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Methotrexate, a High-Affinity Pseudosubstrate of Dihydrofolate Reductase†

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ABSTRACT: Investigations have been made of the slow, tight-binding inhibition by methotrexate of the reaction catalyzed by dihydrofolate reductase from *Streptococcus faecium* A. Quantitative analysis has shown that progress curve data are in accord with a mechanism that involves the rapid formation of an enzyme–NADPH–methotrexate complex that subsequently undergoes a relatively slow, reversible isomerization reaction. From the K_i value for the dissociation of methotrexate from the E–NADPH–methotrexate complex (23 nM) and values of 5.1 and 0.013 min^{-1} for the forward and reverse rate constants of the isomerization reaction, the overall inhibition constant for methotrexate was calculated to be 58 pM. The formation of an enzyme–methotrexate complex was demonstrated by means of fluorescence quenching, and a value of 0.36 μM was determined for its dissociation constant. The same technique was used to determine dissociation constants for the reaction of methotrexate with the E–NADP and E–NADPH complexes. The results indicate that in the presence of either NADPH or NADP there is enhancement of the binding of methotrexate to the enzyme. It is proposed that methotrexate behaves as a pseudosubstrate for dihydrofolate reductase.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. The enzyme has been of considerable pharmacological interest because it is the target for a number of chemotherapeutic agents (Hitchings & Burchall, 1965) such as methotrexate, one of the first folic acid analogues to produce beneficial effects in the treatment of neoplastic disease in man (Blakley, 1969).

Because of its pharmacological importance, much effort has been directed toward an understanding of the nature of the inhibition of dihydrofolate reductase by methotrexate. Unfortunately, in earlier work on methotrexate inhibition, there was a lack of appreciation of the significance of the slow development of the inhibition and a failure to recognize that

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steady-state kinetic theory cannot be applied under conditions where an enzyme–inhibitor complex has a very low dissociation constant. The kinetic theory for tight-binding inhibition under steady-state conditions was developed by Morrison (1969), and subsequently Cha (1975, 1976) derived equations that describe the slow binding of inhibitors to an enzyme. More recently, Williams & Morrison (1978) have discussed the approaches that can be used to study slow and tight-binding enzyme inhibitors.

This report describes a study of the tight-binding inhibition by methotrexate of dihydrofolate reductase from *Streptococcus faecium* A. The results indicate that methotrexate functions as a very slow, very tight-binding inhibitor (Williams & Morrison, 1978). The kinetic theory associated with this type of inhibition is elaborated in detail.

Experimental Procedure

Materials. NADP and NADPH were purchased from P-L Biochemicals, and folic acid was from Calbiochem. Dihydrofolate was prepared by the reduction of folic acid according to the method of Blakley (1960) and stored at −20 °C for not longer than 2 weeks. Methotrexate was obtained from ICN Chemical and Radioisotope Division and purified further by butanol extraction (Blakley, 1957). Concentrations of methotrexate were determined spectrophotometrically at pH 13, by using molar extinction coefficients of 23 250 or 22 100 at 258 or 302 nm, respectively (Seeger et al., 1949). The concentrations of dihydrofolate and NADPH were determined enzymically by using dihydrofolate reductase and a molar extinction coefficient of 12 300 at 340 nm (Hillcoat et al., 1967). Other chemicals were high-purity preparations from commercial sources.

Enzyme Preparation. A methotrexate-resistant mutant strain of *S. faecium* A, provided by Dr. R. L. Blakley, was grown and harvested as described by Nixon & Blakley (1968). The washed cells were suspended in 5 volumes of buffer (pH 7.0) containing 0.05 M Tris, 0.1 M KCl, and 10 mM dithiothreitol and disrupted in a Ribi fractionator at 20 000 psi. All further operations were performed at 4 °C. The cell debris was removed by centrifuging at 30000*g* for 15 min. The extract was made 1.5% (w/v) in streptomycin sulfate, and the precipitate was removed by centrifuging at 30000*g* for 15 min. The supernatant solution was applied directly to a methotrexate–Sepharose column that had been prepared as described by Poe et al. (1972). The column was washed with buffer containing 1 M KCl after which the enzyme was eluted with 0.4 mM dihydrofolate at pH 9.5. After removal of dihydrofolate by chromatography on Sephadex G-75, the enzyme solution was fractionated on a column of DEAE-Sephadex to separate the mutant form of dihydrofolate reductase from the wild-type enzyme which is present only at relatively low concentrations (Nixon & Blakley, 1968). The former enzyme, which was used for all subsequent investigations, was shown by polyacrylamide gel electrophoresis to be homogeneous and completely free of the wild-type enzyme. It was stored at −20 °C in 0.2 M sodium phosphate (pH 7.2) containing 10 mM dithiothreitol.

Enzyme Assays. The activity of dihydrofolate reductase was determined at 30 °C by following the decrease of NADPH and dihydrofolate by absorbance measurements at 340 nm by using a Cary 118 spectrophotometer. Experiments were performed at pH 7.4 in a buffer mixture containing 2-(*N*-morpholino)ethanesulfonate (0.025 M), sodium acetate (0.025 M), Tris (0.05 M), and NaCl (0.1 M). The concentrations of NADPH, dihydrofolate, and methotrexate are given in the text or in the legends to the figures.

Progress Curves. Progress curves were obtained by two different experimental procedures. The slow development of methotrexate inhibition was determined by continuously monitoring the disappearance of NADPH and dihydrofolate after initiation of the reaction by the addition of dihydrofolate reductase (6.0 nM). Reaction mixtures contained buffer mixture (pH 7.4), NADPH (90 μM), dihydrofolate (76 μM), and various concentrations of methotrexate. Experiments to determine the slow recovery of enzymic activity after inhibition of the enzyme by preincubation with methotrexate were performed as follows. Dihydrofolate reductase (0.66 μM) was preincubated for 10 min at 30 °C in the buffer mixture (pH 7.4) containing NADPH (90 μM) and methotrexate (0–1.17 μM). An aliquot of the incubation mixture was then diluted 110-fold into a reaction mixture containing buffer mixture (pH 7.4), NADPH (90 μM), and dihydrofolate (76 μM) to give a final enzyme concentration of 6.0 nM. Recovery of enzyme activity was followed by continuous monitoring at 340 nm.

Fluorescence Measurements. Dissociation constants for the interaction of NADPH and methotrexate with dihydrofolate reductase were determined by measurement of the partial quenching of the enzyme fluorescence that occurs on the binding of these reactants to the enzyme. Experiments were performed at pH 7.4 and 30 °C in an Aminco-Bowman spectrofluorometer by using activation and emission wavelengths of 280 and 350 nm, respectively. Titrations were carried out by serial additions of variable aliquots (0.5–5.0 μL) of concentrated ligand, up to a maximum cumulative volume of 30 μL, to 2.15 mL of buffered enzyme solution that was contained in a quartz cuvette of 1-cm light path. After each addition, the solution was mixed thoroughly and allowed to stand for 2 min before the shutter was opened for the duration of the reading only. The reported wavelengths are uncorrected, but when necessary, relative fluorescence intensities were corrected for dilution. It was not necessary to make any corrections for absorption by the ligands.

Theory and Data Analysis

Progress Curves. Cha (1976) has considered the kinetics of the reaction for a mechanism in which the equilibrium between the enzyme and the substrate(s) is rapidly attained while the equilibrium between the enzyme and an inhibitor is established slowly (mechanism A, Scheme I). It was shown that, if the concentration of inhibitor required for inhibition is relatively low so that the formation of an enzyme–inhibitor complex significantly alters the concentration of free inhibitor, the rate of the enzyme-catalyzed reaction (v) will vary with time (t) according to the equation

$$v = \frac{v_s + [v_0(1 - \gamma) - v_s] \exp(-\lambda t)}{1 - \gamma \exp(-\lambda t)} \quad (1)$$

In eq 1, v_0 is the steady-state rate in the absence of inhibitor while v_s is the steady-state rate in the presence of inhibitor. These rates and the parameters γ and λ are related to the total concentrations of substrate (A), enzyme (E_t), and inhibitor (I_t) as well as to the maximum velocity (V), the Michaelis constant (K_a), and the rate constants k_3 and k_4 by the expressions

$$v_0 = \frac{V(A)}{K_a + (A)} \quad (2)$$

$$v_s = v_0\left[\frac{(E_t) - (I_t) - K_i' + Q}{2(E_t)}\right] \quad (3)$$

$$\gamma = \frac{K_i' + (E_t) + (I_t) - Q}{K_i' + (E_t) + (I_t) + Q} \quad (4)$$

$$\lambda = \frac{k_3 K_a Q}{K_a + (A)} \quad (5)$$

where $K_i' = K_i[1 + (A)/K_a]$, $K_i = k_4/k_3$, and $Q = \{[K_i' + (E_t) + (I_t)]^2 - 4(E_t)(I_t)\}^{1/2}$. While velocity data can be fitted to eq 1, such data must be obtained by subjective estimation of tangents to a curve. The use of the integrated form of eq 1 for analysis of progress curve data is a much more satisfactory procedure. Integration of eq 1 with respect to time gives eq 6, and it will be noted that analyses based on the fitting of data

$$(P) = v_s t + \frac{(1 - \gamma)(v_0 - v_s)}{\lambda\gamma} \ln\left[\frac{1 - [\gamma \exp(-\lambda t)]}{1 - \gamma}\right] \quad (6)$$

to this equation are devoid of subjectivity since the dependent variable (P) is the quantity that is measured experimentally. The general advantages of progress curve analysis have been discussed previously (Duggleby & Morrison, 1977).

Although eq 1 and 6 contain four formal parameters (v_0, v_s, γ, and λ), any particular progress curve will contain information with respect to only three of these parameters. This is because γ is related to v_0 and v_s by the equation

$$\gamma = \frac{(E_t)}{(I_t)}\left(1 - \frac{v_s}{v_0}\right)^2 \quad (7)$$

The fitting of data from a single progress curve to eq 6 and 7 will yield values for v_0, v_s, and λ. If values for these parameters are obtained at different substrate concentrations, then the relationships given in eq 2–5 may be used to calculate values for V, K_a, k_3, and k_4. Alternatively, the data from several progress curves may be combined and analyzed to obtain the best values for these four parameters. Computer programs have been developed for this purpose and they are based on STEPIT which is a nonlinear optimization routine that uses a direct-search strategy (Swann, 1969).

A more complex mechanism which predicts the slow development of inhibition is mechanism B of Scheme I. In this model an enzyme–inhibitor (EI) complex, which is formed rapidly, undergoes a slow isomerization to a second (EI*) complex. Attempts to derive an integrated equation for mechanism B, analogous to eq 6, were unsuccessful for the reasons developed below.

Mechanism B is described by the differential equations

$$\frac{d(E)}{dt} = k_4(EI) + (k_2 + k)(EA) - [k_1(A) + k_3(I)](E) \quad (8)$$

$$\frac{d(EA)}{dt} = k_1(A)(E) - (k_2 + k)(EA) \quad (9)$$

$$\frac{d(EI^*)}{dt} = k_5(EI) - k_6(EI^*) \quad (10)$$

$$\frac{d(P)}{dt} = k(EA) \quad (11)$$

and by the conservation equations

$$(E_t) = (E) + (EA) + (EI) + (EI^*) \quad (12)$$

$$(I_t) = (I) + (EI) + (EI^*) \quad (13)$$

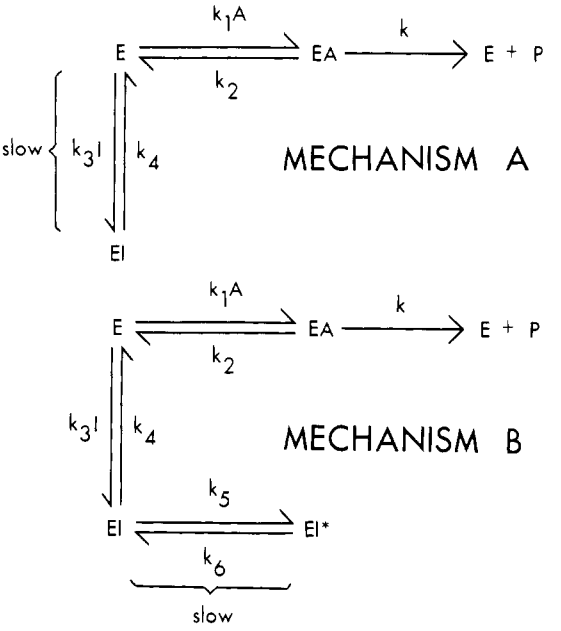
Under conditions where E, EA, and EI are in steady-state equilibrium, eq 8, 9, 12, and 13 may be solved to give

$$(EI)^2 - (EI)[(E') + (I') + K_i'] + (E')(I') = 0 \quad (14)$$

$$(EA) = [(E') - (EI)]/[1 + K_a/(A)] \quad (15)$$

where $(E') = (E_t) - (EI^*)$, $(I') = (I_t) - (EI^*)$, $K_i' = K_i[1 + (A)/K_a]$, $K_i = k_4/k_3$, and $K_a = (k_2 + k)/k_1$. In principle, the above equations may be solved to give (P) as a function of time.

Scheme I



Unfortunately, the attempted solution leads to hopelessly complex algebra and an alternative technique must be sought. For particular values of the various parameters, the equations may be solved by numerical integration to give the expected value of (P) at any given time, provided that the conditions existing at $t = 0$ are specified. The preferred method of defining this boundary condition is to set $(EI^*) = 0$ and $(P) = 0$. The concentrations of E, EA, and EI may be calculated from eq 14 and 15, and thus it is possible to numerically integrate eq 10 and 11 to yield (P) as a function of time.

The aforementioned procedure was modified for analysis of data that were obtained by following the recovery of the activity of enzyme which had been preincubated with inhibitor and then diluted into a reaction mixture. For such experiments the concentration of EI* when $t = 0$ is given by eq 16 and 17,

$$(EI')^2 - (EI')[x(E_t) + x(I_t) + K_i^*] + x^2(E_t)(I_t) = 0 \quad (16)$$

$$(EI^*) = \frac{(EI')}{x}\left(\frac{k_5}{k_5 + k_6}\right) \quad (17)$$

where $K_i^* = K_i k_6/(k_5 + k_6)$, $K_i = k_4/k_3$, and x is the ratio of the final assay volume to the preincubation volume. (EI') represents the sum of (EI) and (EI*).

Chandler et al. (1972) have described the CRICF program which utilizes numerical methods to integrate a system of differential equations and which may be employed to fit progress curve data for a reaction conforming to mechanism B. This procedure has been used to analyze data for the inhibition of dihydrofolate reductase by methotrexate. Each experiment consisted of several (5–10) progress curves, and from each curve 12–17 data points were taken for analysis. The curves were obtained at a single dihydrofolate concentration and different methotrexate concentrations. A value of the apparent Michaelis constant for dihydrofolate of 15 μM was supplied to the program, and the data were used to estimate V, K_i, k_5, and k_6.

Fluorescence Quenching Curves. The fluorescence of dihydrofolate reductase is reduced on binding of substrates or substrate analogues (Freisheim & D'Souza, 1971; Williams et al., 1973b), and this property may be used as a convenient method for determining both the enzyme concentration and the dissociation constants of enzyme–ligand complexes. If the

fluorescence of the free enzyme (E) is F_0 and that of the enzyme–ligand (EL) complex is F_∞, then the enzyme fluorescence (F) at any particular ligand concentration is given by eq 18 where (E_t) represents the total enzyme concentration.

$$F = [F_0(E) + F_\infty(EL)]/(E_t) \quad (18)$$

The dissociation constant (K_d) of the EL complex is given by eq 19 where (L_t) represents the total ligand concentration.

$$K_d = [(E_t) - (EL)][(L_t) - (EL)]/(EL) \quad (19)$$

Equation 19 may be solved for (EL), and the concentration of free enzyme may be calculated from the conservation equation (E_t) = (E) + (EL). If the enzyme fluorescence (F) is plotted as a function of (L_t) (cf. Figure 5), the tangent to the curve at (L_t) = 0 (eq 20) intersects with the asymptote

$$F = F_0 - \left[\frac{F_0 - F_\infty}{K_d + (E_t)}\right](L_t) \quad (20)$$

of the curve ($F = F_\infty$) where (L_t) = (E_t) + K_d. Under circumstances where $K_d << (E_t)$, then the titration curve will approximate two straight line segments which will intersect at (L_t) = (E_t), and the total enzyme concentration may be determined with little uncertainty.

The method of choice for the analysis of fluorescence titration data is to treat F_0, F_∞, (E_t), and K_d as unknown parameters and to obtain estimates of their values by fitting the data to eq 18 and 19 by regression analysis. Such a procedure has been used in the present study. The program developed for this purpose is based on the STEPIT routine that has been referred to above.

Velocity–Titration Curves. In the preceding discussion, reference was made only to the use of fluorescence measurements for determining values of K_d and (E_t). However, use may be made of any other measurement that distinguishes between the free enzyme and the enzyme–ligand complex. If the enzyme–ligand complex is inactive and dissociates slowly, then velocity measurements after preincubation with the ligand will provide an estimate of the free enzyme concentration. This procedure was used to determine the concentration of dihydrofolate reductase.

Computer Programs. The STEPIT program, written by Dr. J. P. Chandler, was obtained from the Quantum Chemistry Program Exchange as part of the STEPT package (QCPE307). The CRICF program has been described by Chandler et al. (1972) and was obtained from these authors. Problem-dependent subroutines were written in Fortran in this laboratory, and details may be obtained from the authors. Programs were run on a Univac 1100/42 computer.

Results

Steady-State Velocity Studies. The steady-state velocities obtained by varying NADPH at different fixed concentrations of dihydrofolate (DHF) yielded an intersecting pattern. The data were fitted to the equation that describes a rapid-equilibrium, random mechanism, and the following values were calculated: $K_{(NADPH)} = 0.72 \pm 0.23$ μM; $K_{i(NADPH)} = 0.76 \pm 0.36$ μM; $K_{(DHF)} = 15 \pm 3$ μM; $K_{i(DHF)} = 16 \pm 9$ μM. The relatively high standard errors are a consequence of the low Michaelis constants for the substrates. At the fixed concentration of NADPH (90 μM) which was used for all subsequent studies, the apparent Michaelis constant for dihydrofolate was calculated to be 15 μM.

Progress Curves in the Absence and Presence of Inhibitor. In the absence of inhibitors, the steady-state velocity of the dihydrofolate reductase reaction is reached immediately (Figure 1, curve A) and maintained until at least 10% of the limiting substrate, dihydrofolate, has been utilized. However, in the presence of methotrexate, there is a time-dependent decrease in the reaction rate which varies as a function of the inhibitor concentration (Figure 1, curves B–D). The development of inhibition by methotrexate occurs in the absence of catalysis as preincubation of the enzyme for several minutes with methotrexate and NADPH resulted in considerable loss of enzymic activity (Figure 2). Thus, when the reaction was started with dihydrofolate, the velocity was slow at first but increased with time. The initial rates obtained after preincubation of the enzyme with several concentrations of methotrexate were used to determine that the concentration of enzyme used for these experiments was 6.0 nM (Figure 3; cf. Theory and Data Analysis). As there was no detectable

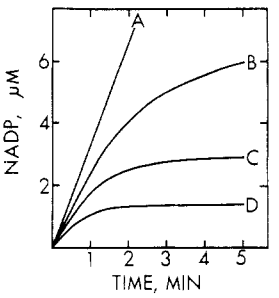


FIGURE 1: Progress curves for the slow development of the inhibition of dihydrofolate reductase by methotrexate. Reactions were started by the addition of enzyme (6.0 nM). The composition of reaction mixtures is given under Experimental Procedure while the concentrations of methotrexate were as follows: (A) 0; (B) 17.6; (C) 35.2; (D) 61.6 nM.

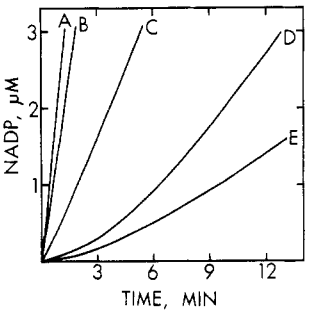


FIGURE 2: Progress curves for the recovery of the activity of dihydrofolate reductase after its incubation with NADPH and methotrexate. Assays were initiated by the addition to reaction mixtures of aliquots of the preincubation mixture which gave a final enzyme concentration of 6.0 nM. Experimental details are given under Experimental Procedure. The final concentrations of methotrexate were as follows: (A) 0; (B) 1.76; (C) 4.40; (D) 6.16; (E) 10.6 nM.

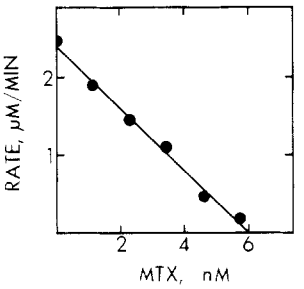


FIGURE 3: Determination of enzyme concentration by titration of dihydrofolate reductase with methotrexate (MTX). The enzyme was preincubated with NADPH and methotrexate after which aliquots of the mixture were taken for determination of residual enzymic activity. Experimental details are outlined under Experimental Procedure. The total concentration of catalytically active enzyme is given by the intercept of the curve with the abscissa.

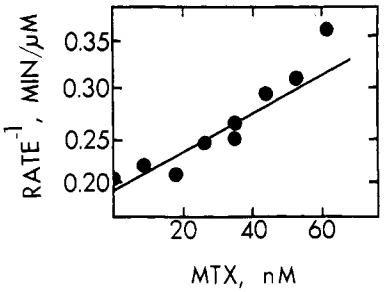


FIGURE 4: Dixon plot of the effect of methotrexate (MTX) on the initial velocities (v_0) of reactions initiated by the addition of dihydrofolate reductase. The initial velocities were determined by fitting progress curve data to eq 6.

Table I: Kinetic Constants of Dihydrofolate Reductase as Determined by Progress Curve Analysis[a]

technique	parameter	value	error
development of inhibition	$V/(E_t)$ (min^{-1})	640	11
	K_i (nM)[b]	23	8
	k_5 (min^{-1})	5.1	1.8
	k_6 (min^{-1})	0.013	0.009
recovery of act. after preincubation	k_6 (min^{-1})	0.013	0.001

[a] Progress curve data, such as those depicted in Figures 1 and 2, were analyzed by fitting to mechanism B (Scheme I) by using the CRICF program. The concentrations of dihydrofolate, NADPH, and dihydrofolate reductase were 76 μM, 90 μM, and 6.0 nM, respectively; the apparent Michaelis constant for dihydrofolate was 15 μM. For the preincubation experiments the error is reported as the standard deviation of five determinations of k_6. The remaining error values are standard errors from overall fits. [b] K_i represents the ratio k_4/k_3.

activity in enzyme solutions that were preincubated with a molar excess of methotrexate, it can be concluded that all catalytically active enzyme will bind this inhibitor.

Analysis of Progress Curve Data. It appears that the initial velocity (Figure 1) varies as a function of methotrexate concentration. To verify this point, the data for each curve of Figure 1 and several other curves were fitted separately to eq 6, and the reciprocals of the resulting values for v_0 were plotted against the methotrexate concentration. The results (Figure 4) indicate that the initial velocity decreases with the inhibitor concentration. The data (Figures 1, 2, and 4) are qualitatively in accord with mechanism B (Scheme I). Analysis of the data (cf. Figure 1) by numerical integration techniques by using the CRICF program of Chandler et al. (1972) showed that the results were also quantitatively in accord with this mechanism. The values obtained for $V/(E_t)$, the dissociation constant of the EI complex (K_i), and the rate constants for the interconversion of EI and EI* (k_5 and k_6) are listed in Table I. E in mechanism B denotes the enzyme–NADPH complex as NADPH was present at a saturating concentration.

An independent and more precise estimate of the value of k_6 was obtained by following the increase in enzymic activity that occurs on dilution of enzyme that has been preincubated with NADPH and methotrexate (Figure 2). The data for five curves with final methotrexate concentrations ranging from 6.2 to 10.6 nM and a final enzyme concentration of 6.0 nM were each analyzed by using the CRICF program. For this purpose, V, K_i, and k_5 were held constant at the values given in Table I. The mean value for k_6 from the five experiments was 0.013 min^{-1} (Table I). When the fixed values of V, K_a, K_i, and k_5 were each varied in turn by ±10%, the value of k_6 varied only from 0.012 to 0.014 min^{-1} and was most sensitive to changes in the value of V which is unlikely to be in error by as much as 10%.

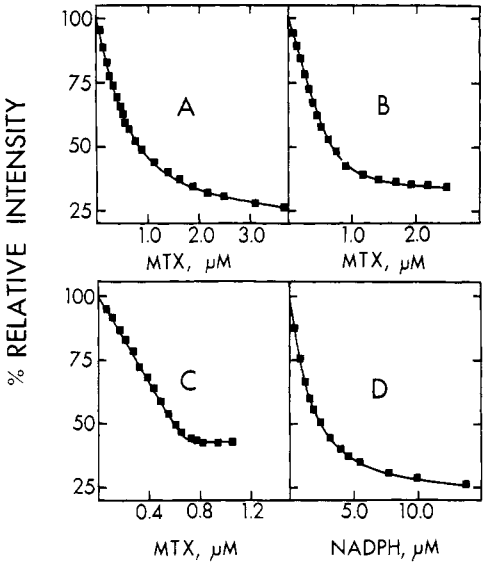


FIGURE 5: Interaction of dihydrofolate reductase with methotrexate (MTX) and NADPH as determined by quenching of enzyme fluorescence. The following additions were made to 0.15 mL of stock enzyme solution which was contained in a total volume of 2.15 mL (cf. Experimental Procedure): (A) none; (B) NADP, 45 μM; (C) NADPH, 3.1 μM; (D) none. The curves represent the best fit of the data to eq 18 and 19 except for (C) where an apparent K_d value of 72 pM was assumed.

In connection with the analysis of the aforementioned inhibition data, it is important to have an accurate estimate of (E_t) as the formation of the enzyme–NADPH–methotrexate complex significantly reduces the concentration of free inhibitor. The value for (E_t) was calculated from the data in Figure 3. This is the preferred method for determining the enzyme concentration.

Binding of Methotrexate, NADPH, and NADP to the Enzyme. The binding of methotrexate to free enzyme, E–NADP, and E–NADPH as well as the binding of NADPH and NADP to free enzyme was determined by following the decrease in enzyme fluorescence that occurs on the formation of enzyme–reactant complexes. The data for the resulting titration curves (Figure 5) were fitted to eq 18 and 19. The dissociation constant for the E–NADP complex was determined to be 12 ± 1 μM (data not shown), while the values for the dissociation constants of the other complexes and the enzyme concentration are listed in Table II. Titration of the free enzyme, the enzyme–NADP complex, or the enzyme–NADPH complex with methotrexate gave similar values for the enzyme concentration. However, the value obtained for enzyme concentration by titration with NADPH was almost twofold higher. Williams et al. (1973a) have made a similar observation using the enzyme from *Escherichia coli*.

The dissociation constant for E–NADPH of 1.0 ± 0.04 μM agrees well with the kinetically determined value of 0.76 ± 0.36 μM. All dissociation constants listed in Table II had quite small errors of estimation except that for the release of methotrexate from the E–NADPH–methotrexate complex. The poor determination of this constant is due to the fact that methotrexate is bound very strongly so that there is little curvature between the two linear sections of the titration curve (Figure 5C). The expected value for the directly determined dissociation constant may be calculated from the expression $K_i k_6[1 + K_{i(NADPH)}/(NADPH)]/(k_5 + k_6)$ when it is assumed that EI and EI* exhibit the same fluorescence properties. The

Table II: Enzyme Concentration and Dissociation Constants for the Interaction of Dihydrofolate Reductase with Substrates and Substrate Analogues[a]

reaction	max quenching[b] (%)	total enzyme (μM)	K_d (nM)
E + MTX[c]	82 ± 1	8.8 ± 0.9	362 ± 25
E-NADP + MTX	69 ± 1	8.4 ± 0.2	64 ± 6[d]
E-NADPH + MTX	57 ± 1	8.4 ± 0.1	0.61 ± 0.41[d]
E + NADPH	80 ± 1	15.5 ± 0.9	999 ± 36

[a] Values were obtained from measurements of the quenching of the enzyme fluorescence (cf. Figure 5) as described under Experimental Procedure. The data were analyzed by fitting to eq 18 and 19 by using the STEPIT program. The errors reported are the mean of the asymmetrical confidence half-intervals and are approximately equal to standard errors. Calculation of these values was performed by using the FIDO routine of the STEPT package. [b] Maximum quenching (%) was calculated by using the expression $100(1 - F_\infty/F_0)$ where F_0 and F_∞ denote the fluorescence of the free enzyme and the enzyme–ligand complex, respectively (cf. eq 18). [c] MTX, methotrexate. [d] Apparent K_d values obtained with concentrations of NADP and NADPH of 45 and 3.1 μM, respectively.

Table III: Dissociation Constants of the Complexes Formed by the Interaction of Dihydrofolate Reductase with Substrates and Substrate Analogues[a]

reaction	technique	K_d (M)
E + DHF[b]	steady-state kinetics	1.6×10^{-5}
E-NADPH + DHF	steady-state kinetics	1.5×10^{-5}
E + MTX[c]	fluorescence quenching	3.6×10^{-7}
E-NADPH + MTX	progress curve analysis	2.3×10^{-8}
E-NADP + MTX	fluorescence quenching	6.4×10^{-8}
E + NADPH	steady-state kinetics	7.6×10^{-7}
	fluorescence quenching	1.0×10^{-6}
E + NADP	fluorescence quenching	1.2×10^{-5}
E → EI*[d]	progress curve analysis	5.8×10^{-11}
	fluorescence quenching	$<10^{-9}$

[a] The data are taken from Tables I and II as well as the text. Dissociation constants are given for the complexes formed by the reactants indicated. A rapid-equilibrium, random mechanism was assumed for the calculation of the dissociation constant of the enzyme–dihydrofolate complex. [b] DHF, dihydrofolate. [c] MTX, methotrexate. [d] The reaction E → EI* represents the binding of methotrexate to E-NADPH, followed by a conformational change in the resultant complex (mechanism B). The dissociation constant associated with this reaction was calculated by using eq 21.

calculated value of 0.072 nM differs by nearly an order of magnitude from the estimated value of 0.61 nM (Table II). However, the curve (Figure 5C) which was drawn by using a K_d value of 0.072 nM is not inconsistent with the data. Indeed, simulation shows that any K_d value less than 1 nM yields theoretical curves that adequately describe the data.

Discussion

The intersecting steady-state velocity pattern indicates that the reaction catalyzed by dihydrofolate reductase from *S. faecium* conforms to a sequential mechanism. It has been reported that the enzyme has an ordered mechanism with NADPH adding before dihydrofolate (Blakley et al., 1971). However, there is evidence that the free enzyme will bind methotrexate (Figure 5) or dihydrofolate (D'Souza & Freisheim, 1972). In addition, dihydrofolate reductase is bound to methotrexate–Sepharose and can be eluted with dihydrofolate. In this connection it should be noted that the dihydrofolate reductases from *E. coli* (Burchall & Chan, 1969) and L1210 murine lymphoma cells (McCullough et al., 1971) exhibit rapid-equilibrium, random mechanisms. It has been assumed, therefore, that dihydrofolate can bind to the free enzyme. However, the major conclusions reached in the present report are not dependent on the nature of the mechanism. Irrespective of whether the reaction has an ordered mechanism with NADPH adding first or a random mechanism, an E–NADPH complex is formed, and all inhibition experiments were performed with NADPH present at a concentration of 125 times its apparent Michaelis constant. Consequently, the kinetic studies have yielded information about the reaction of methotrexate with the E–NADPH complex and not with the free enzyme.

A prolonged transient phase is observed for the inhibition of dihydrofolate reductase by methotrexate (Figure 1), and thus methotrexate exhibits the characteristics of a slow-binding inhibitor. In addition, the concentration of methotrexate required to cause inhibition is comparable to that of the enzyme, and so methotrexate should be classified as a slow, tight-binding inhibitor (Williams & Morrison, 1978). To date, no detailed analyses have been made of this type of inhibition although Cha (1976) has undertaken graphical analysis of data obtained with a slow-binding inhibitor under conditions which mask its tight-binding characteristics. This analysis was based on the assumption that the slow development of inhibition was due to the low magnitude of the apparent first-order rate constant for the reaction of inhibitor with enzyme.

The present results indicate clearly that the inhibition of dihydrofolate reductase by methotrexate is not simply due to their slow rate of interaction. If this were so, the initial velocity of the reaction would be independent of the inhibitor concentration, but the results (Figure 4) show that this prediction is not fulfilled. Consideration was given to an hysteretic mechanism (Frieden, 1970) which involves two enzyme forms which are slowly interconverted but only one of which binds to methotrexate while the other binds only to dihydrofolate. This mechanism was ruled out by the facts that the initial velocity is not independent of methotrexate concentration and there is no detectable lag in assays done in the absence of inhibitor. On the other hand, the data are consistent with a mechanism that involves the rapid reaction of the inhibitor with enzyme to form an EI complex which then undergoes slow isomerization to form an EI* complex (mechanism B, Scheme I).

Analysis of progress curves for the onset of inhibition yielded good estimates of the K_i value for the rapid interaction of methotrexate with the E–NADPH complex (23 ± 8 nM) and the forward rate constant (k_5) for the isomerization of the E–NADPH–methotrexate complex (5.1 ± 1.8 min^{-1}). It was possible for us to obtain an accurate estimate of k_6 by an alternative procedure that involved preincubating the enzyme with NADPH and methotrexate before diluting the mixture and following the recovery of enzyme activity (cf. Figure 2). The alternative procedure for estimating k_6 yielded a value which was similar to, but more precise than, that determined from studies of the onset of inhibition (Table I). Jackson et al. (1977) have studied the slow dissociation of methotrexate from the enzyme–NADPH–methotrexate complex using several different techniques. While their experiments were performed with the enzyme from mammalian sources, the dissociation rate constant which was determined compares well with k_6.

The dissociation constant for the E–methotrexate complex is 44-fold lower than that of the E–dihydrofolate complex (Table III). The enhanced binding of methotrexate, which corresponds to a difference in binding energy of 2.3 kcal/mol, may be due to the formation of an additional noncovalent bond between a group on the enzyme and the N(1) position of the

pteridine ring that is protonated only in the enzyme–methotrexate complex (Erickson & Mathews, 1972; Hood & Roberts, 1977; Subramanian & Kaufman, 1978). The binding of methotrexate and NADPH is synergistic in that the binding of methotrexate to E–NADPH is 16-fold tighter than to the free enzyme (Table III). NADP mimics NADPH in this respect by enhancing the binding of methotrexate (Table III). By contrast, dihydrofolate binds equally well to the free enzyme and to the E–NADPH complex.

The overall inhibition constant (K_i^*) for the combination of methotrexate (I) with the enzyme–NADPH complex (E) is given by the relationship

$$K_i^* = \frac{(E)(I)}{[(EI) + (EI^*)]} = K_i k_6/(k_5 + k_6) \qquad (21)$$

Substituting values of K_i, k_5, and k_6 (Table I), the value of K_i^* is approximately 58 pM which is almost 400 times lower than the K_i value associated with the rapid formation of the E–NADPH–methotrexate complex. Clearly, the tight-binding inhibition by methotrexate is due to the conformational change that the complex undergoes. From the magnitude of the dissociation constants (Table III), it can be deduced that the E–methotrexate and E–NADP–methotrexate complexes do not undergo a slow conformational change as does the E–NADPH–methotrexate complex.

The potency of the inhibition given by methotrexate is undoubtedly due to the structural features that it has in common with dihydrofolate. It would be these features that allow the substrate analogue to combine with E–NADPH and to induce, in the resulting ternary complex, a conformational change which might also be caused by dihydrofolate and which could well constitute the first stage of catalysis. It appears that, in effect, the enzyme recognizes methotrexate as a substrate and initiates catalysis. However, methotrexate will not act as a substrate and so NADPH and methotrexate are locked on the enzyme in the form of an enzyme–transition-state analogue complex. Certainly, the K_i^* value is of a magnitude expected for a transition-state analogue although methotrexate itself does not fall into this category.

The procedure outlined in this report makes possible the determination of the very low dissociation constants which are associated with slow, tight-binding inhibitors and which cannot be determined accurately by standard thermodynamic methods. Further, this procedure, in conjunction with the theory developed by Cha (1975, 1976), enables a distinction to be made between the possible mechanisms for tight-binding inhibition. When tight-binding inhibition involves the rapid formation of an EI complex followed by its isomerization to an EI* complex, values can be calculated for the dissociation constant of the EI complex and the unimolecular rate constants associated with the isomerization reaction.

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