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Methotrexate-Resistant Chinese Hamster Ovary Cells Contain a Dihydrofolate Reductase with an Altered Affinity for Methotrexate†

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ABSTRACT: Previous reports [Flintoff, W. F., Davidson, S. V., & Siminovitch, L. (1976) *Somatic Cell Genet. 2*, 245–261; Gupta, R. S., Flintoff, W. F., & Siminovitch, L. (1977) *Can. J. Biochem. 55*, 445–452] described a series of Chinese hamster ovary cells that were resistant to the cytotoxic action of methotrexate and contained a dihydrofolate reductase that was less sensitive to inhibition by the drug than wild-type enzyme. In this study, binding of labeled methotrexate to the reductase–NADPH complex and separation of free and bound drug by filtration through Sephadex G-25 have been used to demonstrate that clonal isolates of these resistant cells contain a dihydrofolate reductase varying between 2.5- and 6-fold lower in affinity for the drug than the wild-type enzyme. The apparent dissociation constant for the wild-type enzyme is 0.5×10^{-9} M. Using two-dimensional polyacrylamide gel electrophoresis, 11 independently selected resistant isolates have been shown to contain a reductase with a similar overall net charge as the wild-type enzyme. Reductase purified from either wild-type or resistant cells contains two components after isoelectric focusing in polyacrylamide gels. The major component represents about 90% of the total protein and has a p*I* of about 8.0. The minor component representing about 10% of the reductase protein has a p*I* between 7.2 and 7.6.

Previous reports from this laboratory have described a series of Chinese hamster ovary cells (CHO)[1] that have been selected for resistance to the folic acid analogue methotrexate (Mtx) (Flintoff et al., 1976a,b; Gupta et al., 1977). Resistance in class I cells is apparently due to a structural alteration in dihydrofolate reductase, whereas the resistance in class II cells involves a defect in the permeability to Mtx. Class III cells, which were derived from class I cells by a second-step selection in an increased concentration of drug, showed increased levels of the enzyme found in class I cells.

The conclusion that class I cells are resistant because of a structural alteration in the reductase was based on the increased resistance to Mtx inhibition shown by the reductase

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[1] Abbreviations used: CHO, Chinese hamster ovary; DFBS, dialyzed fetal bovine serum; Me_2SO, dimethyl sulfoxide; EMS, ethyl methanesulfonate; HPRT, hypoxanthine phosphoribosyltransferase; IF, isoelectric focusing; K_d, apparent dissociation constant; Mtx, methotrexate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PPO, 2,5-diphenyloxazole.

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activities of mutant cells (Flintoff et al., 1976a) and of the purified enzyme (Gupta et al., 1977). Since Mtx is a stoichiometric inhibitor of reductase (Werkheiser, 1961; Bertino et al., 1964; Perkins & Bertino, 1966; Blakley; 1969; Williams et al., 1973), it might be expected that the enzyme from resistant cells would have an altered affinity for the drug. In this paper, we show that the reductase from class I mutant cells has a decreased affinity for binding Mtx as determined by a binding assay using gel filtration to separate bound and free drug. In addition, we have examined by two-dimensional gel electrophoresis whether the structural change results in a reductase with an altered charge.

Experimental Procedures

Materials. Mtx was purchased from Nutritional Biochemicals and NADPH from Sigma Chemical Co. [3′,5′,9(*n*)-^{3}H]Mtx (specific activity 13.4 Ci/mmol) was obtained from Amersham/Searle Corporation. This compound was periodically checked for purity by paper chromatography using the system described by the supplier. If the purity was less than 90%, the compound was purified by column chromatography using Sephadex G-15 (Whitehead et al., 1975). L-[^{35}S]-Methionine (specific activity 839 Ci/mmol) was purchased from New England Nuclear.

Cell Lines and Cell Culture. Several of the CHO lines used in this study and their nomenclature have been previously described (Flintoff et al., 1976a). Additional isolates displaying the class I Mtx-resistant phenotype were selected spontaneously or from mutagen-treated (EMS or NG) wild-type cells using the procedure previously described (Flintoff et al., 1976a).

The cell line Pro$^-$3 Mtx^{RI} 16-8 is also resistant to α-amanitin, ouabain, and thioguanine and contains a temperature-sensitive leucyl tRNA-synthetase. These markers, however, are not of importance in this particular study and have been omitted from the designation of the line.

The Pro$^-$3 Mtx^{RIII} 1-2 cell line refers to a class III mutant obtained in a second-step selection from the clone Pro$^-$3 Mtx^{RI} 3-3 and contains increased amounts of an altered reductase (Flintoff et al., 1976a; Gupta et al., 1977). The Pro$^-$4 Mtx^{RV} 9-5 line is a mutant obtained in a two-step selection from the wild-type clone Pro$^-$4 and contains about 30 times the level of wild-type reductase.

Cells were maintained in suspension culture at 34 or 37 °C in complete α medium (Stanners et al., 1971) supplemented with 10% fetal bovine serum (Microbiological Associates) according to the methods previously described (Flintoff et al., 1976a).

Preparation of Cell Extracts and Purification of Dihydrofolate Reductase. Cell extracts were prepared as previously described (Flintoff et al., 1976a). Dihydrofolate reductase was purified from wild-type and several resistant cells by chromatography on Sepharose conjugated with Mtx, as previously described (Gupta et al., 1977). All purified enzymes gave a single band of molecular weight 24 000 in sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Determination of [^{3}H]Mtx Binding to Dihydrofolate Reductase. The procedure devised to measure [^{3}H]Mtx binding is based upon the observation that dihydrofolate reductase in the presence of NADPH forms a stable complex with Mtx (Perkins & Bertino, 1966; Freudenthal et al., 1970; Blakley et al., 1971; Otting & Huennekens, 1972). The assay was performed at room temperature (22 °C), and the reaction mixture contained, in a final volume of 0.2 mL, 5 mmol of potassium acetate, pH 5.5; 0.1 μmol of NADPH; 0.1 mg of bovine serum albumin; and either purified reductase or crude cellular extracts as indicated in Table I and the figures. For assays with purified reductase, between 0.5 and 1 μg of protein was used per reaction. With crude cellular extracts ~0.2 to 0.3 mg of protein was used per reaction. Binding was initiated by the addition of labeled drug. Under these conditions, binding was rapid, reaching completion in 3 to 5 min. Therefore, to ensure that binding was at equilibrium, the reaction mixture was allowed to incubate for 10 min prior to the separation of bound and unbound drug.

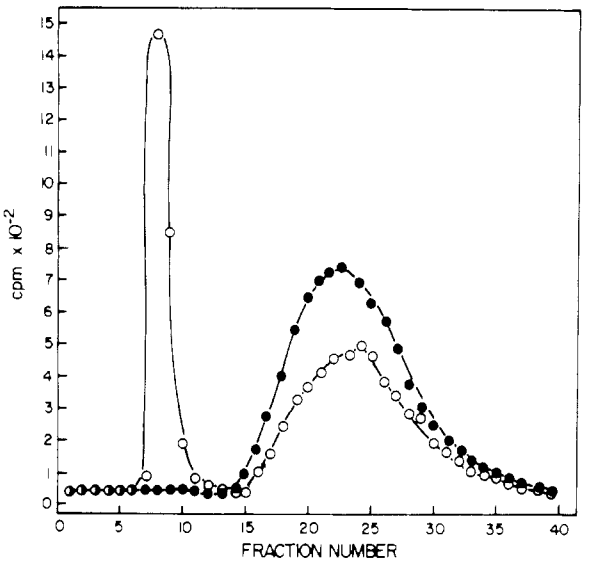

FIGURE 1: Separation of bound and free drug. Reactions were carried out under standard conditions and contained 6 pmol of [^{3}H]Mtx and 0 (●) or 0.2 μg (○) of purified wild-type dihydrofolate reductase. Fractions were collected and counted as described under Experimental Procedures.

Separation of Bound and Free Drug. To separate Mtx bound to dihydrofolate reductase from free drug, gel filtration using Sephadex G-25 was used (Hummel & Dreyer, 1962; Fairclough & Fruton, 1966). Any drug bound to the enzyme is excluded from the column, whereas free drug is included (Figure 1). The reaction mixture was applied to a Sephadex G-25 column (0.7 × 17 cm) equilibrated with 25 mM potassium acetate, pH 5.5, allowed to enter the resin, and washed with 0.2 mL of the column buffer. The material was eluted from the column at a flow rate of 0.7 mL/min. Fractions of 0.24 mL were collected, 6 mL of ACS (Amersham) scintillation fluid was added, and the samples were counted in a Beckman LS350 liquid scintillation system. The excluded volume was obtained in ~6 min. The amount of drug bound was determined from the labeled material excluded from the column. The free drug was determined either by the amount of material in the included peak from the column or by subtracting the bound material from the total input drug. Similar results were obtained in either case.

Determination of the Dissociation Constant (K_d) for the Mtx–Reductase–NADPH Complex. To determine the K_d for the Mtx–reductase–NADPH complex, various concentrations of labeled drug (5 to 80 nM) were added to a constant amount of either purified reductase or crude cell extracts. To minimize the errors in counting, assays were set up to contain varying specific activities of the drug but the same number of counts. The amount of bound and free drug was determined as described above. The reciprocal of the bound drug was graphed as a function of the reciprocal of the free drug (Lo & Sanwal, 1975). At least two determinations were made for each cell

line. To analyze the data, a linear regression analysis employing a programmable Texas Instrument pocket calculator, Model 57, and the program outlined in the Texas Instrument Programming manual was used to give the best straight-line fit to the data points. The intersection of the extrapolation of this line with the abscissa is equivalent to $-1/K_d$ and this value was used to calculate the apparent K_d.

Labeling of Cells and Extract Preparation for Two-Dimensional Gel Electrophoresis. Exponentially growing cells were washed 2 times with α medium lacking methionine, but supplemented with 10% dialyzed fetal bovine serum (DFBS), and incubated in this medium at a concentration of 10^6/mL. After 90 min at 37 °C, the cells were pelleted and resuspended in methionine-free medium supplemented with 10% DFBS and 10 μCi/mL [^{35}S]methionine. After 2 h of incubation, the cells were pelleted and washed 3 times with ice-cold phosphate-buffered saline. Incorporation into trichloroacetic acid insoluble material was on the average 8 cpm/cell. The cell pellet was sonically disrupted and an extract prepared as described above. The extract was dissociated with the O'Farrell urea–NP40–β-mercaptoethanol lysis buffer (O'Farrell, 1975) and loaded onto the gels. Purified enzyme was dissociated with the same lysis buffer prior to electrophoresis.

Two-Dimensional Electrophoresis. The methods employed were similar to those of O'Farrell (1975) with the following differences. The composition of the isoelectric focusing (IF) gels was obtained by mixing ampholytes of pH 2/11 and pH 5/7 in a ratio of 3:2. After we completed the isoelectric focusing run, the pH gradient was measured by means of a microelectrode (Ingold Electrodes Inc.). To estimate the amount of material present in the bands in the IF gels, the gels were fixed and stained with 0.1% Coomassie blue in 50% trichloroacetic acid, destained in 25% ethanol and 7% acetic acid, and scanned at 540 nm using a Gilford spectrophotometer with a linear transport system. The areas under the bands were measured by tracing the perimeters of the peaks, cutting these out, and weighing them.

For the electrophoresis in sodium dodecyl sulfate, an 11% acrylamide gel was used in a vertical slab gel cell (Bio-Rad Labs Model 220).

Fluorography and Autoradiography. Protein spots were visualized by fluorography and autoradiography. The gels were destained, dehydrated, and impregnated with 20% (w/w) PPO in Me_2SO. Following impregnation, the gels were soaked in H_2O for 60 min, dried, and exposed to Kodak X-O mate (RP/R) films at −70 °C according to the methods described by Bonner & Laskey (1974) and Laskey & Mills (1975).

Results

Determination of the Apparent K_d for the Mtx–Dihydrofolate Reductase–NADPH Complex Using Purified Enzyme. Previous work (Flintoff et al., 1976a; Gupta et al., 1977) suggested that the dihydrofolate reductase present in one class of Mtx-resistant CHO cells had an altered affinity for binding the drug. To further examine this possibility, the binding of Mtx to purified enzyme from wild-type and one class I resistant cells was determined.

Preliminary studies indicated that binding of Mtx to dihydrofolate reductase was optimal at pH 5.5 and confirmed earlier reports that the presence of NADPH concentrations in excess of 1×10^{-6} M facilitated this binding (Otting & Huennekens, 1972; Myers et al., 1975; Kamen et al., 1976). Thus, in all assays excess NADPH was present to ensure "tight" binding.

The binding of labeled drug to either dihydrofolate reductase purified from wild-type (Pro$^-$3) or class I resistant cells (Pro$^-$3 Mtx^{RI} 3-3) as a function of increasing concentrations of [^{3}H]Mtx is shown in Figure 2. This figure also shows the binding curve for a 1:1 mixture of the two enzymes. The apparent K_d value for the Mtx–reductase–NADPH interaction can be determined from these data by graphing 1/bound drug as a function of 1/free drug as shown in Figure 3. These graphs indicate that each purified enzyme preparation contained only a single component responsible for the binding of labeled Mtx in the concentration range 5 to 80 nM. As expected, a mixture of the two enzymes gave a biphasic curve characteristic of a two-component system in which the individual components have different binding affinities.

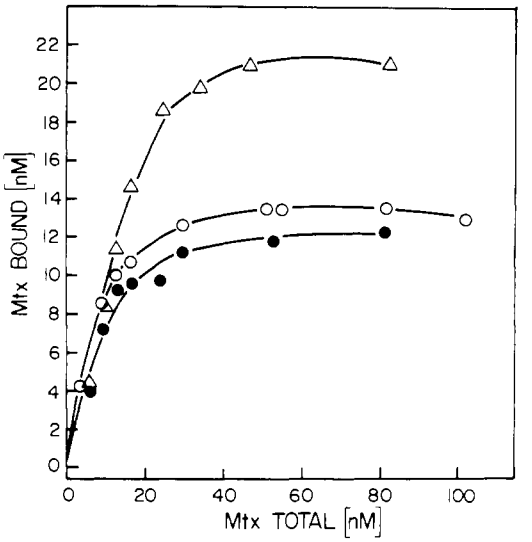

FIGURE 2: Binding of [^{3}H]Mtx to purified dihydrofolate reductase. Increasing concentrations of [^{3}H]Mtx were incubated for 10 min at 22 °C with either 1 μg of dihydrofolate reductase from wild-type Pro$^-$3 cells (○), 1 μg of reductase from resistant cells Pro$^-$3 Mtx^{RI} 3-3 (●), or a mixture of 1 μg of wild-type and 1 μg of resistant enzyme (△) prior to separation of bound and free [^{3}H]Mtx as described under Experimental Procedures.

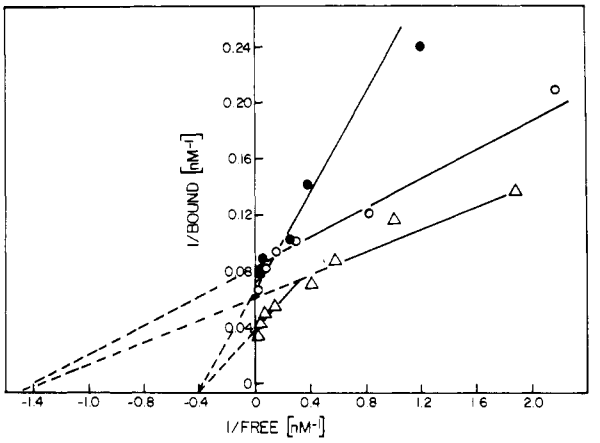

FIGURE 3: Reciprocal plot analysis of [^{3}H]Mtx binding. Reciprocal plot analysis for the binding of [^{3}H]Mtx to dihydrofolate reductase from Pro$^-$3 (○), Pro$^-$3 Mtx^{RI} 3-3 (●) cells, and a 1:1 mixture (△) of the two enzymes.

A decrease in the affinity for binding Mtx was readily apparent for the reductase from the mutant cell line. For the wild-type reductase the apparent K_d was $0.5 \times 10^{-9} \pm 0.1$ M and for the mutant $2.5 \times 10^{-9} \pm 0.3$ M.

Determination of the Apparent K_d for the Mtx–Dihydrofolate Reductase–NADPH Complex Using Crude Cell Extracts. The demonstration that purified reductase had a high affinity for the drug raised the possibility of using crude extracts as a source of the reductase for binding. To examine

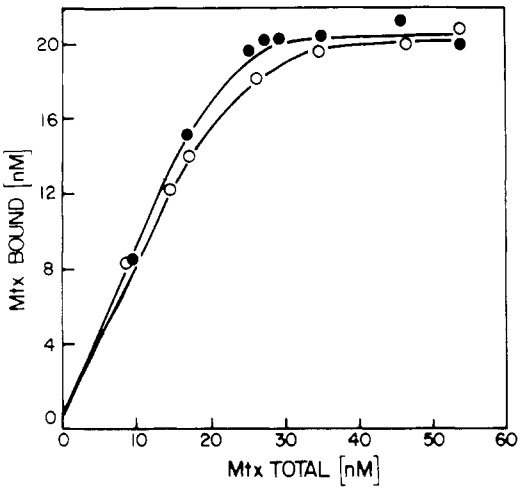

FIGURE 4: Binding of [^{3}H]Mtx to dihydrofolate reductase in cell lysates. Increasing concentrations of [^{3}H]Mtx were incubated for 10 min at 22 °C with cell lysates from Pro$^-$3 (○) (0.25 mg of protein) or Pro$^-$3 Mtx^{RI} 3-3 (●) (0.3 mg of protein) cells prior to separation of bound and free [^{3}H]Mtx as described under Experimental Procedures.

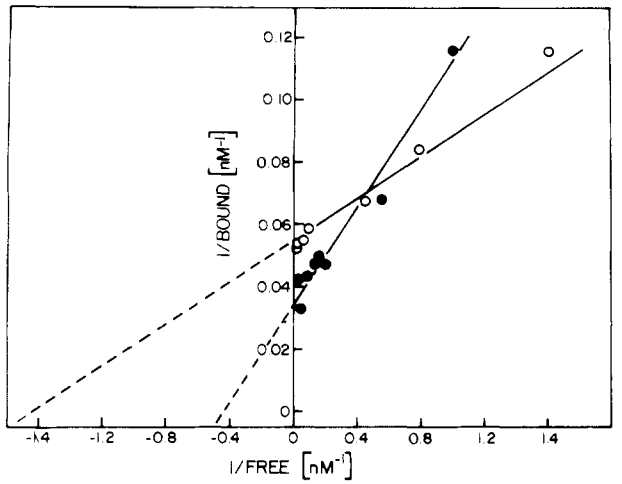

FIGURE 5: Reciprocal plot analysis of [^{3}H]Mtx binding. Reciprocal plot analysis for the binding of [^{3}H]Mtx to dihydrofolate reductase from Pro$^-$3 (○) and Pro$^-$3 Mtx^{RI} 3-3 (●) cells.

this, cell lysates were prepared from Pro$^-$3 and Pro$^-$3 Mtx^{RI} 3-3 cells and used in the binding assays. Figure 4 shows the binding as a function of increasing concentrations of [^{3}H]Mtx. The graphs of 1/bound Mtx as a function of 1/free drug are shown in Figure 5. As with the purified enzymes, these graphs indicate that each cell line contained only a single component responsible for the binding of labeled Mtx. The apparent K_d values calculated from these data are $0.6 \times 10^{-9} \pm 0.1$ M and $2.6 \times 10^{-9} \pm 0.4$ M for the wild-type and mutant enzymes, respectively. These values are similar to those obtained using purified enzymes.

The ability to use crude lysates in the binding assay permitted the screening of several independently selected resistant cells for the binding properties of the reductase present. In all cases, the graphs of 1/bound drug as a function of 1/free drug yielded a straight line, indicating that in each cell line only a single component capable of binding [^{3}H]Mtx, in this concentration range, was present. Table I summarizes the apparent K_d's for the Mtx–reductase–NADPH complexes for the various cell lines. It is apparent that in the class I resistant cells the reductase present has a 2.5- to 6-fold lower affinity for the drug than does the wild-type enzyme.

Table I: Apparent Dissociation Constants for Mtx–Dihydrofolate Reductase–NADPH Complexes

cell line	K_d (M)
Pro$^-$3	0.5×10^{-9} (±0.1)[a]
Pro$^-$4	0.6×10^{-9} (±0.1)
Pro$^-$3 Mtx^{RI} 1-2	1.4×10^{-9} (±0.2)
Pro$^-$3 Mtx^{RI} 3-3	2.5×10^{-9} (±0.3)
Pro$^-$3 Mtx^{RI} 2-6	1.4×10^{-9} (±0.1)
Pro$^-$3 Mtx^{RI} 9-1	1.4×10^{-9} (±0.2)
Pro$^-$3 Mtx^{RI} 4-3	3.4×10^{-9} (±0.4)
Pro$^-$3 Mtx^{RI} 6-3	1.4×10^{-9} (±0.2)
Pro$^-$4 Mtx^{RI} 3-4	3.2×10^{-9} (±0.4)
Pro$^-$3 Mtx^{RI} 10-2	1.3×10^{-9} (±0.2)
Pro$^-$3 Mtx^{RI} 7-1	2.2×10^{-9} (±0.3)
Pro$^-$3 Mtx^{RI} 11-4	2.2×10^{-9} (±0.2)
Pro$^-$3 Mtx^{RI} 16-8	1.4×10^{-9} (±0.2)
Pro$^-$3 Mtx^{RIII} 1-2	2.6×10^{-9} (±0.4)
Pro$^-$4 Mtx^{RV} 9-5	0.6×10^{-9} (±0.1)

[a] The number in parentheses is the standard deviation.

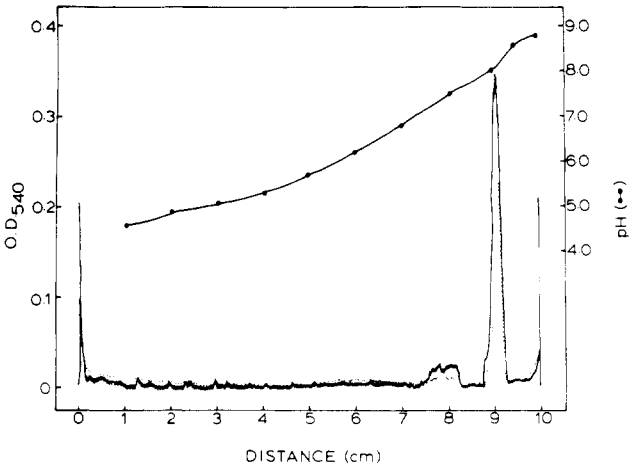

FIGURE 6: Densitometric tracings of purified dihydrofolate reductase after isoelectric focusing. Ten micrograms of purified wild-type dihydrofolate reductase (—) or 8 μg of enzyme from Pro$^-$3 Mtx^{RI} (···) cells was dissociated and introduced onto the IF gels at the cathode as described under Experimental Procedures. Electrophoresis was conducted for 6000 V·h. The gels were stained, destained, and scanned as described under Experimental Procedures.

Isoelectric Point of Dihydrofolate Reductase Purified from Wild-Type and Resistant Cells. Since the resistant cells contained a reductase with a decreased affinity for binding Mtx, it was of interest to determine whether this alteration was also reflected in a charge change of the protein. The purified reductase from either Pro$^-$3 or Pro$^-$3 Mtx^{RI} 3-3 cells was isoelectric focused by electrophoresis in polyacrylamide gels. Figure 6 illustrates the densitometric tracings of the stained gels. In either case, two protein bands are readily visible. Quantitation of the stained protein in each band in either case indicated that 90% of the material was present at an isoelectric point of ~8.0 and the remainder in the range of 7.2 to 7.6.

When a mixture containing 5 μg of wild-type reductase and 5 μg of reductase from the resistant cell line was electrophoresed, only two protein bands were visualized after staining (data not shown), thus indicating that the reductase from the resistant cells had a similar overall net charge as the wild-type enzyme.

Two-Dimensional Gel Analysis of the Reductase from Independently Selected Resistant Cells. Although the reductase from the Pro$^-$3 Mtx^{RI} 3-3 cells did not differ from the wild-type enzyme in overall net charge, it was possible that the reductase from other resistant isolates might. To screen a large number of isolates, the two-dimensional gel system of O'Farrell (1975)

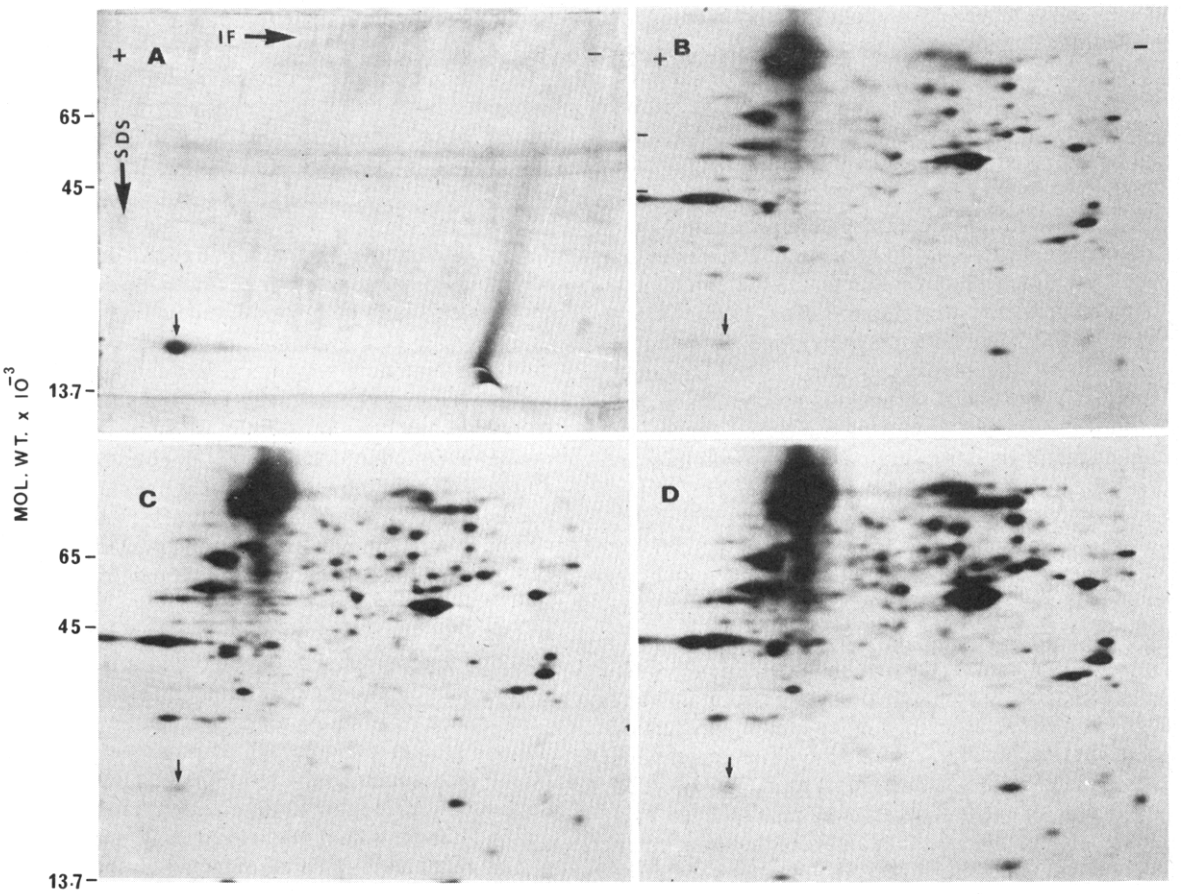

FIGURE 7: Two-dimensional gel electrophoresis. Samples of either purified wild-type dihydrofolate reductase or ^{35}S-labeled cell extracts were dissociated and introduced onto the IF gels at the cathode as described under Experimental Procedures. For crude extracts ~500 000 cpm (about 18 μg of protein) was added to each gel. Electrophoresis was conducted for 6000 V·h. The IF gels were placed onto slab gels, and electrophoresis in the second dimension was carried out for 5 h at 25 mA. (A) Purified wild-type dihydrofolate reductase (10 μg) was fixed and stained with 0.1% Coomassie blue in 50% methanol and 7% acetic acid and destained in 50% methanol and 7% acetic acid. Molecular weight markers in the second dimension were bovine serum albumin (mol wt 65 000), ovalbumin (mol wt 45 000), and ribonuclease (mol wt 13 700). (B) Autoradiogram of an extract from Pro$^-$3 wild-type cells. (C) Autoradiogram of an extract from Pro$^-$3 Mtx^{RI} 2-6 cells. (D) Autoradiogram of a mixture of extracts from Pro$^-$3 and Pro$^-$3 Mtx^{RI} 2-6 cells. Exposure for the autoradiograms was 3 days. The arrow, in each case, indicates the major dihydrofolate reductase component.

was used. Since it was desirable to use crude cell extracts rather than purified reductase from the cell lines, it was necessary to be able to localize the position of the reductase protein on the two-dimensional gel map. For this purpose, experiments with the wild-type enzyme were carried out. Figure 7A shows the two-dimensional map location of the purified wild-type enzyme after staining with Coomassie brilliant blue. The arrow indicates the position of the major reductase component. Figure 7B illustrates the autoradiograph of the two-dimensional polyacrylamide gel map of an extract from wild-type cells. The arrow indicates the position of the major reductase component. The following evidence demonstrates that this spot is the reductase protein. (1) This spot appears at the same molecular weight and isoelectric point as the major component in purified wild-type enzyme. (2) When purified reductase from wild-type cells is added to cell extracts, the major component of the purified reductase coincides with this spot. (3) When extracts of wild-type cells are passed through a column containing Sepharose conjugated with methotrexate (Gupta et al., 1977), the two-dimensional pattern is identical, except that the reductase spot disappears (data not shown).

Using the two-dimensional gel system, 11 independently selected class I resistant cells have been screened to determine whether the reductase present in such isolates has an altered isoelectric point. Figure 7C shows the autoradiogram of the two-dimensional map for an extract from Pro$^-$3 Mtx^{RI} 2-6 cells and Figure 7D shows the autoradiogram for a mixture of extract from wild-type Pro$^-$3 cells and resistant Pro$^-$3 Mtx^{RI} 2-6 cells. The arrow in each figure indicates the position of the reductase protein. Only a single spot in the molecular weight region of the reductase is apparent with the mixture, thus indicating no apparent change in the isoelectric point of the reductase from these resistant cells. A similar analysis with the other isolates indicated that, in all cases examined, there was no apparent major alteration in the overall charge of the reductase protein when compared to the wild-type enzyme.

Discussion

Studies with dihydrofolate reductase from several different sources have indicated that the interaction of Mtx with this enzyme is essentially stoichiometric under certain conditions (Werkheiser, 1961; Bertino et al., 1964; Perkins & Bertino, 1966; Blakley, 1969; Williams et al., 1973). Kinetic experiments on the binding of coenzymes to preformed enzyme–inhibitor complexes and of inhibitor to enzyme–coenzyme complexes indicate that prebinding of NADPH potentiates the binding of Mtx (Perkins & Bertino, 1966; Freudenthal et al., 1970; Blakley et al., 1971; Otting & Huennekens, 1972). Furthermore, the binding of inhibitors to the binary enzyme–coenzyme complex is much stronger than to the enzyme alone (Perkins & Bertino, 1966; Hillcoat et al., 1967). This increased affinity presumably results from a conformational

change in the enzyme brought about by the binding of NADPH, resulting in a stronger interaction of the complex with Mtx (Williams et al., 1979). Thus, it might be expected that mutations to Mtx resistance, in some cases, would involve structural changes in the gene coding for dihydrofolate reductase and be reflected in an altered binding of drug to the enzyme–NADPH complex.

Previous reports from this laboratory have established that CHO cells 8- to 23-fold resistant to Mtx are characterized by the presence of a dihydrofolate reductase 3- to 9-fold less sensitive to inhibition by the drug (Flintoff et al., 1976a; Gupta et al., 1977). In this report, the determination of the equilibrium constant for the binding of Mtx to the enzyme–NADPH complex has provided particularly good evidence that mutations to Mtx resistance displaying the class I phenotype do involve changes in the structure of the dihydrofolate reductase. The apparent K_d value for the binding of drug to the wild-type enzyme was 0.5×10^{-9} M, whereas the reductase from the resistant cells had a 2.5- to 6-fold lower affinity for the drug. This suggests that each of these mutants may have a different alteration in the amino acid sequence of the reductase protein.

It is of interest to note that the cell line Pro$^-$3 Mtx^{RIII} 1-2, which was selected in a two-step process (Flintoff et al., 1976a), contains a reductase with similar binding properties as that present in its parent, Pro$^-$3 Mtx^{RI} 3-3. This agrees with previous data (Flintoff et al., 1976a; Gupta et al., 1977). Also the cell line Pro$^-$4 Mtx^{RV} 9-5, which was also selected in a two-step process (unpublished), contains a reductase similar to the wild-type enzyme. Both the cell lines, however, are much more resistant than either wild-type or class I resistant cells, and preliminary results indicate that both lines contain amplified copies of the gene for dihydrofolate reductase (to be published elsewhere).

The nature of the curves used to determine the equilibrium dissociation constant is of interest with respect to the gene dosage relationships in the cell lines. Because of the large number of recessive mutations obtained in the CHO cell line, it has been postulated that this particular line may have a degree of functional hemizygosity (Siminovitch, 1976). Recent evidence suggests that several functions in this line are present in a single copy (Gupta et al., 1978a,b; Campbell & Worton, 1979). The monophasic nature of the binding curves suggests that only one functional copy of the gene for dihydrofolate reductase is present in these cells. These data agree with previous data on the heat inactivation of purified enzyme (Gupta et al., 1977). It should be pointed out, however, that recent work by Chasin & Urlaub (1979) on the generation of reductase minus mutants in CHO cells indicates that these cells may contain two functional copies of this gene. At present, this difference in results is not understood but may reflect a difference in the clones used.

Although the binding curves appear to be monophasic, it is apparent that both wild-type and at least one resistant cell contain two components: a major one comprising about 90% and a minor one representing about 10% of the total purified protein. Because of the small amount of the minor component present, minor differences in the affinity for drug would not be detected in the system described here. It is possible that the minor component may be a contaminant; therefore, it will be necessary to separate the two components and determine their individual reductase activity, affinity for drug, and peptide maps.

The presence of more than one dihydrofolate reductase component appears to be a common property of both prokaryotic (Gundersen et al., 1972; Poe et al., 1972) and eukaryotic systems (Hänggi & Littlefield, 1974; Kaufman & Kemerer, 1977). Such multiple forms have been readily identified and separated by electrophoresis or chromatography, and the ratio of the forms appears to remain constant during purification procedures (Kaufman, 1971). In microorganisms, two physiological controlled forms coexist inside the cell: the free enzyme and the cofactor-complexed enzyme (Dunlap et al., 1971; Gundersen et al., 1972; Poe et al., 1972; Harding et al., 1970). In eukaryotic systems, similar ligand–enzyme complex mechanisms may be involved, but at least in the case of the baby hamster kidney cell system, two basic enzyme forms, with similar amino acid sequences that can bind to ligands, are present (Hänggi & Littlefield, 1974). These two forms appear to remain in a constant ratio in wild-type cells and cells that greatly overproduce the enzyme (Hänggi & Littlefield, 1974). In the CHO system at present, it is not clear what forms the basis for the two forms observed after isoelectric focusing.

The two-dimensional gel electrophoresis system of O'Farrell (1975) has provided an opportunity to determine whether the dihydrofolate reductase from the resistant cells has a different charge than the wild-type enzyme. This is possible since the spot for the major reductase component is readily distinguishable in the two-dimensional map. However, in the isolates examined in this study, no major charge change in the reductase protein was detected. It is of interest to note that, in HeLa cells containing alterations in HPRT, 1/24 isolates contained a protein with an altered charge (Milman et al., 1976). It is possible that changes in the amino acid sequence of the reductases may be detected by peptide fingerprint analysis with various proteases.

The studies described in this paper and those described by others with different markers in various systems (Ingles et al., 1976; Milman et al., 1977; Steinberg et al., 1977; Boersma et al., 1979; Davidson & Patterson, 1979) have indicated that structural gene mutations can be generated by selection in vitro.

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Conformation of Nucleosome Core Particles and Chromatin in High Salt Concentration†

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ABSTRACT: The conformation of nucleosome core particles and chromatin under different ionic strength conditions has been studied by electron microscopic, hydrodynamic, and spectroscopic techniques. In the range of ionic strength used (6–600 mM), all four core histones were bound to the DNA. The sedimentation coefficient of the core particle decreases from 11.3 in 6 mM NaCl to 9.4 in 600 mM NaCl, and an alteration of the circular dichroic spectrum was observed when the ionic strength was increased. Direct evidence for the alteration of the chromatin structure in high salt was obtained by electron microscopy where a very extended conformation of the nucleosome was observed. The protein cross-linking agent dimethylsuberimidate was used to study the histone–histone proximities in the core particles; our experiments reveal that the same histones are in contact in the extended particles and in the compact native particles.

The structure of chromatin is not static but undergoes structural changes necessary for the functions in which it participates in the cell. The most obvious change is a gross conformational change of the superstructure of chromatin during mitotic condensation of the chromosomes. During interphase, the bulk of chromatin is less condensed and is organized in repeating units or nucleosomes. Electron microscopic observations, as well as nuclease digestion studies, have shown that subtle changes occur at the nucleosomal level during replication or in the transcriptionally active genes [for a review, see Felsenfeld (1978) and Chambon (1977)]. However, the precise structure assumed by chromatin during transcription or replication is not known. In vitro studies of chromatin conformation may help in understanding the properties of chromatin in vivo. Indeed, recent reports have shown that the nucleosome is able to assume different conformations depending on the salt concentration (Griffith & Christiansen, 1977; Oudet et al., 1977; Woodcock & Frado, 1977; Gordon et al., 1978; Dieterich et al., 1979); i.e., at very

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