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Properties of Dihydrofolate Reductase from a Methotrexate-Resistant Subline of Human KB Cells and Comparison with Enzyme from KB Parent Cells and Mouse S180 AT/3000 Cells

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SUMMARY

Dihydrofolate reductases from human KB cells and KB cells resistant to methotrexate (KB/MTX) were purified by affinity chromatography to near homogeneity, and the properties of these enzymes were compared. KB and KB/MTX enzymes were identical with respect to the isoelectric point (7.3) and kinetic constants for NADPH and 7,8-dihydropteroylglutamate (H_2PteGlu) (5.9 μM and 0.67 μM, respectively). 7,8-Dihydropteroylpentaglutamate (H_2PteGlu$_5$) was equivalent to H_2PteGlu as substrate for KB/MTX enzyme as evidenced by K_m and V_{max} values. MTX K_i values were also identical for KB and KB/MTX enzymes (5 pM). The turnover numbers per MTX binding site were slightly different (1360/min for KB/MTX enzyme and 1000/min for KB parent enzyme). The K_i for MTX diglutamate with KB/MTX enzyme was 10 pM, similar to the K_i for MTX. KB/MTX enzyme was activated by potassium chloride with a maximum at 150 mM and showed a double pH profile with maxima at pH 7.2–8.2 and below pH 5. The molecular weight was 20,000 with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Both pteroylglutamate and pteroylpentaglutamate competitively inhibited H_2PteGlu or H_2PteGlu$_5$ reduction with similar K_i values, 0.025 μM and 0.05 μM, respectively. (±)-L-N_5,N_{10}-Methylene-5,6,7,8-tetrahydropteroylglutamate, (±)-L-5,6,7,8-tetrahydropteroylglutamate, and (±)-L-5,6,7,8-tetrahydropteroylpentaglutamate were not inhibitory at 20 μM, whereas N_{10}-formylpteroylglutamate, N_{10}-methylpteroylglutamate, and (±)-L-N_5-methyl-5,6,7,8-tetrahydropteroylglutamate showed inhibition with K_i values of 0.001, 0.13, and 10.1 μM, respectively. Analogues of MTX having substitutions at the α and γ carboxyl groups showed that significant increases in K_i occurred with amidation, esterification, and the addition of a glutamate or aspartate moiety at the α carboxyl group, whereas there was no substantial change in K_i with these same modifications at the γ carboxyl group. The K_m values for NADPH, H_2PteGlu, and H_2PteGlu$_5$ with enzyme from human AML cells were identical with those of the human KB and KB/MTX enzymes (6.2 μM, 0.66 μM, and 0.68 μM, respectively). Direct comparison of KB/MTX human enzyme with S180 AT/3000 murine enzyme showed the murine dihydrofolate reductase to be significantly different in isoelectric point (8.6), kinetic constants for H_2PteGlu (1.7 μM K_m) and H_2PteGlu$_5$ (1.9 μM K_m), K_i value for MTX (110 pM), and turnover number per MTX binding site (570/min).

INTRODUCTION

DHFR[2] (EC 1.5.1.3), which catalyzes the reduction of H_2PteGlu to H_4PteGlu in the presence of NADPH, is a

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[2] The abbreviations used are: DHFR, dihydrofolate reductase, 5,6,7,8-tetrahydrofolate, NAD(P) oxidoreductase; PteGlu, pteroylglutamate, folic acid, folate; PteGlu$_5$, pteroylpentaglutamate; H_2PteGlu, 7,8-dihydropteroylglutamate, dihydrofolate; H_2PteGlu$_5$, 7,8-dihydropteroylpentaglutamate; H_4PteGlu, (±)-L-5,6,7,8-tetrahydropteroylglutamate, tetrahydrofolate; H_4PteGlu$_5$, (±)-L-5,6,7,8-tetrahydropteroylpentaglutamate; MTX, methotrexate, 4-amino-10-methylpteroylglutamate; AML cells, acute myelogenous leukemia cells; ALL cells, acute lymphocytic leukemia cells; KB/MTX, KB cells resistant to MTX; AT/3000, mouse sarcoma (S180) cells resistant to MTX; 5-CH_3-H_4PteGlu, (±)-L-N_5-methyl-5,6,7,8-tetrahydropteroylglutamate; 10-CH_3PteGlu, N_{10}-methylpteroylglutamate; 5,10-CH-H_4PteGlu, (±)-L-N_5,N_{10}-methenyl-5,6,7,8-tetrahydropteroylglutamate; 5,10-CH_2-H_4PteGlu, (±)-L-N_5,N_{10}-methylene-5,6,7,8-tetrahydropteroylglutamate; 10-CHO-PteGlu, N_{10}-formylpteroylglutamate; PBS, phosphate-buffered saline; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

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central enzyme in folate metabolism in that it regenerates tetrahydrofolate from the dihydrofolate product of the thymidylate synthetase reaction. It is the target enzyme for the potent chemotherapeutic agent MTX, and acquired resistance to MTX has been shown to be due to increased levels or altered properties of DHFR and decreased cellular transport of MTX. Although many studies on purified DHFR from bacterial and mammalian sources have been performed (1), very little information about the human enzyme is available. Kinetic properties of the enzyme from cultured human lymphoblast W1-L2 cells and several MTX-resistant sublines were examined recently (2, 3). In addition to an increase in enzyme levels which accompanied MTX resistance, the K_m value for H_2PteGlu and the K_i for MTX were significantly changed. The K_m for H_2PteGlu of the enzyme from the parent cell line was 0.13 μM and the K_i for MTX was 7.3 pM. No studies with dihydrofolate polyglutamates were performed with this enzyme. Coward *et al.* (4) reported on some kinetic properties of the DHFR from human AML cells and ALL cells; they found K_m values for H_2PteGlu of 3.6 and 3.5 μM, respectively. They further demonstrated a substrate preference for the dihydrofolatepolyglutamates as indicated by higher V_{max} and lower K_m values than those for H_2PteGlu. Jarabak and Bachur (5) reported on the properties of DHFR from human placenta, demonstrating a K_m for H_2PteGlu of 0.11 μM and a K_i for MTX of 6.1 pM. They showed small differences in turnover number and K_i for MTX between this human enzyme and enzymes from rat and mouse liver. Because of the conflicting results from various laboratories and the lack of direct comparisons of mouse and human enzymes under identical conditions, we examined the properties of highly purified human DHFR from an MTX-resistant subline of KB cells, including the interactions with H_2PteGlu$_5$, and directly compared its kinetic and physical properties with those of identically purified enzyme from the parent KB cells, human AML cells, and from a murine S180 AT/3000 MTX-resistant subline (6, 7).

MATERIALS AND METHODS

Folyl Compounds

PteGlu and 5-CH_3-H_4-PteGlu were purchased from Sigma Chemical Company (St. Louis, Mo.); 10-CH_3-PteGlu was obtained from American Cyanamid (Princeton, N. J.); MTX was obtained from Nutritional Biochemicals (Cleveland, Ohio); MTX analogues with modifications at the α and γ carboxyl groups, including MTX diglutamate, were generously provided by J. R. Piper, C. Temple, Jr., R. D. Elliott, J. D. Rose, and J. A. Montgomery, of the Southern Research Institute, and A. Rosowsky, C.-S. Yu, and E. J. Modest of the Sidney Farber Cancer Institute. H_2PteGlu was prepared and recrystallized by the method of Blakley (8). Nonreduced pteroylpolyglutamates were prepared and purified by L. Mead, of the Department of Experimental Therapeutics, Roswell Park Memorial Institute, according to the published procedure (9). H_2PteGlu$_5$ was prepared and purified as described (4) except that the initial purification was on DEAE-cellulose, and further purification and desalting on Sephadex G-25 were performed. H_4PteGlu and H_4PteGlu$_5$ were prepared as described (10, 11). The concentration of each compound was determined by UV spectra on the basis of the published values (1).

Other Reagents

All chemicals were reagent grade or better. Reagents and their sources are as follows: Sephadex G-25 (Pharmacia, Piscataway, N. J.); ampholines for isoelectric focusing (LKB, Rockville, Md.); DEAE-cellulose, course mesh, NADPH, and NADP (Sigma Chemical Company); MTX-Sepharose was generously provided by Dr. David Bacchanari of Wellcome Research Laboratories (Research Triangle Park, N. C.). All other chemicals were purchased from Sigma Chemical Company.

DHFR Assay

Enzyme activity was determined spectrophotometrically by measuring the decrease in absorbance at 340 nm which accompanies the reduction of H_2PteGlu to H_4PteGlu and the oxidation of NADPH to NADP (12). Initial rates were measured at 37° with a Leeds-Northrup recorder attached to a Beckman DU spectrophotometer using a Gilford 220 absorbance indicator. One unit of enzyme activity is defined as the conversion of 1 μmole of substrate per minute. Extinction coefficients used for the combined absorbance changes of NADPH-PteGlu and NADPH-H_2PteGlu were 18.3×10^3 M^{-1} cm^{-1} (6) and 12×10^3 M^{-1} cm^{-1} (13), respectively. The standard assay mixture contained 50 mM potassium phosphate (pH 7.5) buffer, 150 mM KCl, 10 mM β-mercaptoethanol, enzyme, 100 μM NADPH, and 20 μM H_2PteGlu in a final volume of 1.0 ml. The reaction was started by the addition of H_2PteGlu. Except where noted in the text, all reactions were linear during the time measured.

Cells

At/3000 cells. The maintenance, preparation, and harvesting of AT/3000 cells were performed as previously described (7).

KB/MTX cells. This is a subline of human KB cells which is 7400-fold resistant to MTX with a 40- to 70-fold elevation of DHFR. They were maintained in RPMI 1640 medium containing 50 μM MTX, 30 μM dThd, and 2 μM folic acid. In order to remove the bound MTX, 2 weeks before harvesting the cells were transferred and maintained in a medium supplemented with 100 μM hypoxanthine, 30 μM dThd, 100 μM glycine, and containing no MTX or folic acid. Harvesting was carried out as described for the AT/3000 cells.

KB parent cells. Wild-type KB cells were maintained in monolayer in RPMI 1640 medium with 10% fetal calf serum and provided as cell pellets by S. Grill, of the Department of Pharmacology, University of North Carolina at Chapel Hill.

After rinsing the flasks with PBS, cells were harvested by scraping them from the flask surface and washing them once with PBS using centrifugation.

Acute myelocytic leukemia cells were processed as previously described (14).

Enzyme Purification

Cell pellets were suspended in 4 volumes of 0.01 M Tris-HCl buffer (pH 7.5) containing 1.5 mM $MgCl_2$ and 3

mM DTT, frozen and thawed three times, sonicated with three 15-sec bursts at maximum setting on a Bronwill sonicator, and centrifuged at 48,000 *g* for 20 min after adjusting the KCl concentration to 0.15 M. The supernatant, designated as the crude extract, was fractionated at 4° with 0–55% ammonium sulfate. The supernatant from this fraction was treated with 55–90% ammonium sulfate and the precipitate was redissolved in a minimal amount of 0.01 M Tris-HCl buffer (pH 7.5) containing 10% glycerol and 2 mM DTT and dialyzed overnight against 1.0 liter of the same buffer at 4°. The dialyzed solution was diluted with 3 volumes of the above buffer and applied to an MTX-Sepharose affinity column at room temperature. After washing with 0.5 M Tris-HCl buffer (pH 8.5) containing 10% glycerol and 2 mM DTT, the enzyme was eluted with a linear gradient of 0–100 μM H_2PteGlu in 0.5 M Tris-HCl buffer (pH 8.5). The DHFR enzyme preparation from KB parent cells was chromatographed twice on MTX-Sepharose since the specific activity of the first MTX-affinity preparation was only 1.0 unit/mg of protein. The contents of the peak tubes were pooled and stored frozen at −70°. This preparation was designated as the MTX affinity preparation and was stable for several months at −70°. Before use in assays, 0.2 ml of each enzyme preparation was chromatographed on small columns (0.9 × 2.6 cm, with a total volume of 1.7 ml) of Sephadex G-25 for desalting and for removal of H_2PteGlu. The elution buffer was 0.05 M Tris-HCl (pH 7.5) containing 10% glycerol and 2 mM DTT. This Sephadex G-25 preparation was used as the enzyme source for most studies.

Further Purification of H_2PteGlu$_5$

H_2PteGlu$_5$ was synthesized and purified as reported previously (4) and was found to contain an impurity which, while not influencing the pH 7 UV spectrum, was a potent inhibitor of DHFR. Further purification and desalting were accomplished by passage through a Sephadex G-25 column (1.5 × 30.6 cm) and eluting with deionized water containing 50 mM DTT. Lyophilized H_2PteGlu$_5$ from the DEAE-cellulose chromatography was dissolved in 8 ml of water containing 50 mM DTT and applied to the Sephadex G-25 column. The column was at room temperature, and fractions (1.86 ml) were put on ice and kept in the dark immediately after collection. H_2PteGlu$_5$ in the peak tubes was lyophilized and stored at −20° under vacuum in sealed ampoules.

Protein Determinations

Protein concentrations were determined by the methods of Lowry *et al.* (15) (crude preparations) and Bradford (16) (purified preparations).

Isoelectric Focusing

Isoelectric focusing of purified enzymes was performed at 800 V in a 110-ml LKB electrofocusing column with cathode on top. LKB ampholines of pH 5–7 and 7–9 were used in a 1:1 mix, 0.8 ml in the light solution and 5.2 ml in the heavy solution. Fractions were 2.0 ml.

Electrophoresis

SDS slab gel electrophoresis was performed according to the procedure of Laemmli and Favre (17). The 12.6% acrylamide-0.34% bisacrylamide gel (pH 8.8) was electrophoresed at 23° and a voltage of 65 V for 24 hr. The gel was stained for protein (17).

RESULTS

Purification. The purification tables for DHFR from KB parent, KB/MTX, and AT/3000 cells are shown in Table 1. Recoveries from the first MTX-Sepharose column were 100% for KB parent, KB/MTX, and AT/3000 enzymes with over-all yields of 28%, 21%, and 13%, respectively, from the crude extract. The second passage through MTX-Sepharose resulted in 78% recovery of the KB parent enzyme. The specific activities of the two human enzymes were the same, 60 units/mg, with a 6900-fold purification of the KB parent enzyme and a 100-fold purification of the KB/MTX enzyme. The specific activity of the AT/3000 enzyme was 30 units/mg with a 5-fold purification. The protein concentration, calculated from the molecular weight and turnover number for each enzyme, agreed with the experimentally determined value, and SDS-polyacrylamide gel electrophoresis of the KB/MTX enzyme (Fig. 4) revealed a single protein band with a molecular weight of 20,000. This evidence, coupled with the similar specific activities of the human enzymes, indicates that the purifications used resulted in nearly homogeneous enzyme preparations.

Isoelectric focusing. The isoelectric points of the purified MTX-affinity preparations from KB parent, KB/MTX, and AT/3000 cells were determined in identical experiments and the results are shown in Fig. 1. KB parent and KB/MTX enzymes had identical isoelectric points of 7.3. The murine enzyme from AT/3000 cells was markedly different with an isoelectric point of 8.6, over one pH unit higher. Preincubation with NADPH did not change the isoelectric profile of KB/MTX enzyme in Fig. 1.

Kinetic properties. Michaelis constants for NADPH and H_2PteGlu were obtained with purified DHFR from each cell line by varying each substrate in the presence of a fixed saturating concentration of the second substrate, 13 μM H_2PteGlu and 100 μM NADPH. The Michaelis constants for H_2PteGlu$_5$ were also determined for KB/MTX and AT/3000 enzyme at a concentration of 100 μM NADPH. These results are presented in Table 2. The KB parent and KB/MTX enzymes are identical in the K_m values for NADPH and H_2PteGlu. In contrast to

TABLE 1

Purification of DHFR from KB parent, KB/MTX and AT/3000 cells

Procedure	Specific activity	Yield	Purification
	units/mg	*%*	*fold*
KB parent			
Cell extract	0.009		
MTX-Sepharose 1	0.99	28	110
MTX-Sepharose 2	62.0	5	6,900
KB/MTX			
Cell extract	0.55		
MTX-Sepharose 1	59.5	21	100
AT/3000			
Cell extract	6.49		
MTX-Sepharose 1	30.1	13	5

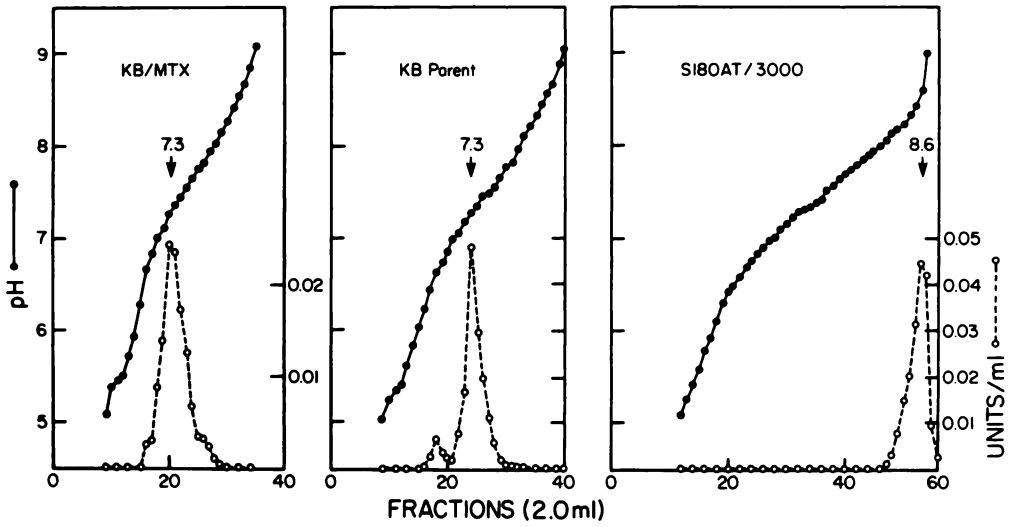


FIG. 1. *Isoelectric focusing of DHFR from KB parent, KB/MTX, and AT/3000 cells*

The MTX-affinity preparation of purified DHFR from each source was applied to the isoelectric focusing column in a pH gradient of pH 5–9 as described under Materials and Methods. ●, The measured pH of the column fractions; ○, the DHFR activity expressed as units per milliliter.

a previous report (4) demonstrating lower K_m values for dihydrofolate polyglutamates with human AML and ALL DHFR, we observed no difference in the K_m values for $H_2PteGlu$ and $H_2PteGlu_5$ with the human MTX-resistant DHFR. The V_{max} values obtained with $H_2PteGlu$ and $H_2PteGlu_5$ were similar (within 20%). The K_m values for $H_2PteGlu$ and $H_2PteGlu_5$ obtained with the murine AT/3000 enzyme in identical experiments were different from those with the human KB/MTX enzyme (1.7 and 1.9 μM, respectively). $H_2PteGlu_5$ had the same K_m value as $H_2PteGlu$ with the murine enzyme.

Interaction with MTX. The interaction at 37° with $H_2PteGlu$ as substrate, of KB/MTX enzyme with MTX was studied by means of the Ackermann and Potter (18) plot using the methods developed by Cha (19). A preincubation time of 15 min was found to be sufficient for equilibration between enzyme and MTX. A parallel experiment was performed with enzyme from AT/3000 cells as well, and both experiments are shown in Fig. 2. Figure 2A shows the plot for KB/MTX enzyme and 2B is that for AT/3000 enzyme. Both plots demonstrate the tight binding characteristics of MTX. Replots of these data are also shown in Fig. 2, where *A-1* is KB/MTX and *B-1* is AT/3000. The replot of the *v* intercept versus MTX concentration yields a slope or turnover number of 1360 min^{-1} per MTX binding site for KB/MTX enzyme (*A-1*) and 570 min^{-1} per MTX binding site for AT/3000 enzyme (*B-1*). These values were corrected for substrate concen-

TABLE 2

Kinetic properties of DHFR from KB parent, KB/MTX, and AT/3000 cells

Michaelis constants were obtained by varying the concentration of one substrate at a constant, saturating concentration of the second substrate. The K_m determinations for NADPH were performed at 13 μM $H_2PteGlu$ with NADPH concentrations varying from 5.3 μM to 53 μM. The K_m determinations for $H_2PteGlu$ and $H_2PteGlu_5$ were performed at 100 μM NADPH with $H_2PteGlu$ and $H_2PteGlu_5$ concentrations varying from 0.69 μM to 6.2 μM with KB parent and KB/MTX enzymes and from 1.4 μM to 12 μM for AT/3000 enzyme.

Enzyme source	K_m NADPH	K_m $H_2PteGlu$	K_m $H_2PteGlu_5$
	μM	μM	μM
AT/3000	5.9	1.7	1.9
KB parent	5.9	0.67	—
KB/MTX	5.9	0.67	0.58

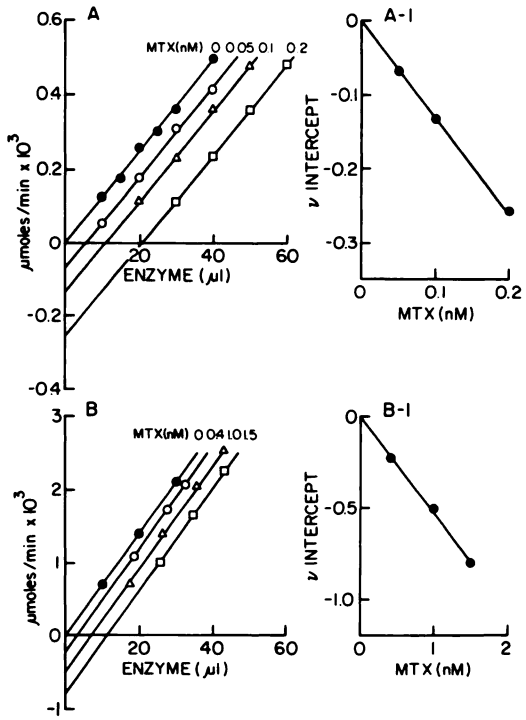


FIG. 2. *Interaction of KB/MTX and AT/3000 enzymes with MTX using Ackermann-Potter plots*

Varying amounts of enzymes were preincubated at 37°, pH 7.5, in 150 mM KCl for 15 min in the presence of several concentrations of MTX, and the initial velocity was measured after the addition of $H_2PteGlu$ at concentrations of 12μM for KB/MTX and 25 μM for AT/3000 enzymes. *A* shows the experiment for KB/MTX enzyme and *B* an identical experiment for AT/3000 enzyme; *A-1* and *B-1* show the replots of the *v* intercept versus MTX concentration for KB/MTX and AT/3000 enzyme, respectively.

tration by multiplying by the term $(K_m + S)/S$ as described (19).

When the data for these two enzymes were plotted as v_o/v_i versus MTX concentration at several enzyme concentrations, the I_{50} values could be estimated and replots of I_{50} versus enzyme concentration [according to the equation (19) $I_{50} = (\frac{1}{2} E_t + K_i)$] yielded straight lines with the K_i values determined from the I_{50} intercepts. These were 0.005 nM with KB/MTX and 0.11 nM with AT/3000 enzymes. These studies demonstrate that the human KB/MTX enzyme is very different from the mouse AT/3000 enzyme in both the turnover number and in the K_i for MTX. A similar experiment with KB parent DHFR (not shown) yielded a turnover number per MTX binding site of 1000 min^{-1} and a K_i for MTX of 0.005 nM.

The interaction of KB/MTX enzyme with MTX diglutamate was also investigated using these techniques and the K_i was found to be 0.01 nM, which is similar to that for MTX. Both MTX-γ-glutamate and MTX-γ-aspartate have been demonstrated to be equivalent to MTX in inhibition of L1210 DHFR (20).

Properties of DHFR from KB/MTX cells. Since the kinetic properties of DHFR from KB/MTX and KB parent cells were identical and differed significantly from those of DHFR from murine AT/3000 cells, we investigated other properties of the KB/MTX enzyme.

KCl Activation and pH profile. Purified KB/MTX enzyme was assayed at varying concentrations of KCl at 37°, pH 7.5, using H_2PteGlu at 3 μM as substrate. The results are shown in Fig. 3A. Enzyme activity steadily increased with increasing KCl, reaching a maximum of 1.6-fold at 0.15 M. The activity decreased sharply with higher concentrations of KCl until control values were reached. No inhibition below control activity was seen up to 0.4 M KCl. Subsequent experiments showed this activation to be due to an increased V_{max} with no change in the K_m for H_2PteGlu.

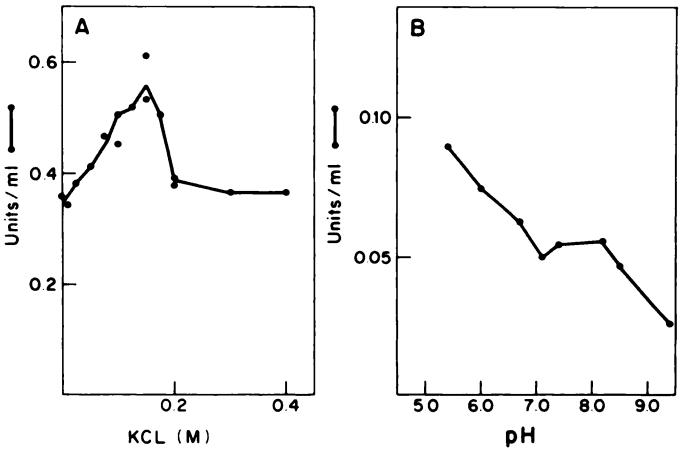


FIG. 3. *KCl activation and pH profile*

A. The assay was carried out at pH 7.5, 37°. Enzyme was preincubated with KCl and 100 μM NADPH for 3 min before the addition of H_2PteGlu as substrate at 3 μM. Control activity in the absence of KCl was 0.36 unit/ml.

B. Assays were carried out at 37° in the presence of 0.15 M KCl. H_2PteGlu at 50 μM was the substrate and the pH was the measured pH of the entire assay mixture.

Figure 3B shows the pH profile at 37° in the presence of 0.15 M KCl with H_2PteGlu as substrate at 50 μM. Potassium phosphate buffer was used and the pH was the measured pH of the assay mixture. This assay could not be used below pH 5 because of the rapid degradation of NADPH in acid. Enzyme activity was maximal at the lowest pH measured (5.4) and decreased up to pH 7, where a second broad optimum occurred between pH 7.2 and 8.2. Both the KCl activation and pH profile are consistent with data obtained for other mammalian DHFRs.

Molecular weight determination. Purified KB/MTX enzyme migrated as a single protein band on SDS-polyacrylamide gel electrophoresis (Fig. 4), with a molecular weight of 20,000 as compared with the marker proteins. Sephadex G-75 chromatography revealed a molecular weight of 18,000 which agreed within 10% with that obtained through SDS-polyacrylamide gel electrophoresis.

Inhibition by PteGlu and $PteGlu_5$. The reduction of H_2PteGlu by KB/MTX DHFR was competitively inhibited by PteGlu and $PteGlu_5$, and K_i determinations were performed by assaying the enzyme at varying levels of H_2PteGlu in the presence of several fixed concentrations of each inhibitor. The NADPH concentration was constant at 100 μM. A K_i determination of $PteGlu_5$ with $H_2PteGlu_5$ as substrate was also done, and the K_i values are shown in Table 3. The inhibition in all cases was competitive, and the K_i values obtained for both PteGlu and $PteGlu_5$ with H_2PteGlu as substrate and for $PteGlu_5$ with $H_2PteGlu_5$ as substrate were similar. These data, in conjunction with those presented in Table 2, indicate that DHFR does not distinguish between the mono- and polyglutamate folyl forms either as substrates or inhibitors.

Inhibition by folyl compounds. Several folate derivatives were tested for inhibition of KB/MTX DHFR using H_2PteGlu as substrate. The results are presented in Table 4. Three different methods for estimating the K_i values were used, depending on the type of inhibition found. PteGlu and $PteGlu_5$ were examined by Lineweaver-Burk analysis as described previously (Table 3). Other compounds which tested as competitive inhibitors (by determining the percentage inhibition at two or more substrate concentrations) were analyzed by measuring the I_{50} value and using the equation for competitive inhibition developed by Cheng and Prusoff (21): $K_i = I_{50}/(1 + S/K_m)$. Two compounds showed tight binding characteristics, and these were analyzed by comparison of the I_{50} value with the I_{50} value for MTX under identical experimental conditions, which included a 15-min preincubation time with enzyme and NADPH. The equation for tight binding inhibition (19), was then utilized in the following way: $I_{50} = E_t/2 + K_i$: since $E_t/2$ was the same for MTX and the compound being studied, the actual equation is $I_{50}^A - I_{50}^B = K_i^A - K_i^B$, where B is MTX and A is the second compound. The resultant K_i value was then corrected for substrate concentration by dividing by $1 + S/K_m$ as recommended for competitive, tightly binding inhibitors (22).

The substrate for thymidylate synthetase, 5,10-CH_2H_4PteGlu, and the products of DHFR, H_4PteGlu, and $H_4PteGlu_5$ were not inhibitory at 20 μM. Strong

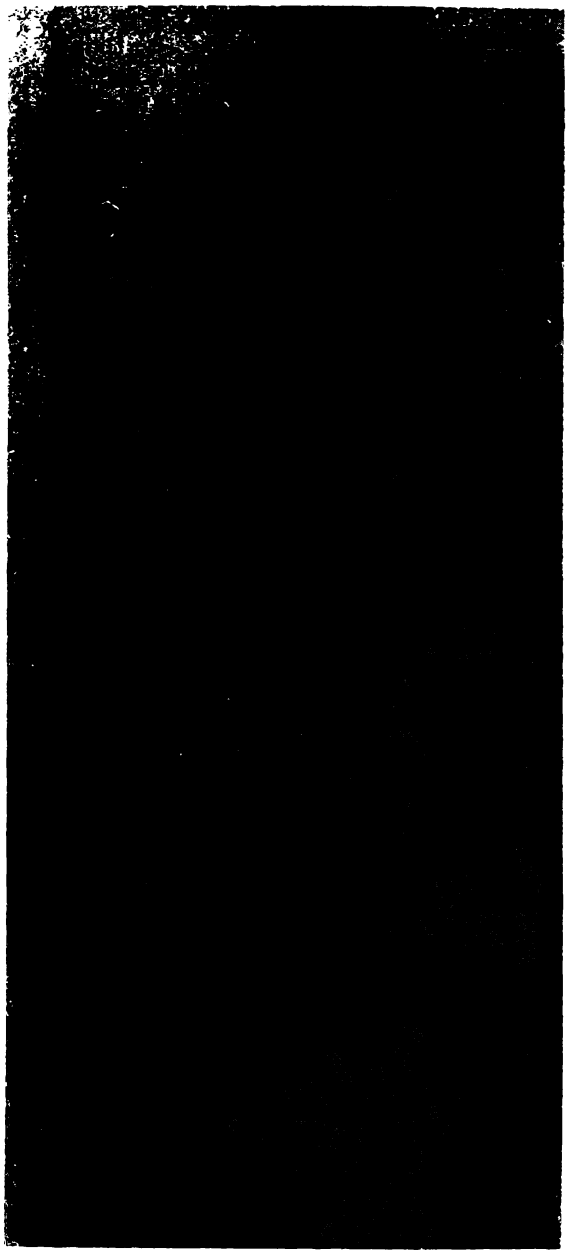


FIG. 4. *SDS-polyacrylamide slab gel electrophoresis of KB/MTX DHFR*

The acrylamide concentration was 12.6%; that of bisacrylamide was 0.34%. Electrophoresis was performed at 23° and 65 V for 24 hr. The purified enzyme (*extreme right*) is seen as a single protein band with an apparent molecular weight of 20,000 as determined by comparison with other markers (from *left*): bovine serum albumin, ovalbumin, lactic dehydrogenase, chymotrypsinogen, ribonuclease A, and lysozyme.

inhibition of DHFR activity was observed with all of the other folyl compounds tested, with K_i values ranging from 0.001 to 10 μM. 10-CHO-PteGlu was treated as a competitive, tightly binding inhibitor, and all other folates tested inhibited competitively with H_2PteGlu. Note that the predominating folate in serum, 5-CH_3-H_4PteGlu, also competitively inhibited the enzyme with a K_i = 10 μM.

Inhibition of KB/MTX DHFR by analogues of MTX. To help elucidate the structure-activity relationship of the tightly binding characteristics of MTX, several MTX analogues (23) with modifications at the α and γ carboxyl groups were examined for inhibition of the purified KB/MTX enzyme. MTX and all analogues were preincubated with enzyme and I_{50} values were obtained. K_i values were calculated according to the equation $I_{50} = ½ E_t + K_i$ as previously described. The results are shown in Table 5. Amidation (b), esterification (c), and the addition of a glutamate (f) or aspartate (g) moiety at the α carboxyl group significantly increased the calculated K_i. In contrast, those same modifications of the γ (h, i) carboxyl groups did not substantially change the estimated K_i. This points out the importance of the α carboxyl group in tightly binding characteristics of MTX. Note that the addition of bulky ethyl groups at the γ carboxyl group (j) also increased the K_i, indicating that this position plays a secondary role in the binding. The K_i for MTX-γ-aspartate (i) was not significantly increased, supporting the observations of Sirotnak *et al.* (20).

Properties of DHFR derived from AML cells. The kinetic parameters for H_2PteGlu, H_2PteGlu$_5$, and NADPH were determined with a preparation of DHFR (specific activity of 3.6 units/mg of protein) from AML. The K_m for NADPH was 6.3 μM, and the K_m values for H_2PteGlu and H_2PteGlu$_5$ were 0.66 μM and 0.68 μM, respectively. These are in close agreement with the values presented in Table 2 for the other human enzymes.

TABLE 3

Inhibition of KB/MTX DHFR by PteGlu and PteGlu$_5$

Initial velocity studies were carried out at 37°, pH 7.5, in the presence of 150 mM KCl. The NADPH concentration was constant at 100 μM. K_i values were determined by Lineweaver-Burk analysis.

Substrate	Inhibitor	K_i
		μM
H_2PteGlu	PteGlu	0.025[a]
	PteGlu$_5$	0.050[b]
H_2PteGlu$_5$	PteGlu$_5$	0.050[c]

[a] PteGlu at 0.20, 0.40, and 0.81 μM; H_2PteGlu from 0.67 to 2.69 μM.
[b] PteGlu$_5$ at 0.06, 0.12, and 0.23 μM; H_2PteGlu from 2.01 to 10.0 μM.
[c] PteGlu$_5$ at 0.09 and 0.18 μM; H_2PteGlu$_5$ from 1.17 to 17.5 μM.

TABLE 4

Inhibition of KB/MTX DHFR by folyl compounds

I_{50} values were determined at 37°, pH 7.5, in 150 mM KCl, 100 μM NADPH, and 15 μM H_2PteGlu. K_i values were calculated as described in the text.

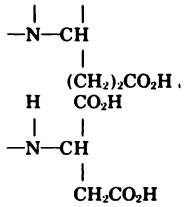
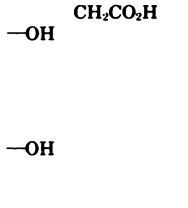
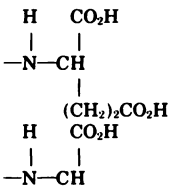
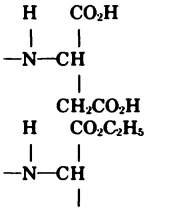
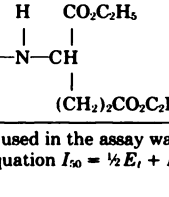
Folate	K_i	Type of inhibition
	μM	
PteGlu	0.025[a]	Competitive
PteGlu$_5$	0.050[a]	Competitive
10-CHO-PteGlu	0.001[c]	—
10-CH_3-PteGlu	0.130[b]	Competitive
5-CH_3-H_4-PteGlu	10.1[b]	Competitive
H_4PteGlu	No inhibition at 20 μM	
H_4PteGlu$_5$	No inhibition at 20 μM	
5,10-CH_2H_4PteGlu	No inhibition at 20 μM	

[a] Lineweaver-Burk analysis.
[b] $K_i = I_{50}/(1 + S/K_m)$
[c] $I_{50} = (E_t/2) + K_i$ in comparison with MTX as described in the text.

TABLE 5

Inhibition of KB/MTX DHFR by analogues of MTX having modifications at the α and γ carboxyl groups

Assays were performed at 37°, pH 7.5, in the presence of 150 mM KCl with $H_2PteGlu$ as substrate at a concentration of 6.0 μM. The NADPH concentration was 100 μM. K_i values were estimated as described in the text.

Compound	α Carboxyl group	γ Carboxyl group	I_{50}[a]	K_i[b]
			nM	nM
a	—OH	—OH	1.1	0.5×10^{-2}
b	$-NH_2$	—OH	21.4	2.0
c	$-OC_4H_9$	—OH	30	2.9
d	—OH	$-NH_2$	1.0	<0.1
e	—OH	OC_4H_9	1.5	<0.1
f		—OH	8.2	0.7
g		—OH	13.5	1.2
h	—OH		1.1	<0.1
i	—OH		1.0	<0.1
j	—OH		2.3	0.12

[a] The concentration of enzyme used in the assay was 2 nM.

[b] Estimated according to the equation $I_{50} = \frac{1}{2}E_t + K_i$ as described in the text.

DISCUSSION

The purified DHFR from KB/MTX cells appeared to be homogeneous as evidenced by a single protein band on SDS-polyacrylamide gel electrophoresis. Furthermore, the specific activities of the two purified human enzymes were identical, 60 units/mg of protein, thus suggesting near homogeneity of these enzyme preparations. The enzymes from the MTX-sensitive KB cells and the MTX-resistant KB/MTX cells were identical in the isoelectric point, K_m values for NADPH and $H_2PteGlu$, and in the K_i value for MTX. This observation in MTX-resistant human cells of an increased DHFR which is identical with that of the parent enzyme confirms the results with the murine S180AT/3000 cell line (7).

The human KB/MTX DHFR was markedly different in side-by-side comparisons from the murine AT/3000 enzyme in several properties, notably its isoelectric point, turnover number, K_m for $H_2PteGlu$ and $H_2PteGlu_5$, and K_i for MTX. These differences indicate a basic structural dissimilarity between mouse and human DHFR. Previous reports with cDNA sequences complementary to AT/3000 DHFR mRNA (24) as well as mouse L-1210/R antibody studies (25) have also indicated a considerable lack of homology between the mouse and human enzyme. A recent report on porcine liver DHFR (26) demonstrates a high degree of similarity between this and other vertebrate reductases and an over-all identity in amino acid sequences of 89%. Thus, the differences observed here between the human KB/MTX DHFR and the murine AT/3000 enzyme are particularly striking. Our unpublished results indicate that there are about 20 differences in the sequence of amino acids between mouse and human DHFR.[3] This is of importance in the extrapolation of enzyme data from the well-studied mammalian systems to the human situation. The results obtained with enzyme from AML cells were similar to those observed with KB and KB/MTX enzymes, suggesting an essential kinetic similarity of human enzymes independent of source.

There was no substrate preference (as indicated by similar K_m and V_{max} values) for $H_2PteGlu_5$ with either the human KB/MTX enzyme or the murine AT/3000 enzyme. In addition, the K_i values obtained for both PteGlu and $PteGlu_5$ with either $H_2PteGlu$ or $H_2PteGlu_5$ as substrate were similar, and the K_i values for MTX diglutamate and MTX were also similar. Thus we conclude that DHFR does not distinguish between the mono- and polyglutamate folyl forms either as substrates or inhibitors. These results are not in agreement with those of Coward *et al.* (4).

Inhibition of the reduction of $H_2PteGlu$ by folic acid and other naturally occurring folates has been observed with enzyme from other sources (13, 27, 28) and with the human placental enzyme (5). Our results show no inhibition by the end-products of $H_2PteGlu$ reduction, $H_4PteGlu$ or $H_4PteGlu_5$, or by the substrate for thymidylate synthetase, 5,10-CH_2-$H_4PteGlu$, at a concentration of 20 μM. All other compounds tested were inhibitory with estimated K_i values ranging from 0.001 to 10 μM. The significant inhibition of $H_2PteGlu$ reduction by substrates and products of other enzymes of folate metabolism suggests a possible regulation of DHFR activity by the over-all pattern of folyl forms in the cell. 10-CHO-PteGlu has been identified as a major folyl form accumulating in rat liver slices incubated in [^{3}H]PteGlu (29), but it is yet to be determined what physiological roles these oxidized formyl compounds play.

Folic acid itself is poorly reduced by DHFR with reported pH-dependent K_m values of 4–70 μM (4, 6, 13, 28). At pH 6.0, folic acid was a substrate for the KB/MTX enzyme, and the V_{max} was one-thirtieth of that obtained with $H_2PteGlu$ at the same pH. It is interesting to note that the K_i value for PteGlu with $H_2PteGlu$ as substrate is very low, 0.025 μM, several orders of magnitude lower than the reported K_m values. This tight binding in the absence of an equivalent catalytic activity leads to speculation of some cellular activation mechanism for folate reduction such as the presence of unknown activators or an enzyme complex which could facilitate the reduction of folic acid in vivo.

The use of 5-CHO-$H_4PteGlu$ as a rescue agent for high-dose MTX chemotherapy introduces high levels of this compound into the cell. It has been shown that it is rapidly converted to 5-CH_3-$H_4PteGlu$ (30), and the ability of this reduced folate to bind to DHFR may aid in the displacement of MTX from the enzyme.

[3] Y. Pan, B. A. Domin, S. Li, and Y.-C. Cheng, unpublished results.

A series of MTX analogues having modification at the α and γ carboxyl groups was examined, and amidation, esterification, or the addition of a glutamate or asparate moiety at the α carboxyl group significantly increased the estimated K_i values whereas these same modifications at the γ carboxyl group did not substantially change the K_i value. This points out the importance of the α carboxyl group for the tight binding of MTX.

In conclusion, the DHFR from human KB/MTX cells is identical with that from the parental KB cells and differs significantly from the enzyme from murine AT/3000 cells according to physical properties and kinetic behavior. Enzyme from AML cells seems similar to these human culture enzymes. However, it is still possible that treatment with MTX could lead to changes in the DHFR of the human tumor system. Caution is advised in extrapolating data obtained with other mammalian or vertebrate DHFRs to the human enzyme. Further work is in progress to ascertain structural differences between the murine and human enzymes to account for their striking dissimilarities in isoelectric point and kinetic properties.

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