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Purification and Characterization of Dihydrofolate Reductase from Methotrexate-Resistant Human Lymphoblastoid Cells†

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ABSTRACT: Dihydrofolate reductase has been isolated from methotrexate-resistant WIL2 human lymphoblastoid cells. This subline produces ca. 150 times more enzyme than the parental drug-sensitive line. The reductase has been purified to homogeneity by methotrexate affinity chromatography, gel filtration, and preparative isoelectric focusing in a yield of 65%. The enzyme has a pI = 7.7 and a molecular weight of ca. 22000. The amino-terminal 27 amino acid residues have been determined, revealing the location of the single cysteine residue at position 6. Reaction of this cysteine with *p*-(hydroxymercuri)benzoate in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) results in a 5-fold increase in enzyme activity. Other agents including KCl, urea, and thiourea also cause enzyme activation. The K_m values for NADPH and dihydrofolate are 0.25 and 0.036 μM, respectively. Mercurial activation of the enzyme results in a 27-fold increase in the K_m for NADPH and a 35-fold increase in the K_m for dihydrofolate.

Dihydrofolate reductase (EC 1.5.1.3) (DHFR)[1] catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. The latter serves as a coenzyme for a number of one-carbon transfer reactions in purine and pyrimidine biosynthesis, including that of thymidylate (Blakely, 1969; Huennekens et al., 1971). The reductase appears to be the major intracellular receptor for the action of 4-amino analogues of folic acid, such as methotrexate. Inhibition of the enzyme by methotrexate depletes the tetrahydrofolate pool, resulting in a decreased synthesis of thymidylate and, in turn, an inhibition of DNA synthesis. Thus, methotrexate has been employed extensively in the chemotherapeutic treatment of leukemias, lymphomas, psoriasis, and other clinical disorders (Bertino & Johns, 1972).

Dihydrofolate reductase has been purified and characterized from a number of bacterial and animal sources, including murine L1210 cells (Perkins et al., 1967; Reyes & Huennekens, 1967), porcine liver (Smith et al., 1979), bovine liver (Rowe & Russel, 1973; Kaufman & Kemerer, 1976), and chicken liver (Kaufman & Gardiner, 1966; Kaufman & Kemerer, 1977). Species differences are apparent in the properties of the various dihydrofolate reductases.

Despite the importance of dihydrofolate reductase as a target for chemotherapy of human neoplasms, there have been few studies of the human enzyme. Dihydrofolate reductase from human placenta (Jarabak & Bachur, 1971) has a molecular weight of 20000–22000, has two pH optima, and is activated by potassium chloride and by urea. The human enzyme has also been isolated from acute myelogenous and

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[1] Abbreviations: MTX, methotrexate (4-amino-10-methyl-4-deoxyfolic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TKEM, 50 mM Tris-HCl, 100 mM KCl, 1 mM EDTA, and 10 mM 2-mercaptoethanol, pH 7.0; NaDodSO$_4$, sodium dodecyl sulfate; MeHgOH, methylmercuric hydroxide; pHMB, *p*-(hydroxymercuri)benzoate; FAH$_2$, dihydrofolic acid; DHFR, dihydrofolate reductase; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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acute lymphocytic leukemia cells (Coward et al., 1974), erythrocytes (Coward et al., 1974), methotrexate-resistant HeLa cells (Morandi & Attardi, 1981), and methotrexate-resistant KB cells (Domin et al., 1982). Kinetic values in the latter studies are difficult to interpret since bound folates were not removed from the isolated enzymes and activating agents were present in the assay systems. The availability of highly purified human dihydrofolate reductase is important not only for the determination of the primary structure of the enzyme but also for X-ray crystallographic studies as well. Detailed information on the molecular interactions of inhibitors with the enzyme should aid in the design of new drugs having potentially greater efficacy in the treatment of human neoplasms. Accordingly, we report in this paper the detailed characterization of highly purified dihydrofolate reductase from MTX-resistant human lymphoblastoid WIL2 cells.

Experimental Procedures

Materials

Cell culture products were purchased from KC Biologicals. Methotrexate (MTX) was a gift from Lederle Laboratories. Dihydrofolate was prepared from commercial folic acid by dithionite reduction (Blakley, 1960) and stored at −20 °C in 10^{-3} N HCl. Sephadexes and AH-Sepharose 4B were obtained from Pharmacia. Iodo[^{14}C]acetic acid (13.6 mCi/mmol) was obtained from Amersham/Searle.

Methods

Enzyme Source. The WIL2 human lymphoblastoid cell line was originated from the spleen of a patient with spherocytic anemia (Levy et al., 1968). Cultures of this cell line (WIL2/S) were a generous gift from Dr. Ralph A. Reisfeld of Scripps Clinic and Research Foundation and are grown in suspension culture in RPMI 1640 containing 5% fetal calf serum and 1% penicillin–streptomycin solution. The WIL2/S parental line has 0.074 nmol of dihydrofolate reductase/10^9 cells.

The source of enzyme for this study was the MTX-resistant subline WIL2/M4 developed in this laboratory by growing WIL2/S cells in the continuous presence of MTX. Starting with an initial MTX concentration of 10^{-8} M, the concentration of inhibitor was repeatedly doubled when the cell generation time approached normal (18 h). WIL2/M4 cells are grown in the presence of 5 μM MTX with a generation time of 24 h to a density of 1.5×10^6 cells/mL and have 11.5 nmol of dihydrofolate reductase/10^9 cells.

Standard Enzyme Assay. Dihydrofolate reductase was assayed spectrophotometrically as previously described (Kempton et al., 1982). Initial rates were derived from the change in absorbance continuously recorded with a Cary Model 219 spectrophotometer.

Protein Determination. Protein concentration was determined by using a modified Lowry procedure (Schacterle & Pollack, 1973).

Gel Electrophoresis. Electrophoresis on 10% polyacrylamide gels with 0.1% $NaDodSO_4$ was performed according to the method of Laemmli (1970). The molecular weight of purified human dihydrofolate reductase was determined by using lysozyme, chymotrypsinogen A, DNase, ovalbumin, bovine serum albumin (BSA), *Lactobacillus casei* DHFR, and chicken liver DHFR as standards.

Purification of Dihydrofolate Reductase from Human WIL2/M4 Cells. Frozen cells (1.96×10^{11}) were thawed, suspended in 400 mL of 10 mM Tris-HCl, pH 7.2, subjected to another freeze–thaw cycle, and centrifuged at 18 000 rpm (40000*g*) for 30 min. The supernatant was dialyzed for 18 h against 6 L of TKEM buffer, pH 7.0, and then centrifuged

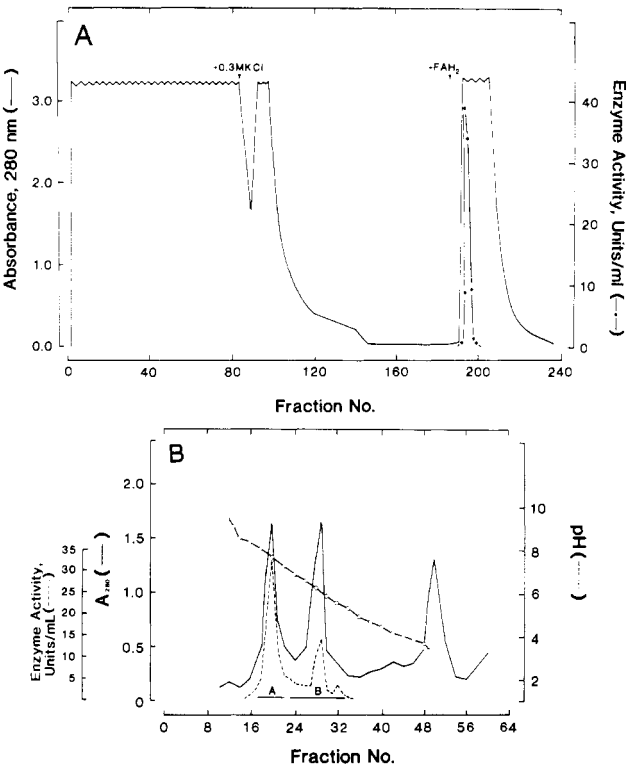

FIGURE 1: (A) Affinity chromatography of human dihydrofolate reductase on MTX–aminohexyl-Sepharose 4B. A freeze–thaw extract of 1.96×10^{11} WIL2 cells containing 655 units of DHFR was applied to 1.5 × 15 cm MTX–aminohexyl-Sepharose 4B column in TKEM buffer, pH 7.0. TKEM buffer containing a 0.3 M increase in KCl concentration was applied at fraction 85. DHFR activity was eluted with the addition of TKEM buffer containing 2 mM FAH_2 at fraction 188; fraction volume, 5.6 mL. (B) Preparative isoelectric focusing of human dihydrofolate reductase. DHFR eluted from the Sephadex G-75 column was focused for 48 h on a 110-mL LKB column in a medium containing sucrose and ampholines (LKB) in a pH 3.5–10 range. After being focused, peaks containing substrate-free DHFR (peak A) and substrate-bound DHFR (peak B) were passed down a 2 × 90 cm Sephadex G-50 column in 0.1 M potassium phosphate buffer, pH 7.5, in order to remove ampholines and sucrose.

at 18 000 rpm for 30 min. Loading of the material onto a 1.5 × 15 cm MTX–aminohexyl-Sepharose 4B column equilibrated with TKEM buffer, pH 7.0, was followed by extensive washing until the A_{280} of the eluant was negligible. Elution of DHFR activity was carried out by the addition of 20 mL of TKEM buffer, pH 7.0, containing 2 mM FAH_2 (Figure 1A). Fractions containing DHFR activity were pooled and concentrated to 5–10 mL in an Amicon ultrafiltration apparatus fitted with a PM-10 membrane. The concentrated sample was then loaded onto a 2 × 86 cm Sephadex G-75 (superfine) column equilibrated with TKEM buffer, pH 7.0. Pooled fractions containing dihydrofolate reductase activity were concentrated to 10–20 mL, dialyzed against 2 L of 10 mM potassium phosphate, pH 7.5, and then subjected to preparative isoelectric focusing (Vesterberg, 1971) on a 110-mL LKB column (Figure 1B). The focusing medium contained sucrose and ampholines in a pH 3.5–10 range. After focusing for 48 h, the eluted fractions containing DHFR activity were pooled and passed down a 2 × 90 cm Sephadex G-50 (superfine) column equilibrated against 0.1 M potassium phosphate, pH 7.5, to remove sucrose and ampholines (data not shown). Enzyme purity and removal of substrate were evaluated by $NaDodSO_4$–polyacrylamide gel electrophoresis and by the UV–vis absorption spectrum, respectively. Substrate-free DHFR (p*I* = 7.7) (Figure 1B, peak A) exhibited virtually no absorbance above 310 nm and an A_{280}/A_{260} ratio of 1.80–1.85.

Table I: Purification of Human Lymphoblastoid WIL2/M4 Dihydrofolate Reductase

	volume (mL)	protein (mg)	reductase activity (units)	sp act. (units/mg)	x-fold purification	yield (%)
crude extract (1.96×10^{11} cells)	384	9600	655	0.068		(100)
affinity chromatography	9	45	595	13.2	194	91
Sephadex G-75	40.5	30	502	16.7	245	77
isoelectric focusing followed by gel filtration (Sephadex G-50)	34	26	424	16.1	236	65

DHFR activity having a pI of 6.2 (Figure 1B, peak B) was substrate-bound enzyme since its UV–vis absorption spectrum produced substantial absorbance above 310 nm. Separation of substrate-free DHFR from substrate-bound DHFR during isoelectric focusing has also been reported for chicken liver DHFR (Kaufman & Kemerer, 1977). Purified enzyme was stored at −20 °C and found to be stable for several months.

Determination of K_m Values for NADPH and Dihydrofolate. The K_m values of NADPH and dihydrofolate for both native and mercurial-activated enzyme were determined in 50 mM Tris-HCl buffer, pH 7.5, 22 °C, by employing a 10-cm path-length cell and an assay volume of 29 mL. Full-scale absorbance was set at 0.10. In the determination of the K_m of NADPH, the initial concentration of cofactor was varied between 0.05 and 4 μM while the initial concentration of dihydrofolate was 0.50 μM for each assay. The reaction was initiated by the addition of 118 ng of enzyme. The average of two determinations of the initial velocity for each NADPH concentration was plotted as the change in substrate concentration per second vs. NADPH concentration in double-reciprocal fashion. Since an initial attempt to determine the K_m of dihydrofolate by the same method was unsuccessful, a single assay was recorded under conditions such that the K_m could be derived from the integrated Michaelis–Menten equation. The concentrations of NADPH and dihydrofolate were initially 15 and 0.15 μM, respectively, in order to minimize product inhibition. After the addition of 89 ng of enzyme, the reaction was monitored for 10 min until exhaustion of substrate occurred. From the resulting progress curve, a plot of $-\Delta t/(\Delta[S])$ vs. $\Delta \ln [S]/(\Delta[S])$ was made (Citri et al., 1976). A value of 100 s was chosen for Δt with $\Delta[S]$ representing the change in dihydrofolate concentration over successive 100-s time intervals. Values of $\Delta \ln [S]$ represent the change in the concentrations between successive time intervals. The K_m was calculated from the product of the slope and the reciprocal of the y intercept while the V_{max} was calculated from the reciprocal of the y intercept.

Amino Acid Analysis. Samples were hydrolyzed in constant-boiling HCl in evacuated, sealed tubes flushed with nitrogen at 110 °C for periods of 24, 48, 72, 96, and 120 h. Analyses were performed on a Durrum Model D-500 amino acid analyzer according to instructions supplied with the instrument, and all results are expressed as molar ratios. Values given under Results are corrected either for the partial destruction of serine and threonine or for incomplete liberation of valine and isoleucine. Values for cysteine and methionine were determined as cysteic acid and methionine sulfone after performic acid oxidation according to Moore (1963). Tryptophan determinations were done according to Edelhoch (1967).

Edman Degradation and Quantitation of [^{14}C](Carboxymethyl)cysteine Groups Present in Dihydrofolate Reductase. Automated Edman degradations were performed as previously described (Kumar et al., 1980). The N-terminal sequence of the intact ^{14}C-carboxymethylated protein was determined by using a single cleavage program designated DMAA I (Beckman No. 102974).

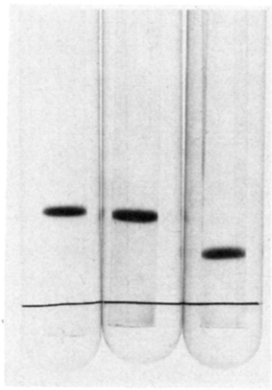

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of purified human, chicken liver, and *L. casei* dihydrofolate reductases. Twenty micrograms each of human (left), chicken liver (middle), and *L. casei* (right) dihydrofolate reductases was subjected to $NaDodSO_4$ electrophoresis on 10% polyacrylamide gels according to the procedure of Laemmli (1970) and stained with Coomassie Brilliant Blue.

Carboxymethylation of the cysteine residue(s) with iodo[^{14}C]acetate was done as previously described (Kaufman et al., 1980). The protein (360 nmol) was treated with a mixture of 3.0 mg of iodoacetic acid and 0.7 mg of iodo [^{14}C]acetic acid (sp act. 13.6 mCi/mmol). A portion of the protein (147 nmol) was dissolved in 600 μL of dimethylallylamine buffer. One 25-μL aliquot was used for amino acid analysis, and two 10-μL aliquots were used to determine radioactivity. With the assumption of a 90% counting efficiency, 256 400 dpm and 136 nmol of protein were applied to the spinning cup for sequence analysis. The radiospecific activity of iodo[^{14}C]acetic acid was 1.02 μCi/μmol.

Results and Discussion

Human MTX-resistant WIL2/M4 cells elaborate ca. 150-fold more dihydrofolate reductase relative to the drug-sensitive parental line (WIL2/S). The overproduction of the reductase in this cell line most probably results from a selection of cells containing amplified DHFR structural genes (Alt et al., 1978). The enzyme from these overproducer cells has been purified to homogeneity by using a four-step procedure involving freeze–thaw extraction, MTX affinity chromatography, Sephadex G-75 gel filtration, and preparative isoelectric focusing in a yield of 65% (Table I). The final step of preparative isoelectric focusing was found to be the only effective method for removing substrate which remained tightly bound to the enzyme after elution from MTX–Sepharose. The substrate-free enzyme has a pI of 7.7 (Figure 1B, peak A) and a molecular weight of ca. 22 000 as determined by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ (data not shown). Figure 2 illustrates the purity of the enzyme as well as comparing its mobility with those of purified chicken liver (M_r 21 650) and *L. casei* reductases (M_r 18 300). Domin et al. (1982) have reported a pI of 7.3 and a molecular weight of 20 000 for a DHFR isolated from a MTX-resistant human KB cell line.

Human dihydrofolate reductase is stoichiometrically inhibited by MTX. A linear decrease in activity vs. drug con-

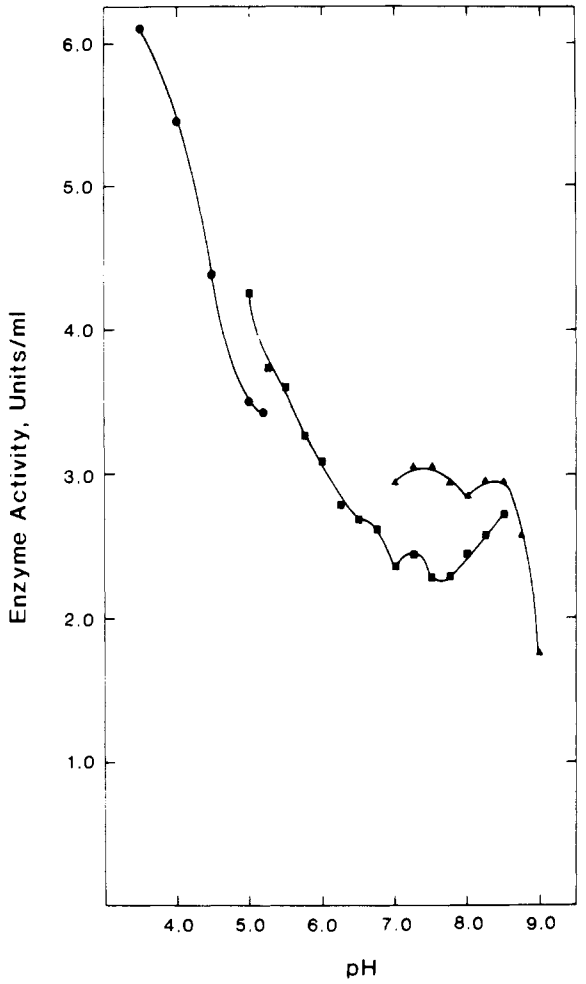

FIGURE 3: Effect of pH on human dihydrofolate reductase activity. Purified enzyme (30 pmol) was assayed in 0.1 M sodium acetate (pH 3.5–5.25), 0.1 M potassium phosphate (pH 5.0–8.5), and 0.1 M Tris-HCl (pH 7.0–9.0) buffers.

centration occurs up to nearly 98% of the total enzyme activity. There is no indication of the synthesis of a reductase with a low affinity for MTX as has been observed in MTX-resistant murine cells (Goldie et al., 1980; Haber et al., 1981) or MTX-resistant Chinese hamster ovary cells (Flintoff & Essani, 1980).

Effect of pH on Enzyme Activity. The effect of pH on enzyme activity with dihydrofolate as a substrate is shown in Figure 3. Human dihydrofolate reductase exhibits three optima, a major one less than pH 4.0 and minor optima at pH 7.25–7.5 and pH 8.25–8.5. Animal dihydrofolate reductases have been reported to possess two pH optima (Reyes & Huennekens, 1967; Smith et al., 1979; Rowe & Russel, 1973; Kaufman & Gardiner, 1966). Human placental (Jarabak & Bachur, 1971) and human KB dihydrofolate reductases have also been reported to have two pH optima. The difference between the pH–activity profiles for the human enzymes may be due to differences in the ionic strengths of the buffers used in the studies (Kaufman & Gardiner, 1966).

K_m Determinations. The detailed experimental procedures for these determinations are given under Methods. From the double-reciprocal plot, the K_m value for NADPH was determined to be 0.25 μM. The K_m value for dihydrofolate was found to be 0.036 μM (Figure 4), which is the lowest value reported for a homogeneous, substrate-free dihydrofolate reductase. If a molecular weight of 22 000 for the human WIL2/M4 dihydrofolate reductase is assumed, a catalytic

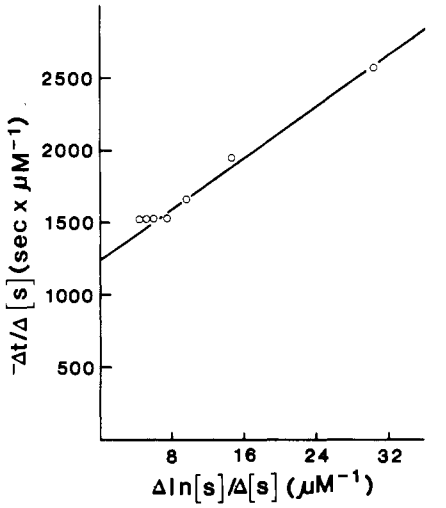

FIGURE 4: Reaction progress curve plotted according to Citri et al. (1976) with FAH_2 undergoing depletion. NADPH concentration (initial), 15×10^{-6} M; FAH_2 concentration (initial), 2.5×10^{-7} M; enzyme concentration, 1.4×10^{-10} M.

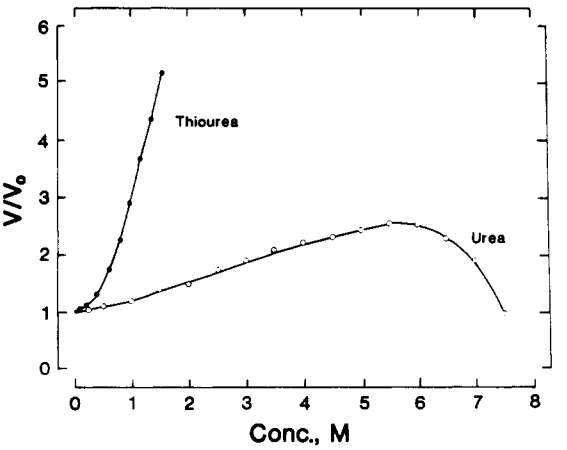

FIGURE 5: Activation of human dihydrofolate reductase in the presence of urea and thiourea. Purified enzyme was assayed in 0.1 M Tris-HCl buffer, pH 7.5, along with the indicated amounts of urea or thiourea. Enzyme (55 pmol) was added last to the complete reaction mixture; V_0, activity of enzyme alone; V, enzyme activity in the presence of activating agent.

coefficient of 358 mol of FAH_2 reduced min^{-1} (mol of enzyme)$^{-1}$ (22 °C) can be calculated.

The K_m values reported for human DHFR range from 2.5 to 5.9 μM for dihydrofolate (Jarabak & Bachur, 1971; Coward et al., 1974; Domin et al., 1982) and from 0.11 to 170 μM for NADPH (Jarabak & Bachur, 1971; Morandi & Attardi, 1981; Domin et al., 1982). Reyes & Huennekens (1967) reported a 4.4-fold increase in the K_m for NADPH from 4.8 to 21 μM for L1210 dihydrofolate reductase in the presence of 0.8 M KCl. Since kinetic determinations for the reductases from HeLa (Morandi & Attardi, 1981), KB (Domin et al., 1982), leukemic, and erythrocytic (Coward et al., 1974) cells were performed in the presence of sufficient KCl to cause enzyme activation, the K_m values in the absence of KCl may actually be lower and thus in closer agreement with those of the placental and WIL2 enzymes.

Enzyme Activating Agents. Human WIL2/M4 dihydrofolate reductase is activated ca. 1.5-fold in the presence of 0.75 M KCl. The human KB enzyme exhibits a maximal 1.6-fold activation in the presence of 0.15 M KCl (Domin et al., 1982) while the human placental enzyme exhibits a maximal 3-fold activation in the presence of 0.6 M KCl (Jarabak & Bachur,

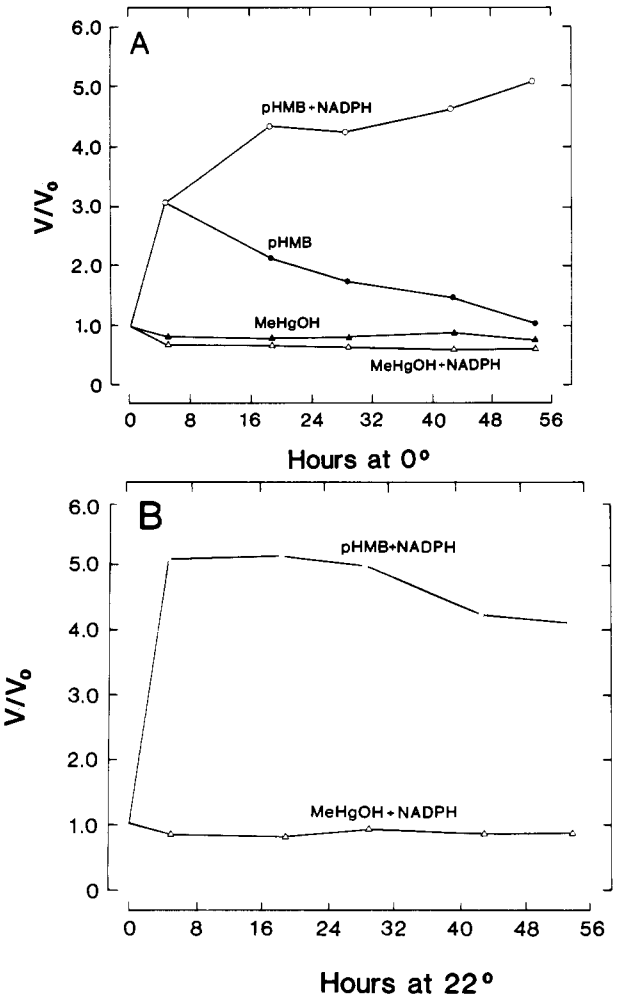

FIGURE 6: Activation of human dihydrofolate reductase in the presence of organomercurials. Purified enzyme (4.4×10^{-6} M) was incubated in 0.1 M potassium phosphate buffer, pH 7.5, at either 0 °C (A) or 22 °C (B), with a 10-fold molar excess of either methylmercuric hydroxide (MeHgOH) or *p*-(hydroxymercuri)benzoate (pHMB) and with or without a 50-fold molar excess of NADPH. Aliquots of enzyme (22 pmol) were removed at the indicated times and assayed in 0.05 M potassium phosphate buffer, pH 7.5.

1971). At urea concentrations between 5.5 and 6.0 M, a 2.5-fold increase in enzyme activity occurs, whereas in 1.6 M thiourea, a 5-fold enzyme activation is observed (Figure 5). The human placental enzyme is maximally activated 1.4-fold in the presence of 2 M urea with inhibition occurring at concentrations of 3 M or higher (Jarabak & Bachur, 1971).

As indicated in Figure 6A, methylmercuric hydroxide (MeHgOH) in the presence or absence of NADPH has no effect on enzyme activity at 0 °C. Incubation of the enzyme at 0 °C with the organic mercurial *p*-(hydroxymercuri)-benzoate (pHMB) results in a 3-fold activation after 4 h followed by slow inhibition thereafter. Prior incubation with NADPH followed by pHMB gives a 5-fold increase in enzyme activity after 54 h at 0 °C (Figure 6A). Incubation of the enzyme at 22 °C with either MeHgOH or pHMB results in 50% or 90% inhibition, respectively, after 8 h (data not shown). In contrast, prior incubation of the enzyme with NADPH at 22 °C followed by either MeHgOH or pHMB produces no change or a 5-fold stimulation in enzyme activity, respectively (Figure 6B). Chicken liver dihydrofolate reductase is activated ca. 12- and 8-fold, respectively, by MeHgOH and pHMB (Kaufman et al., 1980), while bovine (Kaufman et al., 1980) and L1210 (Perkins et al., 1967) reductases exhibit a lesser 1.5–3.0-fold increase in enzyme activity in the presence of

Table II: Amino Acid Composition of Human WIL2/M4 Dihydrofolate Reductase

amino acid	residues[a]
Asx	19.7 (20)
Thr[b]	6.6 (7)
Ser[b]	11.4 (11)
Glx	22.1 (22)
Pro	10.7 (11)
Gly	13.2 (13)
Ala	5.2 (5)
Cys[c]	0.9 (1)
Val	13.1 (13)
Met	5.8 (6)
Ile	8.5 (9)
Leu	19.0 (19)
Tyr	6.6 (7)
Phe	9.8 (10)
His	3.7 (4)
Lys	17.7 (18)
Arg	7.2 (7)
Trp[d]	3.0 (3)
	(186)[e]

[a] Based on hydrolysis times of 24, 48, 72, 96, and 120 h in constant-boiling HCl and assuming 19 leucines per mol of protein. The numbers in parentheses represent the nearest integer. [b] Extrapolated to zero time of hydrolysis. [c] Determined as cysteic acid and methionine sulfone, respectively, according to Moore (1963). [d] Determined according to Edelhoch (1967). [e] Total.

organic mercurials. Incubation of the human placental enzyme in the presence of 8×10^{-7} or 3.3×10^{-5} M pHMB plus NADPH at 25 °C for up to 60 min results in no stimulation of enzyme activity (Jarabak & Bachur, 1971). The reason for the lack of activation of the placental enzyme by pHMB is unclear although an incubation period of greater than 60 min may be required as is the case for the bovine liver enzyme (Kaufman et al., 1980).

Activation of the human WIL2 reductase by pHMB results in a 27-fold increase in the K_m for NADPH and a 35-fold increase in the K_m for dihydrofolate (data not shown). A similar effect is observed when chicken liver reductase undergoes activation by MeHgOH. The K_m for NADPH increases 15-fold from 0.6 to 9.1 μM while the K_m for dihydrofolate increases 10-fold from 0.18 to 1.8 μM (B. T. Kaufman, unpublished results).

Amino Acid Composition. The amino acid composition reveals a preponderance of acidic over basic amino acid residues as indicated in Table II, although some are undoubtedly present as amides. The enzyme contains six methionines, three tryptophans, and a single cysteine residue among other residues.

Since enzyme activation by pHMB presumably involves the mercurial liganded to the sulfur of the single cysteine, it was of interest to determine the location of this residue in the protein sequence.

Amino-Terminal Sequence of Human Dihydrofolate Reductase. For determination of the total number and positions of cysteine residues in human dihydrofolate reductase, iodo-[^{14}C]acetate was used as a radiolabeling agent. The sequence analysis of the N-terminal 27 amino acid residues is indicated in Table III. The results of the radiolabeling indicate that a total of 0.84 equiv of ^{14}C-labeled carboxymethyl groups is incorporated per mol of protein (Table IV). Of the total dpm incorporated, 91% of the label is released at step 6 [*S*-[^{14}C](carboxymethyl)cysteine ([^{14}C]SCM-Cys)] and step 7 (Ile + SCM-Cys overlap). The radiolabel released in the prelap at step 5 and additional overlap at step 8 together with

Table III: Automated Sequence Analysis of ^{14}C-Carboxymethylated Human Dihydrofolate Reductase[a]

step	amino acid identified	yield nmol	yield cpm[b]	step	amino acid identified	yield nmol	yield cpm
1	Val	57	214	15	Gly	4	56
2	Gly	85	62	16	Ile	9	41
3	Ser	26	40	17	Gly	9	47
4	Leu	57	64	18	c		50
5	Asn	32	474	19	Asp	3	49
6	SCM-Cys	24	5820	20	c		41
7	Ile	37	1810	21	Asp	7	40
8	Val	17	479	22	Leu	8	40
9	Ala	23	147	23	Pro	10	36
10	Val	22	77	24	Trp	3	39
11	Ser	10	60	25	Pro	13	41
12	Gln	5	60	26	Pro	5	26
13	Asn	10	47	27	Leu	4	28
14	Met	15	55				

[a] Sequence analysis of 136 nmol of ^{14}C-carboxymethylated protein. The repetitive yield was 90%. [b] Sample aliquots of 10% were taken for the determination of ^{14}C-labeled carboxymethyl groups. The numbers shown are uncorrected for background cpm. [c] From the subsequent sequence analysis of a cyanogen bromide fragment beginning with Gly-15; lysine was determined at position 18 and glycine at position 20.

Table IV: Quantitation of [^{14}C](Carboxymethyl)cysteine Groups Present in Dihydrofolate Reductase

step[a]	corrected dpm released[b]
5	12 650
6	172 530
7	59 710
8	17 680
	262 570[c]

[a] Aliquots of 10 μL (of 100 μL total) were removed from each sequence cycle for counting. [b] The nanomoles of PTH-amino acid released at each cycle was corrected for a 90% repetitive yield. [c] Total.

	5			10			15			20		25
C	V	R	S	L	N	S	I	V	A	V	C	Q	N	M	G	I	G	K	D	G	N	L	P	W	P
B			P		C			S					N		D										
P			P		C			S					N		D										
L			P		C			S					N		D										
H		G			C			S					N		D										

FIGURE 7: Comparison of amino-terminal sequences of animal dihydrofolate reductases. Sequences of chicken liver (C) (Kumar et al., 1980), bovine liver (B) (Lai et al., 1982), pig liver (P) (Smith et al., 1979), mouse L1210 lymphoma (L) (Stone et al., 1979), and human (H) enzymes are aligned at the amino-terminal end and given in single-letter amino acid code with residues identical with the chicken liver enzyme omitted.

that found in steps 6 and 7 accounts for 102% of the radiolabel in the protein. These results clearly establish that human dihydrofolate reductase contains a single cysteine located at position 6 in its amino acid sequence.

As indicated in Figure 7, the amino-terminal sequence of human WIL2/M4 dihydrofolate reductase more closely resembles those from bovine liver, porcine liver, and L1210 cells. The chicken liver reductase has a serine at position 6 and a cysteine at position 11, among other differences (Figure 7). The human enzyme appears to be unique with a glycine at position 2, whereas the other reductases have an arginine at that location. The differences in reactivities toward organic mercurials between the chicken liver reductase and the human, bovine, and L1210 reductases appear to reside in the location and microenvironment surrounding their respective cysteine residues. From a consideration of the known amino-terminal sequences (Figure 7), it is evident that the folding of the polypeptide chain in this region should be quite different between the avian and the other reductases. The bovine, porcine, L1210, and human enzymes all have a proline at position 3, three residues from Cys-6. The avian reductase lacks the helix-breaking residue at position 3, and its cysteine is at position 11 rather than position 6. The relationship between sulfhydryl occupancy by a mercurial and its effects on catalytic activity and active-site conformation, while clearly unique, remains obscure. By contrast, bacterial dihydrofolate reductases do not contain cysteine within the first 50 amino-terminal residues and are not activated by mercurials (Freisheim et al., 1979). Further studies on the interaction of substrates and various inhibitors with WIL2/M4 dihydrofolate reductase are in progress in order to increase knowledge of structure–function relationships between bacterial and animal reductases.

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Reincorporation of Adenosine 5′-Diphosphate/Adenosine 5′-Triphosphate Carrier into Phospholipid Membranes. Phospholipid–Protein Interaction As Studied by Phosphorus-31 Nuclear Magnetic Resonance and Electron Microscopy†

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ABSTRACT: Combined phosphorus-31 nuclear magnetic resonance (^{31}P NMR) and electron microscopic studies were performed on the ADP/ATP carrier protein from beef heart mitochondria. The protein was incorporated into phospholipids by addition of Triton–protein micelles to a lipid suspension or to the dry lipid. All of the phospholipid (egg phosphatidylcholine or mixtures of egg phosphatidylcholine and egg phosphatidylethanolamine) that contributed to the observed ^{31}P NMR signal under these conditions appeared to be in a bilayer configuration. Freeze–fracturing and negative-staining electron microscopy showed unilamellar vesicles and multilayers. An isotropic signal could be attributed to vesicle rotation, judging from its sensitivity to increasing viscosity. The presence of small vesicles was also noticeable in the ^{31}P NMR spectra of planar oriented membranes. In the presence of phosphatidylethanolamine, aggregation of protein particles was observed. Gel chromatography of the protein–Triton–phospholipid mixture revealed that, before Triton removal, large amounts of protein are associated with multibilayers. Separation of loaded and unloaded membranes by centrifugation in D_2O showed that, upon stepwise addition, protein incorporates preferentially into unloaded liposomes. From these findings a mechanism of protein reincorporation was deduced.

The ADP/ATP carrier protein from the inner mitochondrial membrane can be isolated in highly purified form (Riccio et al., 1975a,b). Incorporation of this protein into vesicular phospholipid membranes enhances the affinity for the inhibitor ligands (Krämer & Klingenberg, 1977) and reconstitutes the specific exchange capability for adenine nucleotides to a portion of the incorporated protein. It has been shown recently (Krämer & Klingenberg, 1980) that the transport activity of the ADP/ATP carrier, when incorporated into phospholipid vesicles, is considerably enhanced in the presence of phosphatidylethanolamine (PE).[1] It may be speculated that this effect is due to a specific interaction of PE with the protein molecule or to a structural rearrangement of the membrane that facilitates the function of the ADP/ATP carrier.

Nuclear magnetic resonance proved to be a valuable tool for the study of lipid–protein interactions (Seelig & Seelig, 1980). In particular ^{31}P NMR affords an insight into the order and mobility of the phospholipid head groups in artificial and

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[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; CAT, carboxyatractylate; Mops, 4-morpholinepropanesulfonic acid; NMR, nuclear magnetic resonance.

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