Role of Isomerization of Initial Complexes in the Binding of Inhibitors to Dihydrofolate Reductase†

Raymond L. Blakley* and Lennie Cocco‡

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

Received November 27, 1984

ABSTRACT: Stopped-flow measurements of protein fluorescence quenching when methotrexate (MTX) binds to dihydrofolate reductase (isoenzyme II) of *Streptococcus faecium* (SFDHFR II) analyze as the sum of two differentials: a rapid binding phase and a second phase for which the observed rate constant is independent of methotrexate concentration. Analysis of variation of the ratio of the amplitude of the fast and slow phases with methotrexate concentration indicates that the second phase is an isomerization of the initial binary complex. At pH 7.3, the equilibrium constant for this isomerization is 21.9, and the forward and reverse rate constants are 0.57 and 0.026 s^{-1}, respectively. Similar results were obtained for binding of 3-deaza-methotrexate to SFDHFR II, but the forward rate constant is greater (2.9 s^{-1} at pH 7.3). The equilibrium constants for these isomerizations are pH independent, but the rate constants decrease as the pH is raised, probably due to deprotonation of one or more groups on the enzyme. Analysis of progress curves obtained by the development of inhibition when SFDHFR II is added last to reaction mixtures containing dihydrofolate, NADPH, and MTX gives an association constant for initial reactions of 4.3×10^7 M^{-1}. Since a preliminary estimate of the association constant for the binding reaction is 7.6×10^5 M^{-1}, this suggests an isomerization of the ternary complex(es) with an equilibrium constant of about 56. In addition, analysis of the progress of development of inhibition indicates a further very slow isomerization with equilibrium constant 419 and forward rate constant 2.6 min^{-1}. These two isomerizations transform a modest association constant for initial binding into an extraordinary high one (1.9×10^{10} M^{-1}) and are therefore the key mechanism for tight binding. The rate constant for reversal of the slow isomerization is 0.0062 min^{-1}, corresponding to $t_{1/2}$ = 1.9 h, so that release of MTX from its complex with SFDHFR II can occur no faster than this at pH 7.3 and 20 °C. The slow isomerization does not seem to occur in binary complexes. Binary complex formation by MTX and dihydrofolate reductase from *Lactobacillus casei* is biphasic, but this is due to the presence of two forms of the enzyme which bind MTX at different rates. This may obscure possible isomerization of binary complexes. Isoenzyme I of SFDHFR shows a slower phase due to isomerization of the binary complex at pH 6.0 but not at higher pH.

There is considerable evidence that in the binding of inhibitors such as methotrexate (MTX)[1] to dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3, DHFR) the strong affinity observed is due at least in part to relatively slow processes occurring after the initial binding has occurred (Williams et al., 1979, 1980; Stone et al., 1984). It is assumed that these slower, secondary events are isomerizations of the complex initially formed and that they represent some kind of conformational change in the enzyme, the bound ligand, or both. However, there is presently little information on the nature of these isomerizations. It has also been unclear whether the very tight binding of MTX is due *primarily* to a high association constant for the initial binding step (perhaps with ion pair formation between protonated MTX and the active-site carboxyl as the driving force) or *primarily* to isomerizations of the complex with a quite normal association constant in the initial binding step. The former alternative at first seemed likely because what distinguishes the inhibitors that bind tightly from substrates that bind with normal association constants is that the inhibitors are bound with the pteridine ring protonated at N-1 and the substrates do not (Cocco et al., 1981, 1983; Morrison & Stone, 1983). However, the work of Morrison and his colleagues (Williams et al., 1979) has indicated that a final very slow isomerization of the ternary NADPH–DHFR–MTX complex does make a considerable contribution to the overall association constants for MTX binding in the presence of NADPH, but their results did not provide information about the contribution of any additional faster isomerizations of the ternary complex or about the role of isomerizations for binding of MTX in the absence of NADPH.

In a previous publication (Blakley & Cocco, 1985), we reported results from stopped-flow fluorometry and spectrophotometry on the binding of MTX and 3-deaza-MTX to DHFR from *Streptococcus faecium* and *Lactobacillus casei*. Association constants for the initial binding reaction were calculated from the ratio of rate constants for association and dissociation, and values were found to be quite modest, e.g., 5.8×10^5 M^{-1} for MTX binding to isoenzyme II of *S. faecium* DHFR (SFDHFR II) at pH 7.3 (Blakley & Cocco, 1985). These values are significantly lower than thermodynamic

† 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: Department 58C, 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); 3-deaza-MTX, *N*-[*p*-[[(6,8-diamino[2,3-*b*]pyrazin-2-yl)methyl]methylamino]benzoyl]-L-glutamic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/85/0424-4772$01.50/0

association constants determined by titration of enzyme fluorescence by ligand addition, and these discrepancies, which are especially large when NADPH is also bound to the reductase, indirectly indicate that the high overall association constant must have a significant contribution by one or more isomerizations of the initial complex.

In our stopped-flow studies, we observed that the fluorescence quench or absorbance change that accompanies MTX binding to DHFR analyzes as the sum of two exponentials. The apparent rate constant for the faster phase is concentration dependent and corresponds to the initial binding reaction. Results and conclusions obtained from analysis of it are reported in our previous publication (Blakley & Cocco, 1985). In the present publication, the second, slower phase observed in the stopped-flow experiments is analyzed and shown to be related to an isomerization of the initial complex, though distinct from the additional, much slower isomerization of ternary complexes analyzed by progress of inhibition measurements (Williams et al., 1979). However, in some cases, there is also the complication that a second form of the enzyme exhibits slower binding than the predominant form.

Experimental Procedures

Materials. Dihydrofolate reductase was isolated and purified as previously described (Blakley & Cocco, 1985). Methotrexate was obtained from Sigma, and 3-deazamethotrexate was a generous gift from Dr. John A. Montgomery, Southern Research Institute, Birmingham, AL.

Kinetic Measurements. Kinetic data were obtained with a Durrum D-110 (Dionex Instrument Corp., Sunnyvale, CA) stopped-flow instrument. For fluorescence measurements, excitation was at 290 nm, and emission was measured with the use of a 341-nm filter. A north Star Horizon microcomputer was used to capture, store, and process stopped-flow data in an On-Line Instrument Systems Model 3180 data collection system. The average of four or five traces was used to estimate rate constants. Data were analyzed according to a single-exponential model:

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

or a double-exponential model

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

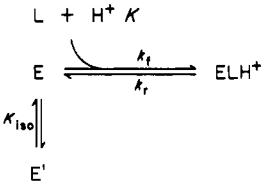
where $F(t)$ is the observed fluorescence at time t, A, A_{fast}, and A_{slow} are amplitude terms, and k, k_{fast}, and k_{slow} are rate constants.

Experiments were carried out at 20 °C in the following 50 mM buffers: pH 5.0–7.3 potassium phosphate; pH 8.1–10.0 Tris-HCl. All mixtures contained 0.5 M KCl (final concentration). Final concentrations of reactants were the following: for fluorescence quenching, 1–4 μM DHFR and 1–20 μM ligand; for absorbance change, 5 μM DHFR and 10–70 μM ligand.

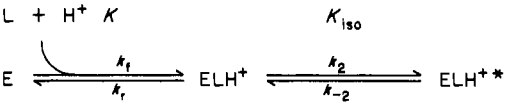
Development of Inhibition Measurements. Experiments were carried out and the data analyzed as described by Williams et al. (1979). Reactions were performed at 20 °C in standard spectrophotometric cuvettes with a 1-cm path length. Mixtures contained 40–50 μM NADPH, 32 μM dihydrofolate, MTX at concentrations between 5 and 60 nM, 50 mM potassium phosphate buffer, pH 7.3, and 500 mM KCl in a final volume of 3.00 mL. Reactions were started by adding SFDHFR II to give a final concentration of 4.64 nM and stirring. The absorbance at 340 nm was recorded on a Cary 219 spectrophotometer and transmitted to a North Star Horizon microcomputer operating on the assay routine of a program provided by On-Line Instrument Systems. Over a 10-min period, 120 data points were collected. Data were transferred to and analyzed on a Data General MV/8000 computer by means of the CRICF program and a Fortran subroutine kindly provided by Dr. Ronald G. Duggleby.

The difference spectrum was obtained as previously described (Blakley & Cocco, 1985).

Scheme I



Scheme II



Results

Slow Phase of MTX Binding to SFDHFR II. As indicated above, the fluorescence quench that occurs when MTX binds to SFDHFR II analyzes as the sum of two exponentials. A representative fit of data to one-exponential and two-exponential models has been published previously (Blakley & Cocco, 1985). The apparent rate constant of the first phase varies with MTX concentration and therefore corresponds to the initial binding reaction (Blakley & Cocco, 1985). However, the apparent rate constant of the slow phase does not vary with MTX concentration and therefore corresponds to an isomerization reaction. Two possible models for this are shown in Schemes I and II. In these schemes, the ligand L (MTX) is represented as being protonated as it binds to give the complex of enzyme and protonated ligand (ELH^+). Conclusive evidence that bound MTX is protonated at all pH values has been obtained by ^{13}C NMR of the complex of [2-^{13}C]MTX with SFDHFR II (Cocco et al., 1981, 1983). Evidence for simultaneous binding and protonation of MTX has been presented in the previous publication (Blakley & Cocco, 1985), and it was shown to result from preferential binding of protonated MTX but with some binding of unprotonated MTX also. In Schemes I and II, binding of both protonated and unprotonated MTX is represented as a single reaction with rate constant k_f.

In Scheme I, the slow phase is due to a slow unimolecular isomerization of uncomplexed enzyme. Only one of the conformers (E) can bind the protonated or unprotonated ligand. The absorbance or fluorescence change in the fast phase corresponds to binding of L and LH^+ with preexisting E. The fluorescence change during the slow phase corresponds to binding of ligand to additional E formed by isomerization of E′. In Scheme II, the fast phase is again due to ligand binding, i.e., formation of ELH^+, and the slow phase to unimolecular isomerization of the protonated binary complex to another conformer, ELH^+*. The isomerization reaction then displaces the equilibrium position in the binding reaction, leading to formation of more ELH^+ from E and L. The observed rate constant for the slow phase in Scheme II should actually show hyperbolic dependence on the inhibitor (L) concentration, according to the equation:

$$k_{obsd} = k_{-2} + k_2/(1 + 1/K[L])$$

where K is the association constant for the fast phase. For

Table I: Concentration-Independent Rate Constants for the Slower Reaction Observed by Stopped-Flow Fluorometry and Spectrophotometry when SFDHFR II Reacts with MTX and 3-Deaza-MTX

pH	values of k_{slow} (s^{-1})	
	MTX	3-deaza-MTX
5.0	1.56 ± 0.42	
6.0	1.33 ± 0.36	2.95 ± 1.70
6.6	1.22 ± 0.39	
7.3	0.60 ± 0.20	2.94 ± 1.55
7.3[a]	1.10 ± 0.32	
8.1	0.34 ± 0.25	1.96 ± 0.62
9.0	0.069 ± 0.03	0.85 ± 0.20
9.0[a]	0.16 ± 0.06	
10.0		0.48 ± 0.06

[a] From absorbance change at 340 nm. Other values are from fluorescence quenching.

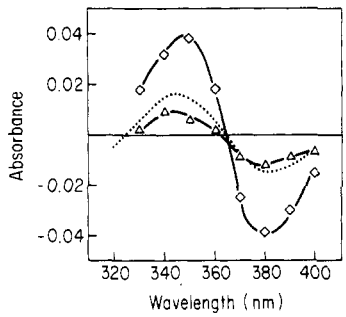


FIGURE 1: Comparison of the amplitudes of the fast and slow phases with the difference spectrum. The amplitudes of the fast phase (◇) and the slow phase (△) were obtained by using a final enzyme concentration of 8.3 μM and an MTX concentration of 10 μM. The difference spectrum (···) was obtained as described under Experimental Procedures.

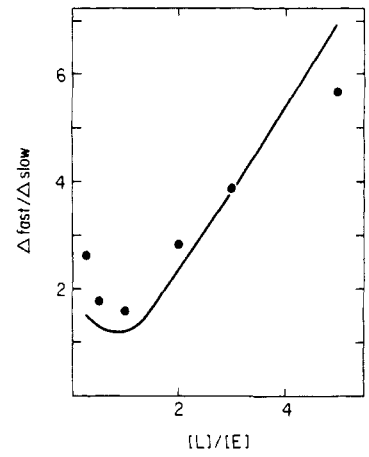


FIGURE 2: Effect of the ligand to enzyme ratio on the relative amplitudes of the fast and slow fluorescent changes ($\Delta_{fast}/\Delta_{slow}$). The curve was calculated for the system $E + L + H^+ \rightleftharpoons ELH^+ \rightleftharpoons ELH^{+*}$ with association constant 4.07×10^5 M^{-1} for the initial binding, equilibrium constant 27 for the isomerization, and enzyme concentration 4 μM. The fast-change amplitude was calculated from the concentration of ELH^+ due to the initial binding equilibrium only. The total amplitude change was calculated from the final concentration of $ELH^+ + ELH^{+*}$ when both reactions are at equilibrium. The slow-phase amplitude change was then calculated by difference. The value of $[ELH^+]$ at equilibrium of the fast (binding) reaction was calculated, assuming participation of protonated ligand, by solving the quadratic $[E_t][LH^+_t] - [ELH^+]([E_t] + [LH^+_t] + 1/K) + [ELH^+]^2 = 0$ where $[E_t]$ is the total enzyme concentration, $[LH^+_t]$ is the total ligand concentration (assumed to become protonated as it reacts), and K is the binding association constant. $[ELH^+]$ at equilibrium of both reactions was obtained by solving the quadratic $[E_t][LH^+_t] - [ELH^+]\{1/K + ([LH^+_t] + [E_t])(1 + K_{iso})\} + (1 + K_{iso})^2[ELH^+]^2 = 0$ where K_{iso} is the equilibrium constant for the isomerization reaction. $[ELH^+] + [ELH^{+*}]$ at equilibrium is given by $[ELH^+](1 + K_{iso})$.

the range of concentrations used and values of K previously reported (Blakley & Cocco, 1985), it can be calculated that k_{obsd} would change 1.15-fold at pH 6.0 and 1.6-fold at pH 7.3. These changes would not be readily detectable in comparison with experimental error. At pH 9.0, k_{obsd} should change by a factor of 3.4 over the concentration range, but such a dependence was not observed.

Since in both these models the quenching of fluorescence is due to additional MTX binding, there should also be an absorbance change due to simultaneous MTX protonation. As may be seen from the results in Table I, this was found to be the case, with a rate constant for the absorbance change not significantly different from that for the fluorescence change. This absorbance change was shown to be due to MTX protonation by comparing the amplitude of the slow phase at various wavelengths with that of the difference spectrum between protonated and unprotonated MTX in the absence of enzyme (Figure 1). In addition, the ratio of the amplitude of the fast and slow changes (Δ_f/Δ_s) was the same, within experimental error, for absorbance measurements as for fluorescence measurements. This suggests that any fluorescence change associated with the isomerization step itself is small.

As previously discussed (Blakley & Cocco, 1985), the association constant for the binding reaction at 20 °C and pH 7.3 calculated from the fast-phase association and dissociation rate constnts (k_f/k_r) is 23-fold lower than the thermodynamic association constant (K_{titr}) determined by fluorescence titration, and this discrepancy is attributed to an isomerization reaction. In the case of Scheme I, however, the ratio of the forms E and E′ is given by the amplitudes of the fast and slow phases. At pH 7.3 in the presence of 10 μM MTX, the slow phase constituted 15.0% ± 0.6% of the total fluorescence change, corresponding to an [E]/[E′] ratio of 5.7. This clearly is unable to account for the discrepancy between the kinetic and thermodynamic estimates of the association constant for the binding reaction.

If the slower reaction is an isomerization of the initial binary complex, as in Scheme II, the association constant from fluorescence titration, K_{titr}, refers to the formation of both ELH^+ and ELH^{+*} as a result of the combined binding and isomerization reactions, and $K_{iso} = K_{titr}/K - 1$. At pH 7.3, $K_{iso} = 13.3/0.58 - 1 = 21.9$. In this mechanism, the observed rate constant for the slower reaction is the sum of the forward and reverse rate constants for the isomerization, $k_2 + k_{-2}$. Since the observed rate constant at pH 7.3 is 0.60 s^{-1} (Table I), the values of k_2 and k_{-2} are 0.57 s^{-1} and 0.026 s^{-1}, respectively. These may be compared with rate constants k_f and k_r for the binding reaction which at pH 7.3 are 0.3×10^6 M^{-1} s^{-1} and 0.52 s^{-1}, respectively. Consequently, although the *observed* rate constant for the fast phase is considerably greater than the *observed* rate constant for the slow phase (3–40 times), the actual rates of the two forward reactions are quite similar over much of the methotrexate concentration range used (1–50 μM). When the ligand is 3-deaza-MTX, the difference is greater between calculated rates for the binding and isomerization reactions. Thus, at 10 μM 3-deaza-MTX, binding at pH 7.3 is 32 times faster than isomerization at equal concentrations of enzyme and enzyme complex, respectively, whereas the ratio is about 6-fold in the case of MTX.

Scheme II predicts that when [L] >> [E] most E will be converted to EL so that the amplitude of the fluorescence change during the fast phase will be much greater than that during the slow phase. The ratio of the amplitudes of the fast

Table II: Rate Constants for Isomerization of Binary Complexes of MTX and 3-Deaza-MTX with SFDHFR II

ligand	pH	K_{iso}[a]	k_{slow} (s^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})
MTX	6.0	11.7	1.33	1.23	0.105
	7.3	21.9	0.60	0.57	0.026
	9.0	35.7	0.069	0.067	0.0019
3-deaza-MTX	6.0	125	2.95	2.93	0.023
	7.3	100	2.94	2.91	0.029
	9.0	90	0.85	0.84	0.009

[a] Calculated from $K_{titr}/K - 1$ (Blakley & Cocco, 1985).

Table III: Rate Constants for the Slower Phase of Formation of the Binary Complex between MTX and LCDHFR at Various pH Values

pH	k_f' ($\times 10^{-5}$ M^{-1} s^{-1})	k_r' ($\times 10^{-1}$ s^{-1})
5.0	8.4 ± 0.3	1.3 ± 2.1
6.0	5.6 ± 0.4	23.7 ± 2.6
7.3	3.2 ± 0.4	6.0 ± 2.1
9.0	1.0 ± 0.1	3.3 ± 0.6
10.0	0.23 ± 0.01	4.8 ± 0.1

and slow changes ($\Delta_{fast}/\Delta_{slow}$) will decrease as [L]/[E] decreases, but when [L] >> [E], $\Delta_{fast}/\Delta_{slow}$ increases again. This is illustrated by the curve in Figure 2, which was calculated for $K = 4.07 \times 10^5$ M^{-1}, the value corresponding to k_f and k_r determined in this experiment, and $K_{iso} = 27$. It may be seen that the experimentally determined values of $\Delta_{fast}/\Delta_{slow}$ fit reasonably well to the curve. The fit is not significantly improved by selecting other values of K and K_{iso}, and the deviation must be attributed to experimental error or to other reactions occurring in a more complex mechanism, or both. In a system such as that described by Scheme I, $\Delta_{fast}/\Delta_{slow}$ should assume a constant moderate value (corresponding to K_{iso}) at high [L]/[E] ratios and become very high when ligand concentrations progressively decrease below enzyme concentration [cf. Dunn et al. (1978)]. It is clear, therefore, that the data favor Scheme II.

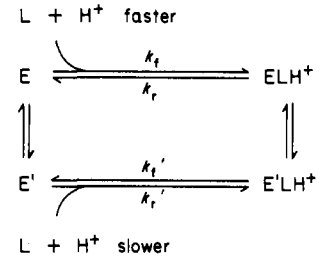
Effect of Ligand and pH on Rate Constants for Isomerization of Binary Complexes. It may be seen (Tables I and II) that the observed rate constant, as well as the forward rate constant, k_2, for the isomerization of the MTX–SFDHFR II complex, decreases significantly as the pH is increased. Between pH 6 and pH 9, these rate constants are decreased 19-fold. The effect of pH on the isomerization reverse rate constant, k_{-2}, is even more marked—approximately 58-fold in this pH range. Effects of pH on the rate constants for isomerization of the 3-deaza-MTX complex are much less marked, but in the same direction.

It should also be noted that just as the rate of binding of 3-deaza-MTX to SFDHFR II is significantly greater than that for MTX, the rate of isomerization is also significantly greater (Table II).

Slower Phase of Binary Complex Formation between MTX and LCDHFR and SFDHFR I. The slower phase of fluorescence quench during binding of MTX to LCDHFR gave a rate constant that increased with ligand concentration and hence indicated reaction of MTX with two forms of the enzyme according to Scheme III. Association and dissociation rate constants k_f' and k_r' were calculated for binding to the second enzyme form E′ and are shown in Table III. For this system, $\Delta_{fast}/\Delta_{slow}$ showed no clear dependence on pH and had a mean value of 1.75.

When binary complex formation between SFDHFR I and MTX was investigated, a slower phase with a rate constant of 3.8 ± 1.2 s^{-1}, independent of MTX concentration, was found at pH 6.0. However, at pH 7.3 and 9.0, the fluorescence change accompanying binding analyzed as a single exponential;

Scheme III



i.e., no slower phase could be seen.

Determination of Kinetic Constants for SFDHFR II by Progress Curve Analysis. Williams et al. (1979) have developed a spectrophotometric method for obtaining rate constants for a very slow isomerization of the MTX–NAPDH–SFDHFR II complex. The method depends on measuring the development of inhibition after enzyme has been added as the last component to the reaction mixture. We have performed similar experiments except that the reaction was performed under the same conditions that we used for the stopped-flow experiments. Despite our use of a lower temperature, we obtained results in excellent agreement with those of Williams et al. and confirmed the occurrence of an isomerization with a rate constant much slower than those that we observed by stopped-flow experiments. Values determined for kinetic constants were as follows: $V/[E_t]$, 650 min^{-1}; K_i, 23 nM; k_{fsi} (forward rate constant for slow isomerization), 2.6 min^{-1}; k_{rsi} (reverse rate constant for slow isomerization), 0.0062 min^{-1}. The association constant for reactions up to the final slow isomerization is $1/K_i = 4.3 \times 10^7$ M^{-1}, and the overall association constant including the slow isomerization is $(k_{fsi} + k_{rsi})/K_i k_{rsi} = 1.9 \times 10^{10}$ M^{-1}.

Discussion

Isomerization of Binary Complexes. In the binding of MTX and 3-deaza-MTX to SFDHFR II, the complexes formed in the initial binding reaction undergo some kind of isomerization, presumably involving a conformational change. The evidence is clear: the second phase of the decrease in the intrinsic protein fluorescence has an observed rate constant invariant with ligand concentration; the relative amplitudes of the fast and slow phases vary with ligand concentration in a manner consistent with binary complex isomerization and inconsistent with isomerization of uncomplexed enzyme, and the association constant determined by fluorometric titration, K_{titr}, is significantly greater (≈20-fold) than that determined from the forward and reverse rate constants for the fast-phase binding reaction.

The nature of this isomerization is unknown except that, as indicated in Scheme II, it apparently prevents dissociation of ligand which can only leave after the complex has reverted to its initial conformation. It might involve relatively subtle rearrangements of side chains, bound water, and inhibitor in the active site, without any noticeable change in the folding of the backbone. This is suggested by the similarity of backbone folding in the MTX binary complex with *E. coli* DHFR and in the ternary complex of LCDHFR (Bolin et al., 1982) and by the rather subtle differences in the binding of stronger and weaker inhibitors to chicken liver and *E. coli* DHFR (Matthews et al., 1985a,b). One possibility is that the rearrangement increases the electrostatic and hydrogen bond interaction between the protonated heterocyclic ring of the inhibitor and the carboxylate of Asp-27. However, this seems rather unlikely since it was found that at pH values where free MTX is unprotonated, binding of MTX to SFDHFR II and

protonation of the ligand were simultaneous (Blakley & Cocco, 1985). Additional protonation of MTX that occurs during the slower isomerization phase (Figure 1) is coincident with additional ligand binding (Table I).

The isomerization that occurs during the slow phase detected by stopped-flow fluorometry is apparently the only isomerization of the binary complexes. There is no very slow isomerization such as occurs with NADPH–E–MTX complexes as shown by analysis of the progress of inhibition curves. The absence of such an additional isomerization is proved by the good agreement (Figure 2) between observed $\Delta_{fast}/\Delta_{slow}$ ratios and those predicted from equilibrium constants obtained from the kinetics of the fast phase and by fluorescence titration which reflects the result of binding and all subsequent isomerizations.

The rate of isomerization of the binary complexes is significantly affected by pH (Table I), but it is not easy to determine what group is ionizing. One possibility is that the analysis of the fluorometric change into fast and slow phases has been incomplete so that some fraction of the large pH effect on the fast (binding) phase is artifactually reflected in the slow phase. A difficulty with this interpretation is that plots of log k_{obsd} vs. pH for the isomerization reactions (not shown) indicate significantly higher pK_a values for this pH dependency than obtained for the binding reaction. For the isomerization of the binary complexes of MTX and 3-deaza-MTX, the pH dependency indicates approximate pK_a values of 7.0 and 8.4, respectively, each of which is about 1 pH unit above the pK_a calculated for the corresponding binding reactions. On the other hand, it is unclear why the isomerization pK_a values differ for the two ligand complexes if the pH effect is due to ionization of one or more groups on the enzyme, though it must be remembered that there are significant differences in the binding of these two ligands (Blakley & Cocco, 1985). It is perhaps significant that although rates of isomerization are apparently pH dependent, the isomerization equilibria are not (Table II).

Isomerizations of Ternary Complexes. An indication that an isomerization of the NADPH–SFDHFR II–MTX ternary complex occurs is obtained from analysis of the progress curve during the development of inhibition. The estimate of K_i is 23 nM, so that the association constant for all steps except the final slow isomerization is 4.3×10^7 M^{-1}. In a preliminary study of the fast phase of MTX binding to the NADPH–SFDHFR II binary complex, the association constant was estimated to be 7.6×10^5 M^{-1} (R. L. Blakley and L. Cocco, unpublished results), so that the value of K_{iso} must be approximately $1/K_iK - 1$ or $(4.3 \times 10^7/7.6 \times 10^5) - 1 = 56$. This is slightly higher than the estimate for K_{iso} for the binary complex at pH 7.3, the value obtained being 22 (Blakley & Cocco, 1985). The data provide no estimate of the rate constants for the isomerization, but an upper limit is provided by k_r', i.e., 0.17 s^{-1}, which is significantly slower than for the binary complex, 0.60 s^{-1} at pH 7.3 (Table I).

Analysis of the progress curve for the development of inhibition also provides evidence, however, that a further slow isomerization of the ternary complex occurs with a forward rate constant, k_{fsi}, of 2.6 min^{-1}. Since the equilibrium constant has a value of 419, the reaction is heavily in favor of the final conformer. This results from an extremely low value for k_{rsi}, the rate constant for the reverse isomerization, 0.0062 min^{-1}.

The final very slow isomerization observed by development of inhibition is silent in stopped-flow measurement of absorbance and fluorescence changes; i.e., no further change was seen with an exponential corresponding to 2.6 min^{-1}. This is to be expected since at experimentally feasible concentrations of enzyme and ligand, the association constant of 4.3×10^7 M^{-1} for preceding stages of the interaction of MTX and DHFR ensures that the formation of complex is already virtually (>97%) complete before the final isomerization begins. Like the first isomerization, the final one would only be observable by an indirect effect on the first (binding) reaction. Nothing is presently known about the structural changes involved in either isomerization, but it should be noted that structural information obtained by X-ray crystallography and NMR must refer to the conformation achieved after the final isomerization.

It is clear from these results that the two isomerizations transform a modest association constant for initial binding (7.6×10^5 M^{-1}) into an extraordinarily high one (1.9×10^{10} M^{-1}), and indeed, it is the occurrence of the isomerization that makes the binding of MTX to SFDHFR II so tight. Williams et al. (1980) have shown that similar slow isomerizations occur for the ternary complexes of many related anti-folates with SFDHFR II, including trimethoprim and aminopterin. Stone et al. (1984) have also shown the occurrence of similar slow isomerizations of ternary complexes of DHFR from chicken liver and *E. coli* where the inhibitor is methotrexate or 5-deazamethotrexate. It is unknown whether the first isomerization occurs in these cases, though the low values obtained for K_i suggest that this might be the case.

It should be noted that the reverse rate constant for the slow isomerization, k_{rsi}, has a value corresponding to a $t_{1/2} = 1.9$ h, so that dissociation of MTX from the ternary complex of SFDHFR II can occur no faster than this at pH 7.3 and 20 °C. Only slightly faster rates have been reported (Stone et al., 1984) for reversal of the slow isomerization step in MTX release from ternary complexes of *E. coli* DHFR and chicken liver DHFR at 30 °C ($t_{1/2} = 0.44$ and 0.58 h, respectively).

The results of Baccanari et al. (1982) on the binding of trimethoprim and its congeners to DHFR from *E. coli*, *L. casei*, and a rodent sarcoma are consistent with some but not all of our conclusions. They found much higher overall association constants (lower dissociation constants) for formation of ternary complexes containing NADPH as well as inhibitor than for binary complexes. This could be explained by the occurrence of the slow isomerization step in the case of ternary but not binary complexes, as we have found in the case of SHDHFR II, but was attributed by Baccanari et al. largely to a slower dissociation rate constant in the reversal of the initial binding step for ternary complexes. In contrast, preliminary stopped-flow results indicate that dissociation rate constants are similar for binary and ternary complexes of SFDHFR II (R. L. Blakley and L. Cocco, unpublished results).

Possible Isomerizations of Complexes of LCDHFR and SFDHFR I. For LCDHFR, any evidence for isomerization of the binary complex was obscured by the occurrence of a slower binding phase presumed to involve a second conformer of the enzyme, and when SFDHFR I was examined, a slow isomerization of the binary complex resulting in the fluorescence quench was observed only at pH 6.0. No investigations of slow isomerizations of ternary complexes have been reported for these enzymes.

Binding of MTX to NADPH–LCDHFR has been investigated by stopped-flow measurement of fluorescence quenching by Dunn & King (1980), who reported only a single phase for this reaction. The rate constant for association (1.9×10^7 M^{-1} s^{-1} at pH 6.5 and 25 °C) is stated to be similar to an unreported value they obtained for binding to the uncom-

plexed enzyme and is also in quite good agreement with our rate constant for the faster phase of association to the binary complex (7.4×10^6 M^{-1} s^{-1} at 20 °C and pH 6.0). Dunn et al. (1978) also reported evidence from stopped-flow fluorescence measurements of NADPH binding to LCDHFR that the enzyme exists in two conformers only one of which binds NADPH efficiently. This is consistent with the proposed binding of MTX to two enzyme forms in Scheme III.

Registry No. DHFR, 9002-03-3; MTX, 59-05-2; 3-deazaMTX, 34561-27-8.

References

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

Blakley, R. L., & Cocco, L. (1985) *Biochemistry* (in press).

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

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

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.

Matthews, D. A., Bolin, J. T., Burridge, J. M., Filman, D. J., Volz, K. W., Kaufman, B. T., Beddell, C. R., Champness, J. N., Stammers, D. K., & Kraut, J. (1985a) *J. Biol. Chem. 260*, 381–391.

Matthews, D. A., Bolin, J. T., Burridge, J. M., Filman, D. J., Volz, K. W., & Kraut, J. (1985b) *J. Biol. Chem. 260*, 392–399.

Stone, S. R., Montgomery, J. A., & Morrison, J. F. (1984) *Biochem. Pharmacol. 33*, 175–179.

Williams, W. W., Morrison, J. F., & Duggleby, R. G. (1979) *Biochemistry 18*, 2567–2573.

Williams, W. W., Duggleby, R. G., Cutler, R., & Morrison, J. F. (1980) *Biochem. Pharmacol. 29*, 589–595.

Photochemical Cross-Linking of $tRNA_1^{Arg}$ to the 30S Ribosomal Subunit Using Aryl Azide Reagents Attached to the Anticodon Loop†

Jan-Kan Chen,‡ Lothar A. Franke,§ Stephen S. Hixson,§ and Robert A. Zimmermann*,‡

Departments of Biochemistry and Chemistry, University of Massachusetts, Amherst, Massachusetts 01003

Received February 11, 1985

ABSTRACT: The 2-thiocytidine residue at position 32 of $tRNA_1^{Arg}$ from *Escherichia coli* was modified specifically with three photoaffinity reagents of different lengths, and the corresponding *N*-acetylarginyl-$tRNA_1^{Arg}$ derivatives were cross-linked to the P site of *E. coli* 70S ribosomes by irradiation. Covalent attachment was dependent upon the presence of a polynucleotide template and exposure to light of the appropriate wavelength. From 4% to 6% of the noncovalently bound tRNA became cross-linked to the ribosome as a result of photolysis, and attachment to the P site was confirmed by the reactivity of arginine in the covalent complexes toward puromycin. Analysis of the irradiated ribosomes by sucrose-gradient sedimentation at low Mg^{2+} concentration revealed that the tRNA was associated exclusively with the 30S subunit in all cases. Two of the *N*-acetylarginyl-$tRNA_1^{Arg}$ derivatives were attached primarily to ribosomal proteins whereas the third was cross-linked mainly to 16S RNA. Partial RNase digestion of the latter complex demonstrated that the tRNA had become attached to the 3′ third of the rRNA molecule. In addition, the tRNA–rRNA bond was shown to be susceptible to cleavage by hydroxylamine and mercaptoethanol.

Affinity labeling provides an effective methodology for investigating the molecular anatomy of ligand binding sites in complex cellular structures such as ribosomes and other components of the translational apparatus (Jakoby & Wilchek, 1977). In this approach, the ligand is first derivatized with an appropriate reactive group. The modified ligand is then allowed to associate with its binding site and a covalent cross-link between the two is established either chemically or photochemically. The use of photolabile substituents is particularly advantageous as the ligand can be positioned and tested for functional activity prior to covalent attachment. Further, by utilization of probes of different lengths and reactivities, binding site constituents located in the immediate vicinity, as well as in the general neighborhood, of the derivatized residue(s) can be identified. Transfer RNAs are well suited for such studies as they contain a number of minor bases that can be modified specifically with the required reagents (Kuechler & Ofengand, 1979). Moreover, because the three-dimensional structure of tRNA is known (Kim, 1979), cross-linking from a variety of positions within these molecules can in principle permit deduction not only of the composition but also of the spatial organization of the sites with which they interact in the course of protein biosynthesis.

Chemical and photochemical labeling techniques have already proved useful in defining the interaction of tRNAs with aminoacyl-tRNA synthetases (Budzik et al., 1975; Schoemaker et al., 1975), elongation factor Tu (Johnson et al., 1978; Duffy et al., 1981), and the ribosomal A and P sites (Kuechler & Ofengand, 1979). For example, the anticodon loop of

† This work was supported by Research Grant GM22807 from the National Institutes of Health.

‡ Department of Biochemistry.

§ Department of Chemistry.

0006-2960/85/0424-4777$01.50/0