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# MULTIPLE FORMS OF L1210 DIHYDROFOLATE REDUCTASE DIFFERING IN AFFINITY FOR METHOTREXATE

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SUMMARY: Dihydrofolate reductase, purified from a Methotrexate-resistant subline (R6) of L1210 mouse leukemia cells, consists of two forms (designated 1 and 2) differing in affinity for the drug. Form 1 is more sensitive to inhibition by Methotrexate. Form 2 is more heat-labile, but it can be stabilized by bovine serum albumin or NADPH. Isoelectric focusing resolves 1 and 2; pI values are 7.4 and 8.2. Forms 1 and 2 comprise about 10 and 90% of the total protein, but 1 has at least a 2-fold higher specific activity. Binding measurements with [$^3$H]Methotrexate provide $K_D$ values of ca. 1 and 27 nM for 1 and 2.

A variety of MTX[1]-resistant cell lines have been reported to contain elevated levels of dihydrofolate reductase, the target enzyme for the drug. In some instances, the enzyme appears to be identical to the wild-type counterpart (reviewed by Morandi and Attardi (1)). Alternatively, other MTX-resistant cells with elevated levels of enzyme contain both wild-type and mutant forms (2-7). The latter are characterized by a decreased affinity for MTX and, in addition, may display alterations in other properties such as amino acid composition, pI value, heat-stability or kinetic parameters.

Previous work from this laboratory has described the purification and properties of dihydrofolate reductase from an MTX-resistant subline (R6) of L1210 mouse leukemia cells (8, 9). Although only a single species was observed when the enzyme preparation was examined by PAGE (9), titration with MTX indicated that multiple forms might be present (10). Further investigation of the problem, described in this communication, has revealed that

[1]Abbreviations: MTX, Methotrexate; $FH_2$, dihydro-folate; BSA, bovine serum albumin; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

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the R6 subline contains at least two forms of the enzyme that differ in affinity for MTX, heat-stability, and pI values.

## EXPERIMENTAL PROCEDURES

Materials. The following were obtained from the indicated commercial sources: NADPH (PL Biochemicals); MTX and BSA (Sigma); [3',5',9-$^3$H]MTX (16 Ci/mmole) (Amersham); cyto-Scint (West Chem); Agarose, marker dyes and Gelbond for isoelectric focusing (FMC); ampholytes (3/10) (Bio-Rad). Nitrocellulose filters ($10^{-2}$ μm pore size, 25 mm diameter) (Sartorius). $FH_2$ was prepared by the method of Blakley (11). Concentrations of MTX, $FH_2$ and NADPH were determined spectrophotometrically.

Dihydrofolate reductase. The MTX-resistant subline (R6) of L1210 cells was maintained in the presence of $10^{-6}$ M MTX. Dihydrofolate reductase was purified from these cells as described previously (9), except that step 5 (chromatography on DEAE-Sephadex) was replaced by affinity chromatography on a 2.5 x 20 cm column of folate-Sepharose (12). The enzyme preparation exhibited a single, sharp band when examined by SDS-PAGE, and the specific activity of various preparations, determined by assay A, ranged from 55 to 60 units/mg protein. Protein concentration was determined by the Bio-Rad method (13).

Assay A: Enzymatic activity. The following components were present in a 1-cm cuvet: $FH_2$, 50 μM; NADPH, 150 μM; KCl, 0.5 M; K-Hepes buffer (pH 7.0), 0.1 M; and enzyme, 1.5-5.0 nM. Volume, 1.0 ml; temperature, 30°. The reaction was initiated by addition of enzyme and the time-dependent decrease in absorbance at 340 nm was monitored using a Beckman DU monochromator with a Gilford optical density converter. Initial rates were linear for at least 5 min. Under these conditions, an absorbance change of 12.3 corresponds to the reduction of 1 μmole of dihydrofolate. Specific activity is expressed as IU/mg protein.

Assay B: Inhibition by MTX. Conditions of assay A were used, except that enzyme and MTX (at the indicated concentrations) were preincubated for 5 min in 0.8 ml of buffer, and the reaction was initiated by the sequential addition of KCl, $FH_2$ and NADPH in a combined volume of 0.2 ml.

Assay C: Inhibition by MTX. Enzyme (500 nM) and MTX (at the indicated concentration) were preincubated (5 min, 30°) in 0.2 ml of 0.05 M K-phosphate buffer, pH 7.0. Aliquots (5 μl) of these mixtures were withdrawn and used for activity measurements via assay A.

Treatment of enzyme preparation with DTNB. Enzyme (4.7 μM), DTNB (8.5 mM) and KCl (0.5 M) in 0.1 M K-Hepes buffer, pH 7.0, were incubated for 2 h at 25° and then dialyzed against 10 mM K-phosphate buffer, pH 7.5.

Electrophoresis. PAGE at pH 8.2 (9) and SDS-PAGE (14) were performed by the indicated methods. Isoelectric focusing utilized the following procedure: enzyme was applied to a 0.05 x 8 x 12 cm gel of 0.5% agarose containing 2% ampholytes. The electrode solutions were NaOH (0.5 M) and acetic acid (0.5 M). The gel was focused for 1.5 h at constant power (2.5 watts). Protein and catalytic activity were detected by the silver-staining (15) and tetrazolium-staining (16) methods.

Binding of [$^3$H]MTX. Enzyme (32.5 nM) was incubated with various concentrations (4-240 nM) of [$^3$H]MTX ($4.7 \times 10^5$ cpm/nmole) for 5 min at 25° in 1.0 ml of 0.05 M K-phosphate buffer, pH 7.0. Solutions were passed through nitrocellulose filters (pre-soaked for 1 h in distilled water), and the latter were placed in scintillation vials containing 8 ml of Cyto-Scint and left at room temperature overnight. Radioactivity was measured using a Beckman liquid scintillation counter, Model LS-233. Corrections (<10%) were made for controls in which enzyme was omitted.

## RESULTS

Detection of multiple forms of L1210 (R6) dihydrofolate reductase by titration with MTX. Preincubation of the enzyme preparation with increasing

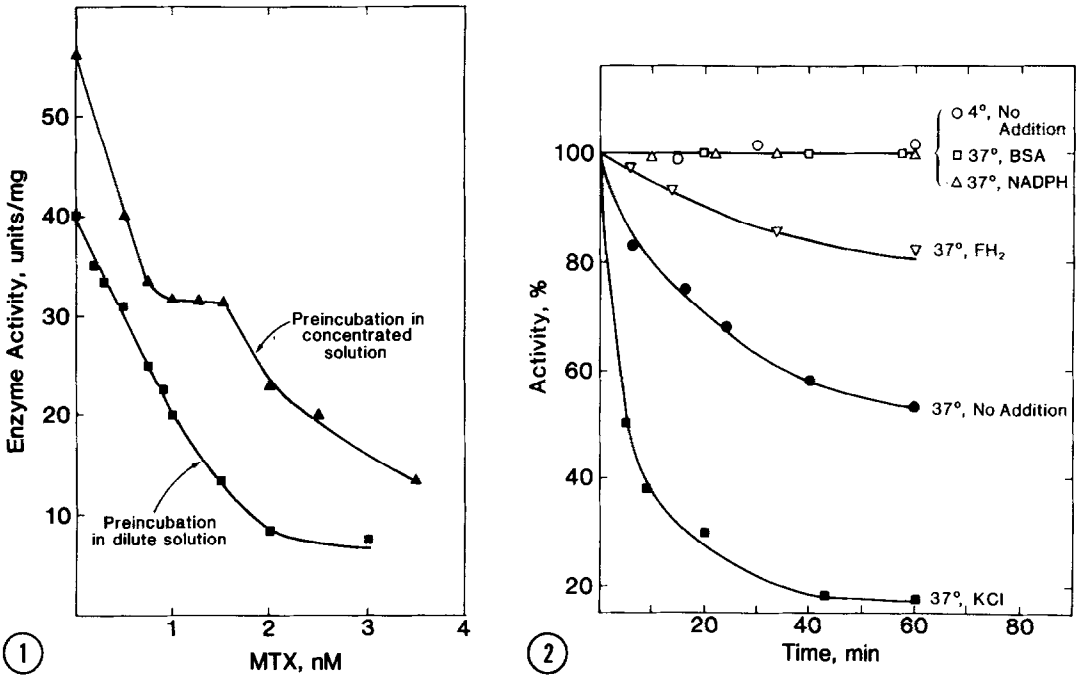


Fig. 1. MTX inhibition of L1210(R6) dihydrofolate reductase. Inhibition measured under conditions of Assay B (■) and Assay C (▲), as described in Experimental Procedures. Results are expressed in terms of residual specific activity (IU/mg protein) as a function of MTX concentration. Final enzyme concentrations were 1.6 and 2.5 nM in Assays B and C.

Fig. 2. Thermal stability of the enzyme preparation and protection by various agents. Enzyme (32.5 nM) in 0.5 M K-phosphate buffer, pH 7.5, was incubated for 60 min at 37° (unless otherwise indicated), and at various times aliquots were monitored for activity via Assay A. Results are expressed as percent of initial (zero-time) activity. NADPH (1 mM), $FH_2$ (1 mM), KCl (0.8 M) or BSA (50 μM) was present as indicated.

concentrations of MTX in a relatively dilute solution (Assay B), resulted in inhibition of activity as shown by the lower curve in Fig. 1. However, when the enzyme was preincubated in a several hundred-fold more concentrated solution (Assay C), activity in the absence of MTX was considerably higher and the titration curve was more complex (upper curve, Fig. 1). These results suggested that the preparation contained at least two forms of the enzyme, differing in stability and in affinity for the drug.

Stability of enzyme preparation. To further investigate the above supposition, stability of the enzyme preparation was examined as a function of temperature and time (Fig. 2). At 4°, full activity was retained over a 60-min period, but at 37° activity was progressively lost. The preparation was completely stabilized, however, by the inclusion of BSA (50 μM)

or NADPH (1 mM). $FH_2$ (1 mM) provided partial protection, glycerol (15%) was ineffective, and KCl (0.8 M) increased the rate of inactivation.

The above results were used to reexamine the MTX titration curves presented in Fig. 1. When preincubation of the enzyme and MTX in dilute solution (lower curve) was repeated, but with BSA present, a response similar to that shown in the upper curve was obtained. Conversely, when preincubation was conducted in concentrated solution (upper curve), but using enzyme that had been heat-treated until ca. 35% of the activity had been lost, a response similar to the lower curve was obtained.

Separation of forms. Isoelectric focusing resolved the preparation into a major band (pI 8.2) and a minor band (pI 7.4). These bands comprised 90 and 10% of the total protein (Fig. 3A). Enzymatic activity was associated with both bands (Fig. 3B); in this instance, however, the minor band accounted for 20% of the total activity. When the enzyme preparation was progressively heat-inactivated prior to analysis, the pI 8.2 band was diminished more readily than the pI 7.4 band in both staining procedures. Alternatively,

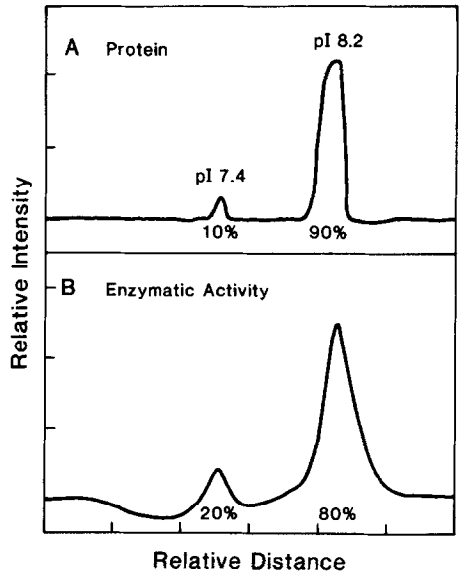


Fig. 3. Resolution of the enzyme preparation via isoelectric focusing. Enzyme (1.63 µg in 5 µl of 0.05 M K-phosphate buffer, pH 7.5) was subjected to isoelectric focusing. A, gel stained for protein and scanned with a Zeineh soft-laser densitometer. B, ditto except that the gel was stained for enzymatic activity prior to scanning.

pre-treatment of the enzyme with increasing concentrations of MTX selectively suppressed the activity of the pI 7.4 band. Based upon these results, the pI 7.4 and 8.2 bands were designated as enzyme forms 1 and 2. The mixture could also be resolved by PAGE at pH 8.2. Under these conditions, the major component (form 2) was seen at $R_f$ 0.69, while the minor component (form 1) moved at the solvent front ($R_f$ 1.0); co-migration of form 1 with the dye marker prevented its detection in the previous investigation (9).

Affinity of forms for MTX. The enzyme preparation was admixed with varying amounts of [$^3$H]MTX and, after equilibrium had been established (ca. 5 min), bound drug was separated by passage through nitrocellulose filters. Fig. 4A shows the concentrations of bound MTX as a function of added drug. The plateau value corresponding to maximum binding (ca. 30 nM) was in good agreement with the concentration of enzyme present (32.5 nM). Replotting the data as the reciprocals of bound vs. free MTX (inset) provided a $K_D$ value of 27 nM. This is considered to be the dissociation constant for form 2,

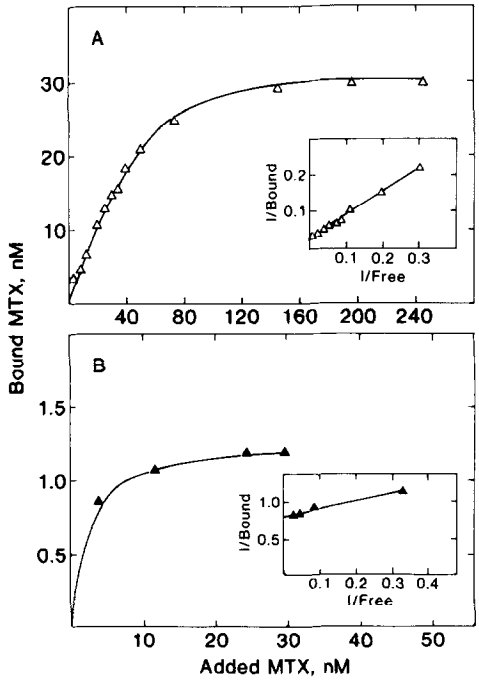


Fig. 4. MTX binding to the enzyme forms. Enzyme-MTX interaction was measured as described in Experimental Procedures. A, untreated enzyme. B, enzyme treated with DTNB. Results are expressed in terms of bound MTX as a function of added MTX. Insets, double-reciprocal plots of bound vs. free MTX.

since it comprises ca. 90% of the MTX-binding capacity of the preparation. It was not possible to obtain an accurate $K_D$ value for form 1 from this experiment, nor was heat denaturation sufficiently selective to remove form 2, leaving form 1 for analysis. This could be achieved, however, by treating the mixture with DTNB, a procedure that inactivates form 2 (as judged by isoelectric focusing). When a preparation treated in this manner was tested with [$^3$H]MTX, analysis of the data revealed a single component with a $K_D$ value of ca. 1 nM (Fig. 4B). This is considered to be the dissociation constant of form 1.

## DISCUSSION

Dihydrofolate reductase from the R6 subline of L1210 cells consists of 10% of form 1 ($K_D$ = ca. 1 nM for interaction with MTX) and 90% of form 2 ($K_D$ = 27 nM). The mixture of these forms accounts for the complex inhibition pattern observed when the preparation is titrated with MTX. The L1210 enzyme may be compared to its counterparts in other MTX-resistant eukaryotic cells containing both wild-type and mutant forms (2-7). Some general conclusions can be drawn regarding the properties of these dihydrofolate reductases: (a) The mutant enzymes have a lower affinity for MTX, although quantitation of this effect varies. (b) Mutant enzymes have different isoelectric points and lower specific activities. (c) Molecular weights of the mutant and wild-type enzymes are not appreciably different. (d) No general pattern is seen in other structural or catalytic parameters such as heat-lability, $K_m$ values, or pH optimum.

It is not yet clear whether 1 and 2 are homogeneous with respect to sub-forms, whether either or both occur in wild-type cells, and whether the forms are interconvertible. Structural differences between 1 and 2 need to be elucidated. These forms may differ in amino acid composition or in conformation. Two forms of the *Escherichia coli* enzyme (17), for example, differ by a single amino acid at position 28. Alternatively, conformational flexibility would be consistent with the observations that mammalian dihydrofolate reductases can be activated (i.e., have their catalytic activity

increased several-fold) by treatment with mercurials, salts, or chaotropic agents (reviewed in 18). A differential response of wild-type and mutant dihydrofolate reductases to activating agents has been observed previously (2,5), and preliminary experiments in this laboratory have shown that form 2, but not 1, of the L1210 (R6) enzyme can be activated.

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