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THERMODYNAMIC CHARACTERIZATION OF THE INTERACTIONS OF METHOTREXATE WITH DIHYDROFOLATE REDUCTASE BY QUANTITATIVE AFFINITY CHROMATOGRAPHY

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Abstract—Affinity chromatography on methotrexate-Sepharose has been used to evaluate dissociation constants for interactions of methotrexate with dihydrofolate reductase from *Lactobacillus casei*. Equilibrium constants of 0.25 μM and 0.6 nM were obtained for dissociation of the inhibitor from the enzyme–methotrexate and enzyme–NADPH–methotrexate complexes, respectively, these estimates being in good agreement with the corresponding published values for dihydrofolate reductase from *Streptococcus faecium*. By employing a different method for evaluating the thermodynamic dissociation constant for the enzyme–NADPH–methotrexate interaction, this investigation provides independent support for the inference drawn from published fluorescence quenching studies that the interaction of methotrexate with dihydrofolate reductase–NADPH complex is governed by a dissociation constant in the vicinity of 600 pM, a value slightly higher than, but approaching, the inhibition constant of 50–60 pM obtained by enzyme kinetic techniques.

Enzyme kinetic studies have shown methotrexate (MTX) to be an extremely potent inhibitor of dihydrofolate reductase from MTX-resistant strains of *Streptococcus faecium* [1, 2] and *Lactobacillus casei* [3]. For enzyme from the former source the inhibition constant of 50–60 pM has been attributed to a rapid, reversible interaction of inhibitor with binary enzyme–NADPH complex, followed by slow isomerization of the ternary enzyme–NADPH–MTX complex so formed [1, 2]. Attempts to verify the magnitude of this extremely small dissociation constant have thus far invariably [1, 2, 4] employed fluorescence quenching as the means of thermodynamic characterization. However, in only one investigation [1] has an actual value, namely 0.6 nM, been ascribed to the equilibrium constant for dissociation of MTX from its complex with dihydrofolate reductase and NADPH; and, as noted by the authors [1], the accuracy of the estimate is poor inasmuch as any value less than 1 nM yields theoretical curves that describe adequately the experimental dependence of fluorescence quenching upon MTX concentration. Such criticism must, of course, be viewed in the light that fluorescence measurements have in the past afforded virtually the only means for thermodynamic characterization of such tight ligand binding.

Since there is clearly a need for thermodynamic studies on the MTX-dihydrofolate reductase system by an alternative procedure, we have explored further the previously noted potential [5] of quantitative affinity chromatography for studying equilibria governed by small dissociation constants. The purpose of the present paper is to report the use of affinity chromatographic procedures for characterizing the dissociation of MTX from enzyme–MTX and enzyme–NADPH–MTX complexes of *L. casei* dihydrofolate reductase, the inhibition of which by MTX is also governed by an inhibition constant in the 50 pM range [3].

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MATERIALS AND METHODS

Chemicals. As in the preceding investigation [3], NADPH (enzymatically reduced) was obtained from the Sigma Chemical Co. St. Louis, MO, the MTX being a Lederle product (American Cyanamid Co. Wayne, NJ); the preparation of dihydrofolate (H_2-folate) from folic acid (Sigma) has also been described therein. The affinity chromatographic matrix for dihydrofolate reductase, prepared by coupling MTX to epoxy-activated Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden), was from the same batch of MTX-Sepharose used for purification of the enzyme [3].

Concentration measurements. Concentrations of NADPH, MTX and H_2-folate were determined spectrophotometrically as outlined in the preceding paper [3]. Molar concentrations of dihydrofolate reductase were again based on titrations of enzymatic activity with MTX. Combination of enzyme concentrations so determined with absorbance measurements at 280 nm on the slightly opalescent solutions yielded an apparent molar absorption coefficient of 38,000 $M^{-1}\,cm^{-1}$. However, correction of the absorbance for light-scattering [6] decreases this value to 24,000, which matches more closely the earlier absorption coefficients of 21,500 [7] and

27,200 [8] for dihydrofolate reductase from *L. casei*. In routine spectrophotometric estimations of enzyme concentration, no corrections were made for effects of light-scattering and, accordingly, the apparent molar absorption coefficient was used for the conversion of absorbance measurements to molar concentrations of dihydrofolate reductase.

Quantitative affinity chromatography. The dissociation constant describing binary complex formation between dihydrofolate reductase and MTX was obtained by a frontal chromatographic procedure with MTX-Sepharose as affinity matrix. Enzyme solution (20 ml; approx. 3 μM) was first dialyzed for 24 hr at 5° against phosphate–chloride buffer (33.5 mM KH_2PO_4–16.5 mM K_2HPO_4–100 mM KCl), pH 6.8, I 0.18, containing 5 mM β-mercaptoethanol and MTX in the range 100–600 nM (4 × 500 ml), in order to establish the free concentration of inhibitor in the solution to be subjected to affinity chromatography [5]. The dialyzed dihydrofolate reductase solution was then applied to a column of MTX-Sepharose (0.8 × 6.8 cm) preequilibrated with final diffusate and thermostatically maintained at 25°. The column eluate, maintained at a flow-rate of 8 ml/hr, was monitored continuously at 280 nm by means of a Uvicord II (LKB Produkter, Uppsala, Sweden). After application of the sample (approx. 20 ml), the column was eluted with more diffusate until the absorbance had decreased to zero, whereupon the column was flushed with 50 mM Tris/HCl–100 mM KCl buffer (pH 8.5) containing 400 μM H_2-folate to ensure complete removal of enzyme prior to preequilibration with the appropriate diffusate for the next experiment. The rationale for quantitative assessment of these elution profiles, which differed markedly from those prevailing in conventional quantitative affinity chromatography [9], is more appropriately considered in conjunction with the presentation of results.

Affinity chromatography on MTX-Sepharose was also used to characterize the interaction of MTX with the enzyme–NADPH complex. Enzyme solution (25 ml; 0.4 nM) in phosphate–chloride buffer (pH 6.5, I 0.18) containing mercaptoethanol (5 mM), polyethylene glycol 6000 (2 mg/ml; BDH Chemicals Ltd.) and NADPH (20 μM) was applied to a column of Sephadex G-25 (2.5 × 20.4 cm) preequilibrated with the same buffer supplemented also with MTX (0.62 or 1.18 nM). Throughout application of the sample and the subsequent elution with more of the MTX-containing buffer, the column was maintained at 25° and a flow rate of 1 ml/min. Since the void volume of the column had been predetermined to be 32 ml, the first 25 ml of effluent was collected in a 25-ml volumetric flask and was discarded. The next 50 ml, containing all of the applied (25 ml) dihydrofolate reductase, was collected in a 50-ml volumetric flask to establish precisely the extent of dilution (2-fold). Experiments with bovine serum albumin (1 mg/ml; Sigma) were used to establish the quantitative recovery of protein in this gel chromatographic equivalent of equilibrium dialysis, while experiments with dihydrofolate reductase in the absence of MTX indicated 95% recovery of enzyme activity under these conditions of extreme dilution. The resulting dihydrofolate reductase solutions with free MTX concentrations of either 0.62 or 1.18 nM were then applied to a 0.3-ml column (0.8 × 0.6 cm) of MTX-Sepharose equilibrated at 25° with the phosphate-chloride/NADPH/polyethylene glycol/mercaptoethanol buffer (pH 6.5) containing the same concentration of inhibitor. The effluent, maintained at a flow rate of either 0.5 or 1.5 ml/min, was monitored enzymatically by introducing H_2-folate (56 μM) and NaOH (5.6 mM): the latter was included to increase the pH slightly (to pH 7) and, hence, facilitate catalysis by decreasing the strength of enzyme–NADPH–MTX complex formation. This automated assay was calibrated by its application to known concentrations of dihydrofolate reductase. Details of the procedure for quantitative analysis are again deferred until after the presentation of results.

RESULTS AND DISCUSSION

Studies of binary complex formation. In the enzyme kinetic investigation of the interaction between MTX and the binary enzyme–NADPH complex of dihydrofolate reductase from *L. casei* [3], the simplifying approximation was made that the concentration of binary enzyme–MTX complex contributes negligibly to the total concentration of dihydrofolate reductase present in reaction mixtures. For enzyme from *S. faecium*, this approximation was justified on the basis that the equilibrium constant describing dissociation of MTX from ternary enzyme–NADPH–MTX complex is some 600-fold smaller than that for dissociation of the enzyme–MTX complex [1]. To verify that a similar situation does indeed prevail for dihydrofolate reductase from *L. casei*, quantitative affinity chromatography has been developed as an alternative technique to fluorescence quenching [1, 2, 4, 10] for the thermodynamic characterization.

In accordance with the envisaged affinity chromatographic procedure [5], dihydrofolate reductase was first dialyzed exhaustively against several changes of MTX solution in order that the concentration of free inhibitor could be identified with that of the MTX solution against which dialysis was effected. The resulting enzyme solution was then subjected to frontal affinity chromatography on a column of MTX-Sepharose equilibrated with final diffusate in anticipation that the elution volume would exhibit dependence on MTX concentration by virtue of competition between free and immobilized inhibitor for enzyme, irrespective of the relative magnitudes of the equilibrium constants for the interaction of enzyme with free and immobilized forms of MTX [5, 9]. However, instead of the advancing elution profiles exhibiting a plateau region corresponding to the composition of the applied mixture [5, 9], a considerably lower enzyme concentration was attained; and, moreover, the position of the eluted boundary showed no dependence upon MTX concentration (Fig. 1). In that regard the elution volume was essentially that of dihydrofolate reductase from an equivalent column of Sepharose 6B.

Whereas the predicted behavior [5, 9] is based on the presumption that chemical equilibrium is main-

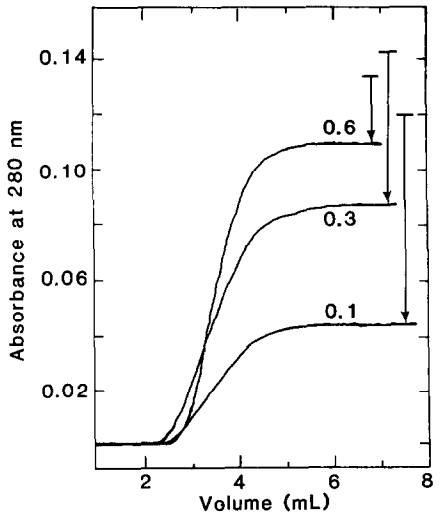

Fig. 1. Advancing elution profiles obtained in quantitative affinity chromatography, on MTX-Sepharose, of mixtures of MTX and dihydrofolate reductase (pH 6.5, I 0.18) in which the indicated concentrations of free inhibitor (μM) were established by prior dialysis. Vertical arrows indicate the extent to which the absorbance (at 280 nm) of the eluted plateau differed from that of the applied solution.

Table 1. Affinity chromatographic evaluation of the dissociation constant for binary complex formation between MTX and dihydrofolate reductase in phosphate–chloride buffer, pH 6.5, I 0.18

Reactant concentration (μM)			Dissoc. const.
MTX*	Free enzyme†	Complex‡	(μM)
0.60	0.66	1.95	0.20
0.60	0.71	1.91	0.22
0.30	1.50	1.58	0.28
0.10	2.05	0.77	0.27
0.10	2.05	0.77	0.27

* Established by prior dialysis.

† Obtained by dividing the difference between applied and plateau absorbances (Fig. 1) by the apparent molar absorption coefficient of 38,000 for enzyme at 280 nm.

‡ Obtained from the plateau absorbance (Fig. 1) and an estimated molar absorption coefficient (assuming absorbance additivity) of 54,500 for enzyme–MTX complex.

tained throughout chromatographic migration, the form of the experimental elution profiles suggests the operation of a rate constant for dissociation of enzyme–MTX complex that is small in relation to the rate of species separation by the affinity chromatographic procedure. Indeed, the form of the elution profiles in Fig. 1 indicates that dissociation of the enzyme–MTX complex is sufficiently slow for effectively no re-equilibration to occur in response to removal of free dihydrofolate reductase by interaction with immobilized MTX on the affinity matrix. In the absence of any such re-equilibration, the elution profile then represents the frontal gel chromatographic behavior of the enzyme–MTX complex present in the applied mixture. Under those circumstances the plateau concentration may be identified with the equilibrium concentration of binary complex. Interpretation of the results on this basis is summarized in Table 1, from which a value of 0.25 ($\pm$0.07) μM is obtained for the dissociation constant describing the interaction of MTX with dihydrofolate reductase from *L. casei*. This value matches fairly closely the dissociation constants of 0.36 μM [1] and 0.39 μM [4] obtained by fluorescence quenching studies of the corresponding interaction for *S. faecium* enzyme under similar, though not identical, conditions of pH, temperature and ionic strength.

Studies of ternary complex formation. Affinity chromatography on MTX-Sepharose has also afforded a means of quantitatively characterizing the interaction of MTX with the binary complex of dihydrofolate reductase and NADPH. However, the above procedure required several modifications because of the much smaller equilibrium constant describing the dissociation of MTX from the enzyme–NADPH–MTX ternary complex, and of the consequent experimental necessity to decrease the dihydrofolate reductase concentration to below 1 nM. (i) Polyethylene glycol (2 mg/ml) was included in the phosphate–chloride–mercaptoethanol buffer (pH 6.5, I 0.18) for stabilization of enzyme activity, presumably by minimization of adsorptive losses. (ii) To ensure effective saturation of NADPH-binding sites on the dihydrofolate reductase, and hence justify consideration of the results solely in terms of the interaction between MTX and enzyme–NADPH complex, the buffer was also supplemented with 20 μM NADPH: dissociation constants of 0.1 μM [7] and 0.8 μM [8] may be inferred from enzyme kinetic studies of the enzyme–NADPH interaction for dihydrofolate reductase from *L. casei*. (iii) Since overnight dialysis led to unacceptably high losses of enzymatic activity, this step was replaced by gel chromatography on Sephadex G-25 in order to define the concentration of free MTX in the enzyme solutions subjected to affinity chromatography. (iv) Use of 0.1 to 0.2 nM dihydrofolate reductase in the affinity chromatography step necessitated resort to a continuous enzymatic activity assay for monitoring the column effluent.

Typical elution profiles in two such affinity chromatography experiments are shown in Fig. 2A, about which the following points are noted. First, these elution profiles bear qualitative resemblance to those shown in Fig. 1 inasmuch as (a) the elution volume was again unaffected by MTX concentration, and (b) the plateau response did not correspond to that for the applied solution. Second, this plateau height is therefore some measure of the equilibrium concentration of enzyme–NADPH–MTX complex; but there is no direct relationship betwen the latter quantity and the measured enzymatic activity in the plateau region, because the concentration of H_2-folate introduced into the automated assay did not completely suppress inhibition. This difficulty was obviated by also measuring the assay response for a sample of the equilibrium mixture subjected to affinity chromatography, whereupon the difference between that response and the plateau response could be attributed to the concentration of unin-

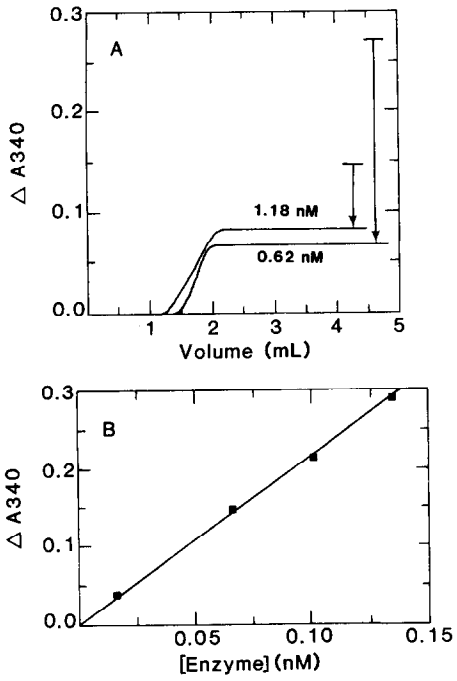

Fig. 2. Investigation of the interaction between MTX and binary dihydrofolate reductase–NADPH complex (pH 6.5, I 0.18) by affinity chromatography on MTX-Sepharose. (A) Elution profiles obtained for mixtures (0.1 to 0.2 nM enzyme) in which the indicated free MTX concentrations (nM) were established by prior gel chromatography on Sephadex G-25, the vertical arrows denoting the difference between the assay responses of the eluted plateau and the applied solution. (B) Calibration plot for interpretation of this difference in assay response in terms of the molar concentration of free enzyme–NADPH complex in the mixture.

hibited enzyme in the equilibrium mixture: this, in turn, could be converted to a molar scale via the calibration plot (Fig. 2B) obtained with a range of dihydrofolate reductase concentrations in the absence of MTX. Third, results of these experiments are summarized in Table 2, from which a value of 0.6 (±0.1) nM is obtained for the equilibrium constant describing the dissociation of MTX from its ternary complex with dihydrofolate reductase and NADPH. An identical value has, in fact, been reported [1] on the basis of fluorescence studies of the corresponding interaction with *S. faecium* enzyme.

CONCLUDING REMARKS

From the methodological viewpoint, a gratifying outcome of this affinity chromatographic investigation of MTX interactions with dihydrofolate reductase is the agreement, between different types of thermodynamic study on enzyme from two bacterial sources, that the equilibrium constant for dissociation of inhibitor from the ternary enzyme–NADPH–MTX complex is some two orders of magnitude smaller than that for its dissociation from the binary complex between MTX and dihydrofolate reductase. By verifying the magnitude of the dissociation constant for the interaction of MTX with the enzyme–NADPH complex of dihydrofolate reductase, this affinity chromatographic study obviously allows greater reliance to be placed on the value of 0.6 nM reported on the basis of fluorescence quenching studies [1]. This confirmation assumes even greater importance in view of the reservations expressed about the reliability of the latter estimate [1]. Indeed, there is doubt whether such tight binding of MTX can be characterized by fluorescence quenching [2, 4], a viewpoint essentially shared by Williams *et al.* [1], who, although reporting the value of 0.6 nM, also stated that the only definitive conclusion to emanate from the fluorescence quenching study was that the dissociation constant was less than nanomolar. Though more cumbersome to perform, the affinity chromatographic technique is more versatile than fluorescence quenching inasmuch as it can be used to determine dissociation constants of virtually any magnitude [5]. In that regard, the inhibition constant of 50–60 pM obtained by enzyme kinetic techniques [1–3] is not encompassed by the estimated uncertainty (±2 SEM) inherent in the present value of 0.6 (±0.1) nM. This estimate of the dissociation constant for the interaction of MTX with binary enzyme–NADPH complex thus approaches,

Table 2. Affinity chromatographic determination of the equilibrium constant for dissociation of MTX from its ternary complex with dihydrofolate reductase and NADPH*

MTX concn† (nM)	Enzyme concentration (nM)		Dissoc. const. (nM)
	Total	Free‡	
1.18	0.095	0.030	0.54
1.18	0.095	0.030	0.54
0.62	0.194	0.099	0.65
0.62	0.194	0.091	0.55

* Experiments were performed in phosphate-chloride buffer (pH 6.5, I 0.18) containing NADPH (20 μM), mercaptocthanol (5 mM) and polyethylene glycol (2 mg/ml).

† Established by gel chromatography on Sephadex G-25 (see text).

‡ Obtained from the difference between absorbance changes for the applied and plateau solutions (Fig. 2A) and the calibration plot (Fig. 2B).

but is slightly larger than, the value of 50–60 pM that characterizes the inhibition of dihydrofolate reductase by MTX.

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