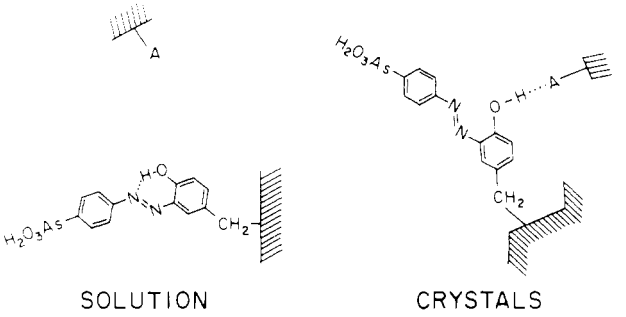

FIGURE 9: Schematic representation of the predominant conformation of the azoTyr-248 residue of azocarboxypeptidase in the solution and crystal phases. The hatched bar represents the protein and the letter "A" an undefined hydrogen-bond acceptor. In the conformation on the left ν^{NN} = 1427 cm^{-1} and $\nu^{\phi N}$ = 1150 cm^{-1}, and in that on the right ν^{NN} = 1456 cm^{-1} and $\nu^{\phi N}$ = 1123 cm^{-1}.

postulated hydrogen bond involves the same proton that is thought to be transferred to the substrate during catalysis.

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Kinetics of Ternary Complex Formation between Dihydrofolate Reductase, Coenzyme, and Inhibitors†

Susan M. J. Dunn‡ and Rodney W. King*

ABSTRACT: The kinetics of ligand binding to dihydrofolate reductase from *Lactobacillus casei* (MTX/R) to form the ternary enzyme–inhibitor–coenzyme complex have been investigated by the stopped-flow fluorescence technique. The fluorescence changes observed when coenzymes or inhibitors bind to the binary complex of the enzyme with the complementary ligand occur in a single fast phase. Under pseudo-first-order conditions the reaction traces could be fitted with precision to a single-exponential decay, and apparent bimolecular rate constants in the range 2×10^6 to 3×10^7 M^{-1} s^{-1} have been measured assuming a bimolecular–unimolecular model. The kinetic constants obtained suggest that prior binding of an inhibitor to the enzyme may, to a minor extent, interfere with coenzyme binding but the rates of inhibitor binding seem to be unaffected by the presence of a bound coenzyme. Dissociation rate constants appear to be less than 1 s^{-1} which suggests that both coenzymes and inhibitors are tightly bound in the ternary complex. An investigation of the effects of pH on the kinetics of ternary complex formation indicated the involvement of ionizable groups in ligand binding, but this shows some ligand dependence. The rates of ligand binding to form the ternary complex are fairly high, but it is unlikely that these associations are diffusion controlled because their measured activation energies of 7.8–14.5 kcal mol^{-1} are higher than expected from reactions whose rates are limited by diffusion in aqueous solution.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (H_2folate)[1] to 5,6,7,8-tetrahydrofolate (H_4folate). The enzyme is therefore necessary for maintaining intracellular pools of THF and its derivatives which are essential cofactors in the many important biosynthetic reactions which require the transfer of one-carbon units. In particular, coupled with thymidylate synthetase, the enzyme

† From the Division of Molecular Pharmacology, National Institute for Medical Research, Mill Hill, London NW7 1AA, Great Britain. *Received August 2, 1979.*
‡ S.M.J.D. held an MRC research studentship. Present address: Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125.

[1] Abbreviations used: H_2folate, 7,8-dihydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; MTX, methotrexate; TMP, trimethoprim; PYR, pyrimethamine.

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is essential for thymidylate biosynthesis and hence for DNA synthesis and cell division.

Dihydrofolate reductases have been isolated from a wide range of bacterial and mammalian species and have been extensively studied not only because of the enzyme's importance in intermediary metabolism but also because it is the target enzyme of a number of clinically important folate analogues which are widely used as antibacterial and antitumor agents [reviewed by Blakley (1969)].

According to the ordered, sequential mechanism thought to characterize most pyridine–nucleotide dependent dehydrogenases, the substrate does not bind to the free enzyme but only to the binary enzyme–coenzyme complex. However, some controversy exists about the kinetic mechanism of dihydrofolate reductase. There have been many reports of the tight binding of both H_2folate and NADPH ($K_d \leq 10^{-7}$ M) to the free enzyme [see Blakley (1969)], and, in addition, it has been shown that the binding of both coenzyme to the binary enzyme–inhibitor complex and inhibitor to the preformed enzyme–coenzyme complex is tighter than to the enzyme alone (Perkins & Bertino, 1966; Hillcoat et al., 1967; Poe et al., 1974). Such mutual stabilization of binding is consistent with a random kinetic mechanism, but it has been suggested (Blakley, 1969; Blakley et al., 1971) that the only process which is kinetically significant is the ordered, sequential mechanism in which NADPH binds first. Some support for this suggestion has come from observations that in some enzyme preparations a conformational change accompanies coenzyme binding (Way et al., 1975; Greenfield, 1975; Cocco et al., 1977).

The relative kinetic significance of the formation of the enzyme–coenzyme and enzyme–substrate complexes has not, therefore, been established. However, it is clear that the binding properties of dihydrofolate reductase are strongly influenced by the presence or absence of a complementary ligand.

We have previously reported the results of a study of the kinetics of coenzyme binding to dihydrofolate reductase isolated from a methotrexate-resistant strain of *Lactobacillus casei* and have shown that the free enzyme exists in at least two interconvertible forms, E_1 and E_2, to one of which, E_1, ligands bind preferentially (Dunn et al., 1978).

This paper describes the kinetics of binding of coenzyme analogues and inhibitors to form the ternary enzyme–coenzyme–inhibitor complex and shows that the kinetics of ternary complex formation appear to be rather simpler than those of binary complex formation.

Experimental Section

Materials

Enzyme. The isolation and purification of dihydrofolate reductase from *L. casei* (MTX/R) has been fully described by Dann et al. (1976). The enzyme used in this investigation was stored at −15 °C in lyophilized form until required. Enzyme concentrations were determined by methotrexate (MTX) titration of enzyme fluorescence and MTX titration of enzymic activity.

Coenzymes. 3-Acetylpyridine-NADP$^+$, deamino-NADPH, and NADPH were obtained from Sigma Chemical Co., Ltd. 1,N^6-Ethenoadenine-NADPH (etheno-NADPH) was prepared as described by Neef & Huennekens (1976). The reduction of 3-acetylpyridine-NADP$^+$ and the assessment of coenzyme purity was carried out as described previously (Dunn et al., 1978).

Inhibitors. MTX was obtained from Nutritional Biochemical Corp. and trimethoprim (TMP) was from Wellcome Laboratories. Pyrimethamine (PYR) and other 2,4-diaminopyrimidine inhibitors were a generous gift from Dr. J. J. Burchall, Burroughs Wellcome Co., Ltd. The structures of these inhibitors are given in Figure 1.

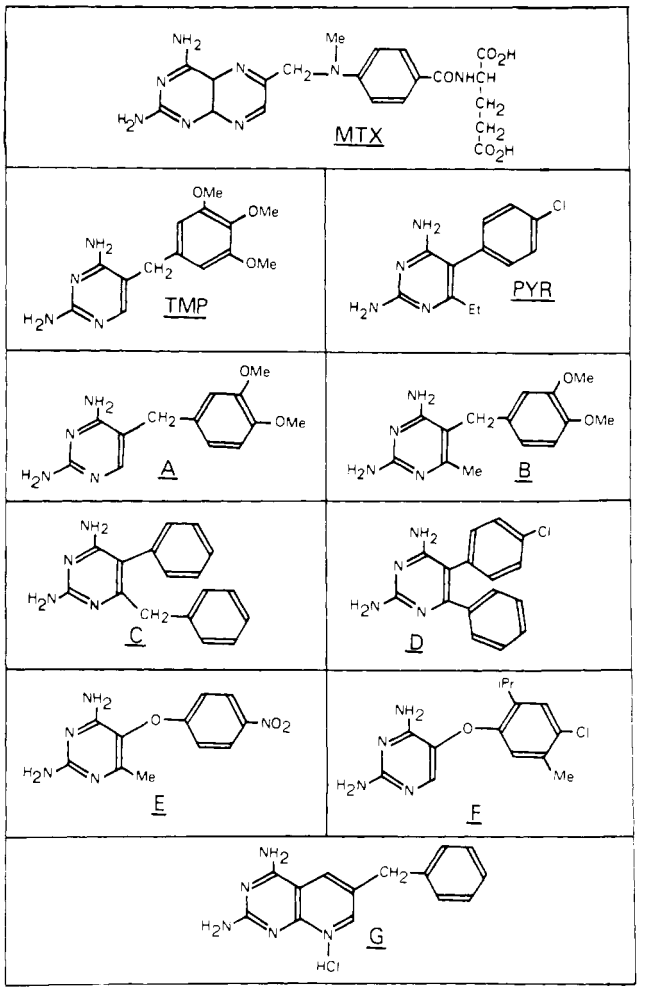

FIGURE 1: Structures of 2,4-diaminopyrimidine inhibitors of dihydrofolate reductase. Abbreviations used: MTX, methotrexate; TMP, trimethoprim; PYR, pyrimethamine. (A) 2,4-Diamino-5-(3′,4′-dimethoxybenzyl)pyrimidine (diaveridine); (B) 2,4-diamino-5-(3′,4′-dimethoxybenzyl)-6-methylpyrimidine; (C) 2,4-diamino-5-phenyl-6-benzylpyrimidine; (D) 2,4-diamino-5-(*p*-chlorophenyl)-6-phenylpyridimine; (E) 2,4-diamino-5-(*p*-nitrophenoxy)-6-methylpyrimidine; (F) 2,4-diamino-5-(2′-isopropyl-4′-chloro-5′-methylphenoxy)pyrimidine; (G) 2,4-diamino-6-benzylpyrido[2,3-*d*]pyrimidine hydrochloride.

Methods

Equilibrium Fluorescence Measurements. Fluorescence emission spectra (uncorrected) were recorded at 25 °C by using a Farrand spectrofluorometer equipped with a 150W xenon (XBO) light source.

Kinetic Measurements. Stopped-flow fluorescence experiments were carried out by using the equipment described in detail by Dunn et al. (1978). The excitation and emission wavelengths used to monitor ternary complex formation are given in the appropriate Results section.

All experiments were carried out at 25 °C unless otherwise stated. Buffer systems used were as follows: pH 5.25–5.5, 15 mM sodium acetate and 500 mM KCl; pH 5.5–7.5, 15 mM Bistris and 500 mM KCl; pH 7.5–8.0, 15 mM Tris and 500 mM KCl.

Some of the 2,4-diaminopyrimidine inhibitors were so poorly soluble in aqueous solution that it was necessary to dilute them from methanolic solution. This was particularly the case with

PYR where the solubility in water was so poor that it was always necessary to use this technique. The concentration of methanol in these experiments did not exceed 1.5% (v/v) and in most cases was much less.

The kinetics of ligand binding to form the ternary complex were investigated by mixing either coenzyme or inhibitor with the preformed binary complex of dihydrofolate reductase and the complementary ligand. The binary complexes were formed by preincubation of dihydrofolate reductase with an excess of the first ligand sufficient to ensure at least 90% saturation of the enzymic binding sites as estimated from the known binding constant of the ligand. The enzyme concentration was 0.5–1.0 μM (final cuvette concentration), and values of the equilibrium dissociation constants of the 2,4-diaminopyrimidine inhibitors, as determined by fluorescence titrations, lay between 4×10^{-7} and 7×10^{-6} M (P. C. Turner and R. W. King, unpublished results). The dissociation constants of the binary enzyme–coenzyme complexes have been published previously (Dunn et al., 1978).

Data Analysis. All kinetic data were analyzed by nonlinear regression using a program written for an HP3000 computer. This program uses the algorithm of Marquardt (1963) which combines the iterative use of the Gauss–Newton and steepest descent procedures for minimizing the sums of squares of residuals. The statistical output included standard errors of the best fit parameters estimated by using the assumption of a linear model about the solution. In all experiments both the raw data and computed best fit curves were plotted so that any deviation from the experimental model could be checked.

The kinetic data were fitted to a single-exponential model:

$$F(t) = Ae^{-k_{app}t} + c$$

where $F(t)$ is the observed fluorescence signal, A is the amplitude of the total signal change, k_{app} is the observed rate constant, t is the time, and c is the data base line at $t = \infty$.

Results

Fluorometric Measurements of Ternary Complex Formation. A necessary preliminary to an investigation of the kinetics of ternary complex formation is the demonstration of conditions suitable for monitoring the binding processes. When dihydrofolate reductase fluorescence is excited at 290 nm, its emission spectrum shows a maximum at 340–350 nm (Figure 2A). The binding of NADPH or one of its analogues quenches this fluorescence, and, in addition, there is an activation of the dihydronicotinamide fluorescence of the coenzyme at 440–450 nm via energy transfer from the protein. This coenzyme fluorescence is also enhanced on binding to the enzyme when it is excited directly at about 350 nm (Figure 2B).

When MTX was mixed with a solution of the enzyme–NADPH complex, the fluorescence of the complex was quenched when it was excited at either 290 or 350 nm as shown in parts A and B of Figure 2, respectively. The fluorescence of the complex was reduced to a level lower than would be expected from the binary complex, which suggests that dihydrofolate reductase, NADPH, and MTX form a nonfluorescent ternary complex. MTX also quenched the fluorescence of the complexes formed between the enzyme and deamino-NADPH, etheno-NADPH, and 3-acetylpyridine-NADPH. Similar results have been reported for the interaction of dihydrofolate reductase from L1210 cells with NADPH and MTX (Perkins & Bertino, 1966). Thus, the binding of MTX to the preformed enzyme–coenzyme complex could be monitored by following the quench of the fluorescence of the complex. The binding of coenzyme to the enzyme–

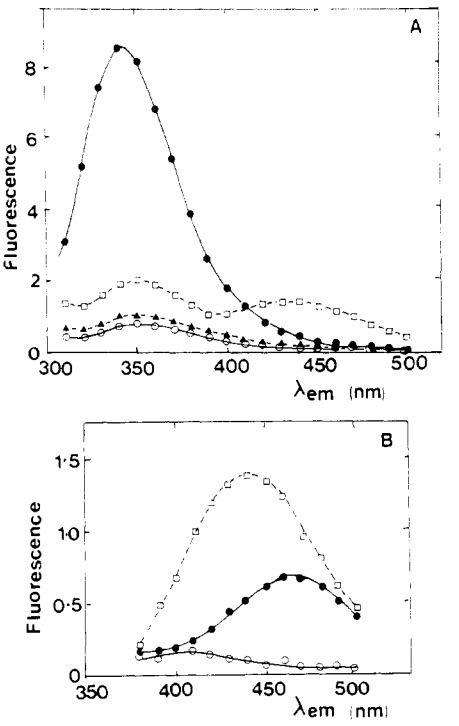

FIGURE 2: Fluorescence spectra of complexes of dihydrofolate reductase, NADPH, and MTX. Spectra (uncorrected) were recorded at pH 6 and 25 °C. The meter multiplier was 0.003. (A) Excitation wavelength was 290 nm. Spectrum of 0.5 μM enzyme alone (●) and in the presence of 0.7 μM MTX (▲), 0.6 μM NADPH (□), and both 0.7 μM MTX and 0.6 μM NADPH (○). (B) Excitation wavelength was 340 nm. Spectrum of 0.6 μM NADPH alone (●) and in the presence of 0.5 μM enzyme (□) and both 0.5 μM enzyme and 0.7 μM MTX (○). Under these conditions MTX alone did not fluoresce detectably.

MTX complex could be monitored by the decay in the fluorescence of the free coenzyme as it formed a nonfluorescent ternary complex.

In contrast to this behavior, the enzyme–NADPH–TMP complex was found to be more fluorescent above 380 nm than either of the two binary complexes when the excitation wavelength was 290 nm. Qualitatively similar results were obtained when the coenzyme was deamino-NADPH or etheno-NADPH, but the fluorescence of the enzyme–3-acetylpyridine complex was quenched by the binding of TMP. Similarly, the enzyme–NADPH–PYR complex exhibited an emission maximum at 425 nm whose amplitude was 50% greater than that exhibited by the enzyme–coenzyme complex and 5 times greater than that of the enzyme–PYR complex.

The fluorescence characteristics of the various ternary complexes were therefore found to be ligand dependent which necessitated the separate evaluation of suitable monitoring conditions for each ligand and complex. A summary of the main conditions used in the kinetic studies is given in Table I.

Effect of Ligand Concentration on the Kinetics of Ternary Complex Formation. The effect of ligand concentration on the kinetics of binding of NADPH to the preformed enzyme–inhibitor complex and of inhibitors to the enzyme–NADPH complex has been investigated at pH 6.5. The fluorescence changes observed in each experiment occurred in a single fast phase and in no case was there any apparent deviation from a single-exponential decay or any evidence of slower processes occurring. When the concentration of the binary complex was kept constant and that of the second ligand was varied, the apparent rate of reaction increased linearly

Table I

Conditions for Monitoring Binding of MTX and TMP to Dihydrofolate Reductase–Coenzyme Complexes

	ligand					
	MTX			TMP		
complex	λ_{ex}	λ_{em}	ΔF	λ_{ex}	λ_{em}	ΔF[a]
E-NADPH	290	449	Q	280	401	E
E-deamino-NADPH	290	435	Q	280	435	E
E-etheno-NADPH	280	401	Q	280	401	E
E-3-acetylpyridine-NADPH	290	449	Q	280	449	Q

Conditions for Monitoring Binding of Coenzyme to Dihydrofolate Reductase–MTX and Dihydrofolate Reductase–TMP Complexes

	complex					
	E-MTX			E-TMP		
ligand	λ_{ex}	λ_{em}	ΔF	λ_{ex}	λ_{em}	ΔF
NADPH	340	449	Q	280	435	E
deamino-NADPH	340	449	Q	280	435	E
etheno-NADPH		*b*		290	401	E
3-Acetylpyridine-NADPH	360	449	Q	280	435	E

Conditions for Monitoring Formation of the Dihydrofolate Reductase–NADPH–PYR Complex

complex	ligand	λ_{ex}	λ_{em}	ΔF
E-NADPH	PYR	290	401	E
E-PYR	NADPH	290	435	E

[a] ΔF = change in fluorescence. Q = quench. E = enhancement. [b] No suitable fluorescence change since the fluorescence of ternary complex is almost identical with that of free etheno-NADPH.

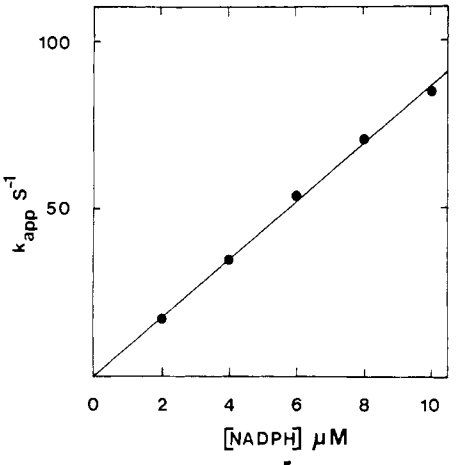

FIGURE 3: Dependence of apparent pseudo-first-order rate constants on ligand concentration. The dihydrofolate reductase–pyrimethamine binary complex reacted with NADPH at 25 °C, pH 6.5, in 15 mM Bistris buffer solution containing 500 mM KCl.

with ligand concentration and showed no evidence of saturation at high concentration.

For a simple association step

$$EL_1 + L_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EL_1L_2$$

the observed rate (k_{app}), under pseudo-first-order conditions, may be approximated by the relationship

$$k_{app} = k_1[L_2] + k_{-1}$$

where k_1 and k_{-1} are the rate constants for the association and dissociation, respectively. Thus, a plot of k_{app} against $[L_2]$ is a straight line of slope k_1 and intercept k_{-1} (Figure 3). Following this scheme, values of association rate constants have been obtained for each ligand at pH 6.5 and 25 °C, and these are given in Table II.

Table II: Association Rates of NADPH Binding to Dihydrofolate Reductase–Inhibitor Complexes and of Inhibitor to the Dihydrofolate Reductase–NADPH Complex[a]

inhibitor[b]	inhibitor binding to the E-NADPH complex k_1 (M^{-1} s^{-1})	NADPH binding to the E-inhibitor complex k_1 (M^{-1} s^{-1})
MTX	1.9×10^7	1.2×10^7
TMP	1.9×10^7	8.5×10^6
PYR	2.2×10^7	6.6×10^6
A	2.1×10^7	8.4×10^6
B	7.0×10^6	1.0×10^7
C	2.9×10^7	7.6×10^6
D		5.4×10^6
E	2.3×10^7	7.9×10^6
F	1.9×10^7	2.5×10^6
G	2.2×10^7	

[a] Values were determined from concentration variation studies at pH 6.5 and 25 °C. [b] Structures are shown in Figure 1.

Table III

Rates of Association of Coenzyme Analogues with Dihydrofolate Reductase–TMP Complexes at pH 6.5 and 25 °C[a]

coenzyme	E-MTX k_1 (M^{-1} s^{-1})	E-TMP k_1 (M^{-1} s^{-1})
NADPH	1.2×10^7	8.5×10^6
deamino-NADPH	5.0×10^6	2.9×10^6
etheno-NADPH		5.6×10^6
3-acetylpyridine-NADPH	5.9×10^6	7.3×10^6

Rates of Association of MTX and TMP with Dihydrofolate Reductase–Coenzyme Complexes at pH 6.5 and 25 °C[a]

complex	MTX k_1 (M^{-1} s^{-1})	TMP k_1 (M^{-1} s^{-1})
E-NADPH	1.9×10^7	1.9×10^7
E-deamino-NADPH	2.7×10^7	2.1×10^7
E-etheno-NADPH	2.9×10^7	2.1×10^7
E-3-acetylpyridine-NADPH	3.0×10^7	2.8×10^7

[a] Values were determined from concentration variation studies.

The rates of binding of the coenzyme analogues NADPH, deamino-NADPH, etheno-NADPH, and 3-acetylpyridine-NADPH to the enzyme–MTX and enzyme–TMP complexes and of inhibitor binding to the various enzyme–coenzyme complexes have also been measured at pH 6.5, and the k_1 values are given in Table III.

In each of these experiments the intercept value of k_{-1} was too small to be measured, meaning that the rates of dissociation of both coenzymes and inhibitors from the ternary complex are very low (<1 s^{-1}). These ligands also bind tightly to the free enzyme, and very low dissociation rates of the coenzyme analogues (Dunn et al., 1978) and of MTX and TMP (R. W. King, unpublished results) from the binary complex have been measured. PYR, on the other hand, has a high dissociation rate constant [approximately 60 s^{-1} at 25 °C (R. W. King, unpublished results)] as measured from the variation in k_{app} with concentration, but it falls to <1 s^{-1} from the ternary complex. Unfortunately, no similar information on the effect of inhibitor presence on coenzyme dissociation rates can be obtained. The binding of the more weakly binding analogue etheno-$NADP^+$ to both free dihydrofolate reductase and to the enzyme–TMP complex has therefore been investigated.

The equilibrium dissociation constant (K_d) of the enzyme–etheno-$NADP^+$ complex was determined in fluorescence titration experiments as 1.2×10^{-5} M at pH 6.0. As a conse-

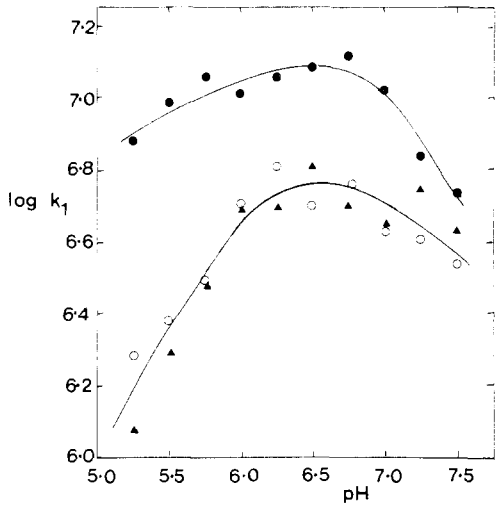

FIGURE 4: Effect of pH on rates of coenzyme binding to the dihydrofolate reductase–MTX complex. Enzyme and MTX concentrations were 0.6 and 1.2 μM, respectively. Values were determined from concentration studies at 25 °C. (●) NADPH; (▲) 3-acetylpyridine-NADPH; (○) deamino-NADPH.

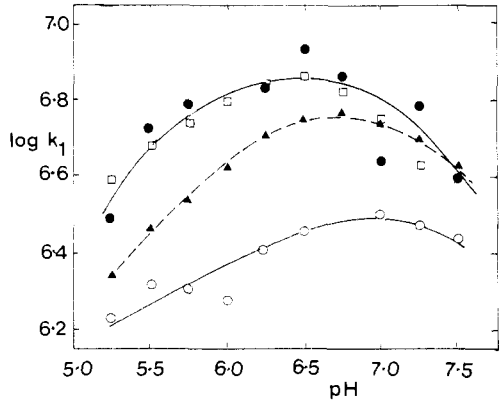

FIGURE 5: Effect of pH on rates of coenzyme binding to the dihydrofolate reductase–TMP complex. Enzyme and TMP concentrations were 0.6 and 2.4 μM, respectively. Values were determined from concentration variation studies at 25 °C. (●) NADPH; (○) deamino-NADPH; (□) etheno-NADPH; (▲) 3-acetylpyridine-NADPH.

quence of this relatively low affinity, the kinetic parameters for the bimolecular association with the enzyme are difficult to measure, but if one assumes a value for k_1 of 1×10^7 M^{-1} s^{-1} on the basis of the very similar association rates measured for other coenzyme analogues (Dunn et al., 1978), a value of 120 s^{-1} can be estimated for k_{-1} from the relationship $K_d = k_{-1}/k_1$. The effect of etheno-NADP$^+$ concentration on its rate of binding to the dihydrofolate reductase–TMP complex has been investigated at pH 6 by using excitation and emission wavelengths of 280 and 401 nm, respectively. The observed k_{app} increased linearly with etheno-NADP$^+$ concentration, and values of 3.6×10^6 M^{-1} s^{-1} for k_1 and 3.2 s^{-1} for k_{-1} have been measured. Thus, the rate of etheno-NADP$^+$ dissociation is probably much lower from the ternary than from the binary complex. The equilibrium dissociation constant calculated from the ratio of rate constants is 8.9×10^{-7} M, which suggests that etheno-NADP$^+$ is bound approximately 10-fold more tightly in the presence of the inhibitor.

Effect of pH on the Kinetics of Ternary Complex Formation. The effect of pH on the kinetics of ternary complex formation has been investigated over the pH range 5.25–7.5. In all these experiments values of k_1 were determined from the dependence of the observed rate on the concentration of

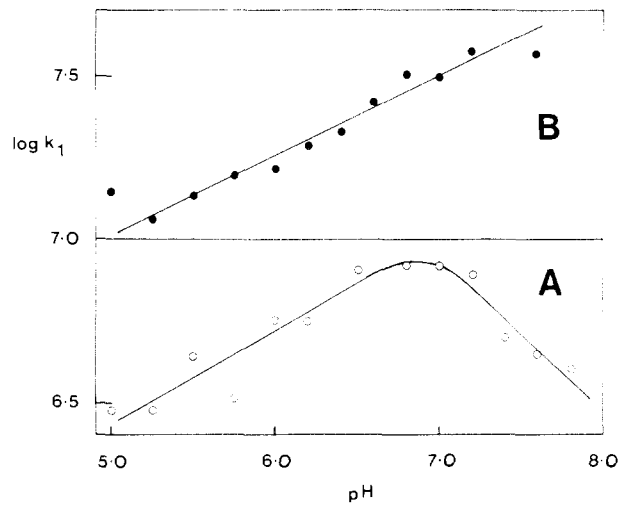

FIGURE 6: Effect of pH on rate of dihydrofolate reductase–NADPH–PYR complex formation. Values were determined from concentration variation studies at 25 °C. (A) Binding of NADPH to the enzyme–PYR complex. Enzyme and PYR concentrations were 1.0 and 10 μM, respectively, except at pH values above 7.0 where the binding constant of PYR decreases sufficiently to make it necessary to add more PYR to ensure saturation. (B) Binding of PYR to the enzyme–coenzyme complex. Enzyme and coenzyme concentrations were 0.5 and 1.1 μM, respectively.

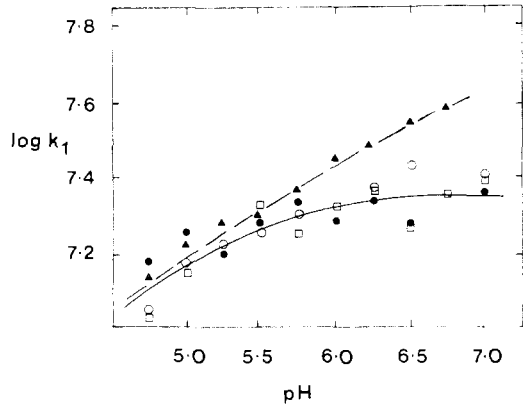

FIGURE 7: Effect of pH on the rate of TMP binding to the enzyme–coenzyme complex. Values were determined from concentration variation studies at 25 °C. Enzyme, 0.6 μM. (●) 1.2 μM NADPH; (□) 2.0 μM etheno-NADPH; (○) 2.0 μM deamino-NADPH; (▲) 2.4 μM 3-acetylpyridine-NADPH.

the second ligand assuming that the interaction is a simple bimolecular association step.

The effect of pH showed some dependence on the nature of both the bound and reacting ligand and is illustrated for coenzyme analogue binding to the enzyme–MTX complex in Figure 4, to the enzyme–TMP complex in Figure 5, and to the enzyme–PYR complex in Figure 6. The data shown in these figures suggest that the dependence of the association rate on pH is not simple but that at least two ionizable groups may be involved in coenzyme binding.

The effect of pH on inhibitor binding to form the ternary complex also showed some ligand dependence. Figure 6, curve B, contains the results of the investigation of the binding of PYR to the enzyme–NADPH complex. A clear dependence of the association rate on pH is observed which can be fitted by a titration curve with a pK of 6.6. The increase in the association rate is nevertheless quite modest, being only just over 3 times between pH 5 and pH 8. The rate of TMP binding to the binary enzyme–coenzyme complex (Figure 7) increased slightly as the pH was raised from 5.25 to 7.5 and was to a certain extent ligand dependent, but the rate of MTX

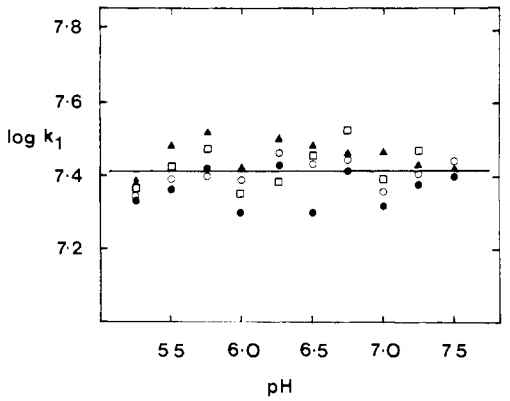

FIGURE 8: Effect of pH on the rate of MTX binding to the enzyme–coenzyme complex. Values were determined from concentration variation studies at 25 °C. Enzyme, 0.6 μM. (●) 1.2 μM NADPH; (□) 2.0 μM etheno-NADPH; (○) 2.0 μM deamino-NADPH; (▲) 2.4 μM 3-acetylpyridine-NADPH.

Table IV: Thermodynamic Parameters for Reaction of Coenzyme Analogues with Dihydrofolate Reductase–Inhibitor Complexes at pH 6.5[a]

coenzyme	E_a (kcal mol^{-1})	$\Delta H^{\ddagger}$ (kcal mol^{-1})	$\Delta S^{\ddagger}$ (cal mol^{-1} deg^{-1})	$\Delta G^{\ddagger}$ (kcal mol^{-1})
Coenzyme Binding to E-MTX				
NADPH	11.2	10.6	9.8	7.6
deamino-NADPH	7.8	7.2	−4.2	8.5
Coenzyme Binding to E-TMP				
NADPH	9.0	8.4	0.6	8.2
deamino-NADPH	11.4	10.8	7.2	8.7
etheno-NADPH	14.5	13.9	18.6	8.4
3-acetylpyridine-NADPH	12.1	11.5	11.4	8.1

[a] Values given are for reaction at 25 °C.

binding showed no obvious pH or ligand dependence (Figure 8).

Effect of Temperature on the Kinetics of Ternary Complex Formation. The effect of temperature on the kinetics of coenzyme analogue binding to the enzyme–MTX and enzyme–TMP complexes and of inhibitor binding to the enzyme–coenzyme complexes has been investigated over the temperature range 10–35 °C at pH 6.5. Kinetic constants were determined by using a single concentration of the second ligand (L_2) under pseudo-first-order conditions in which the concentration of L_2 exceeded that of dihydrofolate reductase in the binary complex by a factor of 10. Under these conditions and when the dissociation rate, k_{-1}, is negligible, k_1 of the fast phase can be estimated from the relationship $k_{app} = k_1[L_2]$. Data points were collected for reaction at eight or more temperatures, and in each experiment the data conformed to the Arrhenius Law in that a plot of ln k_1 against the reciprocal of the absolute temperature was linear with no evidence of curvature. The activation energy, E_a, was calculated from the slope of the Arrhenius plot, and the thermodynamic activation parameters $\Delta H^{\ddagger}$ (enthalpy), $\Delta S^{\ddagger}$ (entropy), and $\Delta G^{\ddagger}$ (free energy) were calculated as described previously (Dunn et al., 1978).

The results of these experiments for coenzyme analogue binding to enzyme–inhibitor complexes are given in Table IV and those for the binding of inhibitors to enzyme–coenzyme complexes are given in Table V.

Discussion

Our previous studies (Dunn et al., 1978) of the kinetics of coenzyme binding to *L. casei* dihydrofolate reductase suggested

Table V: Thermodynamic Parameters for Reaction of Inhibitors with Dihydrofolate Reductase-Coenzyme Complexes at pH 6.5[a]

complex	E_a (kcal mol^{-1})	$\Delta H^{\ddagger}$ (kcal mol^{-1})	$\Delta S^{\ddagger}$ (cal mol^{-1} deg^{-1})	$\Delta G^{\ddagger}$ (kcal mol^{-1})
MTX Binding to the E-Coenzyme Complex				
E-NADPH	9.2	8.6	4.4	7.3
E-deamino-NADPH	10.2	9.6	7.6	7.3
E-3-acetylpyridine-NADPH	10.8	10.2	10.2	7.2
TMP Binding to the E-Coenzyme Complex				
E-NADPH	10.4	9.8	7.5	7.6
E-deamino-NADPH	10.1	9.5	6.7	7.5
E-etheno-NADPH	11.0	10.4	10.0	7.5
E-3-acetylpyridine-NADPH	12.8	12.2	16.4	7.3

[a] Values given are for reaction at 25 °C.

that this enzyme exists in at least two interconvertible forms which we designated E_1 and E_2. Form E_1 predominates at low pH and binds the coenzyme tightly in a rapid bimolecular step, while form E_2 binds the coenzyme weakly, if at all. The kinetic scheme proposed was

$$\begin{array}{ccc} E_1 + L & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & E_1L \\ k_2 \big\uparrow \big\downarrow k_{-2} & & k_4 \big\uparrow \big\downarrow k_{-4} \\ E_2 + L & \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} & E_2L \end{array}$$

In the case of NADPH and its very close structural analogues deamino-NADPH and etheno-NADPH, the binding appears to take place exclusively via the E_1 pathway, E_2 only participating in the reaction after conversion to the E_1 form. With the coenzyme analogues 3-acetylpyridine-NADPH, thionicotinamide-NADPH, and $NADP^+$ and with folate, dihydrofolate, and all the folate analogue inhibitors so far examined (R. W. King, unpublished results), the whole scheme operates.

This scheme is also complicated by ionization effects and by probable further steps, not detected by our technique, leading to more stable forms of the EL complex.

By comparison, the kinetics of binding of coenzymes to the binary enzyme–inhibitor complex and of inhibitors to the enzyme–coenzyme complex are apparently much simpler. Under pseudo-first-order conditions, the reaction traces consisted of a single fast decay whose rate increased linearly with ligand concentration. Within the experimental limits set at the higher concentrations by the optical absorption properties of the reacting system and at the lower concentrations by the sensitivity of the instrument, there were no signs of saturation effects. The fluorescence change could be fitted with high precision to a single-exponential decay. This behavior is consistent with a simple bimolecular association

$$EL_1 + L_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EL_1L_2$$

in which the equilibrium is far to the right, so that dissociation of L_1 from EL_1L_2 need not be taken into account. This proved to be the case as the plots of k_{app} against ligand concentration intersected the k_{app} axis near to the origin, showing that the dissociation rate constant was very small but making an accurate determination of k_{-1} impossible (Figure 3).

By contrast with the free enzyme, it appears that the enzyme–coenzyme or enzyme–inhibitor binary complex exists in a single form, to which the complementary ligand can

rapidly bind. There is some supporting evidence for this from NMR studies (Roberts et al., 1977) and from our own unpublished studies on the effect of preincubation of the enzyme with one of the substrates on the activity of the enzyme. The binding of ligands causes a marked sharpening of the broad lines in the ^1H NMR spectrum of the free enzyme and also in the ^{19}F NMR spectrum of dihydrofolate reductase containing 6-fluorotryptophan or 3-fluorotyrosine. Preincubation of the enzyme with either of its substrates causes a marked pH-dependent increase in the initial catalytic activity when the reaction is initiated by adding the other substrate, compared with the activity when the reaction is initiated by adding pure enzyme to the mixed substrates. These observations are consistent with the interpretation that the free enzyme exists in more than one interconvertible conformation while the binary complexes are essentially in one conformation.

The rate constants obtained for inhibitor binding to the enzyme–NADPH complex are remarkably consistent [and correspond closely with the values for the fast phase binding to the free enzyme (R. W. King, unpublished results)]. Although the values were obtained at a single pH value and are therefore not strictly comparable since it was shown earlier that there is some pH dependence, it is notable that the highest value, 2.9×10^7 M^{-1} s^{-1} for compound C, is only 4 times greater than the lowest, 7.0×10^6 M^{-1} s^{-1} for compound B. This low value is in fact somewhat anomalous as the remaining k_1 values are in a narrow range from 1.9×10^7 to 2.3×10^7 M^{-1} s^{-1}. It is difficult to understand why this value for compound B should be so low on the basis of chemical structure.

The rates of coenzyme binding to the various enzyme–inhibitor complexes are slightly lower than that of 1.6×10^7 M^{-1} s^{-1} measured for the binding to the free enzyme (Dunn et al., 1978). A somewhat more marked inhibitor dependence of k_1 was also found, and these results suggest that the presence of a previously bound inhibitor interferes to a minor extent with coenzyme binding and, thus, that the coenzyme binding site may be a little more restricted in the enzyme–inhibitor binary complex than in the fast-reacting form of the free enzyme. The X-ray structure of the NADPH–MTX–dihydrofolate reductase ternary complex (Matthews et al., 1978) is unfortunately of little assistance in attempting to confirm or deny this since the corresponding X-ray structures of the fast-reacting form of the free enzyme and of the binary complexes are not yet available. The differences noted in compounds A–C and E when they bind to the enzyme–coenzyme complex have disappeared almost completely, and they now lie in the main group between 8.0×10^6 and 10.0×10^6 M^{-1} s^{-1}. Compound F, which has an isopropyl group on the 5-phenoxy function, may cause some small conformational strain in the binary complex in order to accommodate this larger than normal side chain in the 5 position, or, alternatively, it may protrude more toward the nicotinamide binding site. The latter explanation seems most likely as it behaves quite normally when binding to the enzyme–coenzyme complex. MTX itself binds most rapidly to the preformed complex, and the fact that it would be expected on the basis of simple diffusion-limited collision theory to bind more slowly than inhibitors of lower molecular weight must be offset by the molecule's close resemblance to the substrate.

The effect of variation in the coenzyme structure on the rate of binding to enzyme–inhibitor complexes and on the rate of inhibitor binding to the enzyme–coenzyme (or analogue) complexes reveals a similar pattern. Association rate constants for inhibitor binding are little affected by bound coenzyme while the rate constants for coenzyme binding to enzyme–inhibitor complexes are consistently lower than for fast-phase binding to the free enzyme.

What is quite clear from these results is that the binding of neither coenzyme nor inhibitor to the binary complex of the enzyme with the complementary ligand is in any way limited by the dissociation of that ligand. This means that in view of the close structural similarity of the inhibitors and dihydrofolate the initial binding of coenzyme and substrate to *L. casei* dihydrofolate reductase could be a random process and need not, as has been suggested from some steady-state studies with enzyme from other sources (Blakley et al., 1971), require an ordered addition of ligands.

The results of all the experiments on the kinetics of ternary complex formation indicate that the rates of the dissociation of both coenzymes and inhibitors from the ternary complexes are very low. Furthermore, the rate of etheno-$NADP^+$ dissociation appears to be much reduced in the presence of TMP, and the results suggest that this analogue is bound approximately 10-fold more tightly to the enzyme–TMP complex than to the enzyme alone. These findings are consistent with previous reports that the affinity of dihydrofolate reductase for coenzymes, substrates, and inhibitors is greater in the ternary than in the binary complex (Perkins & Bertino, 1966; Hillcoat et al., 1967; Poe et al., 1974; Burgen and Birdsall, personal communication).

The effect of pH changes on the kinetics of ternary complex formation is undoubtedly complicated and not amenable to simple analysis. The data for coenzyme binding (Figures 4–6) show that there is an involvement of ionizing groups in the binding process and that the maximum value of the forward rate constant occurs at pH 6.5–7.0. The shape of the curves suggests the explanation that a pair of ionizing groups control the binding, but attempts to fit a simple exclusive two-group scheme to the data were unsuccessful, giving large errors.

The shape of the log k_1 vs. pH curve is approximately the same for each coenzyme and enzyme–inhibitor combination, although the maximum values differ slightly, dependent on the bound inhibitor. The pK value for the 2′-phosphate group of the coenzymes in free solution is 6.1, and the charge state of the 2′-phosphate group is important in the equilibrium binding of NADPH and NADP (Feeney et al., 1975), the binding of the anion being preferred. The fall off in k_1 below pH 6.5 may be partially explained by the protonation of the 2′-phosphate group in the bulk solution, removing some electrostatic attraction between the ligand and the binding site.

The fall in k_1 above pH 6.5–7.0 demonstrated by all of these coenzymes when binding to preformed binary complexes was not shown by coenzymes binding to the apoenzyme (Dunn et al., 1978). The conclusion can be drawn that a liganded inhibitor modifies the coenzyme binding site in such a way that one or more previously unimportant ionizing groups now affect the association rate significantly. The mechanism whereby this happens is unclear as it could involve direct electrostatic repulsion effects or ionization-induced conformational changes in the binding site.

The binding of MTX to the preformed enzyme–coenzyme complexes shows a high value for the association rate constant essentially invariant with the coenzyme and also independent of the solution pH. Simple rate theory predicts (Czerlinski, 1966; Caldin, 1964) that association rates can be increased by less than 1 order of magnitude by electrostatic attractions, particularly in media of high ionic strength, but may be strongly affected by repulsions, and vice versa for dissociation rate constants. This suggests that the initial binding step is unaffected by the ionization of the N1 nitrogen of MTX which

occurs with a pK of 5.7 (Poe, 1973) and which appears to be responsible for the 1000 times stronger binding of the N1 protonated form of MTX to *L. casei* dihydrofolate reductase than the unprotonated form in the binary complex (Hood & Roberts, 1978). If a similar situation pertains in the ternary complex (unfortunately, the binding constant is too high for accurate measurement), then the effect must manifest itself in a dissociation rate constant which is different for the cationic and neutral forms or in a difference in some subsequent binding step not detected by stopped-flow fluorescence methods, such as that described by Williams et al. (1979) for MTX binding to *Streptococcus faecium* dihydrofolate reductase.

Trimethoprim binding to the binary complex does demonstrate a small dependence (2.0–2.5 times in the range pH 4.5–7.0 dependent upon the coenzyme) on the pH of the solution. This effect is clearly not due to an exclusive discrimination between a charged and uncharged species of the enzyme or the TMP since the change is not large enough over the pH range. A similar situation pertains in the case of pyrimethamine binding where the change in k_1 with pH can be fitted by a titration curve with a pK of 6.6. A curve of this type can be explained by a differential association rate constant for a charged and uncharged species rather than an all or none interaction. The particular ionization involved may be a group in either the enzyme or the ligand. If all ligands bind in approximately the same way, then ionization of an enzymic group should produce a ligand-independent pH dependence. If the ionization is of the ligand, then a correspondence between the measured pK and that of the k_1 vs. pH curve should be apparent. The results with these three inhibitors show that the ligands do not all bind in the same way and that ionization of groups on the enzyme affects different ligands in a different way.

The magnitude of the rate constants for ligand binding to form the ternary complex is fairly large and, as has been noted earlier, spreads across a fairly small range. Differences in binding constants are presumably then reflected in differences in dissociation rates. This is the situation which would be expected on simple theoretical grounds where the rate of association is diffusion controlled while the dissociation depends on the relative strengths of the noncovalent bonds formed between the enzyme binding site residues and the ligand. However, the reality is not quite so simple. The thermodynamic data in Tables IV and V indicate that the initial association is not diffusion controlled since the activation energies recorded between 7.8 and 14.5 kcal mol^{-1} are considerably higher than those of about 4 kcal mol^{-1} expected for diffusion limitation. This suggests that the initial complex may undergo a rapid rearrangement:

$$EL_1 + L_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EL_1L_2^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EL_1L_2$$

In this scheme the species $EL_1L_2^*$ will be present in only a very small concentration relative to the species EL_1 and EL_1L_2, and the bimolecular rate constant obtained from the analysis, $k_{1,app}$, is given by $k_{1,app} = k_1k_2/(k_{-1} + k_2)$. Such a scheme has previously been proposed to explain the kinetic behavior seen when coenzyme analogues bind to the free enzyme (Dunn et al., 1978) and the kinetic behavior of sulfonamide binding to carbonic anhydrase (King & Burgen, 1976).

A comparison of the activation parameters for coenzyme binding to the enzyme–inhibitor complex with those of binding to the free enzyme (Dunn et al., 1978) indicates a significant difference in the entropic contribution to the binding process. Binary complex formation is accompanied by a decrease in entropy which is consistent with a tightening of the enzyme structure during binding or with an increase in polarity which results in increased solvent binding (Laidler, 1955). In contrast, the positive values of ΔS^* for coenzyme binding to the enzyme–MTX and enzyme–TMP complexes suggest that the enzyme assumes a more open conformation or that charge neutralization occurs during binding.

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