Comparison of Solution Structures of Dihydrofolate Reductases and Enzyme–Ligand Complexes Using Cross-Reacting Antibodies†

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ABSTRACT: Polyclonal antibodies against dihydrofolate reductase (DHFR) from the human lymphoblastoid cell line WIL-2/M4 were used as probes to compare the antigenic structures in solution of native DHFRs obtained from a broad range of species and their complexes with substrate, cofactor, and folate antagonist inhibitors. All these antibodies could bind to the denatured human DHFR, indicating that they were specific for the primary structure of this enzyme. Denatured chicken liver and L1210 murine leukemic DHFRs competed for all of the antibodies that bound to the human enzyme, although less effectively than the denatured human enzyme, showing the presence of similar epitopes among the vertebrate enzymes. However, both direct binding and competition experiments showed low antibody cross-reactivities with native chicken liver (8%) and murine (10%) DHFRs, suggesting differences in the disposition of similar epitopes in these enzymes. The *Lactobacillus casei* DHFR showed a low amount (<2%) of cross-reactivity with the antibodies while the same antibodies did not cross-react with the *Escherichia coli* enzyme. DHFR from soybean seedlings competed for a large proportion (70%) of the anti-human DHFR antibodies, indicating a close similarity in the antigenic structures of plant and animal DHFRs. Binary complexes of the *L. casei*, avian, murine, and human DHFRs with dihydrofolate, methotrexate (MTX), trimethoprim (TMP), NADPH, and $NADP^+$ all showed significantly lower antibody binding capacity as compared with the corresponding free enzymes. Further, these ligands inhibited antibody binding to the enzyme to varying degrees. Ternary MTX–NADPH and TMP–NADPH complexes of the enzyme showed a further reduction in antibody binding, but this effect in most cases was not additive with respect to the effects of the individual ligands. This suggests that substrate, cofactor, and anti-folates inhibit the binding of antibodies to DHFR by producing conformational changes in the protein rather than by steric exclusion. Preliminary mapping of the antigenicity of human DHFR using CNBr fragments derived from the enzyme showed that the antigenic domains are predominantly in the sequence 53–111. This fragment also inhibited the binding of most of the cross-reacting antibodies to murine DHFR. Further, most of this antigenicity is not likely to be in the amino-terminal half of this sequence, given the high degree of sequence homology among vertebrate DHFRs in this region and the lower affinity of antibody binding to the denatured murine and avian enzymes as compared with the human enzyme. These results support the concept that differential binding of the antibodies to various DHFR–ligand complexes, in large part, may not be due to direct interaction with the active-site residues (most of which are outside the proposed major antigenic region) but due to ligand-induced conformational changes involving amino acid residues in the sequence 53–111. This sequence should also contain structural features that distinguish the various vertebrate DHFRs.

Dihydrofolate reductase (DHFR)[1] (tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) catalyzes the reduction of dihydrofolate to tetrahydrofolate by NADPH. This is a key reaction in folate metabolism, and DHFR is the primary target for the action of anti-folate drugs in the chemotherapy of cancer and several bacterial diseases. Consequently, extensive studies have been reported on the structure of DHFRs isolated from a variety of bacterial and vertebrate sources and their mode of interaction with a wide variety of substrate analogues [reviewed by Blakley (1984), Hitchings & Baccanari (1984), Montgomery & Piper (1984), and Freisheim & Matthews (1984)].

The detailed structural studies on DHFR include, primarily, X-ray crystallographic data on the bacterial (*Lactobacillus casei* and *Escherichia coli*) (Matthews et al., 1977, 1978, 1979, 1985; Bolin et al., 1982; Filman et al., 1982) and vertebrate (chicken liver, L1210 murine leukemic) (Volz et al., 1982; Matthews & Volz, 1982; Stammers et al., 1983; Matthews et al., 1985) enzymes, with or without bound anti-folate compounds and/or NADPH and also NMR studies of the interaction of amino acid side chains with bound ligand (Feeney et al., 1980a,b; Gronenborn et al., 1981; Birdsall et al., 1984; London, 1984). Both the bacterial and vertebrate enzymes have molecular weights in the range of 18 000–22 000 (Freisheim & Matthews, 1984). The human, bovine, murine, and avian enzymes exhibit about 75–90% sequence homology, and the *L. casei* and *E. coli* enzymes have less than 30% sequence homology, while there is little similarity in primary sequence between the bacterial and vertebrate enzymes

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[1] Abbreviations: DHFR, dihydrofolate reductase; FAH_2, dihydrofolic acid; MTX, methotrexate (4-amino-10-methyl-4-deoxyfolic acid); TMP, trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine]; Ig, immunoglobulin; $NaDodSO_4$, sodium dodecyl sulfate; $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized); NADPH, nicotinamide adenine dinucleotide phosphate (reduced); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HPLC, high-performance liquid chromatography; ELISA, enzyme-linked immunosorbant assay.

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(Freisheim & Matthews, 1984; Blakley, 1984). However, the X-ray crystallographic structures of vertebrate and bacterial DHFRs are strikingly similar. The basic backbone structure of DHFR comprises several strands of β-sheet (seven in the vertebrate enzyme and an additional β-strand in the bacterial DHFR) and four major α-helices with the residues interconnecting these elements being involved in forming loops. Extra residues in the vertebrate enzyme relative to the bacterial enzyme are accommodated in the loop regions. The active site is seen as a pronounced cavity running across one face of the enzyme.

Physical data, together with chemical modification studies [e.g., see Freisheim et al. (1977, 1979), Vehar & Freisheim (1976), Daron & Aull (1982), and Kaufman et al. (1980)] and affinity labeling studies [e.g., see Kumar et al. (1981)], have led to the identification of amino acid residues that are most likely to interact with substrate (or inhibitor) and cofactor. These residues are largely concentrated in the amino-terminal region of the enzyme, which is also a highly conserved sequence. In general, identical or analogous features exist at the active sites of the vertebrate and bacterial enzymes, and dissimilarities in the secondary structures of these enzymes occur distant from the active site.

Ligand-induced conformational changes in DHFR have been invoked to explain observations such as the enhanced affinity of anti-folates to the DHFR–NADPH complex as compared with the apoenzyme. Some evidence for the occurrence of such changes has been obtained from kinetic (Penner & Frieden, 1985), fluorescence (Birdsall et al., 1980), and NMR (Bevan et al., 1983; Birdsall et al., 1984) studies of the bacterial DHFRs and from a comparison of X-ray structures of the holo- and apoenzyme forms of the avian DHFR (Matthews et al., 1985). However, there is little data on the mobility of specific regions of the amino acid sequence of the enzyme. There is also a need to obtain information on the solution conformations of the various DHFRs and to correlate such data with X-ray crystallographic features.

Antibodies of known amino acid sequence specificity can serve as unique probes to study the solution conformations of proteins and to follow structural changes in the native protein. In this paper, we report results of the application of antibodies specific to the primary sequence of DHFR obtained from the human WIL-2/M4 cell line to compare the structures of bacterial, plant, avian, murine, and human DHFRs and of binary and ternary complexes of these enzymes with substrate (FAH_2), cofactor (NADPH and $NADP^+$), and inhibitors (MTX and TMP). We further report preliminary mapping of the antigenic sites on the primary sequence of the human DHFR.

Experimental Procedures

Purification of DHFRs. Human DHFR was purified from a MTX-resistant WIL-2/M4 lymphoblastoid cell line by affinity chromatography on MTX–Sepharose followed by preparative isoelectric focusing to remove bound FAH_2, which was used to elute the enzyme (Delcamp et al., 1983). Murine DHFR was similarly isolated from a MTX-resistant L1210/R81 lymphoma cell line by MTX–Sepharose affinity chromatography followed by isoelectric focusing (Delcamp et al., 1983). Chicken liver DHFR was purified by MTX–Sepharose chromatography, gel filtration on Sephadex G75, and preparative isoelectric focusing to remove enzyme-bound FAH_2 (Kaufman & Kemerer, 1977). DHFR from MTX-resistant *L. casei* cells was purified by the method of Gunderson et al. (1972) as modified by Liu and Dunlap (1974) by ion-exchange chromatography on CM-Sephadex followed by gel filtration on Sephadex G50. *E coli* DHFR was a generous gift from Dr. David A. Matthews, Agouron Institute, La Jolla, CA. DHFR from soybean seedlings was purified to homogeneity by MTX–Sepharose chromatography, gel filtration on Sephacryl S-200, and Blue Sepharose chromatography (S. Ratnam et al., unpublished results). The MTX-resistant cells used above for the purification of DHFR were all overproducers of the enzyme. However, the kinetic properties of these enzymes were identical with those in the corresponding normal MTX-sensitive cells. All DHFRs used in these studies exhibited a single protein band following electrophoresis on 10% $NaDodSO_4$–polyacrylamide gels.

Denaturation of DHFRs. Denatured DHFR from each source was prepared by precipitating the protein with 10% trichloroacetic acid and dissolving the pellet in 50 mM Tris-HCl buffer, pH 7.5, containing 1% $NaDodSO_4$ followed by heating at 100 °C for 3 min. The final concentration of $NaDodSO_4$ in the radioimmunoassays did not exceed 0.05%. This concentration of $NaDodSO_4$, in the presence of 0.5% Triton X-100, did not affect the binding of antibodies to any of the DHFRs.

Production of Antibodies to Human DHFR. A rabbit was immunized with DHFR purified from the human WIL-2/M4 cell line. Protein (1 mg) was injected intradermally at multiple locations on the back, twice, with an interval of 4 weeks, in Freund's complete adjuvant. Four weeks later, a booster dose of 100 μg of protein was injected intraperitoneally in Freund's incomplete adjuvant, and the rabbit was bled 10 days thereafter. The IgG fraction of the antiserum was obtained by precipitating with 37% ammonium sulfate, followed by dialysis against 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM NaCl. The final volume of the IgG solution was the same as the initial volume of antiserum.

Radioiodination of Proteins. Human, murine, avian, and *L. casei* DHFRs were labeled with ^{125}I using Enzymobeads (Bio-Rad) up to a specific radioactivity of (0.5–1) $\times 10^{18}$ cpm/mol.

Solution Radioimmunoassays. The binding of antibodies to ^{125}I-labeled DHFRs was assayed according to Lindstrom et al. (1981) by incubating the appropriate species of ^{125}I-labeled DHFR (10–20 nM) with antibodies (0.01–5 μL) in 100 μL of 10 mM sodium phosphate buffer (pH 7.5)/100 mM NaCl/0.5% Triton X-100 at 22 °C for 1 h along with normal rabbit IgG (final volume 5 μL) and precipitated with a previously titered equivalent amount of goat anti-rabbit IgG (Cooper Biomedical). The immunoprecipitate was washed with 2 $\times$ 1 mL of the above buffer and counted. Nonspecifically bound radioactivity in the immunoprecipitate (<5%) was determined by using normal rabbit IgG instead of antibodies and was subtracted in the final values (Lindstrom et al., 1981).

ELISA. Binding of antibodies to the plant or human DHFR immobilized in microtiter dishes (Immunolon I; Dynatech, Alexandria, VA) was assayed by using various dilutions of antibodies and glucose oxidase labeled goat anti-rabbit IgG and measuring the absorbance at 405 nm in the presence of horseradish peroxidase, β-D-glucose, and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), as described by Hochschwender et al. (1985).

Estimation of FAH_2 and NADPH. FAH_2 or NADPH was estimated by quantitative conversion into product using chicken liver DHFR. FAH_2 or NADPH was diluted into 10 mM sodium phosphate buffer (pH 7.5)/100 mM NaCl/0.5% Triton X-100. A 50-μL aliquot containing 21 nmol of FAH_2 or 16 nmol of NADPH was removed before and after incu-

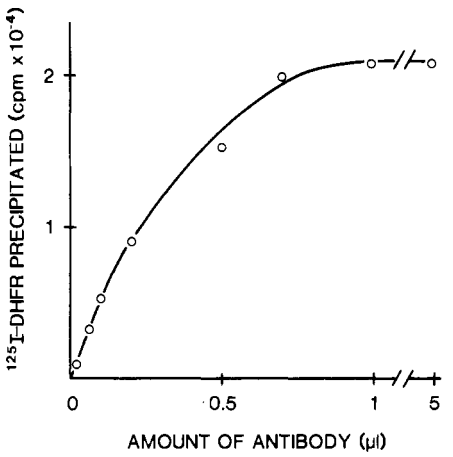

FIGURE 1: Immunoprecipitation of human DHFR. ^{125}I-Labeled human DHFR (20 nM) was taken in each assay (100 μL), and the indicated amounts of anti-human DHFR antibodes were used. The amount of enzyme complexed to antibody was determined by precipitating with goat anti-rabbit IgG, washing, and counting the radioactivity in the precipitate.

bating for 1 h at 22 °C and immediately transferred to a cuvette containing a large excess (5 μg) of chicken liver DHFR and an excess amount of the other substrate or cofactor. The change in absorbance in the cuvette at 340 nm was recorded.

Cyanogen Bromide Fragments. Human DHFR (4 mg) was first subjected to reduction and carboxymethylation of its single cysteine residue using iodoacetic acid as described by Kaufman et al. (1980). It was then incubated with CNBr (40 mg) in 70% formic acid under nitrogen for 20 h at 22 °C. The fragments were initially separated on a Sephadex G50 column (1.5 × 90 cm) equilibrated with 20% formic acid. The first major peak of absorbance at 280 nm was further purified by HPLC on a Waters Bondapak C-18 column (3.9 × 300 mm), initially equilibrated with water/0.1% trifluoroacetic acid. The peptide eluted as a homogeneous peak at 30% 1-propanol/0.1% trifluoroacetic acid (30 mL) using an initial gradient of 0–30% 1-propanol (20 mL). The amino acid composition of this peptide was determined by using a Beckman Model 6300 amino acid analyzer.

RESULTS

Interspecies Cross-Reactivities of Antibodies to Human DHFR. Immunoprecipitation of ^{125}I-labeled human DHFR with various concentrations of antibodies (Figure 1) showed that about 1 μL of antibodies was needed to immunoprecipitate the enzyme completely. The antibody titer, calculated from the linear portion of this curve, was 5 μM. On the basis of the data in Figure 1, we chose to use a subsaturating amount (0.2 μL or 10 nM) of the antibodies in each assay in further experiments involving quantitation of the degree of inhibition of antibody binding produced by the binding of various ligands or peptides to the enzyme. Higher concentrations of the antibodies were required to immunoprecipitate the murine and chicken liver DHFRs. Cross-reactivities of the antibodies with these two enzymes, calculated in terms of the antibody titer against them, relative to the anti-human DHFR titer, gave values of 10% and 8%, respectively, for the native murine and chicken liver enzymes. The antibodies cross-reacted to <2% with ^{125}I-labeled native *L. casei* DHFR (results not shown).

Denatured human DHFR could compete completely for antibodies that bound to the native ^{125}I-labeled enzyme (Figure 2A). Further, the denatured enzyme competed as effectively as unlabeled native enzyme at corresponding concentrations (Figure 2A). This result indicates that the antibodies against

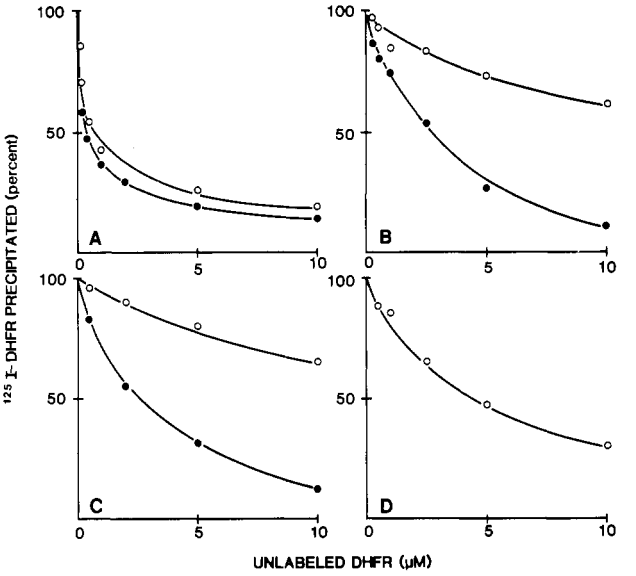

FIGURE 2: Inhibition of antibody binding to ^{125}I-labeled native human DHFR by unlabeled human, chicken, murine, and soybean DHFRs. ^{125}I-Labeled native human DHFR (20 nM) was incubated for 1 h at 22 °C with a subsaturating amount (10 nM) of anti-human DHFR antibodies in the absence and in the presence of various concentrations of unlabeled (A) native (○) and denatured (●) human DHFRs, (B) native (○) and denatured (●) chicken liver DHFRs, (C) native (○) and denatured (●) murine DHFRs, and (D) soybean DHFR. The ^{125}I-DHFR complexed to antibody was precipitated with goat anti-rabbit IgG, washed, and counted.

the native human enzyme are all specific for its primary amino acid sequence.

Due to the low cross-reactivities of the native chicken liver and murine DHFRs with the antibodies, neither enzyme, as expected, could produce complete inhibition of antibody binding to ^{125}I-labeled human DHFR in their native form even at relatively high concentrations (Figure 2B,C). However, when the enzymes from chicken liver and mouse were denatured, they could almost completely inhibit the binding of antibodies to ^{125}I-labeled human DHFR. Although higher concentrations of the denatured avian and murine DHFRs were required to produce 50% inhibition (3.2 and 2.5 μM, respectively) as compared with the denatured human DHFR (0.5 μM), it should be noted that the antibodies bound to denatured chicken liver and murine DHFRs to a significantly greater extent than to the native enzymes (Figure 2B,C).

Interestingly, DHFR purified from a plant source, i.e., soybean seedlings (S. Ratnam et al., unpublished results), produced significant inhibition of antibody binding to the human enzyme (Figure 2D). The high cross-reactivity of these antibodies with the plant enzyme was also tested directly, in an enzyme-linked solid phase immunoassay (ELISA) in which the plant and human enzymes were immobilized in microwells (Figure 3). These results, although not as quantitative as solution radioimmunoassays, point to the similarity in the antigenic determinants of the plant and animal enzymes.

It was not possible to investigate competition for antibodies that bound to ^{125}I-labeled *L. casei* DHFR using unlabeled animal DHFRs since the *L. casei* enzyme has the property of nonspecifically binding to the animal DHFRs and forming aggregates which intercalate with the immunoprecipitate. As indicated in Figure 4, the denatured *L. casei* enzyme could also compete for antibodies binding to the native *L. casei* enzyme. Neither native nor denatured DHFR from *E. coli* could inhibit antibody binding to ^{125}I-labeled *L. casei* DHFR (Figure 4), indicating that these antibodies did not interact with the *E. coli* enzyme. We also observed that the antibodies

Table I: Inhibition of Binding of Antibodies[a] to DHFRs in the Presence of Various Ligands[b]

source of DHFR	% inhibition[c] of antibody binding						
	FAH_2	MTX	TMP	NADPH	$NADP^+$	MTX + NADPH	TMP + NADPH
L. casei	39.6	69.7	20.5	91.9	70.2	100	100
chicken liver	52.7	69.4	44.1	61.1	35.4	82.6	64.9
murine	43.8	71.9	36.6	64.1	45.7	92.6	78.7
human	25.4	32.4	17.9	25.3	19.5	46.8	35.5

[a] Antibody concentration was 10 nM. [b] The ligands were present at supersaturating concentrations (5–10 mM). [c] 100% antibody binding was the amount that bound to free DHFR from each source.

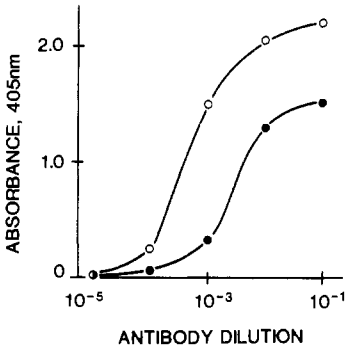

FIGURE 3: Binding of antibodies to immobilized human and plant DHFRs. DHFR (1 pmol/well) from human cells (○) or soybean seedlings (●) was immobilized in polystyrene microtiter wells. Various dilutions of anti-human DHFR antibodies (50 μL) were applied as primary antibody in quadruplicate wells. The wells were further processed as described (Experimental Procedures), and the amounts of specific antibody binding were plotted as the absorbance at 405 nm obtained at the end of the assay. In control experiments in which either no enzyme was immobilized or normal rabbit IgG was used instead of the antibodies, there was negligible absorbance at 405 nm.

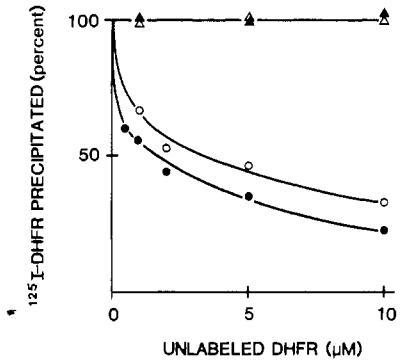

FIGURE 4: Effect of unlabeled *L. casei* and *E. coli* DHFRs on antibody binding to ^{125}I-labeled *L. casei* DHFR. ^{125}I-Labeled *L. casei* DHFR (20 nM) was incubated with anti-human DHFR antibodies (5 μL) for 1 h at 22 °C in the absence and in the presence of unlabeled native (○) or denatured (●) *L. casei* DHFR and native (△) or denatured (▲) *E. coli* DHFR. The *L. Casei* DHFR bound to antibody was precipitated with goat anti-rabbit IgG, washed, and counted.

did not bind to immobilized *E. coli* DHFR in solid phase immunoassays (results not shown).

Binding of Antibodies to Binary and Ternary DHFR–Ligand Complexes. The extent of binding of antibodies against the human DHFR to the DHFRs from various sources was examined after preincubating the enzyme with supersaturating concentrations (5–10 mM) of the substrate (FAH_2), reduced and oxidized cofactor (NADPH and $NADP^+$), and anti-folate inhibitors (MTX and TMP) both as individual components and in combination (MTX plus NADPH, TMP plus NADPH) (Table I). Neither FAH_2 nor NADPH was detectably oxidized under these conditions (see Experimental Procedures). The percent inhibition of antibody binding to ^{125}I–labeled DHFR from various sources in the presence of the ligands was taken as a measure of antibody binding to the

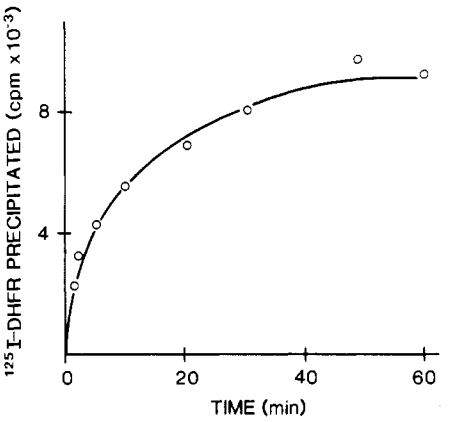

FIGURE 5: Time course of antibody binding to human DHFR. ^{125}I-Labeled human DHFR (20 nM) was incubated with anti-human DHFR antibodies (10 nM) at 22 °C, and at various times, goat anti-rabbit IgG was added, and the immunoprecipitate was washed and counted.

enzyme–ligand complex, considering 100% binding as the value for free, native DHFR in each case.

To justify the assumption that the values in Table I represent antibody binding to the various enzyme–ligand complexes relative to their binding to the free enzyme, the possibility, that free enzyme might be available for antibody binding even in the presence of excess ligand, had to be eliminated. The use of a large excess of the high-affinity ligands over antibodies (5–10 mM as compared with 10 nM antibodies) would not favor the occurrence of a significant concentration of free enzyme. Further proof is provided by a consideration of the relative rates of antibody and ligand binding. While the "on" and "off" rates for the interaction of substrates, cofactor, and anti-folates with DHFR are in the millisecond range, the rate of binding of antibodies to proteins is usually relatively slow. We examined this rate in the present case by monitoring the extent of immunoprecipitation of ^{125}I-labeled human DHFR at an antibody concentration (10 nM) used in our previous experiments (Figure 4, Table I). It is clear from the results shown in Figure 5 that under these conditions, the rate of binding of antibodies is quite slow, requiring nearly 10 min for 50% binding and about 45 min for complete binding. This result indicates that the values reported in Table I are due virtually entirely to antibody binding to the enzyme–ligand complexes, since, if antibodies were to bind to free enzyme alone in the presence of ligand, there should be a proportionate amount of free enzyme available, statistically, for a considerable duration.

Table I shows several interesting features. For each enzyme species tested, the binding of antibodies to the FAH_2–enzyme binary complex is significantly higher than that for the MTX–enzyme binary complex. The TMP–enzyme likewise binds more antibody than the MTX–enzyme. NADPH, in each case, produces reduced antibody binding as compared with $NADP^+$. Further, in at least the chicken liver, murine, and human DHFRs, the inhibitory effects of NADPH and

Table II: Comparison of Amino Acid Compositions of a Purified CNBr Fragment with That of Sequence 53–111 of Human DHFR

amino acid	no. of residues: CNBr fragment[a]	no. of residues: sequence 53–111	amino acid	no. of residues: CNBr fragment[a]	no. of residues: sequence 53–111
Asx	6.0	6	Met	0.5	1
Thr	1.8	2	Ile	1.8	2
Ser	3.3	4	Leu	8.6	9
Glx	7.2	7	Tyr	0	0
Pro	5.6	5	Phe	1.9	2
Gly	3.2	3	His	0.9	1
Ala	3.1	3	Lys	7.0	7
Cys	0	0	Arg	3.4	4
Val	2.1	2			

[a] The number of residues of each amino acid in the CNBr fragment was calculated from its amino acid composition, after assuming a value of 7 for Lys.

MTX or TMP are not additive although these ligands inhibit antibody binding to a lesser extent individually than in combination. In Table I, the percent inhibition of antibody binding produced by ligands is less in each case for the human DHFR as compared with the other DHFRs. This could reflect the fact that there are subpopulations of antibodies that bind to the human DHFR but not to other DHFRs and that the binding of these antibodies may not be affected to a relatively large extent in the presence of ligands.

Mapping of Antigenic Regions on the Primary Structure of Human DHFR. To determine the amino acid sequence specificities of the antibodies, the denatured human DHFR was first subjected to quantitative cleavage with cyanogen bromide. The peptides were first partially purified by gel filtration (Experimental Procedures), and the individual peak fractions in the effluent were pooled and tested for their ability to inhibit the binding of antibodies to ^{125}I-labeled human DHFR. The fraction containing the major inhibitory effect was further purified by HPLC (Experimental Procedures) and identified as peptide 53–111 on the basis of its elution profiles (T. J. Delcamp et al., unpublished results). The amino acid composition of this fragment closely resembles that expected from the amino acid sequence of residues 53–111 of the human enzyme (Blakley, 1984) (Table II), thus confirming the sequence of the fragment.

A mixture of all cyanogen bromide fragments derived from the human DHFR could completely inhibit the binding of antibodies to ^{125}I-labeled human DHFR (results not shown). The effect of various concentrations of peptide 53–111 on antibody binding to both native ^{125}I-labeled human DHFR and native ^{125}I-labeled mouse DHFR is shown in Figure 6. At high concentrations, the peptide inhibited about 80% and 85%, respectively, of the total antibody binding to the mouse and human DHFRs, indicating that the antigenicity of these two DHFRs lies predominantly in this sequence.

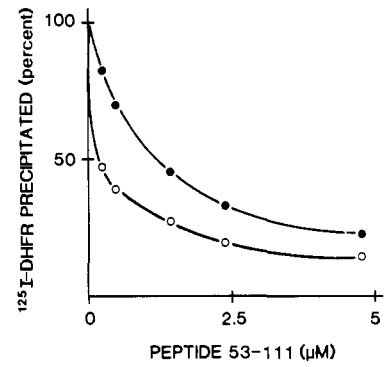

FIGURE 6: Inhibition of antibody binding to human and murine DHFRs by peptide 53–111 of human DHFR. ^{125}I-Labeled human (○) or murine (●) DHFR (20 nM) was incubated with anti-human DHFR antibodies (10 nM) for 1 h at 22 °C in the absence and in the presence of various concentrations of peptide 53–111. The amount of ^{125}I-labeled enzyme bound to antibodies was estimated by precipitation with goat anti-rabbit IgG, washed, and counted.

Discussion

Binding studies with synthetic peptides as well as X-ray crystallographic data on the antigen binding site of immunoglobulin molecules indicate that about six or seven amino acid residues will constitute an antigenic determinant in the primary sequence of a protein (Atassi, 1975; Poljak et al., 1976; Kabat, 1960). Notwithstanding the fact that antigenic sites on proteins could be conformational determinants (Atassi, 1975), we observed that all the antibodies raised against native human DHFR could bind as effectively to its denatured counterpart (Figure 2A). This is not unexpected, since antibodies to synthetic peptides have been found to cross-react at a high frequency with native proteins containing these sequences (Niman et al., 1983). This finding offered the advantage of being able to use anti-human DHFR antibodies as probes to compare structural features of various DHFRs and DHFR–ligand complexes simply in terms of the accessibility of portions of the primary sequence to antibodies.

The fact that the same population of antibodies that bind to the human enzyme in our polyclonal antibody mixture cross-reacts with the murine, chicken, and plant enzymes is evident from the mutual competition between these enzymes and the human DHFR for binding to antibodies (Figure 2B–D). Strikingly, despite the 75% and 90% sequence homology of the human enzyme with the chicken and murine enzymes, respectively (Blakley, 1984), only a small fraction of the antibodies bound to the native chicken and murine enzymes (Figure 2B,C). The observation that denatured chicken and murine enzymes compete for all of the antibodies that bind to the human enzyme (Figure 2B,C) indicates that antigenic determinants that are accessible to antibodies in the native human enzyme either are hidden or are in a different conformation in the chicken and murine enzymes. The amino acid sequences of these determinants, however, are probably not identical in the human DHFR vs. the other vertebrate DHFRs, since higher concentrations of the denatured chicken and mouse DHFRs are required as compared with denatured human DHFR to compete for antibody binding to the native human enzyme (Figure 2A–C). Further, some of these antibodies may have a higher affinity to unique human DHFR sequences.

Current knowledge of the X-ray crystallographic structure of vertebrate DHFR is based almost entirely on data for the chicken liver enzyme, except for a brief report on the structure of the murine enzyme–TMP–NADPH ternary complex at 2.5-Å resolution (Stammers et al., 1983). Given the high sequence homology among vertebrate DHFRs and similarities in their kinetic properties, it is believed that conclusions from high-resolution (2–2.9 Å) X-ray structural data obtained for

the chicken enzyme and its complexes with cofactor and anti-folates (Volz et al., 1982; Matthews & Volz, 1982; Matthews et al., 1985) can be extended to other vertebrate DHFRs as well. The data suggest that the differences in antigenic structures of various vertebrate DHFRs reported herein reflect either structural differences that could possible be detected in their crystal structures or subtle features unique to their solution conformations.

DHFR purified from soybean seedlings showed an unexpectedly high cross-reactivity with the antibodies to human DHFR (Figures 2D and 3). The soybean enzyme, which is being studied in our laboratory, interestingly, exhibits kinetic features which are both similar to and different from those of bacterial and vertebrate DHFRs (S. Ratnam et al., unpublished results). It should be noted that the high degree of antibody cross-reactivity displayed by the plant enzyme does not indicate a similar degree of sequence homology between the plant and animal enzymes but rather represents structural homology restricted to the antigenic determinants which may be a small portion of the total protein. In the case of bacterial enzymes, while the antibodies could immunoprecipitate ^{125}I-labeled *L. casei* DHFR significantly above background (Figure 4) it was not possible to establish the specificity of this interaction in terms of competition with animal DHFRs because of nonspecific protein–protein interactions explained under Results. However, neither native nor denatured *E. coli* DHFR could compete for this binding (Figure 4). This results is consistent with a report (Gronenborn et al., 1984) that antibodies raised against the *L. casei* DHFR did not cross-react with the *E. coli* enzyme.

The significant reduction in the binding of antibodies to binary and ternary DHFR–ligand complexes (Table I) could be the result of (i) direct steric hindrance to the binding of antibodies due to bound ligand or (ii) ligand-induced conformational changes involving antigenic sites on the protein outside the active site. A priori, it would appear unlikely that small molecules could block the binding of a large fraction of antibodies to relatively large domains (about six or seven amino acid residues) or that the antigenic region of DHFR is highly localized at the active site. This view is supported by the observation that the inhibitory effects of anti-folates (MTX and TMP) and NADPH are not additive in ternary complexes of at least the vertebrate DHFRs (Table I). The observation, in binding assays, using nitrocellulose filters, that [^{3}H]MTX bound to human DHFR–antibody complexes as well as to the free enzyme (results not shown) supports this argument. Finally, this concept draws support from the actual mapping of most of the antigenic determinants to amino acid sequence 53–111 of the human enzyme, which is discussed below. Differences in the nature of the structural changes produced in DHFRs by the various ligands in Table I are indicated by differences in the percent binding of antibodies to their complexes with the enzyme. Of particular interest in this regard would be comparison of complexes containing the structurally similar compounds MTX and FAH_2 and also complexes of the bacterial and vertebrate DHFRs containing TMP, which is a highly selective inhibitor of the bacterial enzyme.

There is physical evidence for ligand-induced conformational changes in at least the bacterial DHFR. NMR spectroscopic data show the presence of multiple conformational states of *L. casei* DHFR complexed with folate–NADP$^+$ (Birdsall et al., 1983) and NADP$^+$–TMP (Bevan et al., 1983; Birdsall et al., 1984). In the latter complex, changes have been detected in the environments of histidine residues outside the active site (Birdsall et al., 1984). NMR and fluorescence data suggest

FIGURE 7: Homology in the amino acid sequence region 53–111 of human, chicken, and murine DHFRs. Gaps indicated in the chicken and mouse DHFR sequences indicate identity of residues at these positions with those of human DHFR.

that NADPH induces conformational changes in *L. casei* DHFR that result in enhanced affinity for MTX (Birdsall et al., 1980; Gronenborn et al., 1981; Feeney et al., 1980b; Kimber et al., 1977). More recently, X-ray crystallographic data on TMP complexes of *E. coli* and chicken liver DHFRs showed a large TMP-induced conformational change involving Tyr-31 in the chicken holoenzyme but not in the bacterial enzyme (Matthews et al., 1985). The results reported herein demonstrate the presence of widespread conformational modulations in vertebrate DHFRs.

A unique advantage of using antibody probes in structural studies of DHFR of the type described herein is that specific information may be derived about the disposition of short stretches of the primary sequence in any part of the protein using antibodies specific to these sequences. Antigenic mapping of the vertebrate DHFRs using polyclonal anti-human DHFR antibodies and CNBr-generated fragments of the human enzyme showed that most of the antigenic sites occurred within peptide 53–111 (Figure 6). The high immunogenicity of this sequence is not surprising since it is quite hydrophilic (Figure 7). This sequence was obviously exposed in the human DHFR which was used as the immunogen. Inspection of the aligned primary sequences of the human, chicken, and murine DHFRs (Figure 7) shows major homologies in this region, which explains the complete binding of denatured chicken and murine enzymes to these antibodies and some differences, accounting for the lower affinity of cross-reactivity. It is thus not likely that a large fraction of the anti-human DHFR antibodies are directed against the amino-terminal half of peptide 53–111 which is highly conserved among the vertebrate enzymes (Figure 7). It follows, also, that the observed differences in antigenic structure among native vertebrate enzymes reflect differences in the conformation of the sequence 53–111. In general, most of the active-site residues identified by physical or chemical methods (Freisheim & Matthews, 1984) occur in the amino-terminal portion of DHFR, except for a few interactions involving other amino acid residues, such as hydrogen bonding with Val-115 (Matthews et al., 1985) and the loop formed by Glu_{62}-Lys_{63}-Asn_{64} in the avian enzyme (Volz et al., 1982; Freisheim & Matthews, 1984). The sequence 53–111, therefore, also contains residues which respond to ligand-induced structural changes in DHFR. Further refinement of these data in terms of studying shorter peptide segments, together with NMR and crystallographic structural data, should reveal the significance

of these structural changes resulting from the interaction of ligands with DHFR.

In conclusion, these results emphasize the presence of structural differences among vertebrate DHFRs and similarities between plant and animal DHFRs. They also provide immunochemical data on ligand-induced conformational changes in the enzyme and locate the major antigenic sites involved on the primary sequence of the vertebrate DHFRs. Further, these results demonstrate that fine-scale mapping on the enzyme of antigenic domains of interest, using short peptides, or, alternately, production of antibodies to various peptides should enable us to pinpoint the regions of mobility and structural diversity in various DHFRs and, perhaps, also the exact nature of the structural changes produced by each ligand.

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Registry No. DHFR, 9002-03-3; FAH_2, 4033-27-6; MTX, 59-05-2; TMP, 738-70-5; $NADP^+$, 53-59-8; NADPH, 53-57-6.

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