Methylenetetrahydrofolate Dehydrogenase-Methenyltetrahydrofolate Cyclohydrolase-Formyltetrahydrofolate Synthetase

A MULTIFUNCTIONAL PROTEIN FROM PORCINE LIVER*

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Methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrolase, and formyltetrahydrofolate synthetase from porcine liver have been co-purified more than 500-fold to apparent homogeneity. The inability of three sequential chromatographic procedures followed by affinity chromatography using NADP⁺ or ATP-substituted Sepharose to resolve the three activities demonstrates that they are physically associated. Molecular weight estimates of the native protein by gel filtration (Mₐ = 150,000) and by dodecyl sulfate gel electrophoresis (Mₐ = 100,000) indicate that the native structure is probably a single subunit. Since only one protein band is seen on dodecyl sulfate gels, it is concluded that the three activities are properties of a single polypeptide chain. The kinetic properties of the three activities are described, the most unusual feature being the susceptibility of the cyclohydrolase to competitive inhibition by NADP⁺, NAD⁺, ATP, and folate.

Many tetrahydrofolate-dependent reactions in mammals are catalyzed by enzymes that have been found to be relatively unstable in vitro (1), and consequently have been difficult to study. This property has hampered the development of our understanding of the molecular events involved in the interconversion of "active" 1-carbon units, including the regulation of this important area of metabolism.

One of the potentially important regulatory sites in the interconversion of 1-carbon derivatives is the reaction catalyzed by methylenetetrahydrofolate dehydrogenase. This enzyme interconverts 1-carbon units between the oxidation and reduction states required for thymidine or purine synthesis. Reports that the dehydrogenase from bacteria is inhibited by purine nucleotides prompted attempts at the purification and stabilization of the mammalian enzyme for study (2). At that time it was observed that the dehydrogenase (I) co-purified 100-fold with methylenetetrahydrofolate cyclohydrolase (II) and and formyltetrahydrofolate synthetase (III).

5,10-Methylenetetrahydrofolate + NADP⁺ (I)
⇔ 5,10-methylenetetrahydrofolate + NADPH + H⁺

Enzyme Assays - All the assays were similar to those used previously (2) and involve the spectrophotometric measurement of 5,10-methylenetetrahydrofolate produced or hydrolyzed during the incubations. Methylenetetrahydrofolate dehydrogenase was assayed at 30° in an incubation mixture containing 100 mM potassium phos-
phase, pH 7.3, 200 mM 3-mercaptopropanol, 0.2 mM (+)-tetrahydrofolate, 56 mM formaldehyde, and 7 mM NADP+ in a volume of 1 ml. The reaction was terminated by addition of 1 ml of either 7% trichloroacetic acid or 0.36 M HCl after 2 to 10 min of incubation. After standing at room temperature for 10 min, the absorbance at 350 nm was measured and the production of 5,10-methenyltetrahydrofolate calculated.

Preparation of Substituted Sepharose 4B—Adipic acid dihydrazide was prepared by refluxing 50 ml of diethyl adipate with 100 ml of hydrogen chloride and 100 ml of cold 0.1 M NaHCO3, pH 9.5, and stirred overnight at 4°C. The Sepharose-adipate dihydrazide derivative was washed with 10 volumes each of 0.2 M potassium phosphate, 20% glycerol, and 2 M dithiothreitol, pH 7.3, and the same solution containing 0.2 M potassium phosphate. Fractions containing enzyme activity were pooled, dialyzed against 1.2 M potassium phosphate, pH 7.3, and concentrated by lyophilization. The enzyme preparation was stable for several weeks when stored in ice in the refrigerator.

Affinity Chromatography on NADP+-Sepharose—Enzyme from the previous purification steps, usually after DEAE-Sepharose A-25 chromatography, was exchanged into 0.05 M trichloroacetic acid and redissolved with 20 mM NADP+ in 0.05 M potassium phosphate, 20% glycerol, and 0.08 M KCl, pH 7.3. Enzyme was applied to the column, washed with 50 ml of 0.05 M potassium phosphate, 20% glycerol, and 0.08 M KCl, and eluted by a linear gradient of NADP+ in the wash buffer containing 5 mM NADP+.

Polyacrylamide Gel Electrophoresis—Dodecyl sulfate polyacrylamide gel electrophoresis was carried out by the general procedure of Weber and Osborn (13) using ovalbumin, bovine serum albumin, and phosphorylase a as standards. Samples were either precipitated with 7% trichloroacetic acid and redissolved with 20 μl of 0.1 M NaOH, 3% Na2CO3, or were added in solution to a final concentration of 20% sucrose, 1% 2-mercaptoethanol, 1% dodecyl sulfate. The samples were heated in boiling water for 2 min, stained with 0.04% Coomassie blue and were heated in boiling water for 2 min and stained with 0.04% Coomassie blue. The gel was dried, and the gel cake was placed between two sheets of Whatman 3MM filter paper.
RESULTS

Purification of the dehydrogenase, cyclohydrolase, and synthetase activities is summarized in Table I. No separation of these activities was observed at any stage during the 535-fold purification. The co-purification is illustrated by the results of chromatography of the activities on Sephadex A-25 in 20% dimethylsulfoxide (Fig. 1). This procedure, while resulting in a rather low yield, was essential for the success of subsequent purification steps. Attempts to carry out this type of chromatography using Sephadex A-50 were unsuccessful due to an extremely poor recovery of activity. The enzyme obtained at this stage is generally 300- to 400-fold purified and is stable for about 1 week when concentrated and stored in 20% glycerol, 0.1 M potassium phosphate, pH 7.3.

Despite the extent of purification, the preparation at this stage contained one major and several minor protein bands on dodecyl sulfate gel electrophoresis. The nature of the apparent complex was thus unclear: the activities could be due to separate protein species, or to peptides associated noncovalently, or to covalently linked enzymes. As further tests of the physical association of the enzyme activities, we used isoelectric focusing and affinity chromatography as potential means to both purify and/or separate the activities.

The results of isoelectric focusing are shown in Fig. 2. Only relatively small amounts of protein could be used in the column to avoid aggregation so that a determination of protein in the gradient in the presence of the Ampholines was therefore not possible. However, the three enzymic activities have the same isoelectric point, although only about 10% of the synthetase activity could be recovered. Dodecyl sulfate gel electrophoresis of the peak fraction shows a single protein band (Fig. 3, left). Because of the limits of the amount of protein that could be applied as well as the instability of the synthetase, this step, while of analytical importance, could not be used as a technique for further purification.

Affinity chromatography was attempted as an independent method to further purify or separate the activities; the rationale was to use NADP+, a substrate only of the dehydrogenase, as the affinity ligand. The choice of buffer for this column was important. When the enzyme in 0.05 M potassium phosphate, 20% glycerol, pH 7.3, was applied to an NADP+-Sepharose column equilibrated with the same buffer, the activities appeared almost entirely in the wash. However, if the sample was first solvent exchanged into 0.05 M triethanolamine HCl, 20% glycerol, pH 7.3, on a Sephadex G-25 column, and applied to an NADP+-Sepharose column equilibrated with this buffer, the enzyme was absorbed. Washing with 10-column volumes of 0.06 M KCl in starting buffer resulted in no leakage of enzyme, but the addition of 2 mM NADP+ to this buffer eluted the majority of the enzyme in a single, well defined peak. Because the enzymes are not particularly stable under these conditions, affinity chromatography must be completed as quickly as possible. While the dehydrogenase and cyclohydrolase decrease in activity by about 10% in 4 h, the synthetase loses 50% of its activity during the same time. This instability accounts for the variable synthetase activity of the purified enzyme (Table I).

The elution of the three enzyme activities from the NADP+-Sepharose column is presented in Fig. 4. An NADP+ gradient was produced by the addition of 0.1 M KCl at 11 ml per hour. All three activities were eluted at the same point, while of analytical importance, could not be used as a technique for further purification.

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The elution of the three enzyme activities from the NADP+-Sepharose column is presented in Fig. 4. An NADP+ gradient

![Fig. 1. Chromatography of 5.3 mg of phosphocellulose-purified protein on a column of DEAE-Sephadex A-25 (1.2 x 10 cm) using a phosphate buffer gradient containing 20% dimethylsulfoxide as described under "Experimental Procedures." A, dehydrogenase; B, cyclohydrolase; C, synthetase activities; D, Lowry protein.](image)

![Fig. 2. Isoelectric focusing of 1.5 mg of DEAE-Sephadex A-25-purified protein on a column of phosphocellulose-purified protein on a column of DEAE-Sephadex A-25 (1.2 x 10 cm) using a phosphate buffer gradient containing 20% dimethylsulfoxide as described under "Experimental Procedures." Enzyme activity is expressed as pmol min⁻¹ ml⁻¹ adjusted by the following factors: O, synthetase (25 x); A, cyclohydrolase; B, dehydrogenase (25 x); and C, cyclohydrolase (4 x).](image)

### Table I

**Purification of tetrahydrofolate enzymes**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Enzyme activity</th>
<th>Purification (yield)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant solution</td>
<td>1,100</td>
<td>41,400</td>
<td>560 1,028 1,056</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>500</td>
<td>18,200</td>
<td>379 948 827</td>
<td>1.7 (68)</td>
</tr>
<tr>
<td>Polyethylene glycol 6000</td>
<td>84</td>
<td>6,400</td>
<td>294 659</td>
<td>2.7 (42)</td>
</tr>
<tr>
<td>Sephadex A-50</td>
<td>95</td>
<td>536</td>
<td>147 289 500</td>
<td>20 (26)</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>11</td>
<td>78</td>
<td>95 194 305</td>
<td>90 (20)</td>
</tr>
<tr>
<td>Sephadex A-25</td>
<td>0</td>
<td>7.6</td>
<td>30 50 69</td>
<td>221 (5.5)</td>
</tr>
<tr>
<td>NADP-Sepharose</td>
<td>9</td>
<td>2.8</td>
<td>21 35 61</td>
<td>535 (3.8)</td>
</tr>
</tbody>
</table>

* Calculated for the dehydrogenase.

* Corrected values presented for fractionation steps normally performed on a smaller scale (see "Experimental Procedures").

* Variable due to instability of the synthetase activity.
Multifunctional Protein

FIG. 3. Dodecyl sulfate gel electrophoresis of the enzyme preparation. Left, DEAE-Sephadex purified preparation electrophoresed on 6% polyacrylamide gels, before and after isoelectric focusing. Right, equivalent amounts of protein (10 μg) from phosphocellulose, Sephadex A-25, and NADP-Sepharose steps of purification electrophoresed on 9.5% polyacrylamide gels.

FIG. 4. Chromatography of DEAE-Sephadex A-25-purified enzyme on NADP+-Sepharose. A, approximately 0.5 mg of protein in 0.05 M triethanolamine hydrochloride, 20% glycerol, pH 7.3, was applied to a column (0.7 x 13 cm) and washed with 10 ml of the buffer containing 80 mM KCl. A linear gradient of 10 ml each of starting buffer and buffer containing 2 mM NADP+ was applied and followed with another 10 ml of buffer. Dehydrogenase activity is expressed as 25 μmol min⁻¹ ml⁻¹. B, similar experiment using a column (1 x 5.4 cm) of NADP+-Sepharose washed with 10 ml of 80 mM KCl in starting buffer and eluted with NADP+ as above. Enzyme activities are expressed as μmol min⁻¹ ml⁻¹ adjusted by the following factors: ◆, dehydrogenase (20 ×); □, synthetase (15 ×); and △, cyclohydrolase (6 ×). ○, Lowry protein; ⋯⋯, NADP+.

resulted in elution of all three enzyme activities with the same profile where the peak fraction eluted at 1 mM NADP+. Attempts to elute the enzymes using a KCl gradient resulted in a very broad and ill defined elution profile where about half the enzyme was eluted at 0.25 mM KCl. Further evidence for the specificity of the NADP+-Sepharose column was provided by an attempt to elute the enzyme with NAD1+ which was unsuccessful even with concentrations as high as 12 mM.

Preparative affinity chromatography using a step elution with NADP+ yields the protein with dehydrogenase activity of 7.5 μmol min⁻¹ mg⁻¹ representing a 535-fold purification. A comparison of the dodecyl sulfate gel electrophoresis patterns of the last three purification steps is shown in Fig. 3, right, where the NADP+-Sepharose eluate is represented by one major band of molecular weight 100,000. This compares with estimates of about 136,000 to 150,000 for the native activities by gel filtration on Bio-Gel 0.5m (Fig. 5). The same results were observed using yeast alcohol dehydrogenase (150,000), hexokinase (102,000), alkaline phosphatase (86,000) as standards on an alternate system, Ultragel AcA34. The validation experiment for the dodecyl sulfate gel molecular weight determination indicates that the protein does not have anomalous properties since its position in a plot of free electrophoretic mobility (M₀) against retardation coefficient (Kₐ) falls on a straight line generated by five standard proteins (Fig. 6).

A second type of affinity chromatography was carried out using ATP-Sepharose which has 3.6 μmol of ATP/ml of packed gel. This chromatography, designed to utilize binding at the synthetase site, was not as specific as the NADP+ column (Fig. 7). However, a linear gradient of ATP eluted the three activities together, with the peak fraction appearing at 1.1 mM ATP. Although the activities could be eluted with a gradient of CTP, a 4-fold higher concentration was required, indicating some degree of specificity. When compared with ATP, a relatively high concentration of KCl (100 mM) was required to elute the activities, although this was not as high as that required in the case of the NADP+-Sepharose.

The kinetic properties of the enzyme activities are summarized in Table II. The dehydrogenase is inhibited by NAD1+ and NADPH which are competitive against NADP1+ and by folic...
A, 0 and 5 mM ATP; B, 0 and 5 mM CTP; C, 0 and 0.2 M KCl. The dehydrogenase (50 x 1; C, dehydrogenase (25 x 1, synthetase (12 x 1, and cyclohydrolase (2 x 1; B, buffer, linear gradients composed of 10 ml each of the following in expressed as pmol mini ml-', adjusted by the following factors: A, Synthetic -Tetrahydrofolate dehydrogenase, methylenetetrahydrofolate cyclohydrolase, and formyltetrahydrofolate synthetase have been shown to co-purify more than 500-fold to apparent homogeneity. The inability of three sequential chromatographic steps involving linear gradient elution followed by affinity chromatography or isoelectric focusing to resolve the three activities demonstrates that the enzymic activities are physically associated in some manner. This conclusion is further supported since the affinity chromatography used two different ligands which should preferentially bind to the dehydrogenase or synthetase sites. The elution of the three activities from the NADP+ affinity chromatograft is quite specific as illustrated by comparison of the effectiveness of NADP+, NAD+, and KCl.

The final purified preparation yields a single protein band on dodecyl sulfate gels. This band is the only one seen to increase in intensity during the final steps of the purification (Fig. 3, right), and demonstrates that the protein is composed of only one size of polyepptide chain. Estimates of the native molecular weight by gel filtration gave values of 136,000 to 150,000 while the protein observed on dodecyl sulfate gel electrophoresis is 100,000. It is likely that the native structure is represented by a single polyepptide chain, and the discrepancy in the molecular weights is probably due to the methods employed. The possibility that the native structure contains two peptides (100,000 and 50,000) can be eliminated since both such peptides should be visible on dodecyl sulfate gels where, in fact, only one protein species is observed. The molecular weight determination by gel electrophoresis is probably reliable since the free mobility of the protein band was normal in a plot of $M_w$ versus $K_r$. However, gel filtration often gives erroneous estimates of molecular weight since it basically measures Stokes radius, which is dependent upon frictional coefficient and partial specific volume as well as molecular weight. If the enzyme activities are associated as a result of gene fusion, it can be postulated that the shape of the enzyme could approximate a cylinder-like structure composed of three packed globular regions. This shape would result in a higher frictional coefficient and thus a higher Stokes radius than a sphere of the same molecular weight. More rigorous methods for the molecular weight determinations such as sedimentation equilibrium will be required to finally resolve this problem, but must await better means to stabilize the purified enzyme.

The extent of purification of the complex is very similar to that for another folate enzyme from the same source which has been demonstrated to be homogeneous (15). The turnover numbers for the dehydrogenase and synthetase at 30° are 1700 and 2100, while that for the cyclohydrolase at 22° is 8700, as calculated from values of $V_{max}$ and assuming one site for each reaction. These values are comparable to the turnover number of 3250 reported for the formiminoglutamate-tetrahydrofolate formimino transferase at 37° (16). These numbers are compatible with the conclusion that the major acid, competitive against methylenetetrahydrofolate. The synthetase is not inhibited by either folic acid or NADP+. Surprisingly, cyclohydrolase is inhibited by NADP+, NAD+, and ATP as well as folic acid, where all inhibitors are competitive against methylenetetrahydrofolate. The unexpected inhibition by NADP+ is relatively specific since NAD+ shows 10-fold less affinity for the cyclohydrolase (Table II).

**DISCUSSION**

**Table II**

Kinetic properties of enzymes

Values presented are molar concentrations; the inhibition constants were calculated from double reciprocal plots showing competitive inhibition.

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>$K_i$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dehydrogenase</strong></td>
<td></td>
</tr>
<tr>
<td>NADP$^+$</td>
<td>$1.8 \times 10^{-3}$</td>
</tr>
<tr>
<td>(±)-Methylenetetrahydrofolate</td>
<td>$2.2 \times 10^{-3}$</td>
</tr>
<tr>
<td>NADPH</td>
<td>$6.7 \times 10^{-5}$</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>$2.4 \times 10^{-3}$</td>
</tr>
<tr>
<td>Folic acid</td>
<td>$2.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>ATP</td>
<td>No inhibition</td>
</tr>
<tr>
<td><strong>Synthetase</strong></td>
<td></td>
</tr>
<tr>
<td>(±)-Tetrahydrofolate</td>
<td>$2.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>Formate</td>
<td>$1.6 \times 10^{-2}$</td>
</tr>
<tr>
<td>ATP</td>
<td>$5.6 \times 10^{-3}$</td>
</tr>
<tr>
<td>Folic acid</td>
<td>No inhibition</td>
</tr>
<tr>
<td>NADP$^+$</td>
<td>No inhibition</td>
</tr>
<tr>
<td><strong>Cyclohydrolase</strong></td>
<td></td>
</tr>
<tr>
<td>(±)-Methylenetetrahydrofolate</td>
<td>$1.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>NADP$^+$</td>
<td>$9 \times 10^{-4}$</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>$4.4 \times 10^{-3}$</td>
</tr>
<tr>
<td>Folic acid</td>
<td>$1.4 \times 10^{-4}$</td>
</tr>
<tr>
<td>ATP</td>
<td>$3 \times 10^{-3}$</td>
</tr>
</tbody>
</table>
protein band we observe on gels is responsible for the activities. Attempts to show co-electrophoresis of enzyme activity with protein on polyacrylamide gels was not successful since only traces of activity could be recovered from the gel.

The kinetic properties of the enzymes have not been extensively investigated, but it appears that the dehydrogenase is not inhibited by ATP as has been reported for the enzyme from *Salmonella* (7) and *Clostridium cylindrosporum* (18). Of interest are the effects of inhibitors of the cyclohydrolase: folic acid, NADP+, and ATP are all competitive inhibitors. Inhibition by NADP+ is not reported to occur with the beef enzyme (1) but is seen to be relatively specific for our preparation since NAD+ is 10 times less effective. How the binding site for inhibition by NADP+ relates to the NADP+ substrate site of the dehydrogenase is not clear, although the kinetic constants indicate that two separate sites could be involved. The susceptibility of the cyclohydrolase to inhibition indicates that this enzyme may be of more importance from the standpoint of regulation than the dehydrogenase.

The existence of the trifunctional polypeptide in porcine liver suggests that similar proteins could be observed in other species. Methylene tetrahydrofolate dehydrogenase has been purified to homogeneity from *C. cylindrosporum* (18) and partially purified from several sources including yeast (19), calf thymus (20), and beef liver (1). The enzyme from mammalian sources in particular was reported to be quite unstable: the beef liver enzyme, for example, could be purified 40- to 50-fold but lost all activity after 96 h of storage (1). The yeast enzyme is more stable and has been purified 120-fold. Lázowska and Luzzati (21, 22) have shown that *Streptococcus cerevisiae* contains two molecular forms of the dehydrogenase, one of which is missing in ad 3 mutants. It is of interest that these same mutants have only 10% of the normal cyclohydrolase and synthetase activities. One possible explanation for this phenomenon is that these same enzymes are associated in some similar fashion in yeast and a defect in the dehydrogenase results in destabilization of the synthetase and cyclohydrolase. The best characterized bacterial system is that of *C. cylindrosporum* where Rabinowitz and co-workers have purified and characterized the dehydrogenase, cyclohydrolase, and synthetase which are separate enzyme species (18, 23, 24).

The situation in other mammalian species is as yet not conclusive, although the results of Rowe and Lewis (1) who investigated the same activities in beef liver are not in agreement with our findings in pig liver. These authors reported a 40-fold purification of synthetase that was free of dehydrogenase and cyclohydrolase activities. The preparation was unstable and lost all activity within 72 h. In addition, they reported two forms of cyclohydrolase of about 30,000 and 60,000 daltons, with the latter being considerably more stable. It thus appears that the dehydrogenase, cyclohydrolase, and synthetase are separable species in beef liver. However, it is not clear that the dehydrogenase and cyclohydrolase preparations were free of contaminating folate-enzyme activities. It is possible that instability of the enzymes and the use of different purification schemes for each of the activities combined to give preferential stability to the activity sought. Some of the instability may be due to proteolysis in the crude extracts. The answer to the question of the species specificity of the trifunctional polypeptide obviously must await further investigations.

We have previously demonstrated that another folate-dependent enzyme, formiminotransferase-cyclodeaminase from pig liver, is composed of eight identical bifunctional polypeptides arranged in a circular structure (25) and have now presented evidence in this paper that methylene tetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrolase, and formyltetrahydrofolate synthetase activities are found in a single polypeptide in the liver of the same species. These folate-dependent enzymes are therefore further examples of the class of multifunctional proteins recently reviewed by Kirschner and Bisswanger (5).

Addendum – After submission of this manuscript, a paper appeared by Paukert et al. (26) describing this multifunctional protein from ovine liver.

REFERENCES

Methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase. A multifunctional protein from porcine liver.
L U Tan, E J Drury and R E MacKenzie


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