Methenyltetrahydrofolate Cyclohydrolase Is Rate Limiting for the Enzymatic Conversion of 10-Formyltetrahydrofolate to 5,10-Methylenetetrahydrofolate in Bifunctional Dehydrogenase-Cyclohydrolase Enzymes†

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ABSTRACT: The kinetic properties of three methylenetetrahydrofolate dehydrogenase-cyclohydrolase (D/C) enzymes (the NADP-dependent bifunctional domain of the human cytoplasmic trifunctional enzyme, the human mitochondrial NAD-dependent bifunctional enzyme, and the NAD(P)-dependent bifunctional enzyme from *Photobacterium phosphoreum*) were determined in both forward and reverse directions. In the forward direction, the enzymes possess widely different ratios of k_{cat} C/k_{cat} D, but all channel methenylH_4folate produced by the D activity to the C activity with approximately the same efficiency. A deuterium isotope effect is observed with the human NADP-dependent enzyme in both forward and reverse dehydrogenase assays, consistent with hydride transfer being rate limiting for the interconversion of methenyl- and methyleneH_4folate. However, no kinetic isotope effect is observed for the overall reverse reaction (formylH_4folate to methyleneH_4folate). We devised an assay to measure the reverse cyclohydrolase activity independent of the dehydrogenase, and determined that the k_{cat} (overall reverse) for each enzyme is approximately equal to the k_{cat} for its reverse cyclohydrolase activity. Therefore, the rate-limiting step in the overall reverse reaction is not hydride transfer by the dehydrogenase, but the production of methenylH_4folate catalyzed by the cyclohydrolase. The reverse cyclohydrolase activities of the NADP-dependent D/C and the *P. phosphoreum* enzymes, but not the mitochondrial NAD-dependent enzyme, can be stimulated 2-fold by the addition of 2′,5′-ADP. The results suggest that the cyclohydrolases of the human NADP dependent and *P. phosphoreum* enzymes are optimized to catalyze the reverse reaction in the presence of bound coenzyme. These results imply that essentially all of the methenylH_4folate produced by the cyclohydrolase in the reverse reaction is channeled to the dehydrogenase.

One-carbon units necessary for biosynthetic processes such as purine and thymidine biosynthesis are present in cellular pools of which 5,10-methyleneH_4folate[1] and 10-formylH_4-folate are major constituents (*1*). Interconversion between these forms is catalyzed by two enzyme activities: 5,10-methyleneH_4folate dehydrogenase (D) and 5,10-methenylH_4-folate cyclohydrolase (C) (Scheme 1). In many species, these activities are encoded in a single polypeptide as a bifunctional D/C domain which has been shown to channel substrate from the D to the C activities (*2*). Recently, kinetic and ligand binding studies of the human cytoplasmic D/C domain have shown that the two activities share a single active site (*3*, *4*). As well, D/C domains from a diverse range of species share a significant degree of amino acid sequence identity (*5*), although they differ in nicotinamide adenine dinucleotide cofactor specificity.

The product of the dehydrogenase, 5,10-methenylH_4folate, hydrolyses nonenzymatically to 10-formylH_4folate at a significant rate at neutral pH, bringing into question the in vivo role of the cyclohydrolase activity. Strong and Schirch (*6*) showed that it is possible to produce serine from formate in vitro by using a coupled enzyme system, and recent work in *Saccharomyces cerevisiae* established that one-carbon units from the formylH_4folate pool can be converted to methyleneH_4folate in vivo in yeast that express the wild-type cytoplasmic D/C/S enzyme (*7*). These observations suggest that a major role for the cyclohydrolase may be to catalyze the reverse reaction: the conversion of 10-formylH_4-folate to 5,10-methenylH_4folate.

Recent observations are also consistent with a significant role for metabolic flow in the reverse direction. The equilibrium constant for the overall conversion of methyleneH_4-folate to formylH_4folate was found to be relatively low (*4*), and 10-formylH_4folate binds 20-fold more tightly to D/C301 in the presence of NADP (*3*). In this report we examine the ability of three D/C enzymes to catalyze the reverse reaction in vitro: D/C301, the NADP-dependent bifunctional D/C domain of the human cytoplasmic trifunctional enzyme (*8*, *9*), PPDC, the bifunctional D/C enzyme from *P. phospho-*

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[1] Abbreviations: H_4folate, tetrahydrofolate; D, methylenetetrahydrofolate dehydrogenase; C, methenyltetrahydrofolate cyclohydrolase; S, formyltetrahydrofolate synthetase; NAD(P), nicotinamide adenine dinucleotide (2′-phosphate); 2′,5′-ADP, 2′-phosphoadenosine 5′-phosphate; 5′-AMP, adenosine 5′-monophosphate; MOPS, [3-(*N*-morpholino)propanesulfonic acid]; BSA, bovine serum albumin.

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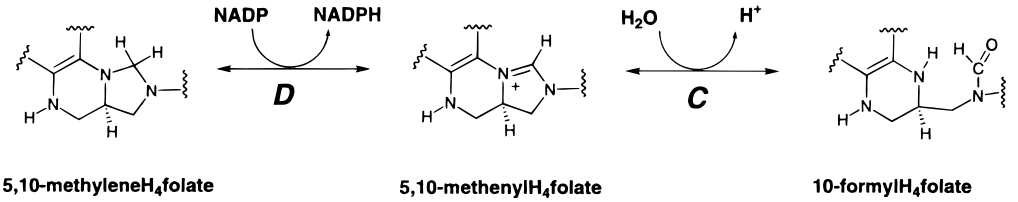
Scheme 1: Reactions Catalyzed by MethyleneH_4folate Dehydrogenase (D) and MethenylH_4folate Cyclohydrolase (C)



reum which can use either NADP or NAD (*5*), and HMDC, the NAD/Mg^{2+}/phosphate-dependent human mitochondrial D/C enzyme (*10*, *11*). In this report, we examine in detail the reverse reactions and channeling of intermediate.

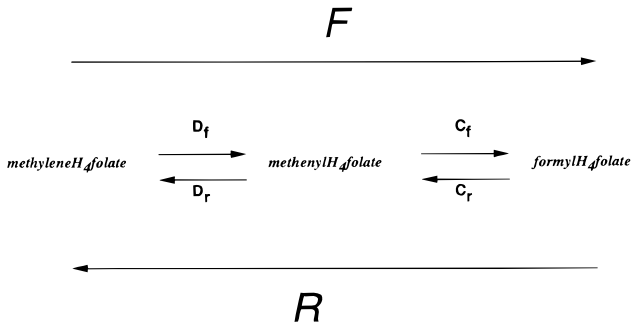
MATERIALS AND METHODS

NADP, NADPH, NADH, and MOPS were purchased from Boehringer Mannheim. NAD and 2′,5′-ADP were obtained from Sigma. (*R*,*S*)-MethenylH_4folate was purchased from B. Schircks Laboratories (Switzerland). (*R*,*S*)-H_4folate was synthesized according to the protocol of Drury et al. (*12*) and stored in sealed glass vials at 4 °C. All other chemicals and reagents were of analytical grade and purchased from BDH. D/C301, HMDC, and PPDC were all expressed in *Escherichia coli* and purified as described previously (*5*, *9*, *13*). Protein concentrations were determined using the Bradford assay (Bio-Rad) with BSA as the standard. Specific activities of the enzymes used in this study were equivalent to those reported previously. proR-NADPD was prepared according to Jeong and Gready (*14*) using [^{2}H]2-propanol (>99% in [^{2}H], Fluka) and a thermostable alcohol dehydrogenase from *Thermoanaerobium brockii* (Sigma). This reaction was allowed to proceed until enzymatic conversion to proR-NADPD was observed to exceed 98%. Enzymatically produced proR-NADPD was then purified on a MonoQ column (Pharmacia) by FPLC using a linear salt gradient. Oxidized and reduced dinucleotide are completely resolved by this system.

Enzyme Assays and Kinetics. All spectrophotometric measurements were performed on a Beckman DU-640 spectrophotometer. Dinucleotide concentrations were determined spectrophotometrically using appropriate molar extinction coefficients (*15*, *16*). MethenylH_4folate concentrations were determined spectrophotometrically ($\epsilon_{350} = 24.9$ mM^{-1} cm^{-1}). Enzymatic reaction rates were determined at 30° C. Generally, substrate concentrations used to determine Michaelis−Menten kinetic constants ranged from 0.5 to 5 times the K_m value for a given substrate. In some instances the range used was limited by the extinction coefficients of the substrates.

The term forward is used to describe reactions assayed in the direction toward formylH_4folate (Scheme 2): the forward dehydrogenase reaction (D_f) is the conversion of methyleneH_4-folate to {methenylH_4folate + formylH_4folate}, and the forward cyclohydrolase reaction (C_f) measures the conversion of methenylH_4folate to formylH_4folate. The term reverse is used to describe the reactions assayed in the direction toward methyleneH_4folate: the reverse dehydrogenase reaction (D_r) is the conversion of methenylH_4folate to methyleneH_4folate, the reverse cyclohydrolase reaction (C_r) is the conversion of formylH_4folate to methenylH_4folate, and the overall reverse reaction (R) measures the conversion of formylH_4-folate to methyleneH_4folate.

Scheme 2: Reactions Involved in the Interconversion of One-Carbon-Substituted Folates[a]



[a] F = overall forward reaction, R = overall reverse reaction, D_f = forward dehydrogenase reaction, C_f = forward cyclohydrolase reaction, D_r = reverse dehydrogenase reaction, C_r = reverse cyclohydrolase reaction.

Forward Dehydrogenase Assay. MethyleneH_4folate dehydrogenase activity in the forward direction was assayed according to Tan et al. (*17*), with the following changes. Assays were performed in a buffer consisting of 25 mM MOPS (pH 7.3), 2 mM potassium phosphate, and 36 mM 2-mercaptoethanol; for assays involving the NAD/Mg^{2+}-dependent mitochondrial enzyme, the assay mix was supplemented with 5mM $MgCl_2$. For a typical {v_o vs [S]} curve, five reaction vessels were prepared, containing increasing concentrations of the varied substrate as well as a fixed concentration of the second substrate. In assays where NAD(P) was the second substrate, its concentration was fixed at a level 10-fold higher than the K_m for the dinucleotide; in assays in which methyleneH_4folate was the second substrate, it's concentration was fixed at 200 μM (once again, greater than 10 times the methyleneH_4folate K_m for each enzyme). Assay mixtures prepared in this manner were preincubated at 30 °C. Diluted enzyme was then added to each mixture, and aliquots were withdrawn at 10 s time points and added to an equivalent volume of 0.36 N HCl. This acidification performs a number of functions: enzyme inactivation, the conversion of all 10-formylH_4folate produced in the reaction to 5,10-methenylH_4folate, and the destruction of NADPH, which absorbs maximally at 340 nm. After a 10 min period of acidification, the absorbance at 350 nm of each sample was measured and the concentration of 5,10-methenylH_4-folate (representing [methenylH_4folate + formylH_4folate]) was calculated. Initial rates were determined from linear plots of [methenylH_4folate] vs time.

Forward Cyclohydrolase Assay. MethenylH_4folate cyclohydrolase activity was assayed according to Tan et al. (*17*) in a buffer consisting of 25 mM MOPS (pH 7.3), 2 mM potassium phosphate, and 36 mM 2-mercaptoethanol. Initial rates were measured continuously by following the disappearance of (*R*,*S*)-5,10-methenylH_4folate substrate at 355 nm and corrected for the nonenzymatic rate of 5,10-

methenylH$_4$folate to 10-formylH$_4$folate conversion under the same conditions. During the continuous spectrophotometric assay, the cuvette holder was constantly heated to 30 °C.

Substrate Channeling. Substrate channeling is defined as the rate of formylH$_4$folate production divided by the rate of [formylH$_4$folate + methenylH$_4$folate] production over a linear time course. Channeling was determined by monitoring the rate of production of 5,10-methenylH$_4$folate from (*R*,*S*)-5,10-methyleneH$_4$folate at 355 nm in a continuous spectrophotometric assay at 30° C; for this assay the extinction coefficient used in the calculation of methenylH$_4$folate production was 29.8 mM^{-1}, which includes the extinction coefficient of NADPH at 355 nm (4.9 mM^{-1}). This rate was then subtracted from the rate of production of [methenylH$_4$folate + formylH$_4$folate] measured in acidified aliquots at 350 nm over the same time course to obtain d[formylH$_4$folate]/dt. Both reactions were performed in the presence of dinucleotide cofactor at 10K_m.

Deuterium Isotope Effect on the Forward Reaction. [*methylene*-^{2}H]-(*R*,*S*)-5,10-MethyleneH$_4$folate was produced by the chemical reaction of [^{2}H]-formaldehyde (>99% in [^{2}H], Cambridge Isotope Laboratories, Inc.) with (*R*,*S*)-H$_4$folate in the D$_f$ reaction buffer at room temperature. Kinetic isotope effects were determined by measuring the initial rate of the forward dehydrogenase reaction in the presence of (*R*,*S*)-5,10-methyleneH$_4$folate and dividing this by the initial rate of the dehydrogenase reaction in the presence of [*methylene*-^{2}H]-(*R*,*S*)-5,10-methyleneH$_4$folate; the dinucleotide cofactor concentration in these assays was fixed at 25K_m. As well, k_{cat} and K_m values for the DC301 D$_f$ reaction in the presence of [*methylene*-^{2}H]-(*R*,*S*)-5,10-methyleneH$_4$folate were determined in order to calculate deuterium isotope effects on k_{cat} and V/K.

Assay of the Overall Reverse Reaction. Assays of the overall reverse reaction were initiated by the addition of enzyme to an assay mix containing 25 mM MOPS (pH 7.3), 36 mM 2-mercaptoethanol, 2 mM potassium phosphate, (*R*,*S*)-10-formylH$_4$folate, and NADPH; in the case of the mitochondrial enzyme, 5 mM $MgCl_2$ and NADH were used. (*R*,*S*)-10-FormylH$_4$folate was prepared by dissolving (*R*,*S*)-5,10-methenylH$_4$folate in a buffer containing 100 mM potassium phosphate (pH 7.3) and 36 mM 2-mercaptoethanol and incubating at room temperature with stirring for 1 h. Initial rates were measured as the rate of disappearance of [methenylH$_4$folate+formylH$_4$folate] upon acidification of aliquots at given time points.

Deuterium Isotope Effects on the Reverse Reaction. Kinetic isotope effects on reverse reactions were determined by dividing the rate of conversion of either (*R*,*S*)-10-formylH$_4$folate or (*R*,*S*)-5,10-methenylH$_4$folate to 5,10-methyleneH$_4$folate in the presence of NADPH by the rate of the same reactions in the presence of an equivalent concentration of NADPD. Dinucleotide concentrations were fixed at 25 times the NADP K_m for D/C301.

Reverse Cyclohydrolase Assay. (*R*,*S*)-10-FormylH$_4$folate was prepared by dissolving (*R*,*S*)-5,10-methenylH$_4$folate in a buffer consisting of 25 mM alanine (pH 8.5) and 36 mM 2-mercaptoethanol. The reaction vessel was flushed with N_2, and the reaction incubated at room temperature for 90−120 min with constant stirring. The mixture was then titrated to pH 8.8 with 10 N KOH. (*R*,*S*)-10-FormylH$_4$folate prepared in this manner was quantitated by measuring OD_{350} of an acidified aliquot of the reaction mixture. The (*R*,*S*)-10-formylH$_4$folate concentration was corrected for the presence of unconverted (*R*,*S*)-5,10-methenylH$_4$folate by measuring the OD_{355} of an unacidified aliquot. At pH 8.8, 98.5% of the initial methenylH$_4$folate was converted to formylH$_4$folate. FormylH$_4$folate was prepared at millimolar concentrations such that small volumes could be added to the assay mixture; this ensured that the pH of the assay mixture was not affected.

The reverse cyclohydrolase assay buffer consisted of 25 mM MOPS (pH 7.3), 2 mM potassium phosphate (and 5 mM $MgCl_2$ in the case of the mitochondrial enzyme), and 36 mM 2-mercaptoethanol. Diluted enzyme was added first to this mixture and the reaction was initiated by the addition of formylH$_4$folate. The production of methenylH$_4$folate was measured at 350 nm in a continuous spectrophotometric assay performed at 30 °C. Reactions were performed in the presence or absence of 0.25 mM and 1 mM 2′,5′-ADP. All enzymatic rates determined in this manner were corrected for the rate of nonenzymatic production of methenylH$_4$folate observed under the same conditions.

Reverse Dehydrogenase Assay. Conversion of methenylH$_4$folate to methyleneH$_4$folate was measured as a decrease in the OD_{350} of aliquots taken from the reaction mixture at 10 s time points and acidified. Reactions progressed at 30 °C in a mixture containing 25 mM MOPS (pH 7.3), 36 mM 2-mercaptoethanol, 250 μM NADPH, and 2 mM potassium phosphate. Initial methenylH$_4$folate concentrations were varied between 50 and 100 μM. This assay is complicated by the concurrent conversion of some methenylH$_4$folate to formylH$_4$folate by the cyclohydrolase; this obviates the ability to determine K_m values for the reverse dehydrogenase activities, and observed rates are likely to be minimal values.

Kinetic Analysis. Initial rates were determined for all enzymatic reactions as the slopes of linear portions of progress curves. These data were fitted to the Michaelis−Menten equation by nonlinear regression using the program Enzfitter (*18*), and standard errors of the fit were less than 25% for K_m and V_{max} values; for the C$_r$ assay, curves with a standard error of the fit of 35% were accepted. Tabulated data represent the averages and standard deviations of experiments performed at least in triplicate. Errors given for ratios of values already possessing standard deviations (i.e., k_{cat}/K_m) were calculated using the formula of propagation of probable errors for the ratio of two independent variables (*19*). V_{max} values for the reverse dehydrogenase reaction were obtained from double reciprocal plots of rates measured at near-saturating concentrations of methenylH$_4$folate.

First-Order Rate Constants. Nonenzymatic conversion of either (*R*,*S*)-5,10-methenylH$_4$folate to (*R*,*S*)-10-formylH$_4$folate or *vice versa* was monitored at 355 nm in continuous spectrophotometric assays performed at 30 °C under standard assay conditions. Data were plotted as log [S] vs time and the first-order rate constants (k_n) were calculated from the slope where slope = $-(k_n/2.3)$.

RESULTS

Catalysis in the Forward Direction. Three bifunctional D/C enzymes were characterized for their ability to catalyze reactions in the forward direction; all enzymes were assayed

Table 1: Kinetic Parameters of the Forward Dehydrogenase Activities for D/C301, PPDC, and HMDC

enzyme	k_{cat} D_f [a] (s^{-1})	K_m methyleneH$_4$folate (μM)	k_{cat} D_f / K_m [b] (s^{-1} μM^{-1})	K_m dinucleotide [c] (μM)	v_H/v_D [d]
D/C301	10 ± 1	9 ± 1	1.1 ± 0.2	22 ± 3	2.8 ± 0.5
PPDC	71 ± 2	10 ± 1	7.5 ± 0.9	38 ± 6	3.6 ± 0.4
HMDC	35 ± 3	12 ± 1	2.8 ± 0.4	507 ± 49	2.3 ± 0.3

[a] D_f = forward dehydrogenase reaction. [b] Calculated using methyleneH$_4$folate K_m values. [c] NADP for D/C301 and PPDC, NAD for HMDC. [d] Deuterium isotope effects determined from reactions in which [NAD(P)] = 10× to 25× K_m.

Table 2: Effect of [*methylene*-^{2}H]MethyleneH$_4$folate on the D_f Reaction of DC301

substrate varied	substrate constant	k_{cat}	K_m	V/K
NADP	[^{1}H]methyleneH$_4$folate	10 ± 1.0	22 ± 3	0.50 ± 0.08
NADP	[^{2}H]methyleneH$_4$folate	3.8 ± 0.4	20 ± 2	0.20 ± 0.03
[^{1}H]methyleneH$_4$folate	NADP	9.4 ± 0.8	11 ± 1	0.85 ± 0.11
[^{2}H]methyleneH$_4$folate	NADP	3.2 ± 0.2	8 ± 1	0.40 ± 0.06

Table 3: Substrate Channeling in the Forward Direction Does Not Correlate with k_{cat} C_f/k_{cat} D_f Ratios

enzyme	k_{cat} C_f [a] (s^{-1})	K_m methenylH$_4$folate (μM)	k_{cat} C_f / K_m (s^{-1} μM^{-1})	k_{cat} C_f/ k_{cat} D_f	% channeling [b]
D/C301	142 ± 8	43 ± 4	3.3 ± 0.4	14 ± 1	51 ± 5
PPDC	152 ± 2	38 ± 2	4.0 ± 0.2	2.2 ± 0.1	58 ± 1
HMDC	145 ± 22	85 ± 19	1.7 ± 0.5	4.2 ± 1.0	57 ± 3

[a] C_f = forward cyclohydrolase reaction. [b] % channeling = [{d(formylH$_4$folate)/dt}/{d(formylH$_4$folate + methenylH$_4$folate)/d*t*}]100.

under the same standard conditions. Reactions involving HMDC were supplemented with 5 mM $MgCl_2$ due to the enzyme's requirement for Mg^{2+}; the presence or absence of $MgCl_2$ has no effect on D/C301 or PPDC. D_f k_{cat} values were observed to vary significantly among the three enzymes (Table 1); in terms of k_{cat}/K_m (folate), PPDC has the most efficient D_f activity. Although significant differences were observed in the D_f k_{cat} values (Table 1), the three enzymes have essentially the same C_f k_{cat} values (Table 3), resulting in widely different k_{cat} C_f/k_{cat} D_f ratios. Notwithstanding these different ratios, the three enzymes channel substrate in the forward direction to approximately the same efficiency (between 50 and 60%) (Table 3).

Experiments using [*methylene*-^{2}H]-(*R*,*S*)-5,10-methyleneH$_4$-folate showed that all three enzymes share dehydrogenase-catalyzed hydride transfer as the rate-limiting step in the forward direction, and that all three enzymes exhibited a kinetic isotope effect of approximately 3. As well, kinetic parameters for DC301 in the presence of [methylene-^{2}H]-(*R*,*S*)-5,10-methyleneH$_4$folate were determined (Table 2). DC301 has approximately the same K_m for the deuterated folate substrate as that for nondeuterated methyleneH$_4$folate, and no significant effect on the K_m for NADP was observed in the presence of the deuterated substrate. The k_{cat} H/k_{cat} D ratios from these data, 2.6 ± 0.4 (NADP as varied substrate) and 2.9 ± 0.3 (methyleneH$_4$folate as varied substrate), compare favorably with the v_H/v_D values quoted in Table 1.

Overall Reverse Reactions. Given that the K_{eq} for the reaction 5,10-methyleneH$_4$folate + NADP ↔ 10-formyl-H$_4$folate + NADPH is 16 at pH 7.3 (*4*), we determined that it was experimentally feasible to study the reverse reactions of the D/C domains in vitro without a requirement for a coupling system to remove methyleneH$_4$folate. Reverse assays were performed for D/C301, PPDC, and HMDC under two experimental conditions. In the first experiment, enzyme concentrations were fixed at levels that yielded identical units of D_f activity (at saturating methyleneH$_4$folate and dinucleotide concentrations) to see whether the three enzymes would catalyze the overall reverse reaction at the same rate. Under these conditions, the following rates for the reverse reaction were observed: DC301, 0.15 nmol/s; PPDC, 0.03 nmol/s; and HMDC, 0.04 nmol/s. In the second experiment, overall reverse reactions were performed at equimolar enzyme concentrations, and the following reverse reaction rates were observed: DC301, 0.15 nmol/s; PPDC, 0.12 nmol/s; HMDC, 0.05 nmol/s. Therefore, at equimolar enzyme concentrations, both DC301 and PPDC catalyze the overall reverse reaction at approximately the same rate, even though their D_f k_{cat} values differ 7-fold.

Kinetic Isotope Effects on the Reverse Reactions. To further investigate whether hydride transfer is rate limiting in the reverse direction, assays of the overall reverse reaction as well as the reverse dehydrogenase (D_r) reaction were performed on D/C301 using deuterated reduced dinucleotide cofactor (NADPD). It has already been established that the dehydrogenase activity of the porcine trifunctional enzyme catalyzes the addition of hydride to the pro-R face of NADP (*20*); therefore, enzymatically prepared proR-NADPD was used in this experiment. The NADPH K_m of D/C301 was determined to be 13 μM, approximately the same as the NADP K_m (data not shown). Kinetic isotope experiments were performed with reduced dinucleotide cofactor levels at 250 μM; considering that this is approximately 25 times the K_m of NADPH, it is extremely unlikely that an increase in K_m for the deuterated cofactor would contribute to the observed isotope effect. When the reverse dehydrogenase assay was performed (using methenylH$_4$folate as the substrate), a kinetic isotope effect was observed in the presence of NADPD [v_H/v_D (methenyl → methylene) = 2.39 ± 0.65], consistent with the isotope effect observed in the forward dehydrogenase assay using [*methylene*-^{2}H]methyleneH$_4$-folate. However, in the overall reverse assay, using NADPD and formylH$_4$folate as substrates, the isotope effect is not

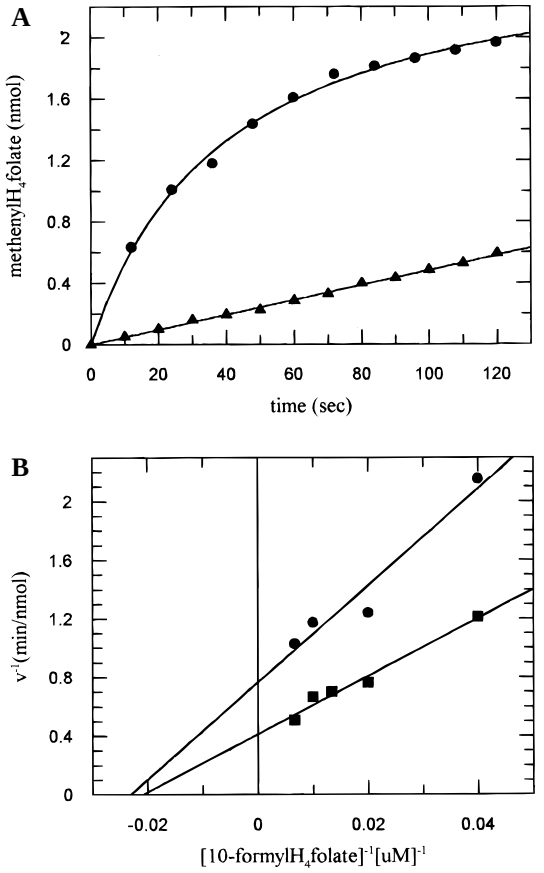


FIGURE 1: Enzymatic catalysis of the production of methenylH$_4$-folate from (*R*,*S*)-10-formyl-H$_4$folate (A) Rates of methenylH$_4$folate production in the presence of excess D/C301 (●), and in the absence of enzyme (▲). (B) Double-reciprocal plot of the reverse cyclohydrolase activity of D/C301; initial rates with varying (*R*,*S*)-10-formylH$_4$folate were measured in the presence (■) or absence (●) of 1 mM 2′,5′-ADP.

observed [v_H/v_D (formyl → methylene) = 1.05 ± 0.06]. Therefore, in the overall reverse direction, it appears that a step prior to dehydrogenase hydride transfer must be rate limiting.

Reverse Cyclohydrolase Activity. Due to the fact that equilibrium between 5,10-methenylH$_4$folate and 10-formylH$_4$-folate lies strongly in the direction of formylH$_4$folate at neutral pH (*21*), it is difficult to experimentally examine reverse cyclohydrolase activity under the standard assay conditions. However, the equilibrium position between methenylH$_4$folate and formylH$_4$folate is highly dependent on pH; we experimentally determined that, at pH 8.8, the proportion of substrate as (*R*,*S*)-10-formylH$_4$folate is 98.5%, and at pH 7.3, the proportion of (*R*,*S*)-10-formylH$_4$folate falls to approximately 90% (data not shown). It is therefore possible to rapidly initiate 10-formylH$_4$folate to 5,10-methenylH$_4$folate conversion at neutral pH by introducing (*R*,*S*)-10-formylH$_4$folate produced at high concentration and high pH to a reaction buffer at pH 7.3. As the reaction approaches equilibrium, approximately 10% of the total folate substrate is converted to 5,10-methenylH$_4$folate. Figure 1A shows both the nonenzymatic and enzymatic conversion of (*R*,*S*)-10-formylH$_4$folate to 5,10-methenyH$_4$folate. Under the experimental conditions used, it would take approximately 3 h for the reaction to reach equilibrium at neutral pH in the absence of enzyme. However, the introduction of excess D/C301 to the reaction allows equilibrium to be achieved after approximately 2 min. By reducing the amount of enzyme used in the reaction, it is possible to measure initial rates and therefore determine kinetic parameters for the reverse cyclohydrolase activity.

Table 4: Effect of 2′,5′-ADP on the Reverse Cyclohydrolase Reaction

	k_{cat} C$_r$[a] (s^{-1})		K_m formylH$_4$folate (μM)	
enzyme	+[b]	−	+	−
D/C301	14 ± 1	5.1 ± 1.3	56 ± 17	51 ± 24
PPDC	16 ± 2	8.0 ± 1.2	35 ± 17	30 ± 12
HMDC	6.2 ± 1.2	5.1 ± 0.8	45 ± 14	45 ± 11

[a] C$_r$ = reverse cyclohydrolase reaction. [b] +/− 1 mM 2′,5′-ADP.

The Effect of 2′,5′-ADP on the Reverse Cyclohydrolase Activity. We were interested in determining if the binding of dinucleotide affects the reverse cyclohydrolase reaction. Since addition of NADPH would prevent us from studying the reverse cyclohydrolase independent of the reverse dehydrogenase, we used 2′,5′-ADP, which is an analogue of the nicotinamide-dinucleotide cofactor yet not a substrate for the dehydrogenase. Figure 1B shows a double reciprocal plot of reverse cyclohydrolase activity of D/C301 as a function of [formylH$_4$folate] in the presence and absence of 1 mM 2′,5′-ADP. It is apparent that 2′,5′-ADP is a strong activator, increasing k_{cat} while not significantly affecting the K_m for 10-formylH$_4$folate. Kinetic parameters for the reverse cyclohydrolase activity were determined for all three enzymes in the presence and absence of 1 mM 2′,5′-ADP (Table 4). Addition of the nucleotide diphosphate to the reaction results in an approximately 2-fold activation for both D/C301 and PPDC. For both enzymes, this activation is due to an increase in the reverse cyclohydrolase k_{cat}, with no significant decrease in the K_m observed for formylH$_4$folate. In contrast, 2′,5′-ADP has no effect on the reverse cyclohydrolase activity of HMDC, which is NAD dependent but has low activity with NADP. Furthermore, addition of 5′-AMP to the assay does not affect the reverse cyclohydrolase activity of HMDC (not shown). In terms of k_{cat} C$_r$/K_m (folate), HMDC clearly has the least efficient reverse cyclohydrolase activity whereas PPDC and D/C301 have k_{cat} C$_r$/K_m (folate) values on the same order of magnitude (Table 5). The activation effect of 2′,5′-ADP on D/C301 and PPDC was also examined at a 2′,5′-ADP concentration of 250 μM and found to be the same as at the higher concentration (not shown). This indicates that the k_{act} for 2′,5′-ADP of these enzymes must be well below 250 μM.

Efficiency of Channeling in the Reverse Direction. A comparison of the reverse kinetic parameters of the three enzymes reveals that the k_{cat} values for overall reverse activity is approximately the same as the k_{cat} values for the reverse cyclohydrolase (Table 5). In order for this to occur, essentially all of the 5,10-methenylH$_4$folate produced by the reverse cyclohydrolase activity must be channeled through the dehydrogenase (in contrast with channeling in the forward direction where only approximately half the intermediate is channeled). This result also shows that the reverse cyclohydrolase activity is rate limiting for the overall reverse reaction. In support of this, k_{cat} values were determined for the D$_r$ reactions of D/C301 and PPDC and found to be approximately 3-fold greater than their respective C$_r$ k_{cat} values (Table 5).

Table 5: Kinetic Parameters for Overall Reverse, Reverse Cyclohydrolase, and Reverse Dehydrogenase Reactions

enzyme	k_{cat} R[a] (s^{-1})	k_{cat} C_r (s^{-1})	k_{cat} D_r (s^{-1})	K_m formylH$_4$folate[b] (μM)	k_{cat} C_r / K_m (s^{-1} μM^{-1})
D/C301	15 ± 2	14 ± 1	36 ± 4	56 ± 17	0.25 ± 0.08
PPDC	17 ± 3	16 ± 2	37 ± 2	35 ± 17	0.45 ± 0.23
HMDC	7.4 ± 0.8	6.2 ± 1.2	n/d	45 ± 14	0.14 ± 0.05

[a] R = overall reverse reaction (k_{cat} determined from a 10-formylH$_4$folate curve in which [NAD(P)H] = 10× to 25× K_m). [b] Determined from reverse cyclohydrolase assays.

Nonenzymatic First-Order Rate Constants. First-order rate constants (k_n) for the nonenzymatic conversion of 5,10-methenylH$_4$folate and 10-formylH$_4$folate were measured under standard assay conditions. A k_n of (7.08 ± 0.07) × 10^{-4} s^{-1} was determined for the conversion of (*R*,*S*)-5,10-methenylH$_4$folate to (*R*,*S*)-10-formylH$_4$folate. The conversion of (*R*,*S*)-10-formylH$_4$folate to (*R*,*S*)-5,10-methenylH$_4$folate had a k_n value of (3.21 ± 0.09) × 10^{-5} s^{-1}.

DISCUSSION

The kinetics of conversion of methyleneH$_4$folate to formylH$_4$folate by D/C enzymes have been well characterized and support the concept of a single or shared site for the two activities (*3*, *4*). However, catalysis of the reverse reactions involved in the production of methyleneH$_4$folate from formylH$_4$folate has not been examined in great detail, due primarily to the difficulty in measuring the conversion of formylH$_4$folate to methenylH$_4$folate since the equilibrium of this reaction at neutral pH lies strongly in the direction of formylH$_4$folate (*21*). Since cellular methenylH$_4$folate concentrations are low, it is highly unlikely that it is a significant substrate for the production of methyleneH$_4$folate.

How, then, do D/C domains catalyze the conversion of formylH$_4$folate to methyleneH$_4$folate? Recent work performed in mutant *S. cerevisiae* strains shows that in strains deficient in cytoplasmic D/C/S activity, one-carbon units from formate do not flow to the products of biosynthetic reactions that require methyleneH$_4$folate as a precursor; furthermore, expression of the cytoplasmic monofunctional NAD-dependent dehydrogenase in these deficient strains does not alleviate this condition (*22*), although it is unclear whether this is due to an absence of the cyclohydrolase or to the change in dinucleotide cofactor specificity. However, it is likely that the cyclohydrolase activity is required to catalyze the overall reverse reaction in vivo. As well, Strong and Schirch (*6*) have shown that it is possible to convert formate to serine in vitro via a coupled system involving a mammalian D/C/S enzyme and serine hydroxymethyltransferase. Although informative, such a system is not ideal to examine the kinetics involved in these enzymatic conversions. To better understand these reverse reactions, we have focused on elucidating the mechanisms of enzymatic conversion of formylH$_4$folate to methyleneH$_4$folate by the bifunctional D/C enzyme without the use of a coupled system.

Rate-Limiting Steps in the Forward and Reverse Reactions. Green et al. (*23*) have shown that the hydride transfer step of the dehydrogenase reaction is rate limiting for the porcine D/C/S enzyme in the forward direction. Here, through experiments with deuterated methyleneH$_4$folate, we confirm that this step is also rate limiting in the dehydrogenase activity of the three D/C enzymes examined. However, the enzymes do not catalyze the overall reverse reaction at rates proportional to their forward dehydrogenase k_{cat} values, which would have been expected if hydride transfer was also rate limiting for the overall reverse reaction. Furthermore, although D/C301 exhibits a kinetic isotope effect in the presence of NADPD in the conversion of methenylH$_4$folate to methyleneH$_4$folate, such an effect is not seen in the conversion of formylH$_4$folate to methyleneH$_4$folate, indicating a different rate-limiting step in the overall reverse direction. There are two major candidates for this rate-limiting step: the cyclohydrolase reaction itself, which involves the dehydration of formylH$_4$folate to produce methenylH$_4$folate, or the efficiency of channeling methenylH$_4$folate from the cyclohydrolase to the dehydrogenase. Because the k_{cat} values of the overall reverse reaction are approximately the same as those of the reverse cyclohydrolase, the cyclohydrolase reaction must be rate limiting. These observations have two important implications. Since forward and reverse reactions have different rate-limiting steps, it is not appropriate to consider the reactions catalyzed by the D/C domain as a single enzymatic mechanism. As well, since the overall reverse reaction has approximately the same turnover number as the reverse cyclohydrolase, essentially all of the methenylH$_4$folate produced by the cyclohydrolase must be channeled to the dehydrogenase.

Forward and Reverse Channeling. The ability of D/C enzymes to channel substrate in the forward direction has already been established (*2*, *9*, *11*). In all cases, between 50 and 60% of the methenylH$_4$folate produced by the dehydrogenase is channeled directly to the cyclohydrolase. However, the result is somewhat surprising since different D/C enzymes vary a great deal in their k_{cat} C_f/k_{cat} D_f ratios. Such a variation suggests that the ability to channel substrate in the forward direction is not simply determined by the kinetic efficiency of the cyclohydrolase relative to the dehydrogenase. It is likely that the uniform channeling efficiencies among these enzymes is due to conserved structural homology at the D/C active site.

In the context of cellular metabolism, the partial channeling observed in the forward direction is probably not of significance since unbound methenylH$_4$folate can hydrolyze nonenzymatically to formylH$_4$folate. If, however, methenylH$_4$folate produced by the reverse cyclohydrolase were to channel poorly to the dehydrogenase, most of the methenylH$_4$folate produced by the cyclohydrolase would nonenzymatically hydrolyze back to formylH$_4$folate, and the production of methyleneH$_4$folate would become very inefficient. By facilitating highly efficient channeling in the reverse direction, the D/C domain circumvents this problem by keeping the methenylH$_4$folate bound to the enzyme throughout the conversion of formylH$_4$folate to methyleneH$_4$folate; the dissociation of the methenylH$_4$folate produced by the cyclohydrolase is much slower than its conversion to methyl-

eneH$_4$folate by NADPH and the D$_r$ activity.

The Role of Dinucleotide in the Reverse Cyclohydrolase Reaction. Previous work on D/C301 has shown that the forward cyclohydrolase activity can be stimulated by about 30% with the addition of 2′,5′-ADP (*3*). We observed a pronounced stimulatory effect of 2′,5′-ADP on the reverse cyclohydrolase k_{cat} (but not on the folate K_m) for both D/C301 and PPDC. This observation and the fact that the 10-formylH$_4$folate K_d is approximately 25-fold lower in the presence of NADP demonstrate that the activities interact significantly and also suggest that a conformational change in the enzyme is induced by the binding of dinucleotide.

The reverse cyclohydrolase activity of the mitochondrial NAD-dependent enzyme, HMDC, is not stimulated by the presence of either 2′,5′-ADP or 5′-AMP. As well, HMDC has a very low k_{cat} C_r/K_m (folate) relative to D/C301 and PPDC. These results are consistent with the proposed metabolic role of HMDC: providing formylH$_4$folate for processes such as mitochondrial protein synthesis. The mitochondrial ratio of NAD to NADH is quite high (*24*), which supports this hypothesized role. In contrast, the high ratios of NADPH to NADP in the mammalian cytoplasm (*25*) suggest that D/C enzymes in these compartments are better poised to catalyze the reverse reactions. As well, these cellular compartments have a number of metabolic requirements for methyleneH$_4$folate which are not shared with the mitochondrion (for a review, see ref *1*).

In summary, we have demonstrated that the bifunctional D/C domain optimizes the conversion of formyl- to methyleneH$_4$folate, where the cyclohydrolase is the rate-limiting step. With NADP-dependent enzymes that are found in cellular compartments where the reverse reaction is physiologically important, the rate-limiting cyclohydrolase activity is enhanced by the binding of the 2′,5′-ADP portion of NADP. The design of the bifunctional site enables essentially complete channeling of intermediate in the reverse direction. The "incomplete" channeling in the forward direction is a consequence of the optimization of the enzyme to maximize the efficiency of the reverse reaction.

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