Structure of the Q67H mutant of R67 dihydrofolate reductase-NADP⁺ complex reveals a novel cofactor binding mode

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Abstract

Plasmid-encoded bacterial R67 dihydrofolate reductase (DHFR) is a NADPH-dependent enzyme unrelated to chromosomal DHFR in amino acid sequence and structure. R67 DHFR is insensitive to the bacterial drug trimethoprim in contrast to chromosomal DHFR. The crystal structure of Q67H mutant of R67 DHFR bound to NADP⁺ has been determined at 1.15 Å resolution. The cofactor assumes an extended conformation with the nicotinamide ring bound near the center of the active site pore, the ribose and pyrophosphate group (PP_i) extending toward the outer pore. The ribonicotinamide exhibits *anti* conformation as in chromosomal DHFR complexes. The relative orientation between the PP_i and the nicotinamide ribose differs from that observed in chromosomal DHFR–NADP⁺ complexes. The coenzyme displays symmetrical binding mode with several water-mediated hydrogen bonds with the protein besides ionic, stacking, and van der Waals interactions. The structure provides a molecular basis for the observed stoichiometry and cooperativity in ligand binding. The ternary model based on the present structure and the previous R67 DHFR–folate complex provides insight into the catalytic mechanism and indicates that the relative orientation of the reactants in plasmid DHFR is different from that seen in chromosomal DHFRs.

Keywords: crystal structure; folate metabolism; NADP⁺; R67 DHFR; symmetric binding; trimethoprim resistance

Supplemental material: see www.proteinscience.org

Dihydrofolate reductase (DHFR, EC 1.5.12.3) catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) utilizing the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. THF is required for the synthesis of thymidylate, purine nucleosides, methionine, and other metabolic intermediates. Therefore, inhibition of DHFR results in cessation of precursors necessary for DNA synthesis, leading to cell death. Trimethoprim (TMP) has been used in the treat-

ment of various bacterial infections. Bacterial resistance to TMP is correlated with the production of novel R-plasmid-encoded DHFRs (Fleming et al. 1972). Type II R-plasmid-encoded R67 DHFR is of special interest because it is genetically and structurally unrelated to chromosomal DHFR.

R67 DHFR and chromosomal DHFRs have distinct structural and kinetic features. At physiological pH, the functional R67 DHFR is a homotetramer ($M_r \sim 8.5$ kDa; 78 amino acid residues per subunit), and at low pH, native tetrameric R67 DHFR dissociates to form inactive dimers (Nichols et al. 1993). By contrast, the chromosomal DHFR is a monomeric enzyme with a core topology that resembles the classic Rossmann-fold usually associated with the binding of NADPH (Bellamacina 1996), whereas

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R67 DHFR has entirely different molecular architecture (five-stranded β -barrel) and belongs to a group of non-classical dinucleotide binding proteins that have a variety of folds. Structures of the dimeric and tetrameric forms of R67 DHFR have been determined (Matthews et al. 1986; Narayana et al. 1995). The tetramer displays a large central pore (Fig. 1A) with three mutually perpendicular and intersecting twofold axes passing through the middle

of the pore. The K_i (TMP) for R67 DHFR is 0.15 mM versus 20 pM for chromosomal DHFR (Howell 2005).

The crystal structure of R67 DHFR with bound folate revealed a novel D_2 -symmetric active site pore (Narayana et al. 1995). In this complex, Gln67 and its symmetry-related residues protrude into the active site pore and are stacked on the bound pteridine rings. The role of Gln67 in ligand binding and catalysis has been investigated by

Figure 1. Cofactor binding and stoichiometry. (A) The enzyme is comprised of four subunits (1–4) shown in gray and black. The simultaneous binding of two cofactor molecules (CPK model without adenosine 2'-phosphate portion) on the front or rear side of the active site pore results in steric clash, primarily between the ribose groups. (B) The protein portions comprised of residues Val66, His67, Ile68, and Tyr69 from four subunits bordering the central active site pore are shown as black and gray ribbons. The *paired* cofactor molecules that approach each other from opposite sides (indicated by arrows that align with a twofold symmetry axis) exhibit minimal stacking of the nicotinamide rings. Vertical bars align with a twofold axis, and the symmetry-related His67 side-chains are shown as van der Waals surfaces. The central dot shows another twofold axis passing through the plane of the paper. (C) Schematic diagram representing the possible modes of cofactor binding. Left and right of the vertical bar denote the front and rear portions of the pore as viewed down one of the two short axes of the active site. There are two binding positions in each half of the pore. The sites represented as horizontal lines are labeled A and B for one half of the pore and C and D for the other half of the pore (*top* panel on the *left*). The center of the pore is depicted as a vertical bar. The three lower panels on the *left* show possible binding modes without steric hindrance. The *bottom* panel on the *left* shows one bound NADP⁺ molecule in site A (see text for details). It can also be bound in either site B or C or D (data not shown). The *right* part of the figure shows different binding modes that are precluded due to stereochemical clashes between the cofactors. It may be noted that binding site A stacks on site D and clashes with site C but is not shown in the figure for clarity (the same applies to site B). The extent of stacking of ligands is larger in the R67 DHFR–folate complex than in the holoenzyme complex.

site-directed mutagenesis and isothermal titration calorimetry (ITC) studies (Park et al. 1997). The introduction of Q67H mutation results in a stable protein with tighter binding for folate, NADPH, and NADP⁺ by 36-, 110-, and 29-fold, respectively, than the wild-type enzyme. Understanding the structural basis of NADPH-dependent reduction of DHF by R67 DHFR is crucial for mechanistic studies of this enzyme. Here, we present the crystal structure of Q67H mutant of R67 DHFR bound to the oxidized cofactor NADP⁺ determined at 1.15 Å resolution.

Results and Discussion

Overall structure

The enzyme folding is similar to the previously published structures (Fig. 1A), with NADP⁺ bound in the central active site (Fig. 1A,B). Least-squares fit of C^α atoms of residues 21–78 in apo R67 DHFR (Narayana 2006) with equivalent atoms in the holoenzyme complex (root-mean-square deviation [RMSD] of 0.30 Å) shows a rigid protein backbone. Steric clashes between the symmetry-related ligands restrict the number of bound cofactors per tetramer (Fig. 1C).

Conformation of the bound cofactor

NADP⁺ binds to R67 DHFR in an extended conformation with the nicotinamide ring near the center of the active site and the remaining portion stretching toward the outer pore. In general, the cofactor in the enzyme-bound form exhibits extended conformation in contrast to the folded architecture in solution. The nicotinamide, ribose, and the pyrophosphate (PP_i) group were seen in a difference Fourier map (Fig. 2A,B,C).

The carboxamide oxygen atom is *cis* to C4 of the pyridine ring as in the chromosomal DHFR (Bystroff et al. 1990). The exocyclic carboxamide moiety is ~25° out of the planar nicotinamide ring, unlike the coplanar geometry observed in chromosomal DHFRs. The orientation of the ribose with respect to the nicotinamide ring is *anti* with χ_N (O4' – C1' – N1 – C2) = –116.2°. The occurrence of *anti* conformation for the ribonicotinamide was surprising because previous NMR studies on a related R388 enzyme as well as R67 DHFR indicated *syn* conformation (Brito et al. 1991; Li et al. 2001). It is likely that the cofactor binds to the enzyme in either *anti* or *syn* conformation, maintaining the A-stereospecificity for the hydride transfer as suggested previously (Narayana et al. 1995). Alternatively, the mutation Q67H may stabilize *anti* conformation.

The nicotinamide ribose adopts a C2'-*endo* geometry as in chromosomal DHFRs. Although there is close resemblance with respect to the nicotinamide-ribose moiety

between R67 DHFR and the chromosomal DHFRs, the conformations of the rest of the cofactor differ markedly. Major deviations occur at torsion angles O4'–C4'–C5'–O5' and C4'–C5'–O5'–PN. Viewing along the virtual PA...PN bond, the phosphate groups are staggered. The adenosine 2'-phosphate moiety is generally ordered in chromosomal DHFR complexes, unlike R67 DHFR.

Cofactor binding stoichiometry

Two combinations, either sites A and D or sites B and C, are free from steric clashes between the cofactors and are designated as *paired sites* for ease of description of interligand interactions (Fig. 1C). The structure reveals that each half of the pore can accommodate only one NADP⁺, resulting in a maximum of two NADP⁺ molecules per tetramer (Fig. 1B). Nonetheless, the stoichiometry for NADP⁺ is 1 per tetramer according to ITC studies (Bradrick et al. 1996; Park et al. 1997). To reconcile this observation, we interpret that due to electrostatic repulsion between the positively charged nicotinamide rings occupying the *paired sites*, the second available site is vacant, leading to one NADP⁺ per tetramer. Assuming NADPH binds in the same orientation as does NADP⁺, we conclude that two NADPH molecules bind per tetramer due to the absence of repulsive forces between the *paired* nicotinamide rings. This result is in accord with previous ITC studies.

Cofactor–protein and cofactor–water–protein interactions

The binding position of NADP⁺ can be divided into three regions. Site A, where the pyridine ring and nicotinamide-ribose are bound; site B, where the PP_i is bound; and site C, where the adenosine 2'-phosphate is bound. Figure 2D shows the hydrogen bonding network involving the cofactor with protein and waters.

Site A

The side-chain of His67 is stacked on the B-face of the nicotinamide. Two pairs of symmetry-related side-chains of His67 form flat surfaces at the top and bottom in the center of the active site. His67 side-chains in each pair are inclined by ~20°. The stacking interaction of the imidazole ring of His67 over nicotinamide and the concomitant van der Waals interactions between the side-chains in the nearby pair of His67 residues may synergistically contribute in large part to the tight binding of the cofactor compared with the wild-type enzyme.

The carboxamide nitrogen N7 of the nicotinamide donates a hydrogen bond to carbonyl oxygen of Val66. The oxygen O7 forms hydrogen bonds with water molecules Wat145, Wat146, and Wat149. The exocyclic oxygen O2'N has water-mediated interactions with

yl oxygen atoms of Val66 and Ile68 (Fig. 2D). The O3'N forms an intramolecular hydrogen bond with O2N and a water-mediated interaction with carbonyl oxygen O2C. The endocyclic oxygen O4' is in van der Waals contact with the side-chain of His67. O2N displays a water-mediated interaction with Asn65 and Ser65:O γ . The phosphodiester oxygen O3' forms a direct hydrogen bond, albeit long (3.2 Å), to Lys69. A single water molecule (Wat144) bridges O5' and

O1N of the phosphate anion interacts with amino acid residues through one or two water molecules. The interacting residues are Lys32, Ser34, Ala36, Tyr46, Thr51, Gly64, Val66, and Ile68. There is an intraligand link between O1N and O5'A via two water molecules, Wat144 and Wat151. The phosphate atom O2N has direct

Site C

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presumably due to a combination of flexibility, static disorder, and partial occupancy owing to symmetric binding. The mobility of this section may be important for its function. For a comparison of NADP⁺ and folate binding environment, see Supplemental materials.

Structure-cooperativity correlation

Previous ITC and solution NOE studies indicate that interligand interactions play a dominant role in binding and catalysis (Bradrick et al. 1996; Li et al. 2001). Two cofactors, one on each half of the tetramer, are bound as described above. Two microscopic K_d ($K_{d1} = 0.027 \mu\text{M}$ and $K_{d2} = 0.62 \mu\text{M}$) were determined for the binding of NADPH to Q67H mutant enzyme, suggestive of two distinct binding sites exhibiting negative cooperativity (Park et al. 1997). Wat149 is conserved in all R67 DHFR structures determined to date. In the absence of the nearby NADPH, Wat149 is involved in hydrogen bonding interaction with the backbone amide of Ile68. Wat149 and the nicotinamide ring are mutually exclusive at a given cofactor binding site due to steric clashes. Therefore, binding of the cofactor entails displacement of Wat149 at a specific binding site. However, in the vacant *paired* site, symmetry-related Wat149 is unperturbed and may serve as an important structural feature for tight cofactor binding through hydrogen bond formation with O7 (Fig. 2D). When the second cofactor binds in the *paired* binding site, it will displace the symmetry-related water molecule Wat149 that is engaged by O7 of the first cofactor via hydrogen bonding. In addition, the second cofactor does not have access to its symmetry-related Wat149 for hydrogen bonding interaction because it was previously displaced by the first cofactor molecule. In other words, the two symmetry-related water molecules (Wat149) associated with the *paired* cofactors are displaced upon binding of the two cofactors. We presume that the first cofactor binding site is stronger due in part to the presence of the hydrogen bonding interaction with Wat149 among other interactions, whereas the binding of the second cofactor exhibits negative cooperativity due in part to loss of a favorable hydrogen bond involving Wat149 in both bound ligands. We differentiate two distinct binding sites for the cofactor based on the differential accessibility of a key water molecule Wat149. For details on the structural basis of cooperativity between the cofactor and folate, see Supplemental Materials.

Modeling ground state of the catalytic ternary complex

A previous model of the ternary complex (Narayana et al. 1995) is revised in light of the present holoenzyme structure. See Supplemental Material for details about the construction and figure of a catalytic ternary model.

Highlights of the model are (1) the A-side of the nicotinamide ring is toward the pore where the pteridine ring resides in the Michaelis complex. This is in agreement with the previous finding that R67 DHFR belongs to the A-stereospecific class of dehydrogenases. (2) Enhanced stacking between pteridine and pyridine rings approximates an *endo* configuration, and the two rings tilt away from each other as suggested for the transition state complex (Andres et al. 1996). (3) The distances C3 (nic)-C7 (fol) and C5 (nic)-C7 (fol) are 3.64 Å and 4.3 Å, respectively, a trend consistent with the interligand NOE data (Li et al. 2001). Several interligand distances are in agreement with those reported in ILOE studies (see Supplemental Materials). (4) The nicotinamide C4, C5, and C6 atoms lie closest to C5 of folate, and the carboxamide group of the nicotinamide is nearest to C7 and N8 of folate in accord with the ILOE data. (5) The exocyclic NA2 (fol) is within hydrogen bonding distance to O2' of the nicotinamide ribose, as noted in ILOE studies. A key feature derived from the modeling studies is that R67 DHFR exhibits a different binding mode for the reactants compared with its chromosomal counterpart.

Materials and Methods

Protein preparation, crystallization, and data collection

Recombinant Q67H mutant of R67 DHFR was expressed and purified as previously described (Park et al. 1997). The N-terminal 16 residues were cleaved using chymotrypsin, yielding a fully active protein of 62 amino acids. The truncated enzyme bound to the cofactor was crystallized at 4°C, employing the protocol described previously for growing R67 DHFR crystals. X-ray data for a single crystal flash cooled at 100 K were collected at MacCHESS beam line F1. The data were processed using the HKL program (Table 1; Otwinowski and Minor 1997).

Table 1. Summary of data collection and structure refinement

Data statistics		Refinement statistics	
Wavelength (Å)	0.9792	R/R _{free} ^b (%)	19.5/21.5
Space group	I4 ₁ 22	Protein atoms	445
Unit cell	a = b = 67.46,	Cofactor atoms	26
dimensions (Å)	c = 52.86		
Resolution range ^a (Å)	20–1.15 (1.24–1.15)	Water molecules	78
Unique reflections	19,048	Average B (Å ²)	
Redundancy	7.1	Overall	14.3
Completeness (%)	86.6 (60.2)	Protein	11.3
Mean I/σ(I)	30.2 (2.4)	Cofactor	27.7
R _{symm} (%)	3.9 (28.5)	Solvent	26.7

^a Values for the highest resolution shell are given in parentheses.

^b R_{free} was calculated for 5% of reflections randomly excluded from the refinement.

Structure refinement

Rigid-body refinement using data to 2.0 Å resolution was initiated using protein atomic coordinates of the native structure (Protein Data Bank [PDB] code 1VIE) with Gln67 modified to alanine. This was followed by cycles of simulated annealing, isotropic B-factor refinement (Brunger et al. 1998), and model building (Jones 2004) with the inclusion of higher-resolution data. In the later stages of refinement, electron density for the His67 side-chain was seen unambiguously in a difference Fourier map ($\sim 6\sigma$). The model was further subjected to refinement with TNT (Tronrud et al. 1987).

A $F_o - F_c$ map was computed using protein and all water molecules except those in the active site. This map revealed features for the nicotinamide ring ($\sim 2.5\sigma$) (Fig. 2A), nicotinamide ribose ($\sim 1.5\sigma$) (Fig. 2B), and the PP_i group ($\sim 3.0\sigma$) (Fig. 2C). An anomalous difference Fourier map using the phases derived from the protein and water atoms yielded two strong peaks corresponding to the PN (5σ) and PA (3σ) atoms in the pyrophosphate (Fig. 2C). There was no density corresponding to adenosine 2'-phosphate of NADP⁺. Difference Fourier maps did not reveal residues 17–20. The final model has an R-factor of 19.5% (Table 1). All main-chain (ϕ , ψ) angles are within the allowed or generously allowed regions of the Ramachandran plot.

Electronic supplemental material

The Supplemental material contains a figure showing a stereo view of a ternary model and a table of distances derived from X-ray/model/ILOE studies. Details related to comparison of NADP⁺ and folate binding environment, molecular basis for cooperativity between cofactor and folate binding, and construction of a catalytic ternary model are presented.

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References

Andres, J., Moliner, V., Safont, V.S., Domingo, L.R., Picher, M.T., and Krechl, J. 1996. On transition structures for hydride transfer step: A

- theoretical study of the reaction catalyzed by dihydrofolate reductase enzyme. *Bioorg. Chem.* **24**: 10–18.
- Bellamacina, C.R. 1996. The nicotinamide dinucleotide binding motif: A comparison of nucleotide binding proteins. *FASEB J.* **10**: 1257–1269.
- Bradrick, T.D., Beechem, J.M., and Howell, E.E. 1996. Unusual binding stoichiometries and cooperativity are observed during binary and ternary complex formation in the single active pore of R67 dihydrofolate reductase, a D₂ symmetric protein. *Biochemistry* **35**: 11414–11424.
- Brito, R.M., Rudolph, F.B., and Rosevear, P.R. 1991. Conformation of NADP⁺ bound to a type II dihydrofolate reductase. *Biochemistry* **30**: 1461–1469.
- Brunger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.S., Kuszewski, J., Nilges, M., Pannu, N.S., et al. 1998. Crystallography and NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr. D Biol. Crystallogr.* **54**: 905–921.
- Bystroff, C., Oatley, S.J., and Kraut, J. 1990. Crystal structures of *Escherichia coli* dihydrofolate reductase: The NADP⁺ holoenzyme and the folate–NADP⁺ ternary complex. Substrate binding and a model for the transition state. *Biochemistry* **29**: 3263–3277.
- Fleming, M.P., Datta, N., and Gruneberg, R.N. 1972. Trimethoprim resistance determined by R factors. *BMJ* **1**: 726–728.
- Hicks, S.N., Smiley, R.D., Hamilton, J.B., and Howell, E.E. 2003. Role of ionic interactions in ligand binding and catalysis of R67 dihydrofolate reductase. *Biochemistry* **42**: 10569–10578.
- Howell, E.E. 2005. Searching sequence space: Two different approaches to dihydrofolate reductase catalysis. *ChemBioChem* **6**: 590–600.
- Jones, T.A. 2004. Interactive electron-density map interpretation: From INTER to O. *Acta Crystallogr. D Biol. Crystallogr.* **60**: 2115–2125.
- Li, D., Levy, L.A., Gabel, S.A., Lebetkin, M.S., DeRose, E.F., Wall, M.J., Howell, E.E., and London, R.E. 2001. Interligand Overhauser effects in type II dihydrofolate reductase. *Biochemistry* **40**: 4242–4252.
- Matthews, D.A., Smith, S.L., Baccanari, D.P., Burchall, J.J., Oatley, S.J., and Kraut, J. 1986. Crystal structure of a novel trimethoprim-resistant dihydrofolate reductase specified in *Escherichia coli* by R-plasmid R67. *Biochemistry* **26**: 8591–8598.
- Narayana, N. 2006. High-resolution structure of a plasmid-encoded dihydrofolate reductase: Pentagonal network of water molecules in the D₂-symmetric active site. *Acta Crystallogr. D Biol. Crystallogr.* **62**: 695–706.
- Narayana, N., Matthews, D.A., Howell, E.E., and Xuong, N-h. 1995. A Plasmid-encoded dihydrofolate reductase from trimethoprim-resistant bacteria has a novel D₂-symmetric active site. *Nat. Struct. Biol.* **2**: 1018–1025.
- Nichols, R., Weaver, C.D., Eisenstein, E., Blakley, R.L., Appleman, J., Huang, T.H., Huang, F.Y., and Howell, E.E. 1993. Titration of histidine 62 in R67 dihydrofolate reductase is linked to a tetramer ↔ two-dimer equilibrium. *Biochemistry* **32**: 1695–1706.
- Otwinowski, Z. and Minor, W. 1997. Processing of X-ray diffraction data collected in oscillation mode. In *Macromolecular crystallography, part A*, Vol. 276 (eds. C.W. Carter and R. Sweet), pp. 307–326. Academic Press, New York.
- Park, H., Bradrick, T.D., and Howell, E.E. 1997. A glutamine 67 → histidine mutation in homotetrameric R67 dihydrofolate reductase results in four mutations per single active site pore and causes substantial substrate and cofactor inhibition. *Protein Eng.* **10**: 1415–1424.
- Tronrud, D.E., Ten Eyck, L.F., and Matthews, B.W. 1987. An efficient general-purpose least-squares refinement program for macromolecular structures. *Acta Crystallogr. A* **43**: 489–501.