Methylenetetrahydrofolate Dehydrogenase-
Methenyltetrahydrofolate Cyclohydrrolase-
Formyltetrahydrofolate Synthetase

A MULTIFUNCTIONAL PROTEIN FROM PORCINE LIVER*

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Methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrrolase, 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. Molar weight estimates of the native protein by gel filtration (M, = 150,000) and by dodecyl sulfate gel electrophoresis (M, = 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 cyclohydrrolase 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 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 cyclohydrrolase (II) and and formyltetrahydrofolate synthetase (III).

\[
\begin{align*}
5,10\text{-Methylenetetrahydrofolate} + \text{NADP}^+ & \rightarrow 5,10\text{-methylenetetrahydrofolate} + \text{NADPH} + \text{H}^+ \\
(\text{I})
\end{align*}
\]

\[
\begin{align*}
5,10\text{-Methylenetetrahydrofolate} + \text{H}_2\text{O} & \rightarrow 10\text{-formyltetrahydrofolate} \\
(\text{II})
\end{align*}
\]

Tetrahydrofolate + HCOOH + ATP
\[
\rightarrow 10\text{-formyltetrahydrofolate} + \text{ADP} + \text{P}_i \\
(\text{III})
\]

The possibility that these three enzymes were associated was of interest not only from the standpoint of understanding folate metabolism in mammals, but also as a system to further assess the functional significance of such protein structures. Enzymes operate in cells at relatively high protein concentrations, probably with specific protein-protein associations and intracellular localization. Model systems using cross-linked or immobilized enzymes (3, 4) have been used as tools to experimentally simulate these features of naturally occurring complexes. Our objective has been to establish that the three folate enzymes, previously shown to co-purify, physically associate, and to attempt to assess its significance in folate-medicated metabolism. We describe in this paper the purification to homogeneity of the enzymes, and present evidence that the three activities do not form a "complex," but are properties of a single polypeptide chain, a multifunctional protein (5).

EXPERIMENTAL PROCEDURES

Materials - Sepharose 4B, DEAE-Sephadex A-50 and A-25, and Sephadex G-25 were obtained from Pharmacia; phosphocellulose P11 from Whatman Biochemicals Ltd.; and Bio-Gel A-0.5m from Bio-Rad Laboratories. Electrophoresis grade acrylamide, bisacrylamide, 2-mercaptoethanol, and hydrazine (+95%) were products of Eastman Chemical Co.; diethyladipate, formaldehyde, and sodium periodate were obtained from British Drug Houses. Folic acid, ATP, dithiothreitol, NAD+, NADP+, and NADPH were supplied by Sigma, and acenocoumarol and polyethylene glycol 8000 by J. T. Baker Co. Ampholines, pH 6 to 8, and Ultragel AcA 34 were from LKB. Dimethylsulfoxide, glycerol, and common chemicals were reagent grade from Fisher Scientific Co.

Tetrahydrofolate was prepared by reduction of a neutral aqueous solution of folic acid at room temperature with 1 atm of hydrogen over platinum (6), followed by purification on DEAE-cellulose (7) by elution with 0.25 M triethanolamine hydrochloride and 0.5 M 2-mercaptoethanol, pH 7.2. After enzymic assay, 10 mm tetrahydrofolate was stored at 4° in 10-ml sealed ampoules. Preparation of (±)-5,10-methylenetetrahydrofolic acid was by the procedure of Rowe (8).

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-
phate, pH 7.3, 200 mM 2-mercaptoethanol, 0.2 mM [(+)-tetrahydrofolate], 20 mM [NADP], 20 mM formate, and 27 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-methylenetetrahydrofolate calculated as described for the dehydrogenase assay.

Cyclohydrolase was assayed by following the decrease in absorbance at 355 nm of an incubation mixture containing 100 mM triethanolamine hydrochloride, pH 8, 20 mM sodium formate, 1 mM ATP and MgCl₂, 50 mM potassium chloride, 0.43 mM [(+)-tetrahydrofolate], and 200 mM 2-mercaptoethanol in a volume of 1 ml. The 10-formyltetrahydrofolate produced was measured as the methenyl derivative after acidification as described for the dehydrogenase assay.

Preparation of Substituted Sepharose 4B—Adipic acid hydrazide was prepared by refluxing 50 ml of diethyl adipate with 100 ml of hydrochloric acid and 100 ml of 20% sodium metaperiodate in 0.02 M sodium phosphate, pH 7.0, and then 1 volume of Sepharose-adipate dihydrazide and stirred gently for 3 h (11). After cooling, the hydrazide was recovered by filtration and recrystallized twice from ethanol/water.

Sepharose 4B was activated with cyanogen bromide by the method of March et al. (12). A slurry of washed Sepharose (50% gel by volume) and 1 volume of 1 M Na₂CO₃ and 0.05 volume of cyanogen bromide (2 g/ml of acetonitrile) was added and stirred for 1 to 2 min. The gel was washed with 10 volumes each of cold 0.1 M NaHCO₃, pH 9.5, water, and 0.1 M NaHCO₃, pH 9.5. The activated Sepharose 4B was resuspended in 1 volume of a saturated solution of adipic acid dihydrazide in 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 NaCl, water, and 0.1 M sodium acetate, pH 5.

Ligand (NADP⁺) was attached to the Sepharose-adipate dihydrazide derivative by the method described for the dehydrogenase (11). For use as enzyme support, 0.02 M NADP⁺ was oxidized for 4 h in the dark with 0.02 M sodium metaperiodate in 0.02 M sodium phosphate, pH 7.0, and then diluted with 4 volumes of 0.125 M sodium acetate, pH 5.0. For small columns, 1.25 volumes of the oxidized NADP⁺ solution were added to 1 volume of Sepharose-adipate dihydrazide and stirred gently for 3 h in the dark at 4°C. After addition of 3.75 volumes of 2 M NaCl, stirring was continued for another 30 min, and the gel was then washed with 10 volumes each of 0.2 M NaCl, water, and the buffer to be used for chromatography. For preparative columns, 2 volumes of NADP⁺ solution were used in the coupling reaction. The amount of NADP⁺ adsorbed was determined spectrophotometrically, and that bound to the resin was estimated by dilution to be 2.4 to 4.5 μmol/ml of packed gel.

Sepharose to which ATP was bound was prepared in the same manner as for ATP-Sepharose (9). About 1.5 mg of protein from the DEAE-Sepharose-A-25 purification step was solvent exchanged and applied to the NADP⁺-Sepharose columns. The loading capacity of the substituted Sepharose was estimated at about 120 μg of DEAE-A-25-purified enzyme/ml of gel by applying excess enzyme so that activity appeared in the eluant. The enzyme units bound were obtained by difference between the activity added to the column and that found in the eluant. The NADP⁺-Sepharose lost approximately 3% bound ligand/day (calculated from the loss of A₂₈₀ while the binding capacity was estimated to decrease by about 5%/day. Greater than 80% of the bound enzyme could be eluted from NADP⁺-Sepharose with 2 mM NADP⁺ in the starting buffer. For analytical experiments, freshly prepared NADP⁺-Sepharose was packed in 1-cm columns ranging in height from 3.4 to 5.4 cm and equilibrated with 0.05 M triethanolamine hydrochloride, 20% glycerol, pH 7.3. Enzyme was applied to the column, washed with 10 ml of 0.06 M or 0.08 M KCl in the same buffer, and then eluted by a linear gradient of 5 to 10 M KCl or NADP⁺ in the starting buffer.

A larger scale column (2.5 × 5 cm) was used with the same buffers to prepare purified enzyme. About 1.5 mg of protein from the DEAE-A-25 purification step was solvated exchanged and applied to the column, and washed with 50 ml of 0.05 M triethanolamine hydrochloride, 0.08 M KCl, 20% glycerol, pH 7.3. Activity was eluted with 30 ml of 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.id were boiled for 2 min after the addition of buffer and 4 ml of 0.06 M KCl in the buffer, and then 1 ml of 0.1 M KCl blue were added after cooling in ice. The reliability of the molecular weight determination by dodecyl sulfate polyacrylamide gel electrophoresis was verified by the method of Banker and Cotman (14).
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 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 solven 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 90% 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

![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; □, cyclohydrolase; ○, synthetase activity; ●, Lowry protein.](http://www.jbc.org/content/111/1/1119/F1.large.jpg)

![Fig. 2. Isoelectric focusing of 1.5 mg of DEAE-Sephadex A-25-purified protein as described under "Experimental Procedures." Enzyme activity is expressed as µmol min⁻¹ ml⁻¹ adjusted by the following factors: ○, synthetase (25 x); □, dehydrogenase (25 x); and ●, cyclohydrolase (4 x).](http://www.jbc.org/content/111/1/1119/F2.large.jpg)

<table>
<thead>
<tr>
<th>Table I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purification of tetrahydrofolate enzymes</td>
</tr>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td>Supernatant solution</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
</tr>
<tr>
<td>Polystyrene glycol 6000</td>
</tr>
<tr>
<td>Sephadex A-50</td>
</tr>
<tr>
<td>Phosphocellulose</td>
</tr>
<tr>
<td>Sephadex A-25</td>
</tr>
<tr>
<td>NADP-Sepharose</td>
</tr>
</tbody>
</table>

* Calculated for the dehydrogenase.

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

* Variable due to instability of the synthetase activity.
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 60 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 M KCl. Further evidence for the specificity of the NADP⁺-Sepharose column was provided by an attempt to elute the enzyme with NAD⁺ 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 (Mₑ) versus retardation coefficient (Kₑ), as described by Banker and Cotman (14), for the tetrahydrofolate enzyme (2), and the standard proteins: 1, β-galactosidase; 2, phospholipase a; 3, catalase; 5, pyruvate kinase; 6, ovalbumin.

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 NAD⁺ and NADPH which are competitive against NADP⁺, and by folic...
Values presented are molar concentrations; the inhibition constants were calculated from double reciprocal plots showing competitive inhibition.

### Table II

**Kinetic properties of enzymes**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Inhibitor</th>
<th>$K_i$</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydrogenase</td>
<td>NADP⁺</td>
<td>$1.8 \times 10^{-3}$</td>
<td>-</td>
</tr>
<tr>
<td>(±)-Methylenetetrahydrofolate</td>
<td>$2.2 \times 10^{-3}$</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cytochrome b559</td>
<td>FAD</td>
<td>$6.7 \times 10^{-5}$</td>
<td>-</td>
</tr>
<tr>
<td>(±)-Methylenetetrahydrofolate</td>
<td>NAD⁺</td>
<td>$2.4 \times 10^{-3}$</td>
<td>-</td>
</tr>
<tr>
<td>Folic acid</td>
<td>ATP</td>
<td>$2.5 \times 10^{-4}$</td>
<td>No inhibition</td>
</tr>
<tr>
<td>(±)-Methylenetetrahydrofolate</td>
<td>ATP</td>
<td>$5.6 \times 10^{-5}$</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Synthetase</td>
<td>(±)-Folic acid</td>
<td>$2.3 \times 10^{-4}$</td>
<td>-</td>
</tr>
<tr>
<td>Folic acid</td>
<td>ATP</td>
<td>$1.6 \times 10^{-2}$</td>
<td>No inhibition</td>
</tr>
<tr>
<td>(±)-Methylenetetrahydrofolate</td>
<td>NAD⁺</td>
<td>$5 \times 10^{-4}$</td>
<td>No inhibition</td>
</tr>
<tr>
<td>ATP</td>
<td>No inhibition</td>
<td>$6 \times 10^{-5}$</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Cyclohydrolase</td>
<td>(±)-Methylenetetrahydrofolate</td>
<td>$1.3 \times 10^{-4}$</td>
<td>-</td>
</tr>
<tr>
<td>Folic acid</td>
<td>ATP</td>
<td>$3 \times 10^{-5}$</td>
<td>-</td>
</tr>
<tr>
<td>ATP</td>
<td>No inhibition</td>
<td>$5 \times 10^{-4}$</td>
<td>-</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate 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⁺-Sepharose column is quite specific as illustrated by comparison of the effectiveness of NAD⁺, NADP⁺, 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 polypeptide chain. Estimates of the native molecular weight by gel filtration gave values of 150,000 to 160,000 while the protein observed on dodecyl sulfate gel electrophoresis is 100,000. It is likely that the native structure is represented by a single polypeptide 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 $M_n$ 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 activity per 100,000 daltons. These values are comparable to the turnover number of 3250 reported for the formimino glutamate-tetrahydrofolate formimino transferase at 37° (16). These numbers are compatible with the conclusion that the major
proteins, attempts to show co-electrophoreses 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. Lazowska 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 cyclohydrodrolase 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 cyclohydrodrolase. 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 cyclohydrodrolase activities. The preparation was unstable and lost all activity within 72 h. In addition, they reported two forms of cyclohydrodrolase of about 30,000 and 60,000 daltons, with the latter being considerably more stable. It thus appears that the dehydrogenase and cyclohydrodrolase are separable species in beef liver. However, it is not clear that the dehydrogenase and cyclohydrodrolase 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 methylenetetrahydrofolate dehydrogenase, methylyltetrahydrofolate cyclohydrodrolase, 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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