Conversion of Arginine to Lysine at Position 70 of Human Dihydrofolate Reductase: Generation of a Methotrexate-Insensitive Mutant Enzyme†

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ABSTRACT: Arginine-70 of human dihydrofolate reductase (hDHFR) is a highly conserved residue which X-ray crystallographic data have shown to interact with the α-carboxylate of the terminal L-glutamate moiety of either folic acid or methotrexate (MTX). The rationale for this study was to introduce a conservative amino acid residue change at position 70 (Arg → Lys) which might function as a titratable group and, thus, reveal possible quantitative changes in ligand binding and kinetic parameters as a function of pH. Such a mutant enzyme (R70K) has been constructed and expressed by using site-directed mutagenesis techniques. This substitution has a dramatic effect on the binding of MTX, which displays a 22 600-fold increase in the dissociation constant (K_D) at pH 7.5 compared to that of the reported wild-type enzyme value. At this pH, the K_D value for dihydrofolate (FAH_2) for the R70K enzyme shows only a 7-fold increase over that for the wild-type hDHFR. The pH profiles of the Michaelis and dissociation constants for FAH_2 and K_D values for MTX for the mutant enzyme all show a 7–8-fold increase from pH 7.5 to 8.5 as compared to its wild-type counterpart. The binding of NADPH or the nonclassical inhibitor trimetrexate (TMQ) to either the wild-type or the mutant enzyme does not show such pH-dependent characteristics. Thus, since FAH_2 and MTX interact with the guanidinium side chain of arginine-70 in the wild-type hDHFR, the replacement of this residue with a lysine in the R70K mutant appears to have resulted in the introduction of a titratable group with a perturbed pK_a value of ca. 8.3. The loss of the positive charge of the ε-amino group of the lysine side chain at high pH results in the observed decrease in substrate and inhibitor binding. The binding of NADPH and TMQ is relatively unaffected since they do not interact directly with the basic side chain of this residue. Other studies of the R70K mutant enzyme in the presence of urea and proteases indicate that the enzyme is in a more open conformation than that of the wild-type hDHFR. This more open conformation of the mutant hDHFR may have resulted in the loss of specific interactions between MTX and the enzyme, explaining the relatively larger increase in the K_D value for MTX at pH 7.5 compared to FAH_2. However, since no further conformational change is indicated at pH 8.5, and the binding of NADPH and TMQ to either enzyme is relatively unaffected by pH, the decrease in the binding of FAH_2 and MTX to the R70K hDHFR at pH 8.5 would appear to be specifically due to the loss of the positive charge of the ε-amino group on the lysine side chain. Arginine-70, therefore, appears to be important in substrate and inhibitor binding in tems of ionic interactions with the bound ligand, and also in the maintenance of the binding site structure.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) (DHFR)[1] catalyzes the NADPH-dependent reduction of dihydrofolate (FAH_2)[1] to tetrahydrofolate (FAH_4).[1] This enzyme plays an important metabolic role since FAH_4 and its one-carbon derivatives serve as coenzymes in purine, pyrimidine, and amino acid biosynthesis. Inhibition of this enzyme results in the depletion of cellular tetrahydrofolates and subsequently cell death. This inhibition forms the basis for treatment of neoplastic and infectious disorders with synthetic folate antagonists, most notably methotrexate (MTX)[1] and trimethoprim (TMP).[1] As a result of its biological and pharmacological importance, DHFR has been the subject of extensive structural and kinetic studies (Freisheim & Matthews, 1984). Chemical modification (Vehar & Freisheim, 1976) and ^{13}C NMR studies (Cocco et al., 1977) on DHFR have implicated the importance of arginine in ligand binding and enzyme activity. Reaction of DHFR from *Lactobacillus casei* with phenylglyoxyal results in a complete loss of enzyme activity. Preincubation of the enzyme with NADPH results in the protection of two arginine residues with complete retention of enzyme activity (Vehar & Freisheim, 1976). This suggests the involvement of an essential arginine at or near the active center of the enzyme. X-ray structural analysis of human DHFR (Oefner et al., 1988; Davies et al., 1990) indicates that folate is directly H-bonded to three conserved hydrophilic residues present at the active site of the enzyme. One of these, arginine-70, is invariant in all vertebrate and bacterial DHFRs of reported sequence (Freisheim & Matthews, 1984) and makes 2 H-bonds with the terminal L-glutamate moiety of either MTX or folate. This laboratory has utilized the technique of site-directed mutagenesis to determine the role of key amino acid residues, and to establish structure–function relationships for

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[1] Abbreviations: MTX, methotrexate (4-amino-10-methyl-4-deoxyfolic acid); DHFR, dihydrofolate reductase; hDHFR, human dihydrofolate reductase; FAH_2, 7,8-dihydrofolic acid; FAH_4, 5,6,7,8-tetrahydrofolic acid; TMQ, trimetrexate [2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]quinazoline]; NADPH, nicotinamide adenine dinucleotide phosphate (reduced); Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; MES, 2-(*N*-morpholino)ethanesulfonic acid; MATS, 50 mM Tris, 25 mM acetic acid, 25 mM MES, and 100 mM NaCl; TMP, trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine].

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human DHFR (hDHFR)[1] (Prendergast et al., 1988, 1989; Huang et al., 1989, 1990). In this study, a mutant was constructed in which Arg-70 was replaced by a lysine residue and the subsequent effects on ligand and inhibitor binding to hDHFR investigated.

Experimental Procedures

Materials. The 17-base oligodeoxynucleotide 5′-TAAGGTAAAATTAATTT-3′ and the sequencing primers were prepared in this laboratory on an Applied Biosystems Model 380 A automated DNA synthesizer. The synthetic oligodeoxynucleotides were purified by electrophoresis using a 15% polyacrylamide gel as described by Applied Biosystems (1984). The oligodeoxynucleotide-directed in vitro mutagenesis system (version 2, code RPN 1523) and [α-^{35}S]ATPαS were both purchases from Amersham. A sequenase kit was purchased from United States Biochemicals (Cleveland). MTX was a generous gift from Dr. J. A. R. Mead (Division of Cancer Treatment, NCI). The Bio-Rad protein assay kit was purchased from Bio-Rad Laboratories, Richmond, CA. Trimetrexate was a generous gift from Dr. L. Werbel of the Warner Lambert Pharmaceutical Research Division, Ann Arbor, MI. FAH_2 was prepared from commercial folic acid by dithionite reduction (Blakley, 1960). NADPH was purchased from Bethesda Research Laboratories. The plasmid pDFR, the expression vector for the hDHFR cDNA (Prendergast et al., 1988), was isolated by using cesium chloride–ethidium density gradient centrifugation on alkaline lysis treated preparations (Maniatis et al., 1982). The C-terminal portion of the hDHFR cDNA, located between the *Eco*RI and *Pst*I sites of pDFR, was cloned into the M13 vector and purified, as described by Amersham (manual of oligonucleotide-directed mutagenesis system version 2), to produce the single-stranded template used for mutagenesis reactions.

Oligonucleotide-Directed Mutagenesis of hDHFR cDNA. The conversion of Arg-70 to Lys-70 was generated by using a protocol developed and described by Amersham (manual of oligonucleotide-directed mutagenesis system 2), with the incorporation of some minor modifications. The 17-base mutagenesis primer was then phosphorylated and annealed with the single-stranded M13 template. The primer was then extended in a reaction mixture containing Klenow polymerase, T4 DNA ligase, dCTPαS, dGTP, and dTTP. The nonmutant strand was removed by selective nicking using the endonuclease NCl, followed by digestion with exonuclease III. The mutant strand was regenerated by polymerization using DNA Pol 1 and ligation to produce double-stranded close circular DNA containing the mutant sequence. An aliquot of this reaction mixture was then used to transform competent TG1 cells, and the mutants were identified by single-base sequencing of 10 randomly picked plaques.

Reconstruction of the Expression Vector for R70K hDHFR. The double-stranded replicative form of the mutant phage was prepared by plasmid preparative protocols. This was followed by digestion with *Eco*RI and *Pst*I to generate a small fragment containing a partial cDNA sequence of hDHFR. This fragment was isolated by electrophoresis on a 0.7% agarose gel. The small fragment band was then cut out and the DNA extracted by electroelution using a UEA model from International Biotechnologies, Inc. The pDFR expression vector was also digested with *Eco*RI and *Pst*I and the large DNA fragment isolated, again using a 0.7% agarose gel followed by electroelution. The large fragment was then mixed with a 10-fold excess of the small fragment in the presence of T4 ligase and incubated at 16 °C overnight. The ligation mixture was then used directly to transform competent JM107 cells, from which the plasmid was isolated and sequenced by using the double-stranded dideoxy sequence method as previously described (Sanger et al., 1977).

Expression and Purification of Wild-Type and R70K hDHFR. The wild-type hDHFR was expressed and purified as previously described (Huang et al., 1989). Purification of the mutant protein was carried out at 4 °C during all steps. Half of the cell pellet from a 14-L culture was resuspended in approximately 250 mL of lysis loading buffer (50 mM Tris-HCl, pH 7.0, 100 mM KCl, 2 mM β-mercaptoethanol, and 1 mM EDTA) with 1 mM phenylmethanesulfonyl fluoride, 2 mg/L leupeptin, and 2 mg/L pepstatin also added. Lysozyme (150 mg/L) in lysis buffer was added and the mixture incubated on ice for 30 min. $MgCl_2$ (1 M, 900 μL) and 24 mg of DNase I were then added, and the suspension was placed on ice for a further 30 min. The mixture was then centrifuged for 30 min at 27000*g*. Ammonium sulfate was added slowly to the supernatant to a final concentration of 90% with gentle stirring overnight. The suspension was then centrifuged at 27000*g* for 30 min, and the resulting pellet was resuspended in 150 mL of standard buffer (50 mM Tris-HCl, pH 7.0, 100 mM KCl, and 2 mM β-mercaptoethanol). The extract was loaded onto a 1.5 × 3 cm MTX aminohexyl-Sepharose 4B column which had been previously equilibrated with standard buffer. The column was then washed with 1–2 L of standard buffer until no protein absorbance at 280 nm was observed. The column was then eluted with FAH_2 (2 mg/mL) in standard buffer at pH 8.0. Fractions containing DHFR activity were pooled and concentrated to 1–2 mg/mL using an Amicon ultrafiltration apparatus fitted with a YM-10 membrane. The concentrated solution was then subjected to gel filtration on a Sephadex G-50 column (1.5 × 20 cm) which had previously been equilibrated with standard buffer. Fractions containing DHFR activity were concentrated to 1–2 mg/mL and subjected to ion-exchange chromatography on a 1.8 × 1.5 cm column containing the anionic resin Bio-Rex 9 (purchased from Bio-Rad Laboratories). The resin had been previously prepared by equilibration in 0.1 N HCl for 18 h, followed by exhaustive washing with water until free of acid. The resin was then resuspended and equilibrated with standard buffer, before loading. The protein was eluted with standard buffer, and 2-mL fractions were collected. The ultraviolet spectrum of each fraction containing enzyme was scanned, from which the dihydrofolate was judged to have been fully removed (Perkins et al., 1967). The concentrated enzyme samples were then stored frozen at −70 °C in standard buffer containing 10% glycerol.

DHFR Assays. DHFR activity was continuously recorded with a Varian Model 219 spectrophotometer, at 22 °C, by monitoring the decrease in absorbance at 340 nm resulting from the enzymatic conversion of FAH_2 and NADPH to FAH_4 and $NADP^+$, respectively. Initial rates could be determined by using a molar absorbance change at 340 nm of 12000 cm^{-1} (Kempton et al., 1982). The concentration of FAH_2 was determined by its absorbance at 280 nm, using a molar extinction coefficient of 2.8×10^4 M^{-1} cm^{-1} at pH 7.5 (Blakley, 1960), and also by use of the enzyme assay system described above. NADPH concentration was also determined in a similar manner using an extinction coefficient of 6.2×10^3 M^{-1} cm^{-1} at 340 nm (Horecker & Kornberg, 1948). Assays were carried out at 22 °C in Tris-HCl, pH 7.5, except for pH-dependent studies where a three-component buffer system was employed containing 50 mM Tris, 25 mM acetic acid, 25 mM MES, and 100 mM NaCl (MATS buffer). This system enabled constant ionic strength to be maintained over

the pH range used in these studies (Williams & Morrison, 1981).

Protein Determination. The concentration of the R70K hDHFR was determined by use of the Bio-Rad protein assay system, using wild-type hDHFR of known concentration as a standard. The protein concentration was also determined spectrophotometrically from its absorption at 280 nm (ϵ_M = 28 000) in the absence of FAH_2 (Huang et al., 1989).

Determination of Equilibrium Dissociation Constants (K_D). The quenching of protein fluorescence which occurs when hDHFR is titrated with various ligands was used to determine the stoichiometry and dissociation constants of various DHFR–ligand/inhibitor complexes (Perkins & Bertino, 1966). Titrations were carried out in MATS buffer at 22 °C using a Perkin-Elmer Model MPF-66 fluorescence spectrometer. The excitation and emission wavelengths were set at 290 and 350 nm, respectively. Fresh enzyme solution was used for each data point with the initial volume at 3.0 mL. The total volume of added ligand was limited to 40 μL.

Data Analysis. Data obtained from K_m determinations by varying the 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:

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

Data obtained from fluorescence titrations were initially plotted as $1/\Delta F$ vs $1/[L]$ to obtain the value of ΔF at a saturating concentration of ligand. At each concentration, the amount of ligand bound to the enzyme was calculated by use of eq 2 (Wong & Frey, 1978) where B = the concentration

$$B = T\left(\frac{1 - \Delta F_{obs}/\Delta F_{max}}{1 - F_E}\right) \quad (2)$$

of bound ligand, T = the total concentration of ligand, ΔF_{obs} = the observed change in fluorescence, ΔF_{max} = the maximum change in fluorescence, and F_E = the quenching coefficient (=fluorescence of the enzyme at saturating ligand concentration/fluorescence of the enzyme without added ligand).

A plot of bound/free vs bound ligand concentration (Scatchard analysis) yielded an approximate K_D value which was then used in eq 3 where F_0 = the initial fluorescence of

$$[A] = K_D(F_0/F - 1) + [E_0](1 - F/F_0) \quad (3)$$

protein, F = the measured fluorescence, [A] = the concentration of ligand added, and [E_0] = the total enzyme concentration.

From the above equation, a theoretical curve of F vs ligand concentration could be plotted, which was then compared to the experimental curve obtained from the actual titration data. Adjustments were then made, if necessary, to the K_D value, to obtain the best possible fit.

Relative Quantum Yield Determinations. The relative fluorescence quantum yields of wild-type and R70K hDHFR were determined with the excitation and emission monochromators set at 6.0 nm. Correction factors were generated by use of the manufacturer's instructions using Rhodamine 101 as a quantum counter. The scan rate was set at 120 nm/min, and the absorbance of each sample did not exceed 0.03 at the excitation wavelength. The relative fluorescence quantum yield was determined by comparing the fluorescence of the samples with that of a tryptophan solution (Teale, 1957).

Results and Discussion

Arginine-70 represents a highly conserved residue, identically located in all bacterial and vertebrate DHFRs. The replacement of this residue by a lysine represents a conservative change, but one in which any resulting differences in enzyme properties would be indicative of the importance of arginine in the mechanism and inhibition of DHFR. The steady-state kinetic properties of the R70K mutant enzyme and wild-type hDHFR are shown in Table I. The specific activity of the R70K DHFR is 3 units/mg, corresponding to a k_{cat} of 105 min^{-1}, a value approximately 7-fold lower than the wild-type k_{cat} of 739 min^{-1} (Prendergast et al., 1988). The pH–rate profiles for R70K and wild-type hDHFR are shown in Figure 1A. The wild-type and R70K profiles both indicate maximum activity at ca. pH 5.0. A drop in activity and enzyme stability is observed below pH 5.0 (data not shown). However, the wild-type enzyme has a second peak of activity between pH 7.0 and 8.0, while the R70K enzyme displays a gradual decrease in velocity as the pH is increased. As indicated in Table I, between pH 7.5 and 8.5, the K_m for FAH_2 for the R70K enzyme increased from a value 4-fold (0.57 μM) to 25-fold (3.72 μM) greater than the corresponding wild-type values (0.118 and 0.151 μM, respectively). At pH 7.5, the R70K enzyme K_m for NADPH (0.074 μM) was 3-fold higher than the corresponding wild-type value (0.024 μM). Unlike the FAH_2 K_m value, no significant increase in the NADPH K_m value was observed at pH 8.5, or above, for either the R70K or the wild-type enzymes. The pH dependence of k_{cat}/K_m for both R70K and wild-type hDHFRs is shown in Figure 1B. The k_{cat}/K_m values for the R70K enzyme were generally lower than those of the wild-type enzyme over the pH range tested.

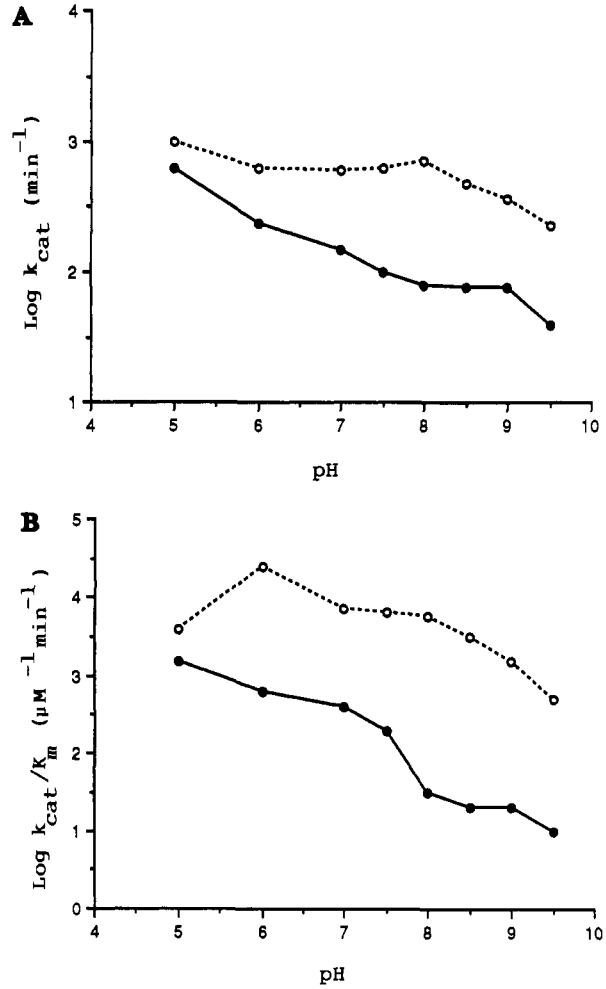

FIGURE 1: (A) R70K (●) and wild-type (○) hDHFR k_{cat} values as a function of pH. The enzymes were assayed over a pH range of pH 5.0–9.5 in MATS buffer at 22 °C. The substrate concentrations used were 30 μM NADPH and 30 μM FAH_2, with an enzyme concentration of 70 nM. (B) $k_{cat}/K_m(FAH_2)$ of wild-type (○) and R70K (●) hDHFR as a function of pH.

Table I: Steady-State Kinetic and Equilibrium Binding Properties of Wild-Type and R70K hDHFRs in the Presence of Various Ligands[a]

	R70K	wild type	R70K/ wild type
sp act (units/mg)	3	20	0.15
k_{cat} (min^{-1})	105	739	0.14
K_m (FAH_2) (μM)			
pH 7.5	0.47	0.12	4.0
pH 8.5	3.72	0.15	24.6
K_m(NADPH), pH 7.5 (μM)[b]	0.8	0.26	3
k_{cat}/K_m (μM^{-1} min^{-1})			
pH 7.5	223	6607	0.03
pH 8.5	21	3162	0.007
K_D(FAH_2) (μM)			
pH 7.5	0.23	0.033	7
pH 8.5	1.90	0.045	42
K_D(NADPH) (μM)			
pH 7.5	0.074	0.024	3
pH 8.5	0.121	0.030	4
K_D(MTX) (μM)			
pH 7.5	0.113	0.000005[c]	22600
pH 8.5	0.744		

[a] All parameters were measured in 50 mM Tris, 25 mM acetate, 25 mM MES, and NaCl (MATS buffer). [b] Determined in 50 mM Tris-HCl, pH 7.5. [c] Wild-type hDHFR value reported by Schweitzer et al. (1989).

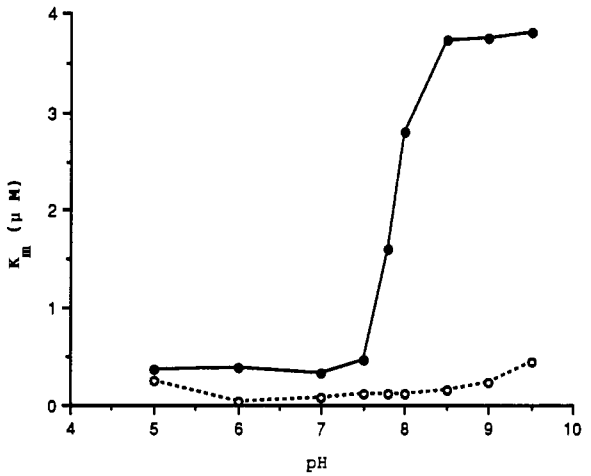

FIGURE 2: K_m(FAH_2) values of wild-type (O) and R70K (●) hDHFR as a function of pH. The K_m values for FAH_2 were determined as 22 °C using MATS buffer, with a NADPH concentration of 30 μM and 5–30 μM FAH_2. The enzyme concentration ranged from 1 to 3 nM.

There is, however, a significant decrease in the k_{cat}/K_m value for the R70K reductase from 223 to 21.2 μM^{-1} min^{-1} at pH 7.5 and 8.5, respectively. The R70K and wild-type DHFR K_m values for FAH_2 as a function of pH are shown in Figure 2. The data in the figure indicate that the major factor contributing to the decrease in the mutant enzyme k_{cat}/K_m from pH 7.5 to 8.5 is a corresponding increase in the K_m value for FAH_2. No further significant increase in the K_m value for FAH_2 for the R70K enzyme was observed above pH 8.5. The wild-type enzyme did not display any pH-dependent characteristics with respect to its K_m values for FAH_2, indicating that this property is unique to the R70K enzyme. The K_m values for NADPH for both the mutant and wild-type enzymes did not show the same pH-dependent changes, with the K_m varying little from pH 7.5 to 8.5.

The data in Figure 3 indicate that the R70K DHFR K_D values for FAH_2 vary from pH 7.5 to 8.5. An increase in the R70K enzyme K_D value for FAH_2 is observed from 0.233 to 1.4 μM, at pH 7.5 and 8.5 (Table I), respectively, with no further increase observed above pH 8.5. The wild-type DHFR K_D values for FAH_2 did not show significant pH dependency

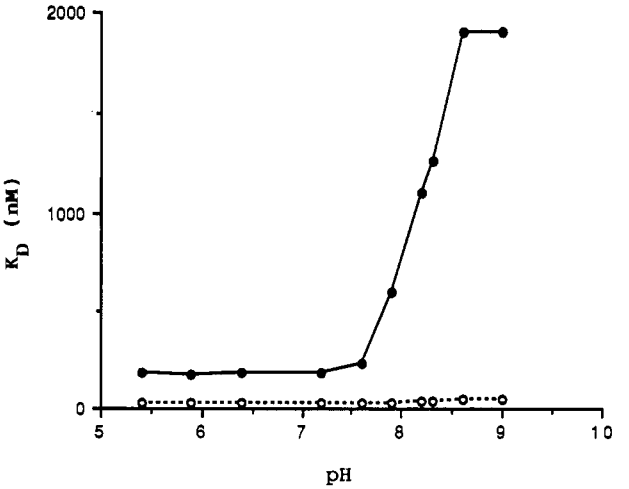

FIGURE 3: R70K and wild-type hDHFR K_D values for FAH_2 as a function of pH. The K_D values were obtained by monitoring the quenching of enzyme fluorescence following the addition of ligand. The titration was carried out in MATS buffer at 22 °C. After the addition of ligand, the mixture was mixed by inverting the curvette several times, and the mixture was then allowed to equilibrate for 1 min before a reading was recorded. The excitation wavelength was 290 nm, and the emission wavelength was 325 nm. The enzyme concentrations used were 50 nM wild-type hDHFR and 110 nM R70K enzyme.

when compared to the R70K enzyme (Figure 3). The R70K K_D profile suggests that binding of FAH_2 to this enzyme is pH-dependent and the resulting observed K_D values are an influence on the K_m values for FAH_2, as suggested by the similarity in the R70K K_m and K_D profiles. The K_D values for NADPH for both R70K and wild-type hDHFRs did not show any significant pH dependency. For the R70K enzyme, the K_D increased from 0.074 μM at pH 7.5 to 0.121 μM at pH 8.5 (Table I). The same proportional increase was observed for the wild-type enzyme NADPH K_D (from 0.024 to 0.032 μM) over the pH range of 7.5–8.5. No further increase in the K_D value for NADPH was observed above pH 8.5 for either enzyme. From these data, it would appear that the factors which confer pH dependency on FAH_2 binding to R70K do not significantly influence the binding of NADPH.

When used in a quenching of protein fluorescence titration with wild-type hDHFR, the inhibitor MTX results in a titration profile in which dissociation of the enzyme–inhibitor complex is so slight that the equivalence point of the curve can be used to accurately determine the enzyme concentration (Perkins & Bertino, 1966). Analysis of the protein fluorescence titration curve obtained when 1.1 μM wild-type enzyme was titrated with MTX at pH 7.5 (see Experimental Procedures) indicated a K_D value of less than 1 nM. It is not possible to accurately determine a K_D value less than 1 nM by using this method. Analysis of the profile obtained from the titration of 1.1 μM R70K mutant DHFR with MTX at pH 7.5 yielded a K_D of 113 nM (Table I), a value 22 600-fold greater than that reported for the wild-type enzyme of 5 pM at pH 7.5 (Schweitzer et al., 1989). Figure 4 shows the pH dependence of K_D values for MTX obtained in this fashion for the R70K enzyme. The profile indicates an increase in the K_D values from pH 7.0 (0.057 μM) to pH 8.5 (0.744 μM), qualitatively reflecting the pattern observed for the R70K K_D profile for FAH_2. These data indicate that while MTX displays a much greater reduction in its binding to the R70K enzyme than FAH_2, both molecules display similar pH-dependent binding profiles.

The substrate binding site of hDHFR occupies an extended cavity on one face of the central β-sheet. The pteridine moiety

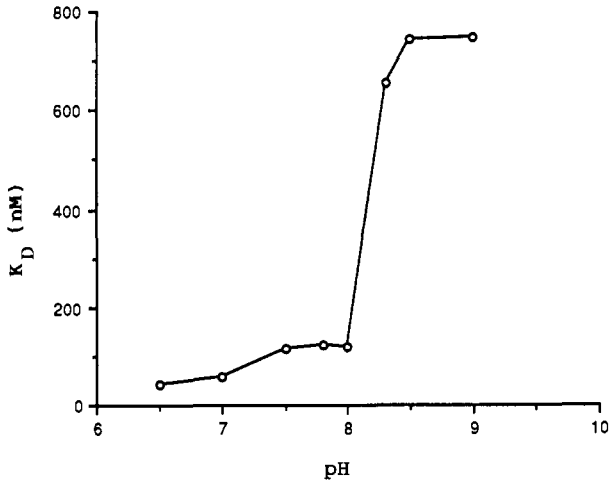

FIGURE 4: K_D(MTX) values for the R70K mutant hDHFR as a function of pH. Titrations of protein fluorescence were carried out in MATS buffer in the pH range of 6.5–9.0. The enzyme concentration was 1.1 μM. After addition of the ligand, the cuvette was inverted several times and the mixture allowed to equilibrate in the dark for 2 min before a reading was recorded. The total volume of added inhibitor did not exceed 40 μL.

of the substrate is located ca. 15 Å deep within this binding site surrounded by backbone carbonyl and polar side chains. This is followed by the *p*-aminobenzyl moiety which interacts with the side chains of five hydrophobic residues. The glutamate portion of the substrate extends toward the surface of the binding cleft where its α-carboxylate interacts strongly with the side-chain guanidinium of Arg-70, via two charge-mediated hydrogen bonds (Davies et al., 1990). The guanidinium group makes additional H-bonds with the –OH of Thr-38 and the carboxyl of Lys-68. Thus, Arg-70 is rigidly oriented in an otherwise hydrophobic environment (Oefner et al., 1988). The replacement of Arg-70 with a lysine appears to have resulted in the introduction of a titratable group into the ligand binding site of the enzyme. Considering the hydrophobic environment surrounding this lysine residue, it is reasonable to assume that the pK_a of its ϵ-amino group might be lowered. Figures 3 and 4 suggest that the apparent pK_a of this lysine is approximately 8.3 and that loss of the interaction between the positively charged basic side chain of this residue and the α-carboxylate of the substrate, or inhibitor, would decrease binding significantly. NADPH binding appears not to be influenced by this titratable group. Examples of highly perturbed pK_a's of groups in proteins include the ϵ-NH_2 group of lysine in acetoacetate decarboxylase. This amino acid is buried in a nonpolar region, resulting in a lowered pK_a of 5.4 (Schmidt et al., 1971). The His-159 of papain with a lowered pK_a of 3.4 is another example (Johnson et al., 1981).

Figure 5A shows the inhibition profile obtained when 50 nM of R70K hDHFR was titrated with MTX at pH 7.5 and at pH 8.5. The pH dependency of inhibition of the R70K mutant enzyme confirms a similar dependency on MTX binding (Figure 4). The additional decrease in inhibitor binding at pH 8.5 is indicated by the observed increase in the concentration of MTX required to cause 50% inhibition of enzyme activity (IC_{50}). A pH profile of the MTX IC_{50} values of the R70K hDHFR was also characterized by a 7–8-fold increase from pH 7.5 to 8.5, (data not shown) reflecting the pattern observed for the FAH_2 and MTX K_D profiles for this enzyme. Trimetrexate (TMQ)[1] is a 2,4-diaminoquinazoline which resembles the 2,4-diaminopteridine moiety of MTX but lacks the *p*-aminobenzoyl-L-glutamate moiety which interacts with Arg-70 of hDHFR. TMQ is a potent inhibitor of wild-

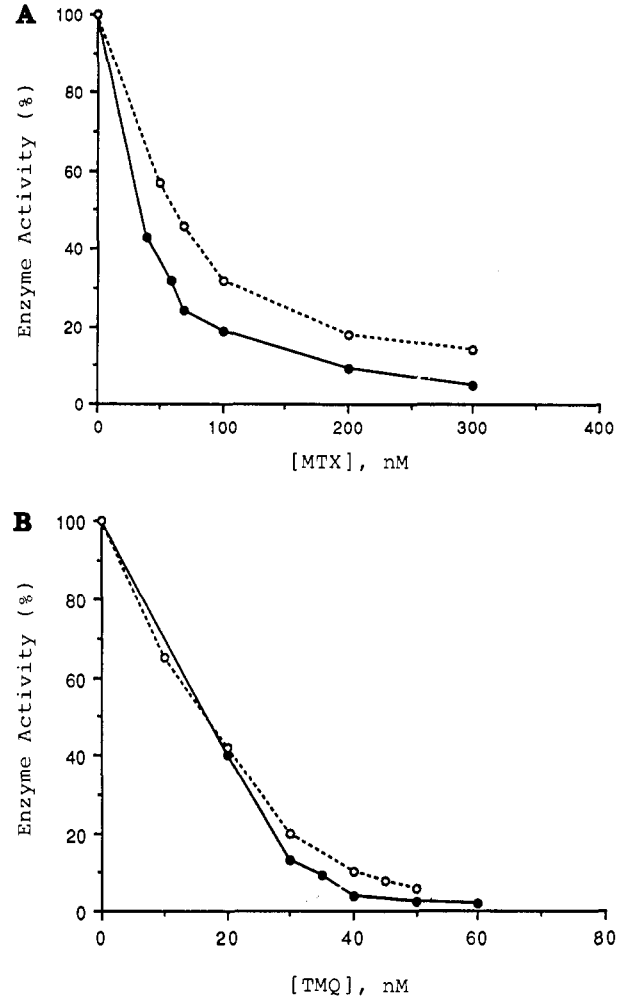

FIGURE 5: Inhibition of the R70K mutant hDHFR with MTX (A) or TMQ (B). The enzyme was incubated with 50 μM NADPH and various concentrations of either MTX or TMQ in MATS buffer at 22 °C. After a 2-min equilibration period, the reaction was assayed following the addition of 50 μM FAH_2. Titrations were carried out at pH 7.5 (●) and pH 8.5 (○) using an enzyme concentration of 50 nM (MTX inhibition) and 40 nM (TMQ inhibition).

type hDHFR. It was therefore of interest to investigate and compare the binding characteristics of TMQ relative to the wild-type and R70K hDHFRs as a function of pH. Figure 5B shows the inhibition profiles obtained when 40 nM R70K hDHFR was titrated with TMQ at pH 7.5 and 8.5. In contrast to the MTX titration, this inhibitor displayed virtually stoichiometric binding with no significant decrease in enzyme inhibition observed at pH 8.5. Fluorometric titration of R70K and wild-type hDHFRs with TMQ at pH 7.5 and 8.5 further indicates that the binding of this inhibitor is unaffected by pH (data not shown). The results indicate that although TMQ binds in the same general binding site as FAH_2 and MTX, it does not possess the same pH-dependent binding characteristics. These results suggest that the binding of TMQ to the R70K enzyme is unaffected by titration of the ϵ-amino group of the lysine side chain and only those molecules such as FAH_2 and MTX which bear a terminal *p*-aminobenzoyl-glutamate moiety interact with this group via charge-mediated H-bonds and display pH-dependent binding properties.

The UV absorption and fluorescence properties of wild-type and R70K hDHFRs are compared in Table II. As expected, both enzymes have the same molar extinction coefficient (28000) at 280 nm. When excited at 280 nm, the fluorescence emission spectra of the native and denatured forms of the R70K DHFR were similar to the corresponding spectra pro-

Table II: Fluorescence Properties of R70K and Wild-Type hDHFRs

	F_{max}[a] (native)	F_{max}[b] (denatured)	relative quantum yield native[a]	relative quantum yield denatured[b]
R70K	325	355	0.037	0.161
wild type	325	355	0.102	0.164

[a] In 0.1 M potassium phosphate, pH 7.0. [b] In 0.1 M potassium phosphate (pH 7.0) + 8 M urea.

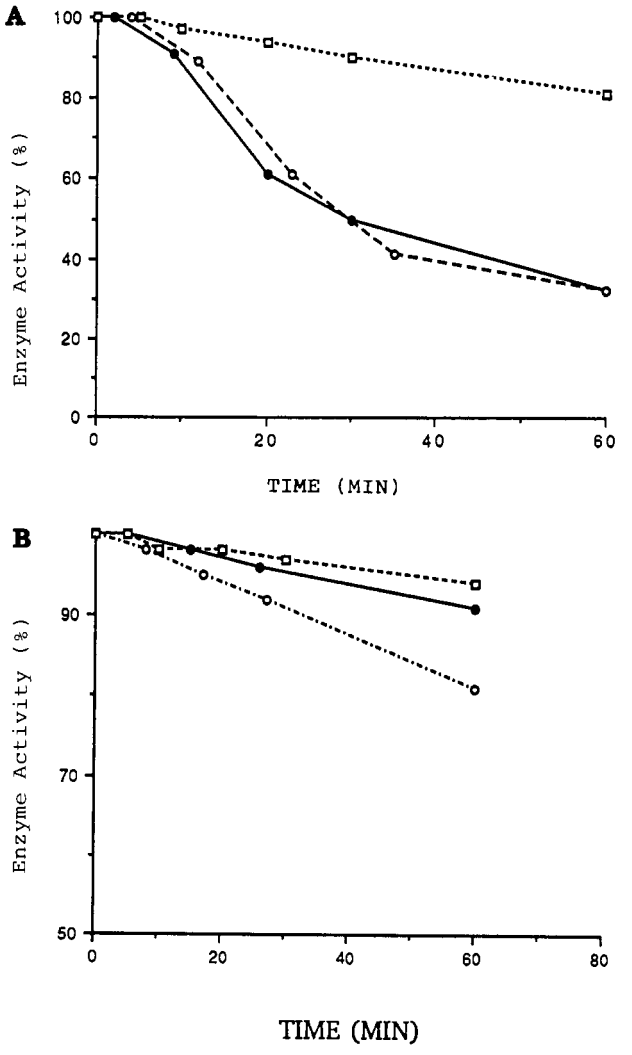

FIGURE 6: Proteolytic digestion of (A) R70K and (B) wild-type hDHFR. The enzymes (113 μg/mL) were incubated with 12 μg/mL either chymotrypsin or trypsin in 0.1 M potassium phosphate, pH 7.0, in the presence of 90 μM NADPH at 30 °C. Aliquots were taken from the reaction mixture at different time intervals and assayed. V_0 = the initial rate of reaction; V = the observed rate of reaction at a specified time interval; (□) = control, (fdt) = chymotryptic digest; (○) = tryptic digest.

duced by the wild-type enzyme (not shown). The relative fluorescence quantum yield of the native R70K enzyme (0.037) was only 36% that of the corresponding wild-type DHFR value at the emission maximum of 325 nm. Treatment of the R70K and wild-type proteins with 8 M urea shifted the emission maximum to 350 nm with an increase in the relative fluorescence quantum yield at this wavelength. Both the denatured wild-type and R70K proteins had approximately the same quantum yield values of 0.164 and 0.161, respectively. These results indicate that one or more of the three tryptophans in R70K DHFR are quenched relative to those in the native, wild-type enzyme. Denaturation of the R70K DHFR, however, results in a similar fluorescence quantum yield at 350 nm as that for the wild-type protein, representing the exposure

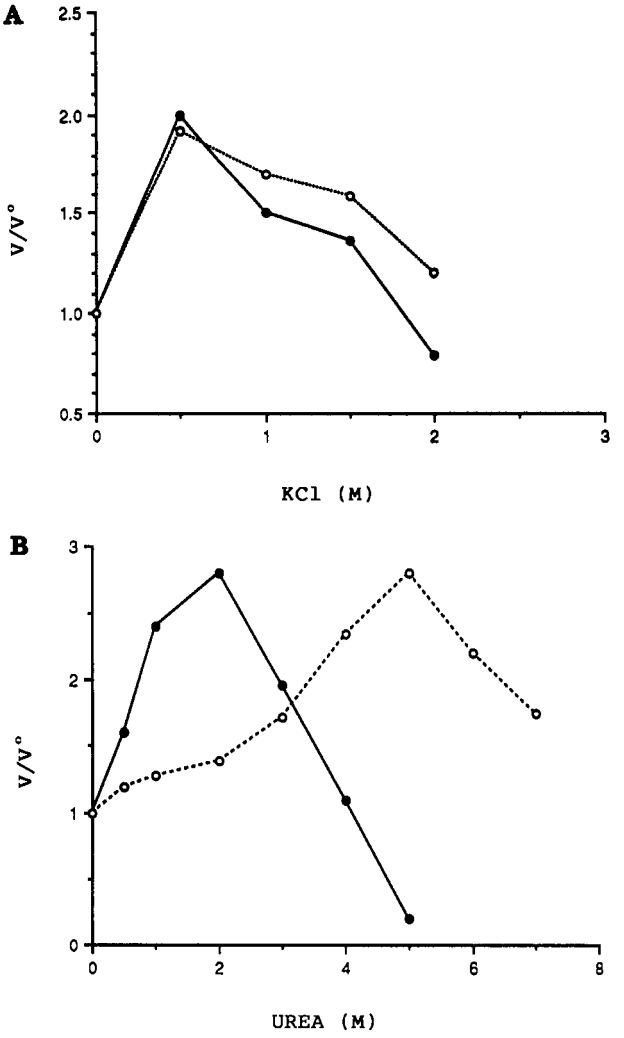

FIGURE 7: Effects of KCl (A) and urea (B) on the activity of wild-type (○) and R70K (●) hDHFR. Each enzyme (13 μg) was assayed in the presence of various concentrations of urea or KCl in 50 mM Tris-HCl, pH 7.0, at 22 °C. V_0 = initial rate of reaction. V = observed rate of reaction at a specified concentration of urea or KCl.

of the enzyme tryptophan residues to solution (Teale, 1957). The quantum yield of the native form of the R70K enzyme remained constant over the pH range 5.0–9.0 (data not shown), suggesting that the conformation of the R70K protein may not be altered by changes in this pH range.

The susceptibility of both wild-type and R70K hDHFRs to proteolytic digestion was investigated by using bovine trypsin and chymotrypsin. The reactions were carried out at 30 °C in 0.1 M potassium phosphate buffer containing 90 μM NADPH. The susceptibility of the R70K and wild-type enzymes to proteolytic digestion is shown in Figure 6A,B, respectively. The control experiment (no protease present) showed that after 60 min at 30 °C, the R70K hDHFR retained 82% of its original activity while the wild-type enzyme possessed 92% activity. The results indicate that R70K is more susceptible to proteolytic inactivation, losing approximately 68% activity when digested with trypsin or chymotrypsin. The wild-type enzyme lost ca. 13% activity due to tryptic digestion, while being largely resistant to chymotryptic digestion. These results suggest that the R70K enzyme may be in a more open conformation and, hence, more susceptible to proteolytic digestion than the wild-type DHFR.

A property of human DHFR is its ability to be activated to different extents by urea and KCl (Delcamp et al., 1983). Figure 7A,B shows the effects of KCl and urea on the activities

of both enzymes. KCl produces the same pattern of activation in both the R70K and wild-type enzymes, with 0.6 M KCl producing a 2-fold enzyme activation (Figure 7A). In the presence of urea, however, the R70K enzyme displayed a different pattern of activation from that observed for the wild-type DHFR (Figure 7B). Maximum stimulation of the R70K enzyme was achieved with 2 molar urea, which caused a 2.8-fold increase in activity, while this concentration resulted in a 1.2-fold increase in wild-type activity. At 5.0 M urea concentration, a 3-fold activation of wild-type hDHFR was observed while concentrations greater than 5 M completely inactivated the R70K enzyme. It would thus appear that the R70K DHFR, while in a more open conformation than its wild-type counterpart, still retains the ability to be activated in a similar manner to that of the wild-type enzyme. However, since the R70K enzyme is inactivated by urea concentrations greater than 5 M, it appears that as a result of its more open or altered conformation, the mutant enzyme is more sensitive to the effects of this chaotropic agent.

Since Arg-70 is known to make additional H-bonds with Thr-38 and Lys-68, the loss of one of these H-bonds may have resulted in a more flexible or open conformation of this mutant enzyme. This altered conformation has presumably affected the K_D values for FAH_2 and MTX at pH values 5.5–7.5; however, as fluorescence studies do not indicate any further conformational changes at pH 8.5–9.0, and since the binding of TMQ to the R70K enzyme is unaffected by pH, it would appear that the observed increases in the FAH_2 and MTX dissociation constants at pH 8.5 are due to titration of the ϵ-amino group of the lysine side chain rather than any further conformational changes in the enzyme. For the R70K mutant enzyme, both FAH_2 and MTX show approximately a 7-fold increase in their respective K_D values at pH 8.5 as compared to those at pH 7.5. The K_m for FAH_2 is also increased by this factor at pH 8.5. However, at pH 7.5, the K_D for MTX for the R70K DHFR is approximately 22 600-fold greater than the reported wild-type enzyme value, while the K_D for FAH_2 is only 7-fold greater than its corresponding wild-type DHFR value at this pH. At pH 8.5, the K_D value for MTX for the mutant R70K DHFR is increased nearly 150 000-fold, assuming that the K_D value for MTX in the case of the wild-type enzyme is essentially unchanged (Table I). It would, therefore, appear that the binding of MTX to the R70K mutant enzyme is dramatically more affected than is FAH_2 binding by the altered conformation of the mutant, but both are affected to the same degree by the titration of the ϵ-amino group of the lysine side chain. MTX is known to bind in an inverse orientation from bound folic acid and is known to be in contact with Glu-30 via ionic H-bonds with the N-1 and 2-amino groups of the pteridine ring (Freisheim & Matthews, 1984). In addition, Ile-7 and Val-115 also interact via H-bonds with the 4-amino group of the inhibitor. The more open conformation of the mutant enzyme may result in the loss of one or more of these unique inhibitor–enzyme interactions, thus greatly reducing the binding of the inhibitor to this enzyme but not affecting the binding of the substrate to the same relative degree.

In conclusion, it would appear that Arg-70 is an important residue in the binding of both substrate and inhibitor in terms of ionic interactions with the bound ligand and also in the maintenance of the structural integrity of the enzyme.

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