Specific arrangement of three amino acid residues for flavin-binding barrel structures in NADH-cytochrome b_5 reductase and the other flavin-dependent reductases

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Abstract The structure of NADH-cytochrome b_5 reductase from pig liver microsomes has been refined to a crystallographic *R* factor of 0.223 at 2.4 Å resolution. A structural comparison between the flavin-binding β barrel domain of NADH-cytochrome b_5 reductase and those of the other flavin-dependent reductases, ferredoxin-NADP$^+$ reductase, phthalate dioxygenase reductase and nitrate reductase, indicated that the overall barrel foldings are similar to each other and that the specific arrangement of three amino acid residues (Arg, Tyr and Ser/Thr) is usually necessary for flavin-binding. These conserved residues overlap each other in their three-dimensional structures and stabilize the flavin-binding site in the four flavin-dependent reductases.

Key words: NADH-cytochrome b_5 reductase; Flavin-dependent reductase; Flavoprotein; Electron transfer; X-ray crystallography

1. Introduction

In an early study of the FAD-binding motif, the β-α-β structure was the only binding motif for the FAD molecule based on the structural investigation of glutathione reductase [1] and *p*-hydroxybenzoate hydroxylase [2]. Both enzymes function as dimeric molecules, and each of their subunits is essentially divided into three domains: one for NADPH binding, another for FAD binding and the last for the subunit interface between the dimer. The NADPH binding and interface domains have quite different structures in the two enzymes, but the structures of the N-terminal FAD-binding domains are very similar.

By comparing structures of FMN-binding enzymes, glycolate oxidase [3] and flavocytochrome b_2 [4], another nucleotide binding motif, the α/β barrel, was identified. The reaction mechanism between these two enzymes is similar; the first step of the reaction is oxidation of the substrate and reduction of the FMN. Both substrates, lactate and glycolate, are α-hydroxy acids. These similarities may require the same structural motif to bind FMN molecules.

Our recent structure determination of NADH-cytochrome b_5 reductase (b5R) from pig liver microsomes [5] revealed a structural homology between the β barrel structures for the binding motif of flavin prosthetic groups in comparison with ferredoxin-NADP$^+$ reductase (FNR) [6], phthalate dioxygenase reductase (PDR) [7] and the FAD-containing fragment of nitrate reductase (NR) [8].

The flavin prosthetic groups in the FNR, PDR, NR and b5R structures are bound to the surface of the β barrel. The FNR molecule accepts two electrons from two molecules of ferredoxin, and then passes on these electrons to the NADP$^+$ in the form of a hydride [9]. The PDR molecule conveys two electrons from NADH to the Rieske type iron-sulfur center of phthalate dioxygenase via FMN and the iron-sulfur center [10]. In both cases, a flavin prosthetic group transfers electrons between a nucleotide and an iron-sulfur center. The FAD-containing fragment of NR accepts electrons from NADH and gives them to the cytochrome *b* domain of NR via the intramolecular electron transport pathway [11]. The b5R molecule accepts two electrons from NADH and then passes them to two molecules of cytochrome b_5 in which heme iron is reduced [12]. In these cases, the FAD molecule acts as an electron carrier from a nucleotide to a heme group. There are some minor differences in these four reductases: (i) NADP$^+$ is the electron acceptor for FNR whereas NADH is the electron donor for PDR, NR and b5R; (ii) the heme group is a prosthetic group which accepts electrons from NR and b5R whereas the iron-sulfur center interacts with FNR and PDR; (iii) FAD is the prosthetic group of FNR, NR and b5R whereas FMN is the prosthetic group of PDR. In spite of these differences, the β barrel core structures for the binding of flavin prosthetic groups are quite similar to each other.

On the basis of the recently solved crystal structure of b5R [5], further crystallographic refinement has been performed to obtain detailed information of the flavin-binding site. In this study we focus on the three conserved residues for flavin binding in the β barrel commonly found in the four flavin-dependent reductases.

2. Experimental

The structure of the hydrophilic domain of b5R has been determined at 2.4 Å resolution [5]. We have proceeded with the further refinement of the structure to obtain more detail. This was done by using the stereochemically restrained least-squares method with the program package PROLSQ [13]. Water molecules were assigned if peaks in the *Fo–Fc* maps were situated at positions forming at least one hydrogen bonding to protein atoms. The current model comprises residues from 3 to 272, an FAD prosthetic group and 48 water molecules. The crystal-

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Abbreviations: FAD, flavin adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NADH, nicotinamide adenine dinucleotide; FMN, flavin mononucleotide; b5R, NADH-cytochrome b_5 reductase; FNR, ferredoxin-NADP$^+$ reductase; PDR, phthalate dioxygenase reductase; NR, nitrate reductase.

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lographic R factor in the resolution range 5.0–2.4 Å is 0.223 for all 10,350 reflections. The stereochemistry of the model is good with r.m.s. deviations for bond lengths of 0.016 Å and bond angles of 3.3°. Individual temperature factors were assigned for all non-hydrogen atoms with a mean value of 18.1 Å^2.

The atomic coordinates of FNR, PDR and NR were obtained from the Brookhaven Protein Data Bank [14]. The structures of the flavin-binding domains of these enzymes were superimposed by the transfer matrices generated as follows. Each flavin molecule of FNR, PDR and NR was manually adjusted to that of b5R so as to overlap in the isoalloxazine plane. Amino acid residues situated near each other between b5R and the other proteins were then picked up and assigned as a 'residue pair'. The number of residue pairs was 59 when b5R was compared with FNR and PDR. On the other hand, the number of residue pairs between b5R and NR was 105, because there were no insertions or deletions between the primary sequences of b5R and NR. The transfer matrices were calculated by minimizing the r.m.s. distances between Cα atoms of the assigned residue pairs. The resulting r.m.s. distances were 1.28 Å between superimposed FNR and b5R, and 1.62 Å between PDR and b5R (for 59 residue pairs), whereas that of NR and b5R was 1.83 Å (for 105 residue pairs).

3. Results and discussion

The amino acid sequences of the flavin-binding domains of FNR, PDR, NR and b5R are shown in Fig. 1. The sequence identities of the flavin-binding domains of FNR and PDR compared to that of b5R are of the same degree, i.e. 16% between b5R and FNR, and 20% between b5R and PDR. On the other hand, the sequence identity between the flavin-binding domains of NR and b5R is much higher, at about 45%. The barrel structures of these enzymes are so compact that the inner space of the barrel is mostly packed by the side chains of the residues in the β strands. In this case, the residues with their side chain inside the barrel may play a more important role in maintaining the barrel structure than those with their side chain outside. However, the side chain of the conserved residues in the β strands are not always located inside of the barrel, as shown in Fig. 1.

The refined structure of b5R is shown in Fig. 2. Superimposed backbone structures of the flavin-binding domains are shown in Fig. 3. The domains overlap well, especially in the regions of the β strands. The relative positions of the flavin prosthetic group and the peptide chain are also the same. The remarkable difference is on the adenine binding loop, which is observed at the top of both Fig. 3A and B. In comparison with FNR (Fig. 3A), different backbone structures of the loop region result from the different conformation of the adenine portion of each FAD. On the other hand, in comparison with

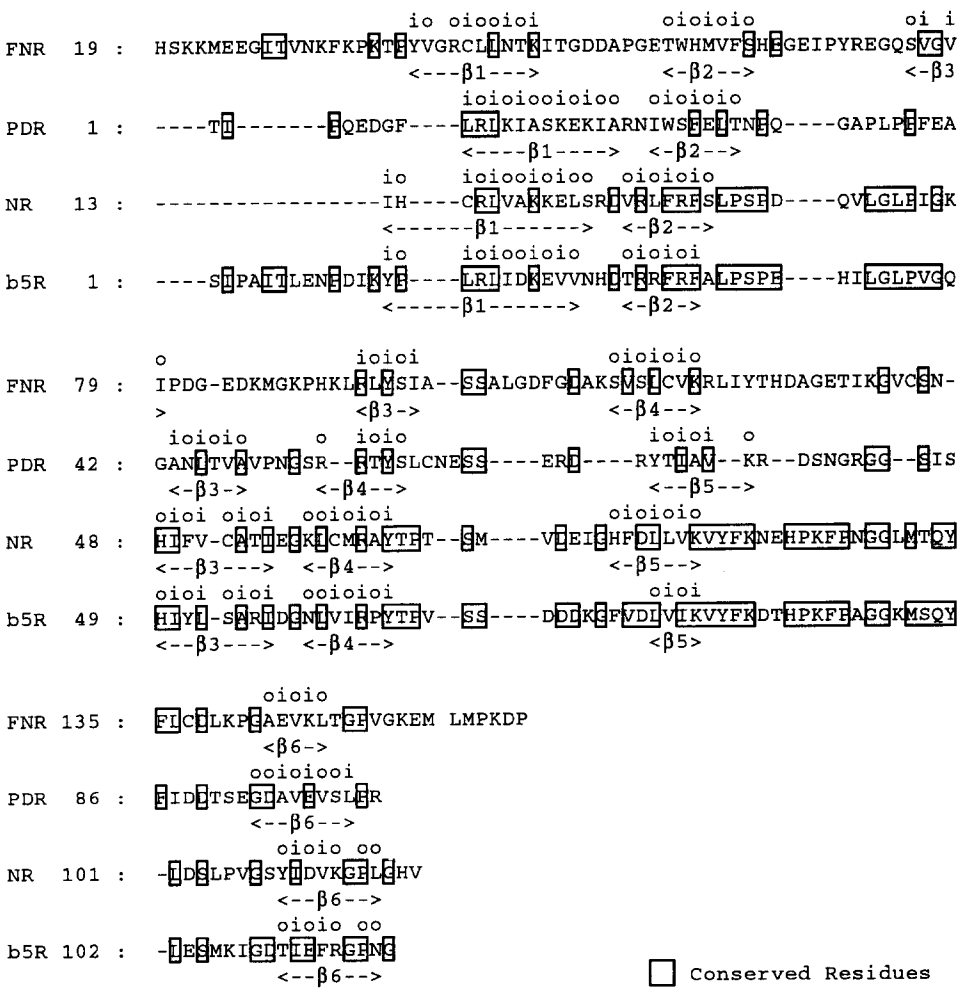

Fig. 1. Primary sequence alignment of flavin prosthetic group binding domains of FNR, PDR, NR and b5R. The residues enclosed in the boxes are conserved between more than two barrel structures. The secondary structure elements are shown below the sequence array. The characters 'i' and 'o' at the upside of the sequence array indicate residues with side chains located 'inside' and 'outside' the barrel, respectively.

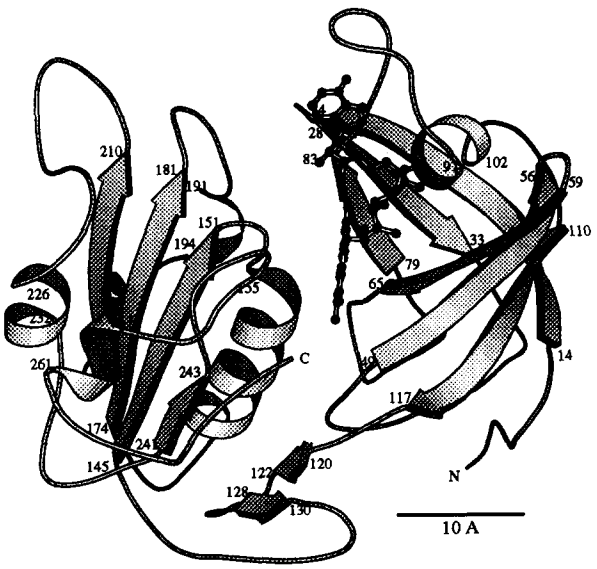

Fig. 2. A ribbon diagram of the refined three-dimensional structure of NADH cytochrome b_5 reductase from pig liver microsomes generated by the program MOLSCRIPT [17]. The FAD molecule is represented by a ball-and-stick model.

PDR (Fig. 3B), the different backbone length in this region is due to the absence of the adenine portion in FMN of PDR. In contrast, most of the Cα atoms overlap well compared with NR (Fig. 3C).

Arg^{63}, Tyr^{65} and Ser^{99} of b5R are found in the corresponding positions of FNR and PDR in the sequence alignment (Fig. 1). It was pointed out that they are in the consensus sequence for binding of the flavin prosthetic group [15]. In NR, Ser is replaced by Thr which has an additional methyl group to Ser. The side chains of these residues are hydrogen bonded directly to the ribitol or phosphate group of the FAD molecule in the b5R structure, and no other side chains are hydrogen bonded. Functionally conserved residues should be located at the same position in the three-dimensional structure. Fig. 4 indicates that these three residues are located at a similar position and overlap well in their tertiary structures between four proteins. The residues with side chains involved in the hydrogen bonds to the flavin molecule, are conserved, which suggests that this specific arrangement of Arg, Tyr and Ser/Thr is usually necessary for the flavin-binding barrel structure. A schematic diagram of the relative positions of flavin prosthetic groups and the important residues is depicted in Fig. 5. Several residues in the fifth β-strand and in the subsequent loop region are hydrogen bonded to the flavin molecule, mostly by their main chain atoms. On the other hand, the residues of Arg and Tyr in the forth strand and the Ser/Thr of the short α helix are hydrogen bonded to the flavin by their side chain atoms to maintain the structure of ribitol and the phosphate group of the flavin. From the view point facing out of the barrel, the left side of the flavin molecule in Fig. 5 is held by the main chain atoms regardless of the type of residue, and the right side is bound to three conserved residues, Arg, Tyr and Ser/Thr. The distances of the hydrogen bonds between the flavin (FAD or FMN) and the three residues (Arg, Tyr and Ser/Thr) are listed in Table 1.

These three conserved residues are also found in the primary sequences of the other FAD dependent reductases, NADPH-sulfite reductase and NADPH-cytochrome *P*-450 reductase [16], although their three-dimensional structures are not yet

Table 1
Comparison of hydrogen bond distances

Hydrogen bond	Distance (Å)			
	FNR [9]	PDR[b] [10]	NR[c] [11]	B5R[a]
OP2 (FAD/FMN[b])–NH1(Arg)	3.5	3.2	2.7	3.3
OP2 (FAD/FMN)–NE(Arg)	2.8	2.9	3.5	2.8
AO1 (FAD)–NH1(Arg)	3.6		3.8	3.5
AO2 (FAD)–NH1(Arg)	3.0		2.8	3.2
O4* (FAD/FMN)–OH(Tyr)	2.7	3.6	3.7	2.5
OP1 (FAD/FMN)–OG(Ser/Thr[c])	2.6	2.8	2.4	2.7

[a] This work.
[b] FMN is a cofactor of PDR.
[c] Ser is replaced by Thr in NR.

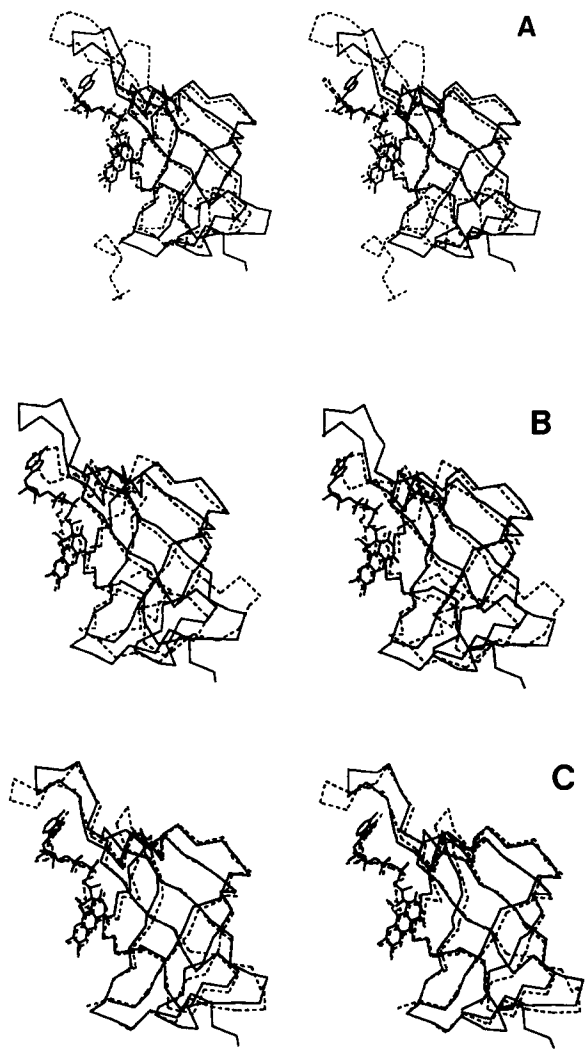

Fig. 3. Stereoviews of the optimally superimposed barrel structures of the flavin prosthetic group binding domains between b5R (solid line) and (A) FNR (dashed line), (B) PDR (dashed line) and (C) NR (dashed line).

Fig. 4. A stereoview of the flavin prosthetic groups and the three related residues, Arg, Tyr and Ser/Thr, superimposed among b5R (yellow, Arg^{63}, Tyr^{65} and Ser^{99}), FNR (red, Arg^{93}, Tyr^{95} and Ser^{133}), PDR (green, Arg^{55}, Tyr^{57} and Ser^{83}) and NR (blue, Arg^{62}, Tyr^{64} and Thr^{98}).

known. The barrel structure and the specific arrangement of the Arg, Tyr and Ser/Thr residues are also expected to be found in these flavoproteins. Furthermore, this structural motif for the binding of the flavin prosthetic group is most likely a common feature for both FAD and FMN.

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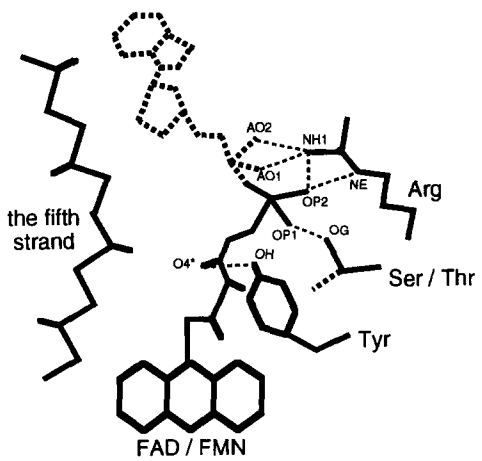

Fig. 5. Schematic diagram of the relative positions of flavin prosthetic groups and the residues involved in flavin binding found in four flavin-dependent reductases. The hydrogen bonds between the side chains and flavin molecules are depicted as thin dashed lines. The hydrogen bonds between the flavin molecule and the residues in the fifth strand are not exactly the same in the four structures and were omitted from the figure. The adenine portion of the FAD molecule is depicted as the bold dotted line which does not exist in FMN of PDR. The covalent bond between $C\beta$ and $C\gamma 2$ of Thr(NR) is also depicted as a bold dotted line and is not included in Ser(FNR, PDR and b5R).

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