Biochemical Pharmacology, Vol. 37, No. 3, pp. 535–539, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
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DIRECT EXPERIMENTAL EVIDENCE FOR COMPETITIVE INHIBITION OF DIHYDROFOLATE REDUCTASE BY METHOTREXATE

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(*Received* 23 *February* 1987; *accepted* 2 *July* 1987)

Abstract—Steady-state enzyme kinetic techniques at very low enzyme concentration (0.4 nM) were used successfully to measure the inhibition constant (53 pM) for the dissociation of methotrexate from the ternary complex of methotrexate, NADPH and dihydrofolate reductase from *Lactobacillus casei*; and to demonstrate unequivocally that the inhibition was, indeed, competitive with respect to dihydrofolate.

Dihydrofolate reductase is inhibited strongly by methotrexate (4-amino-10-methylfolic acid; MTX), a close structural analog of dihydrofolate (H_2-folate). The first attempts to characterize this inhibition by steady-state enzyme kinetics [1] yielded Lineweaver–Burk plots indicative of non-competitive inhibition with respect to H_2-folate; and only indirect evidence of the expected competition between MTX and H_2-folate for the active site. Werkheiser [2] was the first to point out that such results are predicted if the inhibition is both competitive and essentially stoichiometric, a phenomenon now termed tight-binding inhibition. Moreover, Werkheiser [2] showed that inhibition constants measured under such conditions may be gross overestimates of the true values. Because of this situation [3], accurate measurement of an inhibition constant for the MTX-dihydrofolate reductase system had to await the development of theory [4–8] and techniques which allowed assessment of the rate constants for the development and relief of inhibition by analysis of progress curves for product formation in the presence of inhibitor. This procedure of calculating the association and dissociation rate constants to obtain the inhibition constant has been used to characterize the dissociation of MTX from the enzyme–NADPH–MTX ternary complex for dihydrofolate reductase from *Streptococcus faecium* [9, 10]. The kinetic data showed evidence for the rapid, reversible formation of an initial enzyme–NADPH–MTX complex (dissociation constant of 23 nM), followed by slow isomerization to yield a complex for which the overall K_I for MTX dissociation was 58 pM [9] or 53 pM [10, 11]. Confirmation of these findings by direct measurement of the inhibition constant was the objective of the present study. By measuring steady-state velocities in the presence of very low concentrations of both enzyme and MTX, conditions under which the formation of the intermediate complex (K_d = 23 nM) should be insignificant [8], we have been able to demonstrate, for the first time, the competitive nature of the inhibition of dihydrofolate reductase by MTX and to measure directly the inhibition constant for the interaction.

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MATERIALS AND METHODS

Chemicals. Folic acid and NADPH (enzymatically reduced) were obtained from the Sigma Chemical Co., St. Louis, MO; MTX was a Lederle product (American Cyanamid Co., Wayne, NJ). H_2-folate was prepared from folic acid [12] and stored at 5° in 1 mM HCl containing 50 mM β-mercaptoethanol for a maximum of 2 weeks [9]. An affinity chromatographic matrix for dihydrofolate reductase was prepared by coupling MTX to epoxy-activated Sepharose 6B, a product of Pharmacia Fine Chemicals, Uppsala, Sweden.

Concentrations of NADPH and H_2-folate were based on molar absorption coefficients, at pH 7.2, of 6220 at 340 nm [13] and 28,000 at 282 nm [14] respectively. Molar absorption coefficients of 23,200 at 258 and 22,100 at 302 nm for MTX at pH 13 [15] were used to assess concentrations of this substrate analog for dihydrofolate reductase.

Enzyme preparation. Dihydrofolate reductase was prepared from a methotrexate-resistant strain of *Lactobacillus casei* essentially by the method of Dann *et al.* [16], the starting material being an ammonium sulfate fraction obtained from the New England Enzyme Center, Boston, MA. Changes to the published procedure for dihydrofolate reductase purification [16] included: (i) the substitution of DEAE-Trisacryl (LKB Produkter) for DEAE-cellulose and the use of pH 7.5 instead of pH 6.5 for ion-exchange chromatography; (ii) use of 50 mM Tris–HCl buffer (pH 8.5) containing 1 M KCl and 400 μM H_2-folate for elution of the enzyme from an affinity column of MTX-Sepharose; and (iii) removal of the H_2-folate and equilibration of the enzyme with phosphate-

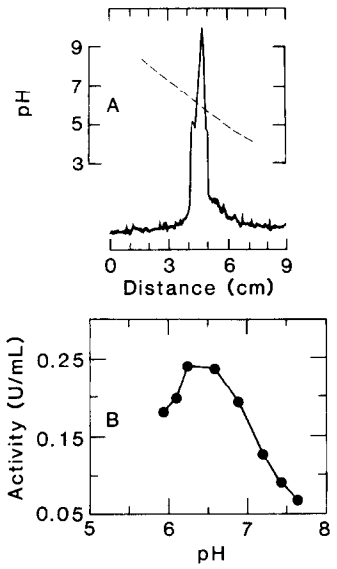

Fig. 1. Effect of pH on electrophoretic and enzymatic properties of dihydrofolate reductase from *L. casei*. (A) Densitometer scan of a silver-stained polyacrylamide gel on which the purified enzyme had been subjected to isoelectric focusing. (B) Determination of the pH optimum for enzymatic activity.

chloride buffer (6.7 mM KH_2PO_4–3.3 mM K_2HPO_4–100 mM KCl), pH 6.5, by zonal gel chromatography on Sephadex G-15. Isoelectric focusing of the purified enzyme on polyacrylamide gel yielded a silver-stained gel in which the major band (Fig. 1A) corresponded to material with an isoelectric point of 5.8, which is intermediate between the values of 5.2–5.5 reported for bacterial dihydrofolate reductases [17] and the pH 6.25 that characterized the enzyme purified by Dann *et al.* [16].

The routine spectrophotometric assay for dihydrofolate reductase [18] entailed observation of the decrease, at 37°, of the absorbance at 340 nm (1-cm cuvettes) of reaction mixtures containing 20 μM NADPH and 20 μM H_2-folate in assay buffer (33.5 mM KH_2PO_4–16.5 mM K_2HPO_4–100 mM KCl, pH 6.5, I 0.18). Molar enzyme concentrations were obtained by titrations of enzymatic activity with MTX on the basis that under these conditions the inhibition is essentially stoichiometric [5]. The pH optimum of 6.2–6.5 (Fig. 1B) confirms the result reported by Gunderson *et al.* [19] but differs considerably from the value of pH 7.3 obtained by the English group [16]: the more acidic value is in keeping with the optima of pH 6.0–6.5 found for other bacterial dihydrofolate reductase preparations [20–22]. Use of different MTX-resistant strains of *L. casei* as the source of dihydrofolate reductase may account, at least in part, for some of the above variations between enzyme preparations [16], the present enzyme being considered to resemble the apoenzyme (fraction I) obtained by Gunderson *et al.* [19]. The absence of NADPH in the present dihydrofolate reductase is inferred from the A_{280}/A_{260} ratio of 1.9, which differs markedly from that of essentially unity for the enzyme–NADPH complex [19]. At pH 6.5 the dihydrofolate reductase solutions were stable for several days at 5°, and they could be stored in the frozen state for several months without loss of activity, as judged by titration with MTX. Potassium fluoride (3 mM) was routinely included in stored samples as a precaution against bacterial contamination.

Enzyme kinetics. Kinetic studies of the inhibition of dihydrofolate reductase by MTX were performed in a Cary 118 spectrophotometer, the cell compartment of which was modified so that a cuvette with a 10-cm pathlength could be thermostatically maintained at 25°. The decrease in absorbance at 340 nm was used to monitor catalysis in reaction mixtures (28 ml) containing enzyme (0.4 nM), NADPH (20 μM), H_2-folate (0.3–5.0 μM) and MTX (0, 0.2 or 0.4 nM) in phosphate-chloride assay buffer (pH 6.5, I 0.18) supplemented with 5 mM mercaptoethanol and also bovine serum albumin (10 μg/ml) to stabilize the extremely dilute enzyme solution, presumably by minimizing adsorption to cuvette surfaces. H_2-folate, enzyme and inhibitor were first preincubated at 25°, and NADPH was then added to initiate catalysis. To allow for the consequences of substrate depletion, [*S*] was taken as the mean value of the H_2-folate concentration prevailing in the period required for delineation of the initial rate.

RESULTS

Titration of enzyme with MTX. When 7.5 nM enzyme was used to effect catalysis in reaction mixtures containing 20 μM NADPH, 20 μM H_2-folate and various concentrations (0–10 nM) of MTX, the essentially stoichiometric binding of inhibitor allowed determination of the exact enzyme concentration by active-site titration (Fig. 2A). If such assays were initiated by addition of enzyme to a cuvette containing substrates and inhibitor, the reaction progress curves were concave downwards, consistent with slow development of the full extent of the inhibition [9]. However, when H_2-folate was the final reactant added, the decrease in A_{340} was linearly time-dependent (Fig. 2B), suggesting instantaneous establishment of an apparent steady state: eventual slight downward curvature was a consequence of substrate (H_2-folate) depletion.

Enzyme kinetic study of MTX inhibition. Despite the 20-fold lower concentration of enzyme (0.4 nM) used in the kinetic studies, the slow velocities were measured successfully by use of a 10-cm pathlength cuvette (Fig. 2C). In the presence or absence of MTX the initial oxidation of NADPH was linearly time-dependent and thus consistent with achievement of a steady state. Results obtained in the absence of MTX yielded an essentially linear Lineweaver–Burk plot (□, Fig. 3), and analysis of the untransformed data by nonlinear regression [23] signified a Michaelis constant for H_2-folate of 0.45 (± 0.06) μM, a value in good agreement with the K_m of 0.36 (± 0.08) μM and the dissociation constant of 0.44 μM reported [16] for the *L. casei* enzyme under similar conditions of pH, ionic strength and temperature.

Lineweaver–Burk plots of results obtained in the

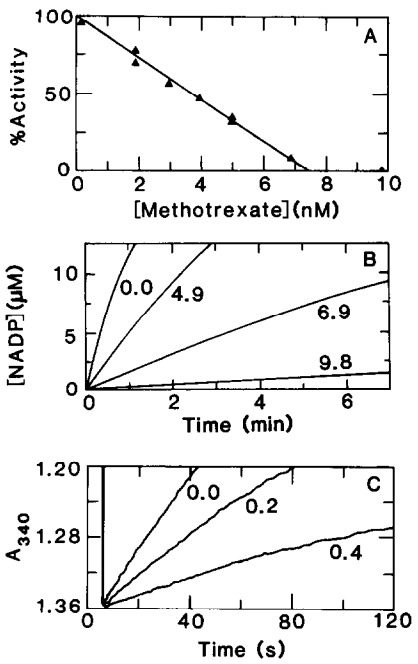

Fig. 2. Studies of the inhibitory effect of MTX on enzymatic catalysis by *L. casei* dihydrofolate reductase (pH 6.5, I 0.18). (A) Active-site titration plot from which the concentration of dihydrofolate reductase in (B) was determined. (B) Progress curves resulting from initiation of catalysis by adding H_2-folate (final concentration 20 μM) to mixtures containing enzyme (7.5 nM), NADPH (20 μM) and the indicated MTX concentrations (nM) in a final volume of 1.015 ml. (C) Progress curves for reaction mixtures containing a 20-fold lower concentration of enzyme (0.4 nM), H_2-folate (2 μM), NADPH (20 μM) and the indicated MTX concentrations (nM) in a final volume of 28 ml.

presence of 0.2 and 0.4 nM MTX (▲ and △, respectively, in Fig. 3) may be extrapolated to a common ordinate intercept coincident with that for uninhibited enzyme—the unequivocal diagnostic of competitive inhibition between MTX and H_2-folate. In that regard the plots should also be curvilinear [4] due to the significant and systematic depletion of free MTX concentration arising from formation of enzyme–NADPH–inhibitor complex in reaction mixtures where the total concentrations of enzyme and MTX are of comparable magnitude. However, from Fig. 3 it is evident that evaluation of kinetic parameters from the expressions presented for the slopes of the initial tangent and final asymptote of such curvilinear Lineweaver–Burk plots (equations (34) and (36) of [4]) places unrealistic demands on the precision with which the nonlinearity and, hence, slopes must be defined. We have therefore sought an alternative means for evaluation of K_I.

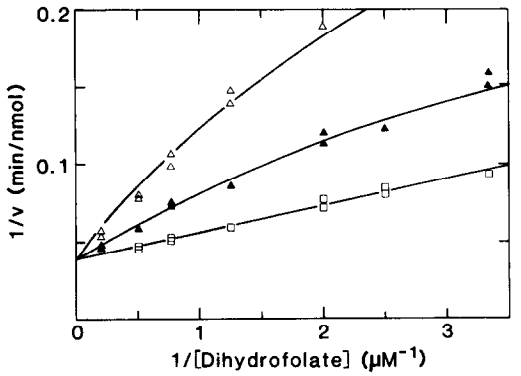

Fig. 3. Lineweaver–Burk plots showing the inhibitory effect of MTX on catalysis by *L. casei* dihydrofolate reductase (pH 6.5, I 0.18). Catalysis was initiated by adding NADPH (final concentration 20 μM) to reaction mixtures containing enzyme (0.4 nM), H_2-folate (0.3–5.0 μM) and zero (□), 0.2 nM (▲) or 0.4 nM (△) MTX. The solid lines are theoretical relationships based on equation (4) and a K_I of 53 pM (see the text).

The concentration of free inhibitor, $[I]$, is related to the total concentration of inhibitor, $[\bar{I}]$, by the expression

$$[I] = [\bar{I}] - f[\bar{E}] \quad (1a)$$

in which $[\bar{E}]$ is the total concentration of enzyme and f the fraction of enzyme inhibited. The latter quantity may be determined experimentally from the relationship

$$f = 1 - (v_i/v) \quad (1b)$$

where v_i and v are the respective initial velocities for a given substrate (H_2-folate) concentration, $[S]$, in the presence and absence of inhibitor. Combination of equations (1a) and (1b) with the classical expression for competitive inhibition of a Michaelis–Menten system then yields

$$v_i = V[S]/[[S] + K_m[1 + \{([\bar{I}] - f[\bar{E}])/K_I\}]] \quad (2a)$$

$$f = 1 - \{v_i(K_m + [S])/V[S]\} \quad (2b)$$

Since the Michaelis constant for H_2-folate and maximal velocity (V) in the absence of inhibitor have already been determined, each measured initial velocity in the presence of total inhibitor concentration $[\bar{I}]$ yields a value of f and hence of the inhibition constant, K_I. Results of such analysis of two sets of results are summarized in Table 1, from which a value of 53 (± 24) pM was obtained for the inhibition constant: a preliminary experiment with a different batch of the same enzyme preparation yielded a similar, though less well-defined K_I (61 ± 51 pM), for competitive inhibition of dihydrofolate reductase by MTX.

Simulation of the dependence of v_i upon $[S]$ in these series of experiments conducted with constant total inhibitor concentration $[\bar{I}]$ requires knowledge of the free inhibitor concentration, $[I]$, appropriate to each value of $[S]$. For this purpose the expressions for total enzyme and total inhibitor concentrations, namely,

$$[\bar{E}] = [E]\{1 + ([S]/K_s) + ([I]/K_I)\} \quad (3a)$$

$$[\bar{I}] = [I]\{1 + ([E]/K_I)\} \quad (3b)$$

Table 1. Enzyme kinetic evaluation of the dissociation constant for inhibition of dihydrofolate reductase by MTX*

	$[\bar{I}] = 0.4$ nM			$[\bar{I}] = 0.2$ nM		
[H_2-folate] (μM)	v_i† (nmol/min)	f‡	K_I§ (pM)	v_i† (nmol/min)	f‡	K_I§ (pM)
2.0	12.8	0.40	66			
2.0	12.6	0.41	62			
1.3	9.4	0.52	46	13.9	0.29	54
1.3	10.2	0.48	59	13.5	0.31	44
0.8	7.2	0.57	47	11.8	0.30	68
0.8	6.8	0.59	40			
0.5	4.9	0.64	38	8.9	0.35	52
0.5	4.9	0.64	38			
0.4				8.2	0.33	71
0.4				8.2	0.33	71
0.3				6.6	0.37	53
0.3				6.3	0.40	36

* Conditions: phosphate–chloride assay buffer (pH 6.5, I 0.18) at 25°; the mixtures also contained NADPH (20 μM), enzyme (0.4 nM), mercaptoethanol (5 mM) and bovine serum albumin (10 μg/ml).

† Initial velocity in the presence of a total concentration $[\bar{I}]$ of MTX.

‡ Calculated from equation (2b) with $K_m = 0.45$ μM and $V = 26.2$ nmol/min (Fig. 3).

§ Determined from equation (2a) with $[\bar{E}] = 0.4$ nM.

are combined to give a quadratic in $[I]$, the only acceptable solution to which is

$$[I] = [-K_I\{1 + ([S]/K_S)\} - [\bar{E}] + [\bar{I}] + \sqrt{\Delta}]/2 \quad (4a)$$

$$\Delta = [K_I\{1 + ([S]/K_S)\} + [\bar{E}] - [\bar{I}]]^2 + 4K_I[\bar{I}]\{1 + ([S]/K_S)\} \quad (4b)$$

Substitution of the consequent value of $[I]$ into the classical expression for competitive inhibition then yields the theoretical value of v_i for assigned values of $[\bar{E}]$, $[\bar{I}]$, $[S]$ and K_S ($= K_m$). Application of this procedure to the present results obtained with 0.2 and 0.4 nM MTX yielded the curvilinear Lineweaver–Burk relationships shown in Fig. 3, which clearly provide adequate descriptions of the experimental results. Figure 3 is therefore considered to provide experimental verification of the prediction that double-reciprocal plots obtained in the presence of tight-binding inhibitors should be curvilinear [4].

DISCUSSION

The dictum [24] that "classical methods of enzyme kinetics based upon the steady-state assumptions are grossly inadequate for determining the inhibition mechanisms or inhibition constants for tight-binding inhibitors" is clearly not strictly correct. A more correct conclusion is that the application of classical methods of enzyme kinetics to tight-binding inhibitors requires use of enzyme and inhibitor concentrations in a sufficiently low range to preclude effectively stoichiometric enzyme–inhibitor complex formation. Such low enzyme concentrations do place technical demands upon the assay methods, demands accommodated in this work by the use of 10-cm pathlength cuvettes for the spectrophotometric assay.

As predicted by Morrison [4], the use of very low enzyme and inhibitor concentrations eliminates the need to consider formation of any unisomerized enzyme–NADPH–MTX complex such as that detected ($K_d = 23$ nM) with dihydrofolate reductase from *S. faecium* [9, 10]. Consequently, the present results do not comment upon the existence or otherwise of that initial complex, and merely measure the dissociation constant for the overall formation of the final enzyme–NADPH–MTX complex, by whatever mechanism it is formed. The equilibrium constant obtained here, by steady-state enzyme kinetics, for the dissociation of MTX from its ternary complex with NADPH and dihydrofolate reductase was 53 pM, which is remarkably close to the values of 58 pM [9] and 53 pM [10] reported for the corresponding dissociation constant from analysis of progress kinetic curves obtained with *S. faecium* enzyme. Such agreement clearly supplies cause for confidence in the two different approaches.

The use of very low enzyme concentrations also appears to have decreased reaction velocities to the extent that any "slow binding" [8] or "slow isomerization" [9] is comparatively insignificant; and to have thereby allowed establishment of an apparent steady-state velocity, as demonstrated in Fig. 2C. In the present analysis, the simplifying approximation has also been made that binary enzyme–MTX complex accounts for negligible proportions of enzyme and inhibitor present in reaction mixtures. This assumption is justified by the use of 20 μM NADPH, which suffices to saturate the NADPH-binding sites given that dissociation constants of 0.1 μM [19], 0.8 μM [16] and 0.7 μM [9] are either obtained or may be inferred for the enzyme–NADPH interaction: the concentration of free enzyme is thus vanishingly small. Moreover, the interaction of MTX with free enzyme is relatively weak, being governed by a dissociation constant in the vicinity of 0.4 μM [9, 11]. Likewise, no account has been taken of any interaction between MTX and the serum albumin

(0.15 μM) included in the assay buffer for stabilization of enzyme activity, since the dissociation constant for the interaction between MTX and bovine serum albumin exceeds 100 μM [25].

For reaction mixtures in which catalysis was initiated by addition of enzyme (7.5 nM), failure to achieve a steady state was indicated by a progressive decrease of reaction velocity in MTX-containing solutions. The fact that no progressive relief of inhibition was observed in such mixtures when H_2-folate was the last reactant added is consistent with the dissociation rate constant for the slow isomerization of enzyme–NADPH–inhibitor complex, namely 0.013 min^{-1}, that has been reported for *S. faecium* dihydrofolate reductase [9]. On that basis, demonstration of inhibition reversal under our conditions would require hours of observation of a reaction which is essentially complete within minutes.

On the basis of current understanding of the mechanism of dihydrofolate reductase [26], its inhibition by MTX should be competitive. In the past that evidence has had to be obtained indirectly by observing dependence of the apparent inhibition constant upon inhibitor concentration [1], or by successful curve-fitting of progress curves to equations based on the assumption that inhibition is competitive [9–11]. The results presented herein are the first to demonstrate unequivocally that MTX does not affect the maximal velocity of the reaction catalyzed by dihydrofolate reductase and, thus, provide the first direct evidence for the competitive nature of the inhibition.

Acknowledgements—We are indebted to the Queensland Cancer Fund and also the National Health and Medical Research Council of Australia for support of this investigation, and to Dr. R. G. Duggleby for criticism of the manuscript.

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