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TIGHT BINDING INHIBITORS—IX

KINETIC PARAMETERS OF DIHYDROFOLATE REDUCTASE INHIBITED BY METHOTREXATE, AN EXAMPLE OF EQUILIBRIUM STUDY*

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Abstract—Equilibrium studies in the presence of methotrexate (MTX), based on the new theories of tight-binding inhibitors and on classical initial velocity analysis, indicated that the reaction mechanism of dihydrofolate reductase *Lactobacillus casei* MTX/R is consistent with a rapid equilibrium random bi–bi and that MTX inhibits the enzyme competitively with respect to dihydrofolate but noncompetitively with respect to NADPH. The kinetic parameters determined at pH 7.3 and 23° were: K_m for DHF, 9.8 ± 1.3 μM; K_m for NADPH, 6.0 ± 1.2 μM; K_d for E·DHF, 5.7 ± 0.7 μM; K_d for E·NADPH, 0.037 ± 0.028 μM; K_d for E·MTX, 1.20 ± 0.15 nM; K_d for E·NADPH·MTX → E·NADPH + MTX, 0.19 ± 0.04 nM; and K_d for E·NADPH·MTX → E·MTX + NADPH, 7.6 ± 5.9 nM; the molar equivalency factor was 3.33 ± 0.44 nM per unit/liter of the enzyme, and the catalytic number was 300 min^{-1}.

In kinetic studies of enzymes in the presence of potent inhibitors, classical experimental techniques based on steady-state kinetics cannot be applied to the determination of inhibition mechanisms or inhibition constants, as pointed out in previous publications [1–5]. The reasons are 2-fold. First, under commonly employed experimental conditions, the transient or non-steady-state phase of the interaction between enzyme and inhibitor is so markedly prolonged that steady-state assumptions are not valid. To accommodate this time-dependency, a term called "decay factor" (λ) has been introduced [1, 4]. Second, the optimal experimental inhibitor concentrations are so low that they are of the same order of magnitude as the molar concentration of the enzyme. Therefore, depletion of the free inhibitor by binding to the enzyme may not be neglected. To account for this effect, a term called "depletion factor" (γ) was also introduced [1, 4]. In addition, it was proposed that the term "apparent inhibition constant" be replaced by "inhibition factor" (F_i), because it is not a "constant" but a function of one or more substrate concentrations. Grouping several terms into F_i makes the expression of complex rate equations simple and more meaningful.

In one of the last publications in this series [1], the theories were extended, and two major types of experimental designs, i.e. equilibrium studies and time course studies, were described. The basic rate equation that accounts for depletion of both free enzyme and free inhibitor by binding and for the relatively slow reactions of association and dissociation involving the inhibitor is:

$$v = \frac{v_s + [v_z(1 - \gamma') - v_s]e^{-\lambda t}}{1 - \gamma' e^{-\lambda t}} \quad (1)$$

where v_z is the zero time velocity, and v_s is the steady-state velocity. If the steady state is ever reached before a significant amount of the substrate is depleted, it can be represented by the equation derived by Morrison [6].§

$$v_s = v_0\{-(F_i - E_t + I_t) + [(F_i + E_t + I_t)^2 - 4E_tI_t]^{\frac{1}{2}}\}/(2E_t) \quad (2)$$

where v_0 is the uninhibited velocity, E_t = total enzyme concentration, and I_t = total inhibitor concentration.

The zero time velocity (v_z) is:

$$v_z = \phi v_0 \quad (3)$$

where, if the reaction is started by addition of the enzyme, $\phi = 1$, and γ' becomes the standard depletion factor γ. On the other hand, if the enzyme is preincubated with the inhibitor until equilibrium conditions have been reached and the reaction is started by addition of the substrate,

$$\phi = \{-(K_{is} - E_t + I_t) + [(K_{is} + E_t + I_t)^2 - 4E_tI_t]^{\frac{1}{2}}\}/(2E_t) \quad (4)$$

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§ The expression of v_s in equation 7 of Ref. 1 was an erratum.

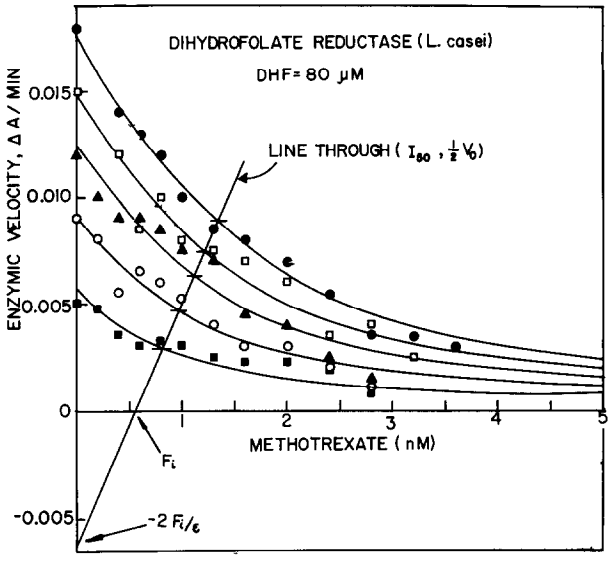

Fig. 1. Example of experiments for determination of inhibition factor and molar equivalency factor. The curves and I_{50} values were those calculated by the least squares method.

If the enzyme catalyzes a multi-substrate reaction and one of the substrates is present during the preincubation, K_{is} in this equation should be replaced by F_i.

In the present publication, the results of equilibrium studies of dihydrofolate reductase (DHFR)* in the presence of methotrexate (MTX) are reported. We have determined the dissociation constants of various binary and ternary complexes (*ES, EI,* and *ESI*), the enzyme molar equivalency factor (ε), and the depletion factor (γ). A preliminary report has been presented [7].

MATERIALS AND METHODS

Materials

NADPH, MTX, and folic acid were obtained from the Sigma Chemical Co. (St. Louis, MO). DHFR, an enzyme from *Lactobacillus casei* MTX/R, was a gift of the New England Enzyme Center at Tufts University. Dihydrofolate was prepared from folic acid by the dithionite method of Blakley [8] and was stored at −20° in small portions as a solid in 0.005 N HCl, 50 μM 2-mercaptoethanol, as described by Poe *et al.* [9]. Just before use, DHF was centrifuged at 3000 *g*, dissolved in a minimal amount of 0.1 N NaOH, and then diluted with the buffer. The buffer used throughout this study consisted of 0.25 M Tris–acetate (pH 7.3), 0.2 M NaCl, 1 mM EDTA, 16 mM mercaptoethanol, and 15% glycerol. The concentration of DHF was determined enzymatically by using DHFR in the presence of excess NADPH and a molar extinction coefficient of 12,300 at 340 nm as described by Hillcoat *et al.* [10].

Methods

The experimental designs and various methods of analysis of data and their theoretical bases for the determination of inhibition factor, enzyme molar equivalency factor, inhibition mechanism, and other kinetic parameters have been described in detail in a previous publication [1]. A synopsis of particular principles and procedures applied in the present work follows.

Experimental procedures. For each experiment, one of the two substrates, either DHF or NADPH, was incubated at room temperature (23°) with various concentrations of DHFR and MTX for a period of 75 min, a time sufficient to establish equilibrium conditions. During this preincubation period, the uninhibited enzyme lost no more than 5 per cent of its activity. The reaction was started by adding a small volume of a mixture of the substrates, enough to bring the final concentrations of both to that of the standard assay conditions, 70 μM. The initial velocity of the DHFR activity was observed by following the decrease in absorbance at 340 nm, as DHF and NADPH were converted to tetrahydrofolate and NADP. It is crucial to measure the velocity at zero time (v_z) before any significant amount of free enzyme, dissociating from the *EI* complex, contributes to catalysis. In our experiments, this was assured by the fact that no appreciable increase in velocity was observed in the first 2 min of the assay period.

Preliminary analysis by the graphical method. The data gathered from each experiment were analyzed graphically to estimate roughly the values of the inhibition factor (F_i) and the enzyme molar equivalency factor (ε). The data were plotted as shown in Fig. 1. The uninhibited velocities were taken as the enzyme concentrations in arbitrary units, e.g. absorbancy change per minute. For each enzyme level, a smooth curve was drawn, and the point

* Abbreviations: DHF, 7,8-dihydrofolate; DHFR, dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP oxidoreductase, EC 1.5.1.3); and MTX, methotrexate.

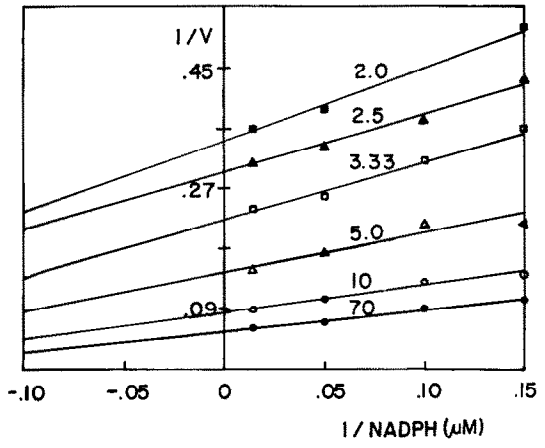

Fig. 2. Initial velocity pattern of DHFR reaction. The number on each straight line indicates DHF concentration in μM.

where the velocity was one-half of the uninhibited velocity (i.e. I_{50}) was marked. Then a straight line drawn through these points was extrapolated. The intercept of this line on the abscissa is the value of F_i and that on the ordinate equals $-2F_i/\varepsilon$ from which ε can be computed.

Least squares procedure for F_i and ε. With the provisional values of F_i and ε, the data were analyzed by the computer program for "equilibrium studies" which, as described in detail previously [1], fits the data points to a family of curves represented by the following equation:

$$v_z = \{-(F_i - \varepsilon v_0 + I_t) + [(F_i + \varepsilon v_0 + I_t)^2 - 4\varepsilon v_0 I_t]^{\frac{1}{2}}\}/2\varepsilon v_0 \quad (5)$$

This equation is similar to equation 2, but it must be emphasized that v_z is the "zero time" velocity after preincubation in the absence of at least one substrate; therefore, the expressions of F_i in the two equations are different. The best values by the least squares criteria and their standard errors were estimated for F_i, ε, v_0, and I_{50}.

Inhibition mechanism and dissociation constants. When the inhibition factors (F_i) had been determined at various concentrations of a substrate, the values of K_{is}, K_m, K'_m and K_{ii} ($= K_{is}K'_m/K_m$) were estimated by a second computer program according to the equation,

$$F_i = K_{is}(1 + S/K_m)/(1 + S/K'_m) \quad (6)$$

where S was the concentration of the substrate present during the preincubation; K_{is}, K_{ii}, K_m and K'_m were, respectively, the dissociation constants for EI, ESI ($\rightarrow ES + I$), ES, and ESI ($\rightarrow EI + S$).

This program also tested automatically the mode of inhibition, i.e. whether the inhibition was competitive, noncompetitive or uncompetitive. The criteria for this test were the null hypotheses, H_0;$1/K'_m = 0$ and H_0;$1/K_{is} = 0$. The probabilities for these null hypotheses were computed by the use of Student's t-test. If $1/K'_m$ was not significantly different from 0, i.e. $K'_m = \infty$, the inhibitior was competitive with the substrate that was present during the preincubation period, and if $1/K_{is}$ was not significantly different from 0, i.e. $K_{is} = \infty$, the inhibition was taken as uncompetitive. Otherwise it was noncompetitive.

RESULTS

Determination of Michaelis constants and reaction mechanism

The classical initial velocity analysis according to Cleland [11], shown in Fig. 2, indicated a sequential mechanism, in agreement with previous investigations, for example that of McCullough *et al.* [12] and that of Burchall and Chan [13]. This result, coupled with the fact that MTX competed with DHF even in the absence of NADPH and that MTX and NADPH enhanced each other's binding to the enzyme as will be shown below, unequivocally established that the bindings of the two substrates, DHF and NADPH, took place in random order. Furthermore, the lack of deviations from linearity of double reciprocal plots within a fairly wide range of

Table 1. Kinetic constants of dihydrofolate reductase from *L. casei* inhibited by methotrexate

K_m or K_d	Data source	Value (nM)	S.E.
K_m for DHF	Fig. 2, K_b	9800.00	1300.00
K_m for NADPH	Fig. 2, K_a	6000.00	1200.00
E·DHF → E + DHF	Fig. 2, K_{ib}	5290.00	2950.00*
	Fig. 3, K_m	5700.00	700.00
E·MTX → E + MTX	Fig. 3, K_{is}	1.20	0.15
	Fig. 4, K_{is}	0.95	0.20
	F_i at S = 0†	1.20	0.20
E·NADPH·MTX → E·NADPH + MTX	Fig. 4, K_{ii}	0.19	0.04
E·NADPH → E + NADPH	Fig. 2, K_{ia}	3200.00	1700.00*
	Fig. 4, K_m	37.00	28.00
E·NADPH·MTX → E·MTX + NADPH	Fig. 4, K'_m	7.60	5.90
E·MTX·DHF → E·MTX + DHF‡	Fig. 3, K'_m	20,000.00	9000.00
E·MTX·DHF → E·DHF + MTX‡	Fig. 3, K_{ii}	10.60	2.98

* No statistically significant difference from that estimated from Fig. 3 or Fig. 4.

† Inhibition factor determined in the absence of any substrate during the preincubation, an average of seven determinations from both Fig. 3 and Fig. 4.

‡ Probability of E·MTX·DHF complex not occurring was 0.977.

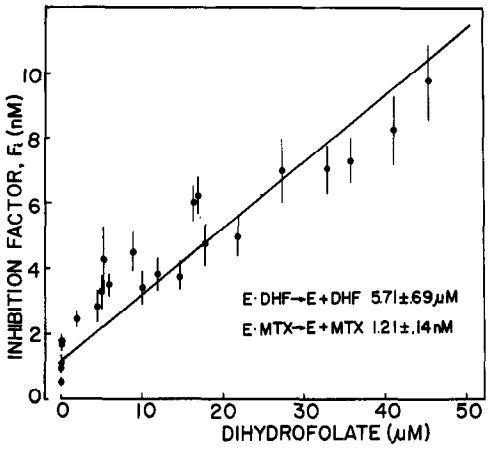

Fig. 3. Inhibition factor as a function of DHF concentration (during preincubation). The straight line is a weighted (n/σ^2) regression line. Standard errors of estimation (N = 25–35) are indicated by the perpendicular lines.

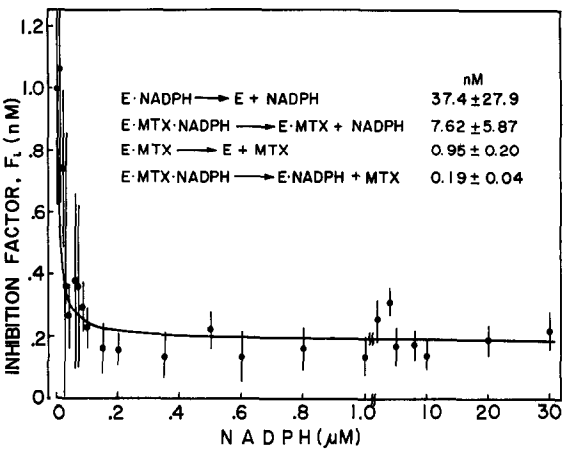

Fig. 4. Inhibition factor as a function of NADPH (during preincubation). The hyperbola was calculated as described in the text. The relatively large standard errors of estimation (N = 25–35) at the steep portion of the curve were due largely to the errors in NADPH concentrations, a small error of which causes a significant difference in the degree of inhibition.

substrate (data not shown) is consistent with, albeit insufficient evidence for, the conclusions of previous investigators [12–14] that the reaction mechanism is a rapid-equilibrium random bi–bi according to Cleland's nomenclature [15]. The data presented in Fig. 2 were analyzed by the statistical method of Cleland [16] in which all experimental points are fitted to families of hyperbolas represented by the rate equation for a rapid equilibrium random bi–bi mechanism.

$$v = \frac{VAB/(K_aK_{ib})}{1 + A/K_{ia} + B/K_{ib} + AB/(K_aK_{ib})} \quad (7)$$

where $K_aK_{ib} = K_{ia}K_b$. The values of kinetic parameters thus calculated are listed in Table 1.

Determination of dissociation constants and inhibition mechanism

The inhibition factors for MTX as a function of DHF concentration during the preincubation are shown in Fig. 3, and as that of NADPH concentration in Fig. 4. For the experiment in which DHF was present during the preincubation (Fig. 3), S was the DHF concentration, and K_{is}, K_m, K'_m, and K_{ii} corresponded, respectively, to the dissociation constants of E·MTX, E·DHF, E·DHF·MTX (→E·MTX + DHF), and E·DHF·MTX(→E·DHF + MTX). It is noteworthy that F_i increased linearly with increasing DHF concentrations. The probability of $1/K'_m$ being 0 was 0.977, indicating that the E·MTX·DHF complex did not occur; therefore, DHF competed with MTX. The dissociation constant of the E·DHF complex, measured as K_m (5.7 ± 0.7 μM) in this experiment, agrees well with that measured as K_{ib} in Fig. 2 (5.3 ± 3.0 μM).

Unlike the results with DHF, when NADPH was included during the preincubation, the relation of the inhibition factor to the NADPH concentration was hyperbolic (Fig. 4). In this case, S represented NADPH concentration; K_m, K'_m, K_{is}, and K_{ii} represented, respectively, the dissociation constants of E·NADPH, E·NADPH·MTX(→ E·MTX + NADPH), E·MTX, and E·NADPH·MTX (→ E·NADPH + MTX). Values of these parameters and their respective standard errors of estimation are listed in Table 1. The dissociation constant of E·NADPH measured as K_m in this experiment and that measured as K_{ia} in Fig. 2 were, respectively, 0.037 ± 0.028 and 3.2 ± 1.7 μM. Despite the apparently great discrepancy between these two estimates, there is no statistically significant difference ($P > 0.30$ at 1 degree of freedom). The value of 0.037 μM, however, may be closer to the true value because the experimental approach in Fig. 4 was more direct than that in Fig. 2, and because it compares more closely with the literature values (Table 2) determined by a stopped flow technique [17] or by a fluorescence quenching method [18].

The fact that the values of K'_m and, therefore, of K_{ii} were not infinite indicates that the ternary complex E·NADPH·MTX occurred and, therefore, that MTX inhibited DHFR noncompetitively with respect to NADPH. The fact that K_{ii} was approximately 6-fold lower than K_{is} signifies that MTX and NADPH (and possibly DHF and NADPH also) enhanced binding of each other by that factor.

The reaction mechanism of DHFR in the presence of MTX and various kinetic constants (nM) may be schematically presented as follows

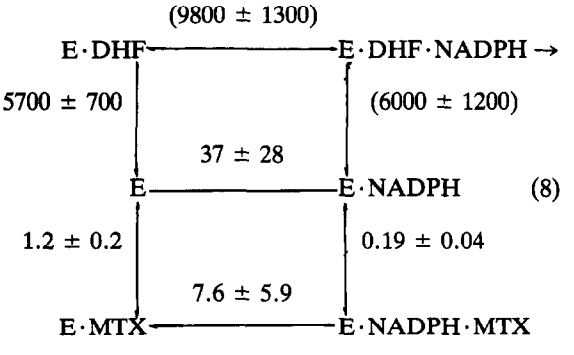

Table 2. Literature values of kinetic parameters of methotrexate-inhibited dihydrofolate reductases

K_m		K_d, DHF	K_d, NADPH		K_d, MTX						
DHF (μM)	NADPH (μM)	E·DHF (nM)	E·NADPH (nM)	E·NADPH·MTX (nM)	E·MTX (nm)	E·NADPH·MTX (nM)	Enzyme source*	Method†	pH	Temp	Ref.
							(Bacterial)				
9.8	6.0	5,700	37	7.6	1.2	0.19	*L. casei* MTX/R	Table 1	7.3	23	
—	—	2,560	—	—	80	—	*L. casei* MTX/R	FQ	?	?	21
—	—	—	10	—	—	—	*L. casei* MTX/R	SFA	6.0	25	17
—	—	—	—	—	4.8	—	*L. casei* MTX/R	FQ	7.5	25	22
—	—	—	—	—	90.9	—	*L. casei* MTX/R	FQ	7.5	25	22
0.36	—	44	—	—	—	—	*L. casei* MTX/R	SSK	7.0	25	18
—	—	—	<10	—	<10	—	*L. casei* MTX/R	FQ	7.2	25	18
0.50	16.6	—	—	—	—	—	*L. casei* MTX/R	SSK	7.3	25	18
—	0.78	—	—	—	—	—	*L. casei* MTX/R	SSK	7.0	25	18
—	—	—	—	—	—	4.76‡	*L. casei* MTX/R	RIB	6.2	23	23
2.56	13.4	—	—	—	—	2.7‡	*D. pneumoniae* ame^r-5	SSK	6.7?	25	24
2.48	16.1	—	—	—	—	2.6‡	*D. pneumoniae* ame^r-4	SSK	6.7?	25	24
2.96	14.1	—	—	—	—	2.9‡	*D. pneumoniae* ame^r-3-5	SSK	6.7?	25	24
12.5	20.0	—	—	—	—	1.0‡	*E. coli* 113-3 MCIM Poona	SSK	7.4	rt	25

Table 2 (*continued*)

K_m		K_d, DHF	K_d, NADPH		K_d, MTX					
DHF (μM)	NADPH (μM)	E·DHF (nM)	E·NADPH (nM)	E·NADPH·MTX (nM)	E·MTX (nm)	E·NADPH·MTX (nM)	Enzyme source*	Method†	pH	Temp	Ref.
—	—	—	20	—	—	—	*E. coli* B (MB1428) MTX/R	UVD	7.2	23	26
—	—	500	—	—	27	—	*E. coli* B (MB1428) MTX/R	UVD	7.2	23	27
—	—	—	—	—	0.36	<0.01	*E. coli* B (MB1428) MTX/R	SSK	7.2	25	28
—	—	1,100	—	<100	—	<100	*E. coli* B (MB1428) MTX/R	CD	7.2	27	29
4.1	—	—	—	—	0.35	—	Mycobacterium sp. 607	?	6.0	25	30
—	—	—	—	—	—	0.06	*S. faecium* A	PCK	7.4	30	31
15.0	0.72	16,000	760	—	—	—	*S. faecium* A	SSK	7.4	30	14
—	—	—	1,000	—	360	—	*S. faecium* A	FQ	7.4	30	14
—	—	—	—	—	—	23.0	*S. faecium* A	PCK	7.4	30	14
7.5	4.9	—	2,300	—	—	—	*S. faecium* var Durans (A)	SSK	7.0	37	32
—	—	—	725	—	—	—	*S. faecium* var Durans (A)	FQ	7.0	20	32
—	—	130,000	—	—	—	—	*S. faecium* var Durans (A)	EQD	7.5	5	32
3.4	12.1	—	12,700	—	—	—	*S. faecium*, wild type	?	?	?	33
5.8	1.34	—	500	—	—	—	*S. faecium*, mutant	?	?	?	33
							(Normal eukaryotic)				
0.5	—	—	—	—	—	2.3‡	Chicken liver	FQ	7.5	37	34
—	—	—	—	—	—	0.5~3.4	Chinese hamster ovary MTX/R	RIB	5.5	22	35
—	—	—	—	—	—	0.004‡	Mouse liver	WWK	7.5	25	36
—	—	—	—	—	—	0.004‡	Rat liver	WWK	7.5	25	36
<0.2	—	—	—	—	—	<0.012‡	Rat liver	WWK	7.5	?	19
—	—	—	—	—	—	<0.03	Rat liver	SSK	6.1	37	20
0.05	0.55	—	—	—	—	0.25‡	Porcine liver	SSK	7.2	25	37
—	—	—	—	—	30	—	Porcine liver	FQ	7.2	23	37

Table 2 (*continued*)

K_m		K_d, DHF	K_d, NADPH		K_d, MTX					
DHF (μM)	NADPH (μM)	E·DHF (nM)	E·NADPH (nM)	E·NADPH·MTX (nM)	E·MTX (nm)	E·NADPH·MTX (nM)	Enzyme source*	Method†	pH	Temp	Ref.
0.11	2.5	—	—	—	—	0.006‡	Human placenta	WWK	7.5	25	36
							(Murine malignant)				
1.3	—	—	—	—	0.67	—	Ehrlich ascites carcinoma	SSK	7.5	37	38
0.16	—	—	—	—	—	0.005‡	L1210 lymphoma	SSK	6.9	30	39
0.54	3.37	—	—	—	—	0.067‡	L1210 lymphoma	SSK	7.5	30	40
0.69	1.36	—	300	—	—	—	L1210 lymphoma	SSK	?	?	33
0.40	5.0	200	50	—	—	—	L1210 lymphoma	FQ	7.5	25	41
0.42	—	—	—	—	—	0.031‡	L5178Y lymphoma	SSK	6.9	30	39
1.21	6.4	—	—	—	—	0.3‡	L5178Y lymphoma	SSK	7.5	30	40
—	—	—	—	—	0.14	—	S180 sarcoma AT/3000	I50	7.5	37	42
0.30	—	—	—	—	—	0.135‡	Yoshida ascites sarcoma	SSK	6.9	30	39
0.96	10.7	—	—	—	—	0.69‡	Yoshida ascites sarcoma	SSK	7.5	30	40
							(Human malignant)				
0.67	11.1	—	—	—	0.04	—	KB MTX/R	I50	7.5	37	42
0.13	3.1	—	—	—	—	0.007‡	WS lymphoblast	SSK	7.2	37	43
2.30	1.4	—	—	—	—	0.367‡	WR8.1 lymphoblast	SSK	7.2	37	43
0.14	—	—	—	—	—	0.007‡	WR9.1 lymphoblast	SSK	7.2	37	43
0.13	—	—	—	—	—	0.007‡	W1-L2 lymphoblast	SSK	7.2	37	39

* *L. cassei, Lactobacillus cassei; D. pneumoniae, Diplococcus pneumoniae; E. coli, Escherichia coli; and S. faecium, Streptococcus faecium.*

† Abbreviations of methods are: CD, circular dichroism; EQD, equilibrium dialysis; FQ, fluorescence quenching; I50, I_{50} method of Cha [2]; RIB, radioisotope binding; SFA, stopped flow apparatus; SSK, steady-state kinetics; PCK, progress curve kinetics; UVD, ultraviolet difference; WWK, kinetic analysis according to Wang and Werkheiser [19] or Werkheiser [20]. It should be noted that the experimental conditions and methods of data analysis varied greatly even within the same category of method.

‡ Values with a double dagger were reported as K_i for MTX determined at a saturating concentration of NADPH. Therefore, they are assumed to represent the dissociation constant for MTX from the ternary rather than the binary complex.

where the numbers in parentheses are Michaelis constants, and all others are dissociation constants.

Enzyme molar equivalency factor and depletion factor

An average of 77 determinations of the enzyme molar equivalency factor was 0.271 ± 0.036 nM per absorbance change of 0.001 per minute. Since the difference in extinction coefficient at 340 nm for the sum of DHF and NADPH was 12,300, 1 unit/liter of DHFR corresponded to 3.33 ± 0.44 nM. One unit is the international unit, i.e. that amount of enzyme catalyzing the conversion of 1 μmole substrate/min. Thus, the catalytic number was 300 min^{-1}.

Values of the depletion factor were calculated from the concentrations of enzyme and inhibitor and from the value of the inhibition factor (1.2 nM in the absence of substrate) by the following expression:

$$\gamma = \{F_i + E_t + I_t - [(F_i + E_t + I_t)^2 - 4E_tI_t]^{\frac{1}{2}}\}/4E_tI_t) \quad (9)$$

The range of enzyme concentrations in the present studies was from 0.005 to 0.05 absorbance change per minute, corresponding to from 1.36 to 13.6 nM, and that of MTX was from 0.5 to 5 nM. In these ranges of enzyme and MTX, values of the depletion factor varied from 0.085 to 0.288, indicating that depletion of free inhibitor and free enzyme by binding (therefore the factor γ in equation 1) may not be neglected as customarily done in the classical steady-state treatment of enzyme kinetics.

Comparison of literature values of kinetic parameters

Although studies on DHFR and its inhibitors, especially folate analogues, are numerous, it appears to be useful to compare values of Michaelis constants and various dissociation constants estimated in the present work to those obtained by others using various methods and different enzyme sources. Table 2 lists such values. Studies reporting K_m values but no K_i value for MTX were not included in this table. It is evident that the kinetic parameters differ greatly from one study to the other, even when enzyme from the same source was used, reflecting at least in part the difficulties involved in determining K_i values for tight-binding inhibitors.

DISCUSSION

Dihydrofolate reductase is one of the most thoroughly studied enzymes and remains an important chemotherapeutic target for which many potent inhibitors are available for investigational as well as therapeutic usage. Since Werkheiser [20] recognized how difficult it is to determine the exact value of K_i for MTX and its mode of inhibition, many authors employing various methods determined the dissociation constants of DHFR·MTX and DHFR·NADPH·MTX complexes, as recently reviewed by Greco and Hakala [44]. Yet an accurate determination of a very low K_i value for an inhibitor of any enzyme still remains a challenge.

The purpose of the present report has been to illustrate how some of the recently developed theories and experimental designs can be applied to a system of tight-binding inhibitors, to determine various kinetic constants. We have employed a large number of assays, only to demonstrate the applicability of the theory and the extent of experimental variations. Once the dissociation constants of E·DHF and E·NADPH are known, however, determination of the dissociation constants of E·I and E·NADPH·I complexes for other tight-binding analogues of folate would require only two determinations of F_i values, i.e. in the absence of substrate during preincubation for *EI* and in the presence of a saturating concentration of NADPH (e.g. 10 μM or greater) for E·NADPH·I. Whenever it is necessary to ascertain the mode of inhibition, F_i should also be determined at two or more concentrations of DHF or NADPH. Thus it is possible to examine a large number of compounds without unduly high costs in terms of time and effort.

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