 (170); the optimum length was n = 5 and n = 4, respectively for methyleneH_4-folate dehydrogenase (163) and thymidylate synthase (175). The affinity of serine hydroxymethyltransferase for $CH_3H_4PteGlu$ increased with the increasing number of glutamates in the coenzyme (171), and

although the heptaglutamate showed the highest affinity, longer derivatives could possibly be preferred. The observation of different affinities within a single source supports the postulated regulatory role of folylpolyglutamates. Moreover, the results of studies in pig liver show that the intracellular predominant glutamate chain length [n = 6 (138)] does not necessarily correlate with the optimal length determined with isolated enzymes, as was suggested by McGuire and Bertino (156).

Another line of evidence also indicates that folylpolyglutamates are physiologically important coenzymes. Certain mutant lines of mammalian cells lack the enzyme folylpolyglutamate synthetase (132,177). When they are supplied with folate, they do not build up the normal intracellular concentration of folates (132,177). They are consequently auxotrophic for the end products of folate metabolism (glycine, purines, thymidine, and methionine) (132,177).

Additional functional advantages of the polyglutamate derivatives have been demonstrated. For example, $H_4PteGlu_5$ and not $H_4PteGlu_1$ provided for channeling between the active sites of the bifunctional transferase-deaminase enzyme (49) which suggested to the authors that the polyglutamate chain could anchor the intermediate on the enzyme while the pteroyl moiety moves between catalytic sites. Since several folate-dependent activities tend to copurify (section 1.2.2), the polyglutamate chain may facilitate such channeling in other site-site interactions. Additional experiments are required to establish more firmly the possible role of folylpolyglutamates in "promoting" channeling in multifunctional proteins and multienzyme complexes. Other specialized functions have been established or postulated for pteroylpolyglutamates, and they have been well summarized elsewhere (156).

1.3 THE BIFUNCTIONAL TRANSFERASE-DEAMINASE

1.3.1 Folate-dependent histidine catabolism: an overview

The reactions involved in histidine catabolism and the properties of folate-dependent enzyme activities in this pathway have been recently reviewed by Shane and Stokstad (178). Some general points will be presented here but our attention will focus on the properties of two folate-dependent activities of the pathway: the formiminotransferase and cyclodeaminase activities.

Degradation of histidine results in the formation of formiminoglutamate in both mammals and microorganisms. Formiminoglutamate is further metabolized to release glutamate. Only in higher species is the utilization of formiminoglutamate catalyzed by folate-mediated reactions (178). In mammals, three enzyme activities were found responsible for the conversion of formiminoglutamate to glutamate and 10-formylH_4folate (102): formiminoglutamate:H_4folate formiminotransferase (reaction D, Fig. 1); 5-formiminoH_4folate cyclodeaminase (E); and 5,10-methenylH_4folate cyclohydrolase (B). The formiminotransferase and cyclodeaminase activities are ubiquitous in mammalian liver and kidney but are absent in other tissues (102,178). Both enzymes are present in filariae (179). The transferase apparently cannot be found in insects and bacteria (178).

The regulation of transferase and deaminase is still not clear. Although these activities are involved in the genesis of one-carbon units, they can also be considered gluconeogenic activities because they produce glutamate (178). Moreover, Scrutton and Beis reported inhibition of gluconeogenesis by formiminoglutamate (107). Stifel et al. (180)

observed that in rat the formiminotransferase activity apparently responds to hormones in a manner consistent with a gluconeogenic function. Similar results cannot be reproduced by other investigators (C. Hansen, M.Sc. Thesis, McGill). The folate-dependent histidine catabolism was found to be sensitive to methionine, thyroxine, and vitamin B_{12} deficiency (178), but there is no evidence that these substances are directly involved in this pathway. It is postulated that the effect of these compounds is exerted rather on the availability of folate coenzymes in cells (85,107,178). The feeding of thyroxine or a deficiency in vitamin B_{12} causes folate impairment: folates are trapped as nonfunctional 5-methylH_4folate derivatives which are poor substrates of pteroylpolyglutamate synthetase (178). Decreased synthesis of folylpolyglutamates and formation of nonfunctional folate derivatives result in lower levels of folates utilized in one-carbon metabolism including histidine oxidation to CO_2 (178). In this context, the level of formiminoglutamate increases. Administration of methionine restores the utilization of folates by means of its conversion to S-adenosylmethionine which is a powerful inhibitor of methylenetetrahydrofolate reductase (reaction H, Fig. 1), the enzyme that produces 5-methylH_4folates (85,144,178,181-183). The existence of folate-dependent transferase and deaminase activities on a bifunctional protein offers potential advantages in terms of regulation and metabolic efficiency.

The transferase and cyclodeaminase were shown to copurify from acetone powders of pig liver by Tabor and Wyngarden (102) and by Slavik et al. (101). The latter group demonstrated copurification through folate affinity chromatography and isoelectric focusing (101). MacKenzie and his co-workers obtained an homogenous crystalline protein using

polyethylene glycol and ammonium sulfate fractionations, and pH precipitation (103). Their purification method (103) was similar to that described by Tabor and Wyngarden (102) but delipidation of liver extract by acetone was circumvented since this procedure could cause a significant loss in enzyme activity, or could yield a modified protein (103). The preparations were homogenous as demonstrated by sedimentation analysis (102,103) and by sodium dodecyl sulfate gel electrophoresis (103). The two enzymes were also found to be associated, presumably as a bifunctional protein, in filarial extracts (179).

Both transferase and deaminase activities can be assayed by spectrophotometric measurement of 5,10-methenylH_4folate, the final product of the sequential reactions (103). The effect of pH, salts, composition of buffers, and various inhibitors on one or the other activity have been summarized in the review already mentioned (178). One striking observation with respect to the effect of salts is the significant increase of the deaminase activity in the presence of the K^+ cation which acts by decreasing the K_m value for 5-formiminoH_4folate (102).

The properties of the pig liver transferase-deaminase have been further investigated by MacKenzie and his co-workers (49,103-105,184-187). The next section reports the results of their studies related to the elucidation of the quaternary structure, the relationship between the two active sites, and the kinetic advantage offered so far by the bifunctional character of the protein.

1.3.2 Formiminotransferase-cyclodeaminase from pig liver

The formiminotransferase-cyclodeaminase is homogenous by sedimentation equilibrium and has a native molecular weight of 5.8×10^5 (103). A single band was obtained after sedimentation analysis in the

presence of 5M guanidine·HCl (M_r = 65,800), after dodecyl sulfate gel electrophoresis (M_r = 62,000) and after electrophoresis in 8M urea (103). These results suggest that the protein is an octamer of similar or identical subunits. Other lines of evidence also support the identity of subunits. Isoelectric focusing in 8M urea (104) gave one major band with an isoelectric point of pH 6.9, and a band of very low intensity. This minor contamination is due possibly to some polypeptides that contain a different amount of non-protein material since the complex contains 2.8% carbohydrate (104). The protein contains 8 methionine residues, and electrophoresis of cyanogen bromide-treated protein revealed eight or nine bands, which is in agreement with the number predicted for identical subunits (104). Amino terminal residues could not be detected indicating that these residues are blocked, but only alanine was obtained as a carboxy terminal residue (104).

The structure of the protein appears unusual with respect to the planar arrangement of the eight subunits. Electron microscopy (104) demonstrated that the protein is a ring-shape molecule both in solution and crystalline forms. The eight subunits could be differentiated by rotational reinforcement of the electron micrographs when rotations of 45° and 90° were carried out (104). The outside and inside diameters of the "donut" are approximately 116 and 53Å, and the diameter of each subunit is about 32Å (104).

Chymotrypsin digestion of the native enzyme in the presence of folate, which sensitizes the deaminase activity to proteolysis, yielded an active fragment (M_r = 80,000) which possessed only the transferase activity (105). Sodium dodecyl sulfate gel electrophoresis of the purified fragment indicated one size of polypeptide of M_r = 39,000

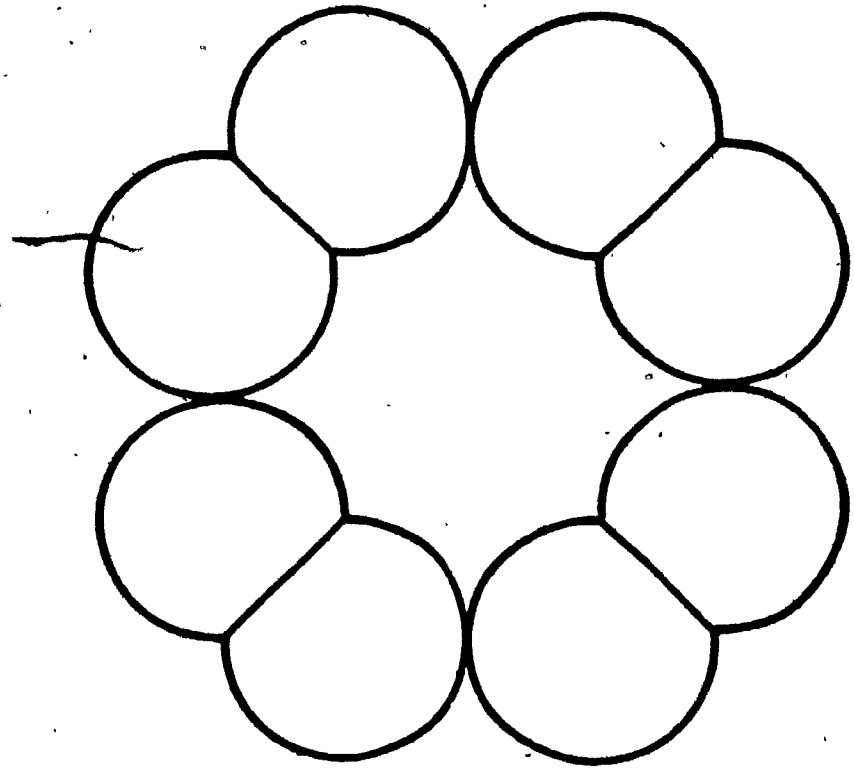
(105). The dimeric structure of the transferase fragment was confirmed by cross-linking with the bifunctional reagent dithiobis(succinimidyl propionate) (105). Cross-linking of the native protein with the short bifunctional reagent difluorodinitrobenzene yielded dimers and tetramers in preference to trimers, which indicates two types of subunit interactions (105). The isolation of a dimeric fragment from the octameric protein and the results of cross-linking support a tetramer of dimers structure for the native enzyme (105). The quaternary structure of the bifunctional enzyme is illustrated in Figure 3.

The identity of the subunits suggests that each polypeptide must contain the two enzymic activities. The activities are apparently due to separate sites since each can be inactivated selectively by chemical modification (49,184). Moreover, the transferase activity is retained after chymotryptic treatment of the native enzyme (102,105) and can be isolated as a separate active fragment (105). Kinetic observations also suggested independent catalytic sites (185). When H_4folate was added to a mixture of transferase active enzyme and unmodified native enzyme, the extra formiminoH_4folate intermediate produced in the assay could result in increased production of methenylH_4folate (185). The results of chemical modification of the bifunctional enzyme with the reagent DTNB indicated that one sulfhydryl/polypeptide is required for deaminase activity (184). It is thus possible that there are eight sites each for transferase and deaminase on the native protein.

Even if the number of catalytic sites and their location on the protein are not clear, a kinetic advantage resulting from the presence of both activities on a single polypeptide could be demonstrated. The product of the transferase is not released in the medium but is trans-

FIGURE 3

Schematic representation of the quaternary structure of transferase-deaminase.



ferred preferentially to the deaminase site when $H_4PteGlu_5$ is used as a substrate (49). When $H_4PteGlu_1$ and H_4PteGu_3 were used, a pronounced lag in the time course of appearance of the final methenyl product indicated that the formimino intermediate had to accumulate to high enough concentration in the medium to result in significant production of methenylH_4-$PteGlu_n$ (49). There was no lag in the time course of appearance of final product with $H_4PteGlu_7$ substrate but the formimino intermediate accumulated, indicating that channeling occured only partially with this substrate (49). To differentiate between a true channeling process and the possibility that formimino$H_4PteGlu_5$ was not detected in the medium because it bound to the deaminase with a high affinity, the experiment was repeated with mixtures of enzyme chemically modified to retain either transferase or deaminase activity (49). When the two activities were on separate molecules, the methenyl product appeared with a distinct lag using $H_4PteGlu_5$ while the time course of appearance of intermediate and final products were unchanged with $H_4PteGlu_1$ (49). These results strongly support channeling between active sites, and the polyglutamate chain has been suggested to act as an "anchor" by interacting with a polyglutamate binding subsite during the transfer of the pteroyl moiety between catalytic sites (49). The channeling is a function of the number of glutamyl residues in the substrate molecule: the mono- and triglutamate are too short to allow binding of the polyglutamate anchor at a subsite and the placement of the pteroyl portion at each of the active sites; the pentaglutamate is of the optimum length to assist channeling between sites, but with the heptaglutamate the anchor is too long such that probability for dissociation approximates probability for transfer (49).

Both activities show greater affinities for the polyglutamate forms of folate derivative than for the monoglutamate (49). The kinetic properties of the native transferase and of the transferase active fragment with the $H_4PteGlu_n$ (n = 1,5,7) substrates indicate that the transferase fragment has lost the specificity for the polyglutamate substrates (105). These data suggest that the protein could have only one polyglutamate binding site per pair of transferase-deaminase catalytic sites, and that this site is closely associated with the deaminase site which has been removed by proteolysis (105). With the bifunctional transferase-cyclodeaminase, the function of the additional γ-glutamyl residues is thus to provide substrates with greater affinities and to assist channeling of an intermediate which has no other metabolic function and which is a potential inhibitor of other folate-mediated reactions (105,185). This enzyme constitutes an ideal system to study the role of both multifunctional proteins and folylpolyglutamates.

1.3.3 Statement of the problem

The overall objective of the research is to understand the mechanism of channeling of folylpolyglutamates between transferase and deaminase active sites. This mechanism must ultimately be reconciled with the unique quaternary structure of the bifunctional protein.

To achieve this goal, two types of approach have been used. The first was concerned with the physical interaction between the transferase and deaminase catalytic sites, and the polyglutamate binding subsites, and involved binding studies. If the two active sites are separate within the polypeptide chain, each activity could bind one folate molecule (8 + 8 folate binding sites); if they are subsites of a single larger site, each catalytic pair would bind only one folate at a given time (8

sites). The folate molecule could also bind to sites formed at a single type of subunit interface (4 sites). In addition, the number of polyglutamate binding subsites may not equal the total number of active sites if there is only one such site per pair of transferase-deaminase activities. Determining the number of polyglutamate binding sites would help to decide whether a monomer (8 or 16 sites) or a dimer (4 sites) is the smallest possible structure required for channeling. The number of folylpolyglutamate binding sites and the specificity of the enzyme for chain length were explored by studying the binding of tritiated $H_4PteGlu_n$ (Chapter 3). The $H_4PteGlu_n$ compounds are substrates of the transferase and inhibitors of the deaminase, and thus bind to each of the active sites on the native octamer. The binding studies required synthesis of the radiolabeled $H_4PteGlu_n$ of high specific activity and the method for their preparation is described in Chapter 2.

The mechanism of channeling was also investigated by a kinetic approach to determine if the efficiency of channeling relates directly to affinity. It is possible that increasing the number of glutamates in the polyglutamate chain allows for a tighter interaction between a folate substrate and the enzyme. As a result, a high concentration of substrate can be created in the vicinity of the active sites, and this local sequestration of substrate could enhance the efficiency of channeling. On the other hand, the process could require an appropriate length of chain to assist the transfer of the intermediate in a favourable position for catalysis. The specificities of the transferase and deaminase activities for their folylpolyglutamate substrates was measured kinetically (Chapter 4) to determine if they reflect the specificity as measured by binding studies. These results were compared with the

polyglutamate specificity for the efficiency of channeling, to establish whether a steric requirement is involved in the mechanism for this process.

CHAPTER 2

Preparation of Tritium Labeled Tetrahydropteroylpolyglutamates of High Specific Radioactivity

PREFACE

The following Chapter will be published in Analytical Biochemistry (1985), Volume 145, under the title "Preparation of Tritium Labeled Tetrahydropteroylpolyglutamates of High Specific Radioactivity", by J. Paquin, C.M. Baugh, and R.E. MacKenzie.

The pteroylpolyglutamates were kindly provided by Dr. Charles M. Baugh of the University of South Alabama, USA. The formiminotransferase-cyclodeaminase enzyme, the transferase activity of which was used to assay the tetrahydrofolates, was purified by Leonora C. Bortoluzzi, a technician in our laboratory. The use of immobilized boronate was suggested by Dr. Drummond Smith. The secretarial assistance of Maureen Caron and Louise Morris for typing the manuscript is appreciated.

SUMMARY

Tritium labeled [6S]-tetrahydropteroylpolyglutamates of high radiospecific activity were prepared from the corresponding pteroylpolyglutamates. Malic enzyme and [2-^{3}H]-D,L-malate were used as a generating system to produce [4A-^{3}H]-NADPH which was coupled to the dihydrofolate reductase catalyzed reduction of chemically prepared dihydropteroylpolyglutamate derivatives. Passage of the reaction mixtures through a column of immobilized boronate effectively removed NADPH, and the tetrahydropteroylpolyglutamates were subsequently purified by chromatography on DEAE-cellulose. Overall yields of the [6S]-tetrahydro derivatives were 18-48% and the radiospecific activities were 3-4.5 mCi.μmol^{-1}.

INTRODUCTION

The natural forms of folate derivatives in cells are the folylpoly-γ-glutamates (1-6). They were first thought to be storage forms of folate but their importance as functional units is now established and has been reviewed recently (7-9). The polyglutamates of the tetrahydrofolate coenzymes are the preferred substrates for several enzymes, in general having much higher affinities and thereby allowing pathways to function at low intracellular folate concentrations (7-13). It is possible that they could play an important role in the regulation of one-carbon metabolism if the flux in different folate-dependent pathways can be affected by specificities of the enzymes for the number of glutamates in the substrates (14-19). Folate metabolism in eucaryotes involves multifunctional enzymes and the polyglutamate chain has been proposed to assist the transfer of the tetrahydropteroyl portion between consecutive active sites in such enzymes (20,21). There is also a possibility for channeling to occur within complexes formed by the non-covalent association of folate-dependent enzymes (22,23).

The role of folylpolyglutamates has received increased attention and the study of their functional advantages can require the use of radiolabeled compounds. Radiolabeled pteroylpolyglutamates have already been used to study the absorption and metabolism of folates (24-26), the biosynthesis of folylpolyglutamates (15,27), and to label a folylpolyglutamate binding site (28). Several synthetic routes to the polyglutamylation of pteroic acid have been published including mixed-anhydride coupling on solid-phase (29) or in solution (30,31), carbodiimide coupling (31,32) and active ester condensation (31). Radiolabeling of pteroylpolyglutamates was done during the condensation reactions by using

labeled pteroic acid (24,25,30) or by incorporating ^{14}C-glutamyl residues (24,29). To function as active coenzyme, the pteroylpolyglutamates must be reduced to the [6S]-tetrahydro form. This paper describes another strategy to prepare tritiated [6S]-tetrahydropteroylpolyglutamates from the unlabeled pteroylpolyglutamates as well as their purification free of labeled NADPH.

MATERIALS AND METHODS

Pteroylpolyglutamates were synthesized as described by Krumdieck and Baugh (29). Cis-oxaloacetic acid, malic enzyme and sodium dithionite were from Sigma. NADPH was purchased from Boehringer Mannheim, glycylglycine from Aldrich Chemicals, DL-alanine from BDH Chemicals and Aquasol-2 cocktail and standardized tritiated toluene were from New England Nuclear. Lactobacillus casei dihydrofolate reductase was a gift from Dr. Roy Kisliuk, Tufts University. Immobilized boronic acid (N-(m-dihydroxyborylaminophenyl)polyacrylamide) and DEAE-cellulose (DE 23) were purchased from Pierce Chemicals Co. and from Whatman, respectively. Common chemicals were reagent grade. Buffers were adjusted to the desired pH at room temperature. Chromatographic steps were carried out at 4°C.

Formiminoglutamate: tetrahydrofolate formiminotransferase (EC 2.1.2.5)-formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4) was purified from frozen pig liver as described previously and the transferase activity (14 μmol min^{-1} mg^{-1}) was used to assay the tetrahydropteroylglutamates (33).

[^{3}H]-D,L-malic acid (8.2 mCi/μmol) was obtained by the catalytic reduction of cis-oxaloacetate with tritium gas in a custom synthesis by New England Nuclear. Although this method will incorporate some label in position 3 in addition to position 2, no effort was made to determine the distribution of tritium in the product. A sample of the labeled malic acid has been chromatographed on DEAE-cellulose and eluted as a single radioactive peak. The tritiated malic acid, provided in methanol solution, was thus used without purification. The concentration of L-malate was obtained by a modification of the method of Hsu and Lardy

(34). Excess malic enzyme was added to a one-ml assay mix containing 1.25 μmole NADP, 4 μmoles $MgCl_2$, 70 μmoles triethanolamine·HCl, pH 7.3 and approximately 0.08 to 0.12 μmoles of malate. The reaction was followed spectrophotometrically until there was no further change in absorbance and the concentration of NADPH produced was measured using using $\varepsilon_{340nm} = 6{,}220\ cm^{-1}\ M^{-1}$. The assay was checked using 0.10 μmole of L-malate from a solution of known concentration. Only one isomer of malate is utilized in the enzymic assay and observed values have been doubled to express all concentrations as D,L-malate.

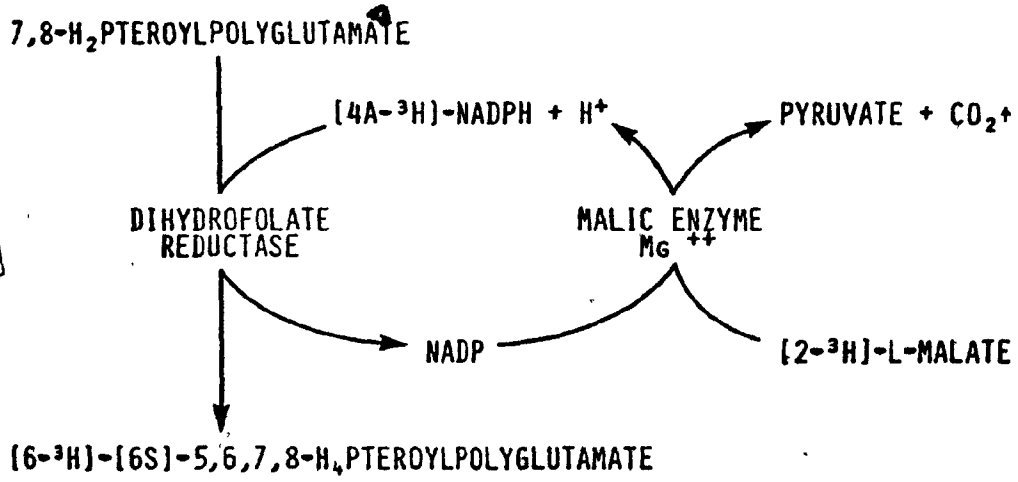
Absorbance measurements were made on a Gilford 2000 recording spectrophotometer. Radioactivity in samples was determined in Aquasol-2 scintillation cocktail using a Beckman LS-250 scintillation counter. Radiodisintegration rates were calculated by use of experimentally determined quench curves comparing counting efficiency with the external standard ratio. Conductivity measurements were done using a Radiometer Copenhagen conductivity meter.

Preparation of tritiated [6S]-tetrahydropteroylpolyglutamates.

(a) Reduction - The pteroylpolyglutamate (5-12 μmoles) was reduced to the dihydro derivative under conditions described by Blakley (35) using 2 ml of sodium ascorbate solution (0.1 g/ml), pH 6.0, and 80 mg of solid sodium dithionite. The dihydrofolate was precipitated by adjusting the solution to pH 2.8 and after 30 minutes was recovered by centrifugation (20 min. at 7,000g, 4°C). The unpurified dihydrofolate was dissolved in one ml of 0.05 M glycylglycine, 0.14 M 2-mercaptoethanol, pH 7.5, and the dihydrofolate solution was adjusted to pH 7.5 with 0.1 N NaOH. The dihydrofolate was then converted to the tetrahydro compound using

FIGURE 1

The enzymatic system for the reduction of dihydropteroylglutamate to tritiated [6S]-tetrahydropteroylglutamate.


7,8-H2PTEROYLPOLYGLUTAMATE
[4A-3H]-NADPH + H+
PYRUVATE + CO2↑
DIHYDROFOLATE REDUCTASE
MALIC ENZYME Mg++
NADP
[2-3H]-L-MALATE
[6-3H]-[6S]-5,6,7,8-H4PTEROYLPOLYGLUTAMATE

dihydrofolate reductase and an NADPH-generating system with malic enzyme as outlined in Fig. 1. From the stock solution of tritiated malic acid, a 2-ml sample containing 30 μmoles of D,L-malic acid was withdrawn, gently dried under nitrogen and redissolved in 2 ml of 0.05 M glycylglycine, pH 7.5. To this working malate solution readjusted to pH 7.5 with 0.1 N NaOH, were added 16 μmoles $MgCl_2$, 0.75 μmoles NADP; 11 units of malic enzyme (as defined by Sigma Chemical Co.), the neutral dihydrofolate solution and 3-5 mg of dihydrofolate reductase (6.6 $nmol.min^{-1}.mg^{-1}$ when assayed as per Mathews et al. (36)). The volume was adjusted to 6.0 ml with 0.05 M glycylglycine, pH 7.5.

The tube containing this final incubation mix was kept protected from light and under vacuum in a 250 ml suction flask which contained 20 ml of 50% v/v 2-mercaptoethanol. After 90 min. at room temperature, the tetrahydrofolate formed was assayed enzymatically with formiminotransferase. The yield of tetrahydropteroylpolyglutamate formed from the corresponding pteroylpolyglutamate ranged between 30 and 60% prior to purification. When removal of [^{3}H]-NADPH was not required, the incubation mix was diluted with an equal volume of 0.36 M 2-mercaptoethanol and directly chromatographed on DEAE-cellulose (step c). Otherwise, the incubation mix was treated as follows:

(b) Chromatography on immobilized boronate - The incubation mix was adjusted to pH 8.5 with 0.1 N NaOH, and solid $MgCl_2$ was added to give a final concentration of 0.1 M. This sample was applied to a column (1 x 18 cm) of immobilized boronate previously equilibrated with elution buffer containing 0.1 M alanine, 0.1 M $MgCl_2$, pH 8.5. The flow rate was reduced to 4 ml/hour for two hours to allow maximal formation of complex

between NADPH and the tetrahedral form of boronate (37). The flow rate was then increased to 40 ml/hour. The early fractions contained the tetrahydropteroylpolyglutamate, were pooled (approximately 12 ml), diluted 5-fold with 0.36 M 2-mercaptoethanol and purified by ion-exchange chromatography.

(c) Ion-exchange chromatography - The diluted sample from (a) or (b) was applied to a 0.7 x 6 cm DEAE-cellulose column in the chloride form, washed with 5-10 ml of 0.36 M 2-mercaptoethanol and eluted using an exponential gradient formed, as already described (20), with 60 ml of 0.075 M triethanolamine·HCl, 0.36 M 2-mercaptoethanol, pH 7.3, in the closed vessel, and 50 ml of the same buffer containing 0.6 M NaCl in the upper reservoir. Fractions absorbing at 298 nm with the same specific activity (cpm per absorbance unit) were pooled and assayed for tetrahydropteroylglutamate. Tritiated [6S]-tetrahydropteroylglutamates were stored at -20°C in 1-ml sealed ampules.

RESULTS AND DISCUSSION

Several approaches to the synthesis of radiolabeled pteroylpolyglutamates have been described previously. For example, condensation of the required number of γ-glutamyl residues to tritiated pteroic acid yields the required products (30). This latter compound was labeled by reductive dehalogenation of diiodopteroic acid using tritium gas in the presence of palladium on charcoal. Radiolabeled pteroylpolyglutamates subsequently can be reduced to the tetrahydro forms. Tritiated tetrahydropteroylmonoglutamate with a specific activity of 0.3 or 3 mCi/μmole has been prepared by the enzymatic reduction of dihydrofolate using chemically (38,39) or enzymatically (40) prepared and purified [4A-^{3}H]-NADPH, or by using a glucose-6-phosphate dehydrogenase system to generate catalytic amounts of [4B-^{3}H]-NADPH (38). A simple method for the preparation of (S)-tetrahydropteroylpolyglutamates of very high specific radioactivity was required to obtain products suitable for binding studies (Paquin and MacKenzie, unpublished). While a glucose-6-phosphate dehydrogenase NADPH generating system is widely used in the synthesis of unlabeled tetrahydrofolates, it is not as useful for synthesis of the tritiated compounds because of the requirement for [1-^{3}H]-glucose-6-phosphate in sufficient amounts. In addition, the radioactivity is diluted by the amount of unlabelled NADP used because the generating system and dihydrofolate reductase have different stereospecificities for NADPH.

The malic enzyme coupled NADPH generating system presents advantages when compared to the above methods: (i) the malic enzyme system satisfies the stereochemical requirement for the "A" side of NADPH, theoretically allowing the complete incorporation of tritium into the tetrahydro-

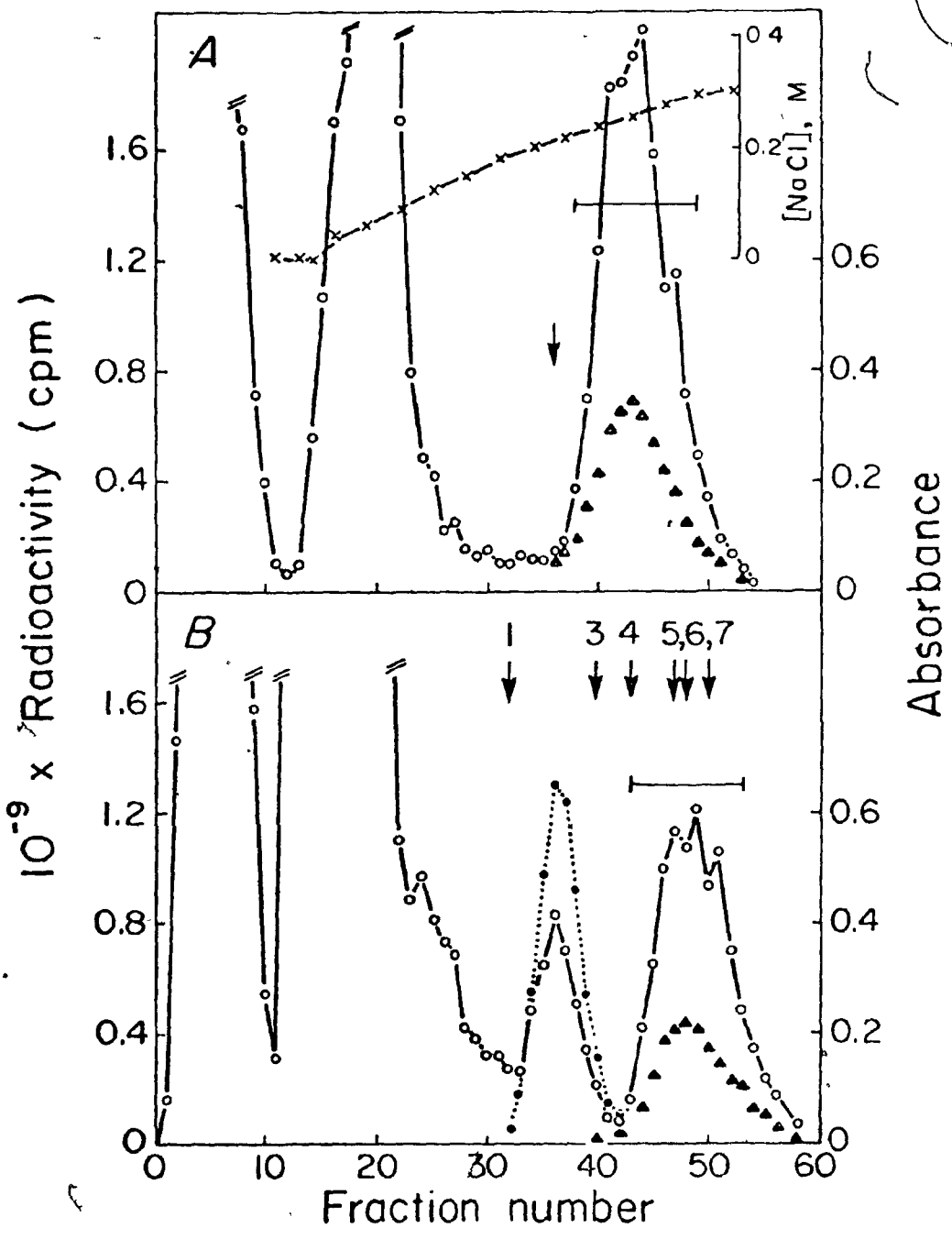
pteroylglutamate, (ii) tritiated malic acid of very high specific activity can be obtained commercially or prepared conveniently (41) in sufficient quantities and at reasonable cost and (iii) the label is incorporated at the last step of the synthesis at position 6 of the molecule (39,42).

The malic enzyme-coupled NADPH generating system in conjunction with the usual folate reduction system using dihydrofolate reductase yielded labeled [6S]-tetrahydropteroylglutamates of very high specific radioactivity. In all cases the excess [^{3}H]-D,L-malate and potentially some labeled [3-^{3}H]-pyruvate were well separated from the tetrahydropteroylglutamates by chromatography on DEAE-cellulose (Fig. 2B). Approximately 80-85% of the radioactivity present in the final reduction incubation mixture was eluted prior to fraction 30. Malate has been found to elute differently from the DEAE-column depending upon the composition of the malate-containing sample applied to the column. An increase in the ionic strength of the sample or particularly the presence of $MgCl_2$, increased the proportion of malate eluting before the application of the gradient.

In contrast to the efficient separation from [^{3}H]-D,L-malate, [^{3}H]-NADPH eluted from the column in the same range of the gradient as the tri- and tetraglutamate compounds. Although contamination of tetrahydrofolates with small amounts of NADPH (or its degradation products) is not often of concern when these compounds are used as substrates for various enzymes, the presence of labeled impurities interferes with their use in equilibrium binding studies. Immobilized boronates have been widely used for the separation and purification of compounds containing 1,2-cis-diol groups (37,43-47). Passing a solution of the tri- or tetraglutamate derivative through a boronate column prior to purification by

FIGURE 2

Purification of tritiated tetrahydropteroylglutamates on DEAE-cellulose. (A) A reaction mix (320 mCi) containing 0.79 μmole [4A-^{3}H]-NADPH, 8.0 μmoles [^{3}H]-tetrahydropteroyltetraglutamate, [^{3}H]-D,L-malate and [^{3}H]-pyruvate was applied to a column of immobilized boronate. Fractions containing the tritiated tetrahydropteroyltetraglutamate were pooled and rechromatographed on DEAE-cellulose with a 0 to 0.33 M exponential gradient of sodium chloride. The arrow shows the elution position of NADPH. (B) A reaction mix (266 mCi) containing 0.75 μmoles [4A-^{3}H]-NADPH, 2.9 μmoles [^{3}H]-tetrahydropteroylhexaglutamate, [^{3}H]-D,L-malate and [^{3}H]-pyruvate was directly chromatographed on DEAE-cellulose under identical conditions as described for A. The arrows show the elution position of tetrahydropteroylglutamates with the indicated number of glutamate residues. The symbols are: radioactivity (o), absorbance at 340 nm (•), absorbance at 298 nm following a 50-fold dilution (▲), salt concentration (x).


A
B
1
3 4
5,6,7
0.4
0.2
0
[NaCl], M
1.6
1.2
0.8
0.4
0
0.6
0.4
0.2
0
10^{-9} x Radioactivity (cpm)
Absorbance
0
10
20
30
40
50
60
Fraction number

ion exchange chromatography removed NADPH as shown in Figure 2A. Both absorbance at 340 nm and radioactivity measurements were at background levels in the fractions where NADPH would normally elute. The NADPH bound to the boronate column was recovered (89%) by elution with 0.05 M glycylglycine, pH 7.0. Boronate tightly binds NADPH and thus offered a simple means to purify labeled or unlabeled folates free from NADPH. Alternatively, the complete separation of tritiated NADPH from tetrahydrofolates would have required repeated ion-exchange chromatography or gel chromatography on Sephadex (48).

The overall yields of purified tetrahydropteroylpolyglutamates obtained from the corresponding folate derivatives are summarized in Table 1. These yields are similar to those observed when NADPH is generated by the glucose-6-phosphate dehydrogenase system (unpublished results). Treatment of the tri- and tetraglutamate derivatives by passage through a column of immobilized boronate prior to purification reduces their overall yield by approximately 10%. The slightly higher specific activity of the tetraglutamate compound was investigated further. Rechromatography of the purified material on DEAE-cellulose failed to show any further resolution and indicated a 98% purity of the original material based on specific activities. The ultraviolet absorption spectrum of each tetrahydropteroylpolyglutamate was characteristic of that of tetrahydrofolate (1). The first-derivative scan between 250 and 390 nm did not detect any dihydrofolate derivatives at 282 nm (1).

The overall yields of radiolabeled tetrahydropteroylpolyglutamates are influenced significantly at the stage of recovery of the dihydro-

TABLE 1

Summary of synthesis of tritiated [6S]-tetrahydropteroylpolyglutamates.

No. of glutamates in $PteGlu_n$	$PteGlu_n$	$H_4PteGlu_n$	
		yield	specific activity
n	μmol	%	$mCi.\mu mol^{-1}$
3	5.6	21[a]	3.2
4	12.2	32[a]	3.9
5	4.8	48	4.5[b]
6	7.1	34	3.3
7	7.1	18	3.0

Abbreviations are: PteGlu, pteroylglutamate; H_4PteGlu, tetrahydropteroyl-glutamate.

a) The incubation mix containing [^{3}H]-[6S]-$H_4PteGlu_n$ and [^{3}H]-NADPH was chromatographed on a column of immobilized boronate before application on DEAE-cellulose.

b) Tritiated [6S]-$H_4PteGlu_5$ was prepared using [^{3}H]-D,L-malate having a specific activity of 9.0 mCi/μmol instead of 8.2 mCi/μmol.

folate derivatives. The longer polyglutamates precipitate less efficiently. It might be possible to increase the overall yield of the heptaglutamate by substitution of gel filtration for pH precipitation, Coward et al. (49) studied the production of dihydropteroylpolyglutamates which they purified by ion exchange chromatography in yields ranging from 27 to 57% for derivatives with 3, 5, and 7 glutamates. The incorporation of additional chromatographic steps and the possibility of dealing with more dilute dihydrofolate solutions for subsequent enzymic reduction diminish the potential advantage of using such procedures. The advantages of the pH precipitation are both an opportunity to concentrate the dihydro-derivatives, and to complete each synthesis and purification within one day.

The final tetrahydropteroylpolyglutamate solutions are 150 to 300 μM in concentration but contain relatively high concentrations of NaCl. Dilution of the tetrahydropteroyl derivatives to the concentration range used in many studies (1-20 μM) would increase only slightly the ionic strength of the resultant solution. Baggott and Krumdieck (14) have pointed out the necessity of considering ionic strength in determining interaction of the polyglutamates with enzymes and this point should be considered for each use of the compounds. If necessary, the tetrahydrofolates can be desalted by gel filtration on Sephadex (29,48).

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CHAPTER 3

Binding of Tetrahydropteroylpolyglutamates to the Native Octameric Transferase-Deaminase

PREFACE

The content of this Chapter has been submitted for publication in the Journal of Biological Chemistry. The article, the first of two characterizing the interaction of folylpolyglutamates with the transferase-deaminase, is entitled "Formiminotransferase-Cyclodeaminase from Pig Liver. 1. Binding of Tetrahydropteroylpolyglutamates to the Native Octamer" by J. Paquin, C.M. Baugh and R.E. MacKenzie.

The pteroylpolyglutamates were kindly provided by Dr. Charles M. Baugh of the University of South Alabama, USA. The preparation of large quantities of the transferase-deaminase enzyme by Leonora Bortoluzzi, a technician in our laboratory, is gratefully acknowledged. Formyltetrahydrofolate synthetase was prepared by Narciso Mejia, another technician in our laboratory, and (R,S)-H_4folate for routine use was prepared by Dr. Robert MacKenzie. Typing of the manuscript by Maureen Caron, and criticism and proofreading by Wendy Findlay are gratefully appreciated.

A part of this chapter has been communicated to the Canadian Federation of Biological Societies (1983) under the title "The binding of folylpolyglutamates to formiminotransferase-cyclodeaminase", and to the Association Canadienne-Française pour l'Avancement des Sciences (1983) under the title "L'utilisation des folylpolyglutamates par une enzyme bifonctionnelle". A part of the results will also be presented to the Canadian Federation of Biological Societies (1985) under the title "Structure and Function of an Octameric, Bifunctional Enzyme".

SUMMARY

The number of binding sites on the bifunctional formiminotransferase-cyclodeaminase for folylpolyglutamate coenzymes was determined by studying the binding of (6S)-tetrahydropteroyl(glutamate)$_n$ (n = 1,3,4,5,6,7) which are substrates of the transferase and inhibitors of the deaminase. The enzyme, a circular tetramer of dimers of identical subunits, binds only four tetrahydropteroylpolyglutamates per octamer, indicating that a dimer is likely its functional unit. Differential inhibition of the transferase and deaminase activities by (6R)-tetrahydrofolate supports the conclusion that the activities are separate catalytic sites, but share a common polyglutamate binding subsite. The affinity of the enzyme for tetrahydropteroylpolyglutamates increases with addition of each glutamyl residue to a total of six; addition of the seventh resulted in a slight decrease in affinity. The large decrease in free energy (5.7 kJ) in binding the fourth glutamate indicates that this residue is particularly important in anchoring the substrate to allow kinetic channeling of the intermediate between the active sites.

INTRODUCTION

It has been widely recognized that intracellular folates exist as polyglutamate derivatives with additional glutamyl residues attached in γ-linkage. Several folate dependent enzymes have been shown to prefer the polyglutamate derivatives; generally an increase in the chain length of the H_4PteGlu coenzyme lowers the Km with lesser effects on Vmax (1-14). This higher affinity makes it possible for pathways to function at the low physiological concentrations encountered inside cells (6). Two additional important functions have been postulated for the folylpolyglutamates: regulation of one-carbon metabolism (6) and promotion of "channeling" (7). The hypothesis that the flux in different folate-dependent pathways can be regulated by specificities of the enzymes for the number of glutamates in the folates arose from two kinds of observations. Several enzymes show different specificities for the length of the chain, within a single species (11-14). In addition, variations in the metabolism have been shown to affect the distribution of folylpolyglutamates of different chain length within a given oxidation level of one-carbon units (15-17). It has been demonstrated that folyl-polyglutamates transfer preferentially between active sites of a bifunctional enzyme, and the polyglutamate chain has been suggested to act as an "anchor" by interacting with a polyglutamate binding subsite during channeling of the pteridine moiety (7). The existence of other multifunctional enzymes (18-23) as well as the copurification of some non-covalently associated enzymes (24-25) in folate metabolism raise the possibility of more extensive transfer of folylpolyglutamates within such complexes.

The bifunctional formiminoglutamate:tetrahydrofolate formiminotransferase (EC.2.1.2.5)-formiminotetrahydrofolate cyclodeaminase (EC.4.3.1.4) utilizes the polyglutamates of folates and thus provides a useful system to study both the metabolic advantages of multifunctional proteins, and the significance of folylpolyglutamates [see ref. (26) for a review on this enzyme]. Physical and electron microscopic studies have indicated that this protein is composed of 8 identical polypeptides arranged as a tetramer of dimers (20) in a circular structure (18). The product of the transferase channels through the deaminase site when the pentaglutamate but not the monoglutamate substrate is used (7).

The unusual quaternary structure of the protein, its bifunctionality and the occurrence of channeling between sites raise questions concerning the number and interaction of the two catalytic sites. The identity of the subunits would suggest that both activities are the responsibility of each monomer but it is not clear as to how many folate binding sites exist on the native octamer. One folate could bind to each transferase and deaminase active site if they are separate (8 + 8 sites); to each catalytic pair (8 sites) if they are subsites of a single larger site; or to sites on each dimer (4 sites) formed at a subunit interface. In addition, the number of polyglutamate binding subsites may not equal the total number of active sites if there is only one such site per pair of transferase-deaminase activities. The number of folylpolyglutamate binding sites and the specificity of the enzyme for chain length were explored using tritiated $H_4PteGlu_n$.

MATERIALS AND METHODS

Pteroylpolyglutamates were synthesized as described earlier (27). [G-^{3}H]Folic acid potassium salt (5 Ci mmol^{-1}) was purchased from Amersham Corp.; [^{14}C]- and [^{3}H]toluene, and Aquasol-2 scintillation cocktail were from New England Nuclear. Common chemicals were reagent grade generally from Fisher. The ultrafiltration cell was purchased from MRA Corporation, Florida, and was provided with PM-10 filters (Amicon) of 7mm diameter. Nitrogen gas of highest purity was from Médigaz (Quebec).

Absorbance measurements were made on a Gilford 2000 recording spectrophotometer. Radioactivity in samples was determined in 10 ml Aquasol-2 scintillation cocktail using a Beckman LS-250 scintillation counter. Radioactivity in each sample was between 1,000 and 200,000 cpm and was counted for 10 or 20 min. Radiodisintegration rates were calculated by use of experimentally determined quench curves comparing counting efficiency with the external standard ratio.

Preparation and assay of native enzyme

Formiminotransferase-cyclodeaminase was purified from frozen pig liver (28,29) and had a transferase specific activity at 30°C of 32-38 μmol min^{-1} mg^{-1} (dry weight) when assayed as described earlier (29). The purified enzyme solution (approx. 2.5 mg/ml) was stored at -20°C and contained 0.1 M potassium phosphate, pH 7.3, 36 mM 2-mercaptoethanol, 40% glycerol. For use in binding studies, volumes from 0.4 to 2 ml were withdrawn from the stock enzyme solution and dialysed overnight against 100 ml of 0.1 M potassium phosphate, pH 7.3, 36 mM 2-mercaptoethanol. The protein content was assayed by the Lowry procedure (30) using bovine serum albumin as standard. The method was modified as per Bensadoun and Weinstein (31) for the presence of interfering substances. The Lowry

assay has been previously found to underestimate the dry weight of the transferase-deaminase by 7% (19) and all results were corrected to dry weight values.

In kinetic studies, the transferase activity was measured as described earlier (19,29) in an assay mixture containing 4.5 mM formimino-L-glutamate, 0.2 M 2-mercaptoethanol, 100 mM potassium phosphate, and 45 mM triethanolamine• HCl, pH 7.3, different concentrations of (6S)-H_4folate, and enzyme in a total volume of 1 ml. Cyclodeaminase activity was assayed by monitoring the rate of formation of 5,10-methenylH_4folate at 355 nm in a 0.5 ml assay mixture containing the same concentration of 2-mercaptoethanol, potassium phosphate and triethanolamine•HCl, pH 7.3, as the transferase mixture above, and different concentrations of (6S)-5-formiminoH_4folate. Because of its lability under the assay conditions (32,33), the substrate was added separately just before the addition of enzyme. Formiminoglutamate was present in the deaminase assay mix because of its presence in the formiminoH_4folate stock solution and was found not to affect the deaminase activity.

Preparation and assay of tetrahydropteroylpolyglutamates

(R,S)-H_4folate for routine use was prepared as before (28). Unlabeled (6S)-H_4PteGlu$_n$ substrates were prepared as previously reported (7). (6S)-[7,9-^{3}H]H_4Folate (0.1 mCi μmol^{-1}) was obtained following the same protocol with the addition of approximately 250 μCi of [G-^{3}H]folic acid. A 2-3 mM solution of unlabeled (6S)-H_4folate was obtained by scaling up the procedure as follows: the enzymic reduction mix contained approximately 90 μmoles unpurified H_2folate, 0.56 mmole glucose-6-phosphate, 4 μmoles NADP, 30 units glucose-6-P dehydrogenase

(as defined by Sigma) and 5-7 mg of dihydrofolate reductase [6.6 nmol min^{-1} mg^{-1} when assayed as per Mathews et al. (34)] in 20 ml of 20 mM Tris•HCl, 0.29 M 2-mercaptoethanol, pH 7.5. After 90 min at room temperature, the sample was diluted with an equal volume of 0.36 M 2-mercaptoethanol and applied to a 0.8 x 12 cm column of DEAE-cellulose in the chloride form, washed with 10 ml of 0.36 M 2-mercaptoethanol and eluted with an exponential gradient formed with 65 ml of 0.075 M triethanolamine•HCl, 0.36 M 2-mercaptoethanol, pH 7.3 in the closed vessel, and 55 ml of the same buffer containing 0.6 M NaCl in the upper reservoir. (6S)-[6-^{3}H]H_4PteGlu$_{3-7}$ (3-4 mCi μmol^{-1}) were prepared following a scheme similar to that for the preparation of unlabeled derivatives except that the NADPH-generating coupled system used malic enzyme, NADP and [2-^{3}H]malic acid (35)[1]. Purified H_4PteGlu$_n$ were assayed using the formiminotransferase with 5 mM formiminoglutamate and 4-5 limiting concentrations of each reduced folate, and were stored in sealed ampoules at -20°C. (6S)-5-FormiminoH_4folate was prepared enzymatically from (6S)-H_4folate [accompanying paper (33)][2] and assayed spectrophotometrically at 350 nm in 0.36 N HCl after its conversion to methenylH_4folate (36). (6R)-H_4folate was prepared by converting the S-isomer of the racemic mixture to 10-formylH_4folate with formyltetrahydrofolate synthetase (EC 6.3.4.3) in a modification (Rios-Orlandi and MacKenzie, unpublished) of the method of Curthoys and Rabinowitz (1). The unreacted D-isomer was purified by chromatography on DEAE-cellulose with a linear gradient of sodium chloride (40 to 250 mM, total volume 60 ml) and assayed in 36 mM 2-mercaptoethanol using ε_{298nm} = 29,400 cm^{-1} M^{-1}.

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[1]Reference 35 is actually Chapter 2 in this thesis.

[2]The accompanying paper (33) is actually Chapter 4 in this thesis.

Filtrate binding assay

Binding was carried out by the "forced dialysis" technique (37) using an ultrafiltration cell originally designed by Paulus (38), with PM-10 filters. The protein-ligand mixtures contained 170-190 μg native transferase-deaminase, 0.06 M potassium phosphate, 0.17 M 2-mercapto-ethanol, 0.03 M triethanolamine·HCl and different concentrations of the tritiated $H_4PteGlu_n$ in a volume of 0.26 ml, pH 7.3. Aliquots of 0.2 ml were added to each of the 8 channels, and a low pressure (5-10 psi) of nitrogen was applied to force the solution through the membrane. When the filtration rates decreased, pressure was increased to 10-20 psi. Three to five consecutive samples (5-30 μl) of filtrate were taken from each channel during the filtration. After each sampling, the residual volume of filtrate was removed by suction. Approximately 2 μl of buffer is retained by the filter (see filter binding assay and Table I) leading to a 5-10% underestimation of the concentration of free ligand in the first microliters of filtrate. The first filtrate sampling was thus not considered in the calculation of free ligand concentration. The radio-activity remained constant in the subsequent filtrate samplings. Greater than 92% of the native enzyme transferase activity was retained after 5 hours; the average time for a complete binding study was about 2 hours. In the absence of enzyme, H_4folate was totally recovered in the filtrate up to a concentration of 500 μM, and the polyglutamate derivatives in the concentration range used for binding studies did not bind to the PM-10 membrane . The very large native transferase-deaminase [$M_r \simeq 5 \times 10^5$ (28)] was easily retained by the PM-10 membrane. A 10 to 40-fold range in concentration of ligands was used in binding studies: 1 to 30 μM for $H_4PteGlu_{4-7}$, and 2.5 to 160 μM for the mono- and the triglutamate

derivatives. For the two latter compounds, the concentration was increased up to 400 μM in some studies. In binding assays, the value of the ionic strength, calculated from the contribution of buffers and salt, varied from μ = 0.113 to 0.139 with the $H_4PteGlu_n$ ligands having n > 4. The variation of ionic strength was greater with the mono- and triglutamate derivatives: the value ranged from μ = 0.113 to 0.168.

Filter binding assay

When direct determination of the amount of bound ligand was required, the binding was measured using the Paulus ultrafiltration method (38). After collection of the filtrate samplings as described above (filtrate binding assay), 40 psi of nitrogen pressure was applied for 3 hours to ensure complete filtration. The membranes were then rinsed from the bottom with 1 ml ethylene glycol as described by Paulus (38), transferred to vials containing 1 ml of water, and soaked overnight. The scintillation cocktail was then added for determination of radioactivity. This procedure was sufficient to dissolve all the radioactivity trapped on filters. Treatment of the membranes with Protosol (NEN) for 8-16 hours was found not to release more radioactivity. The PM-10 membranes retained about 2 μl under the conditions of filtration. As shown in Table I, this small volume appeared to be constant from one channel to another, independent of the folate ligand concentration, and independent of the presence or absence of enzyme as demonstrated by the use of trace amounts of [^{14}C]glycine which did not bind to the membrane. A blank value was determined in each experiment by filtering a ligand sample without protein to correct for the small retained volume. The correction was done as per Markus et al. (39), assuming that the

TABLE I

Control Filtrations through PM-10 Membranes

FOLATE (μM)	Volume Retained -Enzyme[a] (μl)	Volume Retained +Enzyme[b] (μl)
5	2.2	2.0
10	1.7	2.0
40	2.1	1.8
100	1.9	1.9
200	1.7	1.8
400	2.2	1.4

[a]The binding mixtures in absence of enzyme contained varying concentrations of [^{3}H]H_4folate (0.1 mCi μmol^{-1}).

[b]The binding mixtures in the presence of enzyme contained varying concentrations of folate, 1.15 μM of octameric protein, and 4.5 x 10^6 dpm/ml of [^{14}C]glycine (112 mCi mmol^{-1}).

concentration of free ligand was the same in the retained volume as it was in the filtrate.

Treatment of binding data

Moles of bound ligand were obtained directly by measuring the radioactivity present on the filters after correction for retained volume, or indirectly by subtracting the free from the total ligand present initially. Samples of the stock ligand solution were counted with each experiment for determination of the ligand specific activity. The molar binding ratio, R, represents the moles of bound ligand per mole of octameric protein present in the sample [the molecular weight of one subunit was taken as 62,000 (28)] and results were represented as a Scatchard plot (40). The binding parameters were determined by fitting the experimental hyperbolic curve of R versus free ligand concentration using the program KINFIT (41). In the case of curved Scatchard plots, additional binding studies using excess unlabeled ligand were performed to correct for non-specific binding (42).

RESULTS

$H_4PteGlu_n$ are substrates of the transferase and inhibitors of the deaminase activities, and can thus bind to each catalytic site of the bifunctional enzyme. Their binding to the native protein has been studied using the filtrate binding assay (Fig. 1) to determine both the number of sites and the value of K_d for each compound. With $H_4PteGlu_n$ having 4 or more glutamates, the results clearly indicated the presence of approximately 4 high affinity sites on the octamer for the binding of folylpolyglutamates. At least four sites can also bind the triglutamate derivative but with much lower affinity. The limits of the sensitivity of the filtrate binding assay have been reached with this ligand: only 4% of the ligand is bound to the enzyme at high concentration of the ligand. The binding of the triglutamate derivative and also that of the monoglutamate was examined further using the more sensitive filter assay.

Curvature of Scatchard plots for values of $R > 4$ in the binding of $H_4PteGlu_{4-7}$ indicated that some non-specific binding may have contributed to the total binding. The importance of the non-specific binding component was determined with the pentaglutamate derivative by adding excess unlabeled ligand (50 μM) to saturate the high affinity sites. The values of the parameters of the specific binding did not significantly differ from those of the total binding. With $H_4PteGlu_n$ ligands having $n > 4$, the apparent values of the binding parameters determined from their total binding (Table II) are thus a good measure of the values for their specific binding. The reproducibility of the values of the binding parameters had been assessed by additional determinations with the pentaglutamate ligand using different enzyme preparations. The values

FIGURE 1

Scatchard plots of the total binding of [^{3}H]H_4PteGlu$_n$ (n = 3,4,5,6,7) to the native octameric transferase-deaminase. Symbols used are for the different values of n: ● n = 3; □ 4; ■ 5; ▲ 6; and Δ 7. Data were fit by the program KINFIT as described in Materials and Methods. Only values of R/[free] > 0.8 were used except in the case of n = 3 where all the points were taken. Scatchard plot of the total binding of the triglutamate derivative is shown inset with an expanded R/[free] scale.

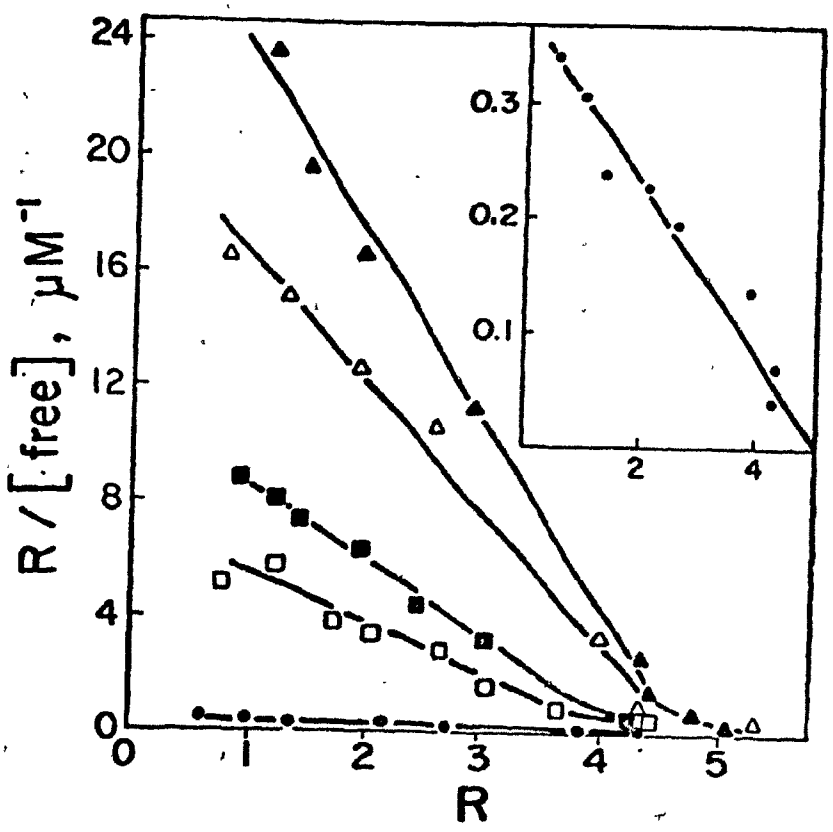

24
20
16
12
8
4
0
0
1
2
3
4
5
R
R / [·free], μM⁻¹
0.3
0.2
0.1
2
4

TABLE II

Binding Parameters of the Transferase-Deaminase with H_4Pteroylpolyglutamates

$H_4PteGlu_n$ (n)	Kd (μM)	Number of sites/octamer
1	35 ± 6	3.2 ± 0.3
3	12 ± 1.6	5.0 ± 0.2
4	0.60 ± 0.05	4.1 ± 0.1
5	0.41 ± 0.02	4.4 ± 0.1
6	0.16 ± 0.01	4.7 ± 0.1
7	0.22 ± 0.01	4.7 ± 0.1

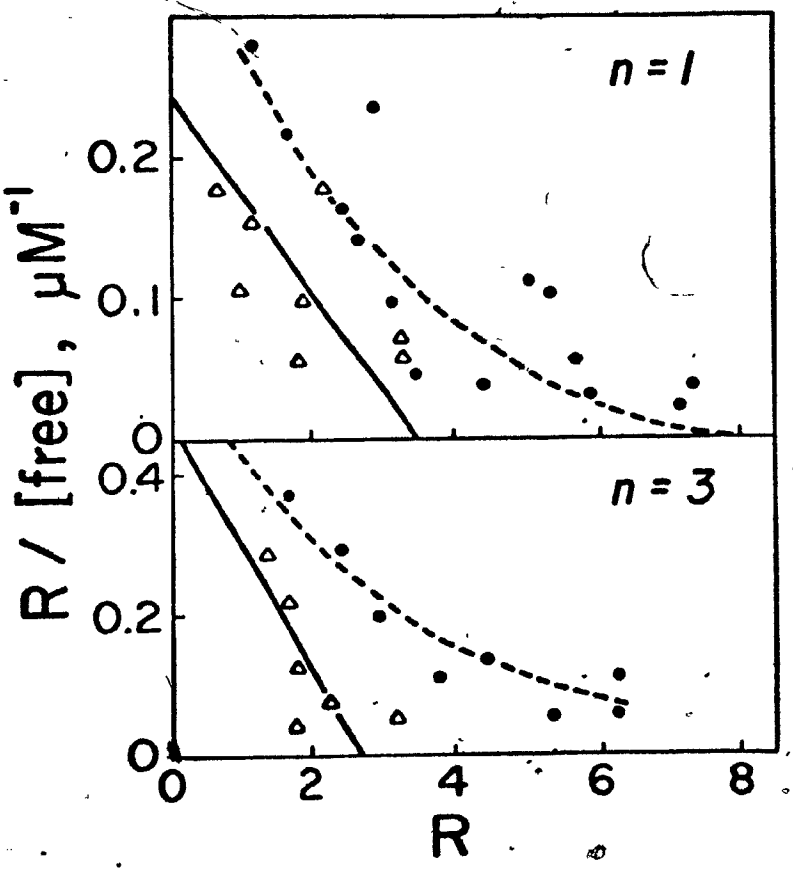
Values were obtained from computer fitting of the total Scatchard plots, and expressed with the standard deviation of the fit.

obtained from five separate experiments were 0.40 ± 0.09 μM for the value of the dissociation constant and 3.9 ± 0.5 for the number of sites.

The total and the specific binding of $H_4PteGlu_1$ and of $H_4PteGlu_3$ to the native enzyme were studied using both the filtrate and the filter assays, the latter permitting the direct measurement of bound ligand. Results by both procedures agreed and an example of a binding study is given for each ligand in Figure 2. In addition, the specific binding for the monoglutamate has been determined in three other separate experiments, using either 75 μM unlabeled folate or 130 μM unlabeled H_4folate to measure the non-specific binding. For the four studies, the values of the binding parameters averaged 3.7 ± 1.1 sites per octamer with a dissociation constant of 10 ± 4 μM. The choice of the concentration of the unlabeled excess ligand is not an easy task, especially when working at the limits in sensitivity of the binding assay, and may result in incomplete separation of the specific and non-specific binding components (43). The contribution of the non-specific binding can also be estimated graphically (43). With the mono- and triglutamate ligands, the values of the non-specific binding parameters obtained by the graphical method were similar to the values obtained directly by addition of an excess of unlabeled ligand. A theoretical curve for the total binding of $H_4PteGlu_1$ was drawn using a two-component binding function (48) for which the parameters of the first component (i.e. specific binding) were experimentally determined. The chosen values for the parameters were as reported in the legend of Fig. 2. Within the same range of free ligand concentration, an essentially identical curve is obtained assuming 8 sites with a K_d value of 500 μM for the second binding component. A theoretical curve is not drawn for the triglutamate ligand because this compound is more

FIGURE 2

Scatchard plots of the total (•) and specific (Δ) binding of $[^3H]H_4PteGlu_n$ (n = 1,3) to the native octameric transferase-deaminase. The specific binding was obtained from the difference between the total and the non-specific binding plots of R vs [free], and fitted using the program KINFIT. Top: The curve of the total binding was drawn using a two-component binding function (48), and assuming the following values: K_{d_1} = 12 μM; K_{d_2} = 200 μM; $n_1 = n_2$ = 4 sites. The non-specific binding was determined by addition of 75 μM unlabeled folate. Values of the specific binding parameters are: 3.4 ± 0.6 sites with a K_d = 14 ± 7 μM. Bottom: The curve of the total binding was drawn by eye. The non-specific binding was determined by addition of 130 μM unlabeled $H_4PteGlu_3$. Values of the specific binding parameters are: 2.8 ± 0.5 sites with a K_d = 6 ± 4 μM.


n = 1
n = 3
R / [free], μM⁻¹
0.2
0.1
0
0.4
0.2
0
0
2
4
6
8
R

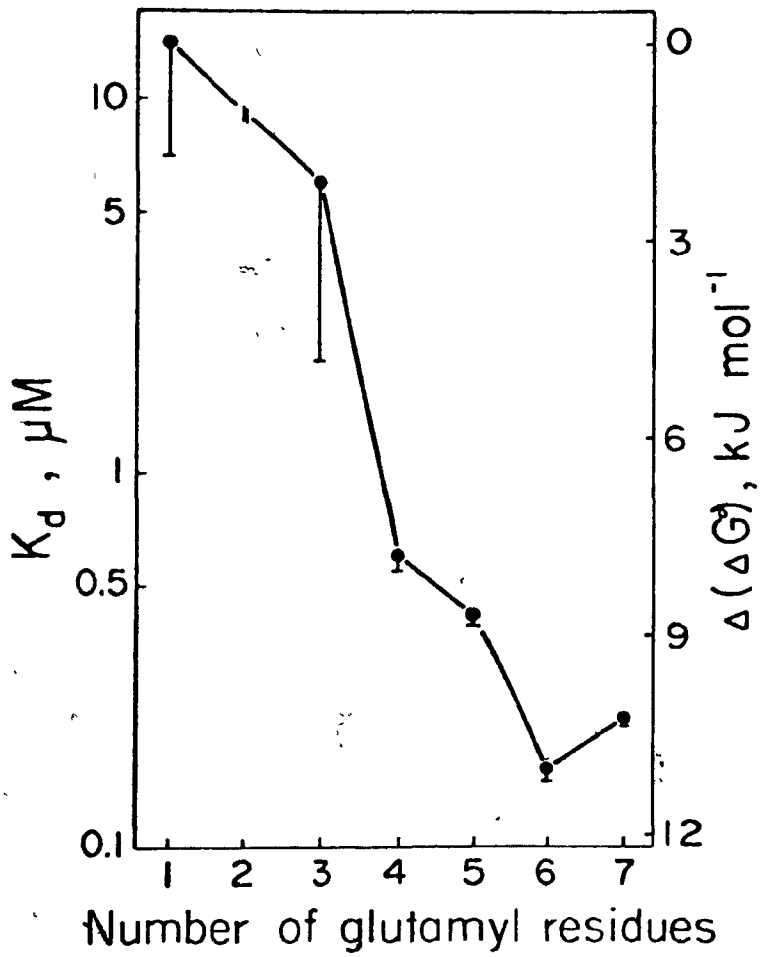
likely to bind to a polyglutamate binding subsite as well as to each catalytic site, thus possibly introducing a third component in the model.

The transferase-deaminase showed a 15- to 50-fold greater affinity for $H_4PteGlu_n$ having 4 or more glutamate residues than for the mono- and the triglutamate derivatives. The highest affinity was observed with the hexaglutamate derivative and a decrease in affinity was obtained by lengthening further the polyglutamate chain. Values of dissociation constants were used to calculate the free energy decrease associated with binding of the glutamyl residues of the polyglutamate chain moiety of $H_4PteGlu_n$ to the native enzyme (Fig. 3). There was little difference in affinity between the mono- and the triglutamate ligands, and binding of the second and third glutamate residues can be associated with a free energy decrease of about 1.05 kJ (0.25 kcal) per residue. A large decrease of 5.7 kJ (1.36 kcal) in free energy is observed by binding a fourth glutamate, while binding of additional glutamyl residues modified the value of the free energy by approximately 1.25 kJ (0.3 kcal) per residue.

One can postulate that the H_4folate ligand lacking the polyglutamate tail could bind to both transferase and deaminase catalytic sites. The low affinity of the enzyme for H_4folate as reflected by the relatively high value of K_d (14 μM) does not allow for an accurate determination of the number of sites within the limits of the binding assays. At least 4 sites on the octamer can bind this monoglutamate ligand. However, it is not clear whether these folate-binding sites are common to both transferase and deaminase catalytic sites or belong to only one type of site. In the latter case, a second type of site of much lower affinity

FIGURE 3

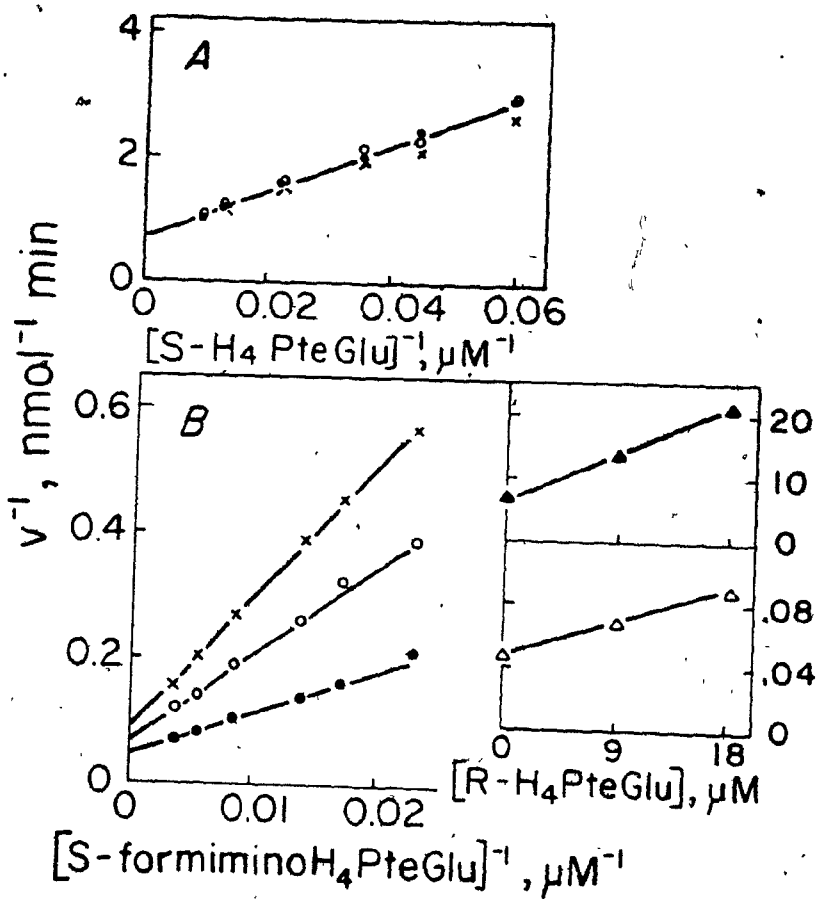
Relative free energy changes associated with the number of glutamyl residues upon binding of $H_4PteGlu_n$ to the native transferase-deaminase. Values of the dissociation constant were taken from Table II for $n > 4$, and from legend of Fig. 2 for $n = 1,3$.


10
5
1
0.5
0.1
K_d , μM
0
3
6
9
12
Δ(ΔG), kJ mol^{-1}
1 2 3 4 5 6 7
Number of glutamyl residues

for folate would not have been detected by the binding assays. A kinetic approach with inhibitors was used to attempt to distinguish two types of pteridine-binding subsite. As shown in Figure 4, the transferase and deaminase activities were not inhibited to the same extent by (R)-H_4folate. This compound is a poor inhibitor of the transferase with an estimated K_i value of 400 μM. In contrast, the deaminase has much higher affinity for this inhibitor which exerts a greater effect on the slopes of double-reciprocal plots than on the intercepts of these plots. Replots of both slopes and intercepts as a function of inhibitor concentration are linear and indicate K_i values of respectively 8 and 21 μM.

FIGURE 4

Double reciprocal plots of the transferase (A) and deaminase (B) activities obtained at different concentrations of the (6R)-H_4folate inhibitor: zero (•), 9 (○) and 18 (×) μM. Data were fitted using the KINFIT program. Replots of slopes (▲) and intercepts (Δ) are shown inset for the deaminase.


A
4
2
0
0
0.02
0.04
0.06
[S-H4 PteGlu]-1, μM-1
B
0.6
0.4
0.2
0
0
0.01
0.02
v-1, nmol-1 min
[S-formiminoH4PteGlu]-1, μM-1
20
10
0
.08
.04
0
0
9
18
[R-H4PteGlu], μM

DISCUSSION

The advantages and the validity of the filter and filtrate binding assays have been discussed by other investigators (37-39,44,45). Sensitivity, rapidity and equilibration within one compartment were important advantages as was the requirement for only small quantities of enzyme and folate ligands. Although the filtrate assay is more rapid, the filter assay can be more accurate in cases of low affinity because it measures bound ligand directly. Scattering in results is also observed with the latter assay because a 10% error exists in the determination of the volume retained by filters (Table I). However, both assays gave similar results in the estimation of binding parameters in the present study. An additional requirement was to maintain the integrity of the binding capacity of the enzyme throughout the filtration process. The constant amount of radioactivity present in the filtrate during the course of the filtration indicated that concentration of enzyme during the procedure, changes in the viscosity of the ligand-protein mixture, or adsorption of protein to the filter did not affect the binding parameters. The data from binding studies were checked on semi-logarithmic plots to ensure that saturation of at least one class of sites was obtained, as described by Klotz (46). Because of the classical Michaelis-Menten kinetics shown by the enzyme with all substrates, cooperativity in binding was not considered in treating any non-linear Scatchard plot.

The results of binding of $H_4PteGlu_n$ derivatives to this bifunctional enzyme composed of identical subunits arranged in an unusual circular structure clearly demonstrated the presence of four high affinity polyglutamate binding sites on the octamer. The number of sites

suggests that the polyglutamate subsite is formed by one type of subunit interface. Because the polyglutamate subsite is required for kinetic channeling, it is likely that at least a dimeric structure is required for channeling to occur. The four sites also suggests that there is one polyglutamate binding site per pair of transferase-deaminase activities.

A more difficult problem arises in attempting to determine the number of catalytic sites. One could postulate that a folate analog could bind to both transferase and deaminase catalytic sites, if it did not contain the polyglutamate portion. If there are indeed two separate catalytic sites, these could show different affinities for the folate ligands. The low affinity of the enzyme for the mono- and triglutamate derivatives suggests that these ligands bind to catalytic sites, but poorly. As indicated by their specific binding, there are at least 4 sites for the binding of the pteroyl portion of folates. The value of K_d for the specific binding of H_4folate [14 ± 7 μM (Fig. 2)] is similar to the value of K_m for the transferase extrapolated to zero formimino-glutamate [18 μM (data not shown)]; the K_i value of the same compound against the deaminase is 64 ± 4 μM (data not shown). Thus it is likely that the specific sites observed with the monoglutamate ligand represent the transferase catalytic activity. The technique would not detect a second class of sites with K_d approximately 60 μM.

Are there then two classes of catalytic sites? No folate ligand was found to have sufficiently low values of K_d to address this question by binding. Instead, evidence for two classes of folate binding sites was obtained by the different extent of inhibition of each catalytic activity using (R)-H_4folate. The non-competitive pattern of inhibition observed

with the deaminase was unexpected although a similar pattern was obtained with methylenetetrahydrofolate dehydrogenase using $PteGlu_n$ inhibitors (13). In the latter case, the folate inhibitor was found to bind to both the folate-substrate site and the $NADP^+$-substrate site. In the case of deaminase, a likely possibility is that (R)-H_4folate binds to the conformer of the enzyme which binds methenylH_4folate, the product of the reaction. In converting 5-formiminoH_4folate to the methenyl derivative, the bridging of nitrogens 5 and 10 requires a significant shape change in the molecule, and consequently an altered enzyme conformation. The S-isomer of H_4folate is simply a competitive inhibitor of this activity (K_I = 64 μM), and perhaps binds only to one enzyme conformation. Despite its more complex characteristics, (R)-H_4folate is a very good inhibitor of the deaminase and a very poor inhibitor of the transferase. That the two activities form separate sites was also suggested by chemical modification of each activity (7), by the isolation of a transferase fragment (20), and by the kinetic observations presented earlier (47), and in the accompanying paper (33), which demonstrate that both activities can function independently and simultaneously. Currently it appears that there are four polyglutamate sites, possibly four transferase catalytic sites and an unknown number of deaminase catalytic sites on the octamer. Because of the limits of the binding assays, a $K_d < 3$ μM is required to accurately determine the total number of binding sites and we were unsuccessful in finding a suitable ligand. Neither (R)- nor (S)-H_4folate, the most promising ligands, meets this condition. In addition, difficulty in the interpretation of binding data may arise due to the complex inhibition pattern observed with the R-isomer.

Although the number of sites cannot be determined with the low affinity ligands, a reasonable estimate of K_d was obtained from their specific binding and used to calculate the variation in relative free energy by binding folylpolyglutamates of different chain length to the enzyme. The large difference of 5.7 kJ (1.36 kcal) in free energy between binding a third and a fourth glutamate to the enzyme suggests that the fourth glutamate is important in anchoring the substrate at the polyglutamate binding subsite during the transfer of the pteroyl moiety between active sites. The free energy profile of the polyglutamate specificity of the transferase-deaminase when compared to the profile of four other folate-dependent enzymes from pig liver (11-14) appears to constitute a fifth pattern in the extent to which the enzyme prefers each polyglutamate derivative versus the monoglutamate. As does methylene-tetrahydrofolate reductase, the transferase-deaminase shows a trend in the specificity constants of the folylpolyglutamates with a maximum at the hexaglutamate derivative (11). Both enzymes have a narrower specificity for folylpolyglutamates than has methylenetetrahydrofolate dehydrogenase (13). However, the observed decrease in free energy between the mono- and the hexaglutamate derivatives is smaller with the $H_4PteGlu_n$ substrates of the transferase-deaminase (10.9 kJ) than with the $H_2PteGlu_n$ inhibitors of the reductase (16.3 kJ). In contrast to the reductase which shows a regular decrease in free energy by adding one glutamyl residue at a time, the transferase-deaminase exhibits a very large decrease (5.7 kJ) upon addition of a fourth glutamyl residue. A similar extensive decrease in free energy upon binding of only one more glutamyl residue is reported for thymidylate synthase [8.8 kJ (14)] and for serine hydroxymethyltransferase [5.0 kJ (12)], but is observed upon

addition of a second glutamyl residue in these two cases. Such a variation between profiles within one species tends to support the possible regulatory role of folylpolyglutamates in folate-dependent one-carbon metabolism. The specificity of the transferase and deaminase activities for their folylpolyglutamate substrates was measured kinetically in the accompanying paper (33) to determine if it reflects the specificity as measured by binding studies. These specificities were also compared to that of efficiency of channeling between sites to better characterize that process.

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CHAPTER 4

Channeling of 5-Formimino-tetrahydropteroylpolyglutamate Between the Transferase and Deaminase Active Sites

PREFACE

This Chapter has been submitted for publication in the Journal of Biological Chemistry as the second paper of two characterizing the interaction of folylpolyglutamates with the transferase-deaminase. The paper is presented under the title "Formiminotransferase-cyclodeaminase from pig liver. 2. Channeling between the active sites" by J. Paquin, C.M. Baugh and R.E. MacKenzie.

The pteroylpolyglutamates were supplied by Dr. Charles M. Baugh of the University of South Alabama, USA. The transferase-deaminase enzyme was prepared by Leonora Bortoluzzi, a technician in our laboratory. (R,S)-Tetrahydrofolate for routine use and methenyltetrahydrofolate are prepared by Dr. Robert MacKenzie. Criticism and proofreading of the article by Wendy Findlay are appreciated, as is the secretarial assistance of Maureen Caron in typing the manuscript.

A part of this work was communicated to the Canadian Federation of Biological Societies (1984) under the title "The Relationship Between the Activities of a Bifunctional Enzyme". The results constitute a part of a communication that will be presented to the Canadian Federation of Biological Societies (1985) under the title "Structure and Function of an Octameric, Bifunctional Enzyme".

SUMMARY

Each activity of the formiminotransferase-cyclodeaminase bifunctional enzyme was examined separately for its specificity for the appropriate tetrahydropteroyl(glutamate)$_n$ substrates where n = 1,3,4,5,6,7. Catalytic efficiency of both activities (V_m/K_m) is much greater with substrates containing four or more glutamates, but this property does not show specificity for a given polyglutamate. With all substrates, V_m of the deaminase is greater than that of the transferase, allowing for potential channeling between these active sites. Only with the long (n > 4) folylpolyglutamates does the enzyme channel the formimino-intermediate between active sites, supporting the concept that the polyglutamate structure acts as an anchor during the transfer of the pteroyl moiety. The specificity for channeling among the physiologically relevant folylpolyglutamates (n > 4) does not correlate with affinity as measured kinetically or by ligand binding. The optimum length of 5 glutamates for complete transfer between sites supports a steric requirement and not simply tight binding to explain the mechanism of channeling. Kinetic observations indicate that transferase and deaminase activities are independent catalytic sites but share a common polyglutamate binding subsite.

INTRODUCTION

5-Formiminotetrahydrofolate: L-glutamate N-formiminotransferase (EC 2.1.2.5)-5-formiminotetrahydrofolate ammonia-lyase (cyclizing) (EC 4.3.1.4) catalyse the following consecutive reactions in the pathway of histidine degradation in mammals:

$$\text{Formiminoglutamate} + H_4\text{PteGlu}_n \rightarrow \text{5-formimino}H_4\text{PteGlu}_n + \text{glutamate}$$

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

The transferase-deaminase enzyme is composed of eight identical subunits (1) arranged in a ring (2) as a tetramer of dimers (3). Although the total number of catalytic sites is still not clear, the transferase and deaminase occur at separate sites. Only four high affinity sites have been observed on the native octamer for the binding of the polyglutamate tail of folylpolyglutamates (4)[1], which suggests that only one polyglutamate binding site exists per pair of transferase-deaminase activities, and raises the potential for site formation between subunits. The enzyme shows increased affinity for $H_4\text{PteGlu}_n$ with increasing numbers of glutamyl residues to a total of six. The relative free energy associated with the binding of $H_4\text{PteGlu}_n$ to the enzyme decreases by approximately 1.25 kJ (0.30 kcal) per glutamyl residue, with one exception. The difference is four-fold greater for binding the fourth glutamyl residue, suggesting that a minimum chain length of 4 glutamates is required for the $H_4\text{PteGlu}_n$ to maintain interaction between the polyglutamate binding site and at least one active site. Channeling of the formimino-intermediate between the transferase and

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[1]Reference 4 is actually Chapter 3 in this thesis.

deaminase sites has been observed when $H_4PteGlu_5$ but not when H_4folate itself is used as substrate (5). The binding of the polyglutamate tail to a subsite on the enzyme was postulated to participate in the transfer of the pteridine portion by "anchoring" the folate coenzyme molecule (5).

Knowing how the covalently associated transferase and deaminase sites interact could help to evaluate the potential transfer of folyl-polyglutamates between other physically associated sites in folate metabolism. In this paper, we have assessed the kinetic specificity of each activity for the number of glutamates in their respective $H_4PteGlu_n$ substrates to better understand channeling between catalytic sites in this particular enzyme. The specificity of the enzyme for folylpolyglutamates as measured by catalytic efficiency (V_m/K_m) has been compared to specificity as measured by efficiency of channeling to determine if simply tight binding or a steric requirement for a given length of the polyglutamate chain is critical in channeling. Kinetic studies have been performed to demonstrate that the transferase and deaminase catalytic sites can function separately and simultaneously.

MATERIALS AND METHODS

Decolorizing charcoal Norit A was from BDH Chemical Co.; Millipore Corp. was the source of Millex-GS 0.22 μm filter units, and CF-25 Centriflo membrane cones were from Amicon Corp. Common chemicals were reagent grade from Fisher Chemical Co.

Formiminoglutamate·hemibarium salt was obtained from Sigma Chemical Co. and converted to the sodium salt by precipitation of the barium with 10% molar excess of sodium sulfate. Formiminoglutamate was assayed using the formiminotransferase assay (6) with 0.25 mM (R,S)-H_4folate. A limiting concentration of formiminoglutamate (between 25 and 125 μM) was incubated in duplicate with 1 unit of the transferase (1 μmol min^{-1}) and the reaction was stopped with 2.0 ml 0.36 N HCl after 30 min incubation at 30°C. These conditions ensure complete reaction; neither additional enzyme nor longer incubations increase the yield. Formiminoglutamate concentration determined by the assay was 90% of the concentration obtained by weight.

Absorbance measurements were made on a Gilford 2000 recording spectrophotometer while fluorescence units were obtained on a Perkin-Elmer LS-5 spectrofluorometer (excitation 350 nm, emission 465 nm). Fluorescence measurements were made with a filter in the emission beam to restrict passage of light to wavelengths > 430 nm.

Preparation and assay of tetrahydrofolate derivatives

(R,S)-H_4Folate for routine assay and (S)-H_4PteGlu$_n$ were synthesized, purified, assayed and stored as described before (1,5) and in the accompanying paper (4). Preparation of (R,S)-5,10-methenylH_4folic acid was by the procedure of Rowe (7). A 1 mM stock solution was prepared in 0.54N HCl, 0.36 M 2-mercaptoethanol and filtered through a 0.22 μM

Millex-GS filter unit. The concentration of methenylH_4folate in the filtered solution was measured in 0.36N HCl using ε_{350nm} = 24,900. In 1.0N HCl, the absorption ratio E_{350nm}/E_{305nm} varied between 2.46 and 2.54 indicating a pure material (8).

(6S)-5-formimino-H_4PteGlu$_n$ (n=1,3-7) were synthesized by converting the corresponding (6S)-H_4PteGlu$_n$ to the formimino derivative using the transferase-active fragment. An incubation mix containing limiting amounts of the tetrahydrofolate (150-2500 nanomoles) and a 50-fold excess of formiminoglutamate in 1.3-1.9 ml of 0.075 M triethanolamine·HCl, pH 7.3, 0.36 M 2-mercaptoethanol was adjusted to pH 6.7 with HCl. The H_4PteGlu$_n$ was completely converted to the formimino product after 2 min at room temperature following the addition of 15 μg of the transferase fragment. The incubation mix was cooled on ice and acidified to pH 3.8 with HCl. The transferase fragment was removed by ultrafiltration through a CF-25 Centriflo membrane. This solution was stable for several hours at 0°C, protected from light (Results). To standardize conditions in kinetic studies, a pH 3.8 blank solution was made containing the same concentrations of triethanolamine·HCl, 2-mercaptoethanol and formiminoglutamate. The formiminoH_4PteGlu$_n$ were assayed by converting them to the 5,10-methenyl derivatives in 0.36N HCl. To complete the conversion, the test tubes were heated in boiling water for 55 sec (9) and cooled in ice. The absorbance at 350 nm immediately after addition of the formiminoH_4PteGlu$_n$ to the acid was used as a blank value (10). Essentially quantitative conversion (> 98%) of H_4PteGlu$_n$ to formiminoH_4PteGlu$_n$ was obtained.

Enzyme and transferase fragment

Formiminotransferase-cyclodeaminase was purified from pig liver (1) and a transferase fragment (24-28 μmol min^{-1} mg^{-1}) was prepared as described earlier (3). Protein determination was as described in the accompanying paper (4).

Assays of transferase-deaminase

For determination of kinetic constants, the transferase was assayed in 1.0 ml of 0.1 M potassium phosphate (pH 7.3), 0.14 M 2-mercaptoethanol, 4.5 mM formiminoglutamate, 15 mM triethanolamine•HCl (pH 7.3), and varying concentrations of the $H_4PteGlu_n$. The reaction was initiated by the addition of enzyme and stopped with 25 μl of 9.6N HCl (spectrophotometric assay) or with 2.5 ml of 0.36N HCl (fluorometric assay) after 5 to 10 min incubation at 30°C. Blanks were prepared in the same assay conditions except that enzyme was replaced by buffer. The test tubes were heated in boiling water for 55 sec and cooled in ice. The 5,10-methenyl$H_4PteGlu_n$ formed in the sample was then measured spectrophotometrically at 350 nm against the corresponding blank. With $H_4PteGlu_{4-7}$, the methenyl final product was assayed using a more sensitive fluorometric method (11) with a Perkin-Elmer LS-5 spectrofluorometer. Samples and blanks were excited at 350 nm (using a slit width of 15 nm) and their emission was measured at 465 nm (using a slit width of 20 nm). Four different concentrations of pure (R,S)-5,10-methenylH_4folate were used as standards and a fluorescence calibration curve was determined for each kinetic run. Standards were measured under the same assay conditions as the samples except that boiling was not necessary. The fluorescence varied linearly with the amount of methenyl-H_4folate from 0 to 0.25 nanomoles when 1 ml of assay mixture is combined

with 2.5 ml of 0.36N HCl. The fluorescence of $H_4PteGlu_n$ is 10 times weaker than that of methenyl$H_4PteGlu_n$ under the assay conditions. This small contribution to fluorescence measurements from the tetrahydrofolates introduced as much as a 10% underestimation of the real velocity of the reaction. However, velocity values are reported without correction. The fluorescence of tetrahydrofolate-containing samples increased slowly with time following boiling, and was thus measured immediately after this treatment. A 5-fold decrease in background level was obtained by using the following procedures. Vessels were treated in boiling 1N HCl whenever possible, carefully rinsed with distilled water, and dried. Also, solutions of 0.36N HCl and of 1 M potassium phosphate (pH 7.3) were treated for 30 min with decolorizing charcoal (1 g per liter of solution) and filtered (Whatman).

The kinetic constants of the deaminase activity were determined at 30°C by monitoring the appearance of methenyl$H_4PteGlu_n$ at 355 nm (1). The 0.5 ml assay mix contained 0.1M potassium phosphate (pH 7.3), 0.3M 2-mercaptoethanol, 67 mM triethanolamine•HCl, and varying concentrations of the formiminoH$_4$PteGlu$_n$. The formiminoH$_4$PteGlu$_n$ substrates were added 30 sec before the addition of enzyme because of their lability under the conditions of assay [(10) and Results]. The final assay mix was pH 7.3, essentially unaltered by the addition of the acidic solution of substrate. Formiminoglutamate was present in the deaminase assay mix (from .03 to 10 mM) because of its presence in the formiminoH$_4$PteGlu$_n$ stock solution. The concentration of formiminoglutamate was not standardized in the assay mix because its presence did not affect the deaminase activity. For example, the velocity curve obtained by varying

the concentration of formiminoH$_4$PteGlu$_4$ was the same in the presence of 0.7 mM or 5.3 mM formiminoglutamate.

The relative ionic strength changed less than 20% in almost all kinetic runs by varying the concentrations of H$_4$PteGlu$_n$ and of formiminoH$_4$PteGlu$_n$. The values calculated from the contribution of buffers and salt ranged from μ = 0.164 to 0.194 in the transferase assay, and from μ = 0.197 to 0.225 in the deaminase assay.

Kinetic constants were calculated by fitting the experimental data to the Michaelis-Menten equation using the program KINFIT (12). An unweighted procedure was used, and no significant difference was seen when points were weighted as $1/v^2$.

The time course for the appearance of the products of the transferase-deaminase used to monitor 'channeling' of the formimino intermediate was determined in an assay mix containing 0.1 M potassium phosphate, 23 mM triethanolamine·HCl, pH 7.3, 0.17 M 2-mercaptoethanol, 4.5 mM formiminoglutamate and 50 μM H$_4$PteGlu$_n$.

The mechanism of channeling was investigated by the addition of either formiminoH$_4$PteGlu$_1$ or formiminoH$_4$PteGlu$_5$ to assay mixtures containing either H$_4$PteGlu$_1$ or H$_4$PteGlu$_5$. Formation of the methenyl product was monitored at 355 nm in an assay mixture containing 67 mM triethanolamine·HCl, 100 mM potassium phosphate pH 7.3, 0.3 M 2-mercaptoethanol, 4.6-4.9 mM formiminoglutamate and the folate substrates in a volume of 0.5 ml. In this series of experiments, the transferase activity was saturated with its H$_4$PteGlu$_n$ substrate. These assays were conducted at room temperature.

RESULTS

The kinetic properties of the formiminotransferase-cyclodeaminase enzyme were determined separately for each activity using the appropriate tetrahydrofolate derivatives as substrates. The transferase activity was examined for substrate specificity using $H_4PteGlu_n$. The K_m values of polyglutamates were very low and were not adequately measured by the spectrophotometric stopped assay because 30% or more of the substrate was converted to product at substrate concentrations below the apparent Km. With these substrates, a fluorescence assay has been adapted using methenylH_4folate as standard. The fluorescence assay was approximately 10 times more sensitive than the spectrophotometric assay and good rates could be obtained with only 5-10% of the substrate converted to product. Because $H_4PteGlu_5$ provided complete channeling (5), formation of the methenyl final product was also determined by direct spectrophotometric monitoring which was found to be 3 to 4 times less sensitive than the fluorescence stopped assay. The values of K_m (2.1 ± 0.5 μM) and V_m (36 ± 2 μmol min^{-1} mg^{-1}) obtained with the pentaglutamate substrate using this assay agreed quite well with the values obtained by the fluorescence assay (Table I) considering the difference in sensitivity of the two methods. The fluorescence assay was thus adopted because of its higher sensitivity and also because the continuous spectrophotometric assay is not applicable to all $H_4PteGlu_n$ [(5) and Results]. Values of V_m were not greatly affected by lengthening the polyglutamate chain; there was at most a two-fold decrease. However, $H_4PteGlu_n$ having 4 or more glutamates have 40 to 80 fold lower values of K_m than do the mono- and triglutamate derivatives (Table I). Values of K_m do not differ

TABLE I

Kinetic Properties of Native Transferase

(6S)-H_4PteGlu$_n$		K_m	V_m
n	N	μM	μmol $min^{-1}mg^{-1}$
1	5	48 ± 14	58 ± 18
3	4	27 ± 3	25 ± 2
4	6	0.7 ± 0.3	29 ± 14
5	5	0.7 ± 0.3	35 ± 10
6	5	0.6 ± 0.2	30 ± 7
7	7	0.7 ± 0.3	41 ± 6

Values are expressed as averages ± S.D. for N separate determinations. Values were obtained from spectrophotometric (n = 1 and 3) or fluorometric (n > 4) stopped assays.

significantly among the longer polyglutamate substrates ($n > 4$) and we cannot distinguish an optimum chain length using this parameter.

The deaminase assay required synthesis of the formimino derivative of $H_4PteGlu_n$ for use as substrate. Because of limited amounts of $H_4PteGlu_n$, the established preparation methods were not practical (9,10,13): chromatography is needed to separate the formimino derivative from the unreacted H_4folate, and during the isolation procedure, the formiminoH_4folate is partly cyclized to the 5,10-methenyl derivative. In addition, purification of the formimino$H_4PteGlu_n$ would have required drastic conditions for elution of the desired compounds (13). Instead, the formimino$H_4PteGlu_n$ were prepared with the approach used by Tabor and Wyngarden for H_4folate (9), except that conditions were found to quantitatively convert the $H_4PteGlu_n$ to their formimino derivatives. This conversion occurred in fact very rapidly, and the reaction mixture could quickly be adjusted to conditions allowing for stability of the product. The formimino$H_4PteGlu_n$ stock solutions were prepared in concentrations varying from 50 to 600 μM and were stable for several hours at pH 3.8, 0°C, according to both chemical assay (Fig. 1) and enzymic assay with the deaminase. The loss of formimino$H_4PteGlu_n$ was mainly due to a very slow cyclization to the methenyl derivative. Although unavoidable, this cyclization was kept to a minimal level. On the other hand, the half-life of formiminoH_4folate at neutral pH and 30°C was estimated to be 53 min (Fig. 1) using the deaminase assay, and is similar to that determined by Rabinowitz (10).

With the deaminase activity, the length of the polyglutamate chain of the formimino$H_4PteGlu_n$ substrates affects both the values of K_m and V_m. The value of the latter decreases 10-fold going from the mono-

FIGURE 1

Stability of 5-formiminoH_4folate at pH 3.8, 0°C (•), and at pH 7.3, 30°C (Δ). Inset are the data at pH 7.3, 30° presented as a semi-logarithmic plot.

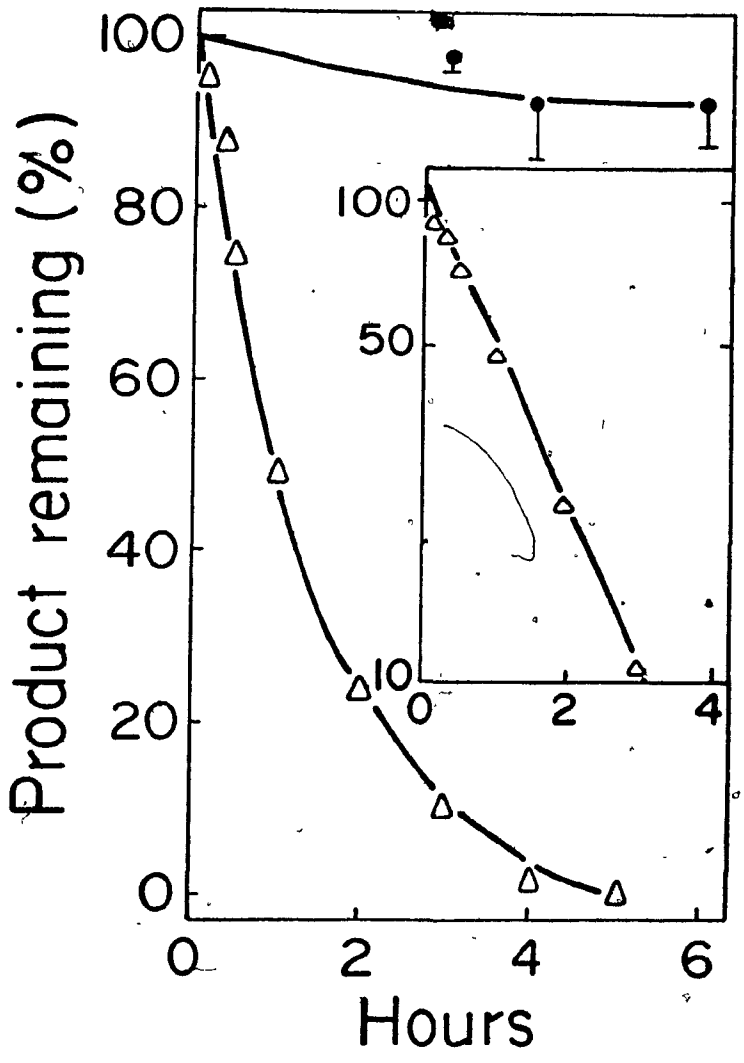

Product remaining (%)
100
80
60
40
20
0
0
2
4
6
Hours
100
50
10
0
2
4

TABLE II

Kinetic Properties of Native Deaminase

(6S)-5-HCNH-H_4PteGlu$_n$		K_m	V_m
n	N	μM	μmol $min^{-1}mg^{-1}$
1	4	149 ± 14	624 ± 53
3	3	21 ± 2.4	232 ± 11
4	4	2.9 ± 0.9	120 ± 8
5	3	2.0 ± 0.7	76 ± 20
6	4	1.2 ± 0.2	67 ± 10
7	3	2.1 ± 0.4	78 ± 8

Values are expressed as averages ± S.D. for N separate determinations.

to the hexaglutamate substrate (Table II). The addition of two glutamates to the monoglutamate substrate decreased the apparent value of K_m by a factor of 7. One more glutamyl residue reduces the value 10-fold, while further increase in the chain length introduces only another factor of 2. The kinetic affinity is clearly the highest with the hexaglutamate substrate. However, because of a parallel decrease in the value of V_m, the catalytic efficiency of the deaminase with this substrate does not differ from those observed with substrates having 4, 5 and 7 glutamates (Fig. 2). In the same manner, the transferase activity does not distinguish between $H_4PteGlu_n$ substrates having 4, 5, 6 and 7 glutamyl residues, in terms of catalytic efficiency. With both activities the discrepancy in catalytic efficiency between short and long polyglutamate substrates is pronounced indicating that $H_4PteGlu_n$ having $n > 4$ are the physiologically relevant substrates.

A major interest in measuring the kinetic specificities of the transferase-deaminase for the $H_4PteGlu_n$ derivatives is to relate these specificities with the efficiencies of channeling of the intermediate i.e. the extent to which the product of the first reaction is preferentially transferred to the second site. With $H_4PteGlu_3$, as well as with the $H_4PteGlu_1$ (5), accumulation of the formimino intermediate was observed and the final methenyl product appeared with a significant lag (Fig. 3). This lag indicated that the intermediate had to accumulate to high enough concentrations before production of the final product can be detected. Although there was accumulation of the intermediate with $H_4PteGlu_4$ and $H_4PteGlu_6$ substrates, the lag in the appearance of the final product disappeared, indicating that a fraction of the formimino intermediate has been preferentially transferred to the deaminase site.

FIGURE 2

Catalytic efficiency of the formiminotransferase (▲) and cyclodeaminase (o) with their respective $H_4PteGlu_n$ substrates containing different numbers of glutamyl residues. Values are averages ± S.D. for 3 to 6 separate determinations.

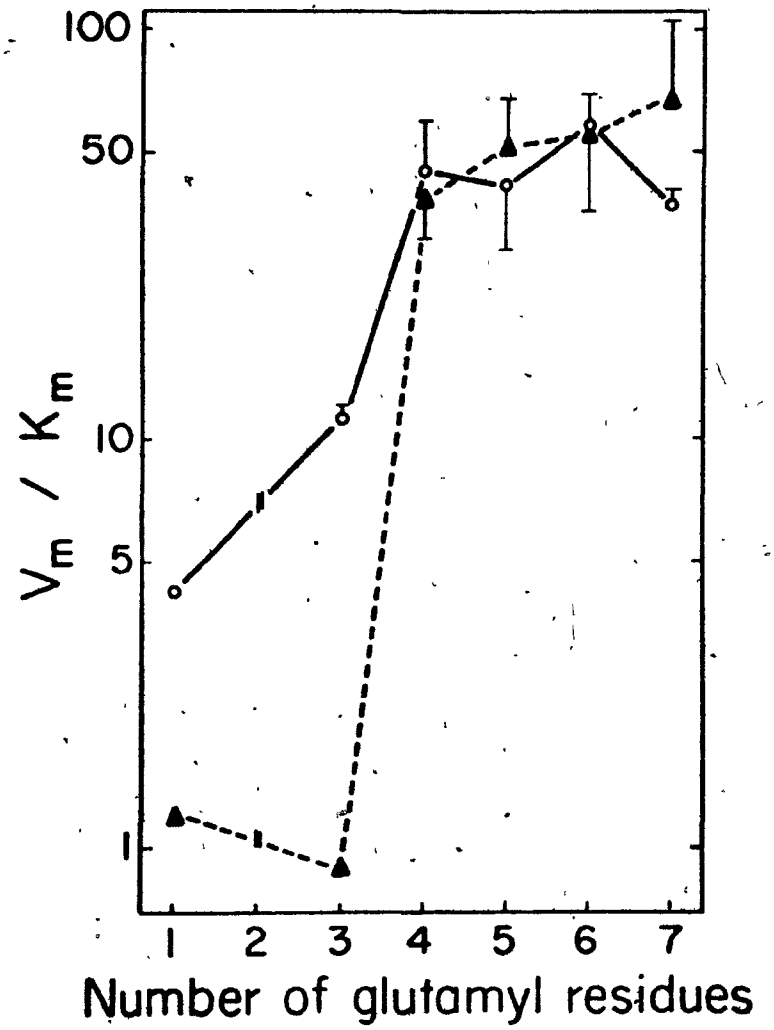
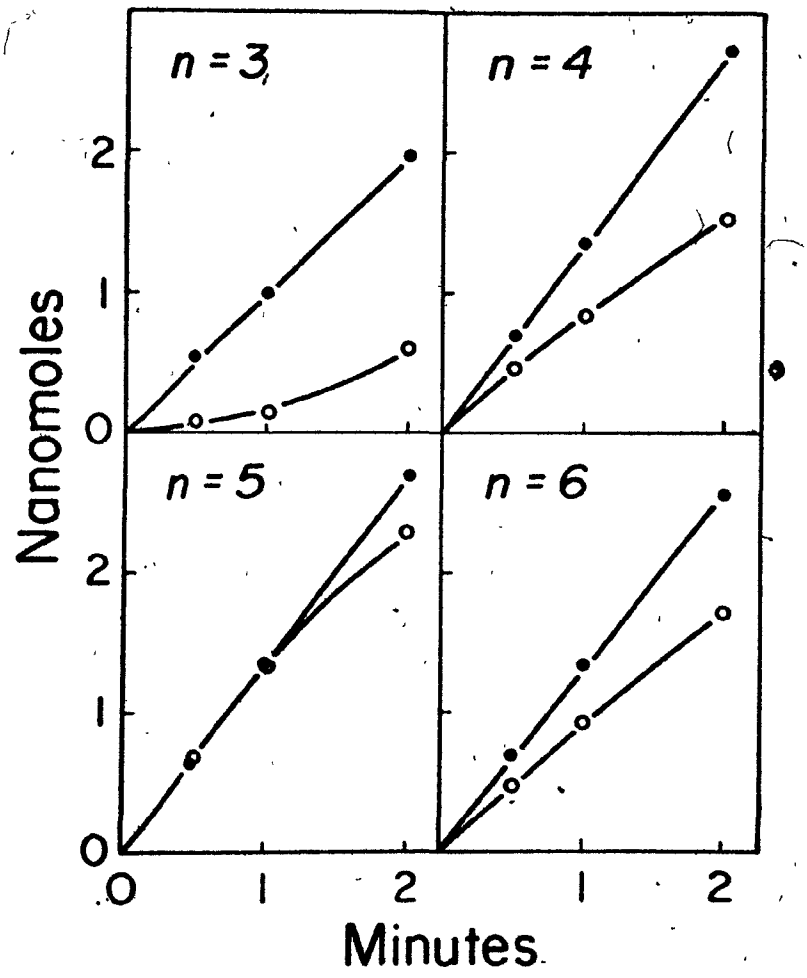

100
50
10
5
1
V_E / K_E
1
2
3
4
5
6
7
Number of glutamyl residues

FIGURE 3

Time course of appearance of products, using 50 μM $H_4PteGlu_n$ (n = 3,4,5,6), 4.5 mM formiminoglutamate, and 42 ng transferase-deaminase. Symbols represent: (●) total methenyl- and formimino$H_4PteGlu_n$, and (o) methenyl$H_4PteGlu_n$ alone.


n = 3
n = 4
n = 5
n = 6
Nanomoles
Minutes
0
1
2
0
1
2
0
1
2

Channeling is complete with the pentaglutamate substrate as indicated by the lack of accumulation of formimino intermediate in the medium. As shown in Figure 4, channeling can occur with n = 4, 5, 6 and 7 glutamates, but the optimum length is clearly 5 glutamates.

Because of instability of the formiminoH$_4$folate and its chemical conversion to methenyl, it has not been possible to examine the properties of the channeling process by the classical approaches of following the enrichment or dilution of radiolabeled products. The question was approached by providing the enzyme with extra formimino intermediate in the assay mixture. Figure 5(A) demonstrates that it is possible to add formiminoH$_4$PteGlu$_1$ to the transferase-deaminase assay where the transferase activity was 90% saturated with H$_4$PteGlu$_1$, and obtain extensive additional production of methenyl product. Similarly, significant addition of formimino intermediate could also occur when at least one component of the H$_4$PteGlu$_n$/formiminoH$_4$PteGlu$_n$ pair was a monoglutamate (B and C) but not when the two components were pentaglutamate derivatives (data not shown). In the latter case, significant addition of the formiminoH$_4$PteGlu$_5$ intermediate could be observed only if it was provided in amounts sufficient to compete with the H$_4$PteGlu$_5$ substrate (D).

FIGURE 4

The efficiency of channeling as a function of the number of glutamates in $H_4PteGlu_n$. Conditions were as in Methods and Figure 3. Efficiency is expressed as a ratio of the initial rate of appearance of methenyl/initial rate of the transferase.

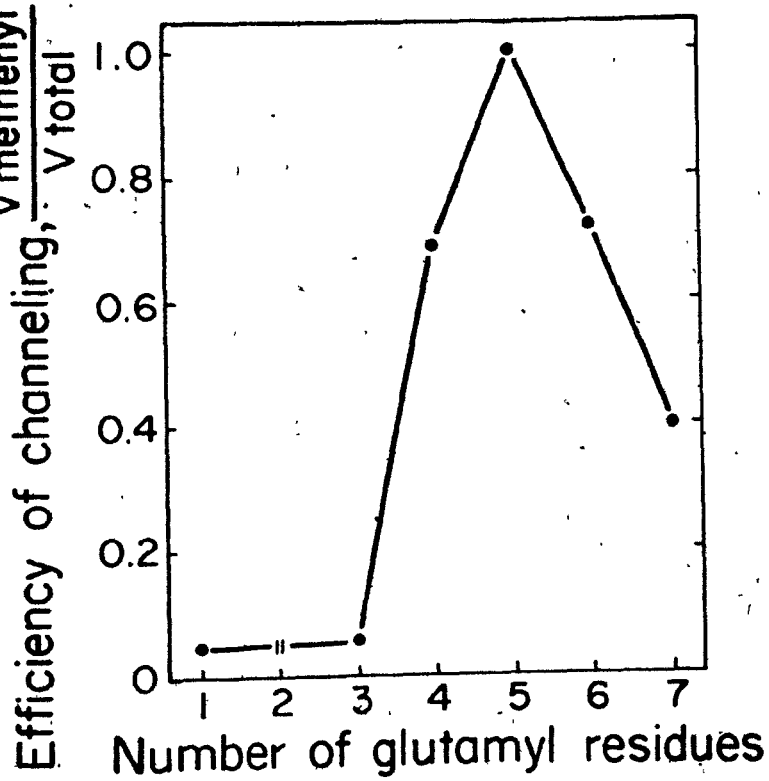
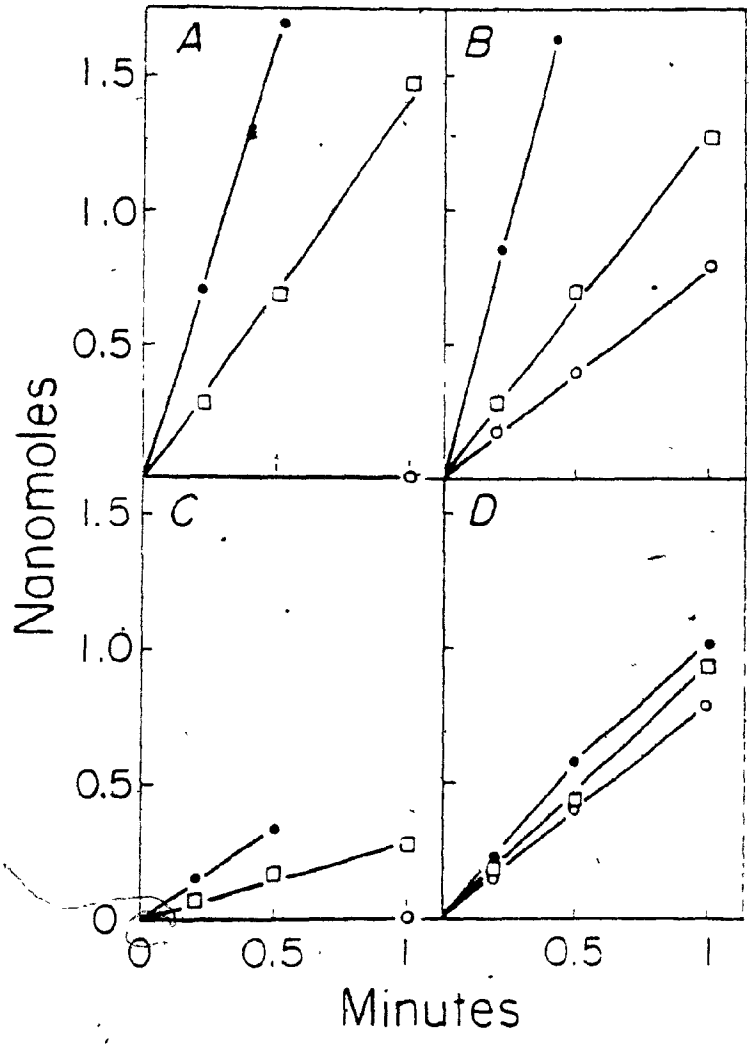

Efficiency of channeling, V methenyl / V total
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
Number of glutamyl residues

FIGURE 5

Rate of appearance of the methenyl$H_4PteGlu_n$ product using 36 ng transferase-deaminase and 4.6-4.9 mM formiminoglutamate reaction mixture containing: A. (o) 450 μM (S)-$H_4PteGlu_1$; (•) 81 μM formimino$H_4PteGlu_1$; (□) both substrates at once. B. a similar experiment with 17 μM (S)-$H_4PteGlu_5$ and 92 μM formimino$H_4PteGlu_1$. C. 462 μM (S)-$H_4PteGlu_1$ and 1.4 μM formimino$H_4PteGlu_5$. D. 36 μM (S)-$H_4PteGlu_5$ and 6.3 μM formimino-$H_4PteGlu_5$.


A
B
C
D
1.5
1.0
0.5
0
0.5
1
Nanomoles
Minutes

DISCUSSION

The polyglutamates of the H_4folate coenzymes are preferred substrates for several enzymes, in general having much lower values of K_m than the corresponding monoglutamate derivatives while the V_m values are only moderately if at all affected (5,14-25). The polyglutamate chain has a similar effect on the kinetic parameters of $H_4PteGlu_n$ with the transferase, thus providing for tighter interaction of the substrates with the enzyme. The values of K_d for binding $H_4PteGlu_n$ to the native enzyme has shown a 20 to 80-fold increase in affinity between short (n = 1 and 3) and long polyglutamate (n = 4, 5, 6, 7) derivatives (4). The catalytic efficiency (V_m/K_m) of each activity discriminates similarly between the short and the long polyglutamates, indicating that folylpolyglutamates with 4 or more glutamates are the physiologically relevant coenzymes of these activities at the low concentrations of folates encountered in the cells.

With the deaminase activity, a decrease in values of V_m (10-fold) accompanied a decrease in values of K_m (100-fold) on lengthening the polyglutamate tail of the substrates, suggests that dissociation of products is slower with polyglutamate than with monoglutamate derivatives. The slower rate of release of products is probably not rate limiting in the overall reaction of the transferase-deaminase since under identical conditions the catalytic turnover of the deaminase is greater than that of the transferase (Fig. 5D).

The mechanism of channeling must be reconciled with the unique tetramer of dimers structure of the native enzyme. Formation of dimers may be needed for channeling since only 4 folylpolyglutamate binding sites have been observed on the octameric protein (4). The large

decrease in free energy observed with the binding of a fourth glutamyl residue to the enzyme suggested this glutamate to be important in anchoring the folate molecule during the movement of the pteroyl moiety between catalytic sites. The observation that channeling only occurs with $H_4PteGlu_n$ having $n > 4$ supports the postulated function for the fourth glutamate. If one assumes tight binding of the α-carboxyl group of residue four, the 'mobile' portion, measured to nitrogen 5 of the $H_4PteGlu$ ring, is about 20-25°A, allowing for a considerable maximal distance between catalytic sites within that radius.

Based solely on kinetic parameters for a two reaction sequence, as presented by Benkovic and his coworkers in a very interesting model (26), channeling in this case could be expected to follow affinity of the deaminase for its formimino substrate, which appears to be maximal for the hexaglutamate. However, the specificity for channeling ($5 > 6 \approx 4 > 7$) does not correlate either with affinity as measured by binding studies ($6 > 7 > 5 > 4$), nor with values of catalytic efficiency ($4 \approx 5 \approx 6 \approx 7$), which suggests that complete transfer of the intermediate between active sites requires an appropriate length of chain, not simply a tighter sequestering of substrate on the enzyme surface. Covalent association between active sites (or possibly strong association generated by a stable quaternary structure) can provide appropriate conditions for kinetic channeling. The active sites of the transferase-deaminase appear thus to be optimally spaced for the pentaglutamate derivative.

As mentioned earlier, classical radiolabeling approaches cannot be used to study the properties of the channeling process with this enzyme. Previously (27) we demonstrated by using mixtures of transferase active

enzyme with unmodified enzyme, that the extra formimino formed in the assay could result in increased production of final methenyl when $H_4PteGlu_1$ but not $H_4PteGlu_5$ substrate was used. This approach permitted study of the behavior of only substrate/intermediate folate pairs with the same number of glutamates. Because formimino$H_4PteGlu_n$ can now be prepared, the use of heterogeneous pairs was also possible. When the native enzyme has its transferase activity saturated with the $H_4PteGlu_n$ substrate, small quantities of exogenous formimino intermediate can be added to the system and increase the production of the methenyl product if at least one of the $H_4PteGlu_n$/formimino$H_4PteGlu_n$ pair is a monoglutamate. These results indicate that the two catalytic sites act independently. The active sites can also be differentiated by chemical modification (5) or by kinetic inhibition using a folate derivative (4). The observation that an increase in the production of methenyl with the pentaglutamate/pentaglutamate pair can occur only at high concentration of added formimino intermediate suggests that the pentaglutamate substrate and the pentaglutamate intermediate compete for the same binding site, namely the polyglutamate binding site. This is consistent with the existence of a single polyglutamate binding site per pair of transferase-deaminase activities as suggested by having only four folylpolyglutamate binding sites on the octamer. Although it is now clear that the transferase and the deaminase sites form two classes of sites, the total number of catalytic sites, and/or the kinetic properties of a monomer or a dimer still remain to be determined before deciding if one or both of the two types of subunit interaction are needed for catalytic activity and channeling.

The transferase-deaminase enzyme provides an appropriate system to

study the transfer of folylpolyglutamates between catalytic sites, within or potentially between subunits, and may thus help to understand the possible channeling of folates between non-covalently associated sites. As far as we are aware, the transferase-deaminase is the only folate-dependent enzyme that completely channels a folate intermediate (28,29). In addition to increasing the efficiency of the overall reactions, channeling with this particular enzymes is also beneficial in reducing the concentration of a labile intermediate for which there is no other known metabolic function and which may compete in other folate-mediated reactions. But, is there a physiological advantage to the clear specificity for the pentaglutamate derivative, which is not the predominant form in pig liver (30)? While there is as yet no clear cut answer to this question, it is interesting that Brody et al. (31) observed that in rats, increased histidine oxidation was associated with an increase in the fraction of cellular folates present as pentaglutamates, with no change in hexa- and heptaglutamate levels. They suggested that efficient histidine catabolism in vivo may have a strong requirement for $H_4PteGlu_5$, an observation consistent with the specificity in efficiency of channeling of the folylpolyglutamates by the transferase-deaminase. The difference between the pentaglutamate and the other polyglutamate derivatives with respect to channeling adds another element to the growing number of observations supporting the possible regulatory function of folylpolyglutamates in folate-dependent one-carbon unit metabolism. In addition to strict specificity in affinities, the regulatory role may also involve defined steric requirements for optimum chain lengths to promote site-site interactions. Enzymes of folate metabolism which show particularly strong binding for a glutamyl

residue(s) distal from the pteridine ring could be considered as candidates for potential site-site interaction via transfer of the pteroyl moiety.

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CHAPTER 5

GENERAL DISCUSSION

The formiminotransferase-cyclodeaminase enzyme catalyzes two sequential folate-dependent reactions. The naturally-occurring folylpolyglutamates certainly have a role with this enzyme. Each activity has higher affinity for the polyglutamate forms of the folate coenzymes as seen in both binding (Chapter 3) and kinetic studies [Chapter 4 and (49)], and shows an increased catalytic specificity with these derivatives (Chapter 4). The folylpolyglutamates thus provide a means to operate at low intracellular folate concentrations. In addition, the use of polyglutamate substrates combined with the bifunctional nature of the protein increases the efficiency of the pathway. With the pentaglutamate substrate, the intermediate formed by the transferase reaction does not leave the enzyme but is transferred preferentially to the deaminase site [Chapter 4 and (49)].

Kinetic observations and the results of binding studies bring out some features of the mechanism of channeling with this particular enzyme. There are four folylpolyglutamate binding sites per octameric protein, and their high affinity for the longer polyglutamate derivatives indicates the importance of the polyglutamate moiety of these ligands in binding (Chapter 3). In addition, only the polyglutamate substrates for which the enzyme exhibits high affinity result in channeling (Chapter 4), thus supporting the concept that the folylpolyglutamate molecule is anchored at a polyglutamate binding subsite during the movement of its pteroyl moiety between active sites (49). The observation of only four polyglutamate binding sites suggests that at least a dimeric structure is necessary for channeling, and that there might exist one such site per pair of transferase-deaminase activities. The results of kinetic studies done with the native enzyme (Chapter 4) and with an active transferase

fragment (105) also support a common polyglutamate binding subsite. The transferase and deaminase sites can function separately and simultaneously [Chapter 4 and (185)] indicating that they form separate sites. This conclusion is consistent with the isolation of a transferase fragment (105) and the results of chemical modifications (49). The presence of additional glutamyl residues on the H_4folate substrates increases the affinity of the enzyme for the coenzymes. This tighter interaction may be seen as a larger effective concentration of substrate at the active sites. However, it has been demonstrated that the efficiency of channeling does not depend only on the maintenance of high local concentration of substrates but also on a specific steric requirement in the length of the polyglutamate chain to assist the transfer of the intermediate in an optimal position for catalysis.

The relationship between catalytic sites of the transferase-deaminase within its unique quaternary structure still needs further investigation. However, the results of the present studies raise interesting aspects of the relationship between active sites in multi-enzyme systems in general, and in folate-dependent pathways in particular. Some comments and perspectives for future studies are developed in the following sections.

5.1 Role of the quaternary structure in catalysis/channeling

The number and the location of the two active sites of the transferase-deaminase enzyme are still not resolved. The observation of four folylpolyglutamate binding sites on the native octamer (Chapter 2) and the occurrence of two types of subunit interaction (105) raise the potential of formation of sites between subunits. The existence of intersubunit sites for catalytic activity has been proposed in several

cases. For example, experimental evidence suggested that the pyridoxal phosphate site of tryptophan synthase from E. coli is formed at the site of interaction of the two β-monomers (188). Similarly, the four folate binding sites of the tetrameric formylH_4folate synthetase from Clostridium cylindrosporum may be formed by the association of the identical monomers (189). The β-ketoacyl synthase activity of both yeast (64) and animal (58) fatty acid synthase requires the juxtaposition of an active cysteine-SH of one α subunit and of a pantetheine-SH of the adjacent α subunit.

One approach which could help to decide whether the transferase and deaminase sites of our enzyme are intrinsic to each monomeric subunit or if they are formed at a subunit interaction involves additional binding studies. It is reasonable to assume that the two activities form separate sites and that a pteroylmonoglutamate ligand lacking the polyglutamate chain would bind only to the pteroyl binding site of each activity. Asking the same type of questions as outlined in Chapter 3, we are faced with three likely possibilities for the number of catalytic sites on the octameric protein. There may exist 4 + 4 sites if each activity is formed at a subunit interface, 8 + 8 sites if each monomer is responsible for both activities, or possibly 4 + 8 sites in a mixed situation. The "4 + 4 sites" would be the clearest situation since the observation of 8 sites for a given activity can result from the formation of two equivalent active centers by one type of subunit association, as has been shown for the mammalian fatty acid synthase (58). The results of binding studies using H_4folate present the possibility of four transferase sites but the affinity of the enzyme for this ligand was not high enough for accurate determination (Chapter 3). Trials to find a folate

ligand of suitable affinity for one or both activities have been unfruitful so far, but folate analogs are potential candidates. The binding of a folate derivative could also be studied in the presence of polyglutamate peptides (either strictly the peptides, or for example, the peptides linked to a p-aminobenzoic acid ring). It has been shown that polyglutamate peptides can offer protection against proteolysis (190). Such protection could possibly occur through a slight conformation change in the protein, and this change could affect the affinity of the enzyme for the pteridine ring of folate ligands. The presence of polyglutamate peptides may appear useful for determination of the number of deaminase sites since it has been suggested that the deaminase site and the polyglutamate binding site are closely associated (105). The number of transferase sites can possibly be determined using analogs of formiminoglutamate which is the other substrate of the reaction; formiminoglutamate itself does not have high affinity with the enzyme. The binding of a formiminoglutamate analog can be studied in the presence of a pteroylpolyglutamate since folylpolyglutamates may affect the affinity of folate-dependent enzymes for their non-folate substrates (section 5.4). The number of catalytic sites could possibly be determined by measuring the incorporation of a folate affinity label into the active sites, as has been done with the trifunctional folate-dependent enzyme (191).

The role of the quaternary structure in activity can also be approached by trying to dissociate and reassociate the subunits of the enzyme. Possibly, conditions can be found for the formation and isolation of stable monomeric and dimeric intermediates. The intermediates could then be assayed for transferase and deaminase activities. Because the octamer is assembled by two types of subunit interaction, two dimeric

species can be obtained, thus caution is needed in interpreting data obtained with intermediates of the dissociation/reassociation processes. One simple way to distinguish the two interactions would be to look for polyglutamate specificity since the four folylpolyglutamate binding sites observed are likely formed at a subunit interface. Studies can also be carried out with the dimeric transferase fragment obtained from chymotryptic cleavage of the protein. Proteolysis could also be used to obtain a deaminase fragment.

One interesting question regarding the interaction of the transferase and deaminase active sites relates to the distance between them in the native enzyme. The sites are not necessarily close since a distance of 20-25Å can easily exist between a polyglutamate binding subsite and each of the catalytic sites (Chapter 4); this distance is slightly less than the size of a subunit [diameter = 32Å (104)]. The distance between active sites can be estimated by energy transfer using fluorescent probes as has been done for example for the tryptophan synthase complex (74).

The importance of quaternary structure for the channeling of an intermediate has been established only in a few cases. The quaternary structure of a complex is obviously required for channeling when the first and the second active sites are on separate polypeptides as is the case for the tryptophan synthase complex (72,74). Dimers but not monomers of the bifunctional UMP synthase can channel the OMP intermediate from one catalytic site to the other (76). In this case, however, the formation of dimers was also apparently necessary for activation of one of the two activities (76). In mammals, the overall synthesis of a fatty acid requires the dimeric form of the multifunctional fatty acid synthase since the active centers of the β-ketoacyl

synthase component are created at the subunit interface (58). One very interesting example of the role of quaternary structure in channeling is that of the yeast fatty acid synthase (64). The native enzyme complex is formed by the association of two multifunctional proteins within an $\alpha_6\beta_6$ structure. The activity of the β-ketoacyl synthase component requires the juxtapositioning of two adjacent α subunits as seen for the mammalian enzyme (64). Even though theoretically an $\alpha_2\beta$ complex could carry out the overall reaction, and even though $\alpha_2\beta_2$ and $\alpha_4\beta_4$ have been detected as intermediate species, the $\alpha_6\beta_6$ structure is the active form of the complex. It has been proposed that only the latter arrangement can ensure the optimal distance between the complementary thiol groups of adjacent α-subunits for catalytic activity of the ketoacyl synthase (64).

According to the results of binding studies with the transferase-deaminase, a dimer is theoretically the minimal structure for realization of channeling. However, as observed for the yeast fatty acid synthase, the octamer may be the only active structure for channeling, especially if the two types of subunit interaction appear to be required for the catalytic activities. The quaternary structure may also be important for regulation or conformational stability.

5.2 Comments on mechanisms and advantages of channeling

A basic aspect of the mechanism of channeling in multienzyme systems relates to the degree of overlap of the active sites. The examples of channeling collected so far (sections 1.1.3 and 5.1) indicate that a unique arrangement is not required for the active sites involved in the transfer of an intermediate. At one extreme, channeling may occur between non-overlapping sites. Indole is channeled between active sites

which are located on different types of polypeptide in the tryptophan synthase complex. As far as I am aware, channeling of an intermediate between separate sites within a monomeric multifunctional protein has not been reported. Whether the juxtapositioning of adjacent identical polypeptides is necessary, as seen for the UMP synthase, cannot be decided because of the lack of examples. In these two examples, the intermediate has been proposed to migrate between active sites within a cleft created by the assembly of the subunits. At the other extreme, both reactions can occur at a common active site. The chorismate mutase-prephenate dehydrogenase enzyme, and the dehydrogenase-cyclohydrolase activities of the trifunctional folate-dependent protein illustrate this situation. Between these two extremes, the multifunctional enzymes fatty acid synthase and formiminotransferase-cyclodeaminase can be regarded as examples of "partial overlap". The catalytic sites in each system are independent but share a common subsite, that is the site of attachment of the "swinging arm" which effects the transfer of intermediate between sites. The transferase-deaminase system differs from the fatty acid synthase system in that the mobile arm interacts with the enzyme most likely through electrostatic forces rather than covalent linkage. This is suggested by the negative charges carried by the polyglutamate chain of folylpolyglutamates, and by the effect of salts on the activity of folate-dependent enzymes with their folylpolyglutamate coenzymes (136,172,192). Whether there exists some limitation in the arrangement of sites for channeling is not yet clear. The study of the mechanism of channeling in other systems can be helpful in this regard.

In addition to increasing the catalytic efficiency of a given pathway by maintenance of high local concentration of substrate at the site

of utilization, the channeling of intermediate may offer other advantages as has been proposed in several cases. The channeling of indole in the tryptophan synthase complex presents a physiological advantage in yeast. At low cell density, mutant strains which could not effect the channeling of indole showed an exaggerated, prolonged lag period preceeding the usual exponential growth (193). The abnormal lag was reduced by addition of low concentrations of indole to the medium (193). The author suggested that the channeling mechanism offers a means to retain indole within the cell. The two catalytic activities of UMP synthase are found on a bifunctional protein only in mammals (76). The channeling of OMP intermediate in this system may be beneficial since mammalian cells have more active pyrimidine nucleotidases than do bacteria (76). The compartmentalized OMP could thus be protected from degradation before its utilization (76). In glycolysis, the channeling of 1,3-diphosphoglycerate occurs via an enzyme-substrate-enzyme complex (77). It is suggested that the formation of this complex could ensure the correct or most effective transfer of this unstable intermediate (77). Thermodynamically, the concentration of this intermediate must be low. In folate metabolism, the formiminoH$_4$folate and the methenylH$_4$folate intermediates are channeled between the transferase-deaminase [Chapter 4 and (49)] and between the dehydrogenase-cyclohydrolase (48,49) activities respectively. Reasons for channeling could relate to the fact that these intermediates are labile, that they are potential inhibitors of other folate-dependent activities, and that they have no other known function (120,185). However, some aspects of their channeling remain obscure. The mono- and polyglutamate forms of methenylH$_4$folate are both channeled between sites but this transfer, although preferential, is not complete (48,49). Only

the long polyglutamate forms of formiminoH$_4$folate are channeled, and complete channeling requires a pentaglutamate [Chapter 4 and (49)]. A deeper comprehension of the mechanisms regulating folate metabolism could help to delineate some metabolic advantage for these different features. In addition, the channeling of the methenyl and formimino intermediates has been studied in only one direction. The extent to which channeling occurs in both directions could give useful information regarding mechansim.

In summary, the examples cited in this section illustrate some of the advantages (outlined in section 1.1.2) which could result from the compartmentation of intermediates in multienzyme systems. These advantages relate to the trapping of important intermediates (e.g. indole), the protection of labile compounds, and the prevention of interference between related or competing pathways. In addition, the channeling process can direct the flux of intermediates toward specific sites for utilization. In this regard, the degree to which enzyme organization occurs in a given pathway could affect the efficiency of this flux. Potential direction of flux through the folate-dependent pathways is discussed in terms of enzyme-enzyme associations in the next section.

5.3 Organization of enzyme activities in folate metabolism

Physical association between enzyme activities has been shown to occur in different pathways of folate metabolism (section 1.2.2). The potential for more extensive association of enzymes in these pathways still exists. A high degree of organization in this metabolism could also involve the interconnection of degradative and synthetic pathways possibly via association of each process with the enzyme activities responsible for the interconversion of one-carbon H$_4$folate derivatives.

The metabolic sequences formed within this organization could be, for example:

1. serine hydroxymethyltransferase → thymidylate synthase → H_2folate reductase.
2. serine hydroxymethyltransferase → methyleneH_4folate dehydrogenase - methenylH_4folate cyclohydrolase → GAR transformylase (or AICAR transformylase).
3. formiminoglutamate:H_4folate formiminotransferase - formimino-H_4folate cyclodeaminase → methenylH_4folate cyclohydrolase → GAR transformylase (or AICAR transformylase).
4. formylH_4folate synthetase → GAR transformylase (or AICAR transformylase).
5. methyleneH_4folate dehydrogenase - methenylH_4folate cyclohydrolase → 10-formylH_4folate dehydrogenase, for the disposal of excess one-carbon units (48,82,96).

The copurification of serine hydroxymethyltransferase, GAR and AICAR transformylases, and the activities of the trifunctional protein (methyleneH_4folate dehydrogenase-methenylH_4folate cyclohydrolase-formylH_4folate synthetase) from avian liver (115,116) suggests that a high degree of organization may occur in folate metabolism. This organization may be important in the regulation of flux through the different pathways and may offer protection to the labile folate intermediates.

Weak interactions between enzymes may not be detected because they do not withstand the conditions of the purification procedures. Approaches to study physical interactions in vitro may involve cross-linking and sedimentation analyses (116). A possible role of folylpolyglutamates in promoting association of folate-dependent enzyme activities

has not been studied and may be a promising avenue of investigation. As has been seen with formiminotransferase-cyclodeaminase (Chapter 4), the polyglutamate chain of folate coenzymes offers two advantages in this regard. The polyglutamate chain tightly anchors the folate molecule on the enzyme surface through interaction with a binding site. If distal glutamyl residues of the chain are involved in this interaction, the pteroyl moiety of the coenzyme can cover a considerable distance from one active site to another. For example, the mobile portion of a folylpolyglutamate, measured from the α-carboxyl group of the fourth glutamyl residue to nitrogen 5 of the pteridine ring, is approximately 20-25 Å. These particular features, tight binding and great mobility of the folylpolyglutamates, could perhaps be sufficient to effect the transfer (channeling) of folate substrates between different enzymes. Such functional site-site interaction could possibly overcome the requirement for strong physical association of the enzyme activities, and could occur through formation of an "enzyme-substrate-enzyme" complex as has been observed in vitro for two glycolytic enzymes, glyceraldehyde-3-P-dehydrogenase and 3-phosphoglycerate kinase (77). The enzymes which show high affinity for distal glutamates of their folylpolyglutamate coenzymes are potential candidates for such association. In pig liver, the formiminotransferase-cyclodeaminase enzyme (Chapters 3 and 4), the activities of the trifunctional protein (49,163), and the activities of serine hydroxymethyltransferase, thymidylate synthase, and methyleneH_4-folate reductase (175) apparently exhibit this feature.

The formiminotransferase-cyclodeaminase enzyme can potentially associate with the trifunctional folate-dependent protein since its final product, methenylH_4folate, is a substrate of the trifunctional enzyme.

The bifunctional protein could also associate with other activities of the histidine degradation pathway. The planar structure of transferase-deaminase is large and could thus offer a good surface area for further enzyme-enzyme association. Its structure is similar to the "equatorial plate-like structure" formed by the multifunctional α-subunits of the yeast fatty acid synthase (64). This equatorial plate is associated with six subunits of another multifunctional protein to form the fatty acid synthase complex (64).

5.4 Specialized functions of folylpolyglutamates

As has been reported previously, the folylpolyglutamates are the functional coenzymes of folate-dependent activities (section 1.2.3). Potentially, they can regulate the flux through the various pathways of folate metabolism via specificities of the enzyme activities for the number of glutamates in their folate substrates [section 1.2.3 and (26)]. Potentially also, they can promote channeling of intermediate through enzyme-enzyme associations (sections 1.2.3 and 5.3). This section briefly covers two other functions of the polyglutamate derivatives: stabilization of enzyme activities, and alteration of the kinetic properties of enzymes (156).

McGuire and Bertino suggested in their review (156) that the binding of folylpolyglutamates to folate-dependent enzymes may protect the enzymes against intracellular degradation. This may affect the cellular levels of enzyme activities. Protection against proteolysis has been demonstrated *in vitro* for *L. casei* thymidylate synthase (156), pig liver formiminotransferase-cyclodeaminase (105), and yeast trifunctional protein (190). In the latter case, protection can even be provided by the use of polyglutamate peptides lacking the pteridine ring (190).

Generally, increased protection is observed with increased length of the polyglutamate chain (190). The results indicate the importance of the polyglutamate moiety in stabilizing enzyme activities.

Folylpolyglutamates can affect the kinetics of a reaction by modifying the kinetic properties of an enzyme activity (156). Changes in K_m for the folate substrate and in V_m have already been reported (sections 1.2.3 and Chapter 4), but the K_m value for the non-folate substrate may also increase or decrease if polyglutamate derivatives of the folate substrates are used (156). The order of addition of substrates and of release of products could possibly differ depending on whether a short- or a long-chain folylpolyglutamate is the coenzyme (156,163). The mode of inhibition could be modified by the use of polyglutamates instead of monoglutamates (156,163). Kinetics of the transferase activity with the monoglutamate form of folate substrate suggested that the reaction involves a rapid equilibrium random mechanism with formation of dead-end complex: enzyme-H_4folate-glutamate (186). This type of mechanism indicates that each substrate (H_4folate and formiminoglutamate) binds independently to a part of the active site of the enzyme, and that either substrate can add first. It may be possible that one order of addition of substrates is greatly preferred in the presence of a folylpolyglutamate substrate. It is also possible that the binding of a polyglutamate derivative of H_4folate could modify the K_m for formiminoglutamate. The significance of such possibilities with the transferase activity remains to be determined.

In conclusion, the role of the quaternary structure of formiminotransferase-cyclodeaminase for catalytic activity and channeling of intermediate is an important aspect of its function, and requires

further study. This bifunctional enzyme constitutes an excellent system to investigate the potential for enzyme-enzyme associations that could occur in folate metabolism, and the role of folylpolyglutamates with such associations. Because of the physical association of its transferase and deaminase activities, this protein leads us into the general area of enzyme organization in cells. Characterizing its properties would help to understand how enzyme organization is useful in terms of metabolic efficiency and regulation.

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