methyl, 3-bromo, and 3-fluoro analogs the predominant product in the Perkow reaction of the β-halo-α-ketoacid with trimethyl phosphite consistently has its $|J_{^{31}POCCH}|$ coupling constant greater than that for the stereoisomer and that the vinyl proton of the predominant product always has a chemical shift at lower field than that for the stereoisomer.

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Formyltetrahydrofolate Synthetase. Substrate Binding to Monomeric Subunits*

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ABSTRACT: Formyltetrahydrofolate synthetase isolated from *Clostridium cylindrosporum* has a tetrameric structure and contains four nucleotide and four folate binding sites. The enzyme can be dissociated into catalytically inactive monomers by dialysis to remove monovalent cations. Substrate binding experiments were performed with the monomeric enzyme. Monomers that can be reassociated and reactivated bind nucleotides with an affinity equivalent to native tetramer. Therefore, the nucleotide site is not altered by dissociation or association processes and is intrinsic to the monomeric subunit. However, the monomeric enzyme does not bind tetrahydroteroyl triglutamate even though the tetrameric form of the enzyme has a high binding affinity for this form of the coenzyme. This suggests that the folate binding site is created by the association of monomers to produce the active tetrameric enzyme. Alteration of the folate binding site during the dissociation process is sufficient to explain the catalytic inactivity of the monomeric enzyme.

Formyltetrahydrofolate synthetase isolated from *Clostridium cylindrosporum* has a tetrameric structure and a molecular weight of 240,000 (Scott and Rabinowitz, 1967). MacKenzie and Rabinowitz (1971) have obtained evidence that the four subunits consist of identical or very nearly identical polypeptide chains. The enzyme behaves as a single species of 60,000 molecular weight during polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate and it gives a single band by isoelectric focusing in the presence of 8 M urea. Previously described substrate binding experiments indicate that there are four nucleotide and four folate binding sites per mole of tetrameric enzyme (Curthoys and Rabinowitz, 1971a,b). This is consistent with the evidence that the subunits are identical and its suggests that the binding sites for both substrates are intrinsic to the monomeric subunit.

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Dialysis of formyltetrahydrofolate synthetase to remove monovalent cations causes the enzyme to dissociate into catalytically inactive monomers (Scott and Rabinowitz, 1967). Addition of certain monovalent cations causes reassociation of monomers to a fully active tetramer. There is an excellent correlation between catalytic activity and the per cent of enzyme present as tetramer. The unusually mild conditions required to effect a change in quaternary structure make this an ideal system for studying the steps involved in dissociation and reassociation processes (MacKenzie and Rabinowitz, 1971).

The reason why oligomeric structure is frequently required for catalytic activity even if all the components of an active site are intrinsic to each monomer is a fundamental and recurring question that is of current interest to many protein chemists (Frieden, 1971). The relative ease of dissociation of this enzyme makes it ideal for investigating this problem. Experiments were therefore designed to measure the ability of the individual monomeric subunits to bind adenine nucleotides and tetrahydropteroyl triglutamate. In *Clostridium cylindrosporum*, the folate coenzymes occur solely as triglutamyl derivatives of pteroic acid (Rabinowitz and Himes, 1960). Competitive binding experiments with the tetrameric enzyme indicate that it has a 200-fold greater binding affinity for the natural triglutamyl coenzyme than for tetrahydrofolate and that both compounds are bound to the same set of binding sites (Curthoys and Rabinowitz, 1971b). If the substrate binding sites are retained in the catalytically inactive monomer, this would prove that each site is intrinsic to the monomeric subunit and is not formed upon their association to give active tetramer. Alternatively, the inability of monomers to bind substrates would provide an explanation for the requirement of the tetrameric structure for enzymic activity.

Experimental Section

Materials. Formyltetrahydrofolate synthetase was purified from *C. cylindrosporum* (Curthoys and Rabinowitz, 1971a) and assayed (Rabinowitz and Pricer, 1962) as previously described.[1] Adenine nucleotides were purchased from Sigma and [^{14}C]ATP and [^{3}H]ADP were purchased from New England Nuclear. *l*-Tetrahydropteroyl triglutamate and *l*-[6,7-$^{3}H_2$]tetrahydropteroyl triglutamate were prepared as recently reported (Curthoys and Rabinowitz, 1971c). The final step in the synthesis of both of these compounds was the repeated lyophilization of the products eluted from a DEAE-cellulose column by an ammonium acetate gradient. Sufficient residual ammonium acetate was present to cause reassociation of monomers in experiments designed to measure their binding of tetrahydropteroyl triglutamate. To circumvent this problem the folate coenzymes were converted to their Tris salts by applying them to a DEAE-cellulose column that was equilibrated with 0.05 M Tris-chloride buffer (pH 7.5). The column was then washed with this buffer and the folate compounds were eluted with 0.5 M Tris-chloride buffer (pH 7.5). All of the tetrahydropteroyl triglutamate solutions were also 0.1 M in 2-mercaptoethanol.

Substrate Binding. The binding of the various substrates was determined by the method of partition equilibrium or by equilibrium dialysis. The partition equilibrium method developed by Gray and Chamberlin (1971) is based on one of the two-phase polymer systems originally described by Albertsson (1960) for the separation of macromolecules. This method was readily applicable to the detailed investigation of nucleotide and folate binding to the active tetrameric form of formyltetrahydrofolate synthetase (Curthoys and Rabinowitz, 1971a,b). Equilibrium dialysis was performed by using the 30-μl dialysis cells and the procedure described by Englund *et al.* (1969). Equilibration of tetrahydropteroyl triglutamate required 24 hr. Therefore, equilibrium dialysis was performed at 3°, to minimize denaturation of the enzyme.

Preparation of Enzyme Monomer. A sample of formyltetrahydrofolate synthetase stored as a crystalline suspension in 0.05 M potassium phosphate–0.05 M 2-mercaptoethanol buffer (pH 7.5), 50% saturated with ammonium sulfate, was centrifuged and the supernatant solution was discarded. The pellet was dissolved in the appropriate volume of 0.05 M Tris-chloride–0.1 mM EDTA–0.05 M 2-mercaptoethanol buffer (pH 8.0), and then dialyzed overnight at 3° against two 1-l. volumes of this buffer. Then it was dialyzed for 3 hr at 3° against 1 l. of 0.05 M Tris-chloride–0.1 mM EDTA buffer (pH 8.0). Finally, it was incubated at 20° for 1 hr. An aliquot of the enzyme was diluted with distilled water and the absorbance at 280 nm was measured. The protein concentration was calculated by using the reported extinction coefficient (Curthoys and Rabinowtiz, 1971a). The enzyme solution was then made approximately 0.1 M in 2-mercaptoethanol.

MacKenzie and Rabinowitz (1971) have shown that there is an excellent correlation between the catalytic activity and the amount of enzyme in the tetrameric form. These studies demonstrated that only the tetrameric form of the enzyme is catalytically active. The monomer solution prepared as described above was then assayed for enzymic activity. It routinely had less than 5% of the specific activity of the tetrameric enzyme. This preparation was then used in the binding experiment. For the purpose of comparison, a binding experiment with fully active tetrameric enzyme was performed simultaneously.

To determine the extent to which the monomer preparation can reassociate, an aliquot was made 0.2 M in NH_4Cl and it was incubated at 20° for 1 hr. The enzymic activity was then redetermined. These conditions result in maximal reactivation (MacKenzie and Rabinowitz, 1971). The specific activity indicated that 70–90% of the monomers could be reassociated. In order to determine if any reassociation occurred during the binding experiment, aliquots from either the phase or the dialysis chamber which contained enzyme were assayed for enzymic activity. The specific activity of the protein was compared with that of tetramer preparation.

The monomeric state of the enzyme was confirmed by sedimentation velocity analysis using a Spinco Model E analytical ultracentrifuge.

Results

Nucleotide Binding to Monomers. Sedimentation velocity experiments showed that monomers of formyltetrahydrofolate synthetase formed very high molecular weight aggregates at temperatures above 30°. To avoid aggregation, monomer and tetramer binding experiments were performed by the partition equilibrium method at 23°. Activity assays showed that there was no significant reactivation of monomers in either the MgATP or MgADP binding experiments.

Catalytically inactive monomers bind MgATP with an affinity equivalent to that of the native tetramer. Scatchard plots of MgATP binding to monomer and tetramer preparations of formyltetrahydrofolate synthetase are shown in Fig-

[1] The concentration of $MgCl_2$ solution used is 0.1 M. This value was inadvertently omitted from the original description.

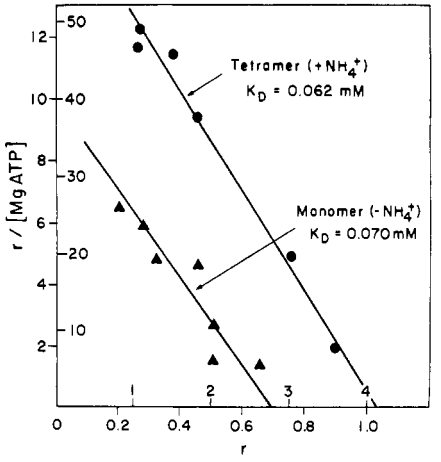

FIGURE 1: Binding of MgATP to monomeric and tetrameric forms of formyltetrahydrofolate synthetase. Data are given as Scatchard plots with r representing moles of MgATP bound per mole of enzyme and r/[MgATP] representing $10^{-3} \times r$ divided by the molar concentration of unbound MgATP. The inside coordinates refer to tetramer binding (●) and the outside coordinates refer to monomer binding (▲). The protein concentrations were (●) 6.5×10^{-5} M, and (▲,) 2.3×10^{-4} M. Partitioning was performed at 23° and pH 8.0.

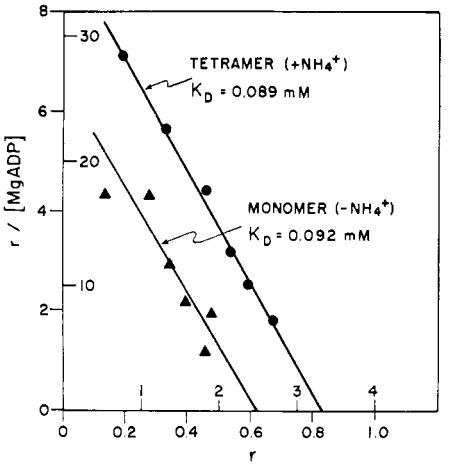

FIGURE 2: Binding of MgADP to monomeric and tetrameric forms of formyltetrahydrofolate synthetase. Data are given as Scatchard plots with r representing moles of MgADP bound per mole of enzyme and r/[MgADP] representing $10^{-3} \times r$ divided by the molar concentration of unbound MgADP. The inside coordinates refer to tetramer binding (●) and the outside coordinates refer to monomer binding (▲). The protein concentrations were (●) 4.9×10^{-5} M and (▲) 1.8×10^{-4} M.

ure 1. Both sets of data fit straight lines. The dissociation constants calculated from the slope of these lines were 0.062 and 0.070 mM for the tetrameric and monomeric enzymes, respectively. There were 4.1 MgATP binding sites/mole of tetramer. However, the monomer preparation had only 0.71 binding site/mole of monomer. This corresponds to 68% of the MgATP sites observed with native tetramer. This apparent decrease in binding sites for monomers was explained by determining the extent to which the monomer preparation could be reassociated and reactivated. Upon incubation with 0.2 M NH_4Cl, 69% of the enzyme was reactivated. This suggests that only the monomers that are still reactivatable and can be reassociated bind MgATP and that the binding affinity is equal to that of the native tetramer.

Identical results were obtained for MgADP binding to monomers. The binding data, plotted according to the Scatchard equation, are shown in Figure 2. MgADP is bound with equivalent dissociation constants to both tetrameric and monomeric preparations of enzyme (0.089 and 0.092 mM, respectively). The enzyme used in this experiment had only 3.3 MgADP binding sites/mole of tetramer. The monomer preparation had only 0.62 site/mole of monomer (75% of tetramer preparation). Incubation of monomers with 0.2 M NH_4Cl resulted in 82% reactivation and approximately 80% tetramer was formed as shown by sedimentation velocity analysis. This again suggests that only the monomers that can be reactivated and reassociated bind MgADP and with an affinity equivalent to that of the native tetramer.

The data for MgATP and MgADP binding are summarized in Table I. The agreement between the dissociation constants for monomer and tetramer binding suggests that the nucleotide binding site of formyltetrahydrofolate synthetase is not altered by dissociation to catalytically inactive monomers. The monomer preparation is more easily denatured than the native tetramer. Such denaturation probably alters the nucleotide binding site. This would explain why only the monomers, which can still be reactivated and reassociated, can bind nucleotides. It therefore appears that the nucleotide binding site is intrinsic to the monomeric subunit and the catalytic inactivity of monomers is not a result of their inability to bind nucleotides.

Binding of Tetrahydrofolates to Monomers. Considerable reactivation of the monomers occurred in the initial experiments designed to measure the binding of tetrahydropteroyl triglutamate to the monomers. Approximately one-half of the monomers that were reactivated when incubated with 0.2 M NH_4Cl were reactivated during the partition equilibrium experiment. The binding observed with the monomer preparations could be accounted for by the extent of reactivation or tetramer formation that occurred during the binding experiment. The remaining undenatured and unreactivated monomers did not bind tetrahydropteroyl triglutamate. The data, although not definitive, suggested that the folate coenzyme was not bound to monomeric subunits. The cause of the enzyme reactivation that occurred in the partition equilibrium experiment was traced to a high concentration of ammonium ions which contaminated the (*l*)-tetrahydropteroyl triglutamate and *l*-[6,7-$^3H_2$]tetrahydropteroyl triglutamate preparations. After the samples were repurified and converted to their Tris salts, the monomer binding experiments were repeated.

Partition equilibrium was used to measure the binding of tetrahydropteroyl triglutamate to monomer and tetramer preparations of formyltetrahydrofolate synthetase. A Scatchard plot of the data obtained with the tetrameric enzyme is

TABLE I: Summary of Nucleotide Binding to Formyltetrahydrofolate Synthetase Monomers and Tetramers.

Ligand	Enzyme	K_D (mM)	No. of Binding Sites/Mole
MgATP	Tetramer	0.062	4.1
	Monomer	0.070	0.71
MgADP	Tetramer	0.089	3.3
	Monomer	0.092	0.62

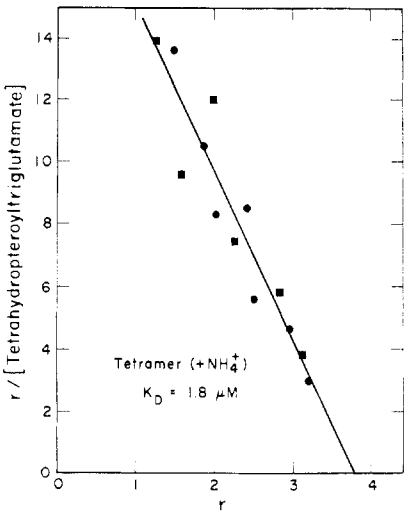

FIGURE 3: Binding of tetrahydropteroyl trigultamate to the tetrameric form of formyltetrahydrofolate synthetase. Data obtained by equilibrium dialysis (■) and by partition equilibrium (●) are given as a Scatchard plot. r represents the moles of tetrahydropteroyl triglutamate bound per mole of enzyme and r/[tetrahydropteroyl triglutamate] represents $10^{-5} \times r$ divided by the molar concentration of unbound tetrahydropteroyl triglutamate. The protein concentrations were (■) 4.64 μM and (●) 3.35 μM.

shown in Figure 3. There were 3.8 sites/mole of tetramer and each had a dissociation constant of 1.8 μM. This agrees well with the values reported previously (Curthoys and Rabinowitz, 1971b). When the same experiment was performed with monomeric enzyme, there was no indication of folate binding. There was no significant shift in the distribution of tetrahydropteroyl triglutamate between the two phases as compared to samples in which the enzyme was omitted. Activity assays indicated that only 8% of the monomers was reactivated during the binding experiments, whereas greater than 90% were reactivatable when incubated with 0.2 M NH_4Cl. The data indicate that monomers which can be reactivated do not bind tetrahydropteroyl triglutamate.

To support this conclusion, the binding experiments were repeated using equilibrium dialysis. The data obtained with the tetrameric enzyme are also shown in Figure 3, and they fit the same line obtained by partition equilibrium. The close agreement between the two methods supports the generalized conclusion that there are four equivalent noninteracting sites for tetrahydropteroyl triglutamate per mole of tetrameric enzyme, but the monomeric enzyme does not bind the folate coenzyme. After complete equilibration, the concentrations of tetrahydropteroyl triglutamate in both chambers of the dialysis cells were equivalent. There was only a slight reactivation (7%) during the binding experiment, and after its completion 81% of the monomers could be reactivated with NH_4Cl. The results obtained with equilibrium dialysis therefore support the previous conclusion that monomers of formyltetrahydrofolate synthetase do not bind tetrahydropteroyl triglutamate even though they may be reassociated and reactivated under appropriate conditions. The lack of a binding site for the folate substrate is sufficient to explain the catalytic inactivity of the monomeric subunits.

Discussion

The tetrameric structure of formyltetrahydrofolate synthetase is required for catalytic activity. Removal of monovalent cations by dialysis caused the *C. cylindrosporum* enzyme to dissociate into catalytically inactive monomers. Experimental evidence indicates that the monomers are identical or very nearly identical (MacKenzie and Rabinowitz, 1971). The binding experiments with the active tetrameric enzyme suggest that there is one nucleotide site and one folate site per monomer (Curthoys and Rabinowitz, 1971a,b). It would therefore appear that all of the elements of the active site are intrinsic to each monomer and it is therefore not obvious why the tetrameric structure is required for catalytic activity.

Suelter (1970) reviewed the evidence that monovalent cations may play an essential role in the reaction mechanism of certain enzymes. If monovalent cations were an essential cofactor in the enzyme mechanism of the tetrameric form of formyltetrahydrofolate synthesis, then inactivation which accompanies dissociation to monomeric subunits may simply be related to the absence of monovalent cations and not due to structural alteration of the enzyme. However, removal of monovalent cations in the presence of substrates results in an altered tetramer (Welch *et al.*, 1968). This form of formyltetrahydrofolate synthetase has an increased K_M for formate, but still has the same maximal catalytic activity. This suggests that the binding of formate is assisted by monovalent cations, but that they are not an essential part of the reaction mechanism.

The catalytic inactivation may result from a conformational change which accompanies dissociation. Even though the binding experiments show that there are four sites for each substrate per mole of tetramer, the binding sites may not be intrinsic to each monomer. One or more of the substrate sites may be formed as a result of conformational changes which are caused by the protein–protein interactions responsible for the association of monomers. A second possibility is that one or more of the substrate sites may be located at the site of interaction between subunits and that the substrate site is composed of segments of polypeptide chains from two distinct subunits. Experimental evidence suggests that the pyridoxal phosphate site of tryptophan synthetase is formed by association of two β-chain monomers (Hathaway and Crawford, 1970) and that the binding of threonine to homoserine dehydrogenase requires the association of monomers (Mankovitz and Segal, 1969).

Experiments with MgATP and MgADP clearly support the conclusion that the nucleotide binding sites of formyltetrahydrofolate synthetase are not altered by dissociation of the enzyme into catalytically inactive monomers. Both monomeric and tetrameric forms of the enzyme bind the nucleotide substrates with equal affinity and there is no significant reactivation during the binding experiment. There is also an excellent correlation between the extent of binding to monomers and the per cent of monomers which can be reactivated by incubation with 0.2 M NH_4Cl. The per cent of monomers, which can no longer be reactivated or reassociated, have probably been irreversibly denatured in such a way that the nucleotide site has been destroyed. These experiments strongly suggest that the nucleotide site is intrinsic to the monomeric subunit and that it is not altered by association or dissociation.

Support for this conclusion comes from the investigation of the mechanism of the reassociation process. MacKenzie and Rabinowitz (1971) have established that there are two distinct processes in which monovalent cations react with monomers. The monovalent cation requirement in the first step was not specific for any particular cation and could be replaced by nucleotides. Concentrations of MgATP or MgADP approximately equivalent to their dissociation constants

produced a half-maximal response in satisfying this requirement. This suggests that nucleotide binding may play an important role in the *in vivo* association of nascent monomers. It also supports the conclusion that the nucleotide binding site exists in an unaltered form in the monomeric enzyme.

In contrast to the results obtained with adenine nucleotides, the experiments performed with tetrahydropteroyl triglutamate suggest that the folate binding sites are substantially altered during the dissociation process. The results of both partition equilibrium and equilibrium dialysis experiments indicate that there are four equivalent, noninteracting binding sites for tetrahydropteroyl triglutamate per mole of tetramer. This suggests that there is one folate site per monomer. However, the results obtained by both methods indicate that the monomeric enzyme does not contain an intact folate binding site. The monomer preparations had no affinity for tetrahydropteroyl triglutamate, even though greater than 80% of the monomers could still be reactivated and reassociated by incubation with NH_4Cl. Such an alteration of the folate binding site is sufficient to explain why the individual monomeric subunits are catalytically inactive.

Since the dissociation and association processes are reversible, the data suggest that the folate binding sites may be formed during the association of nascent monomers to produce active formyltetrahydrofolate synthetase *in vivo*. The subunit interactions resulting from the association may cause conformational changes sufficient to produce the new binding sites. Alternatively, the folate site may be composed of segments of polypeptide chains from the two distinct subunits and is created at the site of interaction between subunits. In either case, the tetrameric structure is essential for the formation of catalytically active formyltetrahydrofolate synthetase.

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A Kinetic Investigation of the Crystallographically Deduced Binding Subsites of Bovine Chymotrypsin Aγ*

David M. Segal

ABSTRACT: A series of seven *N*-acetyl peptide methyl ester substrates was designed and synthesized in order to probe various structural aspects of the chymotrypsin Aγ binding scheme recently proposed from crystallographic considerations by Segal, D. M., Powers, J. C., Cohen, G. H., Davies, D. R., and Wilcox, P. E. ((1971), *Biochemistry 10*, 3728). Kinetics were followed in the pH-Stat, and the K_m's derived from these studies were taken to be a measure of the relative binding strengths of the substrates employed. In all cases the relative magnitudes of the observed binding energies agreed well with those predicted from the model, providing evidence that the crystallographic binding scheme is a good representation of the substrate binding mode during catalysis. The results are also more in accord with the chymotrypsin binding scheme than with an alternative scheme recently proposed for the homologous serine protease, elastase.

Recent X-ray investigations of chymotrypsin Aγ crystals treated with several peptide chloromethyl ketone inhibitors demonstrated that the resultant enzyme–inhibitor complexes involved the formation of an antiparallel β-type configuration between the peptide portion of the inhibitor and an extended stretch of the main chain of the enzyme (Segal *et al.*, 1971a,b). In these studies, the enzyme–inhibitor complexes were also stabilized by the formation of a covalent link between the methylketo portion of the inhibitor and a ring nitrogen of His-57 on the enzyme. Although such a covalent bond would not be formed during catalysis, the model needed only slight alteration in order to make an ester link with Ser-195, which, according to present evidence (Cunningham, 1965) would represent the true catalytic intermediate. It was therefore proposed that the peptide binding scheme deduced from the chloromethyl ketone studies was the same as that occurring

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