“Catch 222,” the Effects of Symmetry on Ligand Binding and Catalysis in R67 Dihydrofolate Reductase as Determined by Mutations at Tyr-69*

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R67 dihydrofolate reductase (R67 DHFR) catalyzes the transfer of a hydride ion from NADPH to dihydrofolate, generating tetrahydrofolate. The homotetrameric enzyme provides a unique environment for catalysis as both ligands bind within a single active site pore possessing 222 symmetry. Mutation of one active site residue results in concurrent mutation of three additional symmetry-related residues, causing large effects on binding of both ligands as well as catalysis. For example, mutation of symmetry-related tyrosine 69 residues to phenylalanine (Y69F), results in large increases in \( K_m \) values for both ligands and a 2-fold rise in the \( k_{cat} \) value for the reaction (Strader, M. B., Smiley, R. D., Stinnett, L. G., VerBerkmoes, N. C., and Howell, E. E. (2001) Biochemistry 40, 11344–11352). To understand the interactions between specific Tyr-69 residues and each ligand, asymmetric Y69F mutants were generated that contain one to four Y69F mutations. A general trend observed from isothermal titration calorimetry and steady-state kinetic studies of these asymmetric mutants is that increasing the number of Y69F mutations results in an increase in the \( K_m \) and \( K_m \) values. In addition, a comparison of steady-state kinetic values suggests that two Tyr-69 residues in one half of the active site pore are necessary for NADPH to exhibit a wild-type \( K_m \) value. A tyrosine 69 to leucine mutant was also generated to approach the type(s) of interaction(s) occurring between Tyr-69 residues and the ligands. These studies suggest that the hydroxyl group of Tyr-69 is important for interactions with NADPH, whereas both the hydroxyl group and hydrophobic ring atoms of the Tyr-69 residues are necessary for proper interactions with dihydrofolate.

Dihydrofolate reductase (DHFR)\(^1\) catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate using NADPH as a cofactor. DHFR activity is necessary for cell survival as tetrahydrofolate is involved in pathways leading to the synthesis of purine nucleosides and other metabolites (1). Chromosomal (Escherichia coli) DHFR is inhibited by the antibiotic trimethoprim. However, plasmid-encoded R67 DHFR provides resistance to the antibiotic. The plasmid-encoded DHFR is unique in that it shows no genetic or structural homologies with chromosomal DHFR (2–4).

Several features of R67 dihydrofolate reductase are unusual. First, as a homotetramer with a monomer length of 78 amino acids, it is one of the smallest enzymes known to self-assemble into an active quaternary structure. Second, the structure possesses 222 symmetry as shown in Fig. 1\(^2\) (2). Third, a pore, 25 Å long, extends through the middle of the enzyme (like a doughnut hole). Fourth, the symmetry, coupled with use of a single active site pore, results in overlapping binding sites for the two different ligands used in the reaction. For example, R67 DHFR binds a total of two molecules, either two NADPH, two folate/DHF, or one NADPH plus one folate/DHF (5). The first two are dead-end complexes, whereas the third is productive. (Interligand cooperativity patterns funnel the enzyme toward the productive ternary complex.) Fifth, site-directed mutagenesis results in four mutations per active site pore and large effects on binding and catalysis. Thus, it is difficult to produce local effects that could allow dissection of how each residue interacts with the ligands as well as the transition state. Although a generalized description of R67 DHFR catalysis exists (5–10), additional detail can be obtained by introduction of asymmetry.

To be able to introduce asymmetry in R67 DHFR, we have constructed a tandem gene array that allows control of the number and location of the mutation(s). Briefly, the tandem gene array contains four in-frame copies of the gene encoding wild-type (wt) R67 DHFR. Transcription and translation yield a monomeric protein (named “Quad3”) mimicking the wild-type enzyme. Quad3 is almost fully active (1.8-fold decrease in \( k_{cat}/K_m \) values), and all physical, binding, and steady-state kinetic studies indicate excellent agreement with wt R67 DHFR behavior (10, 11).

Those residues identified as most important in R67 DHFR catalysis include Lys-32, Gln-67, Ile-68, and Tyr-69 (7, 9, 12). The first asymmetric mutation series constructed to probe binding and catalysis involved the Q67H substitution (10). The companion paper (Hicks et al. (42)) describes construction and characterization of a series of K32M asymmetric mutants. This

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\(^1\) The abbreviations used are: DHFR, dihydrofolate reductase; DHF, dihydrofolate; NMINH, reduced nicotinamide mononucleotide; NADP+/NADPH, oxidized/reduced nicotinamide adenine dinucleotide phosphate; pABA-glutamic acid tail, para-aminobenzoic acid/glutamic acid region of dihydrofolate/folate; ITC, isothermal titration calorimetry; wt, wild-type; Quad3, the product protein of a tandem array of four in-frame R67 DHFR genes; MES, 4-morpholineethanesulfonic acid.

\(^2\) The monomers of R67 DHFR are labeled ABDC going in a clockwise orientation in the crystal structure (1VIE and 1VF in the Protein Data Bank). The residues in monomer A are labeled 1–78, whereas those in monomers B, C, and D are designated 101–178, 201–278, and 301–378, respectively. All four symmetry-related residues are implied when one residue of the homotetramer is described. The corresponding domains in Quad3 are relabeled 1234 (=ABCD) to minimize any confusion and provide consistent nomenclature.
study focuses on the role of Tyr-69 residues.

What is the proposed role for Tyr-69 in R67 DHFR function? Docking studies suggest that these symmetry-related residues may be involved in interactions with both the substrate and cofactor (13). The ring edges of two symmetry-related tyrosine 69 residues dock within van der Waals contact with the pyrophosphate bridge of NADPH as well as with the adenine ribose. Docking studies also predict a possible interaction between the tyrosine hydroxyl and the glutamic acid tail of folate. Kinetic studies of the homotetrameric Y69F mutant also support the hypothesis that Tyr-69 interacts with both DHF and NADPH, because the $K_d$ values for DHF and NADPH are 11- and 17-fold weaker, respectively, than those for wild-type R67 DHFR (9).

In addition, the $k_{cat}$ for the reaction is 2-fold greater than the $k_{cat}$ for wild-type R67 DHFR. A less conservative substitution (Y69H) greatly increased both $K_d$ values as well as decreased $k_{cat}$. Finally, a recent NMR study finds chemical shifts are associated with Tyr-69 upon NADP $^+$ binding (14). A model of the docked R67 DHFR-NADPH-folate ternary complex is shown in Fig. 1E with the positions of the symmetry-related Tyr-69 residues indicated.

Our approach to understanding the role of symmetry-related tyrosine 69 residues in ligand binding and catalysis in this report is 2-fold. First, we generated a series of asymmetric Y69F mutants, including a single mutant (containing one Y69F mutation), three double mutants (each containing two Y69F mutations), a triple mutant, and a quadruple mutant to better understand the specificity of the interactions between Tyr-69 and both DHF and NADPH. This approach allows us to determine if there is a preference for NADPH and/or DHF to interact with wild-type tyrosine 69 residues. Our second approach involved site-directed mutagenesis of tyrosine 69 residues within the homotetrameric enzyme to better understand the type(s) of interaction(s) occurring between symmetry-related tyrosine 69 and the ligands and how these interactions are involved in catalysis.

**EXPERIMENTAL PROCEDURES**

**Construction of Asymmetric Tyr-69 mutants**—Asymmetric Y69F mutants were generated by PCR site-directed mutagenesis as previously described (10). Correct mutations were confirmed by DNA sequencing and the DNA maintained in E. coli STBLII cells to avoid recombination problems (15).

Y69F, Y69K, Y69Q, and Y69L homotetrameric mutants were also generated by PCR-based site-directed mutagenesis. None of the cell lines transformed with these mutants were able to grow in the presence of 20 μg trimethoprim/ml. Because high protein expression was noted for the Y69L mutant, it was pursued.

**Protein Purification and Characterization**—Proteins were purified as previously described (10). The asymmetric Y69F mutants were not as prone to aggregation as the asymmetric K32M mutants (companion paper, Hicks et al. (42)); however, visible turbidity was sometimes noted at the high protein concentrations (~100 μg/ml) used in isothermal titration calorimetry experiments. Thus 0.1 g/liter polyethylene glycol 3350 was added to minimize aggregation as well as increase purification yields of the asymmetric mutants (16). Steady-state kinetic data were collected for each of the mutants to determine the corresponding $K_m$ and $k_{cat}$ values as previously described (Ref. 12 and Hicks et al. (42)).

**Isothermal Titration Calorimetry**—Isotherms were generated for substrate or cofactor binding to each mutant on a MicroCal VP isothermal titration calorimeter at 28 °C as described previously (5, 10). MTH polybuffer (50 mM MES 50 plus 100 mM Tris, plus 50 mM acetic acid; pH 8 (17)) was used for NADPH binding studies, whereas 10 mM Tris plus 1 mM EDTA buffer, pH 8, was used for DHF binding studies. Because DHF is a weak acid, the pH of the DHF solution was titrated to pH 8 prior to ligand binding studies. The data for NADPH binding to the mutants were analyzed by Origin software (Version 5.0) using both the single sites model and the sequential sites model where the stoichiometry was set to two.

**pH Titrations**—Homotetrameric R67 DHFR dissociates into dimers upon titration with acid due to protonation of symmetry-related histidine 62 residues (18, 19). This equilibrium is described by,

$$T + 2nH^+ \rightleftharpoons 2DH_n$$

**RESULTS**

**Nomenclature**—The naming system used to name the asymmetric mutants follows the pattern established by our companion paper (Hicks et al. (42)). Briefly, the Y69F mutation is listed followed by the number indicating the gene copy, which carries the mutation. For example, a single Y69F:1 mutant was constructed where the mutation was placed in gene copy 1. Three double mutants, one triple mutant and one quadruple mutant were also constructed. The double mutants are non-equivalent; this can be seen using the crystal structure for homotetrameric R67 DHFR (Fig. 1, B–D). A model of the NADPH-folate ternary complex that is consistent with NMR and crystallography constraints is shown in Fig. 1E along with the relative positions of Tyr-69, Lys-32, Gln-67, and Ile-68 residues (2, 6, 13, 14).

In addition to Y69F asymmetric mutants, the effects of Y69L mutations in the homotetrameric enzyme were also assessed. This construct is named Y69L R67 DHFR and contains four symmetry-related Y69L mutations.

**Steady-state Kinetics**—To gain a better understanding of the role of Tyr-69 in binding and catalysis, steady-state kinetic data were collected for each of the asymmetric Y69F mutants and are presented in Table I. In general, the $K_m$ values for NADPH and DHF as well as the $k_{cat}$ display a trend contingent upon the location and number of Y69F mutations. A wild-type $K_m$ (NADPH) value is observed for both the Y69F:1 and Y69F:1+3 mutants, whereas the $K_m$ (NADPH) for the Y69F:1+2 and Y69F:1+4 mutants is 3- to 4-fold weaker. The $K_m$ (NADPH) values for the triple mutant and quadruple mutants continue to increase. The kinetic parameters for the Y69F:1+2+3+4 mutant are similar to those for Y69L R67 DHFR (9). Thus, for NADPH binding interactions in the productive, ternary complex, as the number of mutations increases, $K_m$ (NADPH) concurrently increases. The two exceptions to this trend are the Y69F:1 and Y69F:1+3 mutants, which both have wild-type $K_m$ values.

Similar to NADPH interactions in the ternary complex, $K_m$ (DHF) values also tend to increase as the number of mutations is increased. Specifically, the $K_m$ (DHF) for the single and all double mutants is ~2- to 3-fold weaker than the $K_m$ (DHF) for Quad3. In addition, the $K_m$ (DHF) for the Y69F:1+2+3+4 mutant continues the trend of increasing as additional mutations are added, up to the limit associated with the Y69F:1+2+3+4+4 mutant.

Although binding of both NADPH and DHF in the Michaelis complex is, in general, weakened as the number of Y69F mutations is increased, the $k_{cat}$ value increases over a range of ~2-fold. A trend is noted where the $k_{cat}$ increases when two
Y69F mutations occur at the position that corresponds to one dimer-dimer interface in wt R67 DHFR, as occurs in the Y69F:1+4 (see Fig. 1D), Y69F:1+2+3, and Y69F:1+2+3+4 mutants, suggesting this topology may be preferred in the transition state.

**Isothermal Titration Calorimetry**—Although steady-state kinetics provide important insight into interactions between ligands in the Michaelis complex, these $K_d$ values do not necessarily correspond to dissociation constants, because other events can contribute to the observed Michaelis constants (21). Isothermal titration calorimetry (ITC) provides a direct measure of the heat exchange upon ligand binding as well as a direct measure of the fine for the ligand of interest (22, 23). Thus ITC experiments were performed using binary complex conditions where either two NADPH molecules or two DHF molecules can interact with the enzyme. Data for NADPH binding to the Y69F asymmetric mutants are summarized in Table II. These data were analyzed using both the single sites model and the sequential sites model where the stoichiometry was set to two. The $K_d$ and $\Delta H$ values for the first NADPH binding event were similar regardless of the model used to describe the data. However the $K_{d2}$ and $\Delta H_2$ values generated using the sequential sites model varied, most likely because the $K_{d2}$ values are high and fall outside of the detection window afforded by the calorimeter. For accurate results, the $c$...
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| Enzyme variant | $k_{cat}$ | $K_a$ (NADP+) | $K_a$ (DHF) |
|----------------|----------|---------------|-------------|
| WT R67 DHFR | $1.3 \pm 0.1$ | $3.0 \pm 0.1$ | $5.8 \pm 0.1$ |
| Quad3 DHFR | $0.81 \pm 0.02$ | $4.4 \pm 0.4$ | $6.7 \pm 0.4$ |
| Y69F:1 DHFR | $0.98 \pm 0.06$ | $6.4 \pm 0.6$ | $21 \pm 2$ |
| Y69F:1+2 DHFR | $0.96 \pm 0.01$ | $14 \pm 0.8$ | $20 \pm 0.9$ |
| Y69F:1+3 DHFR | $0.56 \pm 0.01$ | $3.1 \pm 0.4$ | $20 \pm 2$ |
| Y69F:1+4 DHFR | $1.9 \pm 0.08$ | $14 \pm 1$ | $28 \pm 2$ |
| Y69F:1+2+3 DHFR | $1.4 \pm 0.04$ | $21 \pm 1$ | $35 \pm 2$ |
| Y69F:1+2+3+4 DHFR | $1.4 \pm 0.04$ | $40 \pm 3$ | $54 \pm 3$ |
| Y69F R67 DHFR | $2.9 \pm 0.1$ | $69 \pm 3$ | $68 \pm 4$ |
| Y69L R67 DHFR | $0.16 \pm 0.01$ | $68 \pm 3$ | $180 \pm 11$ |
| Y9H R67 DHFR | $0.014 \pm 0.002$ | $176 \pm 0.0$ | $46 \pm 4.5$ |

$^a$ Taken from Ref. 39.  
$^b$ Taken from Ref. 10.

Table II

Comparison of NADPH binding constants for Y69F asymmetric mutants and Y69L R67 DHFR as determined by isothermal titration calorimetry

| Complex | $K_a$ (μM) | $\Delta H$ | Stoichiometry |
|---------|------------|------------|---------------|
| Quad3 DHFR | $8 \pm 0.3$ | $-7800 \pm 260$ | $0.83 \pm 0.01$ |
| Y69F:1 DHFR | $10 \pm 1$ | $-6200 \pm 120$ | $0.88 \pm 0.003$ |
| Y69F:1+2 DHFR | $25 \pm 2$ | $-4900 \pm 170$ | $0.49 \pm 0.02$ |
| Y69F:1+3 DHFR | $15 \pm 1$ | $-5200 \pm 300$ | $0.72 \pm 0.01$ |
| Y69F:1+4 DHFR | $9 \pm 1$ | $-7500 \pm 490$ | $0.84 \pm 0.02$ |
| Y69F:1+2+3 DHFR | $25 \pm 2$ | $-5000 \pm 120$ | $0.75 \pm 0.03$ |
| Y69F:1+2+3+4 DHFR | $52 \pm 5$ | $-2400 \pm 210$ | $0.95 \pm 0.11$ |
| WT R67 DHFR$^b$ | $5.0 \pm 0.3$ | $-8600 \pm 200$ | $1.56 \pm 0.14$ |
| Y69F R67 DHFR | $65 \pm 6$ | $-2200 \pm 170$ | $1.1 \pm 0.1$ |
| Y69L R67 DHFR | $75 \pm 0.4$ | $-2800 \pm 40$ | $1.1 \pm 0.1$ |

$^a$ Value describing the first binding site in a two sites model, from Ref. 5.

Similar trends are observed in a graph of total heat versus ligand concentration, however, the individual plots overlay substantially, making visualization difficult.
A conservative Y69L mutation was constructed in homotetramer R67 DHFR, indicating how residue 69 interacts with its ligands, a non-consistent with minimal structural perturbations. The titrations for wt homotrimeric R67 DHFR (solid line) as well as the Y69L mutant (dashed line) are shown. Best-fit values are given in the text.

**Physical Studies**—pH titrations were performed to determine whether the Y69F asymmetric mutations affected the structure as monitored by the equilibrium between the "open" and "closed" conformations of Quad3 mutants (10, 18, 20). The pH titrations (data not shown) indicate the values for all of the asymmetric mutants are within 0.09 pH unit of each other, consistent with minimal structural perturbations.

**Homotetrameric Y69L R67 DHFR**—To gain a better understanding of how residue 69 interacts with its ligands, a non-conservative Y69L mutation was constructed in homotetrameric R67 DHFR. The Y69L mutation affects both ligand binding and catalysis. As indicated in Tables I and II, the $K_m$ (NADPH) values are similar between Y69F and Y69L R67 DHFRs as are the $K_d$ (NADPH) values. In contrast, the $K_m$ (DHF) value is ~2.5-fold weaker for Y69L as compared with Y69F R67 DHFR. The higher $K_m$ value could derive from either a kinetic effect where other events contribute to the observed Michaelis constants (21) and/or a direct effect where the leucine substitution weakens DHF binding. Binding of DHF to Y69L R67 DHFR as monitored by ITC was much weaker as compared with wild-type residues are available in domains 1 and 3 (or 2 and 4) comprise a portion of the NADPH binding site. Previous ITC binding studies indicate that, although R67 DHFR could follow a random mechanism, it prefers to bind NADPH first followed by DHF. The preferred pathway arises from the interligand cooperativity patterns (5). Thus, when mutations are added asymmetrically to weaken binding, it is expected that NADPH will continue to bind first and choose the tightest site available. A comparison of steady-state kinetic values for the asymmetric Y69F mutants supports this binding mechanism, because only when wild-type residues are available in domains 1 and 3 (or 2 and 4) as occurs in the Y69F:1 and Y69F:1-3 mutants is NADPH able to bind to an unperturbed site (this general model is displayed in Fig. 5 of Hicks et al., companion paper (42)). In contrast, when each half pore of these domains contains a Y69F residue (Y69F:1-2, Y69F:1-4, and Y69F:1-2+3), NADPH binding affinity is weakened 3- to 5-fold. Finally, when both residues in domains 1 and 3 (or 2 and 4) are mutated in the Y69F:1-2+3-4 mutant, the binding affinity continues to weaken and mimics that of homotetrameric Y69F R67 DHFR.

What role do the Tyr-69 residues from domains 1 and 3 (or 2 and 4) play in NADPH binding? One possibility is that two tyrosine 69 residues located in different domains on the same side of the pore form direct interactions with NADPH. Support for this proposal comes from a computational model of the ternary complex. In this model (Fig. 1E), contacts are proposed between the ring edge of Tyr-69 in monomer A (= domain 1 of Quad3) and the pyrophosphate bridge of NADPH as well as contacts between the ring edge of Tyr-69 in monomer D (= domain 3 for Quad3) and the adenine ribose of NADPH (13). A second possibility invokes an avidity or multivalency effect. In this scenario, once a site is occupied, the proximity of other symmetry-related sites can enhance binding by reduction of the associated entropy and/or by decreasing the dissociation rate (26–30). This type of effect has previously been proposed to enhance binding in antibodies, extracellular proteins, and carbohydrates (31, 32) and has been the basis for design of multivalent drugs (33, 34).

Because a direct interactions between the -OH of Tyr-69 and NADPH was not predicted in the docked ternary complex model (13), either this model requires revision or water-mediated interactions could be occurring. Alternatively, because the contacts predicted by DOCK between Tyr-69 residues and NADPH largely involve the ring edge of the tyrosine residue, it is possible that one or more dipole-charge interactions may be present (1/distance squared dependence (35)). Because the phenylalanine side chain lacks the -OH group of tyrosine, this dipole moment would be diminished in the Y69F mutant.

Do the $K_d$ data for NADPH binding show the same trend as the $K_m$ values? In general, both data sets show that, as the number of mutations increases, both the $K_d$ and $K_m$ values increase and ITC measurements find the enthalpy becomes less negative. If the $K_m$ values were to exactly mimic the $K_m$ behavior, the $K_d$ (NADPH) for the Y69F:1-3 mutant should be wild-type and the $K_d$ (NADPH) for the Y69F:1-4 mutant would...
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be weaker. Instead, the $K_m$ and $\Delta H$ values for the Y69F:1+4 mutant are more similar to Quad3 values than those for the Y69F:1+3 mutant. However, some differences may occur as these two techniques monitor different complexes, the Michaelis complex for steady-state kinetics and a binary complex where two NADPH molecules bind for isothermal titration calorimetry. Because interactions between two NADPH molecules versus one DHF and one NADPH molecule are likely different, it is possible that these differences are reflected in the values obtained by the two techniques.

**DHF Binding Interactions in the Binary and Michaelis Complex**—Interligand Overhauser effects suggest that NADPH and DHF bind on opposite sides of the pore, allowing stacking between the pterin and nicotinamide rings at the pore’s center. Because NADPH is predicted to bind first and to the tightest site available, DHF would be expected to bind to the enzyme-NADPH complex, and the pABA-glutamic acid tail would then be forced to interact with the amino acid residue available at position 69 in the other half of the pore, either wild-type or mutant, depending on the configuration. This model is supported by steady-state kinetic data for the Y69F:1 and Y69F:1+3 mutants where the NADPH $K_m$ is similar to Quad3 values while the DHF $K_m$ increases. Also, similar $K_m$ (DHF) values are observed for the Y69F:1+2 and Y69F:1+4 mutants where both halves of the pore possess a mutation. The $K_m$ (DHF) value for Y69F:1+2+3 is weaker than that for the single and double mutants as the second half of the pore now contains two mutations.

Support for this model also comes from the ternary complex model where folate was docked into a DHFR:NMMNH complex. (NMNH is a fragment of NADPH that contains only the nicotinamide ring, ribose ring, and a single phosphate group.) From the docking studies, the highest scoring folate conformer is predicted to form a hydrogen bond between the -OH of Tyr-69 (in domain 4 of Quad3) and the $\alpha$-carboxylate of the folate tail. However, other high scoring folate conformers are also predicted with alternate positions for the pABA-glutamic acid tail of DHF. Mobility in the tail region has additionally been observed in both x-ray crystallography and NMR studies (2, 6).

It is therefore possible that interactions occurring between Tyr-69 and folate (or DHF) are weak and/or transient, hence, interligand interactions between bound NADPH and DHF play an important role in DHF binding.

Do these trends continue in the DHF binary complex data? Although we are unable to quantitate differences in binding affinity for the Y69F asymmetric mutants, we can draw qualitative conclusions based on variations in isotherm shape. First, increasing the number of Y69F mutations weakens the binding affinity for DHF as increasing DHF concentrations are required to reach saturation. Also, as the number of Y69F mutations is increased, a more prominent hook is observed in the isotherms, suggesting changes in interligand interactions between the two DHF molecules and/or different enthalpies associated with the two binding events.

**What Role Does Tyr-69 Play in Catalysis?**—Based on steady-state kinetic analyses of Y69F R67 DHFR and the Y69F asymmetric mutant series, one role for Tyr-69 is in ground state kinetic analyses of Y69F:1 and the Y69F asymmetric mutants in this study as well the K32M mutants in Hicks et al. (see companion paper (42)). Variations in $k_{cat}$, $k_{cat}/K_m$ (NADPH), and $k_{cat}/K_m$ (DHF) are plotted by the black, white, and hatched bars, respectively. A negative value indicates an enhancement of the mutant over the wild-type (i.e. Quad3) values.

![Figure 4](http://www.ncbi.nlm.nih.gov/pubmed/21001218)

**FIG. 4. A bar graph describing the effects of asymmetric Y69F and K32M mutations on kinetic parameters.** The difference $\Delta G$ values were calculated using $\Delta G = -RT \ln(\text{kinetic value (Quad3)/kinetic value (mutant)})$ for the Y69F asymmetric mutants in this study as well the K32M mutants in Hicks et al. (see companion paper (42)). Variations in $k_{cat}$, $k_{cat}/K_m$ (NADPH), and $k_{cat}/K_m$ (DHF) are plotted by the black, white, and hatched bars, respectively. A negative value indicates an enhancement of the mutant over the wild-type (i.e. Quad3) values.

These data continue to support the model that Y69F mutations in the homotetramer destabilize binding in the ground state to a greater extent than binding in the transition state (9). Further, this model can be extended such that the Y69F:1+4 double mutant topology appears to provide the least perturbation to ground state binding coupled with the best match to the transition state configuration. Additionally, because the Y69F:1+3 double mutant topology is coupled with a decreased $k_{cat}$ value, it likely provides the worst match (in this series) to the transition state.

**What Types of Interactions Occur between the Ligands and Y69?**—To probe the type(s) of interaction(s) that occur between Tyr-69 and both NADPH and DHF, we have mutagenized Tyr-69 to both conservative and non-conservative amino acid residues. The importance of Tyr-69 in catalysis is emphasized as most mutations at this position are not tolerated without a loss of trimethoprim resistance, which usually correlates with large decreases in protein yield and/or $k_{cat}/K_m$ values. Indeed, our previous study of the Y69H mutant was limited by low yields (9).

Analysis of Y69F R67 DHFR suggests the importance of the hydroxyl group of tyrosine for binding interactions with NADPH and DHF, because its removal results in a 20-fold increase in $K_m$ (NADPH) and a 10-fold increase in $K_m$ (DHF) (9). However, similar $K_m$ (NADPH) and $K_m$ (NADPH) values for Y69F and Y69L R67 DHFR suggest that the hydroxyl moiety is most important for interactions with NADPH. In contrast a 2.5-fold increase in the $K_m$ (DHF) value for Y69L, as compared with Y69F R67 DHFR, suggests that both the aromatic ring and hydroxyl group of Tyr-69 are important for binding DHF. Finally, removal of the hydroxyl group appears to facilitate catalysis (Y69F R67 DHFR), whereas the benzylc ring is necessary for catalysis, because its removal results in an 8-fold decrease in the reaction rate (Y69L R67 DHFR). When the Y69H mutant is included in this comparison (see Table I) (9), a further 2.5-fold increase in $K_m$ (NADPH) values (over Y69L) occurs. This pattern suggests a preference for hydrophobic atoms at this position. Also, $k_{cat}$ decreases substantially in the Y69H mutant, again supporting a hydrophobic preference at position 69. Finally, the $K_m$ (DHF) in the Y69H mutant is in the same range as the Y69F mutant, suggesting some tolerance of...
polar atoms by bound substrate and perhaps a preference for ring edges versus extended side chains.

A Gibbs Free Energy Comparison—One way of dissecting the contribution of active site residues to catalysis is to compare \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) values using a Gibbs free energy approach. Different \( \Delta G \) values were calculated for the Y69F asymmetric mutants in this study as well the K32M mutants in Hicks et al. (see companion paper (42)). As seen in Fig. 4, several asymmetric mutants possess enhanced \( k_{\text{cat}} \) values (Y69F: 1+4, Y69F: 1+2+3, Y69F: 1+2+3+4, or K32M: 1+3) when compared with Quad3, whereas all mutations decrease catalytic efficiency. These variations indicate R67 DHFR has found a solution to the constraints imposed by its 222 symmetry. Because enzyme evolution may involve gene duplication followed by divergence (36–38), what remains to be tested is whether additional mutations in any of the asymmetric mutants could lead to enhanced function. This could involve either introduction of a proton donor (19) or introduction of a specific binding site for enhanced binding by an avidity effect (26–30). (large mutational effects are typically observed in the triple and 4 double mutants (Y69F and K32M series) show similar half-pore.” Subsequent binding of DHF results in its being forced into the “mutant side.” (4) Mutations at the center of the pore (Q67H) are accompanied by NADPH and DHF inhibition, whereas mutations further out on the pore surface (K32M and Y69F) do not. This observation is consistent with the crystallographic R67 DHFR:2folate binary complex and the docked ternary complex model that point to the center of the pore as helping to establish interligand cooperativity patterns.

Use of asymmetric mutations has revealed that one consequence of the “one-site-fits-both” strategy employed by R67 DHFR is a “catch-222” situation, which appears to describe symmetry-related residues that aid ligand binding, but provide too much ground state stabilization. To reach the transition state, some of these interactions likely need to break.

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