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METHYLENETETRAHYDROFOLATE DEHYDROGENASE, METHENYLTETRAHYDROFOLATE CYCLOHYDROLASE AND FORMYLTETRAHYDROFOLATE SYNTHETASE FROM PORCINE LIVER

ISOLATION OF A DEHYDROGENASE-CYCLOHYDROLASE FRAGMENT FROM THE MULTIFUNCTIONAL ENZYME

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Summary

Tryptic digestion of a multifunctional enzyme from porcine liver containing methylenetetrahydrofolate dehydrogenase (5,10-methylenetetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.5), methenyltetrahydrofolate cyclohydrolase (5,10-methenyltetrahydrofolate 5-hydrolase, EC 3.5.4.9) and formyltetrahydrofolate synthetase (formate:tetrahydrofolate ligase, EC 6.3.4.3) activities destroys the synthetase. A fragment containing both dehydrogenase and cyclohydrolase activities has been isolated by affinity chromatography on an $NADP^+$-Sepharose affinity column. The purified fragment is homogeneous on dodecyl sulfate-polyacrylamide gel electrophoresis where its molecular weight was determined as 33 000 ± 1200 compared with 100 000 for the undigested protein. The cyclohydrolase activity retains sensitivity to inhibition by $NADP^+$, MgATP and ATP.

Introduction

Methylenetetrahydrofolate dehydrogenase (5,10-methylenetetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.5), methenyltetrahydrofolate cyclohydrolase (5,10-methenyltetrahydrofolate 5-hydrolase, EC 3.5.4.9) formyltetrahydrofolate synthetase (formate:tetrahydrofolate ligase, EC 6.3.4.3) activities were shown to copurify 100-fold from porcine liver [1] and later were shown to be activities of a multifunctional protein in both sheep [2] and pig liver [3]. Genetic experiments indicate that a similar situation probably occurs in yeast [4—6]. The presence of the three activities in a single polypeptide prompted experiments to attempt to cleave the enzyme and isolate fragments having one

or more enzyme activities to obtain some insight into how the activities are associated. In this paper we demonstrate that the dehydrogenase and cyclohydrolase activities are found in a single polypeptide of only 33 000 daltons indicating at least a very close interaction between these activities, and possibly a partly shared or 'composite' active site.

Materials and Methods

Trypsin and soybean trypsin inhibitor were products of Worthington Biochemicals; $NADP^+$, ATP, ovalbumin, yeast alcohol dehydrogenase and bovine serum albumin were from Sigma Chemicals; 2',5'-ADP-Sepharose was obtained from Pharmacia. All other chemicals were reagent grade.

The trifunctional enzyme containing methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrolase and formyltetrahydrofolate synthetase activities was purified essentially as described previously [3]. A minor modification in the final affinity chromatography on $NADP^+$-Sepharose involved addition of 8 mM potassium phosphate and 5 mM dithiothreitol to the 50 mM triethanolamine hydrochloride, 20% glycerol buffer, pH 7.3. The enzyme was adsorbed onto the affinity column, washed with five column volumes of the buffer containing 30 mM KCl and eluted with buffer containing 2 mM $NADP^+$. The purified enzyme was concentrated with Sephadex G-25 and dialyzed overnight against 50 volumes of 0.1 M potassium phosphate, 20% glycerol, pH 7.3. An alternative method of final purification used 2',5'-ADP-Sepharose instead of $NADP^+$-Sepharose as the affinity column. Enzyme was diluted with 20% glycerol to reduce the potassium phosphate concentration from 0.1 to 0.04 M and was applied to a 2',5'-ADP-Sepharose column equilibrated with 0.06 M potassium phosphate, 20% glycerol, pH 7.3. The column was washed with five column volumes of this buffer and the enzyme was eluted with the buffer containing 2 mM $NADP^+$ and then concentrated as usual. Both procedures yielded enzyme having 4.5—5 units dehydrogenase activity per mg protein. The assays for enzymic activities were as described previously [3] and protein was assayed by the method of Lowry et al. [7].

Tryptic cleavage. Digestion of the native enzyme with trypsin was carried out at 20°C for 1 h in 0.1 M triethanolamine · HCl, pH 7.3, containing 0.5 mM $NADP^+$ using 500 μg trypsin per unit of dehydrogenase activity. When purified enzyme is used, these conditions are approx. 10:1 trypsin:enzyme on a molar basis.

The tryptic fragement was prepared and purified as follows: Enzyme was first solvent exchanged into 0.1 M triethanolamine hydrochloride, pH 7.3, on a Sephadex G-25 column giving a protein concentration of between 62 and 250 μg/ml. Appropriate amounts of trypsin and $NADP^+$ were added. After digestion, two equivalents of soybean trypsin inhibitor and an equal volume of cold 40% glycerol were added and the digest was applied to an $NADP^+$-Sepharose column (1 × 5 cm) equilibrated with 0.05 M triethanolamine hydrochloride, 20% glycerol, pH 7.3. The column was washed with five column volumes of the starting buffer and eluted with (a) 20 ml of 0.3 M potassium phosphate, 20% glycerol, pH 7.3, (b) 10 ml of 2 mM $NADP^+$ in the starting buffer, or (c)

a linear gradient of 13 ml each of buffer and the buffer containing 1.5 mM $NADP^+$. The concentration of $NADP^+$ was measured spectrophotometrically at 259 nm using the extinction coefficient of 17 800 $cm^{-1} \cdot M^{-1}$ [8].

Polyacrylamide gel electrophoresis in dodecyl sulfate was carried out according to the general procedure of Weber and Osborn [9], using undigested trifunctional enzyme, bovine serum albumin, ovalbumin and yeast alcohol dehyhydrogenase as standards.

Results

Purification and experimentation with the dehydrogenase-cyclohydrolase-synthetase enzyme from porcine liver is hampered to some extent by the lability of the protein [3]. However, proteolytic digestion of the multifunctional protein could be carried out only under conditions where the protein structure is not stabilized. Consequently, potassium phosphate buffers or solutions containing glycerol could not be used, and the digestion was carried out in triethanolamine · HCl, pH 7.3, where all the enzymes, but particularly the synthetase, are somewhat labile. $NADP^+$ was added to the buffer to stabilize the dehydrogenase activity of both the native protein and the fragment produced during proteolysis.

Digestion of the trifunctional enzyme with trypsin results in loss of synthetase activity, with optimal results being obtained with rather high amounts of trypsin, 500 μg per unit of dehydrogenase activity, as shown in Table I. Using these conditions for proteolytic digestion of enzyme at different stages of purification, it was observed that the synthetase could be almost completely inactivated, with little change in the dehydrogenase and cyclohydrolase activities (Table II). If $NADP^+$ is excluded, trypsin treatment reduces the dehydrogenase to less than 40% of the original activity after digestion for 1 h. It is not clear if the loss of dehydrogenase is due to proteolytic cleavage, since the greater instability of the fragment must certainly be a contributing factor. $NADP^+$ did not protect the synthetase from proteolysis, but 2 mM MgATP was somewhat effective in this regard.

Attempts at purifying an active fragment with dehydrogenase activity on an $NADP^+$-Sepharose column were successful and the results are shown in Fig. 1.

TABLE I

EFFECT OF TRYPSIN ON THE SYNTHETASE AND DEHYDROGENASE ACTIVITIES

Enzyme was purified through DEAE-Sephadex [3] and was digested with trypsin for 1 h as described in Materials and Methods. The mixture contained 160 μg protein and 0.26 units dehydrogenase activity per ml.

μg trypsin/unit dehydrogenase	Activity (%)	
	Dehydrogenase	Synthetase
0	100	100
5	95	66
50	85	14
500	83	0.7

TABLE II

RECOVERIES OF ENZYME ACTIVITIES FOLLOWING TRYPSIN CLEAVAGE

Native enzyme preparations from different purification steps were used for trypsin digestion. The ratio of trypsin to enzyme activity was kept at the approximate ratio of 500 μg/unit of dehydrogenase activity. The specific activities of the DEAE-Sephadex A-25, $NADP^+$-Sepharose and 2′,5′-ADP-Sepharose enzyme preparations were 1.6, 4.7 and 5.1 units/mg, respectively.

Purification step	Dehydrogenase (%)	Cyclohydrolase (%)	Synthetase (%)
DEAE-Sephadex A 25	109	92	2
$NADP^+$-Sepharose	110	138	5
2′,5′-ADP-Sepharose	79	119	3

The adsorbed enzyme fragment was eluted with a gradient of $NADP^+$ and the enzyme activity peak corresponded to an $NADP^+$ concentration of 0.2 mM (Fig. 1a). In Fig. 1b a step elution using the starting buffer containing 2 mM $NADP^+$ showed that the dehydrogenase and the cyclohydrolase activities copurified. The synthetase activity was undetectable throughout the column.

It appears that there is not a significant change in the dehydrogenase and cyclohydrolase activities after proteolytic cleavage, indicating no major change in the turnover rates of the remaining active sites. Attempts to test this more directly by measuring specific activities of the fragment were only partially

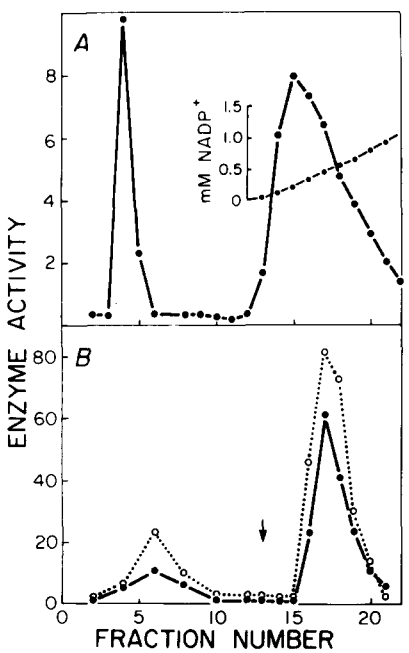

Fig. 1. (A) Enzyme (100 μg) purified through chromatography on DEAE-Sephadex A 25, was treated with trypsin and applied to an $NADP^+$-Sepharose column. The dehydrogenase activity was eluted with a linear gradient of $NADP^+$. (B) Enzyme (200 μg) previously purified on $NADP^+$-Sepharose, was digested with trypsin and applied to a second $NADP^+$-Sepharose column. The fragment was eluted with 2 mM $NADP^+$ as in Materials and Methods. Symbols are: ●, dehydrogenase; and ○, cyclohydrolase. Activities are expressed as units/ml $\times 10^3$.

successful. For example, a fragment isolated from the 2′,5′-ADP-Sepharose column had a dehydrogenase specific activity of 8.7 compared with 5.1 units/mg of the purified native enzyme. Based only on the loss of protein, the predicted activity would have been approx 15 units/mg. While the increase supports the observation that the dehydrogenase activity is now a property of a smaller protein fragment, the actual value must be interpreted cautiously in view of the errors associated with the lability of the protein and the small quantities obtainable.

Dodecyl sulfate polyacrylamide gel electrophoresis of the trypsin-digested enzyme is shown in Fig. 2. The cleavage by trypsin produced an active frag-

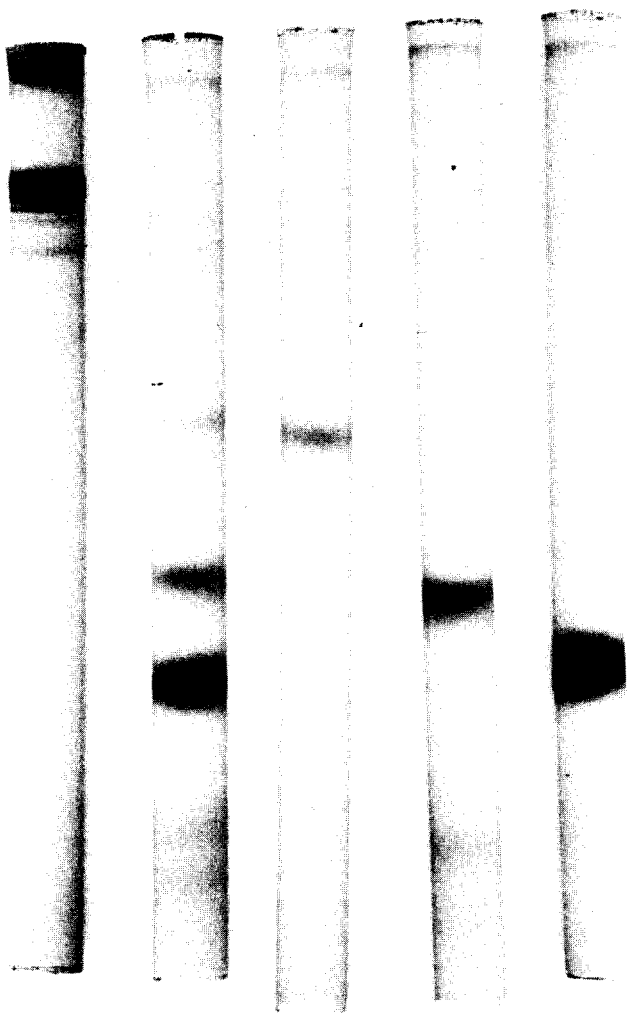

Fig. 2. Dodecyl sulfate gel electrophoresis of tryptic digest on 10% polyacrylamide gels. From the left, the gels represent: undigested protein (10 μg); tryptic digest (70 μg); purified fragment (2.5 μg); trypsin (10 μg) and trypsin inhibitor (10 μg).

TABLE III

STABILITY OF THE TRYPTIC FRAGMENT

Tryptic fragment was prepared by digestion of DEAE-Sephadex purified enzyme, and isolated on $NADP^+$-Sepharose as described in Materials and Methods, by elution with 0.3 M phosphate buffer, 20% glycerol, pH 7.3. Aliquots were stored for 24 h at 4°C under the conditions outlined.

Additions	activity (%)	
	Dehydrogenase	Cyclohydrolase
None	4	9
2 mM $NADP^+$	37	30
2 mM $NADP^+$, 5 mM dithiothreitol	79	61
2 mM $NADP^+$, 5 mM dithiothreitol (sealed flask containing 2-mercaptoethanol)	102	67

TABLE IV

EFFECT OF INHIBITORS ON THE CYCLOHYDROLASE ACTIVITY

An aliquot of $NADP^+$-Sepharose-purified native enzyme was cleaved with trypsin and the active fragment was isolated from a second $NADP^+$-Sepharose column by step elution using 0.3 M phosphate, 20% glycerol, pH 7.3. The substrate (±) methenyltetrahydrofolate, was $4.6 \cdot 10^{-5}$ M in all assays, and results are expressed with standard deviations.

Inhibitor concentration	Inhibition (%)	
	Native enzyme	Tryptic fragment
1 mM $NADP^+$	47 ± 5	30 ± 3
6 mM MgATP	37 ± 5	35 ± 5
6 mM ATP	66 ± 4	75 ± 3

ment as well as some low molecular weight peptides. When the digest was purified on the $NADP^+$-Sepharose column, the enzymically active fragment obtained appears to be homogeneous having a subunit molecular weight of 33 300 ± 120. Both the native enzyme [3] and particularly the purified tryptic fragment are rather labile proteins; this problem is compounded because of the low amounts of protein we can obtain, and thus a requirement to work with dilute protein solutions. Table III shows the results of attempting to store the tryptic fragment for 24 h under different conditions, and it appears that it is extremely easily oxidized. Storage in a test tube held within a suction flask containing 10 ml of 2-mercaptoethanol and sealed under reduced pressure provided the best conditions to maintain enzymic activity.

Certain properties of the cyclohydrolase activity were compared in the native enzyme and the tryptic fragment and are shown in Table IV. It appears that trypsin treatment does not abolish the sensitivity of the cyclohydrolase to inhibition by these three compounds.

Discussion

Several multifunctional proteins are known in mammalian systems [10], including the folate-dependent activities formiminoglutamate:tetrahydrofolate formiminotransferase and formiminotetrahydrofolate cyclodeaminase [11,12]

from porcine liver. The dehydrogenase, cyclohydrolase and synthetase are known to be separable species in *Clostridia* [13—15], although the cyclohydrolase copurifies with the cyclodeaminase from that species [14]. In both sheep [2] and pig liver [3] it is clear that these same three activities are found in a multifunctional protein. The isolation of a homogeneous tryptic fragment with two enzymic activities supports this conclusion. The advantage of multifunctional sequential enzymes may be to "channel" substrates through a metabolic pathway [10]. In our tryptic fragment, the presence of both dehydrogenase and cyclohydrolase activities in a peptide of only 33 000 daltons implies that the active centers must interact closely, and possibly form a composite site, analogous to that of tryptophan synthetase from *Escherichia coli* [16], although in the latter case, the two active centers are on separate polypeptides.

The dehydrogenase-cyclohydrolase activities function to interconvert methylenetetrahydrofolate and formyltetrahydrofolate through the intermediate methenyltetrahydrofolate. Methylenetetrahydrofolate, if not further reduced and used for methionine synthesis, is proposed to be converted to formyltetrahydrofolate which is then oxidized to yield CO_2 and regenerate tetrahydrofolate [17,18]. The three activities of the multifunctional protein can be viewed as reactions designed to produce formyltetrahydrofolate for this 'overflow' pathway, and may be more advantageous for this purpose in vivo when physically associated. The very close physical relationship between the dehydrogenase and cyclohydrolase is consistent with their linear sequence in the metabolic scheme [10], and may effect more efficient tranfer of one carbon fragments between methylene- and formyltetrahydrofolate. Since both methenyltetrahydrofolate and formyltetrahydrofolate are required for purine synthesis, it is probable that some methenyltetrahydrofolate intermediate is made available for this purpose. The functional advantages of this structure will perhaps be made clearer from a detailed investigation of the kinetic properties of the enzymes.

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