Meloche, H. P. (1965) *Biochem. Biophys. Res. Commun. 18*, 277–282.
Meloche, H. P. (1967) *Biochemistry 6*, 2273–2280.
Meloche, H. P. (1970) *Biochemistry 9*, 5050–5055.
Misset, O., & Robillard, G. T. (1982) *Biochemistry 21*, 3136–3142.
Misset, O., Brouwer, M., & Robillard, G. T. (1980) *Biochemistry 19*, 883–890.
Robillard, G. T. (1982) *Mol. Cell. Biochem. 46*, 3–24.
Robillard, G. T., Dooyewaard, G., & Lolkema, J. S. (1979) *Biochemistry 18*, 2984–2989.
Saier, M. H., Schmidt, M. R., & Lin, Ph. (1980) *J. Biol. Chem. 255*, 8579–8584.
Stein, R., Schrecker, O., Lauppe, H. F., & Hengstenberg, W. (1974) *FEBS Lett. 42*, 98–100.
Waygood, E. B., & Steeves, T. (1980) *Can. J. Biochem. 58*, 40–48.
Waygood, E. B., Meadow, N. D., & Roseman, S. (1979) *Anal. Biochem. 95*, 293–304.
Weigel, N., Kukuruzinska, M. A., Nakazawa, A., Waygood, E. B., & Roseman, S. (1982) *J. Biol. Chem. 257*, 14477–14491.
Yoshida, H., & Wood, H. G. (1978) *J. Biol. Chem. 253*, 7650–7655.
Yun, S., & Suelter, C. H. (1979) *J. Biol. Chem. 254*, 1811–1815.

Interaction of Analogues of Nicotinamide Adenine Dinucleotide Phosphate with Dihydrofolate Reductase from *Escherichia coli*†

Stuart R. Stone,‡ Alan Mark, and John F. Morrison*

ABSTRACT: Steady-state kinetic techniques have been used to investigate the pyridine nucleotide specificity of dihydrofolate reductase from *Escherichia coli* as well as the binding of fragments of NADP to the free form of enzyme and to the enzyme–dihydrofolate complex. The reduced forms of nicotinamide hypoxanthine dinucleotide phosphate and acetylpyridine adenine dinucleotide phosphate function as alternative substrates but exhibit maximum velocities that are lower than with NADPH. There are only small differences in the strength of binding of the various pyridine nucleotides to the free enzyme and to the enzyme–dihydrofolate complex. The reduced form of thionicotinamide adenine dinucleotide phosphate is not a substrate, but both the reduced and oxidized forms of this nucleotide act as inhibitors. The inhibitions by these compounds involve their interaction with an enzyme–tetrahydrofolate complex. The kinetic results confirm that the dihydrofolate reductase reaction only approximates to a rapid equilibrium, random mechanism with NADPH as the pyridine nucleotide substrate. But when NADPH is replaced by reduced acetylpyridine adenine dinucleotide phosphate, both kinetic and binding data indicate that the kinetic mechanism of the reaction is truly rapid equilibrium, random. All fragments of NADP which were tested behaved as inhibitory analogues of this nucleotide. From the values of the dissociation constants obtained for their interactions with the enzyme, it is concluded that the 2′- and 5′-phosphoryl groups of adenosine make significant contributions to the binding energy of NADP whereas the nicotinamide moiety does not.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (DHF)[1] to 5,6,7,8-tetrahydrofolate. This enzyme is the site of action of a number of imporant chemotherapeutic agents such as methotrexate, trimethoprim, and pyrimethamine. As a consequence of the clinical importance of the interaction between dihydrofolate reductase and a number of drugs, the enzyme has been studied extensively (Hitchings & Smith, 1980; Gready, 1980; Roth & Cheng, 1982). More recently, a number of investigations have been directed toward the determination of the groups involved with the binding of NADPH and its analogues to dihydrofolate reductase from various sources. Several approaches have been used, and these include X-ray crystallography (Filman et al., 1982; Matthews et al., 1978), rapid reaction techniques (Dunn et al., 1978; Dunn & King, 1980; Cayley et al., 1981), and NMR and fluroescence titration (Way et al., 1975; Birdsall et al., 1980a,b, 1981a; Feeney et al., 1980a; Gronenborn et al., 1981c; Hyde et al., 1980a,b). The majority of studies have been concerned with the formation of either binary enzyme–inhibitor or ternary enzyme–NADPH–inhibitor complexes. Much less attention has been paid to the interaction of NADPH with the enzyme–DHF complex and to groups in the pyridine nucleotide which are of importance for their binding in the ternary complex.

In the present study, steady-state kinetic techniques have been used to determine the substrate specificity of dihydrofolate reductase from *Escherichia coli* and the kinetic mechanism of the reaction with various nucleotides. In addition, determinations have been made of the strength of interaction of analogues and derivatives of NADP(H) with the free en-

† From the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, ACT 2601, Australia. *Received February 29, 1984.*
‡ Present address: Friedrich Miescher Institute, CH-4002 Basel, Switzerland.

[1] Abbreviations: DHF, dihydrofolate; THF, tetrahydrofolate; ADP-ribose, adenosine 5′-diphosphate ribose; ATP-ribose, 2′-phosphoadenosine 5′-diphosphate ribose; APADP, 3-acetylpyridine adenine dinucleotide phosphate; ε-NADP, nicotinamide 1,N^6-ethenoadenine dinucleotide phosphate; NHDP, nicotinamide hypoxanthine dinucleotide phosphate; TNADP, thionicotinamide adenine dinucleotide phosphate; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid.

0006-2960/84/0423-4340$01.50/0

zyme and the enzyme–dihydrofolate complex.

Experimental Procedures

Materials

NADP, NADPH, NAD, and alcohol dehydrogenase from *Leuconostoc mesenteroides* were obtained from Boehringer Mannheim. Adenosine 5′-diphosphateribose (ADP-ribose), 2′-phosphoadenosine 5′-diphosphateribose (ATP-ribose), 3-acetylpyridine adenine dinucleotide phosphate (APADP), nicotinamide 1,N^6-ethenoadenine dinucleotide phosphate (ϵ-NADP), nicotinamide hypoxanthine dinucleotide phosphate (NHDP), and thionicotinamide adenine dinucleotide phosphate (TNADP) were from Sigma. APADP, ϵ-NADP, NHDP, and TNADP were converted to their reduced forms by using ethanol and alcohol dehydrogenase. The reduced coenzymes were purified by the method of Viola et al. (1979). Dihydrofolate was obtained by the chemical reduction of folic acid as described by Blakley (1960). Dihydrofolate reductase was isolated and purified from an overproducing strain of *Escherichia coli* as described previously (Williams et al., 1979; Smith et al., 1982; Stone & Morrison, 1982).

Methods

NADPH and DHF were estimated by using dihydrofolate reductase together with a molar absorbance change for the reaction of 11 800 cm^{-1} at 340 nm (Stone & Morrison, 1982). NADP was estimated enzymically by using glucose-6-phosphate dehydrogenase. Adenosine 2′-phosphate, adenosine 5′-phosphate, adenosine 2′,5′-diphosphate, ADP-ribose, and ATP-ribose were determined spectrophotometrically by using an extinction coefficient of 15 300 M^{-1} cm^{-1} at 259 nm (Dawson et al., 1969). APADP, NHDP, and TNADP, and their reduced forms, were also estimated spectrophotometrically by using the extinction coefficients published by P-L Biochemicals (Circular OR-18). The extinction coefficients used for the estimation of ϵ-NADP and ϵ-NADPH were 9700 M^{-1} cm^{-1} at 265 nm and 7100 M^{-1} cm^{-1} at 332 nm, respectively (Neef & Huennekens, 1976).

Dihydrofolate reductase activity was measured by following the enzyme-dependent rate of decrease in the absorbance at 340 nm with a Cary 118 spectrophotometer. All assays were performed at 30 °C in 0.05 M Tris–0.025 M acetate–0.025 M MES buffer (pH 7.4) containing 0.1 M NaCl. For inhibition studies, the assays contained NADPH and DHF. The inhibitor concentration was varied from zero to levels which yielded significant inhibition. In experiments in which inhibition with respect to NADPH was tested, NADPH concentration was varied from 1.1 to 10 μM, and DHF was held constant between 1.6 and 3.0 μM. When the mode of inhibition with respect to DHF was tested, DHF concentration was varied from 0.89 to 8.0 μM, and NADPH was fixed between 2.0 and 3.3 μM. For alternative substrate studies, the concentration of the reduced coenzyme was varied over a 9-fold range from about one-third to 3 times its Michaelis constant, and molar absorbance changes at 340 nm were 11 800 cm^{-1} for NADPH, APADPH, and NHDP and 12 300 cm^{-1} for ϵ-NADPH.

Data Analysis. Initial velocity data exhibiting linear competitive and noncompetitive inhibition were fitted to eq 1 and 2, respectively, where A is the concentration of the varied

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A} \quad (1)$$

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (2)$$

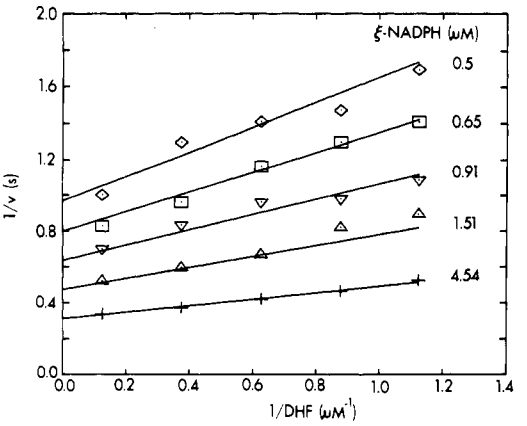

FIGURE 1: Effect of the concentration of ϵ-NADPH on the initial velocity of the dihydrofolate reductase reaction with DHF as the variable substrate.

substrate, I is the concentration of the inhibitor, V is the apparent maximum velocity, and K_a is the apparent Michaelis constant for the varied substrate. K_{is} and K_{ii} are the apparent inhibition constants associated with the effect of the inhibitor on the slopes (K_{is}) and intercepts (K_{ii}) of double-reciprocal plots (Cleland, 1963). Initial velocity data were fitted to eq 3 where

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (3)$$

A and B represent the concentrations of the pyridine nucleotide and DHF, respectively. V denotes the maximum velocity of the reaction, and K_b represents the Michaelis constant for DHF. K_a and K_{ia} denote, respectively, the Michaelis constant and the dissociation constant for the binary complex of the pyridine nucleotide. Initial velocity data for double inhibition experiments were fitted to eq 4 where I and J represent the

$$v = \frac{VA}{K_a[1 + I/K_i + J/K_j + IJ/(\alpha K_iK_j)] + A} \quad (4)$$

concentrations of the two inhibitors; K_i and K_j denote inhibition constants for I and J, respectively, and α represents an interaction coefficient. Data were fitted to eq 1–4 by using weighted, robust, linear regression (Cornish-Bowden & Endrenyi, 1981).

The binary dissociation constants for NHDPH, ϵ-NADPH, and APADPH were also determined thermodynamically by measuring the quenching of protein fluorescence (excitation = 290 nm, emmission = 340 nm) upon addition of the ligand. The method used was as described previously (Stone & Morrison, 1982, 1983). Corrections were made for the absorption of light by the ligand as described by Birdsall et al. (1980a), and the corrected data were used to determine the dissociation constant for the ligand by using nonlinear regression (Stone & Morrison, 1982).

Results

Initial Velocity Studies with Analogues of NADPH. The reduced forms of nicotinamide hypoxanthine dinucleotide phosphate (NHDP), nicotinamide 1,N^6-ethenoadenine dinucleotide phosphate (ϵ-NADP), and acetylpyridine adenine dinucleotide phosphate (APADP) were found to act as alternative pyridine nucleotide substrates for the dihydrofolate reductase reaction. The initial velocity patterns were of the intersecting type, as previously observed with NADPH (Stone & Morrison, 1982), and the pattern obtained with ϵ-NADPH is illustrated in Figure 1. The fitting of the data to eq 3 yielded the values for the kinetic parameters that are listed

Table I: Kinetic Parameters for the Reaction Catalyzed by Dihydrofolate Reductase with Different Pyridine Nucleotide Substrates

pyridine nucleotide	parameter[a]				
	V (s^{-1})	K_{ia} (μM)	K_a (μM)	K_{ib} (μM)	K_b (μM)
NADPH[d]	16.5 ± 0.3	3.8 ± 0.5 (0.51 ± 0.01)[c]	2.5 ± 0.1	0.76 ± 0.09	0.47 ± 0.05
NHDPH	6.4 ± 0.2 (39)[b]	4.8 ± 1.5 (2.8 ± 0.2)[c]	2.1 ± 0.2	0.61 ± 0.23	0.27 ± 0.06
ϵ-NADPH	4.3 ± 0.2 (26)[b]	2.4 ± 0.5 (1.4 ± 0.1)[c]	1.6 ± 0.1	0.77 ± 0.22	0.51 ± 0.08
APADPH	2.4 ± 0.2 (15)[b]	1.6 ± 0.4 (1.2 ± 0.2)[c]	3.0 ± 0.4	0.59 ± 0.19	1.1 ± 0.2

[a] K_{ia} (K_{ib}) and K_a (K_b) represent dissociation and Michaelis constants, respectively, for pyridine nucleotide (A) and DHF (B). [b] Values in parentheses represent the maximum velocity as a percentage of that observed with NADPH. [c] Values in parentheses for K_{ia} were determined by fluorescence titration. [d] Values of parameters are taken from Stone & Morrison (1982).

Table II: Inhibition Constants Associated with the Inhibition of Dihydrofolate Reductase by TNADP and TNADPH

inhibitor	varied substrate	fixed substrate		app inhibn constants		true dissociation constant[a]	
		identity	concn (μM)	K_{is} (μM)	K_{ii} (μM)	symbol	value (μM)
TNADP	NADPH	DHF	3.0	115 ± 26 (79 ± 12)[b]	86 ± 17	K_{I2}	74 ± 15
		DHF	30	84 ± 14		K_I	84 ± 14
					41 ± 7	K_{I2}	41 ± 7
	DHF	NADPH	3.3	121 ± 43	38 ± 4 (58 ± 4)[b,c]	K_i	65 ± 23
	APADPH	DHF	3.0	15 ± 1 (21 ± 3)[b]			
	DHF	APADPH	3.3	68 ± 7		K_i	22 ± 7
					42 ± 5	K_I	20 ± 3
TNADPH	NADPH	DHF	1.6	23 ± 4			*d*
					21 ± 3	K_{I2}	16 ± 2
	DHF	NADPH	3.3	15 ± 3		K_i	8 ± 2
					20 ± 1		*d*

[a] Expressions for the relationships between true and apparent constants were derived from eq 6 [cf. Smith & Morrison (1969)]. Symbols are as defined in the text. [b] Values for true dissociation constants could not be calculated because the expression for the apparent inhibition constants contained two dissociation constants. Values for the latter, determined in other experiments, were used to calculate the apparent inhibition constants which are given in parentheses. [c] A weighted mean value (47 ± 3 μM) of the two values for K_{I2} in the last column was used in the calculation. [d] Values for apparent inhibition constants could not be calculated as a value for K_I was not available.

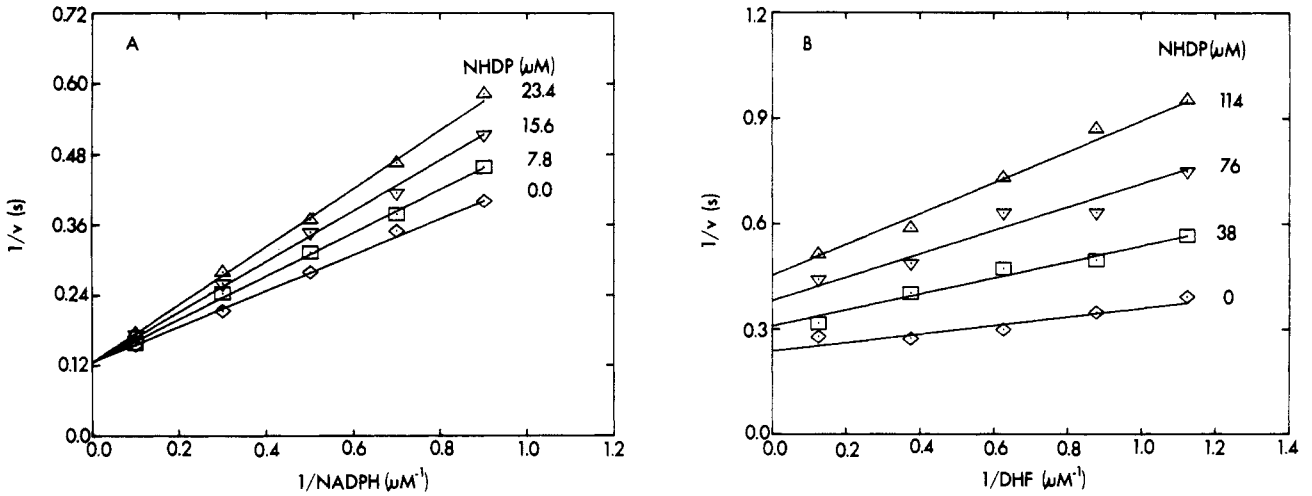

FIGURE 2: Inhibition of the dihydrofolate reductase reaction by NHDP. (A) Linear competitive inhibition with respect to NADPH with DHF concentration held constant at 1.6 μM. (B) Linear noncompetitive inhibition with respect to DHF with NADPH at a fixed concentration of 3.3 μM.

in Table I. At a concentration of 50 μM, and in the presence of saturating DHF, the reduced form of thionicotinamide adenine dinucleotide phosphate exhibited less than 1% of the activity observed at the same concentration of NADPH.

Inhibition of Reaction by Pyridine Nucleotides. The low reaction velocity with TNADPH cannot be accounted for on the basis of its poor ability to combine with enzyme as it acts as a good inhibitor of the reaction with NADPH and DHF as substrates. The inhibition is linear noncompetitive with respect to both substrates (Table II).

NADP, NHDP, ϵ-NADP, APADP, and NAD function as analogues of NADPH, giving rise to inhibitions that are linear competitive with respect to NADPH and linear noncompetitive in relation to DHF. The results obtained with NHDP are shown in Figure 2. By contrast, TNADP behaves in a similar manner to TNADPH in giving rise to inhibitions that are linear noncompetitive with respect to both NADPH and DHF (Figure 3).

Inhibition of Reaction by Fragments of NADP. Fragments of NADP, which include adenosine 5′-phosphate, adenosine

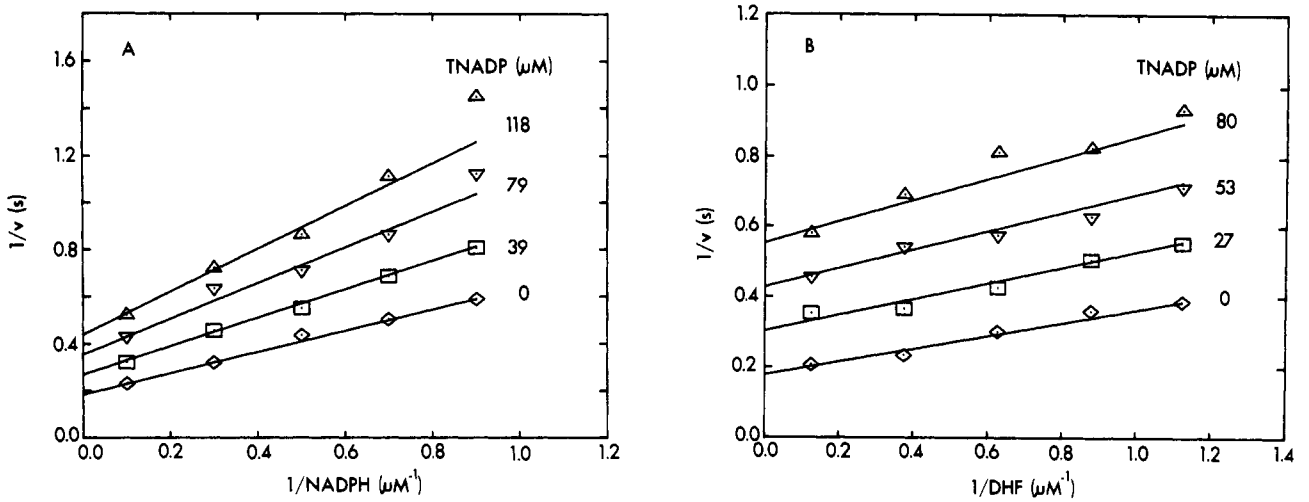

FIGURE 3: Noncompetitive inhibition of the dihydrofolate reductase reaction by TNADP with NADPH (A) and DHF (B) as the variable substrate. The fixed concentrations of DHF and NADPH were 3.0 and 3.3 μM, respectively.

Table III: Dissociation Constants for the Binary and Ternary Complexes Formed by the Interaction of Nucleotides with the Free Enzyme and the Enzyme–DHF Complex

nucleotide	inhibition constant (μM)[a] binary complex (K_i)	ternary complex (K_I)	K_i:K_I ratio
adenosine 5′-phosphate	9700 ± 1100	19000 ± 2000	0.5
adenosine 2′-phosphate	1450 ± 90	1730 ± 680	0.8
adenosine 2′,5′-diphosphate	290 ± 30	170 ± 20	1.7
ADP-ribose	830 ± 60	1600 ± 500	0.5
ATP-ribose	20 ± 2	12 ± 2	1.7
APADP	39 ± 3	38 ± 12	1.0
NHDP	60 ± 7	22 ± 6	2.7
ϵ-NADP	50 ± 9	25 ± 12	2.0
NADP	39 ± 7	17 ± 1	2.3
NAD	1360 ± 170	6760 ± 1400	0.2

[a] The noncompetitive inhibition caused by the nucleotide with respect to DHF was analyzed according to eq 2 to yield values for K_{is} and K_{ii}. Values for the true constants, K_i and K_I, were determined by using the appropriate relationships derived from eq 5 (Smith & Morrison, 1969) together with a fixed concentration for NADPH of 3.0 μM.

2′-phosphate, adenosine 2′,5′-diphosphate, ADP-ribose, and ATP-ribose, act as inhibitory analogues of NADPH (Table III). The inhibition by all derivatives is linear competitive with respect to NADPH and noncompetitive with respect to DHF.

Double Inhibition by TNADP and THF. The data for the variation of the initial velocity of the reaction as a function of the concentrations of both TNADP and THF were fitted to eq 4, and the results are illustrated in Figure 4. The pattern is of the intersecting type, and thus, it can be concluded that both compounds can be present on the enzyme at the same time. By contrast, the double inhibition experiment with NADP and TNADP yielded a parallel pattern (data not shown). Thus, these two pyridine nucleotides cannot be bound simultaneously to the enzyme.

Discussion

In their reduced forms, APADP, NADP, and ϵ-NADP behave as substrates for the reaction catalyzed by dihydrofolate reductase from *E. coli* (Table I). The initial velocity data can be analyzed on the basis that the reaction with each nucleotide, as with NADPH, conforms to a rapid equilibrium, random mechanism (Stone & Morrison, 1982). The results (Table I) then indicate that the presence of one substrate on the enzyme has only a small effect on the binding of the other and that there is little variation in the values of the Michaelis

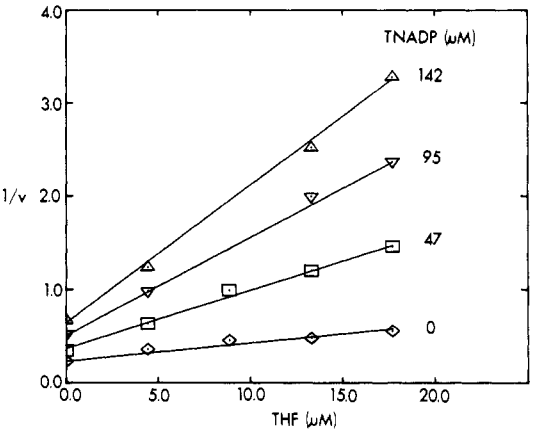

FIGURE 4: Double inhibition of the reaction by TNADP and THF. The fixed concentrations of NADPH and DHF were 33 and 3.0 μM, respectively.

constants for the four nucleotides. Further, they show that the identity of the nucleotide has no effect on the dissociation constant for the enzyme–DHF complex, as expected for a rapid equilibrium, random mechanism, and only a small influence on the Michaelis constant for DHF. However, there are indications that, with NADPH and DHF as substrates, the dihydrofolate reductase reaction only approximates to a rapid equilibrium, random mechanism (Stone & Morrison, 1982; S. R. Stone and J. F. Morrison, unpublished results). Under these circumstances, the kinetically determined values for the dissociation constants of binary enzyme–substrate complexes can be unreliable and differ significantly from those obtained by the utilization of thermodynamic procedures. The difference between the kinetically and thermodynamically determined values for the dissociation constant of the enzyme–NADPH complex (K_{ia}) is recorded in Table I, which also shows that as the maximum velocity of the reaction decreases with the nucleotide so the agreement between the two sets of values improves. Indeed, with APADPH, the two values are not significantly different. Thus, it appears that, for the reaction involving this nucleotide and DHF, catalysis is rate limiting and the kinetic mechanism is truly rapid equilibrium, random.

The small effect of DHF on the binding of pyridine nucleotides to the enzyme, as reflected in the ratio of the values for K_{ia} and K_a (Table I), is in marked contrast to the effect of 2,4-diaminopteridine analogues of folate on the binding of pyridine nucleotides. Unpublished observations in this labo-

ratory indicate that, in the presence of methotrexate, NADPH is bound 50 times more tightly to dihydrofolate reductase from *E. coli*. Synergistic binding of pyridine nucleotides and folate analogues has also been noted by Birdsall et al. (1980a). It appears that such effects are due to conformational changes which occur on the binding of either ligand (London et al., 1979; Feeney et al., 1980a,b; Dunn & King, 1980; Birdsall et al., 1981b; Gronenborn et al., 1981a–c; Dunn et al., 1978; Cayley et al., 1981) and which vary with the identity of the nucleotide (Birdsall et al., 1981b; Gronenborn et al., 1981a).

The relative maximum velocities observed with NADPH, ϵ-NADPH, APADPH, and TNADPH (Table I) are in agreement with the relative activities reported for these compounds by Williams et al. (1977) and Dunn et al. (1978) with the enzyme from *Lactobacillus casei*. However, these two enzymes differ in their ability to utilize NHDPH. Whereas comparable activities are observed for NHDPH and NADPH with the enzyme from *L. casei*, the maximum velocity for NHDPH with the enzyme from *E. coli* is only 40% of that for NADPH. The inability of the dihydrofolate reductases from *E. coli* and *L. casei* to utilize TNADPH could well be due to the difference in the mode of binding of TNADP and NADP (Hyde et al., 1980a,b). A recent report by Feeney et al. (1983) suggests that the thionicotinamide moiety of TNADP does not form any specific interactions with the enzyme but rather extends out into solution.

The oxidized forms of NHDP, ϵ-NADP, and APADP behave similarly to NADP in giving rise to inhibitions that are competitive with respect to NADPH and noncompetitive with respect to DHF (Figure 2, Table III). Such results are as expected for a reaction that approximates to a kinetic mechanism which is of the rapid equilibrium, random type (Stone & Morrison, 1982). On this basis, the inhibitions can be described by eq 5 where A and B represent NADPH and

$$\frac{1}{v} = \frac{1}{V}\left[\frac{K_{ia}K_b}{AB}\left(1 + \frac{I}{K_i}\right) + \frac{K_a}{A}\left(1 + \frac{I}{K_I}\right) + \frac{K_b}{B} + 1\right] \quad (5)$$

DHF, respectively, K_{ia}, K_a, and K_b are as defined in footnote *a* to Table I, and K_i and K_I are the dissociation constants for the enzyme–inhibitor (EI) and enzyme–DHF–inhibitor (EBI) complexes, respectively. The relationships between the true and experimentally determined apparent kinetic constants, as described by Smith & Morrison (1969), were used to calculate the values of the dissociation constants that are listed in Table III. There is little difference in the binding of APADP, NHDP, ϵ-NADP, and NADP to the free enzyme, and the interactions are affected to only a small extent by the presence of DHF on the enzyme. These findings are similar to those with the reduced forms of the pyridine nucleotides. The relatively weak combination of NAD with the free enzyme is further weakened when DHF is bound to the enzyme.

The behavior of the thio analogue of NADP differs from that of the above nucleotides since TNADP acts as a linear noncompetitive inhibitor with respect to both NADPH and DHF (Figure 3). These inhibitions cannot be described by eq 5, and allowance must be made for an additional interaction of TNADP with another enzyme form. One possibility is that TNADP binds to the enzyme–NADPH complex as well as to the free enzyme and the enzyme–DHF complex [cf. Stone & Morrison (1982)]. Such interactions will be described by eq 6 where K_I represents the dissociation constant for the release TNADP from the enzyme–NADPH–TNADP complex and the other symbols are as defined for eq 5. Alternatively, the additional binding of TNADP could be due to its interaction with one of the central complexes, enzyme–NADPH–DHF and enzyme–NADP–THF, or with the enzyme–THF complex which forms after the release of NADP. In either case, the initial velocity equaation would be given by eq 7

$$\frac{1}{v} = \frac{1}{V}\left[\frac{K_{ia}K_b}{AB}\left(1 + \frac{I}{K_i}\right) + \frac{K_a}{A}\left(1 + \frac{I}{K_I}\right) + \frac{K_b}{B}\left(1 + \frac{I}{K_I'}\right) + 1\right] \quad (6)$$

$$\frac{1}{v} = \frac{1}{V}\left[\frac{K_{ia}K_b}{AB}\left(1 + \frac{I}{K_i}\right) + \frac{K_a}{A}\left(1 + \frac{I}{K_I}\right) + \frac{K_b}{B} + 1 + \frac{I}{K_{I2}}\right] \quad (7)$$

where K_{I2} is the dissociation constant for the release of TNADP from a central or an enzyme–TNADP–THF complex and the other symbols are as defined above. The two mechanisms described by eq 6 and 7 can be distinguished on the basis of the type of inhibition given by TNADP with respect to NADPH in the presence of a saturating concentration of DHF (B). Equation 6 predicts linear competitive inhibition while eq 7 predicts linear noncompetitive inhibition. Since the inhibition is still noncompetitive at a concentration of DHF which is about 60 times its Michaelis constant (Table II), the data are in accord with eq 7. This equation was rearranged in the manner described by Smith & Morrison (1969) to determine the relationships between true and apparent inhibition constants. These relationships were then used to calculate dissociation constants for the binding of TNADP to the free enzyme (K_i) and to the enzyme–DHF complex (K_I) as well as to either a central complex or the enzyme–THF complex (K_{I2}) (Table II). The two values which could be calculated for K_{I2} are in fair agreement, and the differences in the values for all three dissociation constants are relatively small. In the presence of a nonsaturating concentration of the changing fixed substrate, it follows from eq 7 that the slope expression with NADPH (A) as the variable substrate and the intercept expression with DHF (B) as the variable substrate contain two dissociation constants. Thus, calculation of values for each constant is precluded. In each case, however, values for the apparent constants can be calculated from the known values of the dissociation constants. The results (Table II) indicate that there is quite good agreement between the experimentally determined and the calculated values.

The inhibition data are qualitatively and quantitatively consistent with a mechanism in which TNADP binds to the free enzyme and the enzyme–DHF complex as well as to either a central complex or an enzyme–THF complex. From the three-dimensional structure of dihydrofolate reductase (Bolin et al., 1982; Filman et al., 1982) it is difficult to envision TNADP binding to the enzyme when both the pteridine and pyridine nucleotide subsites, within the active site, are occupied as in a central complex. The formation of an enzyme–THF–TNADP complex seems more likely to be responsible for the noncompetitive inhibition by TNADP with respect to NADPH (Figure 3). Such a conclusion is in accord with the results of double inhibition experiments which show that an enzyme–THF–TNADP complex can form (Figure 4) whereas an enzyme–NADP–TNADP complex does not. Further, it is in agreement with the finding that substrate activation by NADPH is due to the formation of an enzyme–THF–NADPH complex from which THF is released more rapidly than from the enzyme–THF complex (S. R. Stone and J. F. Morrison

unpublished results). The condition required for the presence of a significant steady-state concentration of enzyme-THF is that the release of THF from the binary complex be slow relative to the other steps of the reaction sequence. The behavior of TNADPH as an inhibitor of the dihydrofolate reductase reaction is similar to that of TNADP. Thus, it can be concluded that the two compounds undergo interactions with the same three forms of the enzyme.

The initial velocity and binding data obtained with APADPH (Table I) indicate that, in the presence of this nucleotide, the dihydrofolate reductase reaction exhibits a rapid equilibrium, random mechanism. This conclusion is supported by the finding that the inhibition pattern given by TNADP with APADPH and DHF as substrates is as expected for such a mechanism (Table II). It appears that with APADPH as the pyridine nucleotide substrate, catalysis is sufficiently rate limiting for the steady-state concentration of enzyme–THF to be kinetically insignificant. Under these conditions, there would be negligible formation of an enzyme–TNADP–THF complex.

Since NADP, ATP-ribose, and adenosine 2′,5′-diphosphate are bound more strongly by dihydrofolate reductase than the corresponding analogues which lack the 2′-phosphoryl group (Table III), it can be concluded that the 2′-phosphoryl group is of importance for the binding of NADP. The binding enhancement is 30–40-fold in the binary complex and 100–400-fold in the ternary complex. It is of interest that the 2′-phosphoryl group of NADPH forms hydrogen bonds and salt bridges with four amino acid side chains of the enzyme from *L. casei* (Filman et al., 1982). Two of these residues (Arg-44 and Gln-65) are conserved in the enzyme from *E. coli* while with the third there is a conservative change from Thr-63 to Ser-63 (Volz et al., 1982; Bennet et al., 1978). The addition of a phosphoryl group to adenosine 2′-phosphate at the 5′-position to form adenosine 2′,5′-diphosphate or of a ribose phosphate moiety to form ATP-ribose further enhances binding. Such enhancements can be accounted for on the basis of the observed hydrogen-bonding and/or hydrophobic interactions of NADPH with the enzyme from *E. coli* (Filman et al., 1982). The nicotinamide moiety of NADP makes little or no contribution to the binding of this nucleotide as judged by a comparison of the dissociation constants for NADP and ATP-ribose (Table III). Similar results are obtained with NAD and ADP-ribose except that the nicotinamide moiety of NAD hinders binding in the ternary complex. Birdsall et al. (1980a) have also reported that the nicotinamide moiety of NADP does not contribute to the binding since NADP and the methyl β-riboside derivative of ATP-ribose have similar dissociation constants for their reactions with the enzyme from *L. casei*. It would appear that the unfavorable effect of having a positively charged nicotinamide ring in the hydrophobic binding site for the pyridine nucleotide is counterbalanced by its specific interactions with groups on the enzyme (Hyde et al., 1980b). The lack of a positive charge on the nicotinamide ring of the reduced pyridine nucleotides could account, at least in part, for their tighter binding in both binary and ternary complexes than the corresponding oxidized nucleotides (Tables I and III). Differences in the environment of bound NADP and NADPH (Hyde et al., 1980b), as well as the conformational changes associated with their binding (Gronenborn et al., 1981c), are factors which can account for the stronger interactions of reduced pyridine nucleotides.

X-ray crystallography and Raman spectroscopy indicate that the carboxamide of the nicotinamide makes three hydrogen-bonding contacts with the enzyme (Filman et al., 1982; Dwivedi et al., 1982). The acetyl group of APADP(H) would be incapable of forming the two hydrogen bonds associated with the amide nitrogen, and yet the tightness of binding of APADP and APADPH differs little, if at all, from that of NADP and NADPH, respectively (Tables I and III). Thus, these bonds do not seem to be improtant in the binding of the pyridine nucleotide. They do, however, appear to be imporant in catalysis since the activity with APADPH is 7-fold lower than that with NADPH (Table I; Filman et al., 1982).

Changes in the structure of the adenine ring had little effect on the binding of the coenzyme. The oxidized and reduced forms of NHDP and ϵ-NADP bind to the enzyme with approximately the same affinity as the respective forms of NADP (Tables I and III). The only direct contacts between the adenine ring and the enzyme from *L. casei* are hydrophobic (Filman et al., 1982). It seems unlikely, however, that these hydrophobic interactions are very strong. The ethenoadenine ring of ϵ-NADP carries a positive charge which would decrease any favorable hydrophobic interactions, and yet this compound is bound as well as NADP to the enzymes from *E. coli* (Table III) and *L. casei* (Birdsall et al., 1980a). The lower activity with ϵ-NADPH and NHDPH suggests, however, that interactions with the adenine ring are imporant for optimal orientation of the pyridine nucleotide for catalysis.

Acknowledgments

We thank Margaret Sneddon for her lively technical assistance.

Registry No. NADPH, 53-57-6; NHDPH, 42934-87-2; ϵ-NADPH, 56973-48-9; APADPH, 2737-69-1; TNADP, 19254-05-8; TNADPH, 38850-22-5; NAD, 53-84-9; NADP, 53-59-8; ϵ-NADP, 56973-46-7; NHDP, 6739-64-6; APADP, 341-67-3; ATP-ribose, 53595-18-9; ADP-ribose, 20762-30-5; adenosine 2′,5′-diphosphate, 3805-37-6; adenosine 2′-phosphate, 130-49-4; adenosine 5′-phosphate, 61-19-8; DHF reductase, 9002-03-3.

References

Bennet, C. D., Rodkey, J. A., Sondey, J. M., & Hirschmann, R. (1978) *Biochemistry 17*, 1328–1337.

Birdsall, B., Burgen, A. S. V., & Roberts, G. C. K. (1980a) *Biochemistry 19*, 3723–3731.

Birdsall, B., Burgen, A. S. V., & Roberts, G. C. K. (1980b) *Biochemistry 19*, 3732–3737.

Birdsall, B., Burgen, A. S. V., Hyde, E. I., Roberts, G. C. K., & Feeney, J. (1981a) *Biochemistry 20*, 7186–7195.

Birdsall, B., Gronenborn, A., Clore, G. M., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1981b) *Biochem. Biophys. Res. Commun. 101*, 1139–1144.

Blakley, R. L. (1960) *Nature (London) 188*, 231–232.

Bolin, J. T., Filman, D. J., Mathews, D. A., Hamlin, R. C., & Kraut, J. (1982) *J. Biol. Chem. 257*, 13650–13662.

Cayley, P. J., Dunn, S. M. J., & King, R. W. (1981) *Biochemistry 20*, 874–879.

Cleland, W. W. (1963) *Biochim. Biophys. Acta 6*, 173–187.

Cornish-Bowden, A., & Endrenyi, L. (1981) *Biochem. J. 193*, 1005–1008.

Dawson, R. M. C., Elliot, D. C., Elliot, W. H., & Jones, K. M. (1969) *Data for Biochemical Research*, Oxford University Press, Oxford.

Dunn, S. M. J., & King, R. W. (1980) *Biochemistry 19*, 766–773.

Dunn, S. M. J., Batchelor, J. G., & King, R. W. (1978) *Biochemistry 17*, 2356–2364.

Dwivedi, C. M., Plante, L. T., Kisliuk, R. L., Pastore, E. J., Verma, S. P., & Wallace, D. F. H. (1982) *Arch. Biochem. Biophys. 213*, 338–340.

Feeney, J., Roberts, G. C. K., Kaptein, R., Birdsall, B., Burgen, A. S. V., & Gronenborn, A. (1980a) *Biochemistry 19*, 2466-2472.
Feeney, J., Roberts, G. C. K., Thomson, J. W., King, R. W., Griffiths, D. V., & Burgen, A. S. V. (1980b) *Biochemistry 19*, 2316-2321.
Feeney, J., Birdsall, B., Roberts, G. C. K., & Burgen, A. S. V. (1983) *Biochemistry 22*, 628-633.
Filman, D. J., Bolin, J. T., Mathews, D. A., & Kraut, J. (1982) *J. Biol. Chem. 257*, 13663-13672.
Gready, J. E. (1980) *Adv. Pharmacol. Chemother. 17*, 37-102.
Gronenborn, A., Birdsall, B., Hyde, E. I., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1981a) *Mol. Pharmacol. 20*, 145-153.
Gronenborn, A., Birdsall, B., Hyde, E. I., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1981b) *Nature (London) 290*, 273-274.
Gronenborn, A., Birdsall, B., Hyde, E. I., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1981c) *Biochemistry 20*, 1717-1722.
Hitchings, G. H., & Smith, S. L. (1980) *Adv. Enzyme Regul. 18*, 349-371.
Hyde, E. I., Birdsall, B., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1980a) *Biochemistry 19*, 3746-3754.
Hyde, E. I., Birdsall, B., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1980b) *Biochemistry 19*, 3738-3746.
London, R. E., Groff, J. P., & Blakley, R. L. (1979) *Biochem. Biophys. Res. Commun. 86*, 779-786.
Matthews, D. A., Alden, R. A., Bolin, J. T., Filman, D. J., Freer, S. T., Hamlin, R., Hol, W. G. J., Kisliuk, R. L., Pastore, E. J., Plante, L. T., Xuong, N., & Kraut, J. (1978) *J. Biol. Chem. 253*, 6946-6954.
Neef, V. G., & Huennekens, F. M. (1976) *Biochemistry 15*, 4042-4047.
Roth, R., & Cheng, C. C. (1982) *Prog. Med. Chem. 19*, 269-331.
Smith, D. R., Rood, J. I., Bird, P. I., Sneddon, M. K., Calvo, J. M., & Morrison, J. F. (1982) *J. Biol. Chem. 257*, 9043-9048.
Smith, E., & Morrison, J. F. (1969) *J. Biol. Chem. 244*, 4224-4234.
Stone, S. R., & Morrison, J. F. (1982) *Biochemistry 21*, 3757-3765.
Stone, S. R., & Morrison, J. F. (1983) *Biochim. Biophys. Acta 745*, 247-258.
Viola, R. E., Cook, P. F., & Cleland, W. W. (1979) *Anal. Biochem. 96*, 334-340.
Volz, K. W., Mathews, D. A., Alden, R. A., Freer, S. T., Hansch, C., Kaufman, B. T., & Kraut, J. (1982) *J. Biol. Chem. 257*, 2528-2536.
Way, J. L., Birdsall, B., Feeney, J., Roberts, G. C. K., & Burgen, A. S. V. (1975) *Biochemistry 14*, 3470-3475.
Williams, J. W., Morrison, J. F., & Duggleby, R. G. (1979) *Biochemistry 18*, 2567-2573.
Williams, T. J., Lee, T. K., & Dunlap, R. B. (1977) *Arch. Biochem. Biophys. 181*, 569-579.

N-Hydroxycarbamate Is the Substrate for the Pyruvate Kinase Catalyzed Phosphorylation of Hydroxylamine†

Paul M. Weiss, Jeffrey D. Hermes, Thomas M. Dougherty, and W. W. Cleland*

ABSTRACT: The true substrate for the pyruvate kinase catalyzed phosphorylation of hydroxylamine at high pH which is activated by bicarbonate is shown to be *N*-hydroxycarbamate, since a lag is seen when the reaction is started by the addition of bicarbonate or hydroxylamine but a burst appears when it is started with a mixture of the two. The lag can be diminished by addition of carbonic anhydrase but not eliminated, showing that CO_2 is an intermediate in the formation of the carbamate and that both the formation of CO_2 and the subsequent reaction of CO_2 with hydroxylamine limit the rate of carbamate formation. The equilibrium constant for the reaction bicarbonate + hydroxylamine ↔ *N*-hydroxycarbamate is 1.33 M^{-1}. The product of the phosphorylation decomposes by loss of CO_2 to *O*-phosphorylhydroxylamine, which is stable at 25 °C between pH 3 and 11 and has $pK_2 = 5.63$ for the phosphate and $pK_3 = 10.26$ for the amino group.

Kupiecki & Coon (1960) first reported that in the presence of bicarbonate, pyruvate kinase would phosphorylate hydroxylamine. The product of the reaction was identified by Cottam et al. (1968) as *O*-phosphorylhydroxylamine by comparison with the same compound prepared by reaction of phosphoramidate and hydroxylamine (Jencks & Gilchrist, 1965). It was reported by Kupiecki & Coon (1960) that in contrast to the physiological reaction between MgADP and phosphoenolpyruvate, the reaction was activated by Zn^{2+} and not by Mg^{2+}. However, Dougherty (1982) has discovered that the reaction is supported by Mg^{2+} at high pH and that V/K decreases a factor of 10 per pH unit below the pK of 9.2 for water in the inner coordination sphere of enzyme-bound Mg^{2+}. Since bicarbonate is an obligate activator, and no alternative one has been found despite an extensive search, the question arises whether it activates the reaction by substrate synergism or whether it combines with hydroxylamine to give *N*-hydroxycarbamate according to reaction 1:

$$HCO_3^- \rightleftharpoons CO_2 + OH^- \quad (1a)$$

$$CO_2 + NH_2OH \rightleftharpoons [HO\text{-}\overset{+}{N}H_2\text{-}COO^-] \xrightarrow{OH^-} HO\text{-}NH\text{-}COO^- \quad (1b)$$

N-Hydroxycarbamate was suggested to be the substrate by Jencks & Gilchrist (1965), but Cottam et al. (1968), while considering it a possibility, were unable to find any evidence either for or against this hypothesis. The overall equilibrium constant for reaction 1 is independent of pH above pH 7.4,

† From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received December 13, 1983.* Supported by a grant from the National Institutes of Health (GM 18938).

0006-2960/84/0423-4346$01.50/0