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A MECHANISM OF RESISTANCE TO METHOTREXATE

NADPH BUT NOT NADH STIMULATION OF METHOTREXATE BINDING TO DIHYDROFOLATE REDUCTASE

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Abstract—Characteristics of methotrexate (MTX) inhibition of dihydrofolic acid reductase (DHFR) enzyme activity and the effects of NADPH and NADH on enzyme–drug interaction were studied. Two highly sensitive assay procedures were used. The first utilized tritium-labeled MTX to measure direct binding properties of the enzyme and the second utilized tritium-labeled dihydrofolate (H_2PteGlu) and folate (PteGlu) to analyze kinetics of reduction of these substrates. NADPH was found to enhance DHFR binding of MTX ($K_d \approx 2.6 \times 10^{-11}$ M), whereas NADH was found to have no effect ($K_d = 3.7 \times 10^{-9}$ M). However, NADH proved to be a good substrate for folate reduction compared to NADPH, especially in low salt buffer. The observation that NADH supports the reduction of folate and dihydrofolate but not MTX binding suggests that natural resistance to MTX could exist if NADH replaces NADPH as the main cofactor for DHFR.

Dihydrofolic acid reductase (DHFR; EC 1.5.1.3), one of the smallest pyridine nucleotide requiring enzymes, functions to reduce dihydrofolate (H_2PteGlu) and, more slowly, folate (pteroylglutamic acid; PteGlu) to tetrahydrofolate (H_4PteGlu). It has been considered the main target site for antifolates such as methotrexate (MTX), which has become a mainstay in the treatment of childhood leukemia, lymphoma, some solid tumors, and nonmalignant disorders such as psoriasis [1]. Cellular resistance to MTX on an induced or natural basis, however, has been observed and is clinically important. Laboratory models for acquired resistance most often involve: (a) increased cellular levels of DHFR, (b) altered DHFR with a decreased affinity for MTX, and (c) decreased transport of MTX. Natural resistance is less well characterized. Our experimental data suggest that natural resistance to MTX may be based upon the specific interactions of DHFR, MTX and pyridine nucleotide.

The interaction between MTX and DHFR has been considered "stoichiometric" because the drug and enzyme have been shown to interact on an equimolar basis [2]. The majority of these data, elaborated using spectrophotometric and fluorometric techniques, also showed that the interaction of drug and enzyme is potentiated by NADPH [3–5]. Enzyme–drug–cofactor complexes have also been demonstrated by polyacrylamide gel electrophoresis [6]. More recently, several laboratories demonstrated the feasibility of using DHFR as a tight binder of MTX in order to develop a radio-ligand binding assay for some antifolates [7–9]. The results of this type of assay also showed that the pyridine nucleotide cofactor is needed only in concentrations approximately equal to the MTX and enzyme in order to ensure "tight binding" [9].

This report further details the interaction of MTX, cofactor and enzyme purified from murine leukemia cells and compares optimal binding and enzyme activity. The results allow the hypothesis that natural resistance to MTX could arise if the enzyme utilizes NADH rather than NADPH as a reducing source, because in this situation MTX binding is decreased approximately 100-fold. This observation is consistent with work of others postulating: (a) multiple forms of DHFR [6], (b) a low affinity, reversible intracellular binder of MTX [10], and (c) the need for MTX to be in excess of the DHFR concentration in order to adequately (>95%) inhibit enzyme activity [11].

MATERIALS AND METHODS

DHFR was purified from murine L1210RR cells. These cells are resistant to MTX because of both elevated DHFR (30- to 40-fold compared to parent line) and a markedly decreased transport of MTX as previously documented [12]. The enzyme is identical to the parent cell enzyme [12]. DHFR was purified to homogeneity using MTX-Sepharose affinity chromatography essentially as described by Whiteley *et al.* [13] and Gupta *et al.* [14]. Briefly, the cell pellet was suspended in 10 vol. of Tris (10 mM, pH 7.5), containing 2.5 mM $MgCl_2$ and 3 mM dithiothreitol (DTT), and freeze-thawed three times; then the enzyme was precipitated selectively with ammonium sulfate. The 0–40% precipitate was discarded, and the supernatant fraction was applied to the MTX-Sepharose column (0.5 × 1.5 cm). Non-tightly bound protein was first eluted with Tris (50 mM, pH

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7.5) containing 2 mM DTT and 1% glycerin and then with 1 M Tris, pH 7.5. The DHFR was eluted in 1 M Tris buffer, pH 8.5, containing 0.5 to 1.0 mM H_2PteGlu. The peak enzyme fractions were identified by [^{3}H]MTX binding and were pooled. Bovine albumin, 0.1 to 0.5 mg/ml, treated with charcoal [9] was added to stabilize the DHFR, and the preparation was dialyzed for 24 hr against frequent changes of 0.02 M Tris buffer (pH 7.5) to lower the salt concentration and remove the H_2PteGlu. The DHFR was judged homogenous by polyacrylamide electrophoresis and by MTX binding [9].

Binding of [^{3}H]MTX to the purified enzyme was analyzed by separating the bound and free drug using selective adsorption of the free MTX by charcoal coated with dextran T-10 as detailed previously [9]. However, to facilitate the assay, the total volume was limited to 0.5 to 1.0 ml so that all reactions could be done in micro test tubes (1.5 ml), and the charcoal was pelleted by centrifuging for 60 sec in a microfuge (Beckman). Under these conditions, exposure of the reaction mixture to the charcoal for 1–3 min at 4° did not significantly change the results. Additional experiments utilizing Sephadex G-25 column chromatography and equilibrium dialysis confirmed the results obtained using coated charcoal.

When less than 95% of the radioactivity was bound by DHFR, present in excess of the MTX, the [^{3}H]MTX was purified on DEAE-cellulose using a linear gradient of NH_4HCO_3 (0.05 to 0.50 M) or high performance liquid chromatography (HPLC) as previously described [15].

DHFR enzyme activity was assessed using the radioassay of Hayman *et al.* [16] with either tritium-labeled H_2PteGlu or PteGlu as the substrate. In this assay, the radioactivity in the blank (tube without enzyme) was 0.5–1% of the total counts, and 15–25% of the radioactivity was converted (reduced) linearly during 10–15 min of incubation at 37° when H_2PteGlu was the substrate and the enzyme concentration was 1×10^{-10} M.

Radioactivity was determined by using a Beckman model L-230 liquid scintillation spectrometer. The samples were solubilized in a solution of Triton X-100/toluene (1/2, v/v) containing 9 g/l of pre-blend 2a70 (R.P.I., Elk Grove, IL) solution. Counting efficiency was 25–30%.

RESULTS

DHFR enzyme activity is known to be affected by both pH and salt concentration [5]. Therefore, the binding of [^{3}H]MTX by DHFR was measured as function of salt and pH in the presence or absence of NADPH (Fig. 1). The initial concentrations of MTX and DHFR were 3×10^{-9} and 2×10^{-9} M respectively. In the presence of excess NADPH, the amount of binding of MTX was optimal (equimolar with DHFR) and was independent of pH and salt in the range shown. In the absence of NADPH, binding was both pH and salt dependent. Additional experiments further showed that MTX binding in salt concentrations between 0.1 and 1 M was not altered appreciably.

To quantitate the change in binding of MTX as a function of cofactor, a Scatchard analysis was done

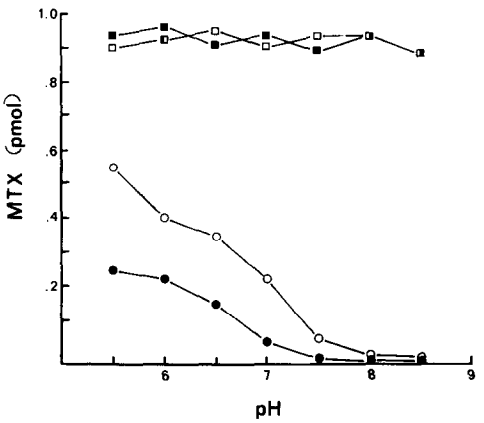

Fig. 1. Binding of [^{3}H]MTX (1.5 pmoles) by DHFR (1 pmole) in the presence and absence of salt (0.15 M KCl) or NADPH (1 μM). The buffer contained 0.01 M potassium phosphate at the pH indicated and dialyzed charcoal-treated albumin (10 mg/ml). The total volume of incubation was 0.5 ml. Incubation was for 15 min at 4°. The reaction was terminated by the addition of 0.2 ml of cold charcoal/dextran, with centrifugation for 60 sec at 8000 *g*. The entire supernatant fraction was transferred to a scintillation vial and counted in 5 ml of scintillant. Results were the same when incubation was carried out at room temperature. Key: (■) NADPH + salt; (□) NADPH; (○) salt; and (●) no addition.

at pH 7.5 in 10 mM potassium phosphate. In the presence of 1 μM NADPH, there was a single class of binding sites with a K_d of 2.6×10^{-11} M (Fig. 2A). In the absence of cofactor, the plot was biphasic, indicating two different binding sites (Fig. 2B). The K_d values were 2.6×10^{-11} and 3.7×10^{-9} M respectively. Since the absicissa intercept was the same in both cases, no binding sites were lost. The lower affinity site accounted for >80% of the total binding. The addition of 50 μM NADH did not alter the results shown in Fig. 2B.

The specificity for nucleotide cofactor was determined by titrating MTX binding as a function of NADPH or NADH concentration, as shown in Fig. 3. No increase in binding was noted with increasing concentrations of NADH, while maximum binding occurred with 2×10^{-8} M NADPH. Additional experiments showed than NAD, NADH and NADP at 1×10^{-5} M, 500 times the minimally effective NADPH concentration, did not increase or decrease MTX binding when added with the NADPH (data not shown).

The stability of the enzyme–NADPH–MTX complex was assessed by a pulse-chase experiment. In the presence of 1000-fold excess of MTX, there was minimal loss (15%) of bound [^{3}H]MTX after 48 hr at 4° when 10^{-9} M enzyme and MTX were preincubated for 10 min in the presence of excess NADPH. The initial rate of binding of [^{3}H]MTX was 3%/sec at starting concentrations of 10^{-9} M enzyme and MTX and excess NADPH. Binding was linear with respect to time until approximately 85% maximum binding of MTX was achieved. Preincubating either MTX or NADPH for 10 min with enzyme did not alter significantly the rate of binding.

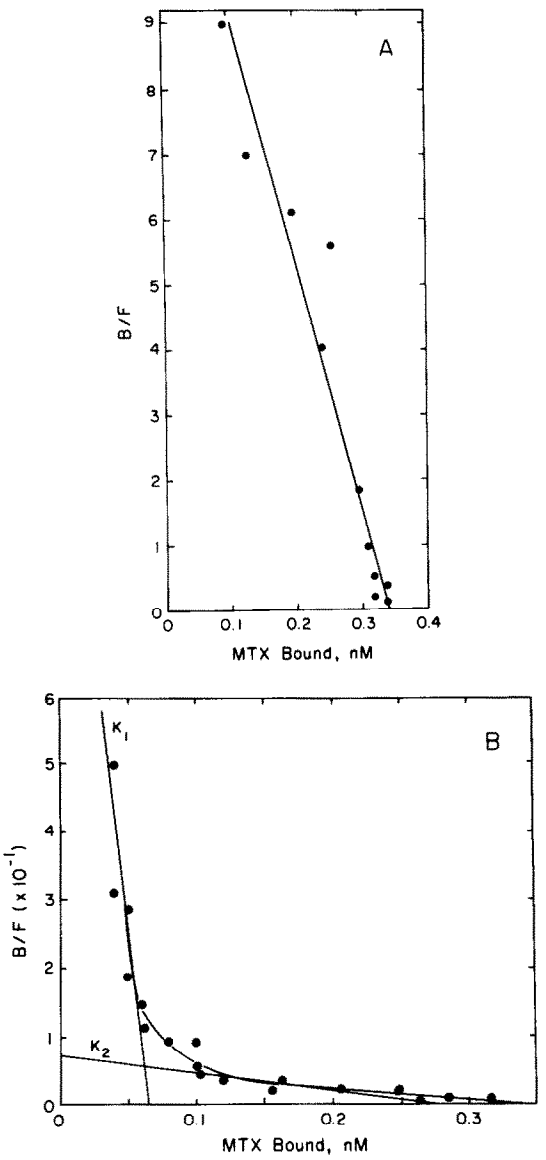

Fig. 2. Scatchard analysis of [^{3}H]MTX binding by DHFR in the presence and absence of NADPH. Panel A depicts binding in the presence of 1 μM NADPH. Panel B depicts binding in the absence of NADPH. The reaction volume was 1.0 ml, and the MTX binding capacity was 0.35 pmole. Incubation was for 15 min at room temperature. The reaction was stopped by the addition of 0.2 ml of cold charcoal/dextran.

Attempts to slow the reaction rate by adding 10–20% (v/v) glycerin also failed to slow the initial velocity.

Some of the enzymatic properties of DHFR from the L1210RR are summarized in Table 1. The data agree well with other laboratories and techniques. The activation by salt and the increase in K_m for NADPH are qualitatively similar to the results of Reyes and Huennekens [17]. The data also clearly show that NADH can be a cofactor for enzyme activity. In the absence of salt, the turnover number for H_2PteGlu was the same in the presence of either NADH or NADPH. NADH also has been shown to be a subtrate for DHFR from chicken liver [18].

The relative efficacies of NADH and NADPH in potentiating inhibition of enzyme activity by MTX are compared in Fig. 4. In this experiment, MTX was preincubated with DHFR and cofactor to allow binding to occur prior to addition of [^{3}H]H_2PteGlu. At equimolar concentrations of MTX and enzyme (1×10^{-9} M), there was no significant enzyme activity when NADPH was cofactor. However, when NADH was the cofactor a 50-fold excess of MTX to enzyme was required to inhibit 95% of the enzyme activity.

DISCUSSION

Previous measurements utilizing fluorescence changes have shown that DHFR forms a stable ternary complex with MTX and NADPH and that the antifolate affinity of such a complex is much greater than that of a binary antifolate–DHFR complex [4]. Our studies confirm that NADPH increases the affinity of DHFR for MTX but also show that NADH does not. A Scatchard analysis done in the absence of pyridine nucleotide or in the presence of NADH revealed two classes of binding sites having K_d of 3.7×10^{-9} and 2.6×10^{-11} M, whereas only the high affinity site was found when NADPH was present. There is, as yet, no explanation for finding two classes of binding sites in the absence of added NADPH, but the higher affinity site may be due to the presence of some NADPH in the enzyme preparation [19]. The inability of NADH to increase the affinity of DHFR for MTX even at a two log-fold higher concentration than the NADPH required for complete binding is shown in Fig. 3. However, as presented in Table 1, NADH is a substrate for enzyme activity. In fact, in a low salt buffer, NADH was equal to NADPH as a reducing source. Therefore, these experimental results show that, although both pyridine nucleotides can serve as substrates for folate reduction, the increased affinity of DHFR for MTX is more dependent upon NADPH than NADH.

The significance of these observations can be visualized in Fig. 4. When NADH was the cofactor, the inhibition of DHFR by MTX was not "stoichiometric". Detection of enzyme activity in the presence of NADH even in a 50-fold excess of MTX allows for the hypothesis that natural resistance to MTX could be a direct consequence of DHFR either having a decreased affinity for NADPH or being in an intracellular milieu where NADH is the available cofactor.

Since it is likely that some DHFR *in situ* is cofactor free [6], this hypothesis also agrees with previously reported studies noting the need for excess MTX to be present intracellularly in order to fully inhibit DHFR [11], i.e. the low affinity MTX binding previously described could be DHFR without NADPH [10]. Confirmation of this mechanism for resistance to MTX must await analysis of the *in situ* state of the DHFR. Knowing that both NADH and NADPH are higher than DHFR in concentration is not sufficient, because subcellular localization of enzyme and cofactors could occur. Therefore, identification

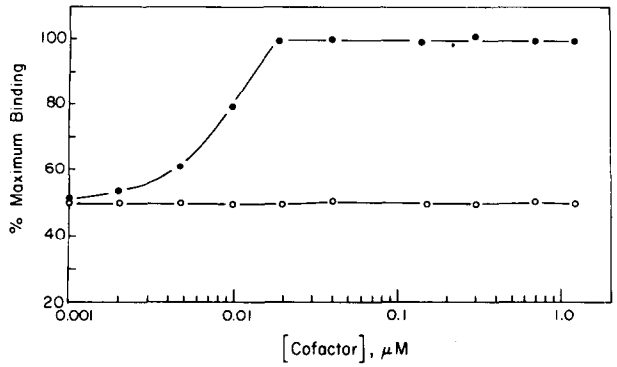

Fig. 3. Specificity of the pyridine nucleotide needed for optimal binding of [^{3}H]MTX by DHFR. The pH was 6.0 so that in the absence of NADPH there would be some binding. The reaction volume was 0.5 ml, and the MTX binding capacity was 6 pmoles. The ratio of MTX bound/NADPH incubated was 1/1.8. Key: (●) NADPH; and (○) NADH.

of *in situ* enzyme or isolation of a DHFR with altered pyridine nucleotide requirements is necessary. This hypothesis, however, may provide an explanation for the discordancy between the level of DHFR in MTX-resistant cells and the observed resistance to extracellular concentration of drug. For example, a 10-fold increase in DHFR can cause a 100-fold increase in cellular resistance to MTX [20].

Table 1. Enzymatic properties of dihydrofolate reductase from L1210RR cells*

Buffer	K_m values for cofactors and substrates NADPH (10^{-6} M)	NADH	H_2PteGlu (10^{-6} M)	PteGlu
pH 7	8	80	1.2	2.0
plus salt†	33	310	1.2	2.0
pH 5	5	60	1.2	2.0
plus salt	33	310	1.2	2.0

Substrate	Turnover number‡ Pyridine nucleotide cofactor§ NADPH	NADH
H_2PteGlu		
pH 7.0	300	300
plus salt	750	31
pH 5.0	400	150
plus salt	1000	62
PteGlu		
pH 7.0	0.7	0.3
plus salt	4	0.1
pH 5.0	7	2.2
plus salt	14	0.6

* Determined with radioassay [16].

† Salt was 0.15 M KCl. Buffer was either 0.01 M potassium phosphate or Tris.

‡ Moles of folate substrate converted to H_4PteGlu per mole DHFR per min at 37°.

§ Pyridine nucleotide and folate concentrations were initially at 10 × K_m.

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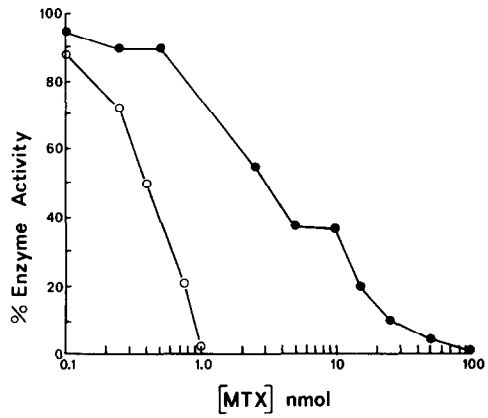

Fig. 4. DHFR enzyme activity as a function of MTX concentration and pyridine nucleotide. Enzyme activity was measured by [^{3}H]H_4PteGlu formation as described in Materials and Methods. In a 1 ml volume, 1 pmole enzyme was preincubated with increasing amounts of MTX for 10 min in the presence of 50 μM NADPH (○) or 500 μM NADH (●), and the enzyme reaction was started by the addition of [^{3}H]H_2PteGlu to a final concentration of 20 μM. Assay conditions were: pH 6.0, potassium phosphate buffer 0.1 M, and pyridine nucleotide as indicated. Enzyme reaction was allowed to proceed for 10 min at 37° and was terminated using folate and acetic acid/$ZnSO_4$ [16].

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