Binding and Interconversion of Tetrahydrofolates at a Single Site in the Bifunctional Methylenetetrahydrofolate Dehydrogenase/Cyclohydrolase†

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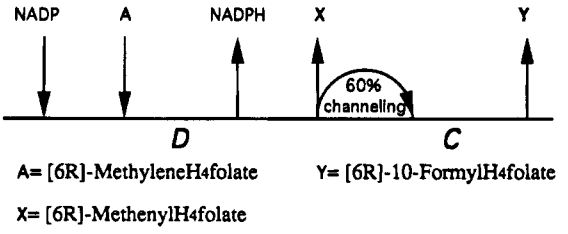
ABSTRACT: The bifunctional dehydrogenase/cyclohydrolase domain of the human NADP-dependent trifunctional methyleneH4folate dehydrogenase/methenylH4folate cyclohydrolase/formylH4folate synthetase (H4folate = tetrahydrofolate) catalyzes two sequential reactions involved in the interconversion of H4-folate derivatives. We have established by equilibrium dialysis that a single H4folate-binding site exists per monomer of the dimeric domain and that the presence of nucleotides has two unexpected effects on H4folate substrate binding. Nucleotides containing a 5′-phosphate cause positive cooperativity in the binding of methyleneH4folate but not of 10-formylH4folate, and NADP increases the affinity for 10-formylH4folate by a factor of 25. The results indicate that dinucleotide preferentially binds before 10-formylH4folate in the reverse cyclohydrolase reaction, and this mechanism increases the efficiency of conversion of 10-formylH4folate to methyleneH4folate. We report new kinetic data that are also consistent with a steady-state random mechanism for this enzyme. To assess whether the enzyme functions at equilibrium *in vivo*, we determined the overall chemical equilibrium constant of $K_{eq} = 16$ for ([10-formylH4folate][NADPH])/([methyleneH4folate][NADP]). Using this value and reported ratios of free dinucleotides and folate derivatives *in vivo*, we estimate that the cytosolic dehydrogenase/cyclohydrolase reactions exist near the equilibrium position. However, the NAD-dependent dehydrogenase/cyclohydrolase reactions in mitochondria are far from equilibrium and are poised toward 10-formylH4folate synthesis. The results of the binding and kinetic studies indicate that the bifunctional nature of the methyleneH4-folate dehydrogenase/methenylH4folate cyclohydrolase domain is designed to optimize the overall reverse reactions *in vivo*.

MethyleneH4folate dehydrogenase (D)[1] and methenylH4-folate cyclohydrolase (C) catalyze two sequential steps in the interconversion of tetrahydrofolate derivatives between different oxidation states of the one-carbon unit (Scheme 1). The activities interconvert the pools of 10-formylH4folate used for purine biosynthesis and methyleneH4folate that is incorporated into thymidylate, methionine, and serine. The two activities exist as monofunctional enzymes in some prokaryotes, although they form a bifunctional enzyme, or a bifunctional domain fused to a 10-formylH4folate synthetase (S) domain, in other organisms (MacKenzie, 1984). A trifunctional D/C/S enzyme exists in the cytoplasm of mammalian cells, and the recombinant human D/C/S enzyme as well as its independently folding D/C domain have been expressed and characterized in this laboratory (Hum & MacKenzie, 1991). The D/C domain is highly homologous to the mammalian mitochondrial bifunctional D/C enzyme (Bélanger & MacKenzie, 1989). A distinguishing feature between these enzymes is their cofactor requirement, where the cytosolic D/C domain requires NADP, while the mitochondrial enzyme requires NAD, Mg^{2+}, and phosphate (Rios-Orlandi & MacKenzie, 1988; Yang & MacKenzie, 1992). The difference in cofactor requirement may reflect the *in vivo* direction of one-carbon flow between methyleneH4folate and 10-formylH4folate, since the redox potentials of the cytosolic NADP/NADPH and the mitochondrial NAD/NADH couples are very different (Sies, 1982). We have determined the overall equilibrium constant between methyleneH4folate and 10-formylH4folate to develop a more quantitative assessment of the effects of cofactor specificity of the D activity on cellular folate pools.

Scheme 1: Sequential Reactions Catalyzed by MethyleneH4folate Dehydrogenase (D) and MethenylH4folate Cyclohydrolase (C)[a]



[a] Order of addition proposed by Cohen and MacKenzie (1978).

The spatial relationship between the sequential D and C activities in D/C domains has long been of interest. In mammalian D/C/S enzymes, the two activities show significant channeling of methenylH4folate. As well, NADP, a substrate of the D activity, inhibits C activity. As a single

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[1] Abbreviations: H4folate, tetrahydrofolate; D, methyleneH4folate dehydrogenase; C, methenylH4folate cyclohydrolase; NAD(P), nicotinamide adenine dinucleotide (2′-phosphate); AADP, 3-aminopyridine adenine dinucleotide phosphate; 2′-AMP, adenosine 2′-monophosphate; 2′,5′-ADP, 2′-phosphoadenosine 5′-phosphate; TCA, trichloroacetic acid; NaOAc, sodium acetate; Tris, tris(hydroxymethyl)aminomethane.

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NADP-binding site exists per D/C domain, the interdependence of the activities on NADP indicates that the active sites are at least in close proximity, if not overlapping (Pelletier & MacKenzie, 1994). These effects, as well as the concurrent chemical inactivation of both activities in porcine (Smith & MacKenzie, 1983), yeast (Appling & Rabinowitz, 1985), and human (J. Pelletier, unpublished results) enzymes, suggested that the two activities may share a common folate substrate-binding site. This hypothesis is consistent with results of affinity labeling of the porcine trifunctional D/C/S enzyme (Smith & MacKenzie, 1985). We investigated the binding of tetrahydrofolate substrates of both the dehydrogenase and the cyclohydrolase to the D/C domain by equilibrium dialysis. The current study makes use of the isolated D/C domain, rather than using the D/C/S enzyme, to eliminate interference from folate binding to the synthetase domain. Our binding and kinetic analyses enable us to redefine the kinetic mechanism of the enzyme and to propose a metabolic advantage for the arrangement of these two activities in a bifunctional domain.

MATERIALS AND METHODS

NADP and NADPH were from Boehringer Mannheim (Laval, QC). All other nucleotides were from Sigma Chemicals (St. Louis, MO), as were glucose-6-phosphate, glucose-6-phosphate dehydrogenase, folic acid, and sodium dithionite. 2-Mercaptoethanol was purchased from Kodak (Rochester, NY), while Tris was from ICN (Mississauga, ON). Dowex 1-X2 anion exchanger was from J. T. Baker Chemicals (Montréal, QC) and immobilized boronic acid from Pierce (Rockford, IL), and matrices used in enzyme purification were from Pharmacia (Montréal, QC). Formaldehyde, potassium phosphate, potassium chloride, magnesium chloride, and TCA were from BDH (Ville St. Laurent, QC). [3′,5′,7,9-^{3}H]Folic acid at 44 Ci/mmol was purchased from Amersham (Oakville, ON). Spectra/Por molecular porous membrane disks from Spectrum (Los Angeles, CA) were used in semimicrodialysis. Glycylglycine was purchased from Aldrich (Milwaukee, WI), while DL-alanine was from General Biochemicals (Chagrin Falls, OH). *Lactobacillus casei* dihydrofolate reductase was a generous gift from Dr. Roy Kisliuk, Tufts University. (6*R*,*S*)-H_4Folate was prepared as described by Drury *et al.* (1975) and stored at 4 °C in sealed ampoules.

Expression and purification of the human D/C/S enzyme and D/C301, the bifunctional dehydrogenase/cyclohydrolase domain of the human D/C/S enzyme, were performed as previously described (Pelletier & MacKenzie, 1994), as were standard enzyme assays unless specified otherwise (Tan & MacKenzie, 1977). In certain instances, the D/C domain was required to be absolutely NADP-free while containing either AADP, 2′-AMP, or 2′,5′-ADP. In such cases, the D/C domain was eluted from the 2′,5′-ADP Sepharose matrix with buffer containing the desired nucleotide. Concentrations of all nucleotides used were quantified spectrophotometrically at neutral pH using the appropriate molar absorptivity coefficients (Windholz, 1976; Anderson & Fisher, 1980).

Enzyme Kinetics. Product inhibition of the dehydrogenase activity of the D/C/S enzyme with NADPH was performed in assay medium containing 25 mM potassium phosphate, pH 7.3 (Pelletier & MacKenzie, 1994). The concentration of NADP was varied from *ca.* 0.5K_d to 10K_d, at constant (*R*,*S*)-methyleneH_4folate = 200 μM, or from *ca.* 1K_d to 10K_d, at constant (*R*,*S*)-methyleneH_4folate = 18 μM, with fixed concentrations of NADPH. Initial rates were measured under conditions where <12% substrate conversion to product had occurred. Assays were carried out in duplicate and repeated using different enzyme preparations. All spectrophotometric measurements were made using a Beckman DU 640 spectrophotometer. The data were fitted to the Michaelis–Menten equation using the nonlinear regression analysis program "Enzfitter" (Leatherbarrow, 1987). Where the data described nonlinear kinetics, the curves were drawn by hand.

Synthesis of (6S)-H_4Folate. Folic acid was chemically reduced to dihydrofolate using sodium dithionite (Blakley, 1960). The product was reduced enzymatically to (6*S*)-H_4folate according to MacKenzie and Baugh (1980) save that the buffer used was as described by Paquin *et al.* (1985). After 2 h at 25 °C, the solution was made nucleotide-free by chromatography on a column containing immobilized boronic acid (Paquin *et al.*, 1985). The pooled fractions were purified by chromatography on DEAE-cellulose in 5 mM Tris·HCl and 0.36 M 2-mercaptoethanol, pH 7.2. The bound sample was washed with the same buffer plus 0.05 M NaCl and eluted with buffer containing 0.25 M NaCl. Yields of 30–35% nucleotide-free (6*S*)-H_4folate were typically obtained. The product was stored at 4 °C in 1 mL sealed glass ampoules.

Synthesis of [^{3}H]-(6S)-H_4Folate. [^{3}H]-(6*S*)-H_4Folate was synthesized by a 2-fold scale-down of the regular (6*S*)-H_4folate synthesis. Folic acid (2.2 μmol) was added to 5.7 nmol of [3′,5′,7,9-^{3}H]folic acid as starting material for the chemical reduction, followed by the enzymatic reduction and subsequent purification over immobilized boronic acid and DEAE-cellulose columns. The majority of the radioactivity eluted from the latter column with the material absorbing at 298 nm. Quantitation of product by methyleneH_4folate dehydrogenase assay using excess D/C domain showed that 0.79 μmol of (6*S*)-H_4folate with a specific activity of 0.137 Ci/mmol was obtained, representing a 36% yield. The product was stored at 4 °C as 200 μL aliquots under an argon blanket in 1 mL sealed glass ampoules.

Synthesis of (6R)-10-FormylH_4folate and (6S)-5-Formyl-H_4folate. (6*R*)-10-FormylH_4folate was synthesized by the method of Ho and Jones (1967), using purified D/C/S as the source of 10-formylH_4folate synthetase activity. (6*S*)-5-FormylH_4folate was synthesized enzymatically using the C activity of the human D/C domain and purified by FPLC using the conditions of Moran *et al.* (1976) (Pelletier and Mackenzie, manuscript in preparation).

Determination of the Equilibrium Constant for MethyleneH_4folate + NADP ↔ 10-FormylH_4folate + NADPH. Both dinucleotides were combined, in varying ratios and in concentrations varying between 7 and 98 μM, with one of the H_4folate reactants over a range of H_4folate concentrations (Table 1). Initial dinucleotide concentrations were determined by spectrophotometry. Initial (6*S*)-H_4folate concentrations were determined by formiminoglutamate:H_4folate transferase assays, as previously described (Drury *et al.*, 1975). Initial 10-formylH_4folate concentrations were determined by acidification of duplicate samples in TCA (final concentration was 3.5%) followed by immersion in boiling water for 55 s, chilling, and microcentrifugation for 5 min. Under these conditions, 10-formylH_4folate was entirely converted to methenylH_4folate; its concentration was deter-

mined spectrophotometrically ($\epsilon_{350} = 24.9\ mM^{-1}\ cm^{-1}$). D/C domain was added to the reactants to establish rapid equilibrium, at 30 °C. When methyleneH$_4$folate was the starting H$_4$folate, (6*S*)-H$_4$folate was used and the equilibration was initiated by addition of 2.5 mM formaldehyde. When 10-formylH$_4$folate was the starting folate, it was prepared as described above and added last. Equilibration was achieved after 30 min, as demonstrated by controls using 2-fold more enzyme and controls allowing extra time (data not shown). The final concentration of 10-formylH$_4$folate was determined as described above. Under these conditions of acidification, NADPH is rapidly decomposed and therefore does not contribute to absorbance at 350 nm. Final concentrations of methyleneH$_4$folate, NADP, and NADPH were calculated by addition or subtraction of the micromolar change in 10-formylH$_4$folate concentrations to (or from) the initial concentrations. Experiments were designed so as to avoid final concentrations of any reactant that were lower than 2 μM in order to reduce error in computation of K_{eq}. Parallel acidification of duplicate samples in 0.5 M NaOAc, pH 3.3, gave identical results, confirming that under these conditions there was no significant formation of 5-formylH$_4$-folate.

Equilibrium Dialysis. H$_4$Folate binding was characterized using a Spectrum equilibrium semimicrodialyzer as described previously (Pelletier & MacKenzie, 1994) except that the buffer contained 0.1% ascorbate. Samples were dialyzed for a minimum of 3 h, as control dialyses with various forms of H$_4$folate showed that equilibrium was achieved under these conditions. H$_4$Folate ligands were used at concentrations *ca.* 5 times higher and lower than K_d. D/C domain was used at a concentration that was generally within a 2-fold range of K_d. The ligands [^{3}H]-(6*S*)-H$_4$folate and [^{3}H]-(6*R*)-methyleneH$_4$folate were used at a specific activity of 0.3–0.7 Ci/mmol at concentrations down to 2 μM and 2 Ci/mmol below 2 μM. Alternatively, nonradioactive (6*R*)-methyleneH$_4$-folate was used and quantified by acidification after reaction with excess D/C domain and NADP. When nucleotide analogues were present, we verified that they did not interfere with quantitation by inhibition of the dehydrogenase activity. When methyleneH$_4$folate was used, formaldehyde was present at 2.3 mM at all times. Potential nonspecific binding was monitored by the addition of 1 mM (*R*,*S*)-methyleneH$_4$-folate to duplicate samples.

When the ligand used was 10-formylH$_4$folate, it was quantified by dilution and acidification with 3.5% TCA (as described above) as well as with 0.5 M NaOAc, pH 3.3 (final concentration), and absorbance at 350 nm was determined. The difference between the concentration determined in TCA and in NaOAc provides the concentration of 5-formylH$_4$-folate that is present. When a combination of methyleneH$_4$-folate and 10-formylH$_4$folate, in a 1:3 ratio, was used, the former was quantified by radioactivity and the latter by spectrophotometry after acidification, since methyleneH$_4$-folate does not contribute to absorbance at 350 nm. When the ligand was 5-formylH$_4$folate, it was quantified by acidification with 3.5% TCA and heating, as described above. Equilibrium dialysis of D/C domain with [^{3}H]NADP was also performed under the conditions described above. The ligand, [^{3}H]NADP, was synthesized and utilized at the specific activity described earlier (Pelletier & MacKenzie, 1994), and dialysis was performed for 7 h. D/C domain was used at a concentration of 4.2 μM. When (*R*,*S*)-10-formylH$_4$-folate was examined for its effect on NADP binding, it was added to both sides of the dialysis cells at a final concentration of 100 μM.

Table 1: Equilibrium Constant Determination for methyleneH$_4$folate + NADP ↔ 10-formylH$_4$folate + NADPH[a]

[H$_4$folate]$_i$[b]	μM	[NADPH]$_i$/[NADP]$_i$	K_{eq}[c]
methyleneH$_4$folate	23	0.51	19
		4.6	17
		56	17
	17	2.0	16
		4.9	16
	12	2.6	13
		6.3	13
		35	12
	9.1	2.0	16
		4.9	16
10-formylH$_4$folate	68	1.0	13
	50	1.0	17
	23	2.7	22
average			16 ± 2.8

[a] Equilibrium was established at 30 °C in 25 mM potassium phosphate, pH 7.3, 35 mM β-mercaptoethanol, and 2.5 mM formaldehyde. The concentration of D/C domain was always 0.01[H$_4$folate]$_i$. [b] *i* indicates initial conditions. [c] K_{eq} = ([10-formylH$_4$folate][NADPH])/([methyleneH$_4$folate][NADP]).

The data were fit to the equation: $[L]_{total} = [L]_{free} \times \{(n[enzyme]/K_d + [L]_{free}) + 1\}$ by nonlinear regression analysis using the program "Enzfitter" (Leatherbarrow, 1987).

RESULTS

Equilibrium Constant. We determined the equilibrium constant (K_{eq}) for ([10-formylH$_4$folate][NADPH])/([methyleneH$_4$folate][NADP]), at 30 °C and pH 7.3, starting with a mixture of the oxidized and reduced forms of NADP plus either one of the H$_4$folate substrates. D/C domain was included to obtain rapid equilibration, at molar concentrations of 0.01[H$_4$folate], which is not sufficiently high for a significant proportion of ligands to be enzyme bound and therefore did not affect the determination of K_{eq}. Under these conditions, there was no significant synthesis of 5-formylH$_4$-folate, as shown by acidification in OAc^- and in TCA. Table 1 shows examples of initial H$_4$folate reactant concentrations and K_{eq} obtained. There is good agreement among all conditions, and K_{eq} of ([10-formylH$_4$folate][NADPH])/([methyleneH$_4$folate][NADP]) was determined to be 16 ± 2.8.

Binding Studies. Previous affinity labeling studies of porcine D/C/S enzyme with carbodiimide-activated folic acid showed that 1 mol was incorporated per monomer of enzyme (Smith & MacKenzie, 1985). We undertook to further test the existence of a shared folate-binding site in the bifunctional domain by the method of equilibrium dialysis, using the natural H$_4$folate substrates. In order to relate these results directly to previous results concerning NADP binding to D/C/S (Pelletier & MacKenzie, 1994), we performed the dialyses under identical conditions except that 0.1% ascorbate was added and the 2-mercaptoethanol concentration was raised from 35 to 100 mM. Equilibrium binding of NADP was repeated under these modified conditions with D/C domain, with no detectable effect on K_d or n.

(6R)-MethyleneH$_4$folate Binding. Equilibrium binding of (6*R*)-methyleneH$_4$folate was undertaken to determine K_d and

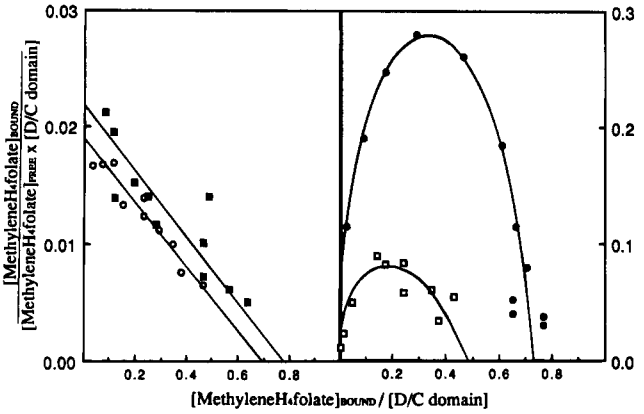


FIGURE 1: MethyleneH$_4$folate binding to the D/C domain. Conditions for dialysis and methods for curve fitting are described in Materials and Methods. The data required no correction for nonspecific binding and are presented as Scatchard plots. Binding was performed in the presence of very low NADP (■) and saturating 2′-AMP (○), AADP (●), or 2′,5′-ADP [□; [methyleneH$_4$folate]$_{BOUND}$/([methyleneH$_4$folate]$_{FREE}$[D/C domain]) is expanded 10 times]. The values determined for n and K_d are listed in Table 2.

Table 2: Effects of Nucleotides on H$_4$Folate Binding

	K_d (μM)		
nucleotide	methylene-H$_4$folate	10-formyl-H$_4$folate	comments[a]
none	39 ± 8[b]	~190[c]	
2′-AMP	36 ± 3		no effect on C activity
2′,5′-ADP	cooperative, n_H = 1.7 K'_d = 300 ± 360		activates C activity
NADP		~7[c]	inhibits C activity
AADP	cooperative, n_H = 1.9 K'_d = 1.6 ± 0.1	7.1 ± 0.9	inhibits C activity

[a] Reference: Pelletier and MacKenzie (1994). [b] Very low NADP concentration present (≤0.3[D/C] domain]). [c] Values extrapolated from inset to Figure 2.

n, under various conditions. It was not possible to entirely remove the nucleotide used in affinity purification of the D/C domain, even by dialysis, and further attempts to remove all the nucleotide resulted in reduced specific activity. Since the concentration of D/C domain used in binding studies was chosen to approximate K_d, the amount of nucleotide included in the enzyme solution itself was significant. Therefore it was not possible to determine K_d for any H$_4$folate substrate in the complete absence of nucleotide. Binding studies were performed with little nucleotide present, in the presence of the natural cofactor NADPH, in the presence of only the adenosine moiety as 2′-AMP or 2′,5′-ADP or with the analogue 3-aminopyridine adenine dinucleotide phosphate (AADP) which is a powerful inhibitor of the D and C activities. Binding was quantified using either [^{3}H]methyleneH$_4$folate or nonradioactive methyleneH$_4$folate. The binding isotherms depicted in Figure 1 show that a single H$_4$folate-binding site exists per monomer of D/C domain. The K_d values determined are listed in Table 2. When [^{3}H]-methyleneH$_4$folate binding was measured in the presence of a very low NADP concentration (estimated to be ≤0.3[D/C domain]) or with saturating 2′-AMP (1 mM), the Scatchard plot was linear (Figure 1, left). However, in the presence of 2′,5′-ADP or AADP, the Scatchard plot was curved (Figure 1, right), indicating the occurence of positive binding cooperativity between the two monomers of D/C domain. The data describing cooperative curves were fit to the Hill

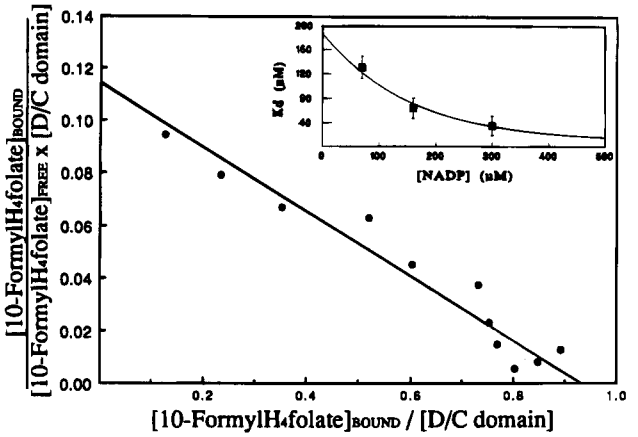


FIGURE 2: 10-FormylH$_4$folate binding to the D/C domain in the presence of saturating AADP. The data required no correction for nonspecific binding and are presented as Scatchard plots. Inset is a replot of K_d(10-formylH$_4$folate) vs the NADP concentration at which binding was performed. The curve was extrapolated to zero, and infinitely high NADP and the limiting K_d values obtained are listed in Table 2.

equation (Enzfitter) and gave n_H = 1.9 in the presence of AADP and 1.7 in the presence of 2′,5′-ADP. In the case of cooperative binding, K_d' is determined (Segel, 1975), which is K_d at 50% fractional saturation of the enzyme (Table 2). The K_d' for methyleneH$_4$folate determined in the presence of saturating AADP was *ca.* 25-fold lower than in the absence of nucleotides or the presence of 2′-AMP. Attempts at determining binding of methyleneH$_4$folate in the presence of 50−200 μM NADPH using both radioactive and unlabeled folate were unsuccessful. This suggests that methyleneH$_4$-folate does not bind readily in the presence of NADPH under the conditions tested. In no case was there evidence of nonspecific binding to the enzyme.

(6R)-10-FormylH$_4$folate Binding. We performed equilibrium dialysis of (6*R*)-10-formylH$_4$folate with various concentrations of NADP present and saturating AADP (30 μM), illustrated as a Scatchard plot in Figure 2. Approximately 15% 5-formylH$_4$folate was formed during the equilibrium dialysis, as determined by postdialysis acidification in TCA and NaOAc (described in Materials and Methods). The 5-formylH$_4$folate was not included in the calculations. Once again, the results confirmed the existence of a single H$_4$-folate-binding site per monomer of D/C domain. The binding of 10-formylH$_4$folate was not cooperative in the presence of NADP or AADP, unlike the binding of methyleneH$_4$folate; the K_d values determined are listed in Table 2. The inset curve in Figure 2 clearly illustrates that the affinity of 10-formylH$_4$folate increases as the NADP concentration is increased. Extrapolation of the curve to 0 NADP gives K_d(10-formylH$_4$folate) of *ca.* 190 μM, while extrapolation to saturating NADP concentrations gives K_d of 7 μM, equal to K_d in the presence of AADP. The K_d value with saturating NADP is currently the best estimate of K_m(10-formylH$_4$folate), as the experimental difficulty in determining this K_m for cyclohydrolase has prevented its accurate measurement. This value is difficult to determine because the equilibrium between methenylH$_4$folate and 10-formylH$_4$folate strongly favors 10-formylH$_4$folate at neutral pH. The strong dependence of K_d(10-formylH$_4$folate) on the concentration of dinucleotide was unexpected, since the proposed ordered substrate addition and release of products depicted in Scheme 1 does not predict this effect.

Although binding was measured at only 20 °C and for a relatively short period of time, 3 h, we determined that a significant concentration of 5-formylH$_4$folate was formed. We established that the D/C domain actually produces 5-formylH$_4$folate with a very low specific activity as a result of the cyclohydrolase activity (Pelletier and MacKenzie, manuscript in preparation). Therefore, unless the synthesis was inhibited by NADP or AADP, the rate of formation of 5-formylH$_4$folate under the conditions used for dialysis exceeded the rate of 10-formylH$_4$folate equilibration. However, as inhibition of synthesis by NADP and AADP is only partial, *ca.* 10−20% 5-formylH$_4$folate was formed during equilibrium binding. It has been suggested by Stover and Schirch (1992) that 5-formylH$_4$folate inhibits both the D and C activities of rabbit liver D/C/S enzyme and may therefore bind to the human D/C domain. This binding would have to be accounted for while calculating binding of 10-formylH$_4$folate. We synthesized (6*S*)-5-formylH$_4$folate enzymatically from 10-formylH$_4$folate, to use in equilibrium dialysis. The results of experiments using up to 190 μM 5-formylH$_4$folate with 30 μM D/C domain and either 50 μM NADP or 30 μM AADP failed to show specific binding to D/C domain. This was further confirmed by lack of inhibition of the dehydrogenase activity with up to 70 μM (6*S*)-5-formylH$_4$folate, using 6, 15, and 200 μM (*R*,*S*)-methyleneH$_4$folate. Therefore, 5-formylH$_4$folate did not compete in 10-formylH$_4$folate-binding determinations, and it was not necessary to account for its contribution to binding, except as it affected the concentrations of 10-formylH$_4$folate.

Binding of MethyleneH$_4$folate and 10-FormylH$_4$folate, in Combination. To establish whether methyleneH$_4$folate and 10-formylH$_4$folate bind at a single site or two independent sites, binding of [^{3}H]-(6*R*)-methyleneH$_4$folate and (6*R*)-10-formylH$_4$folate, combined in a 1:3 ratio, was performed in the presence of saturating AADP. The results were calculated both for the combined H$_4$folates and for each substrate separately. The resulting Scatchard representation (Figure 3) gave $n = 0.6$ for combined H$_4$folates with 0.2 contributed by methyleneH$_4$folate and 0.4 contributed by 10-formylH$_4$folate. The data for each H$_4$folate displayed the expected characteristics with respect to cooperativity and K_d values and, when combined, have the appearance of weakly cooperative binding ($n_H = 1.3$ and $K_d = 5$ μM).

H$_4$Folate Binding. Quantitation of [^{3}H]-(6*S*)-H$_4$folate binding was attempted with 15 μM D/C domain and up to 100 μM (6*S*)-H$_4$folate, in the presence of NADP at concentrations up to 200 μM. There was no evidence of specific binding to D/C domain under these conditions.

Effect of H$_4$Folate on Dinucleotide Binding. We undertook the reciprocal of the experiment illustrating the dependence of 10-formylH$_4$folate binding on the presence of nucleotide, which was to study the effect of 10-formylH$_4$folate on NADP binding to D/C domain. The results, shown in Figure 4, illustrate that 100 μM (6*R*,*S*)-10-formylH$_4$folate has no significant effect on binding of [^{3}H]NADP to the D/C domain.

Product Inhibition of the Dehydrogenase. Cohen and MacKenzie (1978) had previously determined that NADPH (at 200 μM) was a noncompetitive inhibitor against both NADP and methyleneH$_4$folate using partially purified porcine D/C/S enzyme, indicating that NADPH was not the last product released. However, the range of substrate concentrations they used was narrow [(0.5−2.5)K_m of the porcine enzyme]. We repeated the inhibition against NADP using purified human D/C/S and a broader range of substrate concentrations [(0.5−10)K_d of the human enzyme]. The results are illustrated as a double-reciprocal plot in Figure 5. Panel A shows that, with saturating methyleneH$_4$folate (65K_m), NADPH acts as a competitive inhibitor at high NADP concentrations (above 2K_d) but appears to behave as a noncompetitive inhibitor at lower NADP concentrations, giving rise to nonlinear kinetics. Panel B shows that, with methyleneH$_4$folate = 6K_m, NADP acts predominantly as a competitive inhibitor. A replot of the slopes obtained in panel B vs [NADPH] indicates that K_i(NADPH) is *ca.* 6 μM.

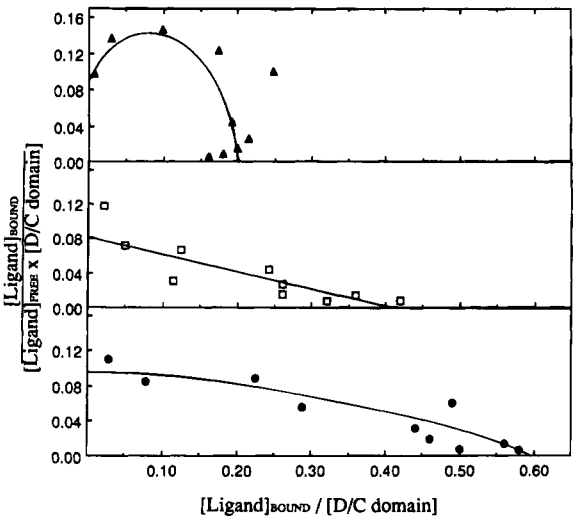


FIGURE 3: [^{3}H]MethyleneH$_4$folate and 10-formylH$_4$folate binding to the D/C domain in the presence of saturating AADP. The H$_4$folates were combined for binding and the data treated for each substrate separately and for both combined. The data required no correction for nonspecific binding and are presented as Scatchard plots of [^{3}H]methyleneH$_4$folate binding in the presence of 10-formylH$_4$folate (▲), 10-formylH$_4$folate binding in the presence of [^{3}H]methyleneH$_4$folate (□), and both H$_4$folate substrates combined (●). The values determined for n, K_d, and n_H are described under Results.

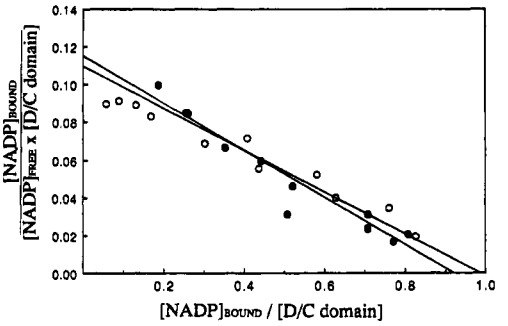


FIGURE 4: NADP binding to the D/C domain. The data required no correction for nonspecific binding and are presented as Scatchard plots. Binding was performed in the presence (○) or absence (●) of 100 μM (*R*,*S*)-10-formylH$_4$folate.

DISCUSSION

"Shared" or Single Catalytic Site. Recognition that the D and C activities occupy a relatively small domain and that NADP, a substrate of the D activity, inhibits the C activity prompted the notion of a shared folate-binding site (Tan & MacKenzie, 1977; Schirch, 1978; Cohen & MacKenzie, 1978). Chemical modification of the porcine, yeast, and human enzymes led to near coincident losses of both

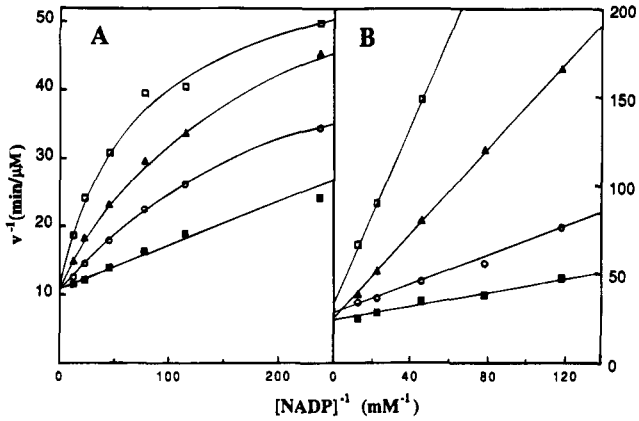
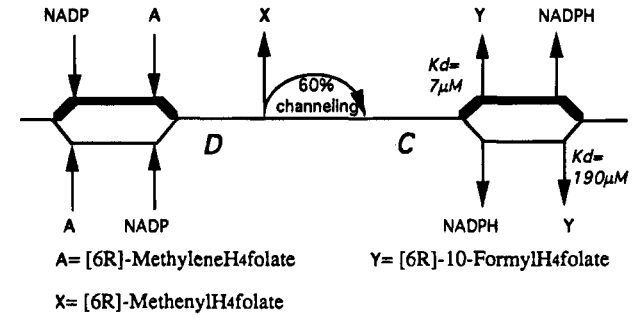


FIGURE 5: Double-reciprocal plot of product inhibition of the dehydrogenase by NADPH. Initial rates with varying NADP concentrations were measured in the absence (■) or presence of 30 μM (○), 82 μM (▲), or 180 μM (□) NADPH, with 200 μM (*R,S*)-methyleneH$_4$folate in panel A or 18 μM (*R,S*)-methyleneH$_4$folate in panel B.

activities (Smith & MacKenzie, 1983; Appling & Rabinowitz, 1985; Pelletier & MacKenzie, 1994). Modification with carbodiimide-activated folic acid also inactivated both activities with the incorporation of a single mole of reagent per subunit (Smith & MacKenzie, 1985). Understanding that the inhibition of C by NADP binding at a single site is partial indicated that it is only the nicotinamide ring that inhibits the C activity (Pelletier & MacKenzie, 1994). Our current results of equilibrium binding of natural isomers of both methylene- and formylH$_4$folate substrates, alone or in combination, indicate that the D/C domain has a single H$_4$folate-binding site and support the notion of a single catalytic site. Systems that channel reactants between active sites are considered dynamic or static systems depending on the associations between participating enzymes (Ovádi, 1991). The D/C domain obviously carries out channeling in a static system as both activities belong to a single polypeptide. It can be considered an extreme example of a static system, as the bound folate reacts at a single site shared by the two activities. Because the enzyme exhibits only 50−60% channeling and because exogenously added methenylH$_4$folate can bind to the enzyme in the presence or absence of dinucleotide, it appears that the site exhibits the characteristics of two separate, consecutive reactions as opposed to a single two-step catalytic mechanism. This is consistent with the existence of monofunctional dehydrogenases (Moore *et al.*, 1974; Ragsdale & Ljungdahl, 1984; Uyeda & Rabinowitz, 1967; West *et al.*, 1993).

Binding of H$_4$Folates. Chemical modification with activated folic acid indicated that this molecule can bind to the enzyme, and we were therefore surprised to find from equilibrium binding experiments that (6*S*)-H$_4$folate and (6*S*)-5-formylH$_4$folate do not. This observation, taken with the fact that the D/C domain has little affinity for polyglutamates (Ross *et al.*, 1984), illustrates that the nature of the one-carbon unit provides stringent H$_4$folate-binding specificity to the enzyme. This raises some concern as to whether the oxidized form, folic acid, binds to the enzyme in the same fashion as the reduced substrates. We determined that a single H$_4$folate-binding site exists per monomer by performing binding studies in the presence of either methyleneH$_4$folate or 10-formylH$_4$folate, or a combination of the two substrates. Saturating the adenosine nucleotide subsite with

Scheme 2: Proposed Order of Substrate Addition and Release[a]



[a] The order is proposed to be steady-state random, with a preferred pathway marked by boldface lines.

2′-AMP did not affect the binding of (6*R*)-methyleneH$_4$folate, but the addition of the 5′-phosphate to the nucleotide, as with 2′,5′-ADP, caused strongly positive cooperative binding to the dimer. The Hill coefficient we determined is close to 2, the value representing perfect cooperativity between two equivalent binding sites. Binding in the presence of AADP shows similar cooperative effects but with *ca.* 25-fold tighter binding (~38/1.8), demonstrating that while the pyridine ring increases affinity it is not required for the cooperativity. The importance of the pyridine ring in binding of H$_4$folates is also underscored by the 25-fold increase in 10-formylH$_4$folate affinity (190/7) resulting from the presence of either saturating NADP or AADP.

We recently demonstrated an effect of the 5′-phosphate of NADP in activating the cyclohydrolase (see Comments column in Table 2) and proposed that this is the result of a conformational change. We now further propose that the putative conformational change elicited by binding of the 5′-phosphate also contributes to the binding cooperativity. The cooperativity is seen only with methyleneH$_4$folate and not with 10-formylH$_4$folate indicating a requirement for a 5,10-bridged structure. Under physiological conditions, it is possible that this property would favor 10-formylH$_4$folate over methyleneH$_4$folate binding, thus favoring the reverse reaction. However, as we have not observed cooperative kinetics with respect to varying methyleneH$_4$folate concentrations, it is difficult to speculate about the physiological significance of binding cooperativity.

Kinetic Mechanism of the Bifunctional Site. 10-FormylH$_4$folate binds very weakly to the D/C domain. However, in the presence of NADP, its affinity is increased to a physiologically relevant range. The binding constants illustrate that for the reverse cyclohydrolase reaction to occur, it would be highly advantageous, although not absolutely obligatory, for NADPH to bind prior to 10-formylH$_4$folate. This contrasts with the kinetic scheme proposed earlier for the porcine enzyme (Scheme 1). We propose that the kinetic mechanism of the D/C is actually a steady-state random bi-bi mechanism (Spector & Cleland, 1981; Segel, 1975), illustrated in Scheme 2. NADP binding is not affected by the presence of 10-formylH$_4$folate although the inverse is true. The results are consistent with a mechanism that employs a preferred order of addition of substrates. The nonlinear product inhibition pattern of NADPH against NADP is consistent with this scheme. NADPH is competitive against very high concentrations of NADP, where NADP binds first (preferred pathway, Scheme 2). However, at lower concentrations of NADP, the inhibition by NADPH

appears noncompetitive because under these conditions methyleneH$_4$folate can also be the first substrate bound (alternate pathway, Scheme 2). This is further illustrated by the fact that at a lower methyleneH$_4$folate concentration, NADPH acts predominantly as a competitive inhibitor (Figure 5B), since the preferred binding pathway prevails at all NADP concentrations used. Due to the narrow range of substrate concentrations used in a previous kinetic analysis of the porcine enzyme (Cohen & MacKenzie, 1978), only the noncompetitive pattern was observed and the random component of binding had not been detected.

In our proposed mechanism in the reverse direction, NADPH binds before the cyclohydrolase reaction occurs although it is not a substrate for this activity. It not only increases the affinity for the 10-formylH$_4$folate but is also already in place to reduce the labile methenyl intermediate when the substrate is cyclized within the active site. The *in situ* generation of methenylH$_4$folate, the substrate for the reverse dehydrogenase reaction, is obligatory since the concentration of free methenylH$_4$folate in cells is vanishingly small due to its equilibrium with 10-formylH$_4$folate which strongly favors the latter (Kay, 1960). This raises an interesting question as to whether the channeling of methenylH$_4$folate might be more efficient in the reverse direction than the 50−60% seen in the forward direction. A high efficiency would prevent cycling between 10-formylH$_4$folate and methenylH$_4$folate. It is nevertheless clear that linking these two reactions in a single site with the properties described here increases the efficiency of the overall reverse reaction.

Metabolic Role(s) of the D/C. The dehydrogenase/cyclohydrolase activities interconvert two major pools of one-carbon donors, formylH$_4$folate and methyleneH$_4$folate. Serine is the main source of one-carbon units incorporated into methyleneH$_4$folate, which the forward reactions convert to 10-formylH$_4$folate for purine biosynthesis (Schirch, 1984). The reverse reaction has been demonstrated to proceed in coupled reactions *in vitro* (Strong & Schirch, 1989) and *in vivo* in yeast (McKenzie & Jones, 1977; Pasternack *et al.*, 1992, 1994), demonstrating the incorporation of formate into the methyleneH$_4$folate pool. The reverse reaction is clearly metabolically important. We wanted a more quantitative estimation of the direction in which this pair of reactions is poised in cells and determined that the overall equilibrium constant of the forward reactions is 16. This determination has the advantage of being independent of quantification of methenylH$_4$folate, which is rapidly converted to 10-formylH$_4$folate at neutral pH. Earlier estimations of the K_{eq} for the separate dehydrogenase gave values of 0.14 (Uyeda & Rabinowitz, 1967), 0.017 (Huennekens & Osborne, 1959), and 7.6 (Yeh & Greenberg, 1965). Better agreement was obtained for the ratio of [10-formyl]/[methenyl] for the cyclohydrolase: 24.4, 26.6 and 11.6 calculated from the data of Kay *et al.* (1960), Tabor and Wyngarden (1959), and Lombrozo and Greenberg (1967), at pH 7.3. Combining the value of 0.14 for the dehydrogenase with the values for the cyclohydrolase gives an overall K_{eq} ranging from 1.6 to 3.4. The value reported by Yeh and Greenberg (1965) for the dehydrogenase (K_{eq} = 7.6) is actually an estimate of the overall reaction and is similar to what we determined.

Much attention has been given to the importance of compartmentalization of folate metabolism [for a review, see Appling (1991)]. What would be the ratio of methyleneH$_4$folate and formylH$_4$folate in the cytoplasm if they were at equilibrium *in vivo*? Assuming that the ratio of free [NADPH]/[NADP] = 42 [calculated using a redox potential = −393 mV and a standard equilibrium midpoint potential of the couple = −343 mV, from Sies (1982)], then the calculated ratio of free [formyl]/[methylene] = 0.4. The actual total cytoplasmic concentrations of 10-formylH$_4$folate and methyleneH$_4$folate + H$_4$folate in rat liver are approximately equal (Horne *et al.*, 1989). This is not unreasonable agreement considering that intracellular folates are largely bound (Strong & Schirch, 1989; Matherly *et al.*, 1990), a condition which cannot be treated in our calculation. Therefore, it appears that the pools of methylene- and 10-formylH$_4$folate are maintained near equilibrium, and changes in cellular requirements for either reactant can efficiently draw from the pool of the other reactant. This would allow for efficient use of both serine and formate as donors to the one-carbon pool.

Using similar assumptions, the ratio of free [formyl]/[methylene] at equilibrium in mitochondria would be predicted to be *ca.* 0.25 for a hypothetical NADP-linked D/C. However, the bifunctional D/C enzyme in mammalian mitochondria is NAD, phosphate, and Mg^{+2} dependent, and this would be expected to shift the equilibrium in favor of the formyl product (Yang & MacKenzie, 1993). Using a ratio of free [NAD]/[NADH] in mitochondria = 0.2 [calculated using a redox potential = −318 mV and a standard equilibrium midpoint potential = −337 mV, from Sies (1982)], the ratio of [formyl]/[methylene] at equilibrium is calculated to be 64. However, the concentration of methyleneH$_4$folate + H$_4$folate is reported to be 5-fold higher than that of 10-formylH$_4$folate in the mitochondria of liver cells (Horne *et al.*, 1989), and therefore this reaction is normally not at equilibrium but is strongly poised in the forward direction. The expression of the mitochondrial D/C enzyme is tightly regulated (Peri & MacKenzie, 1993), and the activity in adult tissues is extremely low at best, preventing attainment of equilibrium. However, when its level is elevated as in transformed cells and fetal tissues, it would allow flow of one-carbon units to 10-formylH$_4$folate presumably to supply the formyl group for the synthesis of formylmethionyl-tRNA^{fmet} to allow protein synthesis to occur in mitochondria.

In conclusion, we have shown that the dehydrogenase and cyclohydrolase activities of the D/C domain share a single H$_4$folate substrate-binding site. The effects of nucleotides on methyleneH$_4$folate and 10-formylH$_4$folate binding, the new kinetic mechanism we propose, and the *in vivo* equilibrium position of the substrates are all consistent with the hypothesis that the bifunctional nature of the cytosolic D/C domain is used to optimize the efficiency of the reverse reactions.

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