Crystal structure of rat liver dihydropteridine reductase

KOTTAYIL I. VARUGHESE*†‡, MATTHEW M. SKINNER§, JOHN M. WHITELEY†, DAVID A. MATTHEWS¶,
AND NGUYEN H. XUONG*§||

Departments of *Biology, §Chemistry, and ||Physics, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0317; †Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037; and ‡Agouron Pharmaceuticals Inc., 3565 General Atomic Court, San Diego, CA 92121

Communicated by David R. Davies, March 23, 1992 (received for review November 4, 1991)

ABSTRACT The structure of a binary complex of dihydropteridine reductase [DHPR; NAD(P)H:6,7-dihydropteridine oxidoreductase, EC 1.6.99.7] with its cofactor, NADH, has been solved and refined to a final *R* factor of 15.4% by using 2.3 Å diffraction data. DHPR is an α/β protein with a Rossmann-type dinucleotide fold for NADH binding. Insertion of an extra threonine residue in the human enzyme is associated with severe symptoms of a variant form of phenylketonuria and maps to a tightly linked sequence of secondary-structural elements near the dimer interface. Dimerization is mediated by a four-helix bundle motif (two helices from each protomer) having an unusual right-handed twist. DHPR is structurally and mechanistically distinct from dihydrofolate reductase, appearing to more closely resemble certain nicotinamide dinucleotide-requiring flavin-dependent enzymes, such as glutathione reductase.

Dihydropteridine reductase [DHPR; NAD(P)H:6,7-dihydropteridine oxidoreductase, EC 1.6.99.7] and the three aromatic amino acid hydroxylases of phenylalanine, tyrosine, and tryptophan play vital roles in the synthesis of the catecholamines, dopamine, epinephrine, and serotonin, and indirectly also influence generation of the melanin pigments. Tetrahydrobiopterin (BH₄) is an essential cofactor in these metabolic pathways and facilitates the monooxygenase activity, which ultimately leads to the hydroxylation of the aromatic amino acid substrates (Fig. 1a). In these reactions phenylalanine, tyrosine, and tryptophan are converted to tyrosine, dihydroxyphenylalanine, and 5-hydroxytryptophan, respectively, and the cofactor undergoes oxidation to a quinonoid dihydro form of biopterin (q-BH₂). q-BH₂ then becomes the substrate for DHPR and in an NADH-mediated reaction is recycled to BH₄.

Defective function of the hydroxylation process has long been recognized clinically in the autosomal-recessive disease hyperphenylalaninemia or phenylketonuria (1). Originally this disease was considered to correlate only with a defective phenylalanine hydroxylase, and although this is still primarily its predominant cause, a small percentage of cases have been identified as arising from defects either in DHPR function (2, 3) or in the biosynthetic route to BH₄ (4). DHPR has always been identified with dihydrofolate reductase (DHFR) (Fig. 1b), the enzymatic target for the chemotherapeutic agent methotrexate, insofar as each requires a reduced dinucleotide cofactor and a dihydropteridine substrate for activity. This report clearly eliminates the commonality of the two enzymes and, moreover, suggests a mechanistic comparison to certain flavin-requiring enzymes, an observation that reemphasizes the similarity of pterin and flavin chemistry proposed earlier by Hemmerich (5), Viscontini (6), and Wessiak and Bruce (7). It should be noted that the protein structure described in this report is that of the rat liver enzyme; however, the

FIG. 1. (a) DHPR reduces q-BH₂ to BH₄, a cofactor for the aromatic amino acid hydroxylases that oxidize BH₄ back to q-BH₂ (R = 1',2'-dihydroxypropyl). (b) DHPR reduces 7,8-dihydropteridine to 5,6,7,8-tetrahydropteridine in the presence of NADPH (R = *p*-aminobenzoylglutamate).

sequence contains only 10 conservative substitutions (8) from that of the human enzyme (9) and, therefore, it is anticipated that any structural prediction made from the rat enzyme will apply to material from the human source as well.

MATERIALS AND METHODS

The crystals of the binary complex DHPR with NADH were grown under the same conditions that produced the monoclinic crystal form (10). Crystals of DHPR were grown at 4°C from hanging 10 μ l drops formed by mixing 5 μ l of protein-NADH solution at 10 mg/ml with 5 μ l of a reservoir solution containing 17% (wt/vol) PEG 4500, 11% (vol/vol) ethanol, and 0.05 M Tris buffer, pH 7.8. The crystals are orthorhombic, space group C222₁ with *a* = 50.10 Å, *b* = 139.13 Å, and *c* = 64.93 Å and contains one subunit per asymmetric unit.

The structure was solved at 2.7-Å resolution with data from four heavy-atom derivatives (Tables 1–3) using standard multiple isomorphous replacement methods and the solvent-flattening methods of Wang (11). The diffraction data were measured at 4°C with graphite monochromated CuK α radiation at the University of California, San Diego, Research Resource (12). In the initial multiple isomorphous replace-

Table 1. Data collection

Data set	Unit cell dimensions			Reflections, no.	<i>n</i>	<i>d</i> min, Å	<i>R</i> _{sym} , %	Overall complete, %	<i>R</i> _{deriv} , %
	<i>a</i>	<i>b</i>	<i>c</i>						
Native	50.10	139.13	64.93	10,097	88,752	2.3	6.0	96.3	
CH ₃ HgBr	50.10	139.33	64.91	10,303	55,501	2.3	7.0	98.2	15.1
K ₂ PtCl ₄	50.13	139.30	64.83	7,129	26,822	2.5	5.0	89.0	8.3
Na ₃ IrCl ₆	49.95	139.08	64.83	6,054	14,593	2.7	5.2	92.3	10.0
<i>cis</i> -Pt(NH ₃) ₂ Br ₂	50.09	139.90	65.10	8,408	27,198	2.4	6.5	91.0	16.5

n, Number of observations. $R_{\text{deriv}} = \sum |F_p - F_{\text{ph}}| / \sum F_p$. $R_{\text{sym}} = \sum_h \sum_{i=1}^N |\bar{I}(h) - I(h)_i| / \sum_h \sum_{i=1}^N I(h)_i$, where $I(h)_i$ is the *i*th measurement of reflection *h* and $\bar{I}(h)$ is the mean value of the *N* equivalent reflections.

ment map good electron density was present for NADH and all but the six amino-terminal amino acids. Intensities from three parent crystals were merged to obtain the native data set, and it was 96% complete to 2.3 Å and 91% complete in the 2.4 to 2.3 Å shell. Four heavy-atom derivatives were prepared: CH₃HgBr cocrystallized in the stoichiometric ratio 1.0:0.8; K₂PtCl₄ soaked for 12 hr at 0.25 mM; Na₃IrCl₆ soaked for 16 hr at 0.2 mM; *cis*-Pt(NH₃)₂Br₂ soaked for 3 days after adding solid powder into the drop. The reservoir solution used in the hanging-drop experiments was also used for crystal mounting and heavy-atom soaking. Hg positions were determined from the isomorphous difference Patterson function, and the positions of the heavy atoms in the other derivatives were located by using Hg phases. The Hg and Ir derivatives each had two sites, whereas K₂PtCl₄ and *cis*-Pt(NH₃)₂Br₂ had three and four sites, respectively, with two Pt sites in common.

Heavy-atom parameters were refined by using the program HEAVY (13). The solvent-flattening calculations were done by using phases computed with multiple isomorphous data from all four derivatives [Hg derivative and *cis*-Pt(NH₃)₂Br₂, 15–2.8 Å; Na₃IrCl₆, 15–3.5 Å; K₂PtCl₄, 15–3.0 Å; and anomalous signals of Hg, 15–3.5 Å]. Initial refinements were done by using simulated annealing with the program X-FLOR (14) and the 8 to 2.7 Å data. Refinements proceeded with all the observed data in the resolution range 8–2.3 Å to give a final *R* of 15.4% with rms deviations for bond lengths and angles of 0.013 Å and 2.3°, respectively. The model described here includes protein residues 5–240, NADH, and 136 ordered solvent molecules. Then eight cycles of PROLSQ (15) refinement were done with the standard restraints that also converged at 15.4%; however, rms deviation in bond lengths were reduced to 0.010 Å. At the conclusion of refinement rms deviations for bond distances and angles were 0.010 Å and 2.3°, respectively.**

**The atomic coordinates and structure factors have been deposited

Further details of the crystallographic analysis will be published elsewhere.

RESULTS AND DISCUSSION

DHPR is an α/β protein with a central twisted β-sheet flanked on each side by a layer of α-helices (Fig. 2). The β-sheet has seven parallel strands and a single antiparallel strand at one edge leading to the carboxyl terminus of the protein. Connections between individual β-strands involve α-helices, except that β_B and β_C are joined by a short stretch of polypeptide in random-coil conformation. Fig. 3 shows that the topology of the backbone folding of DHPR is quite distinct from that for DHFR (16). On the other hand, the first six strands of the central β-sheet in DHPR have the same overall topological connectivity as that found for the coenzyme-binding domains of several other NAD-dependent dehydrogenases. Together with connecting α-helical segments, this structural motif is referred to as a dinucleotide or "Rossmann" fold. The last strand of this nucleotide-binding domain β_F makes an unusual left-handed crossover connection to the adjacent strand β_G. A major function of the two carboxyl-terminal β-strands β_G and β_H is to anchor this complicated left-handed crossover to the canonical dinucleotide fold. This extended loop participates both in stabilizing a *syn* conformation for the nicotinamide base of bound NADH and in formation of the substrate-binding pocket.

NAD-dependent enzymes, such as lactate dehydrogenase (17), malate dehydrogenase (18), liver alcohol dehydrogenase (19), and glyceraldehyde 3-phosphate dehydrogenase (20) are oligomeric proteins with subunits composed of distinct structural domains. Each enzyme contains a dinucleotide-binding domain consisting of 150–200 sequentially contiguous amino acids and a second domain that has evolved to bind substrate and facilitate catalysis. Val-176 at the carboxyl-terminal end

in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (reference IDHR).

Table 2. Heavy atom phasing

		Resolution range/figure of merit (<i>n</i>)								
		Overall/ 0.68 (5741)	8.77/ 0.87 (348)	5.99/ 0.82 (514)	4.81/ 0.81 (622)	4.14/ 0.72 (708)	3.68/ 0.70 (796)	3.35/ 0.66 (872)	3.09/ 0.58 (929)	2.89/ 0.52 (950)
Derivative	Parameter									
CH ₃ HgBr	<i>F</i> _H / <i>E</i>	1.85	3.15	2.68	2.33	1.88	1.82	1.64	1.65	1.43
	<i>F</i> _{anom} / <i>E</i> _{anom}	0.61	1.12	0.93	0.84	0.49	0.32			
	Centric <i>R</i>	0.58	0.60	0.50	0.51	0.54	0.64	0.68	0.64	0.61
K ₂ PtCl ₄	<i>F</i> _H / <i>E</i>	1.26	1.14	1.68	1.80	1.52	1.49	1.37	0.93	0.80
	Centric <i>R</i>	0.64	0.60	0.57	0.62	0.64	0.72	0.66	0.74	0.67
Na ₃ IrCl ₆	<i>F</i> _H / <i>E</i>	0.96	0.92	1.23	1.26	1.24	1.07	0.97	0.70	0.65
	Centric <i>R</i>	0.64	0.57	0.60	0.54	0.66	0.72	0.68	0.73	0.71
<i>cis</i> -Pt(NH ₃) ₂ Br ₂	<i>F</i> _H / <i>E</i>	1.74	1.55	2.27	2.20	1.90	1.64	1.63	1.51	1.45
	Centric <i>R</i>	0.64	0.53	0.46	0.62	0.72	0.82	0.57	0.92	0.90

n, Number of observations. *F*_H, average rms heavy-atom structure factor amplitude. *F*_{anom}, average anomalous dispersion structure factor amplitude of heavy atoms. *E*, rms closure error. Centric *R*, $|F_{\text{ph}}(\text{obs}) - F_{\text{ph}}(\text{calc})| \times 100 / \sum |F_{\text{ph}}(\text{obs}) - F_{\text{ph}}(\text{calc})|$; obs, observed; calc, calculated. *E*_{anom}, rms anomalous closure error.

Table 3. Model refinement

Model	Residues	R factor		B	Data selection, Å
		Initial	Final		
Unrefined model with side-chain assignment for 140 residues	230	54.0	42.0	Overall	8.0–2.7
Partially refined model with all side chains	230	43.0	29.3	Overall	8.0–2.7
Final X-PLOR refinements	236	18.6	15.4	Individual	8.0–2.3
PROLSQ	236	16.4	15.4	Individual	8.0–2.3

of β_F is the last amino acid that forms interstrand hydrogen bonds within the canonical dinucleotide fold of DHPR. A right-handed crossover to the adjacent parallel-strand β_G would require the connecting loop to pass underneath the sheet. By turning above the sheet instead and making an unusual left-handed connection, amino acids 180–186 form a binding surface for the *re*-face of the nicotinamide moiety that must rotate away from the dinucleotide fold as a consequence of the *syn* conformation about the glycosidic bond of NMN. In DHPR, connecting regions between β_D and β_E and between β_E and β_F are also more complicated than corresponding connecting segments in most other dinucleotide-binding domains. These two interstrand connections together with the left-handed crossover between β_F and β_G form the binding site for q-BH₂. Thus, rather than having a separate substrate-binding domain distinct from its dinucleotide-binding domain, DHPR has adopted the strategy of simply elaborating connecting loops within the canonical dinucleotide fold to facilitate substrate binding and catalysis.

Although NADH can assume a folded conformation in solution, it usually binds to enzymes in an extended conformation with the nucleotide bases 10–15 Å apart and the ring planes roughly perpendicular to one another. In DHPR, the center of the bound nicotinamide ring is 11 Å from the center of the adenine pyrimidine ring. The adenine ring is in an anti conformation ($\chi = 155^\circ$), as observed in the other structures, whereas as noted above, the nicotinamide glycosidic bond has a *syn* conformation ($\chi = 67^\circ$). The DHPR-catalyzed transfer of the pro-*S* hydrogen of NADH to N5 of q-BH₂ (21, 22) classifies the reductase as a B-stereospecific dehydrogenase (23) and conforms to the general rule that enzymes transferring the pro-*S* hydrogen bind nicotinamide in a *syn* conformation relative to the attached ribose ring (23–25). Both ribose rings are puckered C2' endo. Rotation about the exocyclic C4'–C5' bond (γ) plays a crucial role in position-

ing the 5'-phosphate group relative to the sugar and base. The +*sc* conformation observed for this dihedral in the NMN portion of NADH orients 05' over the ribose ring, resulting in a more folded structure for this half of the dinucleotide compared with the adenine mononucleotide portion, in which the corresponding torsion angle is in the –*sc* range. The combination of *syn* and +*sc* rotations for χ and γ positions the carboxamide NH₂ 3.1 Å from one of the 5'-phosphate oxygen atoms, resulting in a favorable intramolecular hydrogen-bonding type of interaction, which probably stabilizes this particular overall conformation of the NMN mononucleotide.

DHPR exists in solution as a dimer. In the crystal form reported here the two individual subunits are related by a crystallographic 2-fold axis and, therefore, are necessarily identical. Helices α_E and α_F from one subunit come together with corresponding helices from the other subunit to form a four-helix cluster at the protomer interface (Fig. 4). The four-helix bundle is a common structural motif in proteins. Over a dozen such bundles have been identified, and recent interest has focused both on the topological connectivities between helices (26) and on the energetics of bundle assembly (27). All four-helix clusters described to date are tilted so as to impart an overall left-handed twist. Except for the helical domain in lipovitellin-phosvitin (28), the four adjacent helix pairs in known clusters are antiparallel. Helices α_E and α_F in DHPR are parallel to one another, providing but the second example of a four-helix bundle having two adjacent parallel and two adjacent antiparallel helices. In contrast to all other known four-helical domains, the helix cluster in DHPR has a right-handed twist with an orientation angle between neighboring antiparallel helix axes of around +155° compared with a consensus value of about –163° for this angle in clusters with left-handed twists.

FIG. 2. (a) Ribbon drawing of DHPR with NADH bound to it. (b) Stereoview of the Ca backbone for a DHPR monomer (240 residues) containing bound NADH. The β -strands $\beta_A \dots \beta_H$ are labeled as A $\dots$ H. The major α -helices are assigned nomenclature $\alpha_B, \alpha_D, \alpha_G$, according to the β -strand that follows it—i.e., the helix preceding β_B is called α_B .

FIG. 3. Comparison of the backbone folding of DHPR with DHFR. The α -helices are represented as cylinders, and the β -sheets are represented as triangles.

Recently, Howells *et al.* (29) have reported severe symptoms of phenylketonuria associated with a deficient DHPR enzyme in which a single additional threonine residue is inserted after position 122 of the wild-type human amino acid sequence. In the rat liver enzyme, this would correspond to an insertion after residue 118 near the carboxyl terminus of α_E (Fig. 4) on the backside of the molecule 25 Å from the active site. The last residue of α_E is Leu-122 followed immediately by a type II β turn (residues 123–126), in which residue 126 is also the first amino acid of β_E . We anticipate that protein folding in this region of the human mutant DHPR will be significantly altered because the insertion can be accommodated only by disrupting backbone hydrogen bonding within the tightly linked sequence of secondary structural elements α_E -type II β turn- β_E . The insertion may simply reduce subunit stability or increase sensitivity to proteases. The ability of subunits to form functional dimers could also

be affected because alterations of protein conformation in this region would be expected to directly affect folding of the adjacent contacting loop (residues 160–170) that forms part of the dimer interface.

Near the carboxyl-terminal edge of the dinucleotide fold bounded on one side by the *si*-face of nicotinamide, there is a prominent cleft that must be the binding site for q-BH₂. Protein residues contributing to the formation of this groove come from three extended loops that connect individual β -strands within the central sheet—namely, the polypeptide chain linking β_D to β_E , β_E to β_F , and β_F to β_G . In contrast to DHFR, where the pteridine ring of the substrate fits into a deep pocket, the substrate binding site in DHPR is a surface channel having a U-shaped cross section exposed to solvent at both ends.

To try to understand how substrate binds to DHPR, we used a molecular graphics terminal and attempted to dock the *endo*-isomer of q-BH₂ within the cleft subject to the constraint that the N5 of the substrate should reside ≈ 3.4 Å from C4 of bound nicotinamide. Because of the narrow width of this groove, bounded on the bottom by the nicotinamide base and on the top by the indole side chain of Trp-86, the substrate must slip between these two heterocycles and bind with its pteridine ring nearly parallel to the nicotinamide plane, such that N5 is positioned directly above C4 of the cofactor. The best geometrical fit to the DHPR active site occurs when the *re*-face of the pteridine ring rotates above the nicotinamide in a stacked configuration, allowing the dihydroxypropyl side chain of the substrate to point away from the nicotinamide ring. Unless the loop connecting β_D to β_E changes conformation upon binding q-BH₂, the alternate docking mode, in which the *si*-face of the substrate points toward nicotinamide, appears to be disfavored because of steric crowding between the isopropyl side chain and the cofactor.

Fig. 5 shows that the model predicts that N3 and the 2-NH₂ group of q-BH₂ are exposed to bulk solvent and are not involved in direct hydrogen bonding with DHPR. This interpretation is consistent with kinetic data showing that transient quinonoid species derived from various 2-substituted dihydropteridines, including 2-methyl, 2-methylthio, and 2-desamino analogs are good enzyme substrates (30, 31). The model also suggests considerable tolerance for substitution at the 6 position because the edge of the pyrazine ring is also predicted to bind at the protein surface. This result probably explains why methotrexate is a good inhibitor of this enzyme (32) and why quinonoid dihydropterins derived from com-

FIG. 4. Stereoview of α_E and α_F from each protomer that together form a four-helix bundle at the dimer interface. The intermolecular dyad axis runs approximately horizontal. Also shown is the region around T118 that in a deficient human DHPR associated with phenylketonuria is altered by insertion of an extra threonine residue (see text).

FIG. 5. Stereoview of the active site viewed across the U-shaped cleft, showing a possible binding mode for dihydrobiopterin.

pounds having various 6-alkoxymethyl substituents including long (1-octyl) and branched (*tert*-butyl) side-chain analogs are excellent substrates (33). In DHFR, a conserved carboxyl group protonates the dihydropteridine substrate before hydride transfer from NADPH. There are no amino acid side chains in the vicinity of the DHFR active site that could serve a similar function, suggesting that proton delivery to q-dihydropteridines is mediated directly by solvent.

Finally we call attention to the interesting structural similarity between the *endo*-q-isomer of q-BH₂ and the isoalloxazine ring system of oxidized flavins. Both molecules contain highly conjugated ring systems with identical dispositions of the four ring nitrogens. Moreover, flavin-dependent reductases using nicotinamide dinucleotides as a source of electrons also transfer hydride to N5. In our modeling of how q-BH₂ may bind to DHFR in the presence of NADH we have been led to propose a geometrical relationship between substrate and cofactor very similar to that reported for the oxidized flavin ring of FAD and NADH bound to glutathione reductase (34). A similar model was recently suggested for NADPH and FAD binding at the active site of ferredoxin-NADP⁺ reductase (35) and mercuric ion reductase (36).

We thank Tom Bray for technical assistance in purifying protein; Vic Ashford, Chris Nielson and Don Sullivan for support at the University of California, San Diego (UCSD) multiwire facility and Lynn Ten Eyck for assistance in XPLOR refinement on the San Diego supercomputer. This investigation was supported by Grant RR01644 (UCSD) from the National Institutes of Health, Grant DIR88-22385 from the National Science Foundation (UCSD), the Lucille P. Markey Foundation (UCSD), and Grant CA11778 from the National Institutes of Health (TSRI).

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