C₁-Tetrahydrofolate Synthase from Rabbit Liver

STRUCTURAL AND KINETIC PROPERTIES OF THE ENZYME AND ITS TWO DOMAINS*

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C₁-Tetrahydrofolate synthase is a multifunctional enzyme which catalyzes three reactions in 1-carbon metabolism: 10-formyltetrahydrofolate synthetase; 5,10-methylenetetrahydrofolate cyclohydrolase; 5,10-methylenetetrahydrofolate dehydrogenase. A rapid 1-day purification procedure has been developed which gives 40 mg of pure enzyme from 10 rabbit livers. The 10-formyltetrahydrofolate synthetase activity of this trifunctional enzyme has a specific activity that is 4-fold higher than the enzyme previously purified from rabbit liver. Conditions have been developed for the rapid isolation of a tryptic fragment of the enzyme which contains the methylenetetrahydrofolate dehydrogenase and methylenetetrahydrofolate cyclohydrolase activities. This fragment is a monomer exhibiting a subunit and native molecular weight of 36,000 in most buffers. However, in phosphate buffers the native molecular weight suggests that the fragment is a dimer. Conditions are also given whereby chymotryptic digestion allows the simultaneous isolation from the native enzyme of a large fragment containing the 10-formyltetrahydrofolate synthetase activity and a smaller fragment containing the dehydrogenase and cyclohydrolase activities. The large fragment is a dimer with a subunit molecular weight of 66,000. The small fragment retains all of the dehydrogenase and cyclohydrolase activities of the native enzyme. The large fragment is unstable but retains most of the 10-formyltetrahydrofolate synthetase activity. Kᵣ values of substrates for the two fragments are the same as the values for the native enzyme. The 10-formyltetrahydrofolate synthetase activity of the native enzyme requires ammonium or potassium ions for expression of full catalytic activity. The effect of these two ions on the catalytic activity of the large chymotryptic fragment is the same as with the native enzyme.

We have shown by differential scanning calorimetry that the native enzyme contains two protein domains which show thermal transitions at 47 and 60 °C. Evidence is presented that the two domains are related to the two protein fragments generated by proteolysis of the native enzyme. The larger of the two domains contains the active site which catalyzes the dehydrogenase and cyclohydrolase reactions. Replacement of sodium ion buffers with either ammonium or potassium ions results in an increase in stability of the large domain of the native enzyme. This change in stability is not accompanied by a change in the quaternary structure of the enzyme. The large domain is cold labile, being more stable at 23 than at 0 °C. The property of cold lability does not change over the pH range of 6.5 to 8.0. These results suggest that each subunit of C₁-tetrahydrofolate synthase is composed of a small and large domain. The subunit interface in the native enzyme is primarily associated with the large domain. The large and small domains have little or no interaction with each other with respect to either structural or catalytic properties of the native enzyme.

An important pathway in 1-carbon metabolism is the interconversion of formate and methylenetetrahydrofolate (1). In prokaryotic cells three enzymes catalyze reactions in this pathway. The first enzyme, 10-formyltetrahydrofolate synthetase (EC 6.3.4.3), catalyzes the ATP-dependent conversion of formate and tetrahydrofolate to 10-formyltetrahydrofolate (Reaction 1). The second enzyme, methylenetetrahydrofolate cyclohydrolase (EC 3.5.4.9), dehydrates this product to form 5,10-methylenetetrahydrofolate (Reaction 2). The third enzyme, methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5), uses NADPH to reduce the 1-carbon adduct to 5,10-methylenetetrahydrofolate (Reaction 3). These reactions are reversible and account for the incorporation of formate into the third carbon of serine via the enzyme serine hydroxymethyltransferase (1). In eucaryotic cells Reactions 1 through 3 are catalyzed by a single enzyme. This trifunctional enzyme has been purified from yeast, chicken liver, rabbit liver, porcine liver, and sheep liver (2-11). The name recently given to this protein is C₁-tetrahydrofolate synthase (12). The enzyme from eucaryotic cells is a homodimer with a molecular weight of about 200,000. Recently, it has been shown that proteolytic digestion of this enzyme from hog liver and yeast results in the formation of two fragments with subunit molecular weights of about 30,000 and 70,000 (3, 13, 14). The small fragment contains the activity for Reactions 2 and 3, and the large fragment contains the activity for Reaction 1. The two fragments were not obtained from a single proteolytic digestion. It is not known whether the two fragments occur on overlapping or independent domains.

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We have previously purified and studied C1-tetrahydrofolate synthase from rabbit liver (5). Our results suggested that a single active site catalyzes Reactions 2 and 3 but that a different site is responsible for Reaction 1. We have also shown that the two active sites denature at different temperatures and show different sensitivity to sulfhydryl reagents (15). In our current studies we have found that proteolytic digestion gives two protein fragments from a single digestion which suggests that the enzyme is composed of two nonoverlapping protein domains. Our goal is to try and determine what the advantage is to the cell to have all three activities on a single polypeptide chain. Previous studies with the fragments from other eucaryotic sources did not characterize their kinetic properties. Our initial efforts, which are reported in this paper, are to compare the kinetic properties of the three reactions in the separated fragments with those of the native enzyme. The larger domain which contains the activity for the synthetase activity (Reaction 1) is unstable both in the native enzyme and the isolated fragment. In order to study this enzyme in the native state we needed a rapid purification procedure which would give us sufficient amounts of enzyme for proteolytic digestions and isolation of the domains in a few hours. Our procedure for the purification of the native enzyme and the two fragments is also included in this paper.

Previous investigations with the enzyme from liver have not characterized the structural properties of the fragments and compared them to the structural properties of the native enzyme. The native enzyme is a homodimer, but the subunit structure of the fragments is not known. The number and nature of the domains in the native enzyme have not been determined. In procaryotic cells and yeast, the 10-formyltetrahydrofolate synthetase also shows a requirement for ammonium or potassium ions (16-18). These ions result in a loss of activity during purification. In our procedure gives an active site turnover number of 1000/min which is 4-fold higher than our previous value. This increase in specific activity can be accounted for by two differences from our previous determination. First, we have done our current assays with the optimum concentration of ammonium ion and second, the enzyme is purified in a shorter period of time. Our previous ignorance of the instability of the rabbit synthetase activity in the absence of ammonium or potassium ions resulted in a loss of activity during purification.

Isolation of Catalytically Active Fragments—The rate and extent of proteolytic digestion of C1-tetrahydrofolate synthase is sensitive to the protease used, the pH of the buffer, and the presence of substrates. For the isolation of a fragment which contains the dehydrogenase and cyclohydrolase activities we have found that digestion with trypsin in 50 mM potassium phosphate, 15% glycerol buffer at pH 7.6 with 0.1 mM NADP+ gives optimal results. Under these conditions the synthetase activity is rapidly lost. The active fragment is purified by passing the digestion mixture through an HPLC molecular sieve column (Fig. 1). The protein fragment possessing activity for Reactions 2 and 3 is completely separated from the parent enzyme and trypsin. The fragment gives a single band after SDS-polyacrylamide gel electrophoresis.

Catalytic Properties of the Native Enzyme and the Two Fragments—Our objective was to determine if separation of the two catalytic domains altered their catalytic properties in terms of affinity for ligands and turnover number. Our first experiment was to determine the dehydrogenase and cyclo-

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\begin{align*}
H_4\text{folate}^1 + \text{ATP} + \text{formate} \xrightarrow{\text{synthetase}} 10-\text{formyl}-H_4\text{folate} + \text{ADP} + \text{Pi} \\
10-\text{Formyl}-H_4\text{folate} \xrightarrow{\text{cyclohydrolase}} \text{methylene}-H_4\text{folate} + \text{H}_2\text{O} \\
\text{Methylene}-H_4\text{folate} + \text{NADPH} \xrightarrow{\text{dehydrogenase}} \text{methanol} + \text{NADP}^+ \\
\end{align*}
\]

\(1\) Our current procedure improves on this method by decreasing the time required to purify the enzyme and increasing the yield (Table I). Starting with 10 fresh frozen rabbit livers it is now possible to obtain 40 mg of homogeneous enzyme in about 10 h. Important changes in the procedure are the inclusion of α2-macroglobulin in the homogenization buffer to remove proteases and the use of an Orange A affinity column. A critical step in decreasing the time required to purify the enzyme is the use of a Pellicon cassette system to desalt the protein after the ammonium sulfate precipitation step. The procedure results in a purification of 1500-fold and a yield of 40% based on the activity of Reaction 3 (Table I). We have previously shown that all three activities co-purify (5). The final specific activities are similar to those previously published with the exception of the synthetase activity which in our procedure gives an active site turnover number of 1000/min which is 4-fold higher than our previous value. This increase in specific activity can be accounted for by two differences from our previous determination. First, we have done our current assays with the optimum concentration of ammonium ion and second, the enzyme is purified in a shorter period of time. Our previous ignorance of the instability of the rabbit synthetase activity in the absence of ammonium or potassium ions resulted in a loss of activity during purification.

\[\text{C1-Tetrahydrofolate Synthase} \quad \text{C1-Tetrahydrofolute Synthase} \]

\[\text{H}_4\text{folate} + \text{ATP} + \text{formate} \xrightarrow{\text{synthetase}} 10-\text{formyl}-H_4\text{folate} + \text{ADP} + \text{Pi} \]

\[10-\text{Formyl}-H_4\text{folate} \xrightarrow{\text{cyclohydrolase}} \text{methylene}-H_4\text{folate} + \text{H}_2\text{O} \]

\[\text{Methylene}-H_4\text{folate} + \text{NADPH} \xrightarrow{\text{dehydrogenase}} \text{methanol} + \text{NADP}^+ \]

\[1\text{-Formyl-}H_4\text{folate} + ADP + P_i \]

\[\text{ADP} + \text{Pi} \]

\[\text{methanol} + \text{NADP}^+ \]

\[\text{methionate} \]

\[\text{H}_4\text{folate} + \text{ATP} + \text{formate} \xrightarrow{\text{synthetase}} 10-\text{formyl}-H_4\text{folate} + \text{ADP} + \text{Pi} \]

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\[\text{Methylene}-H_4\text{folate} + \text{NADPH} \xrightarrow{\text{dehydrogenase}} \text{methanol} + \text{NADP}^+ \]
hydrolase activities during the generation of the 36,000 molecular weight fragment by digestion with trypsin. During the digestion aliquots were removed and passed through the HPLC molecular sieve column to determine the extent of digestion. This was compared to the catalytic activity determined by enzymatic assay. As shown in Fig. 4 there is no loss in dehydrogenase and cyclohydrolase activities during the period when 90% of the native enzyme with a molecular weight of 215,000 has disappeared. About 90% of the dehydrogenase-cyclohydrolase activity was recovered in the 36,000 molecular weight fragment after passage through the molecular sieve column. The specific activities of the fragment for the dehydrogenase and cyclohydrolase reactions are essentially the same as in the parent enzyme when compared on the basis of μmol of product/μmol of subunit/min (Table II). This suggests that the removal of the synthetase domain by digestion with trypsin has no effect on the turnover number of the dehydrogenase-cyclohydrolase domain.

A similar experiment was performed with the chymotrypsin digestion. Synthetase activity was compared with the loss of the 215,000 molecular weight parent enzyme. The results show that during digestion there is a gradual loss of synthetase activity. After 102 min of digestion 40% of the activity and 7% of the parent enzyme remain (Fig. 5). During the digestion, 23% of the 280-nm absorbing material has also been lost (Fig. 2). This suggests that some of the decrease in activity is the result of digestion of the synthetase domain. This is confirmed by comparison of the specific activities of the synthetase fragment and parent enzyme. The fragment has 75 to 80% of the catalytic activity of the parent enzyme which is probably a minimum value since some activity may have been lost during the 30-min purification procedure (Table II). We conclude that the specific activity of the isolated large fragment is either unaffected or only slightly diminished by separation from the small fragment. The synthetase domain is unstable in the native enzyme, and attempts to stabilize it with substrates always lead to a decreased rate of digestion.

The next experiments were to determine the $K_m$ values of substrates for the three activities on the respective isolated domains. The results are recorded in Table II. It is clear from these studies that within experimental error there is no change in the $K_m$ values for substrates for any of the three activities.

The 10-formyltetrahydrofolate synthetase enzyme from procaryotic cells has a specific cation requirement (17). Sodium ion gives a catalytically inactive enzyme. The most effective cations with this enzyme are ammonium and potassium ions (16, 17). The rabbit liver synthetase activity shows some activity with sodium ion but is stimulated 6-fold by the addition of either potassium or ammonium ions. The $K_m$ for ammonium ion is 2-fold lower than the $K_m$ for potassium ion (Table II). Studies done with the synthetase fragment show the same effects with potassium and ammonium ions as the

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**Table II**

| Enzyme reaction | Substrate | $K_m$ (μM) | Specific activity (min$^{-1}$) |
|-----------------|-----------|------------|-----------------------------|
|                 | Parent enzyme | Dehydrogenase-cyclohydrolase fragment | Synthetase fragment |
| Methylene tetrahydrofolate dehydrogenase | NADP* | 4 | 4 |
| Methylenetetrahydrofolate dehydrogenase | | 2 | 2 |
| 10-Formyltetrahydrofolate synthetase | MgATP | 67 | 65 |
| | Formate | 166 | 160 |
| | Ammonium ion | 1800 | 2000 |
| | Potassium ion | 4000 | 3300 |

*a* Average of 3 to 5 experimental determinations for enzyme and fragments purified as recorded in Fig. 2.

*b* Units are μmol of product/μmol of subunit/min at 30 °C under standard assay conditions. These are not $V_{max}$ values.

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![Fig. 4](image_url)

**Fig. 4.** Correlation of the loss of C$_1$-tetrahydrofolate synthase protein and dehydrogenase activity during digestion with trypsin. The amount of C$_1$-tetrahydrofolate synthase protein was determined as the area under the 280-nm absorbing peak eluting at 27.8 min from the HPLC elution profile as recorded in Fig. 1. Dehydrogenase activity was determined during the digestion as described under "Experimental Procedures."

![Fig. 5](image_url)

**Fig. 5.** Correlation of the loss of C$_1$-tetrahydrofolate synthase protein and 10-formyltetrahydrofolate synthetase activity during digestion with chymotrypsin. The amount of C$_1$-tetrahydrofolate synthase protein was determined as described for the experiment recorded in Fig. 4. The synthetase activity was determined as described under "Experimental Procedures."
parent enzyme. Optimal activity for synthetase activity is achieved at 25 mM ammonium ion. The rate of activation by either ammonium or potassium ions is complete within the 15 s it takes to mix the solutions in the assay mixture.

Cold Lability—Enzymes which are less stable at 0 °C than at 25 °C are referred to as being cold labile (20-23). In studying C1-tetrahydrofolate synthase, we observed that the synthetase activity is cold labile (Fig. 6A). Two explanations given for this phenomenon are either a change in the pK of a structurally important group with temperature or hydrophobic bonds being of primary importance in maintaining the three-dimensional structure of the enzyme (24). If the cold lability is the result of a change in the pK of a structurally important acidic group, then changing the pH should result in the disappearance of the phenomenon. As shown in Fig. 6B, the cold lability of C1-tetrahydrofolate synthetase does not change with pH. This suggests that the cold lability is due to hydrophobic bonds being important in maintaining the structure of the enzyme. Proteins protect the enzyme from inactivation but they do not alter the cold lability pattern. However, the addition of 10% glycerol does reverse the pattern so that the enzyme is more stable at 0 °C than at 23 °C. The addition of albumin to 1 mg/ml did not protect the enzyme or alter its cold lability.

Cold lability with other enzymes has usually been found to be associated with a change in quaternary structure (20-23). We tested for this possibility by looking for a change in retentive time on a molecular sieve column during cold inactivation. We found that the loss of synthetase activity with time was accompanied with a concomitant loss in the concentration of the dimeric enzyme. However, no smaller fragments or monomers were present. The solution became cloudy suggesting that aggregation had occurred. The insoluble enzyme retained its dehydrogenase and cyclohydrolase activities suggesting that monomer retains complete activity. The sodium ion form of the enzyme, we observed that the sodium ion form of the enzyme approached this question by determining the subunit structure of the enzyme (24). If the cold lability is the result of a change in quaternary structure which is buffer dependent. However, these buffers have no effect on the dehydrogenase and cyclohydrolase activities, suggesting that the monomer retains complete activity. The molecular weight of the native enzyme as determined by sedimentation velocity studies does not change under the conditions described for the small fragment.

Differential Scanning Calorimetry Studies—To further explore the question of the number of domains in the native enzyme, we looked at the change in heat capacity of the enzyme with increasing temperature. Discrete domains have been shown to denature independently in other proteins (25). As shown in Fig. 7, curve B, C1-tetrahydrofolate synthase shows two thermal transitions (Tm). This suggests that the enzyme is composed of at least two separate domains. The question immediately posed is whether these two domains are the same ones which are separated into the fragments observed upon proteolytic digestion of the enzyme. Previous studies had shown that the synthetase activity was more temperature labile than the dehydrogenase and cyclohydrolase activities (5). When we determined the differential scanning calorimeter pattern of the enzyme in the presence of a saturating amount of MgATP, the first Tm was increased from 47 to 51 °C with essentially no change in the second Tm (Fig. 7, curve A). A repeat of the experiment with saturating amounts of NADP+ in place of the MgATP resulted in an increase of the second Tm from 60 to 70 °C with no change in the first Tm (Fig. 7, curve C). These results suggest that the thermal transition at 47 °C is due to the denaturation of a domain which binds MgATP and, therefore, contains the synthetase active site. The thermal transition at 60 °C is due to the denaturation of a domain which binds NADP+ and, therefore, contains the dehydrogenase-cyclohydrolase active site.

SDS-PAGE were 66,000 and 36,000, respectively. The elution times of these two fragments from the molecular sieve column in 50 mM potassium phosphate, 10 mM 2-mercaptoethanol were compared with the elution times of a number of proteins of known molecular weight. By this technique, the large fragment was found to have a molecular weight of 115,000 and the smaller fragment a molecular weight of 74,000. This suggests that both fragments are dimers. The s20,w value was also determined for the fragment containing the dehydrogenase activity by sedimentation velocity studies. The s20,w value of 5.4 which was obtained is consistent with a protein having a molecular weight of about 70,000. A protein with a molecular weight of 36,000 would exhibit an s20,w value in the range of 3.0-4.0 (24). Because the fragment containing the synthetase activity is so unstable, we were not able to determine its s20,w value.

The molecular weight studies reported in the previous paragraph were determined with 50 mM potassium phosphate as the buffer. When buffers were used in which the principal anion was chloride, we observed that the small fragment eluted from the HPLC molecular sieve column and a Sephacryl G-150 column with a Mw of 38,000 to 45,000. The s20,w value decreased from 5.4 to 4.3 in sedimentation velocity experiments. When the fragment is first placed in 50 mM potassium phosphate and a sedimentation velocity pattern is determined, two protein peaks are observed, one at an s20,w of 5.4 and the other at 4.3. We interpret these results to suggest that the small fragment exists in a monomer-dimer equilibrium which is buffer dependent. However, these buffers have no effect on the dehydrogenase and cyclohydrolase activities, suggesting that the monomer retains complete activity. The molecular weight of the native enzyme as determined by sedimentation velocity studies does not change under the conditions described for the small fragment.

Based on the formation of two protein fragments during digestion with chymotrypsin, C1-tetrahydrofolate synthase appears to be composed of at least two domains. The question arises as to which domain(s) forms the subunit interface. We approached this question by determining the subunit structure of the two fragments which are formed upon digestion with chymotrypsin. Digestion with this protease results in the disappearance of the parent enzyme and the concomitant appearance of a large fragment with only synthetase activity and a smaller fragment with both the dehydrogenase and cyclohydrolase activities. The sizes of these two fragments by SDS-PAGE were 66,000 and 36,000, respectively. The elution times of these two fragments from the molecular sieve column in 50 mM potassium phosphate, 10 mM 2-mercaptoethanol were compared with the elution times of a number of proteins of known molecular weight. By this technique, the large fragment was found to have a molecular weight of 115,000 and the smaller fragment a molecular weight of 74,000. This suggests that both fragments are dimers. The s20,w value was also determined for the fragment containing the dehydrogenase activity by sedimentation velocity studies. The s20,w value of 5.4 which was obtained is consistent with a protein having a molecular weight of about 70,000. A protein with a molecular weight of 36,000 would exhibit an s20,w value in the range of 3.0-4.0 (24). Because the fragment containing the synthetase activity is so unstable, we were not able to determine its s20,w value.

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activity. This suggested that these two ions stabilized the domain containing the synthetase active site. We investigated this with the differential scanning calorimeter by looking at the heat capacity change during denaturation for the sodium form of the enzyme. We found that the first \( T_m \) was at 42 °C but that the second \( T_m \) was still at 60 °C. Replacement of sodium ions with either potassium or ammonium ions increased the first \( T_m \) to 47 °C. This suggested that potassium and ammonium ions had the same structural effect on the domain containing the synthetase active site. However, there appears to be a difference between these two ions since the addition of MgATP to the ammonium form of the enzyme does not change the \( T_m \) from 47 °C but its addition to the potassium form increases the \( T_m \) to 51 °C. The different cations had little or no effect on the molar enthalpy change of the synthetase domain.

We conclude from these results that the two fragments generated by proteolytic digestion are related to the two domains that are seen in the denaturation of the enzyme with increasing temperature. The remaining question is to determine if the fragments have the same structural properties as the domains in the native enzyme. This can be answered in part by comparing the denaturation patterns of the fragments in the differential scanning calorimeter. The fragment containing the synthetase activity was so unstable we could not get a denaturation curve for it by this technique. The smaller fragment which contains the dehydrogenase and cyclohydrolase activity exhibited a \( T_m \) at 49 °C. The addition of NADP\(^+\) increased the \( T_m \) to 53.5 °C (Fig. 8). These two values are considerably below the values found for this domain in the native enzyme and suggest that the fragment is structurally different than the native domain.

**DISCUSSION**

The control of 1-carbon metabolism is poorly understood. This is particularly true of the pathway for purine biosynthesis. The immediate donor of formyl groups in this pathway is 10-formyltetrahydrofolate (2). In eucaryotic cells the enzyme which supplies this substrate is part of a trifunctional enzyme which can supply the formyl groups starting either with formate or 5,10-methylenetetrahydrofolate. This enzyme, now referred to as C1-tetrahydrofolate synthase, has been purified from yeast, avian liver, and several mammalian livers (2-6). The enzyme is known to be a homodimer with subunit weights in the 100,000-dalton range. Proteolytic digestion has shown that two fragments can be generated with the smaller fragment, from the amino-terminal end of the molecule, catalyzing the dehydrogenase and cyclohydrodrolase reactions. The larger fragment catalyzes the synthetase reaction. Previous studies have been hampered because only small amounts of the enzyme could be purified and the two fragments could not be generated from a single digestion. It was the purpose of this study to develop a procedure so that sufficient enzyme could be purified to do studies on the catalytic and structural properties of this enzyme. The intent of our research is to elucidate the advantages of having a single enzyme to catalyze the three reactions.

In procaryotic cells, separate enzymes are involved in catalyzing Reactions 1 through 3 (1). The most extensively studied of these procaryotic enzymes is the 10-formyltetrahydrofolate synthetase from *Clostridia* (26). Recently, Staben and Rabinowitz (12) demonstrated that the mammalian C1-tetrahydrofolate synthases immunologically cross-react with antibodies to the yeast and bacterial enzymes. This suggests some structural similarity between these enzymes. Furthermore, the bacterial enzyme is inactive with sodium ion, requiring either potassium or ammonium ions for expression of maximum catalytic activity (16-18). Both the sheep and yeast enzymes of C1-tetrahydrofolate synthase have been shown to be much more active with ammonium and potassium ions. In this paper, we show that the rabbit enzyme is also activated by potassium and ammonium ions. Studies with the *Clostridial* enzymes showed that ammonium and potassium ions had both a catalytic and structural role with the enzyme (18). With sodium ion, the tetrameric enzyme dissociated into inactive monomers. Dissociation was reversed by ammonium and potassium ions. Our differential scanning calorimeter studies with the rabbit enzyme also show that potassium and ammonium ions alter the structure of the synthetase domain.

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**Fig. 7.** Differential scanning calorimeter trace of C1-tetrahydrofolate synthase in 50 mM potassium phosphate, 10 mM 2-mercaptoethanol, pH 7.0. Curve A, the enzyme, 2.5 mg/ml, contained 5 mM MgATP; curve B, enzyme concentration was 3.0 mg/ml with no additions; curve C, the enzyme, 1.5 mg/ml, contained 0.1 mM NADP\(^+\).

**Fig. 8.** Differential scanning calorimeter trace of the dehydrogenase-cyclohydrodrolase fragment. A, the fragment was about 1.5 mg/ml in 50 mM potassium phosphate, 10 mM 2-mercaptoethanol, and 0.1 mM NADP\(^+\) (pH 7.0). B, the same as in A except the buffer did not contain NADP\(^+\).
Although there is not a change in the quaternary structure of the enzyme, both ions increase the $T_m$ from 42 °C for the sodium form to 47 °C for the potassium and ammonium forms.

The results from this study suggest the following model for the structure of C$_1$-tetrahydrofolate synthase from rabbit liver. The enzyme is a homodimer with each subunit containing a large and small domain. Each domain contributes to the subunit interface since separation of the domains by proteolysis results in each domain existing as dimers. However, the small fragment is a dimer only in the presence of phosphate-containing buffers, suggesting that the major forces holding the subunits together lie in the large domain. There appears to be little interaction between the two domains within each subunit since after proteolysis they are readily separated on a molecular sieve column (Fig. 2). Also, the separated domains appear to retain most if not all of their catalytic capabilities (Figs. 4 and 5, Table II). There is no change in $K_m$ values for substrates, and the synthetase fragment retains its sensitivity to monovalent cations (Table III). Further evidence that there is little interaction between the two domains comes from the ligand-binding studies on the differential scanning calorimeter. The results recorded in Fig. 7 show that the binding of MgATP and NADP$^+$ to their respective domains results in conformational changes which stabilize them as evidenced by increased $T_m$ values. However, the conformational change induced by NADP$^+$ binding to the small domain is not transmitted as a change in $T_m$ of the large domain.

Differential scanning calorimetry studies show that the small fragment is structurally different than the corresponding domain in the native enzyme (Fig. 8). However, NADP$^+$ does stabilize the fragment although to a lesser degree than observed with the domain in the native enzyme. The large fragment is so unstable that we have been unable to determine its stability which suggests that it is also structurally different from its respective domain in the native enzyme. One explanation for the decreased stability of the fragments is that proteolysis has removed important structural components of each domain. We are presently studying how much of the protein is lost as peptides during proteolysis.

We are also currently investigating the interaction of tetrahydrofolate compounds with the two domains. It has been proposed that the polyglutamate portion of this molecule may act as an anchor so that the pteridine portion of the molecule can channel between different sites on these multifunctional enzymes without equilibrating with the solvent (14, 27). If this is true, then polyglutamates of tetrahydrofolate should affect the interrelationships of the two domains. This should alter the properties of the enzyme as detected by the techniques of differential scanning calorimetry and proteolytic digestion.

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G-Tetrahydrofolate Synthase Domains

C. Tetrahydrofolate Synthase Domains

**Experimental Procedure**

**Materials.** Fresh frozen rabbit livers were obtained from Rheinlander and Co. (Rheinlander Co. 1970). The livers were homogenized in 10% sucrose solution in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 g for 30 min at 4°C and the supernatant was used. The G-1,4-DT domains were synthesized as previously described. The enzyme was purified from human liver by the method described in this paper through the polyethylene glycol precipitation step according to the method of Bajaj et al. (1969).

**Polymerase activity.** The polymerization of G-1,4-DT by polymerase was achieved by treating the reaction mixture with the enzyme. The reaction mixture contained 0.2 ml of 100 mM potassium phosphate buffer, pH 7.0, 1.0 mM MgCl2, 1.0 mM dNTPs, 50 mM KCl, 1.0 mM dithiothreitol, 100 mM ATP, 200 units of polymerase, and 1000 units of formate dehydrogenase. The reaction was incubated at 37°C for 1 hour in a water bath.

**Purification of C. Tetrahydrofolate Synthase.**

**Preparation of C. Tetrahydrofolate Synthase:**

1. **Homogenization.** The frozen rabbit livers were crushed in a mortar and were homogenized in 3 liters of 20 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 20 mM MgCl2, 1.0% BSA, 1.0 mM DTT, and 1.0 mM EDTA.

2. **Precipitation.** The homogenate was centrifuged at 10,000 g for 30 min at 4°C. The supernatant was dialyzed against a large volume of 20 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 20 mM MgCl2, 1.0% BSA, 1.0 mM DTT, and 1.0 mM EDTA.

3. **Precipitation of C. Tetrahydrofolate Synthase.**

**Purification of C. Tetrahydrofolate Synthase:**

**Table 1:**

| Purification Step | Volume | Protein | 280 nm Specific Absorbance | Recovery | Purification Fold |
|-------------------|--------|---------|---------------------------|---------|------------------|
| 1. Homogenate     | 2400   | 54      | 0.255                     | 100     | 1.0              |
| 2. Supernatant    | 1900   | 53      | 0.128                     | 84      | 1.2              |
| 3. Precipitate    | 1900   | 53      | 0.128                     | 84      | 1.2              |
| 4. Crude Extract  | 150    | 24      | 0.176                     | 51      | 176              |
| 5. Supernatant    | 132    | 12      | 0.124                     | 40      | 1240             |
| 6. IPZ-TK-001    | 61     | 4.5     | 0.155                     | 40      | 1400             |

**Footnote:** For the 5'-3'-polyadenylate tail, poly(A) polymerase activity was measured using 100 ng of poly(A) polymerase, pH 7.0, containing 150 mM NaCl, 20 mM MgCl2, 1.0% BSA, 1.0 mM DTT, 1.0 mM ATP, and 1.0 mM EDTA. The reaction was incubated at 37°C for 1 hour in a water bath.

**Table 1:**

**Figure 1:** Electron micrographs of C. Tetrahydrofolate Synthase from an IPZ-Molecular weight column before and after digestion with trypsin. Wild-type lambda phage C. Tetrahydrofolate Synthase (CYS-1) was subjected to digestion with trypsin at a concentration of 10 μg/ml for 1 hour at 37°C. After digestion, the enzyme was subjected to electrophoresis in a 5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The gel was stained with Coomassie blue, and the bands were visualized by exposure to ultraviolet light. The figure shows the electrophoretic mobility of the enzyme before and after digestion with trypsin.

**Figure 2:** Electron micrographs of C. Tetrahydrofolate Synthase from an IPZ-Molecular weight column before and after digestion with trypsin. Wild-type lambda phage C. Tetrahydrofolate Synthase (CYS-1) was subjected to digestion with trypsin at a concentration of 10 μg/ml for 1 hour at 37°C. After digestion, the enzyme was subjected to electrophoresis in a 5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The gel was stained with Coomassie blue, and the bands were visualized by exposure to ultraviolet light. The figure shows the electrophoretic mobility of the enzyme before and after digestion with trypsin.
Figure 2. Elution pattern of C1-tetrahydrofolate synthase from an HPLC-molecular sieve column before and after digestion with chymotrypsin. A. Elution profile of the enzyme before addition of chymotrypsin. B. Elution profile after digestion with chymotrypsin for 100 minutes. For digestion conditions see the Experimental Procedure section. The protein eluting at 27.6 minutes is undigested parent enzyme which contains all three activities. The protein eluting at 31.3 minutes has only synthetase activity. The protein eluting at 36.5 minutes has only dehydrogenase and cyclohydrolase activities.

Figure 3. An 0.1% polyacrylamide gel electrophoresis pattern of the three proteins eluting from the HPLC-molecular sieve column as recorded in Figure 2. Lanes 4 and 5 are protein standards. Lanes 1 is the parent enzyme before digestion which elutes at 27.6 minutes from the HPLC column. Lanes C and D are the two fragments formed by digestion with chymotrypsin as shown in Figure 2B.

Figure 6. Properties of the cold inactivation of C1-tetrahydrofolate synthetase activity. A. Comparison of synthetase activity at 0°C and 25°C of 0.11 mg per ml solution of enzyme in 1M ammonium phosphate, 5 mM MgCl₂, 0.05 M DTT, 10 mM L-serine, 10 mM L-lysine, 10 mM L-threonine, pH 7.0 at 0°C and 25°C. B. Comparison of synthetase activity after 100 minutes of incubation at 0°C and 25°C as a function of pH. The conditions are the same as described for the experiment depicted in the left panel.