Mechanistic Studies of R67 Dihydrofolate Reductase
EFFECTS OF pH AND AN H62C MUTATION*

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R67 dihydrofolate reductase (DHFR) is encoded by an R-plasmid, and expression of this enzyme in bacteria confers resistance to the antibacterial drug, trimethoprim. This DHFR variant is not homologous in either sequence or structure with chromosomal DHFRs. The crystal structure of tetrameric R67 DHFR indicates a single active site pore that traverses the length of the molecule (Narayana, N., Matthews, D. A., Howell, E. E., and Xuong, N.-H. (1995) Nat. Struct. Biol. 2, 1018–1025). A pH profile of enzyme activity in R67 DHFR displays an acidic pKₐ that is protein concentration-dependent. This pKₐ describes dissociation of active tetramer into two relatively inactive dimers upon protonation of His-62 and the symmetry-related His-162, His-262, and His-362 residues at the dimer-dimer interfaces. Construction of an H62C mutation results in stabilization of the active tetramer via disulfide bond formation at the dimer-dimer interfaces. The oxidized, tetrameric form of H62C R67 DHFR is quite active at pH 7, and a pH profile displays increasing activity at low pH. These results indicate protonated dihydrofolate (pKₐ = 2.59) is the productive substrate and that R67 DHFR does not possess a proton donor.

Dihydrofolate reductase (DHFR, EC 1.5.1.2.3) reduces dihydrofolate (DHF) to tetrahydrofolate using NADPH as a cofactor. DHFR is important in folate metabolism as the reaction product, tetrahydrofolate, is required for the synthesis of thymidylate, purine nucleosides, methionine, and other metabolic intermediates. The chromosomally encoded DHFR from Escherichia coli utilizes a general acid to facilitate catalysis (1) and has specific binding sites for both substrate (DHF) and cofactor (NADPH) (2). It has also been designated a highly evolved enzyme with a calculated efficiency of 0.15 (3). Hydride transfer rates are faster than the rate-determining step, which is release of product, tetrahydrofolate (4). In addition, ab initio quantum mechanical calculations suggest protein-mediated electronic polarization of bound DHF and NADPH may help lower energy barriers and aid catalytic function (5–7). Additionally, an overlap between the binding sites for NADP⁺ and 5-deazafolate in human DHFR has been proposed to facilitate catalysis by compressing the distance between C-6 of DHF and C-4 of NADPH to a separation that is optimal for hydride transfer (8). These studies all indicate that chromosomal DHFR has developed various mechanisms to maximize catalytic efficiency.

A different DHFR has emerged recently due to use of trimethoprim as a clinical drug to treat numerous bacterial infections. Trimethoprim is an active site-directed inhibitor of chromosomal DHFR. Resistance to trimethoprim has been correlated with the production of novel DHFRs encoded by R-plasmids. Type II R-plasmid-encoded R67 DHFR is unrelated genetically to chromosomal DHFRs (9, 10).

To compare this novel R-plasmid-encoded DHFR with its chromosomal counterpart, several crystal structures have been determined. A crystal structure of dimeric R67 DHFR was reported by Matthews et al. (11). More recently, the active homotetramer has been crystalized both as an apoenzyme and in a binary complex with folate (12). Difference Fourier maps describing bound folate indicate the active site is a pore traversing the length of the molecule and that residues from each monomer contribute to the single active site. The center of the pore possesses exact 222 symmetry, indicating R67 DHFR binds DHF and NADPH in an unusual manner. The difference Fourier map for bound folate shows the pteridine ring in the center of the pore. The electron density can be fit by two asymmetric binding sites each present at 1/4 occupancy (12). Due to steric constraints, a maximum of two ligands can bind concurrently (13). A productive model for catalysis proposes binding of DHF in half the pore and binding of cofactor in the other half. The pteridine ring of dihydrofolate and the nicotinamide ring of NADPH encounter each other at the center of the pore. This model of catalysis suggests R67 DHFR uses related sites (due to 222 symmetry) for binding of ligands and each half-pore accommodates either DHF or NADPH. However, DHF and NADPH are bound in slightly different orientations.

The recent evolutionary origin of R67 DHFR coupled with this unusual binding scheme hints that R67 DHFR may not be a very efficient catalyst. To probe the efficiency of the R67 DHFR reaction further and contrast it with the E. coli chromosomal DHFR, a series of experiments have been designed to investigate the pH dependence of the R67 DHFR reaction. As indicated above, a general acid has been proposed to facilitate catalysis in E. coli chromosomal DHFR (1). In a D27S² mutant of E. coli chromosomal DHFR, the proton donor, Asp-27, was removed by site-directed mutagenesis. The D27S² DHFR partially compensated by binding pre-protonated DHF (pKₐ = 2.59); this was reflected in an increased kcat at low pH where protonated dihydrofolate predominates. Alternately, a role for Asp-27 in tautomerization of bound substrate (14) or in alteration of the pKₐ of N-5 for bound substrate (15) has been
proposed. When the crystal structure of R67 DHFR is examined for potential proton donors, the only residue identified in the active site pore is Tyr-69, as well as its symmetry-related residues Tyr-169, Tyr-269, and Tyr-369. Thus we pose the question, does R67 DHFR utilize a proton donor to facilitate catalysis as does chromosomal DHFR?

During a series of experiments to answer the above question, we found that the ionization describing reduction of protonated dihydrofolate is masked by dissociation of tetrameric R67 DHFR to dimers. Thus we additionally ask, what are the relative activities of dimeric and tetrameric R67 DHFR? And can the tetramer be stabilized by site-directed mutagenesis to introduce disulfide bonds across the dimer-dimer interfaces? This paper addresses these questions.

MATERIALS AND METHODS

Construction and Purification of H62C R67 DHFR—The H62C mutation was constructed by standard site-directed mutagenesis techniques using a synthetic R67 DHFR gene (16) and the following oligonucleotide: 5'-TGAGCCGCGGACCTGACAG-3'.

The entire mutant H62C R67 DHFR gene was sequenced to confirm that no additional mutations were present. This mutant gene conferred trimethoprim resistance upon E. coli, indicating that an active DHFR was produced.

H62C R67 DHFR was purified according to the protocol used to purify wild type (wt) R67 DHFR except that E. coli cells were lysed by sonication and reducing agents were omitted from buffers (16). 5 mM EDTA was included in the buffers to minimize air-oxidation of sulfhydryl groups. Purification steps included G75 molecular sieving, DEAE-Fractogel and DEAE-Sephalochromatography, and a Mono-Q column on a Pharmacia FPLC system.

Steady State Kinetics—Steady state kinetic data were obtained with a Perkin-Elmer λ3a spectrophotometer interfaced with an IBM PS2 according to Howell et al. (17). The computer program UVSL3 (Softways, Moreno Valley, CA) was used in data collection and analysis. Assays were performed at 30 °C in a polybuffer containing 50 mM acetic acid + 50 mM MES + 100 mM Tris + 10 mM β-mercaptoethanol (MFA buffer). This buffering system maintains a constant ionic strength between pH 4.5 and 9.5 (18). For the wt R67 DHFR pH profile, the enzyme concentration was held constant at all pH values unless noted. Enzyme was diluted and incubated in the cuvette for 5 min prior to initiation of the essay by addition of 110 mM dihydrofolate and 64 mM NADPH. Non-enzyme hydrolysis rates were measured and subtracted from enzyme rates.

Formation of NADPD, used for measuring isotope effects, involved the reaction of alcohol dehydrogenase from Leuconostoc mesenteroides (Boehringer Mannheim) on 1,1-dideuterioethanol (MSD Isotopes) and NADP+ (Sigma). This reaction was coupled with NADP+ aldehyde dehydrogenase (Sigma) to allow the reaction to approach completion (17, 19). The dehydrogenases were removed from NADPD by ultrafiltration through a YM-10 membrane. Chromatography on a DEAE-Fractogel column (0–4 mM KCl gradient) removed any unreacted NADP+. NADPD was subsequently desalted by chromatography on a Bio-Gel P-2 column and lyophilized.

A model to describe the behavior of R67 DHFR as a function of pH is shown in Scheme I, where T is tetrameric R67 DHFR; DHF is protonated dimer; Kd and αKd are ionization constants; Kc, Ks, and αKc are binding constants; and Ks, describes the linked dissociation and ionization of tetramer to 2 DH (20).

From Scheme I, an equation can be derived to describe the concentration of DHF:

\[
[DHF] = -1 + \left[1 + \left[8K_{overall}(P_{tot})[H]^{n}\right] + [NADPH]K_{d}\right] + [H][HDHF] \frac{[NADPH]K_{d}}{[H]} + \left[\frac{[HDHF][NADPH]K_{c}K_{s}}{(K_{d} + [NADPH])K_{c}K_{s}}\right] + \left[\frac{[HDHF][NADPH]K_{c}K_{s}}{[H][K_{d}]}\right] + \left[\frac{[HDHF][NADPH]K_{c}K_{s}}{[H]^{2}}\right]
\]

(Eq. 1)

The amino acids in the first monomer are labeled 1–78; those in the second monomer, 117–178; the third monomer, 217–278; and the fourth monomer, 317–378. For brevity, when a single residue is mentioned in the text, residues in all four subunits are implied.

\[\text{where [P}_{\text{tot}}\text{]}\text{ describes the total protein concentration and equals DHF} + 2T + 2T \cdot \text{NADPH} + 2T \cdot \text{NADPH} \cdot \text{DFHF} + 2T \cdot \text{NADPH} \cdot \text{DFHF} \cdot \text{DFHF} \text{. (HDHF) describes the concentration of protonated substrate and equals [DFHF}_{\text{total}}/[1 + K_{c}[H]), where }K, K_{c}, \text{ and } K_{s} \text{ represent } K_{c} \text{ values describing binding of DHF and DFHF to E-NADPH, and } K_{s} \text{ describes binding of NADPH to apoenzyme (13).}

The concentration of T-NADPH-DHF can be calculated as shown below.

\[\text{[T-NADPH-DHF]} = \frac{[\text{DHDF}][\text{NADPH}]K_{s,K,s}[K_{c}[H]^{2}]^{2}}{[\text{P}_{\text{tot}}]} \text{ (Eq. 2)}
\]

Enzyme activity can then be described by the fraction of T-NADPH-DHF present multiplied by the pH-independent kcat value.

\[k_{\text{cat}} = k_{\text{cat,max}} \cdot T \cdot \text{NADPH} \cdot \text{DFHF}[\text{P}_{\text{tot}}] \text{ (Eq. 3)}
\]

Three different concentrations of R67 DHFR yielded three different pH-dependent kcat profiles, which were fit globally to Equation 3 using the nonlinear regression program, NONLIN (22).

For H62C R67 DHFR, Kc values for NADPH and DHF were obtained by varying both DHF (4–25 μM) and cofactor (8–80 μM) concentrations at subsaturating levels; primary and secondary plots were calculated according to Cleland (23). The pH profiles for kcat and kcat/Kc were fit to Equation 4,

\[\text{log } k_{\text{cat}} = \text{log } k_{\text{cat,max}} - \log (1 + \chi [P_{tot}]) \text{ (Eq. 4)}
\]

where kcat,max is the pH-independent value of kcat or kcat/Kc and χ values of 10, 5, and 2 represent slopes of 1, 0.5, and 0.2 (24). Fitting with χ permits a non-integral slope. Data were fit using the NLIN program in the Statistical Analysis Systems program package (Sas Institute Inc., Cary, NC).

Circular Dichroism and Gel Filtration—Circular dichroism spectra of wt and H62C R67 DHFRs in 10 mM KH2PO4 at pH 7.0 and 5.0 were obtained at 22 °C using a Jasco J720 spectropolarimeter. Cell path-length was 2 mm. Ten spectra were acquired per sample using 1-μm steps and 2-s integrations, and an averaged spectrum calculated. An essentially flat buffer baseline scan was then subtracted from the average protein scan. The CD data are described as the mean residue ellipticity by taking 111 g/mol as the mean residue molecular weight.

Gel filtration using a Superose 12 HR10–30 column on a Pharmacia FPLC was performed according to Nichols et al. (2). Reducing and non-reducing Ellman’s titrations were done according to Creighton (25) and Riddles et al. (26). Concentrations were determined using the following extinction coefficients: 28,000 M–1 cm–1 at 282 nm for DHF (27); 6220 M–1 cm–1 at 340 nm for DHF (28). The molar extinction coefficient used to assess DHFR reduction of DHF was 12,300 M–1 cm–1 (29). Enzyme concentrations were determined by biuret assays and all proteins were homogeneous according to SDS-polyacrylamide gel electrophoresis.

Oxidation/Reduction Studies of the H62C Disulfide Bond—The disulfide bond stability was assayed according to Sauer et al. (30). Oxidized H62C R67 DHFR was mixed with 8.8 μM oxidized dithiothreitol (Sigma) and varying concentrations of reduced dithiothreitol (0.05–0.1 mM; Life Technologies, Inc.) and incubated at room temperature for 24
Figure 1. pH profiles of $k_{cat}$ in R67 DHFR at three different protein concentrations. $\bigcirc$, $\square$, and $\triangledown$ points correspond to 828 nM, 370 nM, and 166 nM R67 DHFR expressed as dimer. Each point corresponds to an initial rate with 110 $\mu$M dihydrofolate and 64 $\mu$M NADPH present. These ligand concentrations are approximately 20 times the $K_m$ values observed at pH 7.0. The theoretical curves were generated by globally fitting the data to eq 3. Best fit values are given under “Discussion.” Since the basic branch of the pH profile does not show any protein concentration dependence, increasing amounts of enzyme were used to measure activity from pH 5.0 to 9.88 ($\times$3 points); however, these points were not used in the global analysis.

and 48 h. The reactions were quenched by addition of 0.3 volume of 1 M iodoacetic acid. All experiments were performed under a nitrogen atmosphere in TBE buffer (10 mM Tris, 1 mM EDTA, pH 8). The dimer/tetramer concentrations in the quenched samples were analyzed using a Superose 12 HR10–30 column on a Pharmacia FPLC. Peak areas were measured by weighing on a balance.

RESULTS

pH Profile Studies on Wild Type R67 DHFR—The only potential proton donating group in the active site pore is Tyr-69 as well as its symmetry-related residues, Tyr-169, Tyr-269, and Tyr-369. To determine if tetrameric R67 DHFR uses Tyr-69 as a proton donor, we examined a pH profile of R67 DHFR activity. If Tyr-69 were a general acid participating in catalysis, we would expect to observe a bell-shaped profile displaying a $K_a$ of pH 9.5–10.5, corresponding to ionization of tyrosine (assuming no effects due to environment). On the other hand, if R67 DHFR does not use Tyr-69 as a general acid, we would expect to see increasing activity at low pH as the $pK_a$ for protonation of dihydrofolate at N-5 is approached ($pK_a = 2.59$; Ref. 21), similar to the pH profile exhibited by D278E, E. coli chromosomal DHFR (1). Instead of either of these patterns, a pH profile of $k_{cat}$ displays a bell shape as shown in Fig. 1. From the pH profile at high pH, we can conclude that if Tyr-69 is acting as a proton donor, its contribution to catalysis must be minimal since a titration is not observed up to pH 9.88. Activity measurements above this pH were not performed due to the high enzyme concentrations needed. Because of the size of the active site pore (~18 Å wide by ~24 Å long) and consequent solvent accessibility, it seems unlikely that the $pK_a$ of Tyr-69 would be perturbed.

The apparent $pK_a$ value of 6.15 ($P_{tot} = 166$ nM dimer) observed in Fig. 1 suggests histidine may be ionizing and affecting enzyme activity. A single histidine (His-62) occurs per monomer at the dimer-dimer interface. From the 112 symmetry of the tetrameric crystal structure, 2 histidines appear per dimer-dimer interface (i.e., His-62 and His-362 at one interface, and His-162 and His-262 at the second symmetry-related interface). Protonation of His-62 ($pK_a = 6.84$) has previously been found to destabilize tetrameric R67 DHFR and favor dimer formation (20). As the active site pore is lost when tetramer dissociates to dimer, decreased activity results.

Since dissociation of tetrameric R67 DHFR to 2 dimers is a bimolecular reaction, the ionization describing this process should be protein concentration-dependent. To test this, the activity associated with three protein concentrations was monitored as a function of pH. As seen in Fig. 1, the basic portion of the bell-shaped curves overlays fairly well. However the acidic titration clearly shows a protein concentration dependence, consistent with dissociation of active tetramer to less active dimers.

Engineering a Stable Tetramer—Since protonation of His62 is linked to dissociation of active tetramer into inactive or partially active dimers, any potential activity increase at low pH due to protonation of dihydrofolate in solution (p$K_a = 2.59$) will be masked. To investigate whether R67 DHFR utilizes protonated or nonprotonated DHF as the productive substrate, we constructed a stable tetramer to eliminate the $T + 2$H$^+ \equiv 2D_H^+$ ionization from the pH profile.

The distance between the α-carbons of histidines 62 and 362 (or 162 and 262) at the dimer-dimer interface is 9.6 Å in R67 DHFR. The β-carbon distance is 7.8 Å. Sowdhamini et al. (31) have proposed that CA-CA distances of ≤5.5 Å and CB-CB distances of ≤4.5 Å indicate a good potential for disulfide bond formation. However, Matsumura and Matthews (32) have constructed disulfide bridges in T4 lysozyme with CA-CA distances of 8.1 Å. While the distances in R67 DHFR are slightly longer, a H62C substitution was modeled using INSIGHT (Biogen Technologies) and a disulfide bond generated. The model appeared feasible, thus a H62C mutation was constructed in the R67 DHFR gene as described under “Materials and Methods.”

Disulfide Formation in H62C R67 DHFR—A majority of purified H62C R67 DHFR is oxidized and tetrameric as determined by molecular sieving studies and Ellman’s titrations. A reduced, dimeric component can also be observed, which upon extended incubation produces stable, oxidized tetramer. This result indicates the H62C mutation has greatly increased the $K_d$ describing the tetramer $\equiv$ 2 dimers equilibrium, most likely due to the unfavorable distance between cysteines for disulfide bond formation. Previously, the $K_d$ for wt R67 DHFR was estimated to be ≤50 nM by sedimentation equilibrium and 9.72 nM by fitting of protein concentration-dependent pH titration of fluorescence (20). The relatively small dimer-dimer interface areas indicate stacking and hydrogen bond formation between His-62 and His-362 and between His-162 and His-262 are a major determinant of association state.

Disulfide bond formation between H62C R67 DHFR dimers does occur spontaneously in vitro, albeit slowly. Numerous procedures were initially investigated to facilitate disulfide bond formation, for example disulfide exchange (33, 34) or mercuric ion cross-bridging (35). However, the easiest and most efficient method found was to incubate dimeric H62C R67 DHFR at high protein concentrations (~15 mg/ml), pH 8.8, 4 °C for >1 week. Since the $K_d$ for tetramer formation in H62C R67 DHFR is elevated, high protein concentrations were necessary to facilitate tetramer formation and subsequent disulfide cross-linking. Generation of active, cross-linked tetramer was readily monitored by either increased enzyme activity and/or by molecular sieving chromatography and confirmed by Ellman’s titrations (see below). From the chromatography results, two peaks are observed during elution from a Superose 12 HR10–30 column (Pharmacia FPLC), indicating tetrameric and dimeric species are not in rapid equilibrium in H62C R67 DHFR. With increasing time (days → weeks), the first peak increases in area. The first peak was identified as active tetramer (S-S cross-linked) and the second peak as reduced dimer.
by Ellman’s titrations, $K_{av}$ values (and thus predicted molecular weights) and by the first peak possessing virtually all the enzyme activity. To separate active tetramer from dimer, ion-exchange chromatography on a Mono-Q column with a 0.05–0.07M KCl gradient in 10 mM Tris, pH 8 was utilized (Pharmacia FPLC). Purified dimer was maintained in buffer 1 mM dithiothreitol. Alternatively, molecular sieving chromatography on G-75 Sephadex at pH 5 allows separation of reduced, dimer from oxidized tetramer.

Ellman’s titrations were performed to verify the formation of disulfide bonds in tetrameric H62C R67 DHFR. A single sulfhydryl per monomer, Cys-47, occurs on the surface of R67 DHFR. Non-reducing Ellman’s titrations yield 0.78 and 0.99 free sulfhydryls/monomer for wt and oxidized, tetrameric H62C R67 DHFRs, respectively (average of ≥2 determinations). In contrast, reducing Ellman’s titrations yield 1.1 and 2.2 sulfhydryls/monomer (average of 2 determinations) for wt and oxidized, tetrameric H62C R67 DHFR, respectively. These values clearly confirm a stable tetrameric species has been constructed by disulfide cross-linking.

As shown in Fig. 2, a comparison of elution position for wt R67 DHFR from a Superose 12 HR 10–30 column at pHs 5 and 8. Panels A and B show the elution pattern for wt R67 DHFR at pH 8.0 and 5.0, respectively. Panels C and D show the elution pattern for the oxidized, tetrameric H62C R67 DHFR at pH 8 and 5, respectively.

Unusual, however, as both the shape and size of the molecule affect elution position (36).

Circular dichroism studies were performed to assess the effects of the H62C mutation on the secondary structure of R67 DHFR (Fig. 3). The spectra for oxidized, tetrameric H62C R67 DHFR at pH 5 and 7 correlate well with the spectrum of wt, tetrameric R67 DHFR at pH 7.0. However, the spectra for reduced, dimeric H62C R67 DHFR at both pH 5 and 7 show significant differences from the spectrum for wt, dimeric R67 DHFR at pH 5.0. Since dimeric H62C R67 DHFR possesses minimal activity (see below) and is stably folded (37), the change in CD signal must reflect a change in protein conformation/environment. Since His-62 is near Trp-38 at the dimer-dimer interface and His-62 is protonated at pH 5 in wt R67 DHFR, perhaps the H62C substitution affects the contribution of Trp-38 to the CD signal. Woody (38) indicates aromatic side chains can make detectable contributions to the far-UV CD. In contrast to the spectra for wt R67 DHFR, the spectra for oxidized and reduced H62C R67 DHFR do not show pH-dependent changes since they are locked in their respective tetrameric and dimeric forms.

Activity of Tetrameric H62C R67 DHFR—Oxidized, tetrameric H62C R67 DHFR is quite active with $k_{cat} = 74 ± 0.9$ min$^{-1}$, $K_m$(DHF) = 29 ± 0.6 μM and $K_m$(NADPH) = 34 ± 3.0 μM at pH 7.0. For comparison, wt R67 DHFR values are $k_{cat} = 78$ min$^{-1}$, $K_m$(DHF) = 5.8 μM and $K_m$(NADPH) = 3.0 μM at pH 7.0. $K_m$ values increase 5–11-fold, while $k_{cat}$ remains unaffected. One additional difference associated with the H62C mutation is the
observation that DHF inhibition now occurs at high concentrations (≥40 μM at pH 7.0). Substrate inhibition is not observed in wt R67 DHFR; however, it is consistent with nonproductive binding of 2 DHF molecules, which blocks formation of the productive R67 DHFR-DHF-NADPH ternary complex (13).

The availability of a pH stable tetramer allows us the unique opportunity of addressing the proton donation mechanism in R67 DHFR. A pH profile for tetrameric H62C R67 DHFR shows increasing activity at low pH (Fig. 4). This behavior is reminiscent of the pH profile for the mutant D27S E. coli chromosomal DHFR (1) and supports a model for catalysis where R67 DHFR uses protonated dihydrofolate as a substrate. Catalytic activity increases at low pH as the pKₐ for N-5 in dihydrofolate is approached. The values obtained for fitting the kₐₕ profile data to Equation 4 with a fixed pKₐ of 2.59 for protonated DHF (21) are a pH-independent kₐₕ value of 197,000 ± 43,000 min⁻¹ and a slope of 0.64 ± 0.03. Setting the pKₐ is necessary for fitting, since a plateau has not yet been reached and values for kₐₕ at lower pH levels are difficult to obtain due to hydrolysis of NADPH.

At pH 4.95, kₐₕ increases to 2500 ± 110 min⁻¹. Kₐₕ(DHF) increases to 180 ± 11 μM, and Kₐₕ(NADPH) increases to 260 ± 25 μM in oxidized, tetrameric H62C R67 DHFR. The best fit values obtained for the kₐₕ/Kₐₕ(DHF) profile data are a pH-independent kₐₕ/Kₐₕ(DHF) value of 260 ± 53 min⁻¹ μM⁻¹ and a slope of 0.29 ± 0.014.

To confirm that hydride transfer is rate-limiting throughout this pH range for H62C R67 DHFR, NADPD isotope effects were measured. At pH 5.0, ¹⁴N/¹⁵N ratios (kcat using NADPH/kcat using NADPD) = 3.6 ± 0.45 and at pH 7.0, ¹⁴N/¹⁵N = 3.3 ± 0.33. These results indicate hydride transfer is fully rate-determining from pH 5–7. ¹⁴N/kₐₕ(NADPH) at pHs 5 and 7 are 3.8 ± 0.46 and 2.7 ± 0.34, respectively.

Activity of Dimeric H62C R67 DHFR—The activity of dimeric H62C R67 DHFR was assessed under reducing conditions. At pH 7.0, kₐₕ is 2.0 ± 0.12 min⁻¹, Kₐₕ(DHF) = 36 ± 4 μM and Kₐₕ(NADPH) = 74 ± 6 μM. These values compare well with previously determined values for diethyl pyrocarbonate (DEPC)-modified wt R67 DHFR (20). DEPC modification of histidines 62, 162, 262, and 362 resulted in a stabilized dimer and allowed setting of an upper activity limit for dimeric R67 DHFR with kₐₕ, Kₐₕ(DHF), and Kₐₕ(NADPH) values of 3.1 min⁻¹, 40 μM, and 72 μM, respectively (20). The upper limit qualification was due to partial modification of only 2.7 histidines/tetramer. Loss of substantial enzyme activity in both dimeric H62C R67 DHFR and DEPC-modified, wt, dimeric R67 DHFR can be understood in the context of loss of the active site pore upon dissociation of tetrameric R67 DHFR to 2 dimers. From these experiments however, we cannot exclude the possibility of transient tetramer formation induced by ligand binding.

Stability of the H62C Disulfide Bond—To assess the stability of the H62C intersubunit disulfide bonds, the overall Kₐₕ for their reduction/oxidation was monitored. The reduction/oxidation reaction is as follows.

$$ T_{2S-8} + 2DTT_{red} \rightleftharpoons 2D_{2S} + 2DTT_{ox} $$

**REACTION 1**

where T₂S₈ represents a tetramer connected by two disulfide bonds and D₂S₈ represents dimer. The Kₐₕ for this equilibrium was calculated using the following equation.

$$ K_{a} = \frac{[D_{2S}]^{2}[DTT_{ox}][T_{2S-8}]}{[T_{2S-8}]^{2}[2DTT_{red}]} $$

**(Eq. 5)**

The dimer and tetramer concentrations were obtained by analysis of the respective peak areas after passing the sample over a Superose 12 HR 10–30 FPLC column. Equilibrium was reached by 24 h, as the results for the 24 and 48 h incubations were equivalent. A potential intermediate in the oxidation/reduction reaction is a tetramer with one oxidized dimer-dimer interface and one reduced interface (T₂S₈ + 1₈-8); however, this species cannot be separated from a tetramer possessing two oxidized interfaces. Therefore, the resulting Kₐₕ is an overall value representing both oxidation/reduction reactions. The first reaction describes formation of an intermolecular disulfide bond (K₁) between two dimers, while the second equilibrium constant approximates formation of an “intramolecular” disulfide bond (K₂), since the first disulfide bond serves to link the
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The pH-independent $K_{a}$ values predicted for wt and H62C R67 DHFRs show a 15-fold difference (2,910,000 min$^{-1}$ versus 197,300 min$^{-1}$, respectively). The origin of this difference likely lies in the fits for these two different data sets, as the slope describing protonation of free and bound DHF was allowed to vary for the H62C R67 DHFR data but was constrained to 1 for the wt R67 DHFR data. If the slope (i.e. the number of H$^{+}$ adding to DHF and E-NADPH-DHF in Scheme I) is treated as a variable and the wt R67 DHFR data re-fit globally, the fit values obtained are $K_{\text{overall}} = 2.66 \times 10^{-10}$ M (2.53 $\times 10^{-10}$ to 2.77 $\times 10^{-10}$, 67% confidence interval), $k_{\text{cat(max)}} = 93,000$ min$^{-1}$ (77,200–110,000, 67% confidence interval) and slope = 0.794 (0.784–0.805, 67% confidence interval; fit not shown). The use of a non-integral slope for both wt and H62C R67 DHFR data results in only a 2-fold difference in $k_{\text{cat(max)}}$ values, consistent with a similar mechanism associated with each DHFR variant. A non-integral slope likely reflects minor effects of additional ionizations on the catalytic activity and/or protein conformation.

A Comparison of Chromosomal and R-plasmid DHFRs—As indicated in the Introduction, E. coli chromosomal DHFR appears to be a well evolved enzyme. In striking contrast to catalytic function in chromosomal DHFRs, our kinetic results indicate that R67 DHFR is not very efficient as it does not possess a proton donor. Also, in a model of a productive ternary complex based on the crystal structure and difference Fourier maps as well as solution binding studies, either DHF or NADPH can occupy the same half pore, although with different orientations (12, 13). These observations lead us to wonder what factors R67 DHFR utilizes to promote hydride transfer. It is interesting to speculate that the elements most likely involved in facilitating catalysis are: binding interactions for the substrate and cofactor, a cavity effect that may affect dielectric constants in the pore; and third, an orientation effect that restricts accessible conformations (39, 40).

A mutant D27S E. coli chromosomal DHFR was previously constructed using site-directed mutagenesis techniques to determine the effect on catalysis of removing the proton donor (1). An increase in activity at low pH was also observed. When the catalytic efficiencies ($k_{\text{cat}}/K_{m}$) for R67 DHFR and D27S E. coli chromosomal DHFR are compared at pH 7.0, R67 DHFR is 30 times more efficient, as $k_{\text{cat}}$ for the D27S E. coli chromosomal DHFR at pH 7.0 is 24.6 min$^{-1}$ and $K_{m}$ (DHF) = 56 $\mu$M. The relative agreement of rates (25 versus 74 min$^{-1}$) in D27S chromosomal E. coli DHFR and R67 DHFR may indicate a limit to the activity feasible without a proton donor present.

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