Binding to the 2′,5′-ADP Subsite Stimulates Cyclohydrolase Activity of Human NADP$^+$-Dependent Methylenetetrahydrofolate Dehydrogenase/Cyclohydrolase†

Joelle N. Pelletier and Robert E. MacKenzie*

Department of Biochemistry, McGill University, Montréal, Québec, Canada H3G 1Y6

Received November 10, 1993®

ABSTRACT: The bifunctional dehydrogenase/cyclohydrolase domain of the human trifunctional methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase catalyzes two sequential reactions with significant channeling of the intermediate, methenyltetrahydrofolate. Equilibrium dialysis established that a single, high-affinity NADP$^+$ binding site exists per monomer of the dimeric enzyme. Kinetic characterization of NADP$^+$ binding to the dehydrogenase using analogs as inhibitors demonstrated that affinity for this substrate is due almost exclusively to binding at the 2′,5′-ADP subsite. The same structural specificities for binding are exhibited by these analogs in their effects on the cyclohydrolase. Both NADP$^+$ and its 3-aminopyridine analog AADP partially inhibit the activity of the cyclohydrolase when assayed with added methenyltetrahydrofolate as substrate. However, under the same conditions, the cyclohydrolase is actually activated by 2′,5′-ADP; activation requires the presence of the 5′-phosphate since 2′-AMP binds but does not activate. Nicotinamide ribose monophosphate (NMN) has no detectable effect either alone or in combination with 2′,5′-ADP. The results are consistent with the existence of a shared dehydrogenase/cyclohydrolase active site proximal to the 2′,5′-ADP subsite. NADP$^+$ reduces the rate of the fully activated cyclohydrolase by 2-fold. Inhibition appears to be due to the loosely bound nicotinamide ring interacting with the common folate subsite, resulting in only partial inhibition by NADP$^+$. The interaction of 2′,5′-ADP with the cyclohydrolase suggests a potential role for this portion of the molecule in promoting the efficiency of the channeling of endogenously generated methenyltetrahydrofolate.

The enzymes 5,10-methyleneH_4folate[1] dehydrogenase, 5,10-methenylH_4folate cyclohydrolase, and 10-formylH_4folate synthetase are involved in the interconversion of tetrahydrofolate derivatives between the different oxidation states of the one-carbon unit required for the biosynthesis of methionine, thymidylate, and purines. These activities exist as monofunctional enzymes in some prokaryotes, although the dehydrogenase and cyclohydrolase form a bifunctional protein in others (MacKenzie, 1984). In higher eukaryotes the dehydrogenase and cyclohydrolase activities (Scheme 1) are found in one domain, which is fused to a larger synthetase domain, forming a trifunctional enzyme. Most methyleneH_4folate dehydrogenases are NADP$^+$-dependent, although monofunctional dehydrogenases in yeast and some prokaryotes are NAD$^+$-dependent (Moore *et al.*, 1974; Ragsdale & Ljungdahl, 1984; Barlowe & Appling, 1990). In transformed and embryonic mammalian cells, there exists a bifunctional dehydrogenase/cyclohydrolase enzyme that is dependent on both NAD$^+$ and Mg^{++} for activity (Mejia & MacKenzie, 1985; Rios-Orlandi & MacKenzie, 1988; Yang & MacKenzie, 1992). This enzyme is found in mitochondria, while its NADP$^+$-dependent trifunctional counterpart is localized in the cytosol (Mejia & MacKenzie, 1988).

Separate expression of the dehydrogenase/cyclohydrolase domain of the cloned human trifunctional enzyme (Hum & MacKenzie, 1991) produces a protein that folds independently and retains the same kinetic properties. This demonstrates that the dehydrogenase/cyclohydrolase domain is functionally independent and suggests that its association with the synthetase may be the result of linkage through gene fusion. However, simple gene fusion does not effectively describe the method of association of the dehydrogenase and cyclohydrolase activities. The domain in which they reside has approximately the same subunit size as that of all monofunctional dehydrogenases characterized to date (Moore *et al.*, 1974; Ragsdale & Ljungdahl, 1984; Barlowe & Appling, 1990; Wohlfarth *et al.*, 1991). Substrate addition has been proposed to occur in an ordered, bi–bi sequence initiated by NADP$^+$ binding (Cohen & MacKenzie, 1978). Channeling of the product of the dehydrogenase to the cyclohydrolase active site occurs in the trifunctional enzymes from different sources (Cohen & MacKenzie, 1978; Wasserman *et al.*, 1983; Hum & MacKenzie, 1991) as well as in a bifunctional enzyme (Rios-Orlandi & MacKenzie, 1988). NADP$^+$, substrate of the dehydrogenase activity, inhibits the cyclohydrolase activity of the porcine enzyme (Smith & MacKenzie, 1983). This interdependence of the activities indicates that a functional interaction occurs between the two activities that share a small domain.

Our interest in the relationship between the dehydrogenase and cyclohydrolase activities of the human enzyme stems from evidence suggesting that these sites are overlapping or may share a single active site. Both activities in the porcine (Smith

† This work was supported by MRC Canada Grant MT4479. J.N.P. is a recipient of studentships from the Joint Fonds pour la Formation de Chercheurs et l'Aide à la Recherche/National Science and Engineering Research Council of Canada and more recently from MRC Canada.

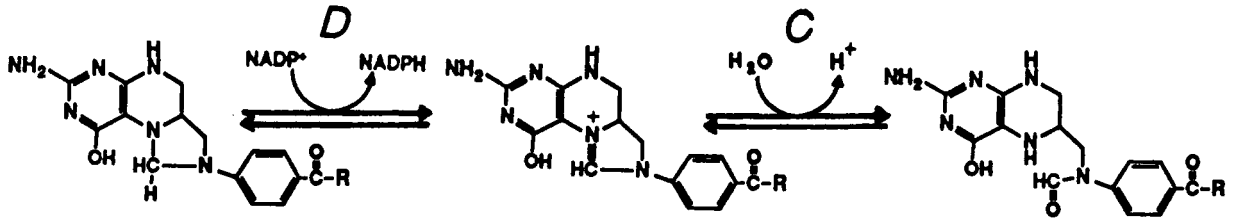
* Address correspondence to this author: telephone (514)398-7270; fax (514)398-7384.

® Abstract published in *Advance ACS Abstracts,* February 1, 1994.

[1] Abbreviations: D/C/S, methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase; H_4folate, tetrahydrofolate; NAD(P)$^+$, nicotinamide adenine dinucleotide (2′-phosphate); AADP, 3-aminopyridine adenine dinucleotide phosphate; NMN, β-nicotinamide ribose monophosphate; 2′,5′-ADP, 2′-phosphoadenosine 5′-phosphate; 2′-ATP-ribose, 2′-monophosphoadenosine 5′-diphosphoribose; 2′-GMP, guanosine 2′-monophosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEP, phosphoenolpyruvate.

0006-2960/94/0433-1900$04.50/0

Scheme 1: Sequential Reactions Catalyzed by 5,10-MethyleneH$_4$folate Dehydrogenase (D) and 5,10-MethenylH$_4$folate Cyclohydrolase (C) Activities[a]



[a] R represents the polyglutamate tail.

& MacKenzie, 1985), yeast (Appling & Rabinowitz, 1985), and human (this laboratory, unpublished results) enzymes are simultaneously inactivated upon chemical modification, suggesting that their catalytic sites share residues in common. Affinity labeling of the porcine enzyme has shown that the two activities share a common folate binding site (Smith & MacKenzie, 1985). There are few examples described in enzymes where two activities share a domain, existing either in close proximity (Palosaari *et al.*, 1991) or sharing one active site (Knight *et al.*, 1990; Kühn-Velten *et al.*, 1991; Minami *et al.*, 1992). Little is known about the mechanism of evolution of such domains, although their existence has generated much interest because of the substantial kinetic advantage that such a close functional association of activities can offer. Using kinetic analysis, we characterize the remarkable interdependence that exists between the two activities with special attention given to novel nucleotide effects on the cyclohydrolase activity.

MATERIALS AND METHODS

Pyruvate kinase, Hepes, $NADP^+$, and NADPH were purchased from Boehringer Mannheim (Laval, Quebec); all other nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO), as was malate dehydrogenase. Nicotinamide adenine (2,8-^3H)-dinucleotide at 23 Ci/mmol was purchased from ICN (Mississauga, Ontario). NAD kinase and PEP were from Calbiochem (San Diego, CA). 2-Mercaptoethanol was from BDH (Ville St-Laurent, Quebec), Kodak (Rochester, NY), and ICN. All gel matrices used in enzyme purification were from Pharmacia (Montréal, Quebec). Dowex 1-X2 was from J. T. Baker Chemical Co. (Montréal, Quebec). Spectra/Por molecularporous membrane discs from Spectrum (Los Angeles, CA), molecular mass cutoff 12–14 kDa, were used in semimicrodialysis. Ammonium sulfate and ammonium formate were Fisher Certified A.C.S. (Nepean, Ontario). Formaldehyde, potassium phosphate, potassium chloride, magnesium chloride, and Folin and Ciocalteu reagent were from BDH. (6*R*,*S*)-H$_4$folate was prepared as described by Drury *et al.* (1975) and stored at 4 °C in sealed glass ampules. (6*R*,*S*)-5,10-methenylH$_4$folate was synthesized as described previously (Rios-Orlandi *et al.*, 1986). The dry powder was stored at –20 °C. For use, a stock solution was made daily in 36 mM HCl, on ice, and filtered.

Enzyme Expression. Human trifunctional enzyme and the bifunctional dehydrogenase/cyclohydrolase domain D/C301 were expressed using a modified pBluescript-SK$^+$ expression vector/*Escherichia coli* K38 cell system (Hum & MacKenzie, 1991) and later using *E. coli* BL21-DE3 cells. In the latter case, expression was obtained by growing the cells in a New Brunswick Scientific BIOFLO IIc fermentor at 37 °C in Terrific Broth plus 100 μg/mL ampicillin to an OD_{600} = 1.0–1.5 before inducing expression by the addition of 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG). Cells were grown for a further 2.5 h until OD_{600} = 6.0 and harvested by centrifugation for 30 min at 4500*g*. Snap-frozen cell pellets were stored at –80 °C; typically, 10 g of cells was obtained/L of culture.

Enzyme Purification. Purification of the human trifunctional enzyme and of the dehydrogenase/cyclohydrolase fragment D/C301 on 2',5'-ADP Sepharose was performed at 4 °C essentially as described by Hum and MacKenzie (1991). For kinetic experiments, the enzyme was made $NADP^+$-free by desalting on a 1 × 28 cm Sephadex G-25 column in 50 mM potassium phosphate, pH 7.3 (all buffers used in purification contain 20% glycerol, 40 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mM benzamidine unless otherwise specified). The trifunctional enzyme was concentrated using Amicon Centricon-30 or Centriprep-30 concentrators, and the D/C301 fragment was concentrated using Centriprep-10 concentrators, as specified by the manufacturer. In some cases where highly concentrated $NADP^+$-free enzyme was required, it was eluted from the 2',5'-ADP Sepharose column with 7 mM $NADP^+$ in 20 mM potassium phosphate, pH 7.3. The enzyme was applied directly to a 1.5 × 2 cm column of heparin-Sepharose, washed with 5 column vols of 20 mM potassium phosphate and 20 mM KCl and eluted in 20 mM potassium phosphate and 500 mM KCl. Protein concentrations were determined by the method of Bradford (1976) using BioRad dye reagent concentrate or by the modified Lowry technique (Markwell *et al.*, 1981). The enzyme was stored in 50 mM potassium phosphate, 40% glycerol buffer at –20 °C and, if $NADP^+$-free, was used for a maximum of 2 weeks. For equilibrium dialysis, concentrated trifunctional enzyme (>1 mg/mL) was stored in 50 mM potassium phosphate, 30% glycerol buffer at –80 °C, where it is stable for over 1 month in the presence or absence of $NADP^+$. The porcine liver trifunctional enzyme was purified according to the previously established protocol (Smith & MacKenzie, 1983).

Enzyme Assays. Standard dehydrogenase and cyclohydrolase assays used during enzyme purification were performed as previously described (Tan & MacKenzie, 1977). However, in order to be able to directly compare results obtained for the two enzymatic activities, a single assay medium was selected for all kinetic experiments. This medium contained 25 mM potassium phosphate, pH 7.3, and was chosen because it does not alter the kinetic properties observed under standard assay conditions and does not cause excessive nonenzymatic hydrolysis of 5,10-methenylH$_4$folate during cyclohydrolase assays. Unless specified otherwise, for kinetic experiments, standard fixed substrate concentrations were 200 μM (6*R*,*S*)-methyleneH$_4$folate and 200 μM $NADP^+$ for the dehydrogenase assay and 90 μM (6*R*,*S*)-methenylH$_4$folate for the cyclohydrolase assay.

Concentrations of all nucleotides used in kinetic experiments were quantified spectrophotometrically at pH 7.3 using the appropriate molar absorptivity coefficients (Windholz, 1976; P-L Biochemicals, Inc., 1973; Anderson & Fisher, 1980). In

kinetic experiments, the concentration of a single component was varied from approximately 0.5 × K_d to 10 × K_d (unless limited by high absorbance values) while keeping all other components constant for each assay. Initial rates were measured under conditions where less than 10% substrate conversion to product had occurred. All spectrophotometric measurements were made using a Beckman DU-7 spectrophotometer. Data from all kinetic experiments were fit to the Michaelis–Menten equation using the non-linear regression analysis program "Enzfitter" (Leatherbarrow, 1987). Assays were carried out in duplicate, and the results of 3–5 separate experiments were averaged. The percent maximal inhibition or activation of cyclohydrolase activity was calculated both as activity at saturating nucleotide concentration (40 × K_d) and as determined from the fit to the Michaelis–Menten equation.

Preparation of [^{3}H]NADP. [^{3}H]NAD, diluted with unlabeled NAD to 0.11 μmol, was phosphorylated with 0.0016 unit of NAD kinase and 0.5 μmol of ATP in the presence of 50 units of pyruvate kinase and 0.5 μmol of PEP. The reaction volume was 100 μL, in 50 mM Hepes, 25 mM magnesium chloride, and 20 mM potassium chloride. After 3 h at 37 °C, a further 0.0008 unit of NAD kinase was added (20 μL), and the reaction was allowed to continue for 2 h, at which time it was terminated by immersion in boiling water for 1 min. The reaction mix was clarified by microcentrifugation for 10 min.

Purification of [^{3}H]NADP$^+$. The supernatant was applied to a 1.2-mL column of Dowex 1-X2 formate, and the unreacted [^{3}H]NAD was eluted with 0.5 N formic acid. A stepwise ammonium formate gradient to 2N eluted [^{3}H]NADP$^+$ [modification of the method of Bernofsky and Gallagher (1975)]. The product was quantified by spectrophotometry (ϵ_{260} = 18.0) as well as by malate dehydrogenase assay. Spectrophotometric and enzymatic assays agreed within 2%. Pure [^{3}H]NADP$^+$ was obtained with a 90% yield and specific activity of 1.25 Ci/mmol. It was lyophilized, and aliquots were stored in water at −80 °C.

Equilibrium Dialysis. NADP$^+$ binding was characterized using a Spectrum equilibrium semimicro dialyzer, with 12–14-kDa cutoff dialysis membranes. The ligand, [^{3}H]NADP$^+$, was diluted with unlabeled NADP$^+$ to a specific activity of approximately 66 mCi/mmol up to 10 μM (final concentration in cell) and 7 mCi/mmol between 10 and 50 μM. Its concentration was determined by spectrophotometry, and an aliquot was used to determine specific radioactivity. The ligand was introduced in both sides of the cells. D/C/S, at a concentration of 7.1 or 7.3 μM and specific activity of approximately 9 units/mg, was introduced in one side of the cells. The dialysis was performed in buffer containing 10% glycerol, 25 mM potassium phosphate, pH 7.3, 50 mM potassium chloride, 0.5 mM PMSF, and 35 mM 2-mercaptoethanol at 20 °C, and the cells were rotated at 20 rpm for 7 h. Potential nonspecific binding was monitored by the addition of 390 or 430 μM unlabeled NADP$^+$ to duplicate samples. Affinity for 2′,5′-ADP was determined in a competitive binding assay by the addition of 9.1 μM unlabeled 2′,5′-ADP to both sides of a duplicate set of cells in an otherwise standard NADP$^+$ binding experiment. The data were fit to the equation: $[L]_{total} = [L]_{free} \times \{(n[D/C/S]/K_d + [L]_{free}) + 1\}$ by nonlinear regression analysis using the program Enzfitter.

Modification with Phenylglyoxal. Chemical modification of the D/C301 fragment was performed with 5 mM phenylglyoxal at 20 °C in 50 mM potassium phosphate, pH 7.3, containing 20% (v/v) PEG 200 and 1 mM EDTA (no mercaptoethanol). Reactions were terminated by diluting suitable samples directly into the appropriate enzyme assay mix.

Table 1: Kinetic Constants of MethyleneH4folate Dehydrogenase[a]

compd	K_m (μM)	K_i (μM)
(6*R*,*S*)-methyleneH4folate	2.8 ± 0.7	
NADP	5.7 ± 1.5	
AADP		0.43[b]
2′,5′-ADP		41[b]
		32 ± 9[c]
2′-AMP		35[b]
5′-AMP		–
2′-GMP		–
NMN		–

[a] Values are given as the mean ± standard deviation for the results of 3–5 experiments performed in duplicate. Dashes (–) indicate that no effect was seen at concentrations up to 500 μM. [b] K_i was obtained from the intercepts of Dixon plots. [c] Calculated from K_m(apparent) = K_m(1 + [2′,5′-ADP]/K_i) at 5 and 10 μM 2′,5′-ADP.

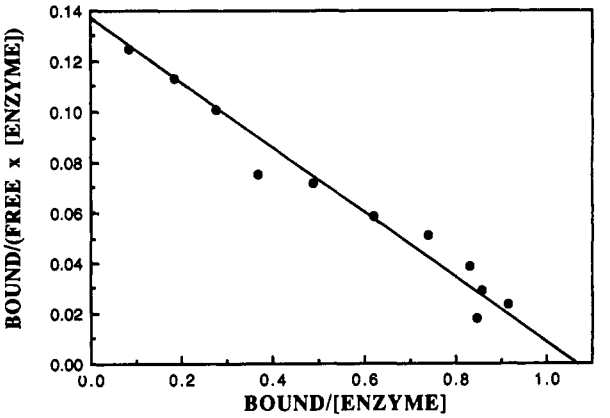


FIGURE 1: NADP$^+$ binding to D/C/S. There is no detectable nonspecific binding of NADP$^+$ under these conditions (described in Materials and Methods), and the uncorrected data are presented as a Scatchard plot. A single high-affinity NADP$^+$ binding site exists per monomer (n = 1.07 ● 0.04) with K_d = 7.8 ± 0.9 μM.

RESULTS

MethyleneH$_4$folate Dehydrogenase. The kinetic interdependence of the dehydrogenase and cyclohydrolase activities was previously illustrated with the porcine enzyme both by the channeling of the folate intermediate and by the inhibition of cyclohydrolase activity by NADP$^+$. To further explore this interaction, we first characterized the kinetic properties of the recombinant human dehydrogenase for its substrates, as shown in Table 1. The K_m for NADP$^+$ was shown to be independent of the methyleneH$_4$folate concentration used (15, 30, and 200 μM, data not shown). We performed a binding analysis with [^{3}H]NADP$^+$ to determine K_d and the number of binding sites. The Scatchard plot illustrated in Figure 1 shows that a single NADP$^+$ binding site exists per monomer of enzyme.

To determine specificity for NADP$^+$ binding, various analogs of NADP$^+$ which differ in the pyridine ring substituent, in the purine ring, or analogs representing portions of the molecule were used as inhibitors. The major kinetic results are listed in Table 1. Aminopyridine adenine dinucleotide phosphate (AADP) does not act as a substrate of the dehydrogenase; in fact, it is an excellent competitive inhibitor of the dehydrogenase. The 2′,5′-ADP moiety of NADP$^+$ is also a competitive inhibitor of the dehydrogenase. From

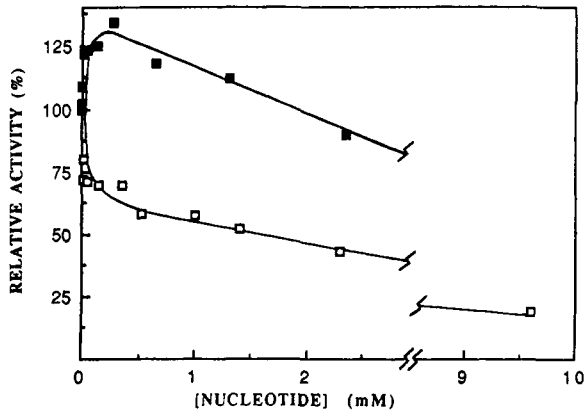


FIGURE 2: Effect of millimolar concentrations of nucleotides on cyclohydrolase activity. Activity was quantified in the presence of increasing concentrations of NADP+ (□) or 2',5'-ADP (■).

comparison of the values of K_i in Table 1, it appears that a large part of the affinity for NADP+ binding is contributed by the 2',5'-ADP moiety. Inhibition with 2',5'-ADP performed under standard conditions (200 μM methyleneH4folate) exhibits the same K_i and competitive character as inhibition performed with 20 μM methyleneH4folate present (data not shown). We also determined that K_i = 22 μM by 2',5'-ADP competition of [3H]NADP+ binding in equilibrium dialysis, where K_d(apparent) = $K_d\{1 + [2',5'\text{-ADP}]/K_i\}$. The 2'-phosphate is essential, as shown by the competitive inhibition by 2'-AMP but not 5'-AMP. Free nicotinamide mononucleotide (NMN) does not inhibit dehydrogenase activity. As well, alteration of the purine ring results in the loss of binding, as seen by using 2'-GMP.

MethenylH4folate Cyclohydrolase. Because the nature of the NADP+ inhibition of cyclohydrolase is poorly understood, we wanted to determine if NADP+ binding responsible for the inhibition has the same kinetic characteristics as NADP+ substrate binding for the dehydrogenase. We used some of the same analogs to examine the specificity of inhibition of the cyclohydrolase. At very high concentrations (millimolar) both NADP+ and 2',5'-ADP inhibit the cyclohydrolase, but their effects were found to be biphasic (Figure 2). At concentrations which represent physiologically relevant levels (micromolar), NADP+ inhibits but, to our surprise, 2',5'-ADP stimulates the cyclohydrolase activity.

Characterization of the effects at low nucleotide concentrations, shown in Figure 3, illustrates that NADP+ inhibits the cyclohydrolase with a K_i of 4.9 μM, similar to its K_m for the dehydrogenase activity; the inhibition is maximal at approximately 40%. Activation by 2',5'-ADP results in a maximum of 130% cyclohydrolase activity, with a K_{act} of 5.6 μM. This activation constant was found to be independent of the concentration of methenylH4folate used (25–90 μM, data not shown). A summary of the kinetic effects is given in Table 2. The presence of the 5'-diphosphoribose group, as in 2'-ATP ribose, does not significantly change the affinity. Taking the 2',5'-ADP-stimulated level of cyclohydrolase activity as being optimal, the additional presence of the NMN moiety of NADP+ results in a 2-fold decrease of activity, to 46% (i.e., 60%/130%) of the optimal level.

AADP is as potent an inhibitor of the cyclohydrolase activity as it is for the dehydrogenase, with a maximal inhibition of approximately 78%, i.e., to 17% of the optimal level. 5'-AMP, NMN, and NAD+ were found not to inhibit the cyclohydrolase. Significantly, 2'-AMP, which is an inhibitor of the dehydrogenase (Table 1), does not affect the cyclohydrolase activity.

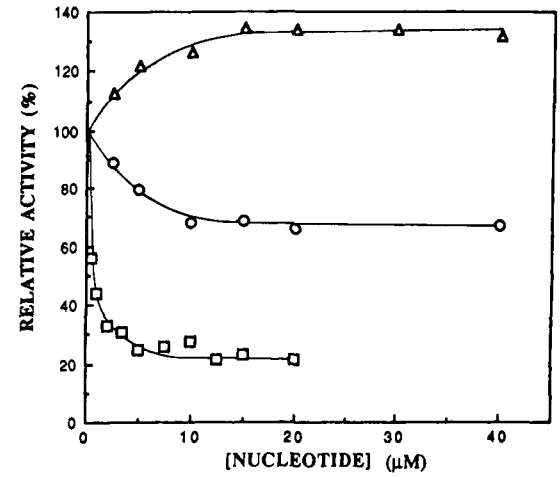


FIGURE 3: Effect of micromolar concentrations of nucleotides on cyclohydrolase activity. Cyclohydrolase activity was quantified in the presence of increasing concentrations of 2',5'-ADP (△), NADP+ (○), or AADP (□). The K_i and K_{act} values were determined by nonlinear regression analysis.

Table 2: Kinetic Constants of MethenylH4folate Cyclohydrolase[a]

compd	K_m (μM)	K_i (μM)	K_{act} (μM)	max effect (%)
(6*R*,*S*)-methenyl-H4folate	16.4 ± 0.2			
NAD		–	–	
NADP		4.9 ± 0.3		40 (inhibition)
AADP		0.35 ± 0.11		78 (inhibition)
2',5'-ADP			5.6 ± 1.8	29 (activation)
2'-ATP ribose			8.3 ± 4.2	27 (activation)
2'-AMP		–	–	
5'-AMP		–	–	
NMN		–	–	

[a] Values are given as the mean ± standard deviation for the rate constants of 3–5 experiments performed in duplicate. Dashes (–) indicate that no effect was seen at concentrations up to 500 μM.

Mechanisms of Stimulation and Inhibition of Cyclohydrolase Activity. The effects on cyclohydrolase were observed under assay conditions where the enzyme is less than optimally saturated with methenylH4folate. We therefore determined whether the activation or inhibition by the nucleotides were effects on K_m or V_{max}. Activation by saturating 2',5'-ADP (100 μM) is the result of an increase by a factor of 1.3 in both V_{max} and K_m (data not shown), confirming the 129% maximal activation effect measured (Table 2). Inhibition by NADP+ at three fixed concentrations (0, 6.2, and 47 μM) showed a noncompetitive type of inhibition with a decrease in V_{max} but no change in K_m (data not shown). We also confirmed the previously reported uncompetitive inhibition pattern of AADP inhibition of cyclohydrolase activity (Smith & MacKenzie, 1983; this study, data not shown).

Binding of 2'-AMP to Enzyme. From kinetic experiments with 2'-AMP, we observed that it is an inhibitor of the dehydrogenase activity but has no apparent effect on cyclohydrolase activity. In Figure 4, we show that while 2'-AMP itself has no effect on cyclohydrolase activity, it effectively competes both with NADP+ inhibition and 2',5'-ADP activation of cyclohydrolase activity.

Protection of Phenylglyoxal Modification by Nucleotides. The kinetic and binding studies measured the affinity of 2',5'-ADP by competition against NADP+; we examined protection of phenylglyoxal modification of the D/C301 fragment in order to estimate the affinity of the enzyme for 2',5'-ADP in the absence of NADP+. The D/C301 domain, which

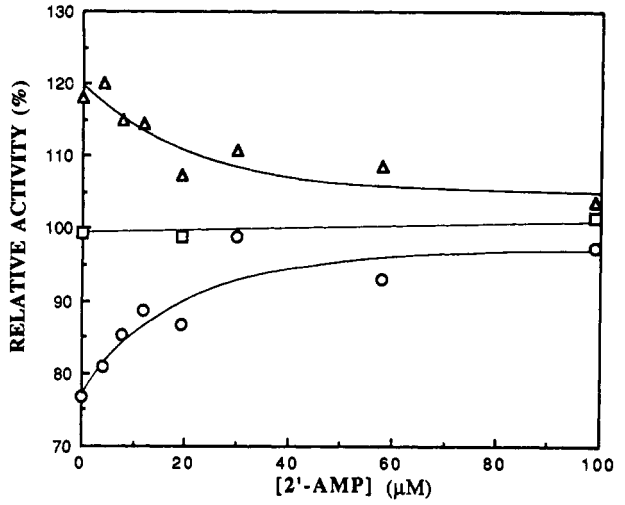


FIGURE 4: Effect of 2′-AMP on cyclohydrolase activity. Cyclohydrolase reaction rates were determined in the presence of increasing 2′-AMP concentrations, either alone (□) or in the presence of 20 μM 2′,5′-ADP (△) or 20 μM $NADP^+$ (○). K_d, calculated as described in Results, is approximately 4 μM against 2′,5′-ADP and 3 μM against $NADP^+$.

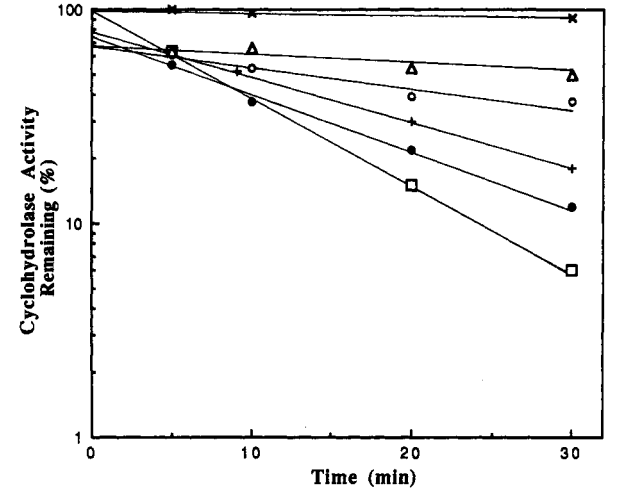


FIGURE 5: Protection of phenylglyoxal modification of D/C301 fragment cyclohydrolase activity by 2′,5′-ADP. The D/C301 fragment was modified in the absence (□) or in the presence of 5 (●), 10 (+), 30 (○), and 500 μM (△) 2′,5′-ADP. There was no significant loss of activity in the absence of phenylglyoxal (×). The modification rates, calculated from the slopes, were plotted versus [2′,5′-ADP] to obtain K_d = 8.3 μM.

represents only one-third of the protein sequence, exhibits the same kinetic properties as we have described for the full-length protein (data not shown). This domain was used for chemical modification to avoid indirect effects on the dehydrogenase that might accompany modification of the arginines in the synthetase domain. Figure 5 illustrates that loss of cyclohydrolase activity is partially protected by 2′,5′-ADP. The half-maximal concentration for protection, derived from rate of modification versus ligand concentration under the conditions stated in the methods, is 8.3 μM. The dehydrogenase activity is protected by 2′,5′-ADP with a half-maximal concentration of 9.1 μM. These values are very similar to the protection of the dehydrogenase and cyclohydrolase activities by $NADP^+$ with values of 4.3 and 6.3 μM, respectively.

DISCUSSION

The present investigation has enabled us to further examine the interdependence of two enzymatic activities in close physical association. The efficient interaction of the dehydrogenase and cyclohydrolase activities is made evident by the preferential channeling of the product of the dehydrogenase reaction to the cyclohydrolase active site (Cohen & MacKenzie, 1978; Hum & MacKenzie, 1991), resulting in a substantial kinetic advantage. However, inhibition of the cyclohydrolase activity by the dinucleotide substrate of the dehydrogenase activity appears to counter the improvement in kinetic efficiency which results from their close physical association. We have investigated these apparently opposite effects by analysis of $NADP^+$ binding, focusing on resolving nucleotide interactions with the dehydrogenase active site and their effects on cyclohydrolase activity. All kinetic constants of the human enzyme are reported here for the first time. Because methenyltetrahydrofolate is also a substrate for the cyclohydrolase, kinetic analysis of the reverse dehydrogenase reaction with NADPH is not feasible. Interestingly, the Michaelis constants for the substrates of the dehydrogenase and cyclohydrolase reactions are 3–10-fold lower than those reported for other homologous enzymes [MacKenzie (1984), Mejia *et al.* (1986), Barlowe *et al.* (1989), and Yang and MacKenzie (1992)], except for the rabbit trifunctional enzyme (Schirch, 1978) where the values are in the same range. There is no highly conserved consensus sequence for dinucleotide binding (McKie & Douglas, 1991) in the domain. This is not exceptional as there exists an increasing number of dinucleotide-binding proteins where nonconsensus binding sequences have been reported (Adams *et al.*, 1981; Carne & Walker, 1983; Paulauskis & Sul, 1989).

Dinucleotide Binding. One single high-affinity dinucleotide binding site exists per monomer of enzyme as shown by the binding analysis. These results are consistent with the kinetic analyses as $NADP^+$ binds with approximately the same affinity both as a substrate for the dehydrogenase and as an inhibitor of the cyclohydrolase. The standard free energy of binding $NADP^+$ to the enzyme at 20 °C (from $\Delta G° = -RT \ln K_d$) is 28.7 kJ, mostly provided by the 2′,5′-ADP moiety of the dinucleotide. Substitution of aminopyridine for the nicotinamide ring (AADP) adds an additional 10-fold increase in affinity for the enzyme, or 8.4 kJ over $NADP^+$, both with respect to the dehydrogenase and the cyclohydrolase. NMN alone has no measurable affinity for the enzyme.

The kinetic pattern of $NADP^+$ inhibition of the cyclohydrolase is biphasic. Smith and MacKenzie (1983) reported a K_i for $NADP^+$ inhibition of the porcine cyclohydrolase of 109 μM. However, the value of K_d obtained in the same study, from protection against chemical modification, was much lower (4 μM). Moreover, the authors did not recognize that the high-affinity binding results in only 40% maximal inhibition, an observation which significantly increases our understanding of the system. The two phases of inhibition with $NADP^+$ described in this study with respect to the human enzyme also occur in the porcine enzyme (results not shown) and explain the discrepancy where the earlier value of 109 μM is intermediate between those of the high and low affinity sites.

2′,5′-ADP Subsite. The 2′,5′-ADP moiety is of primary importance for $NADP^+$ binding to the enzyme. Specificity for this binding shows that the purine ring must be adenine and that the 2′-phosphate must be present since neither 5′-AMP nor NAD^+ are inhibitors of the dehydrogenase. Binding of 2′-AMP to the enzyme is not increased by the addition of the 5′-phosphate since comparison of the 2′-AMP and the 2′,5′-ADP binding affinities, either against the dehydrogenase or the cyclohydrolase, shows that they are approximately equal.

However, the presence of the 5′-phosphate activates the cyclohydrolase by 30%. It is possible that any extra binding energy is used to mediate a conformational change that allows maximal turnover of the cyclohydrolase. The presence of a diphosphoribose group at the 5′-position does not further enhance the affinity of the adenosine nucleotide or the extent of activation of the cyclohydrolase.

We have examined the binding of 2′,5′-ADP by both kinetic and competitive binding methods. The K_d values determined from direct effects of 2′,5′-ADP on dehydrogenase or cyclohydrolase approximate the K_d values for $NADP^+$. However, when measured by competition against $NADP^+$, the K_d values for 2′,5′-ADP appear 3–7-fold higher. The kinetic and binding data show that 2′,5′-ADP binding is purely competitive against $NADP^+$, and it appears that a small contribution of the nicotinamide moiety assists in $NADP^+$ binding.

Interaction of NMN with the Active Site. The bound $NADP^+$ interacts with the two active sites that we propose share a single folate binding site. In a structural analysis of the dihydrofolate reductase–$NADP^+$ holoenzyme crystal, Bystroff *et al.* (1990) show that while the 2′,5′-ADP moiety is ordered, the NMN moiety is disordered. We propose that such an arrangement also exists in the dehydrogenase/cyclohydrolase domain of the human trifunctional enzyme where most of the binding involves the 2′,5′-ADP moiety, away from the catalytically active portion of the molecule. This model restricts the distance between the dehydrogenase and cyclohydrolase active site(s) to the distance to which the NMN moiety can reach. Since evidence obtained from affinity labeling experiments suggests that only one folate binding site exists (Smith & MacKenzie, 1985), the active sites must overlap proximal to the 2′,5′-ADP binding subsite. Coincident protection of the two activities against chemical modification by $NADP^+$ or 2′,5′-ADP further supports the existence of a shared active site.

The inhibitory effects of the nicotinamide portion of the molecule on cyclohydrolase activity should be considered relative to the fully activated cyclohydrolase (i.e., with the 2′,5′-ADP subsite occupied). Addition of the NMN moiety to 2′,5′-ADP results in a decrease of the optimal activity to 46%, while the 3-aminopyridine substituent reduces the activity to about 17%. These results illustrate the full effect of the 'loosely bound' pyridine ring portion of the dinucleotides. Subsequent references to the inhibition of cyclohydrolase will be relative to the fully activated form.

Inhibition by $NADP^+$ is not competitive toward methenylH_4folate because the folate binding site is distinct from the high-affinity 2′,5′-ADP subsite. Inhibition is caused by the linked nicotinamide moiety, and this is reflected in the noncompetitive pattern. The mechanism of inhibition is unproven. The NMN could be interfering with formation of a productive E–methenylH_4folate complex in a "dynamic" fashion. In this case maximal inhibition (54%) would be determined by the occupancy of the cyclohydrolase site by the mobile nicotinamide ring. The alternative explanation is that the NMN moiety binds and causes a conformational change that decreases k_{cat} by about 54%. The marginal additional effect of the nicotinamide moiety on 2′,5′-ADP binding affinity supports the first interpretation. When the binding of the substituted pyridine ring is enhanced, as seen with AADP, the inhibition is much more severe, with a maximal inhibition of 83%. The inhibition is not complete because, while it is possible to saturate the 2′,5′-ADP subsite by increasing the concentration of AADP, the inhibition of the cyclohydrolase is limited by the residency of the pyridine ring in the cyclohydrolase site. The apparent uncompetitive nature of the inhibition suggests that a dead-end complex of E–AADP–methenylH_4folate is formed.

Although kinetic analyses of multienzyme systems where channeling occurs have been performed (Sauro & Kacser, 1990; Ovádi *et al.*, 1989), there exist few analogous analyses describing the kinetics of noncovalent channeling between activities that belong to a single polypeptide chain. Earlier work has shown that the channeling of the folate intermediate (Cohen & MacKenzie, 1978; Hum & MacKenzie, 1991) is essentially independent of the polyglutamate tail length (MacKenzie & Baugh, 1980). This evidence is consistent with channeling within a single bifunctional site in the D/C/S, without translocation of the pteroyl ring of the folate. This contrasts with channeling of the folate substrate in the bifunctional enzyme formiminoglutamate:H_4folate formiminotransferase:formiminoH_4folate cyclodeaminase where the optimally long pentaglutamate tail acts as an anchor, allowing the pteridine ring to move between two separate pteroyl binding sites (MacKenzie & Baugh, 1980; Paquin *et al.*, 1985). Coincident protection against chemical modification (Smith & MacKenzie, 1983; this study, data not shown), the inactivation of both activities with the incorporation of a folate affinity label (Smith & MacKenzie, 1985), and the data presented in this paper are also consistent with the existence of an active site shared by the dehydrogenase and the cyclohydrolase. However, the enzyme chorismate mutase-prephenate dehydrogenase serves as a precautionary tale: long thought to possess a shared active site (Heyde, 1979), it has recently been shown to possess two "similar but distinct" active sites (Turnbull & Morrison, 1990). Our current analysis of the complex interaction between two activities offers new evidence to support the existence of a shared active site.

The binding of $NADP^+$ to the bifunctional domain has two opposite effects on the cyclohydrolase activity. The 2′,5′-ADP moiety increases the turnover of the cyclohydrolase by 30%, while the addition of the NMN portion causes a 2-fold inhibition from that optimally active state, the global result of which is moderate inhibition of cyclohydrolase by $NADP^+$. It is important to point out that this inhibition is against exogenously added methenylH_4folate. If the inhibition is actually due only to interference with formation of a productive complex with added methenylH_4folate, the effect with regards to channeling of endogenously generated intermediate could be quite different. In fact, our observations indicate that it would be beneficial for the enzyme to retain NADPH following the dehydrogenase reaction so that the 2′,5′-ADP moiety could increase the k_{cat} of the cyclohydrolase and thus promote the efficiency of channeling. The nature of the interaction between these activities is consistent with the notion of the chemically uncomplicated cyclohydrolase activity having evolved as a second function in a preexisting monofunctional dehydrogenase domain where it shares the folate binding site proximal to bound $NADP^+$. This is consistent with the fact that the bifunctional domains are not larger than the monofunctional dehydrogenase enzymes. Structural analysis is required to confirm that a bifunctional active site proximal to bound $NADP^+$ exists and to fully understand the efficiency in the design of this bifunctional domain.

ACKNOWLEDGMENTS

We thank Narciso R. Mejia for purification of the porcine trifunctional enzyme; Dr. Edward A. Meighen, Dr. Joanne Turnbull, and Dr. W. Wallace Cleland for helpful discussions and comments; and Bio-Méga/Boehringer Ingelheim Re-

search Inc. for generously giving us access to an equilibrium microdialyzer.

REFERENCES

Adams, M. J.; Archibald, I. G., Helliwell, J. R., Jenkins, S. E., & White, S. W. (1981) in *Structural Studies on the Molecules of Biological Interest* (Dodson, G., Glusker, J. E., & Sayre, D., Eds.) Clarendon Press, Oxford.

Anderson, B. M., & Fisher, T. L. (1980) *Methods Enzymol. 66*, 81–87.

Appling, D. R., & Rabinowitz, J. C. (1985) *Biochemistry 24*, 3540–3547.

Barlowe, C. K., & Appling, D. R. (1990) *Biochemistry 29*, 7089–7094.

Barlowe, C. K., Williams, M. E., Rabinowitz, J. C., & Appling, D. R. (1989) *Biochemistry 28*, 2099–2106.

Bernofsky, C., & Gallagher, W. J. (1975) *Anal. Biochem. 67*, 611.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Bystroff, C., Oatley, S. J., & Kraut, J. (1990) *Biochemistry 29*, 3263–3277.

Carne, A., & Walker, J. E. (1983) *J. Biol. Chem. 258*, 12895–12906.

Cohen, L., & MacKenzie, R. E. (1978) *Biochim. Biophys. Acta 522*, 311–317.

Drury, E. J., Bazar, L. S., & MacKenzie, R. E. (1975) *Arch. Biochem. Biophys. 169*, 662–668.

Heyde, E. (1979) *Biochemistry 18*, 2766–2775.

Hum, D. W., & MacKenzie, R. E. (1991) *Protein Eng. 4*, 493–500.

Knight, S., Andersson, I., & Brändén, C.-I. (1990) *J. Mol. Biol. 215*, 113–160.

Kühn-Velten, W. N., Bunse, T., & Förster, M. E. C. (1991) *J. Biol. Chem. 266*, 6291–6301.

Leatherbarrow, R. J. (1987) *Enzfitter*, Biosoft, Cambridge, U.K.

MacKenzie, R. E. (1984) in *Folates and Pterins: Chemistry and Biochemistry of Folates* (Blakley, R., & Benkovic, S., Eds.) Vol. 1, pp 255–306, John Wiley and Sons, New York.

MacKenzie, R. E., & Baugh, C. M. (1980) *Biochim. Biophys. Acta 611*, 187–195.

Markwell, M. A., Hass, S. M., Tolbert, N. E., & Bieber, L. L. (1981) *Methods Enzymol. 72*, 296–303.

McKie, J. H., & Douglas, K. T. (1991) *FEBS Lett. 279*, 5–8.

Mejia, N. R., & MacKenzie, R. E. (1985) *J. Biol. Chem. 260*, 14616–14620.

Mejia, N. R., & MacKenzie, R. E. (1988) *Biochem. Biophys. Res. Commun. 155*, 1–6.

Mejia, N. R., Rios-Orlandi, E. M., & MacKenzie, R. E. (1986) *J. Biol. Chem. 261*, 9509–9513.

Minami, M., Bito, H., Ohishi, N., Tsuge, H., Miyano, M., Mori, M., Wada, H., Mutoh, H., Shimada, S., Izumi, T., Abe, K., & Shimizu, T. (1992) *FEBS Lett. 309*, 353–357.

Moore, M. R., O'Brien, W. E., & Ljungdahl, L. G. (1974) *J. Biol. Chem. 249*, 5250–5253.

Ovádi, J., Tompa, P., Vértessy, B., Orosz, F., Keleti, T., & Welch, G. R. (1989) *Biochem. J. 257*, 187–190.

P-L Biochemicals, Inc. (1973) *Ultraviolet Absorption Spectra of Pyridine Nucleotide Coenzymes and Coenzyme Analogs*, Circular OR-18, P-L Biochemicals Inc., Milwaukee, WI.

Palosaari, P. M., Vihinen, M., Mäntsälä, P. I., Alexson, S. E. H., Pihlajaniemi, T., & Hiltunen, J. K. (1991) *J. Biol. Chem. 266*, 10750–10753.

Paquin, J., Baugh, C. M., & MacKenzie, R. E. (1985) *J. Biol. Chem. 260*, 14925–14931.

Paulauskis, J. D., & Sul, H. S. (1989) *Biochem. Biophys. Res. Commun. 158*, 690–695.

Ragsdale, S. W., & Ljungdahl, L. G. (1984) *J. Biol. Chem. 259*, 3499–3503.

Rios-Orlandi, E., & MacKenzie, R. E. (1988) *J. Biol. Chem. 263*, 4662–4667.

Rios-Orlandi, E., Zarkadas, C. G., & MacKenzie, R. E. (1986) *Biochim. Biophys. Acta 871*, 24–35.

Sauro, H. M., & Kacser, H. (1990) *Eur. J. Biochem. 187*, 493–500.

Schirch, L. (1978) *Arch. Biochem. Biophys. 189*, 283–290.

Smith, D. D. S., & MacKenzie, R. E. (1983) *Can. J. Biochem. Cell Biol. 61*, 1166–1171.

Smith, D. D. S., & MacKenzie, R. E. (1985) *Biochem. Biophys. Res. Commun. 128*, 148–154.

Tan, L. U. L., Drury, E. J., & MacKenzie, R. E. (1977) *J. Biol. Chem. 252*, 1117–1122.

Turnbull, J., & Morrison, J. F. (1990) *Biochemistry 29*, 10255–10261.

Wasserman, G. F., Benkovic, P. A., Young, M., & Benkovic, S. J. (1983) *Biochemistry 22*, 1005–1013.

Windholz, M., Ed. (1976) *The Merck Index, An Encyclopedia of Chemicals and Drugs*: 9th ed., Merck & Co., Inc., Rahway, NJ.

Wohlfarth, G., Geerligs, G., & Diekert, G. (1991) *J. Bacteriol. 173*, 1414–1419.

Yang, X.-M., & MacKenzie, R. E. (1992) *Protein Expression Purif. 3*, 256–262.