Kinetics of Substrate, Coenzyme, and Inhibitor Binding to *Escherichia coli* Dihydrofolate Reductase†

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ABSTRACT: Reduced nicotinamide adenine dinucleotide phosphate (NADPH), folate, dihydrofolate, and the inhibitors trimethoprim and methotrexate bind rapidly and reversibly to both dihydrofolate reductase isoenzymes isolated from *Escherichia coli* RT500. The coenzyme and substrates appear to bind to only one of the mixture of two forms of the isoenzyme present at equilibrium, while the inhibitors bind to both forms. The proportions of the two forms are different for the two isoenzymes and are pH dependent in each case. The measured association rate constants for substrates and inhibitors lie in the range (1–2) × 10^7 M^{-1} s^{-1} at 25 °C but are unlikely to be diffusion controlled. The rate constant for NADPH binding is 2 × 10^6 M^{-1} s^{-1}. The formation of binary complexes takes place through a multistep mechanism. A minimum of three steps is required to explain the kinetic results. An equilibrium between two or more forms of the enzyme–ligand complex governs the overall dissociation. The stability of this equilibrium is largely responsible for the tighter binding of inhibitors relative to substrate or coenzyme and also for the different binding strengths of inhibitors to the isoenzymes.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) is an enzyme of considerable pharmacological interest. It is the target enzyme for several antifolate drugs such as the antitumor agent methotrexate (MTX)[1] and the antimalarial trimethoprim (TMP). Furthermore, the inhibitory pattern of these antifolates varies considerably from species to species, and it is partly from this that the great utility of these drugs arises.

Dihydrofolate reductase isolated from *Lactobacillus casei* has been the subject of extensive stopped-flow kinetic studies to establish the mechanism of binding of substrates and inhibitors (Dunn et al., 1978; Dunn & King, 1980; R. W. King et al., unpublished results). This enzyme was shown to be an equilibrium mixture of at least two interconvertible conformers, only one of which formed tightly bound binary complexes. The binary complexes, on the other hand, exist in only one form and bind the complementary ligand in a fast, but not diffusion-limited, step.

The dihydrofolate reductase purified from *Escherichia coli* B RT500 is a mixture of two isoenzymes, named form I and form II, which differ by only one unit of charge (Baccanari et al., 1977). The molecular weight of the isoenzymes from their amino acid analysis is ~18 200 (Stone et al., 1977) and is identical for each isoenzyme (Baccanari et al., 1977). Some steady-state kinetic measurements have been reported which indicate an inhibitor-dependent difference in binding to the two forms of 5–80-fold.

We have made stopped-flow kinetic measurements of both the association and dissociation rates of ligands to and from the purified forms of the *E. coli* enzyme. The results obtained from the formation and dissociation of binary complexes of both forms of the enzyme are compared with each other and with those obtained from the *L. casei* enzyme.

Experimental Procedures

Materials

Enzyme. *E. coli* B RT500 (a TMP-resistant strain of *E. coli* generously donated by Dr. J. J. Burchall, Burroughs-Wellcome) was grown in 400 L of culture following the small-scale procedure described by Baccanari et al. (1975) except that the glucose concentration was increased to 1.5% (w/v), giving a 5-fold improvement in the yield of cells.

The harvested wet cells (4 kg) were then treated to produce a mixture of the pure isoenzymes by the procedure described for *L. casei* dihydrofolate reductase in Dann et al. (1976) with the following modifications. (i) The gradient used to elute the enzyme from the first DEAE-23 column was from 100 mM KCl/25 mM potassium phosphate buffer, pH 6.5, to 1 M KCl/25 mM potassium phosphate buffer, pH 6.5. (ii) Folate was finally removed from the purified isoenzyme mixture on a column of DEAE-cellulose by using 25 mM potassium phosphate/500 mM KCl buffer, pH 8.5, for elution.

The isoenzymes were separated by affinity chromatography using an MTX resin. Mixed isoenzymes (300 mg) in 50 mL of 100 mM potassium phosphate/1 M KCl buffer, pH 7.0, were pumped onto a 40-mL column of affinity resin preequilibrated with 50 mM potassium phosphate/100 mM KCl buffer, pH 7.0. The column was then washed with 300 mL of 200 mM potassium borate/1 M KCl buffer, pH 9.0, and the enzymes were eluted with 1 mM folic acid/50 mM Tris-HCl/500 mM KCl buffer, pH 8.0. The effluent was passed through a hollow-fiber dialysis unit circulated with 10 mM potassium phosphate buffer, pH 6.0, to remove excess folate. The elution was monitored by assaying the fractions at pH 5.0 and 7.0. Form II, which is 5 times more active at pH 5.0 than at pH 7.0, eluted first. Form I, which is 5 times more active at pH 7.0 than at pH 5.0, eluted in a second, well-separated peak. The isoenzyme pools were lyophilized, and bound folate was removed by further chromatography on DEAE-23 cellulose and Sephadex G-25 as described for the mixed isoenzymes. The purified isoenzymes were stored in the lyophilized form at −15 °C. Overall recovery of activity from the wet cells was 36%, yielding 400 mg of form I and 850 mg of form II.

It has been shown that the enzyme can exist in multiple forms through oxidation of the sulfhydryl groups (Baccanari

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[1] Abbreviations used: H_2folate, 7,8-dihydrofolate; MTX, methotrexate; TMP, trimethoprim; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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Table I: Equilibrium Affinity Constants (K_a) from Fluorescence Quenching Experiments at pH 7.0 and 25 °C

ligand	% quench	K_a (M^{-1}) form I	K_a (M^{-1}) form II
NADPH	65 ± 5	$7.0 \pm 0.4 \times 10^5$	$9.0 \pm 0.4 \times 10^5$
H_2folate	60 ± 5	$2.0 \pm 0.2 \times 10^6$	$2.0 \pm 0.2 \times 10^6$
folate	55 ± 5	$6.0 \pm 0.4 \times 10^5$	$3.0 \pm 0.2 \times 10^6$
MTX	90 ± 5	$>>10^8$	$>>10^8$
TMP	20 ± 5	$>10^8$	$1.0 \pm 0.2 \times 10^7$

et al., 1977). In our hands, the enzyme was not oxidized or aggregated as judged by polyacrylamide gel electrophoresis of samples in the presence and absence of dithiothreitol. The precaution of adding 1 mM dithiothreitol was, nevertheless, followed to ensure that the sulfhydryl groups were fully reduced.

Trimethoprim, folic acid, dihydrofolic acid, and NADPH were obtained from Sigma. Methotrexate was obtained from Nutritional Biochemical Corp.

Unless otherwise stated, the buffer systems used were the following: pH 4.5–5.5, 15 mM sodium acetate and 500 mM KCl; pH 5.5–7.5, 15 mM bis-tris and 500 mM KCl; pH 7.5–8.5, 15 mM Tris and 500 mM KCl.

Methods

Equilibrium dissociation constants were measured as described by Dunn et al. (1978) using a Perkin-Elmer MFP 44A spectrofluorimeter.

Reaction rate measurements were made with a Durrum D110 stopped-flow apparatus operating in the fluorescence mode (Durrum Instruments Corp.). Descriptions of modifications to the apparatus and data analysis are given in Dunn et al. (1978). Experimental conditions are quoted with the results.

Results

Equilibrium Fluorescence Measurements. Excitation of either form of *E. coli* dihydrofolate reductase at 290 nm produces a maximum uncorrected fluorescence emission at 338 nm. Addition of coenzyme, substrates, or inhibitors causes quenching of the fluorescence to varying extents as shown in Table I. The binary complex of NADPH with the enzyme also gave rise to an emission maximum at 435 nm resulting from protein-to-ligand energy transfer as described by Velick (1958) for dehydrogenase enzymes.

The fluorescence quenching which accompanies ligand binding was used to determine equilibrium affinity constants and to monitor the rates of association and dissociation of the ligands. A 1:1 overall stoichiometry of binding was assumed for analysis of the results, and we have found no evidence for multiple binding sites with any of the ligands used. The results of equilibrium experiments carried out at pH 7.0 and 25 °C are reported in Table I.

Trimethoprim binds more tightly to form I than to form II, as do some trimethoprim analogues (R. W. King, unpublished results). The affinity constant for methotrexate is too high to be measured by this method, but the differential binding of the isoenzymes to the methotrexate affinity resin during the purification procedure clearly indicates that the form I isoenzyme binds MTX more tightly (Cayley et al., 1979). Baccanari et al. (1977) have reported a similar effect on inhibition constants for TMP and three other powerful inhibitors. In contrast, the substrates show a smaller differential binding to the isoenzymes, and there is no difference in coenzyme binding. It should, nevertheless, be borne in mind that while the apparent K_m for NADPH is very similar to both isoenzymes, the K_m for H_2folate is 8.9 μM for form I but 0.65 μM for form II (Baccanari et al., 1977).

Scheme I

$$E_1 + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_1L^*$$

Scheme II

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

The affinity constants for NADPH and folate have been examined over the pH range 5–7.5 and the results show that K_a is independent of pH for both ligands and both isoenzymes.

Kinetic Properties: Qualitative Description. When the enzyme was reacted with greater than equimolar concentrations of NADPH, dihydrofolate, or folate, a fast quenching of the enzyme fluorescence was observed, followed by a much slower quenching process. The rate of the fast process was dependent on the ligand concentration while the rate of the slow process (except for H_2folate reacting with form II) showed no concentration dependence. Both the rapid quenching (under pseudo-first-order conditions) and the slow process fitted single-exponential decay curves with precision.

In contrast, the inhibitors MTX and TMP caused only a rapid quenching of the enzyme fluorescence. In the case of MTX, this decay did not fit satisfactorily to a single-exponential decay curve but could be fitted with precision by summing two exponentials, as shown in Figure 1. Here the k_{app} values of the two exponentials differ by a factor of only 2–4 times and are strongly correlated. Both apparent rates are concentration dependent. The rather poor signal/noise ratio combined with the small amplitude of the signal change for TMP binding made discrimination between a single- or double-exponential fit impossible, even after averaging 16 transients.

Effect of Ligand Concentration on the Fast Quenching Process. NADPH, Folate, and Dihydrofolate. When the enzyme concentration was kept constant at 0.5–0.25 μM (final cuvette concentration) and the ligand concentration was increased from 1 to 10 μM, the observed first-order rate constant increased linearly with no sign of saturation. This behavior is typical of a bimolecular association reaction opposed by a unimolecular dissociation reaction as shown by

$$E + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EL$$

where the measured rate constant, k_{app}, is related to the forward and backward rate constants k_1 and k_{-1}, respectively, by

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

Values of k_1 and k_{-1} can then be obtained from a plot of k_{app} against [L] as shown in Figure 2.

Inhibitors. Under similar conditions to those described above for the coenzyme and substrates, MTX binding to the enzyme also gave a linear relationship between the two apparent rate constants and the inhibitor concentration.

The Slow Quenching Process. The ligand-concentration-independent slow process which occurs when coenzyme or substrates bind to the enzyme is typical of a reaction scheme involving a unimolecular isomerization. Two minimal schemes (Schemes I and II) which can encompass this behavior have previously been proposed to explain the very similar kinetic

Table II

A. Fast-Phase Kinetics of Ligand Binding to *E. coli* DHFR[a]

ligand	form I		form II	
	k_1 (M^{-1} s^{-1})	k_{-1} (s^{-1})	k_1 (M^{-1} s^{-1})	k_{-1} (s^{-1})
NADPH[d]	$1.9 \pm 0.2 \times 10^6$	7.5 ± 0.5	$1.7 \pm 0.2 \times 10^6$	2.4 ± 0.5
H_2folate[d]	$18 \pm 2 \times 10^6$	28 ± 2	$15 \pm 2 \times 10^6$	13 ± 1
folate[b,d]	$5 \pm 1 \times 10^6$	35 ± 3	$4.2 \pm 1 \times 10^6$	20 ± 2
MTX 1[c]	$4 \pm 1 \times 10^6$	≤ 1	$3.3 \pm 0.5 \times 10^6$	≤ 1
MTX 2[c]	$16 \pm 4 \times 10^6$	10 ± 1	$17 \pm 2 \times 10^6$	3.3 ± 0.5
TMP[d]	$20 \pm 3 \times 10^6$	15 ± 2	$12 \pm 1 \times 10^6$	12 ± 1

B. Rate Constants for Slow-Phase Binding

ligand	k, form I (s^{-1})	k, form II (s^{-1})
NADPH	$3.3 \pm 0.3 \times 10^{-2}$	$2.5 \pm 0.3 \times 10^{-2}$
H_2folate	$5.4 \pm 0.4 \times 10^{-2}$	
folate	$6.1 \pm 0.5 \times 10^{-2}$	$4.9 \pm 0.5 \times 10^{-2}$

[a] Values were determined from concentration dependence studies at pH 7.0 and 25 °C. [b] Values were determined at 10 °C. [c] Analyzed as the sum of the two exponentials. [d] Analyzed as a single exponential.

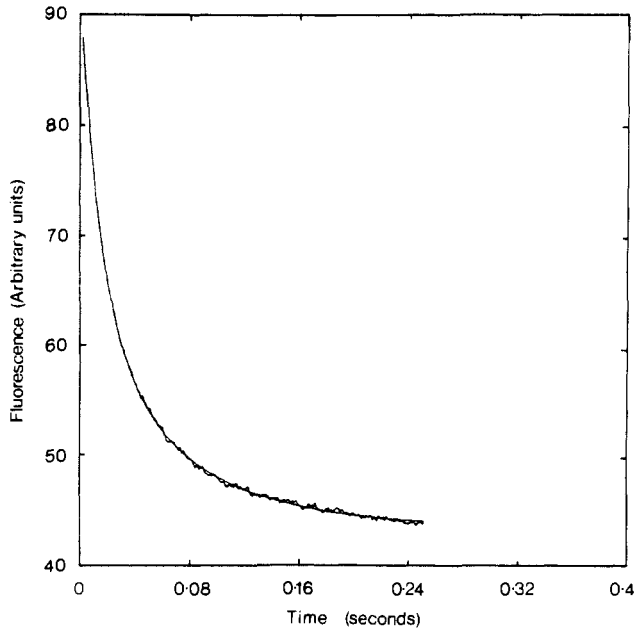


FIGURE 1: Binding of MTX to *E. coli* dihydrofolate reductase form I at pH 7.0, 25 °C. Data fitted to summation of two exponential decays by nonlinear least-squares regression.

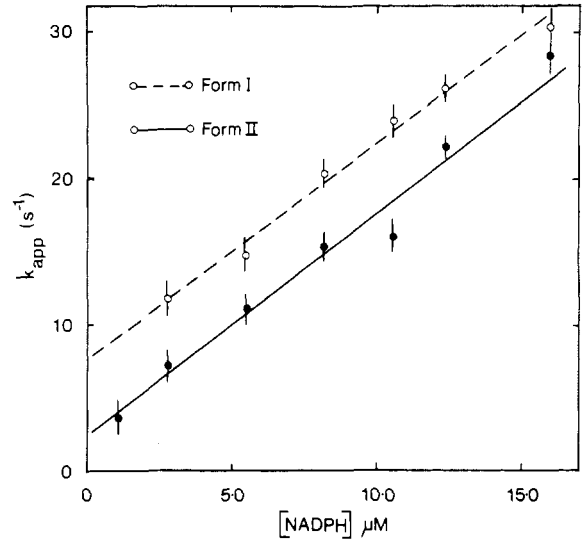


FIGURE 2: Dependence of apparent rate constant on the concentration of ligand. Binding of NADPH to both isoenzymes at pH 6, 25 °C.

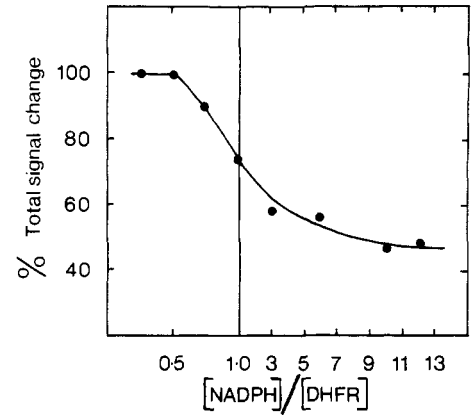


FIGURE 3: Percentage of total signal change due to fast phase of the reaction between NADPH and form I isoenzyme. Results plotted as a function of the mole ratio of NADPH to enzyme.

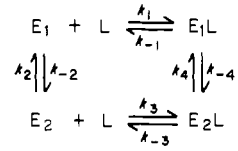
behavior of NADPH binding to dihydrofolate reductase from *L. casei* (Dunn et al., 1978). In these schemes, E_1 and E_2 represent different conformations of the free enzyme, and E_1L and E_1L^* represent different conformations of the enzyme–ligand complex.

A series of experiments was carried out to confirm which of these schemes applies to the *E. coli* enzymes. At a constant enzyme concentration, the relative concentration of NADPH was progressively decreased below equimolar. The percentage of the total signal change due to the fast process increased from 50% to 100% as the molar ratio fell from 10 to 0.5, which is exactly what would be expected from Scheme II if E_1 and E_2 exist in approximately equal amounts (Figure 3).

The kinetic data for MTX binding can also be explained by Scheme III if both forms of the enzyme bind the inhibitor rapidly so that the complex formation is complete before isomerization is possible.

The rate constants obtained from plots of k_{app} against ligand concentration are given in Table II. MTX, TMP, and H_2-folate have very similar association rate constants, and it is probable that the k_1 value for folate at 25 °C is also of this magnitude. Folate has a very fast dissociation rate constant,

Scheme III



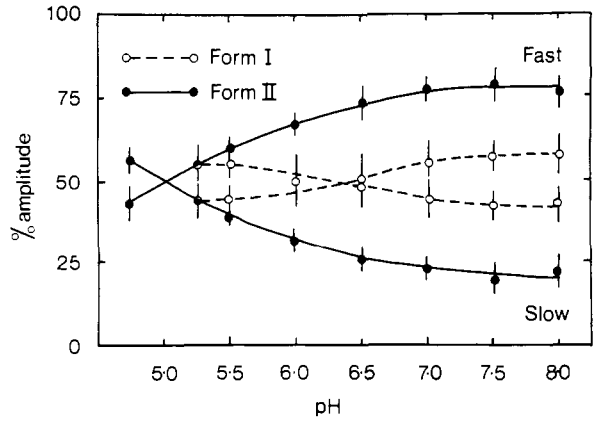
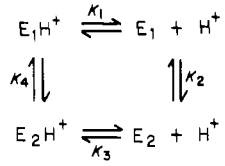


FIGURE 4: Variation in the relative amplitudes of the fast and slow phases of binding as a function of pH. NADPH binding to both isoenzymes (25 °C).

Scheme IV



35 s^{-1} at 10 °C, making pseudo-first-order measurements of k_{app} at 25 °C too rapid for our apparatus. However, the value measured at 10 °C for k_1 is still 2–2.5 times greater than that for NADPH binding at 25 °C.

The dissociation rate constants show a much wider variation, from <1 to 35 s^{-1}. For all the ligands, the off rate constant obtained from these experiments is smaller for form II than for form I. The calculated affinity constants (k_1/k_{-1}) are invariably smaller than the measured equilibrium values, suggesting that a further binding step, not detected by the fluorescence quenching technique, must take place. In general, the contribution of this step to the overall binding energy must be greater for complexes of form I and is ligand dependent.

The rate constant for the slow phase (Table II, part B) was found to lie in the narrow range between 0.025 and 0.061 s^{-1}. The fact that this value is not constant and that it was found to be somewhat concentration dependent for H_2folate binding to form II means that Scheme II is an oversimplification, and it is probable that some formation of E_1L takes place via the E_2L pathway for the coenzyme and substrates.

Effect of pH on Binding Kinetics. ^1H NMR studies have shown that the histidine C-2 proton pH profiles are no longer reversible when the enzyme has been exposed to pH 8.5 or higher (P. J. Cayley, unpublished results). Also, the activity of the enzyme rapidly decreases below pH 4.5, so the range in which pH variation studies can be made is restricted to pH 4.5–8.0.

The relative amplitudes of the fast and slow phases of the binding reaction with coenzyme or substrates vary with pH. The results for both forms of the enzyme binding to NADPH are shown in Figure 4. Unlike *L. casei* dihydrofolate reductase, where the largest proportion of the fast phase is at low pH, the fast-phase amplitude increases as the pH is raised. This effect is shown by both forms of the enzyme but is much more pronounced for form II. The same results are obtained when H_2folate is used instead of NADPH as the ligand.

These results can be interpreted in terms of a single ionization of the protein which has a different pK in the E_1 form to that in the E_2 form. Scheme III can be adapted to include pH effects as seen in Scheme IV.

Table III: Equilibrium Constants and pK Values Determined from Analysis of Relative Amplitudes of Fast and Slow Phases at 25 °C

enzyme	pK_1	pK_3	K_2	K_4
form I	6.4 ± 0.2	6.7 ± 0.2	0.7	1.3
form II	5.2 ± 0.2	5.9 ± 0.2	0.3	1.5

Table IV: Dissociation Rate Constants Measured from Competition Experiments at pH 7.0 and 25 °C

ligand	form I (s^{-1})	form II (s^{-1})
H_2folate	9.5 ± 3	11 ± 3
folate	16.5 ± 2	51 ± 5
TMP	0.28 ± 0.04	3.7 ± 0.4

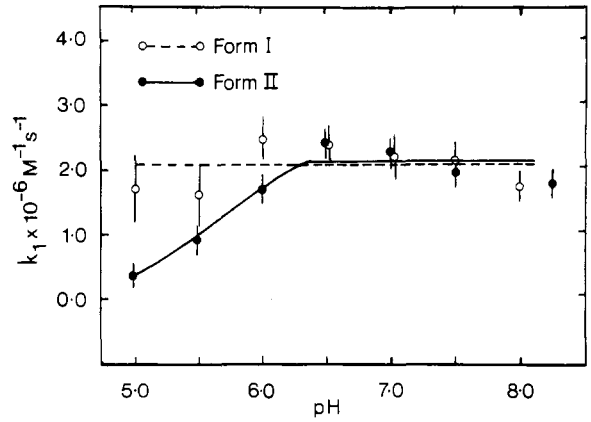


FIGURE 5: Dependence of association rate constant on pH for NADPH binding to both isoenzymes at 25 °C.

Substrates and coenzymes bind rapidly and tightly to E_1 and E_1H^+, while inhibitors bind to all four species. As noted above, the equilibrium binding of NADPH or folate to the enzyme does not appear to be affected by pH in this range, so the ionization of this group affects only the relative proportions of the two forms of the enzyme.

The pK values and equilibrium constants derived from the data in Figure 4 are given in Table III.

The rate constants for fast-phase binding were found to be independent of pH for folate or H_2folate binding to either isoenzyme. The rate constant for coenzyme binding to form I was independent of pH between 5.0 and 8.0, but, for form II, the rate constant decreased sharply with pH below 6.0 (Figure 5).

Dissociation Rate Constants from Competition Experiments. When a solution of an enzyme–ligand complex (EL_1) is mixed with a solution containing a different ligand, L_2, which binds at the same binding site, the following reactions take place:

$$EL_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E + L_1$$

$$E + L_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EL_2$$

If $k_{-1} \ll k_2[L_2] \gg k_1[L_1]$, fluorescence changes are attributable to the conversion of EL_1 into EL_2 and are single exponential with a rate constant k_{-1}. The dissociation rate constants for all the ligand complexes, except E–MTX, have been measured by this method by using MTX as the competing ligand. MTX is most suitable since it binds very tightly to the enzyme and has a high quenching efficiency at 340 nm (Table I).

The results (Table IV) show that these rate constants are smaller than those determined from concentration-dependence

Table V: Equilibrium and Rate Constants Obtained Using Scheme V

	form I			form II		
	NADPH	H_2folate	TMP	NADPH	H_2folate	TMP
K_k (M^{-1} s^{-1})	2.5×10^5	6.4×10^5	1.3×10^6	7.1×10^5	1.2×10^6	1×10^6
K_c	3.7	4.2	>127	0.7	1.2	12.3
k_f (s^{-1})		40	>36		13.2	46
k_b (s^{-1})		9.5	0.28		11	3.7

studies and that the values of k_{-1} are lower for form I than for form II, as would be expected from the equilibrium K_a measurements.

Discussion

The binding of substrates or coenzyme to *E. coli* dihydrofolate reductase (DHFR) appears to follow a similar kinetic scheme to that proposed for *L. casei* DHFR. The biphasic-reaction curve observed under pseduo-first-order conditions indicates a rapid bimolecular association of ligand with one conformation of the enzyme and a concurrent slow reequilibration of the free enzyme, resulting in further complex formation with the fast-binding form. The major difference between the two schemes is that the proportion of the fast-binding form of the *E. coli* isoenzymes increases with pH whereas with the *L. casei* enzyme it decreases.

Inhibitor binding shows no slow fluorescence quenching, but the fast phase of MTX binding can be resolved into two components. In order that the mechanism should be consistent, these ligands must be able to bind rapidly and tightly to both conformational species, with the binding of free enzyme completed before significant interconversion can take place. This is not exactly similar to binding of inhibitors to dihydrofolate reductase from *L. casei* where the differential affinity of inhibitors for the two species leads to a more complicated fluorescence decay (R. W. King, unpublished results).

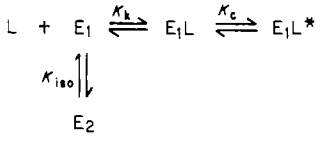
Supporting evidence for the formation of two enzyme–inhibitor complexes comes from ^{1}H NMR experiments. Two proton signals of approximately half-intensity are observed for the C-2 protons of several histidine residues in the enzyme–TMP and enzyme–MTX binary complexes. These proton signals exhibit behavior consistent with the enzyme's possessing two slowly interconverting pH-dependent conformational forms (Cayley, 1979).

NMR studies of the ternary complexes of dihydrofolate reductase from *L. casei* MTX/R with NADP–TMP, NADP–pyrimethamine, and NADP–folate also clearly demonstrate the existence of two conformations of the complex (B. Birdsall and A. Gronenborn, unpublished results). This is demonstrated both by the ^{1}H signals from two of the histidine residues and by the nicotinamide protons.

The population of the E_1 and E_2 states is clearly much more pH dependent in the form II enzyme than in the form I enzyme. The major chemical difference between these two isoenzymes is the replacement of a leucine in form I by an arginine (D. Stone, personal communication). The positively charged arginine side chain influences the equilibrium between the two conformational forms of the enzyme, causing a lowering of approximately one unit in the pK of the group responsible for the pH dependence. This effect may be due to direct competition between the arginine side chain and protons for a neighboring nucleophilic residue.

The measured association rate constants are very similar to those obtained with ligand binding to *L. casei* DHFR in both binary and ternary complexes (Dunn et al., 1978; Dunn & King, 1980). In those cases, the requirement for a $\Delta H^{\ddagger}$ value for association of less than 5 kcal/mol was not met, and so diffusion control was ruled out. It is probable that, in view of the close similarity in the binding mechanisms of the two enzymes, the association between ligands and the *E. coli* isoenzymes is also not diffusion controlled. This would require that at least a two-step mechanism is involved in the formation of the E_1L complex. Such a mechanism has previously been described to account for the kinetic behavior of sulfonamide binding to carbonic anhydrase (King & Burgen, 1976). The association rate constant of NADPH is at least 1 order of magnitude lower than even the most pessimistic estimates of diffusion control would predict.

Scheme V



The insensitivity to pH changes of the rate constant for folate or H_2folate binding shows that their initial binding reaction is not dependent on ionic interactions with binding site residues ionizing in this pH range or on ionization of the ligand. The same is true for coenzyme binding to form I. For form II, the decrease in rate constant below pH 6.0 may possibly be explained by unfavorable interaction with an ionizing group brought about by conformational changes in the coenzyme binding site resulting from the remote amino acid substitution.

The discrepancy between the equilibrium affinity constants and those calculated from the measured kinetic parameters suggest that the binding scheme is yet more complicated. Scheme II predicts that the measured equilibrium affinity constant will be smaller than the kinetically determined affinity constant by the factor $k_2/(k_2 + k_{-2})$. Thus, at pH 7.0 and 25 °C, the ratio of k_1 to k_{-1} (obtained by plotting k_{app} against ligand concentration) should have exceeded the measured equilibrium constant by factors of 1.7 and 1.3 for forms I and II, respectively. In fact, in cases where comparison is possible, these factors vary from 0.79 for NADPH binding to form II down to <0.013 for TMP binding to form I. These results indicate that a further binding step, not detected by the fluorescence quenching technique, takes place after the formation of the E_1L complex. The disparity between the dissociation rate constants measured by displacement methods and those obtained from plots of k_{app} against ligand concentration also indicate that the rate-limiting step in the displacement reaction is not the dissociation of the E_1L complex. Economy of hypotheses suggests that we include one further step in Scheme II to give Scheme V, where K_k is the kinetically determined affinity constant for binding L to E_1, K_c = $[E_1L^*]/[E_1L]$ is the equilibrium constant for a conformational change of the complex, and K_{iso} = $[E_1]/[E_2]$ is the isomerization equilibrium constant. The overall measured equilibrium constant K_c is then related to K_c by

$$K_c = \frac{K_c(1 + K_{iso})}{K_k K_{iso}} - 1$$

Values of K_{iso} for both forms are available from the data of Figure 4. Values of K_e and K_k for NADPH, H_2folate, and TMP can be obtained from the equilibrium and kinetic data in Tables I and II. If we assume that the dissociation rate constants measured by competition (k_b) are those for the conversion of E_1L^* to E_1L, then values of the rate constant for the formation of E_1L^* (k_f) can be calculated. These calculated values are shown in Table V.

These results clearly indicate that the tight binding of the TMP relative to the substrate or coenzyme in the binary complex is due largely to the stability of the E_1L^* form of the complex. Williams et al. (1979, 1980) have used measurements of inhibition of the catalytic activity of the enzyme to demonstrate that a similar process is responsible for tight binding of inhibitors in the ternary complex. Hood & Roberts (1978) thought it likely that conformational differences between substrate and inhibitor binary complexes with the enzyme are responsible for a large part of their difference in binding energy. It seems likely that the E_1L to E_1L^* transition is also the manifestation of either a conformational readjustment of the complex or a positional change in the ligand.

Arguments similar to those used above may be applied to Scheme III, although we have insufficient data to make quantitative comparisons.

The results in Tables II and V also demonstrate that even if other steps occur between E_1L and the formation of E_1L^* the major factor governing the difference in affinity of the two isoenzymes for trimethoprim is the transition of E_1L^* to E_1L. This may reflect a difference in the energy of interaction of the inhibitor with the side chains of the protein since the leucine residue which is replaced forms part of the binding site for the methoxy groups of TMP. Alternatively, it may result from a difference in the energetics of an enzyme conformational change, although this seems less likely since a similar effect is not seen with dihydrofolate.

The formation of binary complexes between dihydrofolate reductase and the ligands described is thus at least a three-step process. The formation of E_1L takes place via a transitory intermediate, and a further step leads to the formation of E_1L^*. In the case of MTX, which binds strongly to both E_1 and E_2, parallel reaction pathways through E_1L and E_2L appear to lead to a final equilibrium between E_1L^* and E_2L^*.

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Mapping Adenosine Cyclic 3′,5′-Phosphate Binding Sites on Type I and Type II Adenosine Cyclic 3′,5′-Phosphate Dependent Protein Kinases Using Ribose Ring and Cyclic Phosphate Ring Analogues of Adenosine Cyclic 3′,5′-Phosphate†

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ABSTRACT: A series of adenosine cyclic 3′,5′-phosphate (cAMP) derivatives containing modifications or substitutions in either the 2′, 3′, 4′, or 5′ position or the phosphate were examined for their abilities to activate type I isozymes of cAMP-dependent protein kinase (PK I) from rabbit or porcine skeletal muscle and type II isozymes of cAMP-dependent protein kinase (PK II) from bovine brain and heart. The studies revealed that the activation of both PK I and PK II isozymes requires a 2′-hydroxyl group in the ribo configuration, a 3′ oxygen in the ribo configuration, and a charged cyclic phosphate. The two isozymes appeared to differ in those portions of their respective cAMP-binding sites that are adjacent to the 4′ position of the ribose ring and the 3′ position, 5′ position, and phosphate portion of the cyclic phosphate ring.

The original proposal of Kuo & Greengard (1969) that the diverse actions of adenosine cyclic 3′,5′-phosphate (cAMP)[1] on eukaryotic physiology are all mediated through cAMP-dependent protein kinases is still the principal working hypothesis for the mechanism of action of cAMP (Rosen et al., 1977). Many cAMP analogues can efficiently activate various

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[1] Abbreviations used: cAMP, adenosine cyclic 3′,5′-phosphate; DEAE, diethylaminoethyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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