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KINETICS OF METHOTREXATE BINDING TO DIHYDROFOLATE REDUCTASE FROM *NEISSERIA GONORRHOEAE*

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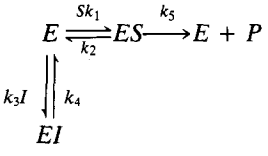
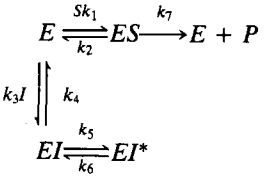
Abstract—The kinetics of methotrexate inhibition of dihydrofolate reductase from *Neisseria gonorrhoeae* have been investigated. Methotrexate was shown to be a tight-binding inhibitor (K_i = 13 pM) competitive with dihydrofolate. However, "stoichiometric" or "pseudoirreversible" inhibition could not be demonstrated. Progress curves of inhibited assays quickly attained steady state regardless of the order of substrate addition, indicating that methotrexate association and dissociation processes were rapid. Kinetic techniques were used to measure the rate of methotrexate dissociation from the enzyme–NADPH–methotrexate ternary complex. At 30°, the first-order off-rate constant (k_{off}) was calculated to be 0.56 min^{-1}. This value is ~40-fold greater than the dissociation rate constant of methotrexate for *Escherichia coli* dihydrofolate reductase. At lower temperatures, progress curves of methotrexate-inhibited gonococcal enzyme assays displayed marked increases in both curvature and the time to reach steady state. At 9°, the methotrexate dissociation rate was slow enough (k_{off} = 0.04 min^{-1}) so that initial velocities of the reaction could be measured, and under these conditions methotrexate inhibition was shown to be "stoichiometric".

Methotrexate is often described as a tight-binding, "stoichiometric", "pseudoirreversible" inhibitor of dihydrofolate reductase [1–6]. Tight binding refers to the high affinity of the enzyme for methotrexate, with K_i values commonly in the 5–60 pM range. Stoichiometric implies that essentially complete inhibition of enzyme activity is observed when $I_t \approx E_t$,† and pseudoirreversible indicates that the dissociation of inhibitor is slow compared to the time scale of the assay. The association of methotrexate with enzyme has also been observed to be a slow process [5, 7]. Recently, Cha [8–10] and Morrison [11] developed kinetic theory to describe the non-steady-state conditions of enzyme reactions in the presence of slow, tight-binding inhibitors. Two basic mechanisms apply for competitive inhibitors [8]:



Mechanism A



Mechanism B

Time-dependent development of inhibition in enzyme-started reactions occurs when k_3 is not diffusion-limited and/or the inhibitor concentration is low (Mechanism A) or when the first-order rate constant (k_5) for the formation of *EI** is low (Mechanism B). Similarly, time dependent increases in reaction velocity are observed in substrate-started reactions when the value of k_4 or k_6 (in Mechanisms A and B respectively) is low. Pseudoirreversible, stoichiometric methotrexate inhibition of dihydrofolate reductase occurs in dihydrofolate-started assays for two reasons. First, methotrexate has a high affinity for the enzyme and, second, progress curve transients are so slow that the initial velocity is dependent upon the enzyme distribution in the preincubation mixture. In this study, we show that the time course of methotrexate inhibition of dihydrofolate reductase from *Neisseria gonorrhoeae* is unusually fast for a tight-binding inhibitor (K_i = 13 pM). Under normal assay conditions (at 30°), dissociation of methotrexate from the enzyme–methotrexate–NADPH complex was rapid, and progress curves often attained steady state within 30 sec. Pseudoirreversible, stoichiometric methotrexate inhibition was only demonstrated at low temperature where progress curves displayed a marked increase in the time to reach steady state.

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† Abbreviations: I_t, total inhibitor (free + bound); E_t, total enzyme (free + bound); E, free enzyme; S, free substrate; I, free inhibitor; P, product; k_n, first- or second-order rate constants where odd numbers denote forward rates and even numbers reverse rates; k_{on}, the second-order association rate constant for binding to the ternary complex; k_{off}, the first-order methotrexate dissociation rate constant for the ternary complex; K_i, the methotrexate dissociation constant of the ternary complex; K_s, Michaelis constant; v_o, reaction velocity in the absence of inhibitor; v_i, reaction velocity in the presence of inhibitor; $t_{\frac{1}{2}}$, half-time; and R_f, relative mobility.

MATERIALS AND METHODS

Folic acid was purchased from Calbiochem (La Jolla, CA). Dihydrofolate was prepared as described by Blakley [12], and methotrexate was supplied by Nutritional Biochemicals. NADPH was from the Sigma Chemical Co. (St. Louis, MO), and all other chemicals were of reagent grade. [3′,5′,7′,^{3}H]-Methotrexate (12.8 Ci/mmole) was from Amersham (Arlington Heights, IL). Prior to use, the compound was purified by thin-layer chromatography on Analtech cellulose MN 3400 plates in 0.2 M ammonium acetate. Several ultraviolet absorbing bands were observed, and the one corresponding to pure methotrexate ($R_f = 0.65$) was eluted from the cellulose with 0.2 M ammonium bicarbonate. Rechromatography of the lyophilized sample yielded a single peak of radioactivity at $R_f = 0.65$. Samples were counted for radioactivity in Aquasol-2 (New England Nuclear, Boston, MA) using a Packard Tri-Carb model 3385 liquid scintillation counter.

Dihydrofolate reductase was isolated from *N. gonorrhoeae* T47 (D. Baccanari and R. Tansik, unpublished results) and *Escherichia coli* RT500 [13] and purified to homogeneity. Gonococcal enzyme concentrations were calculated from enzymic activity and methotrexate inhibition assays (performed at either 30° or 9°), assuming 1 mole of methotrexate bound per mole of enzyme.

Enzyme assays. The standard assay for dihydrofolate reductase activity was performed in 0.1 M imidazole chloride (pH 6.4 for the gonococcal enzyme and pH 7.0 for the *E. coli* enzyme) with 70 μM NADPH, 45 μM dihydrofolate and 12 mM mercaptoethanol in a final volume of 1.0 ml at 30°. One enzyme unit is defined as the amount of enzyme needed to reduce 1 μmole of dihydrofolate per min, based on a molar extinction coefficient of $12.3 \times 10^3\ M^{-1}\ cm^{-1}$ [14]. The decrease in 340 nm absorbance was monitored using a Gilford model 250 recording spectrophotometer set at 0.1 or 0.2 full scale. Reaction velocities were usually measured 30–60 sec after adding dihydrofolate. Enzyme concentrations (0.1 to 0.2 nM) were chosen so that a total 340 nm absorbance change of about 0.06 to 0.1 was observed during an 8- to 10-min assay. This corresponds to a maximal substrate (dihydrofolate) depletion of 18%. In some instances, particularly to illustrate progress curves of methotrexate-inhibited reactions, total absorbance changes of 0.18 were observed. However, the reaction rates of uninhibited control assays were linear under the same conditions.

Determination of methotrexate k_{off}. The rate constant (k_{off}) for methotrexate dissociation from the enzyme–methotrexate–NADPH ternary complex was determined at temperatures between 9° and 30° using the computer program described by Jackson *et al.* [15]. Enzyme (0.1 to 0.8 nM), NADPH (70 μM) and methotrexate (0.3 to 12 nM) were preincubated for 10 min (longer preincubation times did not change the shape of the progress curves), and the reaction was started by the addition of dihydrofolate. The 340 nm absorbance data points were then collected for a 15–30 min time period using a microcomputer attached to the spectrophotometer. During this period <20% of substrate was converted into product. The curves were analyzed for initial velocity, half-maximal velocity, steady-state velocity, and time to reach half-maximal velocity. These values, along with the enzyme, dihydrofolate and methotrexate concentrations, the dihydrofolate K_m and methotrexate K_i, were used to compute the dissociation rate constant. Between five and eleven determinations of k_{off} were made at each temperature, and the standard error of each reported value ranged from 6 to 15%.

RESULTS

Ackermann–Potter analysis of gonococcal dihydrofolate reductase. Stoichiometric inhibition by tight-binding inhibitors such as methotrexate is often demonstrated by an Ackermann–Potter analysis [16]. In this procedure, enzyme is preincubated with inhibitor, and the reaction is started by adding the competing substrate. With pseudoirreversible inhibitors the initial velocity is dependent upon the enzyme distribution in the preincubation mixture. However, determinations of initial velocities of methotrexate-inhibited gonococcal enzyme assays were complicated by a slight curvature in the progress curve that often occurred within 30–60 sec after adding dihydrofolate. In a few instances, depending upon the enzyme to inhibitor ratio, this velocity increase was observed throughout the first 2 min of a 10-min assay. When the linear-inhibited velocities were used in an Ackermann–Potter analysis (Fig. 1), the plot was composed of lines that intersected the origin. A similar plot was obtained when "initial" velocities were measured by drawing tangents to the curved reaction tracings at time = 0 (data not shown). These results are characteristic of reversible inhibition and indicate either that methotrexate is not a tight-binding inhibitor of gonococcal dihydrofolate reductase (i.e. under the assay conditions $E_t \gg K_i$) or that equilibrium was reached

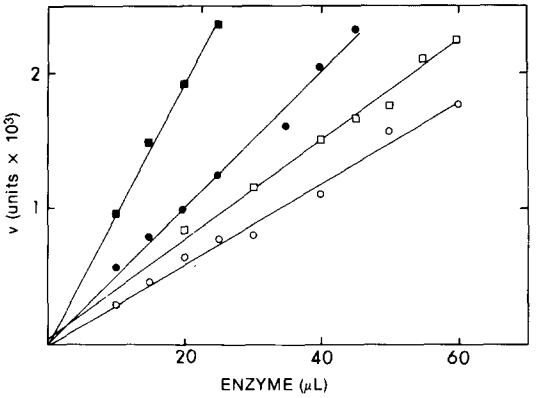

Fig. 1. Ackermann–Potter plot of gonococcal dihydrofolate reductase inhibition by methotrexate at 30°. Various amounts of enzyme were preincubated with 70 μM NADPH and 0 (■), 0.25 nM (●), 0.51 nM (□) or 0.76 nM (○) methotrexate for 2 min. Reactions were started by the addition of dihydrofolate (final concentration = 45 μM in the 1.0 ml assay mixture). Although progress curves were followed for 5–10 min, most of the velocities were measured within the first minute of the assay. See Results for further details.

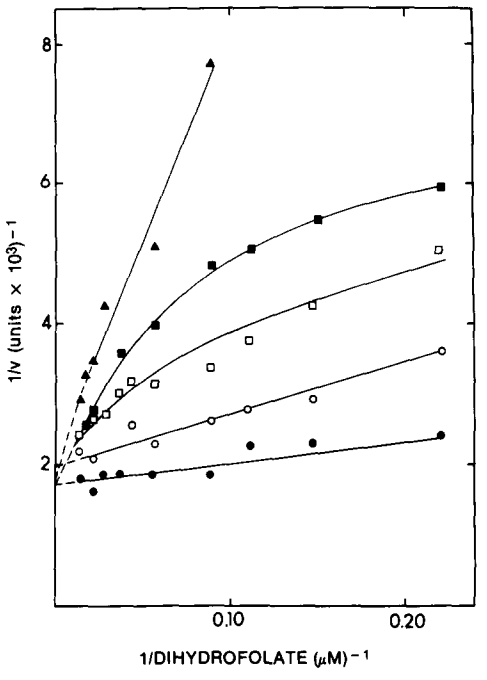

Fig. 2. Methotrexate inhibition of dihydrofolate binding to gonococcal dihydrofolate reductase. The reciprocal of the steady state velocity was plotted versus the reciprocal of the dihydrofolate concentration (4.5 to 67 μM) at methotrexate concentrations of 0 (●), 20 pM (○), 40 pM (□), 80 pM (■) and 160 pM (▲). The enzyme (95 pM), NADPH (70 μM) and methotrexate were incubated at 30° for 2 min, and the reaction was started by the addition of dihydrofolate.

among all enzyme species before the velocity could be measured. Both these possibilities were examined in more detail.

Methotrexate inhibition constant. A plot of reciprocal initial velocity versus reciprocal dihydrofolate concentration at various levels of methotrexate is shown in Fig. 2. The family of lines having an asymptote and a concave-down curved region near the ordinate results from depletion of free inhibitor by enzyme binding (i.e. $I_t \approx E_t$) and indicates that methotrexate is a tight-binding competitive inhibitor [17]. This double-reciprocal analysis is useful for identifying tight-binding inhibitors, but its equations are complex and not commonly used for calculating kinetic parameters.

The Henderson analysis [18] which accounts for depletion of free inhibitor by binding was chosen to evaluate the methotrexate K_i value of the gonococcal enzyme. This method is one of a number capable of determining low K_i values [19] and was selected because it had been employed successfully with other dihydrofolate reductases and does not require a complex computer analysis [18]. The equation for a competitive inhibitor is:

$$\frac{I_t}{(1-v_i/v_o)} = K_i\,(1 + S/K_s)\,(v_o/v_i) + E_t \qquad (1)$$

Plots of $I_t/(1-v_i/v_o)$ versus v_o/v_i result in straight lines with slopes of $K_i(1 + S/K_s)$ and an ordinate intercept of E_t. The experiment shown in Fig. 3 was performed at methotrexate concentrations varying from 0.5 to 2.4 nM, and the data were analyzed using the QUADFIT program of Henderson [20]. Methotrexate K_i values were calculated from the slope of the Henderson plot, the dihydrofolate K_m (2 μM) and the assay concentration of dihydrofolate (45 μM). A total of seven different experiments at three dihydrofolate concentrations and enzyme concentrations varying from 0.1 to 0.5 nM resulted in an average K_i value of 13.0 pM with a standard deviation of ±26%. The standard error of each individual value was <12%. For comparison, similar analyses of *E. coli* dihydrofolate reductase have shown that methotrexate is a pseudoirreversible inhibitor with a K_i value of 21 pM [21]. Therefore, it was concluded that the linear Ackermann–Potter plot observed with gonococcal dihydrofolate reductase was not the result of weak methotrexate affinity.

Progress curves of methotrexate inhibition. Tight-binding and slow-binding inhibitors result in nonlinear progress curves because both association and dissociation processes are slow compared to the time scale of the assay [8, 11]. The effect of methotrexate on the linearity of the dihydrofolate reductase assay was determined with the enzymes from *N. gonorrhoeae* and *E. coli* (Fig. 4). With the dihydrofolate-started reactions, the initial velocity of the inhibited *E. coli* enzyme assay was low; then the rate slowly increased for 14–16 min until it reached steady state. The gonococcal enzyme reaction tracing (dihydrofolate start) was also nonlinear, but in this case steady state was attained in 2–3 min. The reaction tracings for the enzyme-started assays show there were also significant differences in the time course for methotrexate association with the two enzymes. It should be emphasized that the exact shapes of the progress curves and the times to reach steady state were dependent upon the relative proportions of enzyme and inhibitor in the assay. The

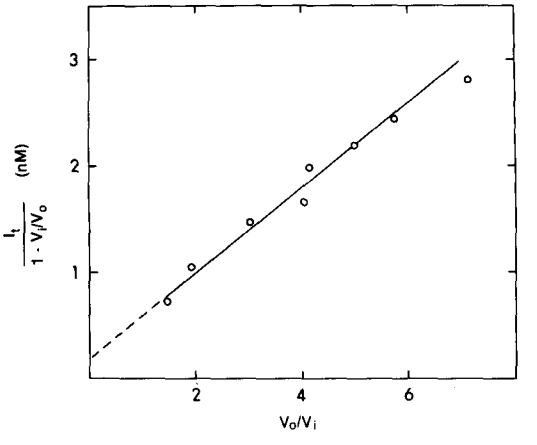

Fig. 3. Henderson plot of methotrexate inhibition. Enzyme, NADPH (70 μM) and methotrexate (0.25 to 2.4 nM) were preincubated at 30° for 2 min. The reactions were started by the addition of dihydrofolate at 45 μM final concentration, and steady-state velocities were measured. Percent inhibition of the enzyme assay ranged from 48 to 85%. Statistical analysis [20] of this data set resulted in a methotrexate K_i value of 17 ± 1.3 pM and an E_t value (y-intercept) of 192 ± 84 pM.

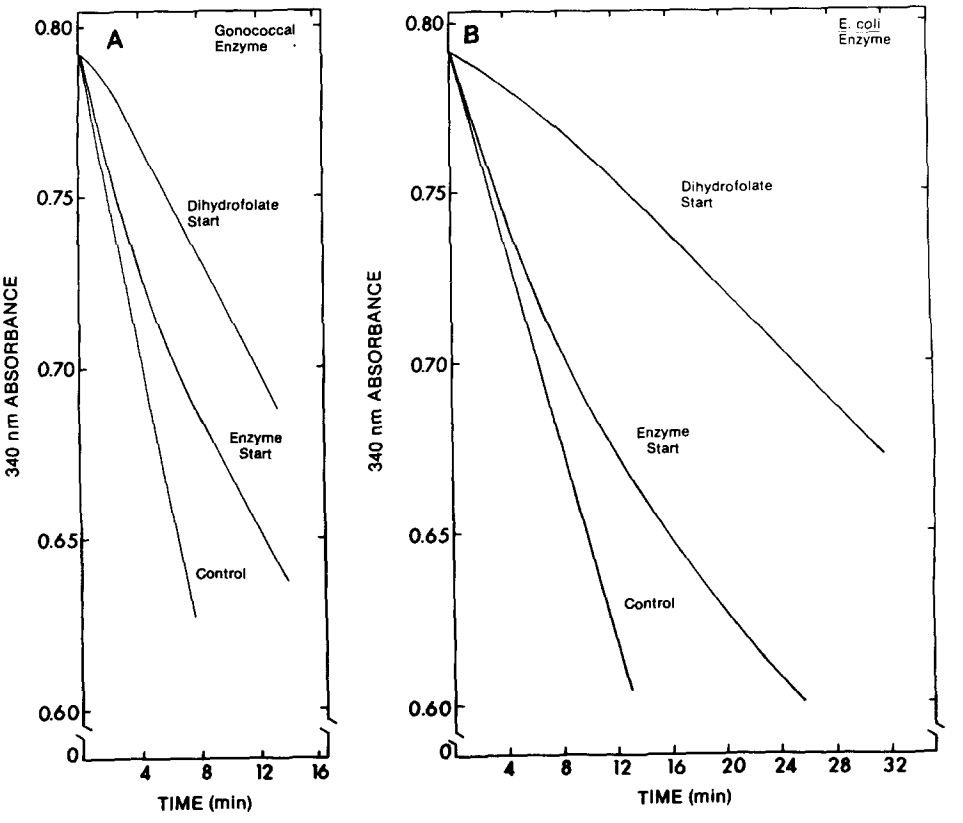

Fig. 4. Progress curves of (A) *N. gonorrhoeae* dihydrofolate reductase and (B) *E. coli* dihydrofolate reductase inhibited by methotrexate. (A) The complete reactions contained 0.1 nM gonococcal dihydrofolate reductase, 70 μM NADPH, 45 μM dihydrofolate and 0.25 nM methotrexate in a final volume of 1.0 ml. All the components except one (either enzyme or dihydrofolate) were preincubated for 10 min at 30°, and the reaction was started by adding the missing component. The control reaction lacked methotrexate and was started by the addition of dihydrofolate. (B) The protocol was the same as in (A) except that the reaction mixtures contained 0.3 nM *E. coli* dihydrofolate reductase and 0.75 nM methotrexate.

tracings shown in Fig. 4 were an extreme case chosen to illustrate the differences between enzyme and dihydrofolate-started reactions with the two enzymes. More highly inhibited gonococcal enzyme assays showed significantly less curvature and, in most cases, the velocities were linear within 30 sec of adding dihydrofolate.

Determination of methotrexate k_{off}. The rates of methotrexate dissociation from the two enzymes were quantitated using the method of Jackson *et al.* [15]. In this technique, the concentrations of enzyme, substrates and inhibitor plus their affinity constants and data from the progress curves are used to calculate the first-order constant for methotrexate dissociation (k_{off}). Analyses of methotrexate dissociation from dihydrofolate-started assays of gonococcal and *E. coli* dihydrofolate reductase yielded k_{off} values of 0.56 min^{-1} and 0.015 min^{-1} respectively. The dissociation half-times ($t_{\frac{1}{2}}$), calculated from the expression $t_{\frac{1}{2}} = 0.69/k_{off}$, were 1.2 min for gonococcal dihydrofolate reductase and 46 min for the *E. coli* enzyme.

An alternative approach to the study of methotrexate dissocation from dihydrofolate reductase involved measuring the rate at which [^{3}H]methotrexate dialyzed from the enzyme–[^{3}H]methotrexate–NADPH ternary complex (Fig. 5). The initial reactant concentrations ($E_t > I_t$ and $E_t > K_i$) were chosen to ensure that essentially all the radiolabeled inhibitor was bound in the ternary complex which was then mixed with unlabeled inhibitor and dialyzed. The basic assumption was that, upon dissociation from the enzyme, [^{3}H]methotrexate was diluted by a large excess of unlabeled inhibitor and had a low probablility of reassociation. The radioactive inhibitor could then be dialyzed from the enzyme in a conventional manner. Figure 5 shows the dialysis of the *E. coli* and gonococcal enzyme ternary complexes and a control which lacked enzyme. Although no attempt was made to quantitate the methotrexate k_{off} values from these data, the experiment serves as an independent verification that *E. coli* dihydrofolate reductase retains methotrexate in the ternary complex much longer than the gonococcal enzyme.

Temperature dependence of k_{off} *and pseudo-irreversible inhibition.* The temperature dependence of methotrexate dissociation from gonococcal dihydrofolate reductase was also studied. Assays similar to those in Fig. 4A but performed at lower temperatures displayed marked increases in curvature and time to reach steady state (data not shown).

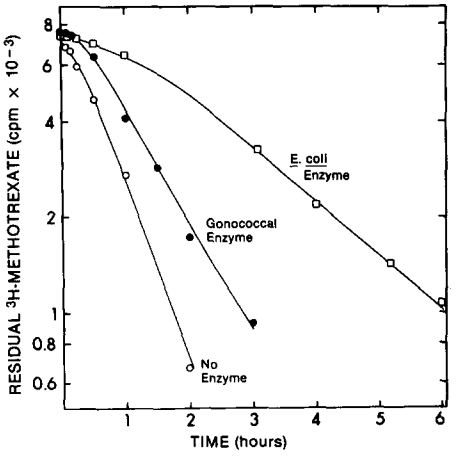

Fig. 5. Dissociation of [^{3}H]methotrexate from gonococcal dihydrofolate reductase. NADPH (80 μM) and [^{3}H]methotrexate (12 nM, 0.6 μCi) in a total volume of 5 ml of 100 mM imidazole chloride buffer, pH 6.4, 5% glycerol were preincubated for 10 min at 23° in the absence or presence of 400 nM *E. coli* or gonococcal dihydrofolate reductase. A 4000-fold molar excess of unlabeled methotrexate was added, and the sample was dialyzed against 1 liter of 80 μM NADPH in 100 mM imidazole chloride buffer, pH 6.4. Aliquots of the control lacking enzyme (○), the gonococcal ternary complex (●) and the *E. coli* ternary complex (□) were withdrawn from the dialysis bag at various time intervals and counted for radioactivity.

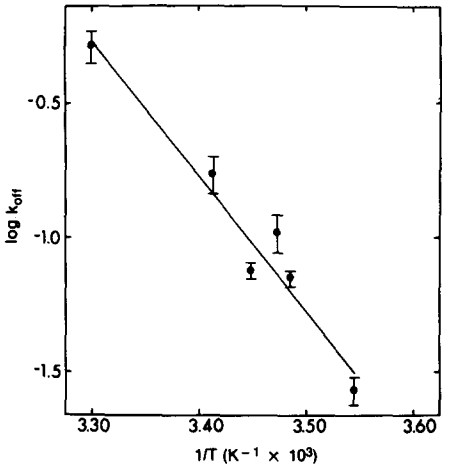

Fig. 6. Temperature dependence of the methotrexate dissociation rate constant. The first-order rate constant for dissociation (k_{off}) of methotrexate from the gonococcal enzyme–NADPH–methotrexate complex was determined between 9° and 30°. The straight line is a nonweighted regression line of log k_{off} vs $1/T$. The standard error of each estimation (N = 5–11) is indicated by the perpendicular line.

The dihydrofolate K_m and methotrexate K_i did not change significantly with temperature between 9° and 30°, and the 30° values (2 μM and 13 pM respectively) were used to calculate methotrexate k_{off} at all temperatures (Fig. 6). The relationship between log k_{off} and $1/T$ is expressed by the Arrhenius equation:

$$\log k_{off} = \frac{E_a}{-2.3\,R}\,\frac{1}{T} + \log A \qquad (2)$$

where E_a is the energy required to raise the ternary complex to a transition state for methotrexate dissociation, R is the gas constant, and A is a constant composed of kinetic energy and probability factors. E_a was calculated to be 23 kcal/mole.

The slower methotrexate dissociation rates at low temperature allowed ample time to measure initial velocities of inhibited reactions. For example, at 9°, $k_{off} = 0.04\ min^{-1}$ which corresponds to a $t_{\frac{1}{2}}$ of 17 min. An Ackermann–Potter analysis performed at this temperature resulted in a series of curves with parallel asymptotes that intersect the E_t-axis (Fig. 7). This pattern is consistent with pseudoirreversible inhibition of gonococcal dihydrofolate reductase by methotrexate. However, one anomaly associated with this assay is that the calculated enzyme concentrations were often greater than the values obtained from Henderson analyses. An example can be seen with the data in Fig. 3 where extrapolation of the line to the y-axis resulted in an enzyme concentration of 0.19 nM. When the same enzyme was subjected to a methotrexate titration at 9°, a value of 0.49 nM was obtained. The reason for this difference is not known but may be related to the fact that the extrapolated Henderson analyses intercept the y-axis close to the origin. Under these conditions, small changes in the slopes of the lines have little effect on K_D but can change the value of E_t several-fold.

DISCUSSION

Kinetic analyses of enzyme assays and dialysis experiments with radiolabeled ligand were used to show that the dissociation of methotrexate from *N. gonorrhoeae* dihydrofolate reductase was fast when compared to the reductases from other sources.

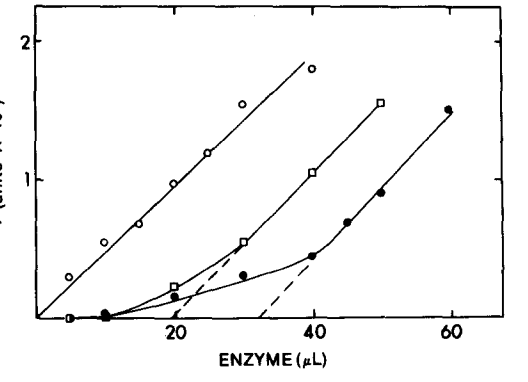

Fig. 7. Ackermann–Potter plot of the reaction of gonococcal dihydrofolate reductase with methotrexate at 9°. Various amounts of the enzyme were preincubated with 70 μM NADPH and 0 (○), 0.25 nM (□) or 0.38 nM (●) methotrexate for 10 min. The reactions were started by the addition of dihydrofolate (final concentration = 45 μM in the 1.0 ml assay mixture). Progress curves were monitored for 10–12 min and showed a marked degree of nonlinearity with the velocity gradually increasing throughout the assay period. Initial velocities of the first minute of each assay were measured.

Table 1. Methotrexate affinity constants and first-order off-rate constants for various dihydrofolate reductases

Enzyme source	Methotrexate K_i (pM)	Methotrexate k_{off}* (min^{-1})
N. gonorrhoeae	13	0.56
E. coli	20	0.015
S. faecium†	58	0.013
L1210‡	5.3	0.014
W1-L2‡	7.3	0.016
WR8.1‡	367	0.1

* All k_{off} values were determined at 30°.
† Data from Williams *et al.* [5].
‡ Data from Jackson *et al.* [15].

Table 1 lists the methotrexate binding constants and dissociation rate constants for several enzymes. The dihydrofolate reductases from *E. coli*, *Streptococcus faecium*, L1210 and W1-L2 cells have high affinities for methotrexate with K_i values that range from 5 to 60 pM, and all have k_{off} values $\sim 0.015\ min^{-1}$. This combination of low K_i and slow dissociation is responsible for their apparent stoichiometric inhibition by methotrexate. Although the gonococcal enzyme also has a high affinity for methotrexate ($K_i = 13$ pM), its dissociation rate constant is elevated 40-fold. This results in rapid equilibrium among all enzyme species in inhibited assays and, consequently, methotrexate does not appear to inhibit stoichiometrically. The dihydrofolate reductase from WR8.1 cells has a dissociation rate constant ($0.1\ min^{-1}$) similar to that of the gonococcal enzyme (Table 1), but this is not unexpected. WR8.1 is a mutant subline of W1-L2, and its dihydrofolate reductase has a methotrexate K_i 50-fold greater than that of the parent strain [15].

The rate of methotrexate dissociation from gonococcal dihydrofolate reductase decreased with decreasing temperature, and at 9° the gonococcal methotrexate k_{off} value ($0.04\ min^{-1}$) was similar to that of the other reductases (at 30°). Under these conditions, an Ackermann–Potter analysis yielded results consistent with pseudoirreversible inhibition by methotrexate.

The progress curves of enzyme-started reactions (Fig. 4) show that the rate of methotrexate association with gonococcal dihydrofolate reductase is also fairly rapid. Steady state was attained within 8 min, whereas the *E. coli* enzyme-started reaction did not reach steady state within the 24-min assay. Williams *et al.* [22] used stopped-flow kinetics to measure an *E. coli* enzyme-methotrexate second-order association rate constant (k_{on}) of $9 \times 10^8\ M^{-1}\ min^{-1}$. Although the gonococcal enzyme methotrexate k_{on} was not directly measured, it must be very high. An estimate from the expression $K_i = k_{off}/k_{on}$ (k_4/k_3 in Mechanism A), where $K_i = 13 \times 10^{-12}$ M and $k_{off} = 0.56\ min^{-1}$, results in a k_{on} value of $4.3 \times 10^{10}\ M^{-1}$. This approaches the diffusion control limited k_{on} value ($\sim 6 \times 10^{10}\ M^{-1}\ min^{-1}$) estimated for the interaction of a protein with a small molecule ligand assuming ~5% of the collisions are at the active site [23]. Cha [10] has shown that association rate constants of some tight-binding adenosine deaminase inhibitors can be measured without the use of special equipment, and Williams *et al.* [5] analyzed the slow transitions of *S. faecium* dihydrofolate reductase progress curves to show that the methotrexate inhibition kinetics are consistent with Mechanism B. However, the rates of the gonococcal enzyme-methotrexate association and dissociation processes (at 30°) are too fast to be evaluated by these techniques. Preliminary studies at 9° indicate that the initial velocities of enzyme-started reactions are independent of methotrexate concentration, suggesting that gonococcal enzyme inhibition may be described by Mechanism A (D. Baccanari and R. Tansik, unpublished results).

It is interesting to compare methotrexate inhibition of gonococcal dihydrofolate reductase with tight-binding inhibitors of other enzymes. For example, deoxycoformycin inhibits human adenosine deaminase with a K_i value of 2.5 pM and has a dissociation rate constant of $4 \times 10^{-4}\ min^{-1}$ [24]. Also, 2-C-carboxy-D-arabinitol 1,5-biphosphate is a tight-binding inhibitor of ribulosebisphosphate carboxylase/oxygenase with an overall binding constant of ≤ 10 pM and a dissociation rate constant estimated to be $3 \times 10^{-5}\ min^{-1}$ [25]. These enzymes have affinity constants similar to that of methotrexate for *N. gonorrhoeae* dihydrofolate reductase, but their dissociation rate constants are 10^3- to 10^4-fold less than the gonococcal enzyme–methotrexate K_{off} value. In another comparison, the dissociation rate constant of erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) from calf intestinal adenosine deaminase ($k_{off} = 0.28\ min^{-1}$ [24]) is similar to that of methotrexate from gonococcal dihydrofolate reductase ($k_{off} = 0.56\ min^{-1}$). However, the corresponding EHNA K_i value is 6.9 nM, about 10^3-fold greater than the methotrexate K_i. These examples show that the dissociation of methotrexate from gonococcal dihydrofolate reductase is faster than commonly observed with other tight-binding inhibitors.

Inhibitors can be grouped into four broad categories on the basis of the relationship between their affinity constants and the rate at which steady state is attained [26]. The largest group is composed of inhibitors that follow classical Michaelis–Menten kinetics. They have relatively high K_i values and rapidly establish equilibrium in the enzyme assay. Weak-binding inhibitors that are also slow binders form the second category. For example, the K_i value of 1-deazamethotrexate for *S. faecium* dihydrofolate reductase is high (0.58 μM), yet the compound shows slow-binding inhibition kinetics, compatible with mechanism B [7]. Several examples have already been given for the third category which is composed of slow, tight-binding inhibitors. Finally, those inhibitors that have a high enzyme affinity and rapidly attain steady state belong to the fourth group. Although the inhibition of gonococcal dihydrofolate reductase by methotrexate does not "instantaneously" attain steady state on the time scale of the assay, its rate is significantly faster than other enzyme–inhibitor interactions with comparable K_i values and, to the best of our knowledge, may be the first example of a relatively fast, tight-binding inhibitor.

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