Role of Lysine-54 in Determining Cofactor Specificity and Binding in Human Dihydrofolate Reductase†

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ABSTRACT: Lysine-54 of human dihydrofolate reductase (hDHFR) appears to be involved in the interaction with the 2′-phosphate of NADPH and is conserved as a basic residue in other species. Studies have suggested that in *Lactobacillus casei* dihydrofolate reductase Arg-43, the homologous residue at this position, plays an important role in the binding of NADPH and in the differentiation of K_m values for NADPH and NADH. A Lys-54 to Gln-54 mutant (K54Q) of hDHFR has been constructed by oligodeoxynucleotide-directed mutagenesis in order to study the role of Lys-54 in differentiating K_m and k_{cat} values for NADPH and NADH as well as in other functions of hDHFR. The purpose of this paper is to delineate in quantitative terms the magnitude of the effect of the Lys-54 to Gln-54 replacement on the various kinetic parameters of hDHFR. Such quantitative effects cannot be predicted solely on the basis of X-ray structures. The K_m for NADPH for the K54Q mutant enzyme is 58-fold higher, while the K_m for NADH for K54Q is only 3.9-fold higher than that of the wild type, indicating that the substitution of Lys-54 with Gln-54 decreases the apparent affinity of the enzyme for NADPH dramatically, but has a lesser effect on the apparent affinity for NADH. The ratio of K_m(NADH)/K_m(NADPH) decreases from 69 in the wild-type enzyme to 4.7 in the K54Q enzyme, suggesting that Lys-54, among other interactions between protein side-chain residues and the 2′-phosphate, makes a major contribution in terms of binding energy and differentiation in K_m values for NADPH and NADH. $NADP^+$ is a competitive inhibitor of NADPH for the K54Q enzyme as it is for the wild-type hDHFR; however, the K_i for the K54Q enzyme is 30 μM, which is 81-fold higher than the value of 0.31 μM for the wild-type enzyme. On the basis of determinations of the association (k_{on}) and dissociation (k_{off}) rate constants, the K_D for $NADP^+$ for the K54Q hDHFR–dihydrofolate complex is elevated 125-fold as compared to that for its wild-type counterpart. NAD^+ has no inhibitory effect on either wild-type or K54Q enzyme. The k_{cat} using NADPH as cofactor is elevated 2.5-fold, while the k_{cat} with NADH is increased 1.7-fold in the K54Q enzyme compared to values observed for the wild-type enzyme. The K_m for dihydrofolate is also elevated 5.6-fold over that determined for the wild-type enzyme. Agents at concentrations that show activating effects on the wild-type enzyme such as potassium chloride and urea all inactivate the K54Q enzyme. There appear to be no gross conformational differences between wild-type and K54Q enzyme molecules as judged by competitive ELISA using peptide-specific antibodies against human dihydrofolate reductase and from protease susceptibility studies on both wild-type and K54Q mutant enzymes. The pH–rate profiles using NADPH for K54Q and wild-type enzymes show divergences at certain pH values, suggesting the possibility of alteration(s) in the steps of the catalytic pathway for the K54Q enzyme.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) (DHFR)[1] catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (FAH_2).[1] This enzyme is necessary for maintaining the intracellular pools of tetrahydrofolate (FAH_4)[1] and its derivatives, which are essential cofactors in many biosynthetic reactions. DHFR is a target enzyme for a group of antifolate drugs that are widely used as antitumor and antibacterial agents, such as methotrexate (MTX)[1] and trimethoprim (TMP).[1] Because of its biological and pharmacological importance, DHFR has been the subject of extensive structural and kinetic studies.

One of the common features of DHFRs thus far examined is the discrimination between NADPH and NADH. NADH can serve as a cofactor for most DHFRs from different sources; however, in every case the K_m value is much higher for NADH than for NADPH, and in most cases the V_{max} using NADH is only 10–30% that for the reaction with NADPH. In certain cases DHFRs have no activity when NADPH is replaced by NADH (Blakley, 1984).

^{31}P NMR studies on NADPH and $NADP^+$ bound to the *Lactobacillus casei* reductase have been interpreted to indicate that the 2′-phosphate is in the dianionic state in both enzyme–$NADP^+$ and enzyme–NADPH complexes and that the

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[1] Abbreviations: MTX, methotrexate (4-amino-10-methyl-4-deoxyfolic acid); DHFR, dihydrofolate reductase; hDHFR, human dihydrofolate reductase; FAH_2, dihydrofolic acid; FAH_4, tetrahydrofolic acid; TMP, trimethoprim; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized); NADPH, nicotinamide adenine dinucleotide phosphate (reduced); NAD^+, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; IPTG, isopropyl β-D-thiogalactopyranoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; MATS, 50 mM Tris, 25 mM acetate, 25 mM MES, and 100 mM NaCl; ELISA, enzyme-linked immunosorbent assay.

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pK_a of the 2′-phosphate group must differ by at least three units from its value in free NADPH (Feeney et al., 1975). These authors have suggested that an electrostatic interaction between the phosphate group and a cationic group or groups makes a major contribution to the 100-fold difference in the K_m values for NADPH and NADH observed for DHFR. X-ray crystallographic studies of *L. casei* and *Escherichia coli* DHFR have indicated that the 2′-phosphate makes hydrogen bonds and salt bridges to three conserved residues, among them is Arg-43 (*L. casei*) which is involved in two standard hydrogen bonds with two of the three oxygens of the 2′-phosphate (Filman et al., 1982). X-ray structural studies of hDHFR have shown that the interactions between the 2′-phosphate and hDHFR are very similar to those in the bacterial DHFRs (Oefner et al., 1988). The corresponding residue for Arg-43 (*L. casei*) in hDHFR is Lys-54. The homologous residue in most other DHFRs of known sequence is either lysine or arginine, except in T4 phage DHFR which, surprisingly, has an alanine at this location based on homology alignments (Kraut & Matthews, 1987). Although all of the above results suggest the importance of Lys-54 or its corresponding residues in NADPH binding and in differentiating K_m values for NADPH and NADH for various DHFRs, it is not known how the differences in K_m values in NADPH and NADH relate to k_{cat} for these two coenzymes. It has been shown that the activation of hDHFR by organomercurial compounds (Delcamp et al., 1983) or by certain amino acid substitutions (Huang et al., 1989; Prendergast et al., 1989) is associated not only with increases in k_{cat} but also with increases in K_m values for both NADPH and FAH_2 as well. Thus, the higher K_m value for NADH may or may not be a consequence of the lower k_{cat} for this coenzyme compared with those for NADPH. In an attempt to determine the contribution of Lys-54 in differentiating K_m and k_{cat} for NADPH and NADH in hDHFR, Lys-54 was replaced by glutamine (K54Q hDHFR) by oligonucleotide-directed mutagenesis. This substitution also allowed an investigation into the role of this particular residue in protein structure as well as other quantifiable kinetic parameters of hDHFR that cannot be predicted from the structure of this enzyme.

Experimental Procedures

Materials. Restriction endonucleases, T4 polynucleotide kinase, and T4 ligase were purchased from Bethesda Research Laboratories; oligodeoxynucleotide-directed in vitro mutagenesis system version 2 and [α-^{35}S]ATPαS were purchased from Amersham; a Sequenase kit was purchased from United States Biochemicals; a Geneclean kit was purchased from BIO 101 Inc. NADPH, $NADP^+$, NADH, and NAD^+ were purchased from Sigma. MTX was a generous gift from Dr. John A. R. Mead (Division of Cancer Treatment, NCI); FAH_2 was prepared from commercial folic acid by dithionite reduction (Blakley, 1960). The 19-base oligodeoxynucleotide probe used for mutagenesis 5′-ATTATGGGTCAGAAGACCT-3′ and the three sequencing primers were synthesized in this laboratory by using an Applied Biosystems Model 380A automated DNA synthesizer and purified by electrophoresis on a 15% polyacrylamide gel as described by Applied Biosystems (1984). The plasmid pDFR, an expression vector for the hDHFR cDNA (Prendergast et al., 1988), was isolated according to alkaline lysis protocols, followed by cesium chloride–ethidium bromide density gradient centrifugation (Maniatis et al., 1982). The single-stranded template for mutagenesis was obtained by cloning the C-terminal portion of the hDHFR cDNA between the *Eco*RI and *Pst*I sites of pDFR into M13 vector (Thompson, unpublished) and purified as described by Amersham (manual of oligonucleotide-directed mutagenesis system version 2).

Oligonucleotide-Directed Mutagenesis of hDHFR cDNA. Conversion of the Lys-54 codon (AAG) to the Gln-54 (CAG) was carried out according to a single-stranded protocol described by Amersham (manual of oligonucleotide-directed mutagenesis system version 2, code RPN 1523) with some minor modifications. The phosphorylated mutagenesis primer was annealed with the single-stranded recombinant M13 and extended by Klenow polymerase in the presence of T4 DNA ligase and dCTPαS as well as dATP, dGTP, and dTTP. Then the nonmutant strand was selectively nicked by *Nci*I and digested by exonuclease III. The mutant strand was then repolymerized with DNA Pol I and ligated to generate double-stranded closed circular DNA. An aliquot of the reaction mixture was used directly to transform competent TG1 cells. The mutants were identified by single-base sequencing of 10 randomly picked plaques.

Reconstruction of the Expression Vector for K54Q hDHFR. The RF form of the positively identified phage was prepared by a standard plasmid-preparative method and digested with *Eco*RI and *Pst*I. The small fragment, which contains the partial cDNA sequence of hDHFR, was isolated on 0.7% agarose gel, the band was then cut out, and the DNA was extracted from the agarose by using the Geneclean kit. The plasmid pDFR was also digested with *Eco*RI and *Pst*I, and the large fragment was isolated on a 0.8% agarose gel followed by electroelution. The two fragments were then mixed with the small fragment being 10-fold in excess of the larger one and ligated with T4 ligase at 16 °C overnight. The ligation mixture was then used directly to transform competent JM107 cells. The plasmid was then isolated and sequenced by the double-stranded dideoxy sequence method as previously described (Sanger et al., 1977).

Expression and Purification of the Wild-Type and K54Q hDHFR. The wild-type hDHFR was expressed and purified as previously described (Huang et al., 1989) except that Zubay's medium (KH_2PO_4, 10 g; K_2HPO_4, 28.9 g; yeast extract, 10 g; glucose, 10 g; and thiamin, 10 mg/L) was used for growth of the bacterial cells. The K54Q mutant hDHFR was expressed in the same way as the wild-type hDHFR. The purification steps were carried out at 4 °C. Half of the cells from the 14-L culture were resuspended in 300 mL of buffer A (50 mM Tris-HCl, pH 6.5, 1 mM EDTA, and 10 mM β-mercaptoethanol) with 1 mM phenylmethanesulfonyl fluoride and 2 mg/L leupeptin (lysis buffer). Lysozyme (150 mg in 7 mL of lysis buffer) was added to the suspension, followed by incubation on ice for 15 min. $MgCl_2$ (1 M, 900 μL) and 24 mg of DNase I were added, and the suspension was placed on ice for another 15 min. The suspension was then centrifuged at 12000 rpm for 15 min. To the supernatant was slowly added ammonium sulfate to a final concentration of 85% with gentle stirring overnight. Following centrifugation at 18000 rpm for 30 min, the pellet was resuspended in 150 mL of lysis buffer and loaded onto a 1.5 cm × 3 cm MTX–aminohexyl-Sepharose 4B column equilibrated with buffer A. After being extensively washed with buffer B (buffer A + 500 mM KCl) and buffer A, the column was eluted with FAH_2 (2 mg/mL in buffer A, pH 8.0). The fractions containing DHFR activity were pooled and concentrated to ca. 2 mg/mL in an Amicon ultrafiltration apparatus fitted with a YM-10 membrane and stored at −70 °C or subjected to a Sephadex G-50 column (1.5 cm × 20 cm) equilibrated with 50 mM Tris-HCl and 10 mM β-mercaptoethanol, pH 7.5, to remove excess FAH_2. The fractions containing DHFR activity were

used for all studies described herein and stored at 4 °C for no more than 3 days. Enzyme purity was evaluated by SDS–PAGE.[1] To the purified, wild-type hDHFR was added FAH_2 to a final concentration of 2 mg/mL, and the mixture was then subjected to gel filtration on a Sephadex G-50 column prepared as above for the K54Q mutant. The fractions containing DHFR activity were used to obtain data for the wild-type enzyme.

Protein Determination. Enzyme concentration was determined by both MTX titration (Williams & Morrison, 1981) and UV absorption at 280 nm (E_M = 28 000) in the absence of FAH_2 (Huang et al., 1989). MTX concentration was determined spectrometrically at 258 nm (E_M = 23 250) and 302 nm (E_M = 22 100) (Seeger et al., 1949).

Kinetic Studies. Dihydrofolate reductase activity and initial reaction rates were measured as previously described (Huang et al., 1989) as were FAH_2, NADPH, and NADH concentrations. Tris-HCl (50 mM), pH 7.5, was used in all the assays except for pH–rate studies, where a three-component buffer system was used (50 mM Tris, 25 mM acetate, 25 mM MES, and 100 mM NaCl, MATS buffer) to maintain constant ionic strength over the pH range examined (Williams & Morrison, 1981). Measurements to obtain the K_i value for $NADP^+$ were made from initial velocity experiments and calculated by using either Dixon plots or double-reciprocal plot methods.

Determination of Equilibrium Dissociation Constants (K_D Values) for $NADP^+$. All measurements were made in 0.05 M Tris-HCl and 0.025 M 2-mercaptoethanol, pH 7.5, at 20 °C. For $NADP^+$ binding to the hDHFR–FAH_2 binary complex, association rate constants (k_{on}) for wild-type and K54Q enzymes and the dissociation rate constant (k_{off}) for the K54Q reductase were determined on the basis of relaxation measurements as described in Appleman et al. (1990). The value of k_{off} for wild-type hDHFR was too small to determine accurately by relaxation and was therefore measured in the following experiment. A solution containing 200 μM NADPH was placed in one drive syringe of a stopped-flow instrument (Applied Photophysics SF.17MV). A second solution containing 12 μM wild-type hDHFR, 16 μM $NADP^+$, and 10 μM FAH_2 was admixed and placed in the second drive syringe. Catalysis was initiated by mixing equal volumes of these two solutions (final concentrations: 6 μM enzyme, 8 μM $NADP^+$, 5 μM FAH_2, and 100 μM NADPH). Since FAH_2 is substoichiometric with enzyme, a single turnover of enzyme is sufficient to convert all of this substrate to product, and the k_{obs} for the reduction of FAH_2 was 4.3 s^{-1}. The rate constant for dissociation of $NADP^+$ from wild-type hDHFR·$NADP^+$·FAH_2 (k_1 in Scheme I) is less than k_{obs} because alternate pathways occur in which FAH_2 rather than $NADP^+$ dissociates from this complex and lead to the formation of the ternary substrate complex (Scheme I).

ELISA. The binding of wild-type and K54Q enzymes to antibodies generated against cyanogen bromide peptides 15–52, 53–111, and 140–186 derived from hDHFR was assayed by using the competitive ELISA assay described by Ratnam et al. (1988) except that 50 mM Tris-HCl, pH 7.5, was used instead of 10 mM sodium phosphate buffer (pH 7.5)/100 mM sodium chloride.

Data Analysis. Data obtained for K_m determinations by varying FAH_2 or NADPH concentration were fitted to eq 1 by a nonlinear regression program to yield values for V_{max} and the Michaelis constant K_m, as well as V_{max}/K_m.

$$V = V_{max}[S]/(K_m + [S]) \quad (1)$$

The best-fit value of k_{off} for $NADP^+$ in the wild-type enzyme·$NADP^+$·FAH_2 ternary complex was calculated with the program CRICF (Chandler et al., 1972) and equations appropriate for a simplified version (Scheme I) of the complete wild-type hDHFR kinetic scheme (Appleman et al., 1990).

Table I: Comparison of Kinetic Properties of the Mutant K54Q and Wild-Type hDHFRs

	K54Q	wild type	K54Q/ wild type
k_{cat} (s^{-1}), NADPH	18.5	7.4	2.5
k_{cat} (s^{-1}), NADH	2	1.2	1.7
k_{cat} (%), NADH/NADPH	11	16	
K_m (μM), NADPH	15	0.26	58
K_m (μM), NADH	71	18	3.9
K_m (x-fold), NADH/NADPH	4.7	69	
K_m (μM), FAH_2	0.18	0.032	5.6
k_{cat}/K_m (s^{-1} μM^{-1}), NADPH	1.2	28.5	0.043
k_{cat}/K_m (s^{-1} μM^{-1}), NADH	0.028	0.067	0.42
k_{cat}/K_m (%), NADH/NADPH	2.3	0.24	
K_i (μM), $NADP^+$	30	0.37	81

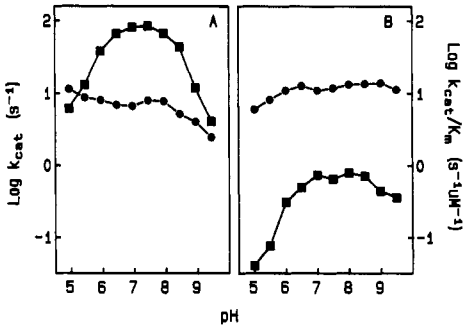


FIGURE 1: pH dependence of k_{cat} (●) and k_{cat}/K_m (NADPH) (■) for the K54Q DHFR (B) and the wild-type enzyme (A). The reaction conditions were 50 μM FAH_2, 1.1 nM enzyme, and 12–200 μM NADPH for the K54Q enzyme and 25 μM FAH_2, 64 nM enzyme, and 0.5–3 μM NADPH for wild-type enzyme at 22 °C in MATS buffer.

Results and Discussion

The K54Q hDHFR mutant cDNA was constructed according to a single-stranded protocol, and the expression vector pDFR was reconstructed to express the mutant cDNA (see Experimental Procedures). The entire coding region for the K54Q mutant was determined by double-stranded dideoxy sequence analysis. The results (data not shown) verified that the correct substitution of Lys-54 → Gln-54 had occurred and demonstrated that no other alteration in the hDHFR coding region was present. Both wild-type and K54Q were expressed and purified to homogeneity as determined by SDS-PAGE (data not shown).

Human DHFR, as with most DHFRs studied, discriminates between NADPH and NADH. With NADH as the coenzyme at pH 7.5, the k_{cat} for the wild-type enzyme is only 16% that observed with NADPH, while the K_m is 69-fold higher than that for NADPH. The K_i for $NADP^+$ for the wild-type enzyme at pH 7.5 is 0.37 μM (Table I), while NAD^+ had no inhibitory effect on the enzyme (data not shown). The pH–rate profiles for the wild-type enzyme using NADPH and NADH are shown in Figures 1 and 2, respectively. With NADPH, the profile shows two pH optima, one at ca. pH 5 and another at ca. pH 7.5. The double pH optima profile is shifted to a single optimum centered at ca. pH 6.5 when NADH is used as the coenzyme. These differences in pH–rate profiles may reflect differences in the kinetic pathways for these two coenzymes in hDHFR.

The steady-state kinetic properties of the K54Q mutant hDHFR are shown in Table I. The mutant has a k_{cat} value

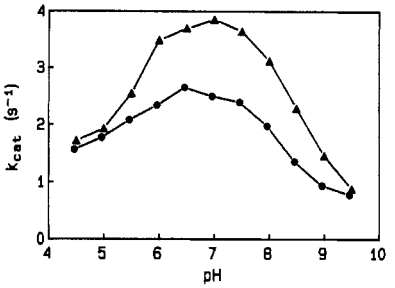


FIGURE 2: pH dependence of k_{cat} with NADH as cofactor for K54Q (▲) and wild-type enyzme (●). The reaction conditions were 200 μM NADH, 46 μM FAH_2, and 32 nM enzyme in MATS buffer at 22 °C.

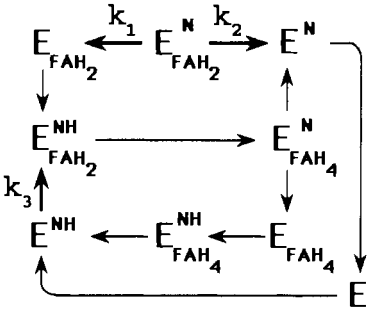
Table II: Kinetic Binding Constants for $NADP^+$ for Wild-Type and K54Q Human DHFRs in Enzyme–Dihydrofolate Binary Complexes[a]

enzyme	$k_{on} \times 10^{-6}$ (M^{-1} s^{-1})[b]	k_{off} (s^{-1})	$K_D \times 10^{-6}$ (M)[c]
WT	74 ± 5	3.8 ± 0.3[d]	0.051 ± 0.005
K54Q	4.1 ● 0.2	26.3 ± 2.6[b]	6.4 ± 0.07
K54Q/WT	0.06	6.9	125.5

[a] Except where noted these values were calculated from time courses of fluorescence transients accompanying ligand binding (relaxation) and from time courses of absorbance or fluorescence transients accompanying ligand dissociation and binding of other ligands (competition). Differences in buffer composition between this and our previous study (Appleman et al., 1990) are responsible for the altered kinetics of $NADP^+$ binding to the wild-type hDHFR·FAH_2. [b] From relaxation measurements as described in Appleman et al. (1990). [c] $K_D = k_{off}/k_{on}$. [d] Calculated from competition with NADPH (see Experimental Procedures).

of 18.5 s^{-1} with NADPH as cofactor and a k_{cat} of 2 s^{-1} with NADH as cofactor. These values are 2.5-fold and 1.7-fold higher than the corresponding k_{cat} values for the wild-type enzyme, respectively. The K_m for NADPH for K54Q is 58-fold higher than that of the wild-type DHFR, while the K_m for NADH for K54Q is only 3.9-fold higher than that of the wild-type enzyme. It is this decrease in the apparent affinity for NADPH that makes this coenzyme a much less "suitable substrate" for the K54Q enzyme as judged by the first-order rate constant k_{cat}/K_m compared to the wild-type DHFR, while the "suitability" of NADH for both the mutant and wild type are of the same order of magnitude. The 58-fold increase in K_m for NADPH for K54Q compared to that of the wild-type enzyme also indicates that, among the interactions between amino acid side chains and the 2′-phosphate, Lys-54 plays a major role in contributing to the binding energy. It accounts for ca. 93% of the ratio of K_m(NADH)/K_m(NADPH) observed for the wild-type hDHFR (4.7 for K54Q hDHFR; see Table I). It has been shown that $NADP^+$ behaves as a classical competitive inhibitor with respect to NADPH with the wild-type human enzyme (Prendergast et al., 1989). In the case of the K54Q mutant enzyme, $NADP^+$ is also a competitive inhibitor with a K_i value of 30 μM, while NAD^+ has no inhibitory effect on the enzymic activity of either K54Q or the wild-type enzyme. As indicated in Table II, the binding of $NADP^+$ is decreased ca. 125-fold in the K54Q·FAH_2·$NADP^+$ ternary complex, based on measurements of association (k_{on}) and dissociation (k_{off}) rate constants. For the K54Q mutant enzyme the k_{on} is decreased 18-fold, while the k_{off} is increased ca. 7-fold (Table II). These results indicate that the apparent affinity for both NADPH and $NADP^+$ has been drastically decreased in the K54Q mutant enzyme. In addition, the K_m for FAH_2 is elevated in the K54Q mutant hDHFR ca. 5.6-fold compared to that in the wild-type enzyme. Differences in the catalytic pathways for the K54Q versus

Scheme I[a]



[a] Symbols: E, wild-type hHDFR; NH, NADPH; N, $NADP^+$; FAH_2, dihydrofolate; FAH_4, tetrahydrofolate.

wild-type hDHFR could explain the increase in K_m for FAH_2, as could alterations in the interaction of FAH_2 with the enzyme–NADPH complex. Alternatively, the FAH_2 binding domain might be directly affected by the amino acid substitution due to the overlapping of the two binding domains although it is believed that Lys-54 in hDHFR does not interact with FAH_2.

A simplified kinetic scheme operative for wild-type hDHFR at concentrations of enzyme, substrates, and $NADP^+$ used in the determination of k_1, the rate constant for $NADP^+$ dissociation from the wild-type hDHFR·$NADP^+$·FAH_2 complex, is shown in Scheme I. Steps that limit the rate of catalysis are depicted in bold arrows. Other operative steps (thin arrows) are sufficiently fast that they do not limit the overall process. Processes not specifically depicted (such as FAH_2 dissociation from hDHFR·NADPH) are sufficiently slow compared to other steps that they need not be considered here. The value of k_2 (for FAH_2 dissociation from E·$NADP^+$·FAH_2) was measured by competition with methotrexate as described in Appleman et al. (1990) and equals 0.5 s^{-1} in the buffer system employed herein. The value of k_3 (for FAH_2 binding to E·NADPH) is 1.5×10^8 M^{-1} s^{-1} (Prendergast et al., 1989).

Despite the 58-fold increase in K_m for NADPH, the k_{cat} for the K54Q enzyme is elevated 2.5-fold compared to that of the wild-type enzyme. It has been shown that activation of hDHFR is often associated with a general loosening of the overall conformation of the protein and a single amino acid substitution can cause such a conformational change (Huang et al., 1989). The same approaches that had been used to reveal a conformational change in a W24F mutant hDHFR (Huang et al., 1989) have been utilized to address the question of possible structural alterations. These techniques include competitive ELISA using peptide-specific antibodies against hDHFR (Ratnam et al., 1988) and protease susceptibility studies. As indicated in Figure 3A–C, in all three cases of the antibodies tested, wild-type and K54Q enzymes compete for the antibodies, inhibiting their binding to the immobilized denatured enzyme to about the same extent. Since the antigenicity of any region of a protein molecule is correlated with the accessibility (Novotny et al., 1986) and conformational flexibility (Westhof et al., 1984) of that region, the results of the ELISA suggest that there is no significant conformational difference between the wild-type and K54Q mutant. Protease susceptibility experiments also support this notion. Figure 4 demonstrates that, under the experimental conditions, in the absence of NADPH, both wild-type and K54Q have about the same sensitivity to protease inactivation (Figure 4B,D). These results suggest that the overall structural features of the two enzyme molecules are basically quite similar.

It is interesting that whereas K54Q hDHFR shows similar responses to the antibodies and proteases as compared to that

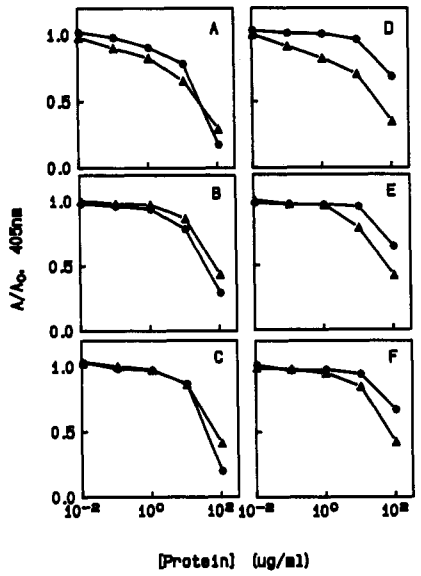


FIGURE 3: Competitive immunoassays using antisera to peptides 15–52 (A and D), 53–111 (B and E), and 140–186 (C and F). ELISA assays were carried out with denatured hDHFR as the immobilized antigen. Antisera to cyanogen bromide peptides 15–52, 53–111, and 140–186 were used at 1/100, 1/200, and 1/1000 dilutions, respectively. The antisera (50 μL) were preincubated for 2 h at 22 °C in the absence and in the presence of various concentrations of wild-type (●) and K54Q (▲) DHFRs with (D–F) or without (A–C) the addition of 100 μM NADPH. The antibody solutions were then applied directly onto the microtiter plate and assayed as described under Experimental Procedures. A_0 = absorbance in the absence of either wild-type or mutant DHFRs during the preincubation. A = absorbance in the presence of various forms of wild-type or mutant DHFRs at the indicated concentration.

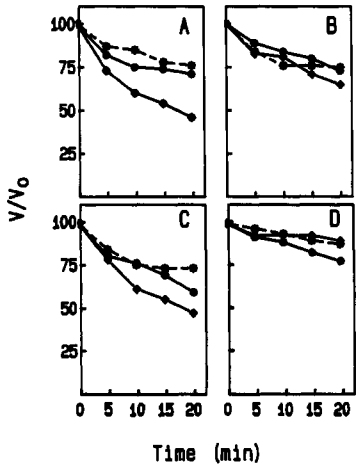


FIGURE 4: Proteolytic susceptibility and thermal stability (*) of the K54Q enzyme (A), K54Q enzyme + NADPH (B), wild-type enzyme (C), and wild-type enzyme + NADPH (D) to chymotrypsin (◆) and *Staphylococcus aureus* V8 protease (●). The DHFRs (150 μg/mL) were incubated with a 6 μg/mL quantity of the proteases in the presence or absence of 150 μM NADPH at 30 °C in 50 mM Tris-HCl and 10 mM 2-mercaptoethanol, pH 7.5. Aliquots were removed from the incubation mixture at the time intervals indicated and assayed. V_0 = DHFR activity at time 0. V = DHFR activity at the indicated time. * = enzyme without proteases.

for the wild-type enzyme in the absence of NADPH, in the presence of NADPH the K54Q enzyme shows quite different responses as compared to that for the wild-type. It has been demonstrated that the binding of ligands, such as NADPH, reduces the overall conformational mobility of hDHFR, thereby resulting in a reduction in the binding of the antibodies to the enzyme (Ratnam et al., 1988). As indicated in Figure 3, in the presence of 100 μM NADPH (panels D–F), the

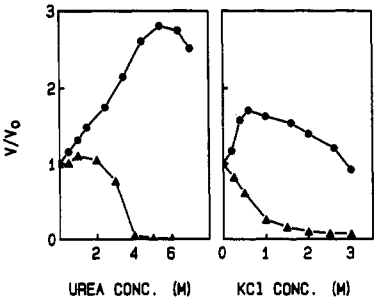


FIGURE 5: Effects of urea and potassium chloride on the enzymic activity of wild-type (●) and K54Q (▲) DHFRs. The enzymes were assayed in the presence of various concentrations of urea or potassium chloride as indicated in 50 mM Tris-HCl, pH 7.5, at 22 °C. V_0 = DHFR activity in the absence of urea or potassium chloride. V = DHFR activity at the indicated concentration of urea or potassium chloride.

binding of the wild-type enzyme to all three of the antibodies is reduced compared to the binding in the absence of NADPH (Figure 3A–C). Such an effect is not observed for the K54Q mutant enzyme. As shown in Figure 3, the binding of the K54Q enzyme to the antibodies is the same in the absence or presence of 100 μM NADPH. This insensitivity in the binding of the K54Q enzyme to antibodies in the presence or absence of NADPH reflects either that the binding of NADPH does not reduce the overall conformational flexibility of the K54Q enzyme as is the case for wild-type enzyme or that the binding of NADPH is so weak that the constraints on the conformational flexibility of the enzyme upon NADPH binding are insignificant. In the protease susceptibility experiments, NADPH protects wild-type enzyme from protease inactivation as well as thermal denaturation (Figure 4C,D), whereas NADPH only protects K54Q from protease inactivation, but not from thermal denaturation (Figure 4A,B). These results suggest that the conformational constraints imposed upon the K54Q enzyme molecule caused by NADPH binding are much weaker compared to that of the wild-type enzyme, presumably due to the weaker binding of NADPH to K54Q hDHFR.

The thermal stability of the substrate-bound K54Q enzyme in comparison with the substrate-bound wild-type enzyme is also shown in Figure 4. In the absence of NADPH, both K54Q and wild-type enzymes lose ca. 25% of their original activity after incubation for 20 min at 30 °C. In the presence of 150 μM NADPH, the wild-type enzyme loses only ca. 15% of the original activity. The presence of NADPH had no effect on the mutant enzyme, i.e., a loss of ca. 25% of the original activity after 20 min of incubation at 30 °C. However, the apoenzyme form of the K54Q mutant is extremely unstable. Attempts have been made to remove the bound substrate (FAH_2) from the affinity-purified K54Q enzyme by isoelectric focusing, which has been used to obtain substrate-free wild-type enzyme (Delcamp et al., 1983; Huang et al., 1989). After removal of the ampholines, following isoelectric focusing, by Sephadex G-50 gel filtration, the substrate-free K54Q enzyme loses ca. 60% of its original activity when stored at 4 °C overnight. Due to the instability of the K54Q apoenzyme, it was impossible to determine the K_D value for NADPH in an enzyme·NADPH binary complex by equilibrium methods.

The pH–rate profiles for the K54Q enzyme with either NADPH or NADH as a cofactor are shown in Figures 1 and 2. When NADH is used as cofactor, the pH–rate profile for the K54Q enzyme has the same pattern as for the wild-type enzyme, but the pH optimum has shifted from pH 6.5 for the wild type to pH 7 for the mutant. The pH–rate profile for K54Q enzyme with NADPH as cofactor, however, is quite different from that of the wild-type hDHFR. The two pH

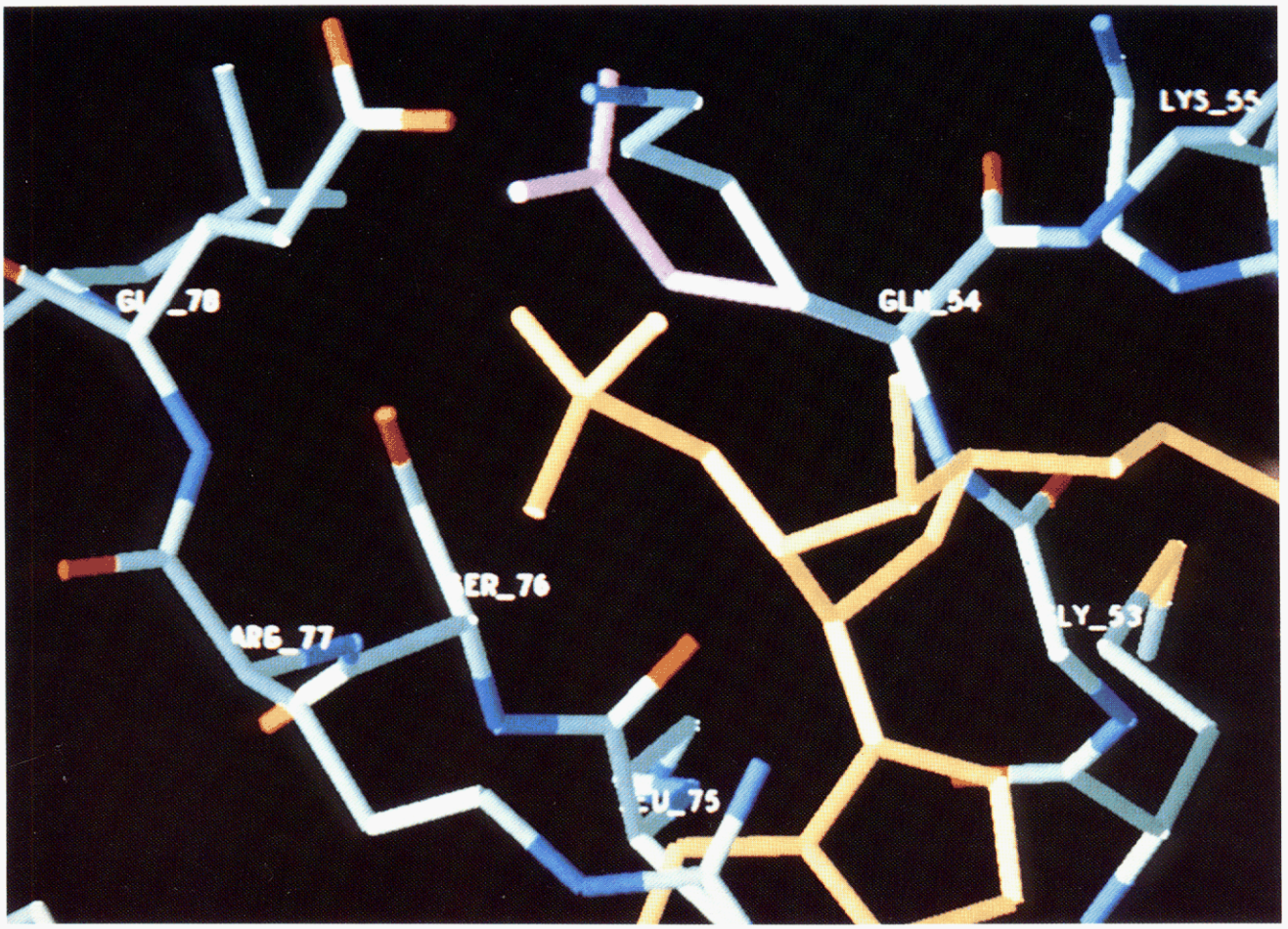


FIGURE 6: Complex of native vertebrate DHFR and $NADP^+$ in the vicinity of the 2′-phosphate. $NADP^+$ is shown in yellow. Atoms of the native protein are shown in "atom colors" (oxygen, red; nitrogen, blue; carbon, green). Coordinates are from the Brookhaven Protein Data Bank (Bernstein et al., 1977) for the chicken liver DHFR·$NADP^+$ complex (Volz et al., 1982; dataset 8DFR). All residues in this picture are identical in human and chicken liver DHFRs. In the native structure there is a strong hydrogen bond between the side chain of Lys-54 and one oxygen of the 2′-phosphate (N···O, 2.5 Å; N–H···O angle, 160°). The side chain of Gln-54 (in purple) is shown in its lowest energy conformation as predicted by the AMBER molecular mechanics force field (Weiner et al., 1984). Although the amide nitrogen can make a close approach to the phosphate oxygen (N···O, 3.3 Å), the N–H···O angle (112°) indicates a rather poor hydrogen bond.

optima have shifted from pH 5 and pH 7.5 for the wild-type enzyme to pH 6.5 and pH 9 for the mutant, and unlike the wild-type enzyme, which shows a rate enhancement at acidic pH, the catalytic rate for the K54Q mutant decreases as the pH is decreased from 6.5 to 5.0. The pH profiles of k_{cat}/K_m for NADPH were also determined as shown in Figure 1. In the entire pH range examined, the k_{cat}/K_m values for NADPH for K54Q were lower than that for the wild-type due to the increases in the K_m values for the mutant, while the patterns show some divergence at alkaline pH. The changes in these pH profiles indicate possible alterations in the catalytic pathway for the mutant enzyme as compared to that for wild type. It is possible that these alterations in the catalytic pathway of K54Q account for the increase in k_{cat} for this mutant enzyme despite the 58-fold increase in K_m for NADPH. Further kinetic studies on the K54Q mutant would be helpful to pinpoint these alterations, given the knowledge of the kinetic scheme for the wild-type human enzyme (Appleman et al., 1990).

Human DHFR is among those DHFRs that can be activated to different extents by certain concentrations of potassium chloride and urea (Delcamp et al., 1983). When the enzyme is activated either by treatment with organic mercurial compounds (Barbehenn & Kaufman, 1982) or by an amino acid substitution at a certain position (Huang et al., 1989), potassium chloride causes inhibition of enzyme activity, while urea at low concentration stimulates enzyme activity slightly. However, urea at concentrations higher than 2 M inhibits enzyme activity. The responses of the K54Q enzyme to potassium chloride and urea compared to those for wild-type enzyme are shown in Figure 5. Again, for the K54Q mutant, potassium chloride inhibits enzyme activity, whereas urea at low concentration activates slightly but causes enzyme inhibition at higher concentrations.

The interactions made by Lys-54 and Gln-54 with the 2′-phosphate of $NADP^+$ are indicated in Figure 6. That the replacement of lysine at position 54 with a glutamine should produce a 125-fold decrease in the binding of $NADP^+$ in the K54Q enzyme·FAH_2·$NADP^+$ ternary complex is not surprising. Lys-54 donates a hydrogen bond and forms an ionic interaction with the 2′-phosphoryl moiety. Gln-54, in contrast, is neutral and may not be able to form a strong hydrogen bond. The increase in K_D for the binding of $NADP^+$ to the hDHFR·FAH_2 complex corresponds to the loss of 2.8 kcal/mol in ΔG_{bind} for K54Q enzyme as compared to its wild-type counterpart.

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