Biochimica et Biophysica Acta, 522 (1978) 311—317

BBA 68357

METHYLENETETRAHYDROFOLATE DEHYDROGENASE-METHENYLTETRAHYDROFOLATE CYCLOHYDROLASE-FORMYLTETRAHYDROFOLATE SYNTHETASE FROM PORCINE LIVER

INTERACTION BETWEEN THE DEHYDROGENASE AND CYCLOHYDROLASE ACTIVITIES OF THE MULTIFUNCTIONAL ENZYME

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(Received July 29th, 1977)

Summary

Methylenetetrahydrofolate dehydrogenase (5,10-methylenetetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.5) one of the activities of a trifunctional folate-dependent enzyme from porcine liver, uses an ordered kinetic mechanism as determined from initial velocity, product inhibition and dead-end inhibition studies. The final product released from the dehydrogenase is methenyltetrahydrofolate. However, from the time course of appearance of products it is observed that the methenyltetrahydrofolate, rather than equilibrating with the solution, is converted preferentially to formyltetrahydrofolate by the cyclohydrolase, (5,10-methenyletrahydrofolate 5-hydrolase (decyclizing), EC 3.5.4.9) demonstrating a functional interaction between these two enzymic activities.

Introduction

Methylenetetrahydrofolate dehydrogenase (5,10-methylenetetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.5) (I), methenyltetrahydrofolate cyclohydrolase (5,10-methenyltetrahydrofolate 5-hydrolase (decyclizing), EC 3.5.4.9) (II) and formyltetrahydrofolate synthetase (formate:tetrahydrofolate ligase, EC 6.3.4.3) (III) were shown to copurify from pig liver [1] and to have some type of physical interaction in yeast as determined from genetic studies [2—4]. Sub-

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sequently the activities have been shown to comprise multifunctional proteins in both sheep [5] and pig liver [6]. Multifunctional proteins have more than one catalytic and/or binding site per polypeptide, and the activities are often consecutive in a metabolic scheme [7] as is the case with this trifunctional enzyme:

$$5,10\text{-methylene}H_4\text{folate} + NADP^+ \overset{I}{\rightleftharpoons} 5,10\text{-methenyl}H_4\text{folate} + NADPH$$

$$5,10\text{-methenyl}H_4\text{folate} + H_2O \overset{II}{\rightleftharpoons} 10\text{-formyl}H_4\text{folate}$$

$$10\text{-formyl}H_4\text{folate} + ADP + Pi \overset{III}{\rightleftharpoons} \text{formate} + ATP + H_4\text{folate}$$

One of the apparent metabolic advantages of a multifunctional enzyme is the possibility of "channeling", a direct transfer of an intermediate between two enzyme domains, without equilibration with the solution. Such an interaction between the dehydrogenase and cyclohydrolase activities is supported by the kinetic mechanism of the dehydrogenase, as well as by the time course of appearance of the products of both reactions.

Materials and Methods

$NADP^+$, NADPH, ATP and folic acid were from Sigma; formaldehyde was obtained from British Drug Houses. Tetrahydrofolate was prepared by reduction of an aqueous, neutral solution of folic acid at atmospheric pressure with hydrogen and platinum [8] and purified by chromatography on DEAE-cellulose (Whatman DE-23) [9] but using triethanolamine · HCl pH 7.2 rather than Tris · HCl as eluant. After enzymic assay, 10 mM (±) tetrahydrofolate was stored at 4°C in 10 ml ampoules. Other chemicals were reagent grade from Fisher Scientific Co.

The trifunctional enzyme containing dehydrogenase, cyclohydrolase and synthetase activities was purified from pig liver as described previously [6] through chromatography on Sephadex A25 in 20% dimethylsulfoxide and had a dehydrogenase specific activity of 2.2—3 μmol · min^{-1} · mg^{-1}. Enzyme purified to this stage was used for kinetic studies for one week during which time it retained about 90% of its original activity. Standard enzyme assays were as described previously [6] and were modified as required with respect to substrate concentrations, and contained 0.17—0.22 μg of protein per assay. Concentrations of $NADP^+$ were determined by the absorbance of freshly prepared solution using $\epsilon_{259} = 17\,800\ M^{-1} \cdot cm^{-1}$ [10].

Attempts to observe "channeling" of the methenyl intermediate involved the following modifications to the enzyme assays: 100 mM triethanolamine · HCl, pH 7.3 was used instead of phosphate buffer to enhance the stability of the intermediate methenylH_4folate, and incubations were carried out at room temperature. The dehydrogenase produces stoichiometric amounts of methenylH_4folate and NADPH. Acidification of the assay mix destroys the NADPH, stabilizes the methenylH_4folate and converts any formylH_4folate to methenylH_4folate, and thus the absorbance at 350 nm is a measure of the total methenylH_4folate produced by the dehydrogenase. When following this same reaction at 355 nm in neutral solution, the contribution of NADPH to the absorbance was cor-

rected using ϵ_{355} (NADPH) = $4.9 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ to obtain the net production of methenylH_4folate.

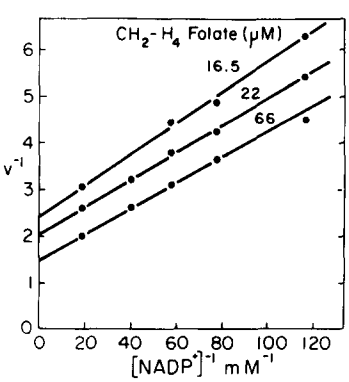
Results

Kinetic mechanism of the dehydrogenase

Double reciprocal plots of the initial velocities with either methyleneH_4folate or $NADP^+$ as the variable substrate at fixed concentrations of the other substrate are shown in Figs. 1 and 2. The intersecting plots in both cases indicate a sequential kinetic mechanism.

To determine the order of addition of substrates and release of products, both product and dead-end inhibition studies were carried out. In the case of product inhibition, only NADPH could be used since methenylH_4folate is a substrate for the cyclohydrolase and, in addition, is hydrolyzed chemically under the assay conditions. Using NADPH as an inhibitor against $NADP^+$ (Fig. 3) and methyleneH_4folate (Fig. 4), two non-competitive plots were obtained. This observation eliminates a simple random mechanism since all product inhibition patterns are competitive in such a case [11,12]. However, NADPH could function both as a product and a dead-end inhibitor by formation of a complex with free enzyme. For this situation to exist, the inhibition patterns with one product should yield either two competitive plots or one competitive and one noncompetitive plot [11]. Neither situation is observed and the mechanism is therefore a sequential, ordered one. The product inhibition results allow us to obtain the order of release of products. Since NADPH is not competitive against either substrate, it does not bind to the same enzyme form as these substrates, and thus cannot be the last product released in the reaction. The product inhibition results are consistent with two sequential ordered mechanisms differing only in their order of addition of substrates, but where the release of NADPH must occur prior to that of methenylH_4folate.

The order of addition of substrates was determined using folic acid as a



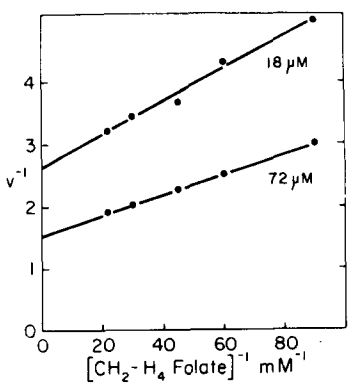
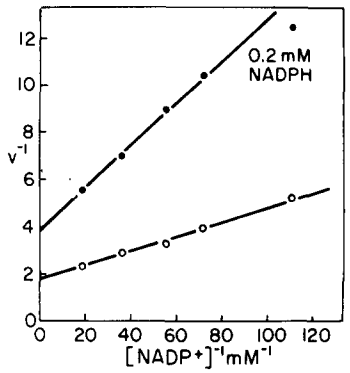


Fig. 1. Effect of $NADP^+$ concentration on the initial velocity of the dehydrogenase reaction at the fixed concentrations of methyleneH_4folate indicated.

Fig. 2. Effect of methyleneH_4folate on the initial velocity of the dehydrogenase reaction at fixed concentrations of $NADP^+$.



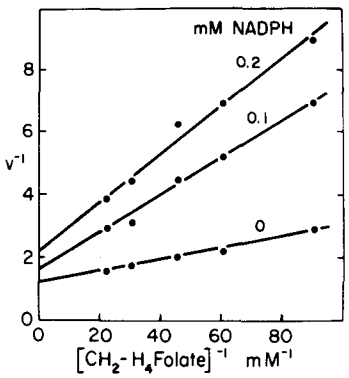
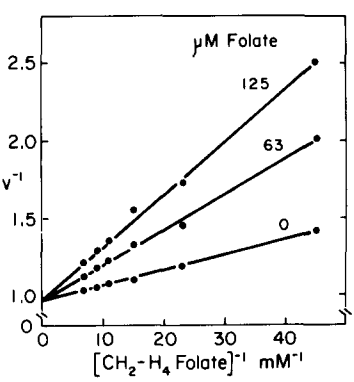


Fig. 3. Product inhibition of the dehydrogenase by NADPH with $NADP^+$ as the variable substrate and methyleneH$_4$folate hel constant at $1 \cdot 10^{-4}$ M.

Fig. 4. Product inhibition of the dehydrogenase by NADPH with methyleneH$_4$folate as variable substrate and $NADP^+$ held constant at $9 \cdot 10^{-5}$ M.

dead-end inhibitor. Folate is a competitive inhibitor against methyleneH$_4$-folate (Fig. 5) and is uncompetitive with respect to $NADP^+$ (Fig. 6). Replotting the data of Fig. 6 as a plot of s/v vs. s [13] shows that all lines intercept on the ordinate at a common value of K_m/V (Fig. 7) giving a further graphical check on the uncompetitive nature of the inhibition by folate. These results are consistent with an ordered mechanism in which $NADP^+$ binds first and folate binds subsequent to the formation of the $NADP^+$ · enzyme complex, where the two enzyme · ligand complexes are not reversibly connected downstream in the reaction sequence under conditions of the initial velocity measurements. All the kinetic data are in agreement with the following ordered mechanism for the dehydrogenase:



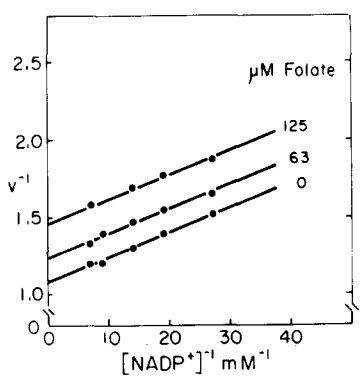


Fig. 5. Inhibition of the dehydrogenase by folate with methyleneH$_4$folate as the variable substrate and $NADP^+$ held constant at $9 \cdot 10^{-5}$ M.

Fig. 6. Inhibition of the dehydrogenase by folate with $NADP^+$ as the variable substrate and methyleneH$_4$-folate held constant at $1 \cdot 10^{-4}$ M.

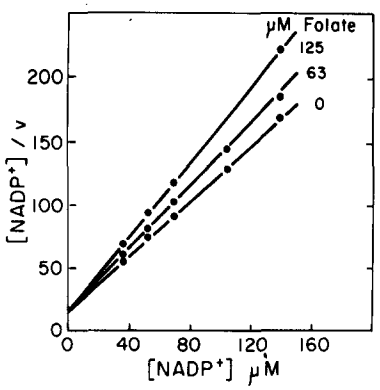


Fig. 7. The data of Fig. 6 presented as a Hanes plot.

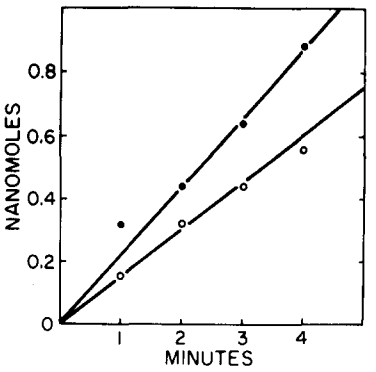


Fig. 8. Rate of appearance of methenylH_4folate (○) and formylH_4folate (●) with 22 μM methyleneH_4-folate and 36 μM $NADP^+$ as substrates.

"Channeling" of methenylH_4folate

The ordered kinetic mechanism of the dehydrogenase with release of methenylH_4folate as the last product is consistent with the possibility of channeling of this enzyme-bound intermediate through the cyclohydrolase reaction. The time course of appearance of methenylH_4folate and 10-formylH_4folate when the enzyme was incubated with the substrates of the dehydrogenase is shown in Fig. 8. No lag in production of formylH_4folate could be observed under these conditions, indicating that the methenylH_4folate product does not equilibrate with the medium before reacting with the cyclohydrolase. However, the production of formylH_4folate from the methenyl derivative can occur chemically, as well as by subsequent binding of released methenylH_4folate to the cyclohydrolase site. The combined chemical and enzymic rates observed with meth-

TABLE I

RATE OF APPEARANCE OF PRODUCTS OF THE DEHYDROGENASE AND CYCLOHYDROLASE ACTIVITIES

Assay conditions are as described in Materials and Methods.

Conditions		Rate of appearance (nmol/min)	
		MethenylH_4folate	FormylH_4folate
MethyleneH_4-folate	22 μM	0.12 *	0.18
$NADP^+$	36 μM		
B. MethenylH_4folate	63 μM **	—	1.6
$NADP^+$	36 μM		
C. Calculated from (B) for MethenylH_4folate concentrations observed in Fig. 8.			
MethenylH_4folate = 0.3 μM		—	0.008
MethenylH_4folate = 0.5 μM		—	0.012

* The dehydrogenase activity is 0.3 nmol · min^{-1} measuring both methenyl- and formylH_4 as products.

** Approximately 0.5 K_m.

enylH$_4$folate added at the highest concentration observed in Fig. 8 gives an initial rate of formation of 10-formylH$_4$folate of only about 10% of that observed in the assay, as shown in Table I. Therefore, the steady state concentration of methenylH$_4$folate is not sufficient to account for the observed rate of production of formylH$_4$folate. The combined actions of the dehydrogenase and cyclohydrolase can be represented as follows with the intermediate methenyl compound preferentially hydrolyzed by the cyclohydrolase:

Discussion

The dehydrogenase, cyclohydrolase and synthetase comprise multifunctional proteins in sheep and pig liver [5,6]. To more fully understand the catalytic properties of this protein we have investigated the kinetic mechanism of the dehydrogenase and its interaction with the cyclohydrolase. The kinetic properties have been used in a qualitative manner to establish the general kinetic mechanism by elimination of several possibilities. Initial velocity studies indicate that the kinetic mechanism of the dehydrogenase is sequential, and product inhibition patterns rule out random mechanisms. In addition, inhibition by NADPH is inconsistent with ordered mechanisms where NADPH is the final product released; therefore methenylH$_4$folate must be released last. A possible Theorell-Chance mechanism with NADP$^+$/NADPH as the central pair is also eliminated because of the non-competitive inhibition observed with NADPH.

Dead-end inhibition with folate showed that the order of substrate addition is NADP$^+$ followed by methyleneH$_4$folate. Purification of this trifunctional enzyme utilizes an NADP$^+$ affinity column [6] which also demonstrates that this ligand can bind prior to methyleneH$_4$folate. The ordered addition of substrates is consistent with the results and is not unusual with other dehydrogenases [12], while the ordered release of products is consistent with the subsequent utilization of the methenyl derivative by the cyclohydrolase reaction.

MethenylH$_4$folate produced by the dehydrogenase is not entirely equilibrated with the medium; approximately 60% is converted preferentially to formylH$_4$folate by the cyclohydrolase. This conclusion is supported by the lack of a lag period in the production of formylH$_4$folate as well as the observation that the rate of the cyclohydrolase reaction with the methenyl compound accumulated in the assay mix is not sufficient to account for the rate of production of the final product. It should be noted that these studies have been carried out with the available tetrahydrofolate derivatives, not the polyglutamate forms normally found in cells, which might be even more efficient in "channeling". Tan and MacKenzie [14] have recently demonstrated that proteolytic cleavage of the trifunctional polypeptide of mol. wt. 100 000 yields a bifunctional dehydrogenase-cyclohydrolase fragment of mol. wt. 33 000 that can be purified by affinity chromatography on NADP$^+$ Sepharose. Thus both catalytic and physical properties indicate a close association between these two enzymic activities.

The nature of the "association" of the two activities is not yet clear. It is

possible that the methenylH_4folate produced at a dehydrogenase domain preferentially interacts with a separate but proximal cyclohydrolase domain. It is also possible that the two activities share a single domain consisting of one (or two) folate-binding sites. It is significant that in previous studies [6] $NADP^+$ was found to be an inhibitor of the cyclohydrolase reaction; perhaps the presence of $NADP^+$ in the domain prevents it from expressing cyclohydrolase activity. Although the exact nature of the physical interaction is not resolved, a catalytic function appears to be to "channel" the intermediate methenyl derivative through to formylH_4folate. The latter compound is a substrate for formylH_4-folate dehydrogenase, which catalyzes a reaction Krebs and co-workers [15,16] have recently suggested is the overflow pathway for one-carbon units produced in excess of biosynthetic requirements. The properties of the trifunctional protein described here could make it very efficient in producing the substrate for such an outlet pathway.

Acknowledgement

This work was supported by operating grant MT4479 from the Medical Research Council of Canada. We appreciate the cooperation of Olivier Bienvenue Ltd. in supplying fresh pig livers.

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