Ligand-Induced Structural Constraints in Human Dihydrofolate Reductase Revealed by Peptide-Specific Antibodies†

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ABSTRACT: Peptides from human dihydrofolate reductase (DHFR) generated by cyanogen bromide cleavage and corresponding to residues 15–52, 53–111, 112–125, and 140–186 (carboxyl terminus) were purified and used to immunize rats. Titration of the immune sera against *denatured* human DHFR by solid-phase immunoassay showed that peptides 15–52 and 140–186 were relatively highly immunogenic, unlike the *native* enzyme which is most immunogenic in the sequence 53–111. The antisera were specific for the corresponding peptides used for immunization. Antibodies to peptides 15–52, 53–111, and 140–186 cross-reacted with native human DHFR in solution in competition assays. However, the binding of nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) and the inhibitors folate and methotrexate, both in binary and in ternary complexes with the enzyme, caused a striking reduction in binding of antibody. Using a sensitive radioactive assay, it was found that antisera to peptides 15–52 and 140–186, both of which exhibited a high antibody titer, caused significant inhibition of DHFR. Because peptide 140–186 does not include any active-site residues, it is concluded that at least in this case all the antibodies bound to regions outside the active site. Since comparison of the X-ray structures of the chicken liver DHFR holoenzyme with the apoenzyme reveals no changes in secondary structural elements (α-helices and β-sheets), the reduction in antibody binding to DHFR–ligand complexes must not involve epitopes within these structures. These results, in light of the recent concept that antibodies to peptides cross-react with corresponding sequences in the native protein more frequently when these regions exhibit conformational flexibility, indicate that ligand binding reduces the overall conformational mobility of vertebrate DHFR. Such a ligand-induced effect, in the flexible loop regions of the DHFR molecule, might be a physical basis for the synergism between NADPH and dihydrofolate or methotrexate in binding to the enzyme.

Dihydrofolate reductase (DHFR)[1] (tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) from vertebrate and bacterial sources is a single polypeptide chain of M_r 18K–22K. The importance of DHFR as the target for anti-folate drugs in the treatment of cancer and some bacterial infections, together with the relative simplicity of its structure, has resulted in extensive physicochemical, kinetic, and structural characterization of this enzyme [reviewed in Freisheim and Matthews (1984), Hitchings and Baccanari (1984), Montgomery and Piper (1984), and Blakley (1984)]. While the vertebrate DHFRs display extensive sequence homology among themselves and little homology with the bacterial enzymes (Freisheim & Matthews, 1984), DHFRs from both sources display a remarkable conservation of structural features as determined by X-ray crystallography. Thus, the chicken liver DHFR consists of seven strands of β-sheet and four major α-helices with the residues interconnecting these elements involved in forming loops (Freisheim & Matthews, 1984). The active site is formed by a pronounced cleft running across one face of the enzyme with active-site residues largely restricted to the amino-terminal half of the primary structure (Freisheim & Matthews, 1984).

A critical factor in the efficacy of interaction of substrate or anti-folates and NADPH with the enzyme is the mutual enhancement (up to several thousandfold) of their affinity for the enzyme via structural changes induced in the active site (Freisheim & Matthews, 1984; Hitchings & Baccanari, 1984; Montgomery & Piper, 1984; Blakley, 1984). The presence of different conformational states of DHFR in its free form vs complexes with NADPH or anti-folates has been established in terms of displacement of specific amino acid residues from NMR and fluorescence data (Bevan et al., 1983; Birdsall et al., 1980, 1984; Gronenborn et al., 1981; Feeney et al., 1980) for bacterial DHFR and by X-ray crystallography for bacterial and vertebrate DHFRs (Matthews et al., 1979, 1985a,b). Results from this laboratory have recently demonstrated that polyclonal antibodies to human DHFR which are largely directed against the sequence 53–111 are sensitive probes of ligand-induced conformational changes in the enzyme (Ratnam et al., 1986). Peptide-specific antibodies are attractive tools to study protein conformational changes in solution where different parts of the protein molecule display multiple conformations or segmental mobility. In this report, we extend our previous studies to include antibody probes directed against various parts of the DHFR molecule including the carboxyl-terminal portion.

Experimental Procedures

Source of DHFR. Human DHFR expressed in *Escherichia coli* strain JM107 using a plasmid vector (pDFR) (Prendergast

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[1] Abbreviations: DHFR, dihydrofolate reductase; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; IgG, immunoglobulin G; FAH_2, dihydrofolate; MTX, methotrexate (4-amino-10-methyl-4-deoxyfolic acid); trimethoprim, 2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine; $NADP^+$, 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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et al., 1987) was purified by methotrexate (MTX) affinity chromatography, gel filtration on Sephadex G-75, and isoelectric focusing to remove bound dihydrofolate (FAH_2) (Delcamp et al., 1983).

Preparation of Peptides. Human DHFR was reduced and carboxymethylated and subjected to cyanogen bromide cleavage. The resulting fragments were separated by gel filtration on Sephadex G-50 followed by HPLC on a Waters Bondapak C-18 column as described previously (Ratnam et al., 1986; Kaufman et al., 1980). The amino acid compositions of the peptides were determined with a Beckman Model 6300 amino acid analyzer.

Immunization. Peptides were coupled to bovine serum albumin (BSA) using glutaraldehyde (Reichlin, 1980), and the conjugates were injected into rats in Freund's complete adjuvant intradermally and intraperitoneally, in three doses of 30 nmol of peptide at 2-week intervals, followed by bleeding, 2 weeks later.

ELISA. Human DHFR, denatured by precipitation with 10% trichloroacetic acid followed by boiling in 1% sodium dodecyl sulfate ($NaDodSO_4$) and diluting to <0.05% $NaDodSO_4$, was immobilized in microtiter dishes (0.1 μg/well). Various dilutions of antisera in 10 mM sodium phosphate, pH 7.5, containing 150 mM NaCl, 0.5% Tween-20, and 1% nonfat dry milk were assayed in these wells using glucose oxidase conjugated goat anti-rat 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) as described by Hochschwender et al. (1985). Since the antisera used could contain antibodies to BSA, which was used in the immunizations, BSA was replaced by 1% nonfat dry milk as a quenching agent in the buffers used in the immunoassays. All the assays were done in quadruplicate, and normal rat sera were used in negative controls. Negative controls were also included, using the antisera in the absence of immobilized DHFR.

Enzyme Assay. DHFR was assayed by using a radioactive assay in the presence of [^{3}H]FAH_2 and NADPH as described by Hayman et al. (1978) and modified by Rodenhuis et al. (1986) but without 2-mercaptoethanol in the assay mixture. [^{3}H]FAH_2 was prepared by reducing [3′,5′,7,9-^{3}H]folic acid with sodium dithionite (Hayman et al., 1978). The reaction mixture (0.2 mL) contained 50 mM Tris-HCl buffer, pH 7.0, 0.1 mM NADPH, 0.2 M KCl, 0.1 mM [^{3}H]FAH_2 [(3–5) × 10^6 cpm/μmol], and 1 mg/mL BSA. The reaction was initiated by the addition of NADPH and allowed to proceed at 37 °C for 4 min; then 25 μL of 0.11 M folate and 25 μL of 25% acetic acid were added, and the reaction tubes were immediately placed on ice for 10 min. The precipitate was pelleted and the supernatant counted for radioactivity.

Results

Purity of Cyanogen Bromide Fragments. The peptides derived from human DHFR were eluted as single symmetrical peaks from the HPLC column (results not shown). The amino acid compositions of the peaks were compared with the expected compositions for different regions of the DHFR sequence (Hitchings & Baccanari, 1984) in order to identify the fragments (Table I). It is clear from the results indicated in the table that the peptides isolated closely resemble in composition the sequences 15–52, 53–111, 112–125, and 140–186.

Relative Immunogenicity of the Peptides. Figure 1 shows the binding profiles of the various antisera to denatured human DHFR immobilized in microtiter wells. Peptides 15–52 and 140–186 elicited relatively high antibody titers, and peptide 112–125 was very poorly immunogenic, while peptide 53–111 showed moderate immunogenicity. The binding of these antisera to *native* DHFR immobilized in microtiter wells was identical with that indicated for the denatured enzyme (Figure 1). Antisera to peptides 15–52, 53–111, and 140–186 were,

Table I: Amino Acid Analysis of Purified Cyanogen Bromide Peptides of Human Dihydrofolate Reductase

amino acid	peptide 15–52	sequence 15–52	amino acid	peptide 53–111	sequence 53–111
Asx	3.6	4	Asx	6.0	6
Thr[c]	2.3	3	Thr[c]	2.0	2
Ser[c]	1.8	2	Ser[c]	3.8	4
Glx	3.7	4	Glx	7.0	7
Pro	2.9	3	Pro	4.6	5
Gly	3.9	4	Gly	3.1	3
Ala	0.4	0	Ala	(3.0)[a]	3
Cys	ND[d]	0	Cys	ND	0
Val	2.1	2	Val	2.0	2
Hse[b]	1.1	2	Hse[b]	0.5	1
Ile	1.6	2	Ile	1.9	2
Leu	(3.0)[a]	3	Leu	8.6	9
Tyr	1.1	1	Tyr	0	0
Phe	1.9	2	Phe	1.9	2
His	0.2	0	His	0.9	1
Lys	2.0	2	Lys	6.6	7
Arg	3.5	3	Arg	3.8	4
Trp	ND	1	Trp	ND	1

amino acid	peptide 112–125	sequence 112–125	amino acid	peptide 140–186	sequence 140–186
Asx	0.1	0	Asx	5.6	6
Thr[c]	0	0	Thr[c]	1.1	1
Ser[c]	1.8	2	Ser[c]	1.8	2
Glx	1.0	1	Glx	9.3	10
Pro	0	0	Pro	3.0	3
Gly	2.0	2	Gly	2.3	2
Ala	(1.0)[a]	1	Ala	0.2	0
Cys	ND	0	Cys	ND	0
Val	2.7	3	Val	3.1	3
Hse[b]	0.6	1	Hse[b]	0	0
Ile	0.7	1	Ile	1.9	2
Leu	0	0	Leu	(4.0)[a]	4
Tyr	0.9	1	Tyr	3.6	4
Phe	0	0	Phe	3.5	4
His	0	0	His	0.1	0
Lys	0.9	1	Lys	5.5	6
Arg	0	0	Arg	0.3	0
Trp	ND	1	Trp	ND	0

[a] The number of residues of each amino acid in the above fragments was calculated from its amino acid composition, after normalizing to an amino acid residue value in parentheses. [b] Hse = homoserine + homoserine lactone. [c] Ser and Thr values are corrected for 10% and 5% destruction, respectively. [d] ND, not determined.

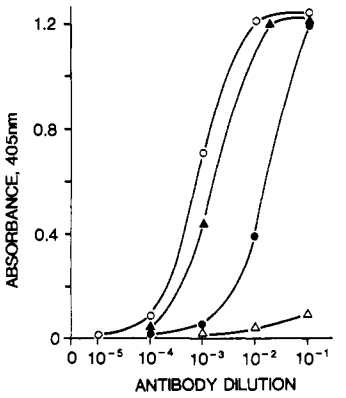

FIGURE 1: Titration of antisera to peptides 15–52 (○), 53–111 (●), 112–125 (△), and 140–186 (▲) of human DHFR. Denatured human DHFR (0.1 μg) was immobilized in quadruplicate ELISA assays, and antibody binding was monitored by the absorbance at 405 nm (see Experimental Procedures). Controls with normal rat serum were taken at various dilutions.

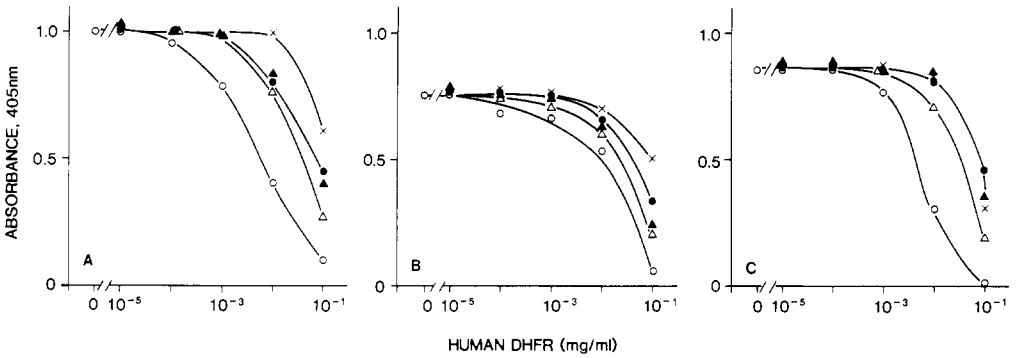

FIGURE 2: Competitive immunoassays using antisera to peptides 15–52 (A), 53–111 (B), and 140–186 (C). ELISA assays were carried out with denatured human DHFR as the immobilized antigen. Antisera to peptides 15–52, 53–111, and 140–186 were used at 1:1000, 1:200, and 1:1000 dilutions, respectively. The antisera (50 μL) were preincubated in 10 mM sodium phosphate, pH 7.5, containing 150 mM NaCl, 0.5% Tween-20, and 1% nonfat dry milk for 2 h at 22 °C in the absence and in the presence of various concentrations of native human DHFR alone (○) or the enzyme in the presence of 5 mM folate (△), MTX (●), NADPH (▲), or MTX plus NADPH (×). The antibody solutions were then applied directly onto the microtiter plates and assayed as described under Experimental Procedures. Virtually identical results were obtained when the concentration of each ligand was reduced to 0.1 mM.

Table II: Binding of Antisera to Human DHFR after Preincubation with Various Peptides[a]

	peptide immunogen		
competing peptide	15–52	53–111	140–186
15–52	0.0	0.73	0.55
53–111	0.87	0.02	0.88
140–186	0.55	0.58	0.01

[a] Antisera to peptides 15–52, 140–186 (50 μL of a 1:1000 dilution), or 53–111 (50 μL of a 1:100 dilution) were incubated overnight at 4 °C with 1 nmol of each peptide in 10 mM sodium phosphate (pH 7.5)/150 mM NaCl/0.5% Tween-20/1% nonfat dry milk and then assayed against immobilized human DHFR (see Experimental Procedures). The values are expressed as absorbance at 405 nm.

Table III: Concentration[a] of Native Human DHFR or DHFR–Ligand Complexes Required for 50% Inhibition of Antibody Binding to Denatured DHFR

peptide immunogen	ligand				
	none	folate	MTX	NADPH	MTX + NADPH
15–52	0.006	0.04	0.071	0.071	>0.1
53–111	0.025	0.05	0.09	0.063	>0.1
140–186	0.006	0.05	>0.1	0.095	0.09

[a] Expressed in milligrams per milliliter. The values shown in this table are derived from Figure 2.

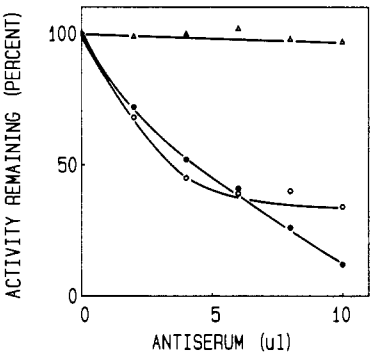

FIGURE 3: Inhibition of DHFR activity by antisera to peptides. Human DHFR (9 pmol) in 50 μL of 50 mM Tris-HCl, pH 7.0, containing 1 mg/mL BSA was incubated either in the absence or in the presence of 2–10 μL of normal rat serum (△) or antisera to peptide 15–52 (●) or peptide 140–186 (○) at 22 °C for 4 h, in duplicate tubes. The residual enzyme activity was assayed as described under Experimental Procedures.

therefore, employed in further experiments involving their use as structural probes of DHFR.

Specificity of Antibodies. Antibodies to peptides 15–52, 53–111, and 140–186 were preincubated with a large excess of each of the above peptides and assayed by ELISA against immobilized DHFR. The results indicated in Table II show that under the assay conditions each antiserum specifically bound to the peptide used for immunization as indicated by the virtually complete inhibition of binding to the immobilized antigen.

Binding of Antibodies to Native DHFR and Enzyme–Ligand Complexes. The binding of antibodies to DHFR in solution was monitored by competition ELISA assays in which denatured human DHFR was immobilized. The binding of antibodies, at an appropriate dilution, to the immobilized antigen was assayed in the presence of various concentrations of native human DHFR or in the presence of the enzyme preincubated with constant, saturating concentrations (0.1 or 5 mM) of MTX, NADPH, folate, or MTX plus NADPH (Figure 2A–C). As indicated in the figure, in the case of all three antisera, native DHFR competed for the antibodies, inhibiting their binding to the immobilized, denatured enzyme in a concentration-dependent manner. Preincubation of the native human enzyme with MTX, NADPH, or folate significantly inhibited antibody binding to the native enzyme to various degrees, shifting the curve to the right in all cases (Figure 2A–C). Further, for all antisera, maximum inhibition of antibody binding to the native enzyme was produced when MTX and NADPH were both bound in a ternary complex; this inhibition was greater than that produced by either MTX or NADPH alone for antisera to peptides 15–52 and 53–111. In no case did the ligand alone have any detectable effect on antibody binding to denatured DHFR immobilized in the microtiter plates. This clearly indicates that the above effects were due to the specific binding of ligands to the native DHFR in solution. Further, at a ligand concentration of 5 mM, there was either a marginal or an insignificant decrease in antibody binding to the native enzyme as compared with the values obtained at 0.1 mM ligand. The data in Figure 2 are depicted in Table III in terms of the concentration of *native* human DHFR needed to inhibit antibody binding to *denatured* DHFR by approximately 50% either in the absence or in the presence of various ligands. These values, for the various DHFR–ligand complexes, range from 2-fold to over 20-fold greater than the values for the apoenzyme.

Interaction of Ligands with DHFR–Antibody Complexes. Native human DHFR (0.18 μM) was incubated either with various amounts of normal rat serum (control) or with the antipeptide sera and then assayed for enzyme activity by a very sensitive radioactive assay using [^{3}H]FAH_2. As shown in

Figure 3, while the normal serum had no effect on DHFR activity, antibodies to peptide 15–52 inhibited the enzyme almost completely in a concentration-dependent manner. Antibodies to peptide 140–186 inhibited the enzyme to a maximum level of ca. 65%. At the concentrations tested (2–10 μL of antiserum in 50 μL of reaction mixture), antibodies to peptide 53–111 did not significantly inhibit enzyme activity.

Discussion

The various criteria suggested for the antigenicity of any region of a protein molecule include accessibility (Novotny et al., 1986), hydrophilicity (Hopp & Woods, 1981), conformational flexibility (Westhof et al., 1984), and protrusion (Thornton et al., 1986). In the particular case of sequential or continuous antigenic determinants, as opposed to discontinuous or conformational determinants, there is substantial evidence for a very strong correlation between the mobility of these determinants in the native protein and their antigenicity (Westhof et al., 1984; Tainer et al., 1984, 1985). Antibodies made primarily against peptides recognize corresponding epitopes in native proteins when these regions are free to assume different conformational states as is the case for the free peptides. These regions usually correspond to loops and ridges on the protein's surface (Barlow et al., 1986). DHFR displays conformational flexibility as it assumes different conformational states in solution (Kraut & Matthews, 1986). The X-ray structure of the protein also shows several regions lacking ordered secondary structure such as loop regions connecting α-helices and β-sheets that could cause the protein to be flexible (Freisheim & Matthews, 1984; Kraut & Matthews, 1986). The present work was prompted by recent findings from this laboratory that antibodies to human DHFR that are predominantly directed against the sequence 53–111 bound to a lower extent to binary and ternary enzyme–ligand complexes than to the free enzyme (Ratnam et al., 1986).

The cyanogen bromide peptides isolated herein were homogeneous and corresponded to the sequences 15–52, 53–111, 112–125, and 140–186 (carboxyl terminus) (Table I). The antibodies elicited against the peptide–BSA conjugates were specific for the corresponding peptides (Table II). The antisera with higher titers, i.e., those against peptides 15–52, 53–111, and 140–186 (Figure 1), were used to investigate structural changes in DHFR induced by the binding of NADPH, MTX, and folate, and, indeed, all three ligands significantly inhibited antibody binding with the three antisera (Figure 2A–C, Table III). The inhibition occurred to various extents with the different ligands used, and in most cases, ternary complexes bound less antibody than the binary complexes (Table III), suggesting conformational changes involving different parts of the protein molecule for the cofactor and anti-folates. The fact that residues in at least the carboxyl-terminal sequence, 140–186, do not overlap with the active site according to X-ray crystallographic data (Freisheim & Matthews, 1984) precludes direct interference by the ligands with antibody binding. The value of using competition assays of the type described (Figure 2) is that the competing protein is not subjected to possible local denaturation as would occur in direct immunoassays either by immobilization or by radioiodination. In view of the inhibitory effects of ligands on antibody binding to different regions of DHFR–ligand complexes, it might be expected that the antibodies would, in turn, inhibit enzymic activity in the enzyme–antibody complex by preventing a ligand-induced conformational change. It is also possible, of course, that antibody binding to regions outside of the active site on native DHFR can directly induce an altered conformation, leading to a loss in enzyme function. Since the total titer of specific polyclonal antibodies in an antiserum is usually low (<1 μM), it is essential to use a relatively low concentration of enzyme in an assay to ensure a significant occupancy of an individual epitope by the corresponding antibody. Accordingly, a sensitive radioactive assay was used which revealed enzyme inhibition by antibodies to peptides 15–52 and 140–186. Antibody binding to peptide 140–186 resulted in a partially active complex with DHFR as indicated by the plateau in Figure 3. Our inability to detect significant inhibition of DHFR by antibodies to peptide 53–111 under these conditions could, of course, simply reflect the relatively low antibody titer.

Studies of ligand-induced conformational changes in DHFR by kinetic (Penner & Freiden, 1985; Dunn & King, 1980) and NMR or fluorescence (Bevan et al., 1983; Birdsall et al., 1980, 1984; Gronenborn et al., 1981; Feeney et al., 1980; Kimber et al., 1977) methods have focused on the bacterial enzymes. In these cases, changes have been localized in the environments of amino acid residues outside the active site upon the binding of $NADP^+$, NADPH, and anti-folates such as trimethoprim and MTX. X-ray data (Kraut & Matthews, 1986; Matthews et al., 1985a,b) have been less discerning, understandably, because of the loss of these features in crystal packing. However, a recent comparison of the X-ray structure of the chicken liver holoenzyme with the apoenzyme showed a major structural perturbation of residues 91–96 contained in the loop connecting β-strand D and α-helix E. Such a change is absent in the *E. coli* DHFR although a structural counterpart for this loop does exist, emphasizing the occurrence of different types of conformational modulations in bacterial vs vertebrate DHFRs. The data also indicate that binding of NADPH causes a movement of Leu-22 in the chicken enzyme to a position favorable for interaction with MTX. This is a clear example of how conformational changes mediate the cooperative binding of ligands to vertebrate DHFR. However, such crystal structural data are largely restricted to minor displacements of residues within the active site. Since antigenic sites must be mobile for recognition by peptide-specific antibodies, ligand binding should at least restrict the mobility of some regions of DHFR. These regions are likely to involve protein segments that do not form stable secondary structures, i.e., sequences within the loop regions. Since the cyanogen bromide fragments used in this study were up to 59 residues in length, antisera against them may contain antibodies against secondary structural elements (Al Moudallal et al., 1985). However, such antibodies are still likely to bind to the DHFR–ligand complexes since X-ray crystallography of such complexes indicates that the α-helices and β-strands remain intact (Kraut & Matthews, 1986). Consistent with this hypothesis is a recent report (Hammond et al., 1986) for the *Lactobacillus casei* DHFR, from ^1H NMR combined with X-ray data, that coenzyme binding produces conformational changes in the loop formed by residues 13–23 and a "rigid body" movement of an α-helix C.

The dynamics of conformational changes in the DHFR molecule in solution is central to our detailed understanding of the synergism in cofactor and substrate or inhibitor binding to the enzyme. Our results suggest the possible role of regions of the protein distant from the active site in communicating the effects of ligand binding. Since antibody probes can be directed against virtually any accessible region on a protein molecule (Benjamin et al., 1984), detailed information concerning these processes can be obtained by using antibody probes against short (six residue) synthetic peptides within flexible regions of DHFR and by designing mutant forms of

the enzyme with deletions and/or substituents in these regions. In conclusion, these results suggest that the flexibility of certain loop regions of the DHFR molecule may be a critical factor in one or more events in ligand binding and/or catalysis.

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