Recombinant 10-formyltetrahydrofolate dehydrogenase catalyses both dehydrogenase and hydrolase reactions utilizing the synthetic substrate 10-formyl-5,8-dideazafolate

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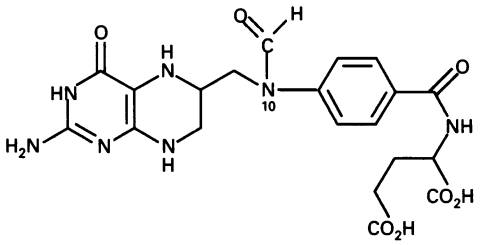
10-Formyltetrahydrofolate dehydrogenase (EC 1.5.1.6) is a bifunctional enzyme, displaying both $NADP^+$-dependent dehydrogenase activity for the formation of tetrahydrofolate and CO_2, and $NADP^+$-independent hydrolase activity for the formation of tetrahydrofolate and formate. A previous report [Case, Kaisaki and Steele (1988) J. Biol. Chem. **263**, 1024–1027] claimed that dehydrogenase and hydrolase activities were products of separate cytosolic and mitochondrial forms of this enzyme. Here we report that recombinant 10-formyltetrahydrofolate dehydrogenase carries out both enzymic reactions, proving that a product of a single gene, i.e. one protein, not two, has both activities. The stable synthetic analogue 10-formyl-5,8-dideazafolate can substitute for the labile natural substrate, 10-formyltetrahydrofolate, in both reactions. This was shown with both native and recombinant rat liver enzyme. The K_m values for 10-formyl-5,8-dideazafolate were half of those for 10-formyltetrahydrofolate in both the dehydrogenase and hydrolytic reactions. The $V_{max.}$ values were similar for both substrates. Both dehydrogenase and hydrolase reactions were dependent on the presence of 2-mercaptoethanol. The pH optima were 7.8 and 5.6 for the dehydrogenase and hydrolase reactions respectively, consistent with the presence of two active sites in the enzyme.

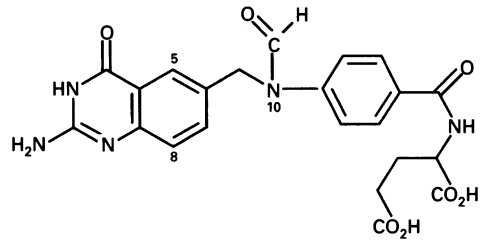
INTRODUCTION

The enzyme 10-formyltetrahydrofolate dehydrogenase (10-FTHFDH) (EC 1.5.1.6) catalyses the NADP-dependent oxidation of 10-formyltetrahydrofolate (10-HCO-H_4PteGlu) to tetrahydrofolate (H_4PteGlu) and CO_2. Although early work [1–3] showed that the enzyme also catalyses an NADP-independent hydrolysis of 10-HCO-H_4PteGlu to H_4PteGlu and formate, it has been claimed [4] that the two activities reside separately on mitochondrial and cytoplasmic proteins. 10-FTHFDH is abundant in liver cytosol, comprising about 1% of the total protein [5]. Its physiological role is probably to recycle any 10-HCO-H_4PteGlu not required for purine synthesis to H_4PteGlu, where it is available for other one-carbon reactions [6]. Rat liver 10-FTHFDH has been cloned [7] and the derived amino acid sequence revealed an N-terminal domain (residues 1–203) that is 24–30% identical with a group of glycinamide ribonucleotide transformylases (EC 2.1.2.2) from different species and a C-terminal domain (residues 417–902) that is about 46% identical with a series of NAD-dependent aldehyde dehydrogenases (EC 1.2.1.3).

The natural substrate for rat 10-FTHFDH is 10-HCO-H_4PteGlu$_5$, but the monoglutamate also serves as a good substrate [1,3]. An inconvenience associated with use of 10-HCO-H_4PteGlu is its susceptibility to oxidative degradation [8]. In order to prevent this, high concentrations of 2-mercaptoethanol are required in reaction mixtures. Figure 1 shows the analogue 10-formyl-5,8-dideazafolate (10-FDDF), which can substitute for 10-HCO-H_4PteGlu as a formyl donor for glycinamide ribonucleotide transformylase [9]. This analogue is stable to oxidation in air. We therefore sought to determine whether 10-FDDF could substitute for 10-HCO-H_4PteGlu in the reactions catalysed by 10-FTHFDH.



10-Formyl-5,6,7,8-tetrahydrofolate (10-HCO-H_4PteGlu)



10-Formyl-5,8-dideazafolate (10-FDDF)

Figure 1 Structures of 10-formyltetrahydrofolate and 10-formyldideazafolic acid

Abbreviations used: 10-FTHFDH, 10-formyltetrahydrofolate dehydrogenase; 10-HCO-H_4PteGlu, 10-formyltetrahydrofolate; H_4PteGlu, tetrahydrofolate; 10-FDDF, 10-formyl-5,8-dideazafolate; DDF, 5,8-dideazafolate; 2-ME, 2-mercaptoethanol.

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MATERIALS AND METHODS

Materials

5,8-Dideazafolate (DDF) and 10-FDDF were obtained from Dr. John B. Hynes, Department of Pharmaceutical Chemistry, Medical University of South Carolina. (6-*RS*)-10-HCO-H_4PteGlu was prepared from (6-*RS*)-5-HCO-H_4PteGlu (Sigma) by the method of Rabinowitz [10]. Purified rat liver 10-FTHFDH was prepared as described by Cook and Wagner [5,11].

Enzyme expression

The cDNA corresponding to the coding region of rat liver 10-FTHFDH was subcloned into pVL 1393 baculovirus vector (Invitrogen, San Diego, CA, U.S.A.) through the *Eco*RI restriction site. A 433 bp fragment between *Xba*I and *Nco*I restriction sites, including the whole 5′ non-coding sequence of cDNA, was removed and replaced with a 271 bp PCR fragment, containing the coding sequence. This construct was expressed in Sf9 insect cells by using the MaxBac expression system (Invitrogen) according to the manufacturer's directions. Analysis of the culture media and cells after infection with recombinant baculovirus showed that about 70 % of the expressed 10-FTHFDH was released into the media when the infected cells were grown in monolayer.

Enzyme purification

Recombinant enzyme was purified from the culture media by affinity chromatography on a column of Sepharose–5-formyltetrahydrofolate [5,11]. A column (1.5 cm × 10 cm) was packed with about 8.0 ml of settled gel and equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, containing 10 mM 2-mercaptoethanol (2-ME) and 1 mM NaN_3 (buffer 1). 2-ME and NaN_3 were added to 200 ml of medium to give concentrations of 10 mM and 1 mM respectively and applied to the affinity column. The column was then washed with buffer 1 (100 ml), followed by the same buffer containing 1 M KCl (100 ml). The enzyme was then eluted from the column with buffer 1 containing 1 M KCl and 20 mM folic acid. The eluate was passed through a column of Bio-Gel P-6DG (Bio-Rad) equilibrated with buffer 1 at pH 6.2 to remove excess folate. The eluate was concentrated to approx. 5 ml. Additional purification was done on a DEAE-cellulose DE-52 column with the use of a linear gradient (0–0.5 M) of NaCl. The enzyme peak was collected, concentrated and desalted to a column of Bio-Gel P-6DG. Glycerol was added to the enzyme solution to a final concentration of 25 % (v/v) and samples were stored at −20 °C. The purified enzyme gave only one band when analysed by SDS/PAGE.

Measurement of enzyme activity

This was performed at 30 °C in a Perkin–Elmer Lambda 4B double-beam spectrophotometer. For measurement of dehydrogenase activity, the reaction mixture contained 0.05 M Tris/HCl, pH 7.8, 100 mM 2-ME and various amounts of $NADP^+$ (0.25–100 μM). The substrate was either 10-HCO-H_4PteGlu or 10-FDDF (0.5–12 μM), and 1 μg of enzyme was added in a final volume of 1.0 ml. For measurement of hydrolase activity, $NADP^+$ was omitted. The reaction was started by the addition of enzyme and read against a blank cuvette containing all components except enzyme. Appearance of product was measured at either 295 nm (DDF) or 300 nm (H_4PteGlu) by using a molar absorption coefficient of 22.2×10^3 for DDF [9] or 21.7×10^3 for H_4PteGlu [4]. Addition of $NADP^+$ provided a measure of both dehydrogenase and hydrolase activity. Hydrolase activity measured in the absence of $NADP^+$ was subtracted from the total activity to give the dehydrogenase activity. Dehydrogenase activity was also measured independently by using the increase in absorbance at 340 nm due to production of NADPH and the molar absorption coefficient of 6.2×10^3.

Analysis of kinetic data

Initial reaction rates were used to determine the respective enzyme activities. Kinetic parameters were derived by using KCat (Biometallics Inc., Princeton, NJ, U.S.A.), which determines kinetic parameters from the Michaelis–Menten equation by using non-linear regression.

H.p.l.c. analysis of reaction products

Reaction mixtures were filtered through Centricon microconcentrators (10 μm pore; Amicon) to separate enzyme from the reaction products. Reaction products were analysed by the modified method of Horne et al. [12] using an Ultrasphere IP C-18 column (4.6 mm × 150 mm, 5-μm-diam. particles; Beckman) and a Spectra-Physics SP8700 solvent delivery system. The elution solvents were (A) 7 mM tetrabutylammonium phosphate and (B) 25 % (v/v) ethanol in solvent A. Elution conditions were a linear gradient of 0–100 % solvent B at 1 ml/min. Samples were diluted with buffer A before injection on to the column. Peaks were detected at 214 nm and collected for spectra analysis.

Preparation of 10-[^{14}C]formyl-DDF

10-FDDF labelled with ^{14}C in the formyl group was prepared by formylation of DDF as described by Smith et al. [9], by using sodium [^{14}C]formate (Amersham International, U.K.). First, 0.5 ml of sodium [^{14}C]formate (100 μCi) was evaporated to dryness and redissolved in 40 μl of 95–97 % formic acid. Then 1 μmol of DDF was added, and the mixture was heated to 90 °C for 90 min in a sealed tube (Pierce, Rockford, IL, U.S.A.). Formic acid that had not reacted was removed by evaporation of the reaction mixture to dryness, followed by redissolving the residue in 50 μl of 6 M acetic acid. This was repeated three times. Finally, the product was dissolved in 0.2 M Tris/HCl buffer, pH 7.8. Spectral properties of the product were identical with authentic 10-FDDF. The specific radioactivity of the product was approx. 1 μCi/μmol.

Measurement of $^{14}CO_2$ production

The production of CO_2 during the dehydrogenase reaction was measured by using the 10-[^{14}C]formyl-DDF. The reaction mixture contained 0.05 M Tris/HCl, pH 7.8, 0.1 mM $NADP^+$, 100 mM 2-ME, 0.05 mM 10-[^{14}C]formyl-DDF (~ 0.1 μCi) and 40 μg of enzyme in a total volume of 2 ml. The reaction was carried out in a 25 ml plastic Erlenmeyer flask sealed with a rubber cap. After incubation at 30 °C for 90 min, each cap was fitted with a glass rod that had a ground-glass surface to hold 10 μl of 10 M NaOH suspended above the reaction mixture. Then 1 ml of 5 M H_2SO_4 was injected through the rubber cap to acidify the solution and liberate CO_2. After 15 min, the caps were removed from the flasks and the NaOH was washed into scintillation vials with 1 ml of water. Then 10 ml of Bio-Safe II scintillation fluid (RPI) was added to the vial and radioactivity was counted.

Table 1 Kinetic parameters of 10-FTHFDH

The results of a typical experiment are shown.

	Dehydrogenase reaction		Hydrolase reaction		$NADP^+$	
Substrate	K_m (μM)	$V_{max.}$ (μmol/min per mg)	K_m (μM)	$V_{max.}$ (μmol/min per mg)	K_m (μM)	$V_{max.}$ μmol/min per mg)
10-HCO-H_4PteGlu	5.5	0.260	11.0	0.090	0.88	0.160
10-FDDF	3.2	0.144	5.8	0.085	0.86	0.123

RESULTS

Dehydrogenase and hydrolase activities of 10-FTHFDH

DDF has an absorbance maximum at 295 nm. The conversion of 10-FDDF into DDF was followed by measurement of the increase in A_{295} in the presence (dehydrogenase plus hydrolase activity) or in the absence of $NADP^+$ (hydrolase activity). The dehydrogenase activity was measured by the increase in A_{340} due to production of NADPH. Both reactions take place with 10-FDDF as substrate. The K_m values of 10-FDDF for the dehydrogenases and hydrolase reactions were 3.2 μM and 5.8 μM respectively. The corresponding $V_{max.}$ values were 0.144 and 0.085 μmol/min per mg respectively for the dehydrogenase and hydrolase activities. The K_m value for $NADP^+$ was 0.86 μM, with a $V_{max.}$ of 0.123 μmol/min per mg. These values are shown in Table 1 for the recombinant enzyme in comparison with corresponding values obtained with the natural substrate 10-HCO-H_4PteGlu. The kinetic parameters for the enzyme isolated from rat liver and for the recombinant enzyme were the same. When 10-FDDF was used as the substrate in the dehydrogenase reaction, both K_m and $V_{max.}$ were about one-half of the values obtained when 10-HCO-H_4PteGlu was used as substrate. The values for $NADP^+$ were unaffected. With regard to the hydrolase activity, when 10-FDDF was used as substrate the K_m value was also one-half of that obtained with 10-HCO-H_4PteGlu, but the $V_{max.}$ was unchanged. Comparison of the results obtained with the recombinant form of the enzyme and the native enzyme show no essential difference between the two forms.

Influence of 2-ME on enzyme activities

Previous studies in our laboratory (R. J. Cook and C. Wagner, unpublished work) indicated that 2-ME had a strong influence on the hydrolase activity of 10-FTHFDH. Figure 2 shows that both reactions are highly dependent on the presence of 2-ME, with the hydrolase activity displaying perhaps a greater sensitivity.

Dependence of enzymic activities on pH

The pH-dependence of both the dehydrogenase and hydrolase reactions was measured. This was carried out in phosphate buffer, which was titrated to different pH values over a large pH range, to avoid complications arising from different buffer salts. Figure 3 shows that the dehydrogenase activity is greater at alkaline pH, whereas the hydrolase activity is greater at acid pH.

Identification of dehydrogenase and hydrolase reaction products

10-FDDF and DDF were easily separated by h.p.l.c. Reaction mixtures for the hydrolase, dehydrogenase and control (no-enzyme) activities were set up and allowed to proceed for 60 min at 30 °C. Samples (100 μl) of the reaction mixtures were diluted with solvent A and analysed by h.p.l.c. as described in the Materials and methods section. In the absence of NADP (hydrolase activity) two peaks were found (Figure 4a), eluting at 43 and 46 min. Spectral analysis of these peaks showed that the peak at 43 min was 10-FDDF, and the other was DDF. A control reaction without enzyme and $NADP^+$ had only one peak, at 43 min, corresponding to 10-FDDF (Figure 4b). When the

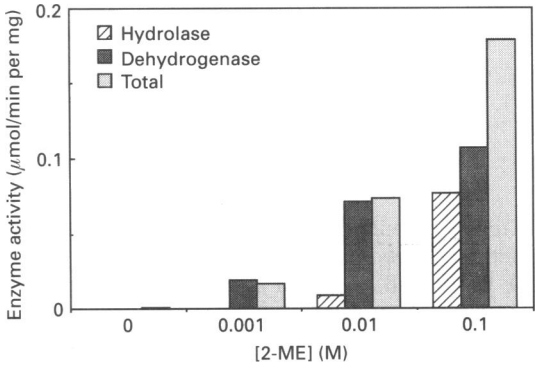

Figure 2 Dependence of 10-FTHFDH activity on the concentration of 2-ME

Enzyme activity was assayed as described in the Materials and methods section, in the presence of various concentrations of 2-ME.

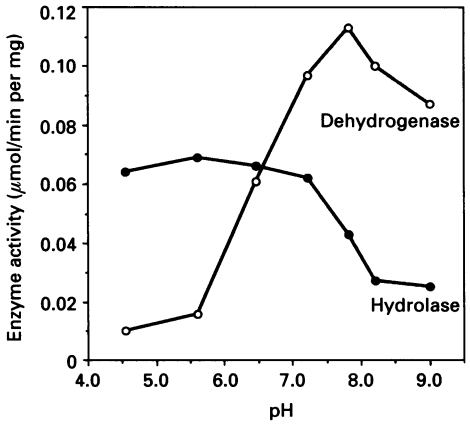

Figure 3 Dependence of 10-FTHFDH activity on pH

Enzyme activity was assayed as described in the Materials and methods section in phosphate buffer at various pH values: ○, dehydrogenase activity; ●, hydrolase activity.

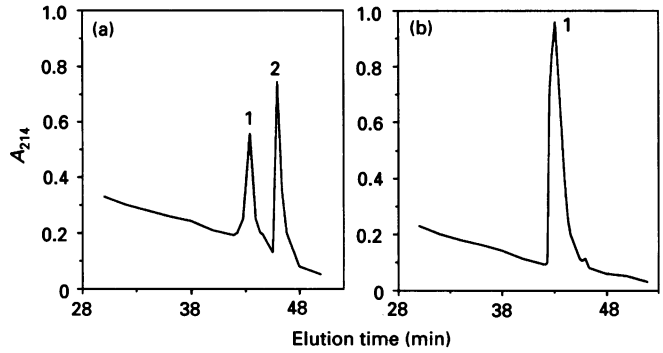

Figure 4 H.p.l.c. analysis of products of the hydrolase reaction

(**a**) Reaction mixture after hydrolase reaction; (**b**) control for hydrolase reaction (no enzyme was added). Peak 1, 10-FDDF; peak 2, DDF. Details of the h.p.l.c. method are given in the Materials and methods section.

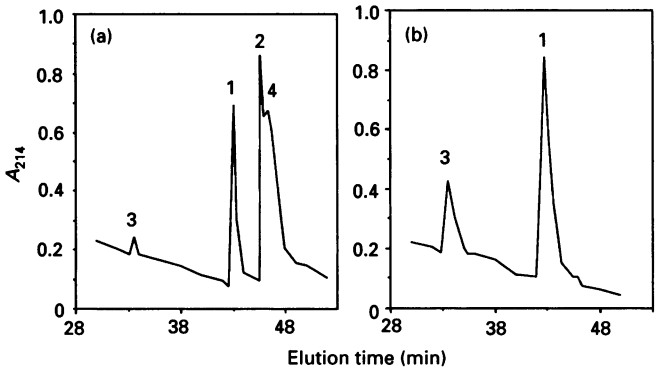

Figure 5 H.p.l.c. analysis of products of the dehydrogenase reaction

(**a**) Reaction mixture after dehydrogenase reaction; (**b**) control for dehydrogenase reaction (no enzyme was added). Peak 1, 10-FDDF; peak 2, DDF; peak 3, $NADP^+$; peak 4, NADPH. Details of the h.p.l.c. method are given in the Materials and methods section.

reaction mixture contained $NADP^+$ (dehydrogenase and hydrolase activities), two additional peaks were seen (Figure 5a). One peak was eluted at 24 min and was identified as $NADP^+$, and the second new peak appeared as a shoulder on the side of the DDF peak at 47 min and was identified as NADPH by spectral analysis. A control reaction without enzyme showed only the peaks at 24 and 43 min corresponding to $NADP^+$ and 10-FDDF (Figure 5b). In the presence of enzyme, the peaks corresponding to 10-FDDF were decreased (compare peak 1 in Figures 4a and 4b, 5a and 5b). The $NADP^+$ peak was also decreased in the presence of enzyme (Figures 5a and 5b).

Measurement of CO_2 production

In order to be sure that the product of the dehydrogenase reaction was CO_2, a microdiffusion experiment was carried out using a scaled-up reaction mixture, with 10-[^{14}C]formyl-DDF synthesized from [^{14}C]formate and DDF as described in the Materials and methods section. The reaction was allowed to proceed for 90 min at 30 °C. $^{14}CO_2$ was liberated and counted for radioactivity as described in the Materials and methods section; approx. 11000 d.p.m., corresponding to 10 nmol of CO_2, was produced during the incubation, which corresponds to about a 10% conversion of the 10-FDDF in the reaction. This was a qualitative indication that $^{14}CO_2$ was produced during the dehydrogenase reaction. A control reaction without enzyme produced no $^{14}CO_2$.

DISCUSSION

These studies show that 10-FDDF can serve as a substrate for both enzyme reactions catalysed by 10-FTHFDH. H.p.l.c. analysis and microdiffusion experiments showed that 10-FDDF was converted into DDF in the absence of $NADP^+$, and was converted into DDF and CO_2 in the presence of $NADP^+$. This analogue was previously shown to be a substrate for glycinamide ribonucleotide transformylase [9], an enzyme which exhibits sequence identity with 10-FTHFDH and which also uses 10-HCO-H_4PteGlu as its natural substrate. With glycinamide ribonucleotide transformylase, 10-FDDF proved to be extremely useful, because it has no asymmetric centre at position 6 of the pterin ring, unlike 10-HCO-H_4PteGlu (Figure 1). Use of the racemic (*RS*) mixture of 10-HCO-H_4PteGlu led to the erroneous conclusion that it was not the substrate of the enzyme. The unnatural (*S*) form of the 10-HCO-H_4PteGlu is a potent inhibitor of glycinamide ribonucleotide transformylase, whereas the natural (*R*) form is a substrate. With 10-FTHFDHF, the use of 10-HCO-H_4PteGlu as substrate has obscured the role of 2-ME in the enzymic reactions that this bifunctional enzyme catalyses. 10-HCO-H_4PteGlu is one of the most labile of the reduced folate coenzymes [8], and is generated and stored in the presence of 2-ME. The usual reaction mixtures contain significant amounts of 2-ME. Preliminary studies (R. J. Cook and C. Wagner, unpublished work) showed that the rate of the hydrolase reaction catalysed by 10-FTHFDH was dependent on 2-ME. The deaza analogue is not reduced, and does not require a reducing agent to protect it from oxidative degradation. The data provided in Figure 2 clearly show the dependence of the hydrolytic reaction on the presence of 2-ME. The dehydrogenase reaction is only slightly less dependent on 2-ME. A possible reason for the 2-ME requirement may be to reduce disulphide bonds that have formed during isolation and purification of the enzyme. It is also possible that 2-ME may be directly involved in the reactions through the formation of an intermediate complex with the enzyme and/or substrate. It is probable that natural reductants, such as glutathione, substitute for 2-ME within the cell.

The dideaza analogue is a good substrate for both the dehydrogenase and hydrolase activities of 10-FTHFDH. In the dehydrogenase reaction the analogue binds more tightly, but has a lower $V_{max.}$, as was observed for glycinamide ribonucleotide transformylase [9]. This may result from a difference in the orientation of the 10-formyl group in the two compounds. In the natural compound the 10-formyl group lies close to the N-5 position, which may permit hydrogen-bonding of the proton of the formyl group. Such hydrogen-bonding is impossible for the dideaza analogue, because there is no nitrogen in position 5. In both the hydrolytic and dehydrogenase reactions the affinity of the enzyme is twice as high for the dideaza analogue as for the natural substrate. Computer analysis of the conformations of 10-HCO-H_4PteGlu and 10-FDDF show that the heterocyclic ring system of the dideaza analogue has a planar structure, whereas in 10-HCO-H_4PteGlu the carbon atom in position 7 protrudes beneath the plane of the ring. Also, when the two compounds are compared in an extended conformation, the overall length of 10-HCO-H_4PteGlu is greater than that of the analogue. It is apparent that such conformational differences have contributed to the differences in affinity of the two substrates.

Because 10-HCO-H_4PteGlu is unstable at lower pH values, the use of the dideaza analogue also provided an opportunity to study the effect of pH on both activities. The existence of different pH optima for the dehydrogenase and hydrolase reactions suggests that they are taking place by separate mechanisms. These may occur at separate sites in this large multi-domain enzyme. This conclusion is consistent with results obtained by us (R. J. Cook and C. Wagner, unpublished work) and by Schirch et al. [13] showing a differential sensitivity of the dehydrogenase and hydrolase activities to proteolytic enzymes.

In addition, these studies are in contrast with those of Case et al., who reported that the dehydrogenase and hydrolase activities of 10-FTHFDH were products of separate cytosolic and mitochondrial forms of this enzyme [4]. Here we clearly show that both activities are associated with the product of a single gene.

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