Binding of Methotrexate to Dihydrofolate Reductase and Its Relation to Protonation of the Ligand†

Raymond L. Blakley* and Lennie Cocco‡

Division of Biochemical and Clinical Pharmacology, St. Jude Children's Research Hospital, Memphis, Tennessee 38101

Received November 14, 1984; Revised Manuscript Received March 18, 1985

ABSTRACT: Stopped-flow spectrophotometry and stopped-flow fluorometry have been used to study the binding of methotrexate (MTX) and 3-deazamethotrexate (3-deazaMTX) to dihydrofolate reductase (DHFR) isoenzymes from *Streptococcus faecium* and from *Lactobacillus casei*. The absorbance change and fluorescence quenching that occur when MTX binds to DHFR isoenzyme II from *S. faecium* (SFDHFR II) are both biphasic and give similar apparent rate constants for both phases. The faster phase has an apparent rate constant that is dependent on MTX concentration and therefore corresponds to the initial binding reaction. From the concentration dependence it has been calculated that the association rate constant is 3.0×10^5 M^{-1} s^{-1} at 20 °C and pH 7.3, and the association constant (equilibrium constant) under these conditions is 5.8×10^5 M^{-1}. By examination of the amplitude of the fast-phase absorbance change at various wavelengths, it has been determined that the absorbance change occurring in the fast phase is due to MTX protonation. Within the limits of the method it was thus not possible to detect a difference in the rates of binding and of protonation of MTX. The MTX association rate constant is pH dependent, decreasing 330-fold as the pH is decreased from 5.0 to 9.0. The data fit well to a curve generated by assuming a single ionization with a pK_a of 6.0 and a pH-independent association rate constant 1000-fold greater for binding of protonated MTX to SFDHFR II than for binding of unprotonated MTX. In the case of 3-deazaMTX binding the data fit to a curve corresponding to a pK_a of 7.5 and 200-fold higher pH-independent rate constant for the protonated species than for the unprotonated. Binding of MTX to isoenzyme I of *S. faecium* DHFR and to DHFR from *L. casei* occurs with association and dissociation rate constants quite similar to those for binding to SFDHFR II. However, the binding of the protonated form of MTX is favored by a factor of only 200-fold in the case of *S. faecium* DHFR isoenzyme I and only 7-fold in the case of *L. casei* DHFR, despite the fact that the overall association constants for the initial binding of MTX to the three types of DHFR are comparable. Thus, the relative rates of binding of protonated MTX and unprotonated MTX to DHFR vary with the source of the enzyme and hence its detailed structure. Thermodynamic association constants determined by fluorescent titration are significantly higher than those calculated from ratios of rate constants for association and dissociation of complexes. This implies occurrence of important isomerizations of the complexes after the initial binding reaction. The data also suggest that the protonated ligand–active site carboxylate interaction has a limited effect on the rate of inhibitor binding in the case of MTX binding to DHFR from *L. casei* and to isoenzyme I from *S. faecium*, though it greatly increases the binding rate in the case of SFDHFR II. However, even in the latter case the ion pair interaction does not result in an unusually high association constant for the initial binding reaction.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3; DHFR)[1] catalyzes the NADPH-dependent reduction of H_2folate to H_4folate. The metabolic importance of DHFR, which stems from the fact that derivatives of its product, H_4folate, have coenzyme functions in one-carbon metabolism, has inspired extensive research on inhibitors of the reductase, several of which are widely used as antibacterial or antitumor agents.

An aspect of major interest regarding 2,4-diamino heterocyclic inhibitors of DHFR like methotrexate (MTX) has been the molecular basis for their tight binding. Evidence of many kinds (reviewed recently; Blakley, 1984) indicates that the major factor in the tighter binding of such inhibitors, as compared with the substrate, H_2folate, and other folate derivatives, is the ability of the inhibitors to become protonated at N-1 and form an ion pair with an active site carboxylate group (the side chain of Asp-27 in the case of the *Streptococcus faecium* enzyme). Interaction of the protonated heterocyclic ring of the inhibitor and the carboxylate group of the enzyme side chain is very strong as shown by a marked shift in the pK_a of the inhibitor when bound (Cocco et al., 1981a, 1983; Stone & Morrison, 1983). The strength of the interaction is attributed to the hydrophobic environment of the active site which increases the ionic interaction and to hydrogen bonding between the enzyme carboxylate and N-1 and the 2-amino group of the inhibitor.

† This research was supported in part by U.S. Public Health Service Research Grant R01-CA-31922 (R.L.B.), Cancer Center Core Grant P30-CA-21765 (R.L.B.), and American Lebanese Syrian Associated Charities (R.L.B. and L.C.).

‡ Present address: Abbott Laboratories, Abbott Park, IL 60064.

[1] Abbreviations: DHFR, dihydrofolate reductase; SFDHFR I, *Streptococcus faecium* dihydrofolate reductase isoenzyme I; SFDHFR II, *S. faecium* dihydrofolate reductase isoenzyme II; LCDHFR, *Lactobacillus casei* dihydrofolate reductase; MTX, methotrexate (4-amino-4-deoxy-10-methylpteroylglutamic acid); 1-deazaMTX, 1-deazamethotrexate [*N*-[*p*-[(5,7-diaminopyrido[3,4-*b*]pyrazin-3-yl)methylamino]-benzoyl]-L-glutamic acid]; 3-deazaMTX, *N*-[*p*-[[(6,8-diaminopyrido-[2,3-*b*]pyrazin-2-yl)methyl]methylamino]benzoyl]-L-glutamic acid; H_2-folate, 7,8-dihydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; $\Delta G^\ddagger$, free energy of activation; $\Delta H^\ddagger$, enthalpy of activation; $\Delta S^\ddagger$, entropy of activation; k_f, association rate constant; k_r, dissociation rate constant; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/85/0424-4704$01.50/0

In solution at physiological pH inhibitors like methotrexate and trimethoprim are present mostly as the unprotonated form, whereas in the complex with DHFR they are protonated even up to pH 10 (Cocco et al., 1981a, 1983). The process of complex formation therefore involves both protonation and binding of the inhibitor, and it is of some significance to determine the order and relative rates of these processes. We have investigated the formation of inhibitor complexes of *S. faecium* DHFR isoenzyme II (SFDHFR II) by stopped-flow spectrophotometry and by stopped-flow fluorometry, in order to obtain information on these aspects of DHFR inhibitors. Previous applications of stopped flow to the binding of inhibitors to *Lactobacillus casei* DHFR (Dunn & King, 1980) and to *Escherichia coli* DHFR (Cayley et al., 1981) did not specifically address this question. We have also made less detailed studies of MTX binding to DHFR from *L. casei* and to DHFR isoenzyme I from *S. faecium* (SFDHFR I). Compared with SFDHFR II, SFDHFR I has narrower substrate specificity (folate is not a substrate) but considerably higher turnover number with H_2folate as substrate (Blakley, 1984). There are major differences in the amino-terminal portion of the sequence that contributes many of the active site residues.

Our results indicate that, in the case of SFDHFR II, the protonated ligand binds much more rapidly to the reductase than unprotonated ligand does, whereas SFDHFR I and LCDHFR bind these two forms of the ligand at less dissimilar rates.

Experimental Procedures

Materials. Dihydrofolate reductase was isolated and purified from the following sources: *S. faecium* var. durans strain A (Blakley et al., 1978), *S. faecium/F* (Nixon & Blakley, 1968; Freisheim et al., 1972), and *L. casei* (Cocco et al., 1981b). Methotrexate was obtained from Sigma, and 1-deazamethotrexate and 3-deazamethotrexate were generous gifts from Dr. John A. Montgomery, Southern Research Institute, Birmingham, AL.

Kinetic Measurements. Kinetic data were obtained by the use of a Durrum D-110 (Dionex Instrument Corp., CA) stopped-flow apparatus operating in either the absorbance or fluorescence mode. In most cases the formation of the complex was followed either by measuring absorbance at 340 nm or by using an excitation wavelength of 290 nm and monitoring the quenching of the protein intrinsic fluorescence with a 341-nm interference filter. In certain cases other wavelengths were used for absorbance measurements. Reaction traces were collected with an On-Line Instruments Systems Model 3180 data collection system. This system is based on a North Star Horizon microcomputer and is used to capture, store, and process the stopped-flow data. The average of four or five traces was routinely used to estimate the rate constants.

All experiments were carried out at 20 °C unless otherwise stated. Buffers used were the following: pH 5.0–7.3, 50 mM potassium phosphate plus 500 mM KCl; pH 8.1–10.0, 50 mM Tris-HCl plus 500 mM KCl. For absorbance measurements the final enzyme concentration was 5 μM, and ligand concentrations were in the range 10–70 μM. For measurement of fluorescent changes, the final enzyme concentration was 1–4 μM, and ligand concentrations were in the range 2–20 μM.

Data Analysis. Kinetic data were analyzed by using either a single-exponential model

$$F(t) = A \exp(-kt) + \text{base line}$$

or a double-exponential model

$$F(t) = A_{fast} \exp(-k_{fast}t) + A_{slow} \exp(-k_{slow}t) + \text{base line}$$

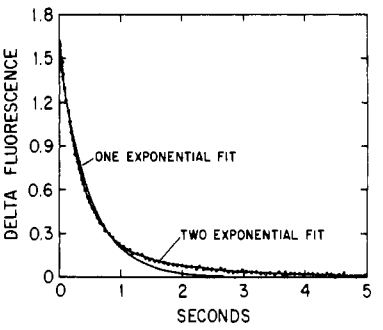


FIGURE 1: Fit of data for the fluorescence decrease on mixing MTX and SFDHFR II to single-exponential and double-exponential models. The reaction was performed at 20 °C in 50 mM potassium phosphate buffer, pH 7.3, containing 500 mM KCl. Final concentrations of reactants were the following: SFDHFR II, 4 μM; MTX, 12 μM. Many of the data points have been omitted to make the fit of the double-exponential line clearer. The fit of omitted points was as good as for those shown.

where $F(t)$ is the observed fluorescence at time t, A, A_{fast}, and A_{slow} are amplitude terms, and k, k_{fast}, and k_{slow} are rate constants. The exponential fit portion of the On-Line Instrument System data reduction program was modified for this purpose.

Fluorescence Titrations. Fluorescence titrations were performed on a Perkin-Elmer MPF-44B spectrofluorometer at 20 °C unless otherwise indicated and with the same buffers used in the kinetic experiments. Excitation was at 290 nm (slits at 8 nm), and emission was measured at 350 nm (slits at 12 nm). Titration of 2 μM enzyme was performed in 3 mL of the same buffers used for the stopped-flow studies. A solution of tryptophan with an absorbance similar to the enzyme solution was used to determine the correction for internal filter effects during the titrations. Corrected fluorescence intensity data were computer fitted as previously described (Blakley et al., 1983) in order to calculate the association constant, K_{titr}.

UV Difference Spectroscopy. UV difference spectra were obtained on either a Cary 14 or a Cary 219 spectrophotometer using the 0–0.1 absorbance scale. The sample compartment contained one cuvette with 4.3 μM SFDHFR II and 5 μM MTX (or 30 μM 1-deazaMTX) and a second cuvette containing only buffer. The reference compartment contained 4.3 μM SFDHFR II in one cuvette and 5 μM MTX (or 30 μM 1-deazaMTX) in a second cuvette. The buffer was the same as used for stopped-flow studies at pH 7.3, and the temperature was 20 °C.

Results

Binding of MTX to SFDHFR II. The absorbance change that was observed at 340 nm as a function of time after mixing of SFDHFR II and MTX in the stopped-flow spectrophotometer was analyzed as the sum of two exponentials (Figure 1). The observed rate constant for the fast phase was dependent on the ligand concentration and therefore clearly corresponds to the initial phase. The characteristics and significance of the second phase will be considered in a subsequent publication.

The concentration dependence of the fast phase requires a bimolecular reaction as shown in Scheme I, where k_f is the association rate constant and k_r is the dissociation rate constant. The observed rate constant for the fast phase (k_{obsd}) can be approximated by $k_{obsd} = k_f[L] + k_r$ under pseudo-first-order conditions. A plot of k_{obsd} vs. [L] gives a straight line with a slope of k_f and intercept of k_r. The kinetic pa-

Table I: Rate Constants for the Fast Reaction Involved in Binary Complex Formation between SFDHFR II and MTX at Various pH Values

pH	k_f[a] ($\times 10^{-6}$ M^{-1} s^{-1})	k_r[a] (s^{-1})
5.0	4.98 ± 0.22	3.54 ± 1.37
6.0	3.42 ± 0.10	1.34 ± 0.59
6.6	2.03 ± 0.05	1.29 ± 0.29
7.3	0.30 ± 0.008	0.52 ± 0.11
7.3	0.32 ± 0.04[b]	0.57 ± 1.34[b]
8.1	0.11 ± 0.003	0.18 ± 0.02
9.0	0.015 ± 0.002	0.22 ± 0.01
9.0	0.017 ± 0.003[b]	0.046 ± 0.01[b]

[a] From concentration dependence of apparent rate constant for fast phase. [b] From absorbance change at 340 nm. Other values are from fluorescence quenching.

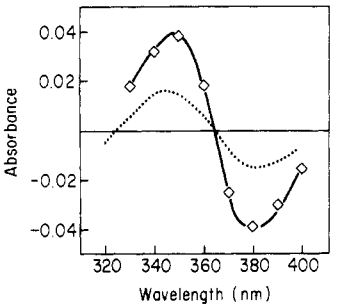


FIGURE 2: Comparison of the amplitude of the fast phase with the difference spectrum. The amplitude of the fast phase (◇) as measured by stopped-flow absorbance spectrophotometry was obtained by using a final enzyme concentration of 8.3 μM and MTX concentration of 10 μM. The difference spectrum (···) was obtained as described under Experimental Procedures.

rameters for MTX binding to SFDHFR II were obtained by analysis of such data and are listed in Table I.

Scheme I

$$E + L \underset{k_r}{\overset{k_f}{\rightleftharpoons}} EL$$

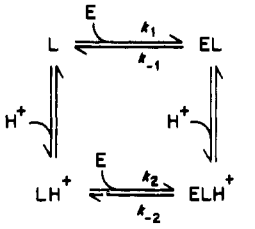
The amplitude of the absorbance change in the fast phase was determined at various wavelengths in the range 330–400 nm and closely follows the difference spectrum obtained with a mixture of SFDHFR II and MTX in the sample compartment and unmixed solutions of these compounds in the reference compartment (Figure 2). This difference spectrum is due to MTX binding to DHFR and has previously been studied and attributed to binding of MTX in the protonated state (Erickson & Mathews, 1972; Poe et al., 1974; Hood & Roberts, 1978; Subramanian & Kaufman, 1978; Cocco et al., 1981b). The amplitude–wavelength relationship in the stopped-flow spectrophotometry therefore indicates that the fast phase corresponds to appearance of bound, protonated MTX and that protonation is coincident with binding.

When the reaction of MTX with SFDHFR II was followed by monitoring quenching of intrinsic protein fluorescence, a biphasic change in fluorescence was observed; the observed rate constant for the fast phase increased with ligand concentration. The values of k_f and k_r obtained by this method agreed well with those obtained by absorbance measurements (Table I). This close agreement confirms that binding of MTX and its protonation are simultaneous in the fast phase.

The connection between binding and protonation in the fast phase can be explained by Scheme II.

Absorbance change monitors the formation of ELH^+, fluorescence quenching monitors the formation of bound ligand ($EL + ELH^+$), and either pathway from L to ELH^+ might be operational provided the protonation of EL to ELH^+ is as rapid as the binding step.

Scheme II



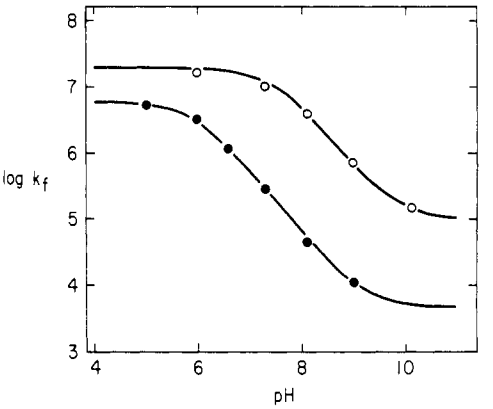


FIGURE 3: Effect of pH on the association rate constant, k_f, for MTX and for 3-deazaMTX binding to SFDHFR II. The experimental points are compared with curves calculated for log $[(k_1 + k_2R)/(1 + R)]$ where log $R = pK_a - pH$, and for the upper curve $pK_a = 7.5$, $k_1 = 1 \times 10^5$ M^{-1} s^{-1}, and $k_2 = 2 \times 10^7$ M^{-1} s^{-1}, and for the lower curve $pK_a = 6.0$, $k_1 = 5 \times 10^3$ M^{-1} s^{-1}, and $k_2 = 6 \times 10^6$ M^{-1} s^{-1}.

pH Dependence of MTX Binding. In order to determine the relative contribution of the two pathways for ELH^+ formation in Scheme II, the effect of pH on the observed and calculated rate constants was determined with the results shown in Table I. On the basis of values obtained by stopped-flow fluorometry, the association rate constant, k_f, shows a 330-fold decrease as the pH is increased from 5.0 to 9.0 whereas the dissociation rate constant shows a 16-fold decrease.

According to Scheme II, the overall association rate constant, k_f, should be related to association rate constants, k_1 and k_2, for binding of the unprotonated and protonated ligand, respectively, by the relation

$$k_f = (k_1 + k_2R)/(1 + R)$$

where $R = [LH^+]/[L]$, log $R = pK_a - pH$, and K_a is the acid dissociation constant for unbound MTX. This relation assumes that protonation of EL is fast compared with EL formation. According to this equation, at low pH (high R) the value of k_f approaches k_2, while at high pH k_f approximates k_1. The overall dissociation rate constant k_r is given by the sum of k_{-1} and k_{-2}.

Estimates of the pH-independent rate constants were obtained by simulating the pH dependence of k_f shown in Table I, with the assumption of the equation above. As shown in Figure 3, a good fit to the data is obtained with a pK_a of 6.0, $k_1 = 5 \times 10^3$ M^{-1} s^{-1}, and $k_2 = 6 \times 10^6$ M^{-1} s^{-1}. It is evident that the protonated form of MTX binds much more rapidly to SFDHFR II than the unprotonated. At pH 7.3, 98.4% of the protonated MTX complex is formed by way of binding protonated MTX even though the latter constitutes only 4.8% of the MTX in solution at this pH.

pH Dependence of 3-DeazaMTX Binding. The pH dependence of k_f for MTX is consistent with preferred binding of protonated MTX as described above. However, an alternative explanation, also consistent with the data, is that a proton dissociates from a group on the enzyme with a pK_a of

Table II: Rate Constants for the Fast Phase of Binary Complex Formation between SFDHFR II and 3-DeazaMTX at Various pH Values

pH	k_f[a] ($\times 10^{-6}$ M^{-1} s^{-1})	k_r[a] (s^{-1})
6.0	18.01 ± 0.89	24.88 ± 5.61
7.3	9.32 ± 0.40	19.67 ± 1.88
8.1	3.43 ± 0.16	4.20 ± 0.89
9.0	0.94 ± 0.03	3.67 ± 0.19
10.0	0.18 ± 0.02	2.78 ± 0.22

[a] From concentration dependence of apparent rate constant.

Table III: Rate Constants for Binary Complex Formation between SFDHFR I and MTX at Various pH Values

pH	k_f[a] ($\times 10^{-6}$ M^{-1} s^{-1})	k_r[a] (s^{-1})
6.0	5.20 ± 0.21	5.35 ● 1.27
7.3	0.95 ± 0.14	0.14 ± 0.96
9.0	0.045 ± 0.009	0.33 ± 0.05

[a] From concentration dependence of apparent rate constant for fast phase.

6.0 and that this results in less favorable binding of MTX. The rather close agreement between the reported pK_a for MTX of 5.73 (Cocco et al., 1981a) and the calculated pK_a for the pH dependence of k_f makes this seem unlikely, but to distinguish between these possibilities, the pH dependence of k_f for 3-deazaMTX was also examined (Table II). This ligand has a pK_a of 7.4 (Elliott et al., 1971).

As in the case of MTX, stopped-flow fluorometric changes were analyzed as the sum of two exponentials, the fast phase having a concentration-dependent observed rate constant. It was found that the association rate constant, k_f, decreases 95-fold with increasing pH, but the dissociation rate constant shows a much smaller decrease (9-fold). Analysis of the dependence of k_f on pH as in the case of MTX gave a good fit (Figure 3) for the equation corresponding to Scheme II, with the values pK_a = 7.5, $k_1 = 1 \times 10^5$ M^{-1} s^{-1}, and $k_2 = 2 \times 10^7$ M^{-1} s^{-1}.

The fact that the pK_a defined by the pH dependence of k_f again corresponds to the pK_a of the ligand, and is significantly different from the value obtained with MTX as the ligand, clearly indicates that it is protonation of the ligand that has the major effect on k_f and not protonation of the enzyme. The good fit of the data to the simulated curves in Figure 3 indicates that in the case of both MTX and 3-deazaMTX there can be relatively little effect on k_1 and k_2 of ionization of enzyme groups. However, the effects of pH on k_r ($=k_{-1} + k_{-2}$) are perhaps due to complex effects of pH on the enzyme. It is also of interest that rate constants for binding of unprotonated and protonated 3-deazaMTX are 20 times and 3 times greater, respectively, than the corresponding constants for MTX.

MTX Binding to SFDHFR I. Stopped-flow observation of fluorescence decrease due to binding of MTX to SFDHFR I gave biphasic kinetics at pH 6.0 but showed no slow phase at pH 7.3 and 9.0. Values for association and dissociation rate constants for the fast phase (Table III) are quite comparable with those for SFDHFR II, although k_f is slightly higher in the case of SFDHFR I. The value of k_f decreases 116-fold with increase of pH from 6.0 to 9.0, suggesting preferred binding of protonated MTX by this isoenzyme also, but this aspect of the binding was not investigated further. The limited data obtained will fit a curve of the type shown in Figure 2 with a pK of about 6, k_2 of about 6×10^6 M^{-1} s^{-1}, and k_1 of about 3×10^4 M^{-1} s^{-1}.

MTX Binding to LCDHFR. MTX binding to LCDHFR showed biphasic kinetics as determined by fluorescence changes observed by stopped flow with reaction mixtures at pH values in the range 5.0–10.0. The observed rate constants for the fast phase of binding to LCDHFR are dependent on ligand concentration, and calculated values of k_f and k_r at pH 5.0 (Table IV) are only slightly higher than those for MTX binding to SFDHFR II at this pH (Table I). However, since k_f is only slightly dependent on pH, with a 5-fold decrease as the pH is raised from 5 to 10 (Table IV), binding of MTX to LCDHFR is much faster than binding to SFDHFR II at high pH. The dissociation rate constant, k_r, shows complex apparent pH dependence.

Table IV: Rate Constants for the Fast Phase of Binary Complex Formation between LCDHFR and MTX at Various pH Values and 20 °C

pH	k_f[a] ($\times 10^{-6}$ M^{-1} s^{-1})	k_r[a] (s^{-1})
5.0	8.82 ± 0.75	4.5 ± 4.5
6.0	7.40 ± 0.32	25.0 ± 3.4
7.3	4.49 ± 0.70	17.22 ± 2.02
9.0	1.75 ± 0.10	4.42 ± 0.59
10.0	1.74 ± 0.09	9.18 ± 0.58

[a] From concentration dependence of apparent rate constant for fast phase.

Table V: Association Constants for Binary Complexes of MTX and 3-DeazaMTX with SFDHFR II

pH	K[a] ($\times 10^{-6}$ M^{-1})	K_{titr}[b] ($\times 10^{-6}$ M^{-1})	K_{iso}[c]
	MTX		
6.0	2.55	32.3	11.7
7.3	0.58	13.3	21.9
9.0	0.068	2.5	35.7
	3-DeazaMTX		
6.0	0.72	90.9	125
7.3	0.47	47.6	100
9.0	0.26	23.8	90

[a] From k_f/k_r determined for the fast phase in stopped-flow measurements of fluorescence change. [b] From fluorescence titration. [c] From $(K_{titr}/K) - 1$. K_{iso} is the equilibrium constant for the isomerization of the binary complex (Scheme III).

Binding of 1-DeazaMTX to SFDHFR II. 1-DeazaMTX is a poor inhibitor of DHFR compared with either MTX or 3-deazaMTX, the concentration for 50% inhibition being 300–400-fold higher than for MTX (Montgomery et al., 1971). The pK_a (4.5) is considerably lower than that for MTX, and little of the charge would be located at the 1-position. We found that the association constant for binary complex formation with SFDHFR II as determined by fluorescence titration is 2.6×10^5 M^{-1} [value not in tables; cf. 13.3×10^6 M^{-1} for MTX (Table V)]. 1-DeazaMTX was found to exhibit no absorbance change when bound to DHFR. This was determined by difference spectroscopy under conditions in which 90% of the ligand present was bound to the enzyme and indicates that the bound ligand is not protonated. In stopped-flow fluorometry experiments no fluorescence change could be detected, despite the observation of some small degree (≈20%) of fluorescence quenching in titration experiments. This may be due to a high value of k_{obsd} which results in attainment of equilibrium within the dead time of the instrument. Since $k_{obsd} = k_f[L] + k_r$, a value of k_f similar to that for MTX, combined with a relatively high k_r, would result in the postulated high value of k_{obsd}.

The association constant obtained by fluorescence titration for folate binding is 2×10^5 M^{-1} at 25 °C. As in the case of 1-deazaMTX, there is no absorbance change on binding, and no fluorescence change could be observed at 25 °C by stopped flow, presumably for the same reason as in the case of 1-deazaMTX. However, when preliminary stopped-flow ex-

periments were carried out at 12 °C, a biphasic change of fluorescence was observed, the fast phase having a concentration-dependent rate constant. From the concentration dependence, approximate values of (1.6 ± 0.36) × 10^5 M^{-1} s^{-1} and 3.42 ± 2.35 s^{-1} were obtained for the association and dissociation rate constants, respectively. These values correspond to an approximate association constant of 4.7 × 10^5 M^{-1}. Although folate is not bound in protonated form (Cocco et al., 1981), the association rate constant is considerably higher than those for unprotonated MTX (5 × 10^3 M^{-1} s^{-1}) and for unprotonated 3-deazaMTX (1 × 10^5 M^{-1} s^{-1}). This is probably connected with the fact that folate binds with the pteridine ring turned over with respect to the side chain [evidence reviewed by Blakley (1984)].

Activation Parameters for MTX Binding to SFDHFR II. The effect of temperature on the kinetics of binding of MTX to SFDHFR II was investigated over the temperature range 10–35 °C at pH 7.3. The rate constants were determined under pseudo-first-order conditions, with $k_f[L] \gg k_r$, and the association rate constant could be estimated from $k_{obsd} = k_f[L]$. The slope of a plot of ln k_f vs. the reciprocal of the absolute temperature was linear and yielded an activation energy, E_a, of 5.9 kcal mol^{-1}. From this value, the other activation parameters could be calculated according to the method of Dunn et al. (1978). For the reaction at 20 °C, $\Delta H^{\ddagger}$ = 5.3 kcal mol^{-1}, $\Delta S^{\ddagger}$ = −15.1 cal mol^{-1} deg^{-1}, and $\Delta G^{\ddagger}$ = 9.8 kcal mol^{-1}.

Discussion

Relation of Protonation to Binding. A primary objective of this investigation was to determine the sequence of events by which MTX and other antifolates form complexes with DHFR in which the inhibitor is protonated when bound. We have shown that ligand binding to the enzyme, as measured by quenching of protein fluorescence, and protonation of the ligand, as indicated by absorbance changes (Figure 2), are simultaneous within the resolution of the stopped-flow method. This is consistent with selective binding of protonated MTX or with very rapid protonation of bound unprotonated MTX (Scheme II). In the case of MTX binding to SFDHFR II the large change with pH in the association rate constant for the fast phase of binding [330-fold over 4 pH units (Figure 3)] clearly indicates that selective binding of protonated ligand is much the more important route at most pH values (including physiological pH). With this enzyme the rate constant for binding of protonated MTX (6 × 10^6 M^{-1} s^{-1}) is almost 1000 times greater than that for unprotonated MTX, and in the case of 3-deazaMTX the ratio is about 200.

Effect of Ion Pair Formation on Initial Binding Rates. Although rate theory predicts that association rates should be increased by less than 1 order of magnitude by electrostatic attractions, particularly in media of high ionic strength (Dunn & King, 1980), the much larger association rate constants for MTX and 3-deazaMTX binding to SFDHFR II when the ligands are protonated are presumably due to the attraction between the charge on the protonated ligand and that of the carboxylate of the active site aspartate (Asp-27). Perhaps in the rate-limiting step in binding there is limited contact between solvent molecules and the interacting ion pair.

The effect of the ratio of the rate constants for binding of protonated and unprotonated forms (k_2/k_1, Scheme II) on the pH dependence of the association rate constant is shown in Figure 4. It may be seen that the ratio of values of the observed constant k_f at accessible pH extremes approximates the ratio of k_2/k_1. Consequently, it is clear from Tables III and IV that in the case of binary complex formation by SFDHFR I the preference for binding of the protonated form

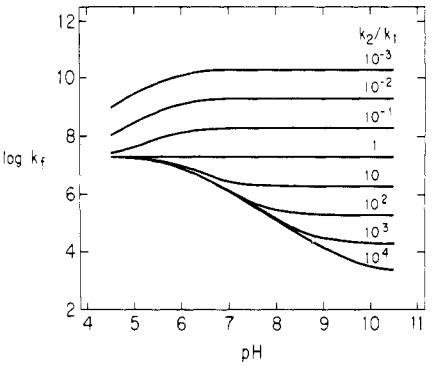


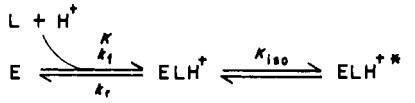
FIGURE 4: Effect of association rate constants k_2 and k_1 (for binding of protonated and unprotonated ligand, respectively) on the pH dependence of the observed association rate constant, k_f. The value of log k_f was calculated from the expression log k_f = log $[(k_1 + k_2R)/(1 + R)]$ where log R = pK_a − pH. The value used for pK_a for the ligand is 5.8, k_2 = 2 × 10^7 M^{-1} s^{-1}, and k_2/k_1 values are as shown.

of MTX is less than for SFDHFR II and is slight for LCDHFR. Yet k_f is very similar at pH 6.0 for MTX binding to all three enzymes, and it is well established in the case of the binary complex of LCDHFR that the bound MTX is protonated (Cocco et al., 1983). It seems likely therefore that although attraction between protonated MTX and the active site carboxylate plays a role in MTX binding in all cases, it is not rate determining in the case of MTX binding to LCDHFR and only partially so in the case of SFDHFR I, though it plays an important role in the case of SFDHFR II.

Binding of Unprotonated MTX. Although in all cases examined more MTX binds to DHFR at physiological pH as the protonated form than as the unprotonated, binding of the latter is nevertheless significant in binding to LCDHFR and SFDHFR I. As argued above, after binding of unprotonated MTX, protonation must occur rapidly. Our results do not provide evidence as to whether the carboxyl of Asp-27 is the immediate proton donor. However, it seems unlikely that this carboxyl is significantly protonated in free DHFR around neutral pH. Thus, no other charged or H-bonding groups are found in close proximity to the active site aspartate in the model derived from X-ray crystallography of the *L. casei* DHFR ternary complex with MTX and NADPH, or in such a model where side-chain changes are made in accordance with the SFDHFR II sequence. The backbone conformation and side-chain orientations may be different in SFDHFR II and may also be modified by differences in salts, ionic strength, etc. between our conditions and those used in crystallization. However, reported comparisons between other DHFR structures suggest that this would not be great enough to place a strongly perturbing group near Asp-27. Possibly, partial exclusion of water due to the presence of bound nucleotide could raise the pK_a of Asp-27, but this is doubtful. Furthermore, Subramanian & Kaufman (1978) have shown by calorimetry that after solutions of MTX and chicken liver DHFR are mixed at pH 7.4, a proton is abstracted from the solvent. Nevertheless, Stone & Morrison (1983) concluded from data on the binding of MTX to *E. coli* DHFR that this process requires the active site carboxyl to be protonated. This should favor binding of unprotonated MTX over protonated, contrary to our results, and the source of the discrepancy is unclear.

Effect of Ion Pair Interaction on the Binding Equilibrium. The strength of the interaction of the active site carboxylate with the protonated N-1 of MTX, pyrimethamine, and trimethoprim is demonstrated by the fact that the pK_a for each of the bound ligands is shifted by several pH units to a value greater than 10 (Cocco et al., 1983). This and other evidence

Scheme III



has led to the assumption that the very low inhibition constants of these ligands for DHFR from various sources are due to this interaction, and it is often implied that the equilibrium constants for the binding of these ligands are very high. In support of this view thermodynamic association constants (determined by fluorescence titration, difference spectra titration, CD titration, or equilibrium dialysis) are in the range 10^6–10^8 M^{-1} for binary complex formation and for ternary complex formation in the range 10^9–10^{12} M^{-1} as determined by kinetic methods, radioisotope binding, fluorescence titration, or equilibrium dialysis [for review, see Blakley (1984)].

However, our calculations of the association constant for the *initial* binding reaction, derived from k_f/k_r, are of the order of 10^5 M^{-1} for binary complexes of MTX and of 3-deazaMTX (Table V). Only slightly higher values (1.0×10^6 and 1.6×10^6 M^{-1}) have been obtained for association constants for binding of trimethoprim to isozymes of *E. coli* DHFR (Cayley et al., 1981). Furthermore, our k_f/k_r values for binary complexes of MTX and 3-deazaMTX were 23 and 100 times lower than the fluorescence titration constants (Table V), clearly indicating that at least one subsequent isomerization must occur after ligand binding, according to Scheme III. Calculations of K_{iso} are given in Table V. The values do not vary greatly with pH. A similar isomerization was inferred for *E. coli* DHFR by Cayley et al. (1981). The isomerization reaction clearly has an important part in determining the effective or thermodynamic association constant, measured by such methods as fluorescence titration, for MTX and 3-deazaMTX binary complexes. It is worth noting that in the case of folate binding to SFDHFR II the association constants determined from k_f/k_r and from fluorescence titration appeared to agree within experimental error, so that in this case no isomerization seems to occur.

Formation of the ion pair appears to occur during the initial binding reaction, as indicated by the absorbance change coincident with the fluorescence quench, even at pH 9 (Table I). However, from the above data it appears that this alone does *not* determine a particularly favorable equilibrium constant for binding. The high values of thermodynamic association constants appear to depend rather on the subsequent isomerizations of the ligand–reductase complex. The nature of these isomerizations cannot be determined from the above data but will be discussed further in subsequent publications.

ACKNOWLEDGMENTS

We thank Patricia Morgan for valuable technical assistance and Vicki Gray for expert typing of the manuscript.

REFERENCES

Baccanari, D. P., Daluge, S., & King, R. W. (1982) *Biochemistry 21*, 5068–5075.

Blakley, R. L. (1984) in *Folates and Pterins. Vol. 1. Chemistry and Biochemistry of Folates* (Blakley, R. L., & Benkovic, S. J., Eds.) pp 191–253, Wiley, New York.

Blakley, R. L., Cocco, L., London, R. E., Walker, T. E., & Matwiyoff, N. A. (1978) *Biochemistry 17*, 2284–2293.

Blakley, R. L., Crane, A., Cocco, L., & Baugh, C. E. (1983) *Adv. Exp. Med. Biol. 163*, 1–18.

Bolin, J. T., Filman, D. J., Matthews, 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.

Cocco, L., Groff, J. P., Temple, C., Jr., Montgomery, J. A., London, R. E., Matwiyoff, N. A., & Blakley, R. L. (1981a) *Biochemistry 20*, 3972–3978.

Cocco, L., Temple, C., Jr., Montgomery, J. A., London, R. E., & Blakley, R. L. (1981b) *Biochem. Biophys. Res. Commun. 100*, 413–419.

Cocco, L., Roth, B., Temple, C., Jr., Montgomery, J. A., London, R. E., & Blakley, R. L. (1983) *Arch. Biochem. Biophys. 226*, 567–577.

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.

Elliott, R. D., Temple, C., Jr., Frye, J. L., & Montgomery, J. A. (1971) *J. Org. Chem. 36*, 2818–2823.

Erickson, J. S., & Mathews, C. K. (1972) *J. Biol. Chem. 247*, 5661–5667.

Freisheim, J. H., Smith, C. C., & Guzy, P. M. (1972) *Arch. Biochem. Biophys. 148*, 1–9.

Hood, K., & Roberts, G. C. K. (1978) *Biochem. J. 171*, 357–366.

Montgomery, J. A., Elliot, R. D., Straight, S. L., & Temple, C., Jr. (1971) *Ann. N.Y. Acad. Sci. 186*, 227–234.

Nixon, P. F., & Blakley, R. L. (1968) *J. Biol. Chem. 243*, 4722–4731.

Poe, M., Greenfield, N. J., Hirshfield, J. M., & Hoogsteen, K. (1974) *Cancer Biochem. Biophys. 1*, 7–11.

Stone, S. R., & Morrison, J. F. (1983) *Biochim. Biophys. Acta 745*, 247–258.

Subramanian, S., & Kaufman, B. T. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3201–3205.