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METHYLENETETRAHYDROFOLATE DEHYDROGENASE-METHENYLTETRAHYDROFOLATE-CYCLOHYDROLASE-FORMYLTETRAHYDROFOLATE SYNTHETASE. AFFINITY LABELLING OF THE DEHYDROGENASE-CYCLOHYDROLASE ACTIVE SITE

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Methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase are inactivated in parallel by carbodiimide-activated folic acid in an NADP-dependent reaction. Modification with tritium-labelled reagent resulted in the incorporation of 1 mole ^{3}H-folate per mole polypeptide, which demonstrates that these activities share a single folate binding site.

The three folate-dependent enzyme activities, methylenetetrahydrofolate dehydrogenase (E.C. 1.5.1.5), methenyltetrahydrofolate cyclohydrolase (E.C. 3.5.4.9) and formyltetrahydrofolate synthetase (E.C. 6.3.4.3) occur as a trifunctional enzyme in mammalian (1,2,3) and avian (4) liver as well as in yeast (5,6).

The results of a number of investigations of the trifunctional enzymes from various sources are consistent with the presence of a combined dehydrogenase-cyclohydrolase active-site (3,7-11) but no direct evidence for this has been obtained. With the enzyme from pig liver, chemical modification with two different reagents caused essentially coincident loss of these two activities, but the number of residues or sites modified was not determined (9). We report here the results of affinity labelling studies of the porcine trifunctional enzyme which show that the dehydrogenase and cyclohydrolase activities utilize a common folate binding site.

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MATERIALS AND METHODS

The trifunctional enzyme was purified from porcine liver as described by Tan et al. (1) and modified by Smith & MacKenzie (9). Prior to limited proteolysis experiments, enzyme was further purified by gradient elution from a cellulose phosphate column (19 x 1.5 cm) using a 160 ml gradient of 60-200 mM potassium phosphate pH 7.3/20% (v/v) glycerol/35 mM 2-mercaptoethanol. The three enzyme activities were assayed as previously described (1) except that the 2-mercaptoethanol concentration was 140 mM. Protein determinations were by the method of Lowry et al. (12) after sample preparation as described by Bensadoun and Weinstein (13).

Activated folic acid was prepared in a similar manner to (14) by reaction of 5 mM folic acid with 15 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in dimethylsulfoxide for 30 mins at 23°C.

Reaction of the trifunctional enzyme with activated folate in solution was performed at 0°C in 60 mM potassium phosphate pH 7.3 containing 20% (v/v) dimethylsulfoxide and any required ligands by adding aliquots of activated folate at 15 min intervals to a concentration of 20 μM each. The reaction of activated folate with 2',5'AADP Sepharose bound enzyme was performed at 4°C in 60 mM potassium phosphate pH 7.3 containing 20% (v/v) dimethylsulfoxide, 0.1 mM EDTA and 10 μM NADP with 40-80 μM activated folate. After ten minutes of reaction the column was washed with fresh buffer and the cycle repeated the required number of times.

Limited proteolysis of 2',5'-ADP Sepharose-bound enzyme was carried out by introducing 100 μg/ml trypsin (TPCK-treated) (Worthington) in 0.1 M triethanolamine HCl pH 7.3/0.1 mM EDTA into the column and stopping the flow for 3 hours at 4°C. After this time the column was washed with fresh buffer and the 33K molecular weight dehydrogenase-cyclohydrolase fragment formed eluted with 2 mM NADP in 0.2 M potassium phosphate pH 7.3/20% (v/v) dimethylsulfoxide/0.1 mM EDTA.

The incorporation of radioactive reagent was determined by coprecipitating 10-15 μg of labelled enzyme with 100 μg BSA in ice-cold 10% (w/v) trichloroacetic acid, collecting the resulting precipitate on Whatman GF/C glass-fibre filters, followed by solubilization of the collected precipitate with 1 ml of NCS (Amersham) overnight at room temperature and the radioactivity measured by scintillation counting in 10 ml econofluor (NEN) after the addition of 35 μl glacial acetic acid to quench chemiluminescence. The counting efficiency was determined by addition of a [^{3}H]-toluene internal standard (NEN).

RESULTS

The susceptibility of the activities of the trifunctional enzyme to inactivation by carbodiimide-activated folate was tested in the presence and absence of NADP, a substrate of the dehydrogenase reaction which had previously been shown to affect the binding of folate at this active-site (9). In the absence of NADP none of the enzyme activities are particularly sensitive to inactivation. However, when the modification is performed in the presence of NADP, the dehydrogenase and cyclohydrolase activities are considerably more sensitive to inactivation and are lost in a parallel manner (Fig. 1). Moreover,

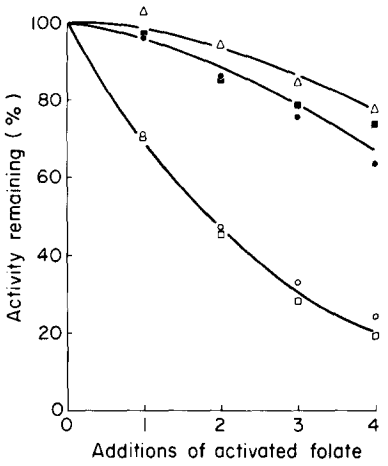

Fig. 1 Inactivation of the trifunctional enzyme with activated folate. The trifunctional enzyme was modified in solution as described in 'Materials and Methods, using 20 μM additions of activated folate. The reaction was performed in the absence of NADP monitoring the cyclohydrolase (■) and dehydrogenase (●) activities and in the presence of 200 μM NADP assaying the cyclohydrolase (□) and dehydrogenase (○). The effect on the synthetase (Δ) activity was the same in the absence and presence of NADP.

the dehydrogenase and cyclohydrolase activities are protected to the same extent both by folate and by pteroyltetraglutamic acid (Table 1). These results, in conjunction with the parallel loss of the activities suggested that reaction of this reagent was occurring at a single folate site.

Affinity labelling of the trifunctional enzyme could lead to modification of not only the dehydrogenase-cyclohydrolase region of the enzyme, but also the

TABLE 1

Protection of the Activities of the Trifunctional Enzyme Against Inactivation

	Percent Activity Remaining		
Addition	Dehydrogenase	Cyclohydrolase	Synthetase
None	24.5	26.1	59.6
Folate	52.3	54.8	67.5
$Pte(glu)_4$	76.3	77.4	91.6

Enzyme was modified in the presence of 100 μM NADP as described in 'Materials and Methods' in the absence of other added ligands and in the presence of 200 μM folate or 200 μM $Pte(glu)_4$. The activities are expressed as percentages of a control to which no reagent was added.

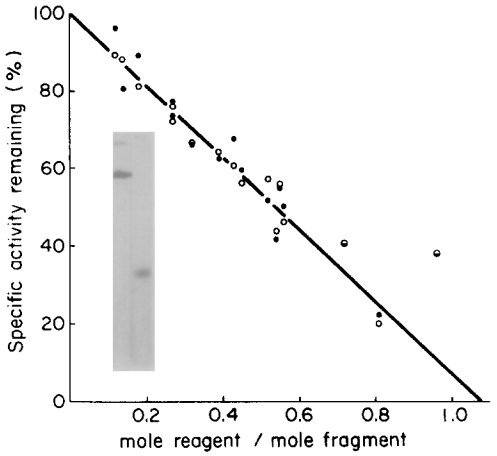

Fig. 2 Incorporation of [^{3}H] reagent into reversibly immobilized enzyme. Trifunctional enzyme reversibly immobilized on 2',5'-ADP Sepharose was modified with activated [^{3}H]-folate, then limited proteolysis with trypsin was performed as in 'Materials and Methods'. A plot of the residual specific activity as a percent of control versus the incorporation of radioactive ligand into the 33K dehydrogenase-cyclohydrolase domain is shown; cyclohydrolase activity (●), dehydrogenase (○) or if the activities coincide (⊖). Inset are dodecyl sulfate polyacrylamide gels of 5 μg loadings of trifunctional enzyme and the 33K dehydrogenase-cyclohydrolase domain formed by limited proteolysis.

synthetase. In order to simplify the analysis of the data we measured the incorporation into the 33K dehydrogenase-cyclohydrolase fragment produced by trypsin proteolysis of the native enzyme.

To measure the incorporation of the affinity label into the dehydrogenase-cyclohydrolase domain, the trifunctional enzyme was modified with [^{3}H]-labelled reagent while reversibly immobilized on the 2',5'-ADP sepharose affinity column followed by limited proteolysis and elution of the fragment. The use of reversibly immobilized enzyme allowed the facile removal of hydrolyzed reagent between cycles of modification and of the trypsin used to form the 33K dehydrogenase-cyclohydrolase fragment from the native protein. The total incorporation into the domain was then found from plots of the residual specific activities after modification versus incorporation of reagent. The data for both the dehydrogenase and the cyclohydrolase activities lie on the same line (Fig. 2) and give an estimate of 1.08 mole reagent incorporated/mole fragment at complete inactivation. Thus a single modification with this reagent inactivates both the dehydrogenase and the cyclohydrolase activities.

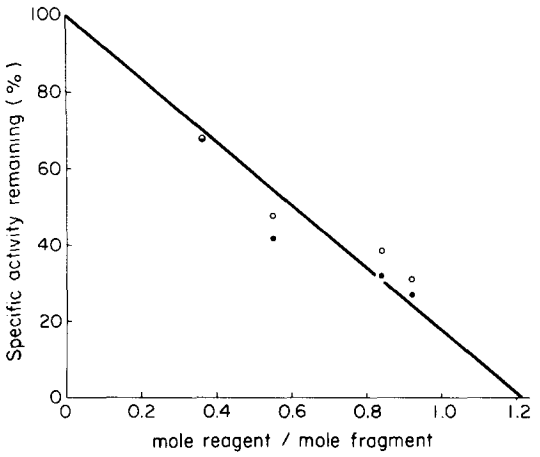

Fig. 3 Incorporation of radioactive reagent into the dehydrogenase-cyclohydrolase domain. Following limited proteolysis of the trifunctional enzyme the dehydrogenase-cyclohydrolase domain produced was modified in solution with activated [^{3}H]folate as in 'Materials and Methods'. The graph shows the percent specific activity remaining versus incorporation of reagent into the proteolytic fragment for the cyclohydrolase (●) and dehydrogenase (○) activities.

A further incorporation experiment was also performed by first forming the dehydrogenase-cyclohydrolase domain by limited proteolysis of 2',5'-ADP sepharose bound trifunctional enzyme, eluting the domain thus formed with NADP and performing the modification in solution. Using this method the dehydrogenase and cyclohydrolase activities were again equally sensitive to inactivation and the total incorporation of reagent was 1.22 mole/mole of domain as shown in Figure 3.

DISCUSSION

A number of lines of evidence from protein chemistry (limited proteolysis and modification with group specific reagents) (7,9,11) and kinetics (substrate channelling and inhibition data) (8,9,10) have suggested that the dehydrogenase and cyclohydrolase activities of the trifunctional enzyme are intimately related and may share a common folate sub-site at the active-site. To obtain more direct evidence for the occurrence of a combined active-site we have used affinity labelling with carbodiimide-activated folic acid.

We have shown that modification of the trifunctional enzyme with this reagent causes coincident loss of the dehydrogenase and cyclohydrolase activities and that the inactivation is dependent on the presence of NADP during the modifi-

cation. The requirement for NADP is in accord with previous results obtained using diethylpyrocarbonate modification of this enzyme which showed a tighter binding of folate to the active-site in the presence of NADP (9).

If the dehydrogenase and cyclohydrolase share a common folate site then incorporation of 1 mole reagent/mole active site should fully inactivate both these activities. In order to test this we measured the incorporation of reagent into the dehydrogenase-cyclohydrolase domain in two ways. Modification of the reversibly immobilized trifunctional enzyme prior to generation of the 33K dehydrogenase-cyclohydrolase fragment gave an incorporation of 1.08 mole/mole of fragment with inactivation of both activities. Very similar results were obtained when the modification was performed in solution after the limited proteolysis, both activities being lost with incorporation of 1.22 mole reagent/mole fragment. These experiments provide the most direct evidence to date that a single folate site is implicated in both the dehydrogenase and cyclohydrolase reactions. This combined site is consistent with the kinetic mechanism and substrate channelling (8), as well as with the observation that these two activities cannot function simultaneously (9), and indicates that on binding of NADP the dehydrogenase reaction is catalyzed, and, upon release of NADPH, the site is then capable of cyclohydrolase activity.

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REFERENCES

1. Tan, L.U.L., Drury, E.J. and MacKenzie, R.E. (1977) J. Biol. Chem. 252, 1117-1122.
2. Paukert, J.L., D'Ari-Straus, L. and Rabinowitz, J.C. (1976) J. Biol. Chem. 251, 5104-5111.
3. Schirch, L. (1978) Arch. Biochem. Biophys. 189, 283-290.
4. Caperelli, C.A., Chettur, G., Lin, L.Y. and Benkovic, S.J. (1978) Biochem. Biophys. Res. Comm. 82, 403-410.
5. Paukert, J.L., Williams, G.R. and Rabinowitz, J.C .(1977) Biochem. Biophys. Res. Comm. 177, 147-154.
6. Suarez de Mata, Z. and Rabinowitz, J.C. (1980) J. Biol. Chem. 253, 2569-2577.
7. Tan, L.U.L. and MacKenzie, R.E. (1977) Biochim. Biophys. Acta 485, 52-59.
8. Cohen, L. and MacKenzie, R.E. (1978) Biochim. Biophys. Acta 522, 311-317.

9. Smith, D.D.S. and MacKenzie, R.E. (1983) Can. J. Biochem. Cell Biol. 61, 1166-1171.
10. Wasserman, G.F., Benkovic, P.A., Young, M. and Benkovic, S.J. (1983) Biochemistry 22, 1005-1013.
11. Schirch, L., Mooz, E.D. and Peterson, D. (1979) in Chemistry and Biology of Pteridines (Kisliuk, R.L. and Brown, G.M., eds), pp.495-500, Elsevier/North Holland Publishing Co., New York.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
13. Henderson, G.B. and Potuznik, S. (1982) Arch. Biochem. Biophys. 216, 27-33.
14. Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241-250.