Crystal structures of the naturally fused CS and cytochrome b_5 reductase (b_5R) domains of Ncb5or reveal an expanded CS fold, extensive CS– b_5R interactions and productive binding of the NAD(P)⁺ nicotinamide ring

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Ncb5or (NADH-cytochrome b_5 oxidoreductase), a cytosolic ferric reductase implicated in diabetes and neurological diseases, comprises three distinct domains, cytochrome b_5 (b_5) and cytochrome b_5 reductase (b_5R) domains separated by a CHORD–Sgt1 (CS) domain, and a novel 50-residue N-terminal region. Understanding how interdomain interactions in Ncb5or facilitate the shuttling of electrons from NAD(P)H to heme, and how the process compares with the microsomal b_5 (Cyb5A) and b_5R (Cyb5R3) system, is of interest. A high-resolution structure of the b_5 domain (PDB entry 3lf5) has previously been reported, which exhibits substantial differences in comparison to Cyb5A. The structural characterization of a construct comprising the naturally fused CS and b_5R domains with bound FAD and NAD⁺ (PDB entry 6mv1) or NADP⁺ (PDB entry 6mv2) is now reported. The structures reveal that the linker between the CS and b_5R cores is more ordered than predicted, with much of it extending the β -sandwich motif of the CS domain. This limits the flexibility between the two domains, which recognize one another via a short β -sheet motif and a network of conserved side-chain hydrogen bonds, salt bridges and cation– π interactions. Notable differences in FAD–protein interactions in Ncb5or and Cyb5R3 provide insight into the selectivity for docking of their respective b_5 redox partners. The structures also afford a structural explanation for the unusual ability of Ncb5or to utilize both NADH and NADPH, and represent the first examples of native, fully oxidized b_5R family members in which the nicotinamide ring of NAD(P)⁺ resides in the active site. Finally, the structures, together with sequence alignments, show that the b_5R domain is more closely related to single-domain Cyb5R proteins from plants, fungi and some protists than to Cyb5R3 from animals.

1. Introduction

Cytochrome b_5 reductase (Cyb5R) is present in all living organisms and serves as a structural module in a wide variety of redox enzymes. Of the four isoforms of Cyb5R that exist in animals, three (Cyb5R1–Cyb5R3) are single-domain proteins with high sequence homology (Zhu *et al.*, 1999; Supplementary Fig. S9). Cyb5R3 plays key roles in methemoglobin reduction (Kitao *et al.*, 1974) and fatty-acid desaturation (Holloway, 1971), while much less is known about Cyb5R1 and Cyb5R2. The fourth isoform (Cyb5R4), known as Ncb5or (NADH cytochrome b_5 oxidoreductase; Zhu *et al.*, 1999), is a

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flavohemoprotein that is implicated in diabetes, neurological diseases and iron homeostasis (Xie *et al.*, 2004; Zhu *et al.*, 2004; Wang *et al.*, 2011; Stroh *et al.*, 2016, 2018). Ncb5or differs from Cyb5R3 in several notable ways. Firstly, it comprises three distinct domains in an arrangement that is unique to Ncb5or, cytochrome b_5 (b_5) and cytochrome b_5 reductase (b_5 R) redox domains separated by a CHORD–Sgt1 (CS) domain, and it also features a 50-residue N-terminal region [Fig. 1(a); Garcia-Ranea *et al.*, 2002; Zhu *et al.*, 1999]. CS domains exist in diverse proteins (Garcia-Ranea *et al.*, 2002) and are commonly involved in protein–protein interaction (Supplementary Table S1). Secondly, the b_5 R domain contains notable gaps and insertions relative to Cyb5R3 (Zhu *et al.*, 1999). Finally, it is able to utilize either NADH or NADPH as the source of electrons to reduce FAD (Zhu *et al.*, 2004).

Although full-length Ncb5or has proven to be resistant to crystallization, we have succeeded in obtaining a high-resolution X-ray crystal structure of the b_5 domain (1.25 Å resolution; PDB entry 3lf5; Deng *et al.*, 2010), which exhibits substantial differences in fold and heme environment in comparison to microsomal b_5 (Cyb5A). We now report high-resolution structures of the CS– b_5 R construct with bound FAD and NAD⁺ or NADP⁺ (2.05 and 2.15 Å resolution, respectively). The structures reveal the nature of the interactions between the CS and b_5 R domains of Ncb5or, provide a structural explanation for the apparent lack of discrimination of the b_5 R domain for NADH and NADPH (Zhu *et al.*, 2004) and represent the first example of a fully oxidized b_5 R with NAD⁺ or NADP⁺ bound with the nicotinamide rings perched above the isoalloxazine ring of FAD. The results also advance our long-term goal of elucidating how the three domains of Ncb5or work together to facilitate electron transfer from flavin to heme in comparison to the better characterized intermolecular microsomal system (Cyb5R3/Cyb5A; Shirabe *et al.*, 1998; Kawano *et al.*, 1998).

2. Materials and methods

2.1. Limited proteolysis studies of Ncb5or

Full-length recombinant human Ncb5or carrying eight extra residues (MGHHHHHH) was prepared to homogeneity as described previously (Zhu *et al.*, 2004) and was concentrated to 13 mg ml^{−1} (in 10 mM Tris–HCl pH 7.0, 200 mM NaCl). Limited proteolysis was performed by mixing 10 µl Ncb5or with an equal volume of 10 µg ml^{−1} trypsin (in 10 mM HEPES pH 7.5, 500 mM NaCl) and incubation at 8°C for 48 h. The products were separated on SDS–PAGE and visualized by Coomassie Blue [Supplementary Fig. S1(a)]. To identify the cleavage site, the resultant stable fragments of Ncb5or (bands 1 and 2) and undigested control (band 3) were subjected to peptide mapping (5 h incubation with chymotrypsin) by liquid chromatography–tandem mass spectrometry analysis (LC–MS/MS) as described previously (Koen *et al.*, 2013). The results, which are shown in Supplementary Fig. S1(b), are based on the assumption that chymotrypsin and trypsin specifically cleave at the C-terminus of Phe/Leu/Trp/Tyr and Lys/Arg

residues, respectively. In contrast to band 3 with all peptides (*i.e.* full-length Ncb5or), bands 1 and 2 lack the residues prior to the 170–181 peptide (EGPSYPSYDWF) but include all subsequent residues, and thus were identified as the CS– b_5 R fragment resistant to limited proteolysis.

2.2. Purification and crystallization of CS– b_5 R

The expression and purification of recombinant CS– b_5 R was performed as described previously (Deng *et al.*, 2010). Briefly, the cDNA corresponding to residues 164–521 of human Ncb5or was subcloned into the NcoI and BamHI sites of pET-19b vector and a six-His tag was added to the N-terminus using PCR primers. The CS– b_5 R protein was expressed in *Escherichia coli* BL21(DE3) pLysS–RARE cells with 0.5 mM IPTG induction at 17°C overnight and purified to homogeneity by Ni²⁺–NTA chelation, ion-exchange and size-exclusion column chromatography using HisTrap HP, HiTrap Q HP and Superdex 200 Increase 10/300 GL columns at flow rates of 1.0, 1.0 and 0.4 ml min^{−1}, respectively. Purified CS– b_5 R was concentrated to 23 mg ml^{−1} in 5 mM K₂HPO₄ pH 7.4 supplemented with 0.2 mM FAD for crystallization screening. All crystallization experiments were conducted in 96-well Compact Jr (Rigaku Reagents) sitting-drop vapor-diffusion plates at 20°C using equal volumes of protein and crystallization solution equilibrated against 75 µl of the latter. Crystals that formed thin plate clusters were obtained after four days from Wizard Classic 3 & 4 screen (Rigaku Reagents) condition B8 [10% (w/v) PEG 6000, 100 mM HEPES pH 7.0]. Refinement screening was carried out using the Additive HT screen (Hampton Research), in which 10 µl of the 96 additives was dispensed into each reservoir of a Compact Jr plate along with the Wizard Classic 3 condition B8 to give a volume of 100 µl. This yielded a refinement screen that contained 10% of the additives relative to their stock concentrations. Single crystals displaying a thin plate morphology were obtained from conditions containing additives F3 [condition 1: 10% (w/v) PEG 6000, 100 mM HEPES pH 7.0, 300 mM NDSB-195] and F4 [condition 2: 10% (w/v) PEG 6000, 100 mM HEPES pH 7.0, 200 mM NDSB-201]. To prepare complexes with NAD⁺ (Sigma, catalog No. N3014) and NADP⁺ (Sigma, catalog No. N3139), single crystals were transferred into solutions of condition 1 supplemented with 5 mM NADP⁺ and condition 2 supplemented with 5 mM NAD⁺ and incubated for 1 h. Samples were transferred to a fresh drop composed of 80% crystallization solution and 20% glycerol and were stored in liquid nitrogen.

2.3. Data collection and structure refinement

X-ray diffraction data were collected on beamline 17-ID at the Advanced Photon Source using a Dectris PILATUS 6M pixel-array detector. Intensities were integrated using XDS (Kabsch, 1988, 2010) via *autoPROC* (Vonnrhein *et al.*, 2011) and the Laue class analysis and data scaling were performed with AIMLESS (Evans, 2011). The Matthews coefficient (Matthews, 1968; $V_M = 2.7 \text{ Å}^3 \text{ Da}^{-1}$, 54% solvent) indicated that a single molecule was present in the asymmetric unit.

Figure 1 CS- b_5 R-NAD structure. (a) Schematic diagram of the domain arrangement of Ncb5or. (b) Asymmetric unit showing the CS domain (red) and b_5 R subdomains colored by the FAD-binding (green) and NAD-binding (magenta) regions. Linker regions between subdomains are colored gray. The FAD and NAD⁺ molecules are rendered as blue and gold spheres, respectively. (c) Secondary-structure annotation. The colored bars above each segment are color-coded to match the domains as shown in (b). Residues highlighted in yellow are part of the purification tag and the underlined residues at the N-terminus were disordered and could not be modeled in the structure. Residues that make up the linker regions between domains or subdomains are both italicized and underlined. The C-terminal residue Ala521 is ordered in the NAD⁺ complex but not in the NAD⁺ complex.

Structure solution was conducted by molecular replacement, using ‘as-isolated’ crystals of CS- b_5 R that had not been soaked with NAD⁺ or NADP⁺, by first placing the b_5 R domain followed by the CS domain, and the top solutions were obtained in space group $P2_1$. This was performed as follows. Automated molecular replacement was carried out with *MORDA* (Vagin & Lebedev, 2015), which generated a search model based on the structure of cytochrome b_5 reductase from the slime mold *Physarum polycephalum* (PDB entry 2eix; Kim *et al.*, 2007). Initial automated model building and refinement of the b_5 R domain were conducted with *ARP/wARP* (Langer *et al.*, 2008) and *REFMAC* (Murshudov *et al.*, 2011), respectively, which converged at R and R_{free} values of 0.42 and 0.48, respectively. This model was used as a fixed partial solution and the CS domain was placed using *MOLREP* (Vagin & Teplyakov, 2010) and the coordinates of an unrelated CS-domain structure (PDB entry 2xcm, chain C, residues Ala150–Lys241; Zhang *et al.*, 2010) as the search model, which was approximately 23% identical. The contrast and corrF scores for the solution were 5.3 and 0.61, respectively, which indicated that a correct solution had been obtained. Automated model building with *ARP/wARP* and *REFMAC* converged at R and R_{free} factors of 0.23 and 0.27, respectively. This model was used for molecular replacement with *Phaser* (McCoy *et al.*, 2007) against the diffraction data obtained from the NAD⁺- or NADP⁺-soaked crystals. Structure refinement and manual model building were conducted with *PHENIX* (Adams *et al.*, 2010) and *Coot* (Emsley *et al.*, 2010), respectively. Disordered side chains were truncated to the point at which electron density could be observed. Structure validation was conducted with *MolProbity* (Chen *et al.*, 2010) and figures were prepared using the *CCP4mg* package (Potterton *et al.*, 2004). The angles between the mean planes of FAD and NAD⁺/NADP⁺ and the butterfly bend angles of FAD were calculated using *Olex2* (Dolomanov *et al.*, 2009).

The initial structure solution was carried out using ‘as-isolated’ crystals of CS- b_5 R which had not been soaked with NAD⁺ or NADP⁺. Following initial refinement, prominent difference electron density consistent with an FAD molecule was observed. After successive rounds of model

building and refinement, large regions of electron density were present near the FAD that were consistent with an NAD⁺ or NADP⁺ molecule that was most likely to have been acquired from the expression host. The identity could not be determined with certainty since the electron density was weak owing to partial occupancy. Therefore, crystals were soaked in the presence of NAD⁺ (CS-*b*₅R-NAD) and NADP⁺ (CS-*b*₅R-NADP), which allowed structure determination of these cofactors with full occupancy. The final refinement and model statistics are given in Table 1. The refined atomic coordinates and experimental structure factors of CS-*b*₅R-NAD and CS-*b*₅R-NADP have been deposited in the Worldwide Protein Data Bank (PDB entries 6mv1 and 6mv2, respectively).

3. Results and discussion

3.1. General comparison of CS-*b*₅R-NAD and CS-*b*₅R-NADP

The overall structure of CS-*b*₅R-NAD is depicted in Fig. 1(*b*), along with the secondary-structure annotation. These annotations relative to the CS-*b*₅R construct used for structural analysis are provided in Fig. 1(*c*). The CS-*b*₅R-NAD and CS-*b*₅R-NADP structures are nearly identical: superposition with *GESAMT* (Krissinel, 2012) yielded an r.m.s.d. of 0.21 Å between C^α atoms for 355 aligned residues. As such, the hydrogen-bonding interactions are highly similar. The binding modes of FAD and of the NAD⁺ and NADP⁺ molecules are also virtually identical, and no major conformational differences in the surrounding amino acids are observed. The structural descriptions and comparisons in the following sections are largely based on the CS-*b*₅R-NAD structure, with differences in CS-*b*₅R-NADP noted where relevant.

3.2. The Ncb5or CS domain features an expanded β-sandwich motif

The structure of the Ncb5or CS domain (including its connection to the *b*₅R domain) is depicted in Fig. 2(*a*), along with the corresponding structure of the CS domain of the human co-chaperone p23 [PDB entry 1ejf; Fig. 2(*b*); Weaver *et al.*, 2000]. The p23 protein and all other structural homologues of the Ncb5or CS domain identified in a search conducted using the *DALI* server (Holm & Laakso, 2016) feature a four-on-three antiparallel β-sandwich fold. Superpositions of some of these proteins with the Ncb5or CS domain are shown in Supplementary Fig. S2. Fig. 2 and Supplementary Fig. S2 show that the Ncb5or CS domain differs from its structural homologues in featuring additional β-sheet structure involving residues Gly256–Pro267. These residues form two strands (β8 and β9) separated by a five-residue loop that align in an antiparallel fashion with β5 on the three-stranded face of the β-sandwich. Well defined structure extends even beyond Pro267, with Arg268 and Thr271 anchoring a classic type I β-turn. In previously published structures that include CS domains, the polypeptide emanating from β7 generally extends away from the core fold or interacts with it in a

Table 1

Crystallographic data for CS-*b*₅R structures.

Values in parentheses are for the highest resolution shell.

	CS- <i>b</i> ₅ R-NAD	CS- <i>b</i> ₅ R-NADP
Data collection		
Unit-cell parameters (Å, °)	$a = 56.15, b = 68.63, c = 57.60, \beta = 97.5$	$a = 56.26, b = 68.84, c = 57.63, \beta = 97.4$
Space group	$P2_1$	$P2_1$
Resolution (Å)	43.90–2.15 (2.22–2.15)	47.97–2.05 (2.11–2.05)
Wavelength (Å)	1.0000	1.0000
Temperature (K)	100	100
Observed reflections	80292	92745
Unique reflections	23683	27467
$\langle I/\sigma(I) \rangle$	10.8 (1.7)	11.8 (1.8)
Completeness (%)	99.8 (99.7)	100 (100)
Multiplicity	3.4 (3.3)	3.4 (3.4)
$R_{\text{merge}}^{\dagger}$ (%)	8.5 (67.9)	7.7 (60.6)
$R_{\text{meas}}^{\ddagger}$ (%)	10.1 (81.1)	9.1 (71.3)
$R_{\text{p.i.m.}}^{\S}$ (%)	5.4 (43.8)	4.9 (38.0)
$CC_{1/2}^{\P}$	0.997 (0.776)	0.996 (0.830)
Refinement		
Resolution (Å)	34.31–2.15	36.34–2.05
Reflections (working/test)	22465/1189	26069/1370
R factor/ $R_{\text{free}}^{\dagger\dagger}$ (%)	18.3/23.0	17.4/22.4
No. of atoms		
Protein	2753	2777
FAD	53	53
NAD ⁺ or NADP ⁺	44	48
Water	132	221
Model quality		
R.m.s. deviations		
Bond lengths (Å)	0.009	0.009
Bond angles (°)	1.080	1.100
Average B factor (Å ²)		
All atoms	38.2	34.7
Protein	38.0	34.4
FAD	37.5	34.5
NAD ⁺ or NADP ⁺	43.3	36.6
Water	40.9	39.0
Coordinate error (maximum likelihood) (Å)	0.25	0.22
Ramachandran plot		
Most favored (%)	95.8	98.0
Additionally allowed (%)	4.2	2.0

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity measured for the i th reflection and $\langle I(hkl) \rangle$ is the average intensity of all reflections with indices hkl . [‡] R_{meas} is the redundancy-independent (multiplicity-weighted) R_{merge} (Evans, 2006, 2011). [§] $R_{\text{p.i.m.}}$ is the precision-indicating (multiplicity-weighted) R_{merge} (Diederichs & Karplus, 1997; Moore *et al.*, 2001). [¶] $CC_{1/2}$ is the correlation coefficient of the mean intensities between two random half-sets of data (Karplus & Diederichs, 2012; Evans, 2012). ^{††} R factor = $\sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$; R_{free} is calculated in an identical manner using a randomly selected 5% of reflections that were not included in refinement.

different manner. For example, in the structure of human p23 shown in Fig. 2(*b*) two residues distantly separated from the end of β7 align with β1 in a parallel fashion (Weaver *et al.*, 2000). As a result of the additional secondary structure in the Ncb5or CS domain, only three residues separate it from the start of the core *b*₅R domain fold (Tyr275). This provides a compelling explanation for the resistance of the CS-*b*₅R linkage to proteolysis as described in Section 2.1.

3.3. The *b*₅R domain exhibits insertions and deletions in comparison to Cyb5R3

The structure of the core *b*₅R domain of Ncb5or (NADP⁺ complex), comprising residues Tyr275–Ala521, is compared

with the human Cyb5R3 structure (PDB entry 1umk; Bando *et al.*, 2004) in Figs. 3(a) and 3(b). The closest structural homolog of the Ncb5or b₅R domain, as revealed in the automated molecular-replacement process described in Section 2.2, is the single-domain b₅R protein from the slime mold protist *P. polycephalum* (PDB entry 2eix; Kim *et al.*, 2007). Superposition of the Ncb5or b₅R domain with *P. polycephalum* b₅R and three other reductases is shown in Supplementary Fig. S3.

The amino-acid sequence alignment of the Ncb5or b₅R domain, human Cyb5R3 and *P. polycephalum* b₅R in Fig. 3(c) shows that the Ncb5or b₅R domain exhibits notable deletions and insertions in comparison to human Cyb5R3 and other mammalian Cyb5R3 proteins (Supplementary Fig. S9). For example, Ncb5or has a much longer polypeptide separating the fourth and fifth strands (β 14 and β 15) of the β -sheet in the FAD subdomain [shown in orange in Figs. 3(a) and 3(b)]. The side chains of Leu333 and Leu334 in this well ordered loop engage in distinct hydrophobic packing with α 2 and/or α 3 in the NAD subdomain [Figs. 3(d) and 3(e)], interactions that have no equivalent in Cyb5R3. On the other hand, Ncb5or lacks the characteristic three-stranded antiparallel β -sheet motif that comprises the hinge region between the FAD and NAD subdomains in Cyb5R3 [highlighted in red for human Cyb5R3 in Fig. 3(b); italicized in the amino-acid sequence alignment in Fig. 3(c)]. A loop between two strands in this Cyb5R3 motif contains two of the four lysine residues known to be involved in electrostatic recognition of cytochrome b₅ (Lys162 and Lys163; the other two are Lys41 and Lys125; Asada *et al.*, 2009; Shirabe *et al.*, 1998). In place of this motif in Ncb5or is an extended polypeptide that has 15 fewer residues, none of which are in the vicinity of Lys162 and Lys163 in Cyb5R3.

One further deletion and one additional insertion result in distinctly different environments for the two nucleotide units of FAD in Ncb5or versus Cyb5R3. Cyb5R3 proteins feature a conserved surface loop that provides a 'lid' for the adenine

moiety of FAD [shown in forest green in Fig. 3(b) and Supplementary Fig. S4(b)]. Recognition elements include π -stacking between the adenine ring and the side chains of the conserved residues His117 and Phe120, and a hydrogen bond between the α -CO of residue 113 and the adenine NH₂ group. The corresponding loop in Ncb5or is nine residues shorter [Fig. 3(a); Supplementary Fig. S4(b)] and provides no interactions with the FAD adenine ring, which has a different orientation to that in Cyb5R3 and is fully exposed to bulk solvent. The additional insertion affects the FAD isoalloxazine ring and occurs at the C-terminus of the protein. In Cyb5R3 proteins (and all other known b₅R family members) the polypeptide sequence ends with a Phe or Tyr residue with its side chain packed against a conserved proline [Phe300 and Pro185 in human Cyb5R3, rendered with yellow C atoms in Fig. 3(b)]. Ncb5or has analogous packing interactions between Phe519 and Pro404 but features two additional C-terminal residues, thus ending with a conserved Phe519-Thr520-Ala521 [also rendered with yellow C atoms in Fig. 3(a)]. In the CS–b₅R–NADP structure, the Ala521 side chain packs against the 7- and 8-methyl groups of the FAD isoalloxazine ring (electron density for Ala521 in the NAD⁺ complex could not be located), whereas in Cyb5R3 proteins the carboxylate group of the C-terminal Phe residue occupies this space. This may reflect a different redox environment for flavin in Ncb5or, or a different mechanism for controlling electron transfer from heme to the flavin.

P. polycephalum b₅R shares with Ncb5or the absence of a three-stranded β -sheet between the FAD and NAD subdomains and of a recognition pocket for the FAD adenine ring, but does not feature the insertions noted above that distinguish Ncb5or from Cyb5R3 and other b₅R proteins. BLASTP (Altschul *et al.*, 1990, 1997) searches carried out for the purposes of this study showed that Cyb5R from *P. polycephalum* and other protists is closely related to the single-domain Cyb5R (type 1) proteins from plants and fungi (Supplementary Fig. S9). On the other hand, the closest structural homolog to Cyb5R3 from animals is the b₅R domain of nitrate reductase from green plants.

3.4. CS–b₅R interdomain interactions

The expanded fold of the Ncb5or CS domain, detailed in Section 3.2, contributes to CS–b₅R interactions by limiting interdomain flexibility. Specific interactions between the CS and b₅R domains are highlighted in Fig. 4. Three contiguous residues on the edge of β 3 in the CS domain, Val197, Asp198 and His199, engage in hydrogen-bonding interactions with contiguous b₅R

Figure 2

(a) Ribbon diagram of the CS domain in CS–b₅R–NAD, highlighting the β 8 and β 9 strands spanning Gly256–Pro267 and the connection to β 10 of the b₅ domain (green). (b) Crystal structure of the CS domain of human co-chaperone p23 (PDB entry 1ejf).

domain residues Ser300, Thr301 and His302, which are located in a long loop between the second and third strands of the core b_5R domain fold. Thr301 and His302 are annotated as comprising strand β_{12} in the b_5R domain on the basis of their interactions with β_3 , which include mutual hydrogen bonds between the α -CO and α -NH groups of Val197 (CS) and His302 (b_5R). A third backbone-backbone hydrogen bond occurs between the α -NH group of His199 (CS) and the α -CO group of Ser300 (b_5R), but the motif is capped by another one between the α -CO group of His199 and the side chain of Ser300. The β_3 - β_{12} interactions also include a hydrogen bond between the side chain of Asp198 (CS) and Thr301 (b_5R). The Asp198 side chain also engages in a salt bridge with the side chain of Arg276 in β_{10} (the first strand in the b_5R domain), and this network of side-chain interactions is extended by hydrogen bonding of the Arg276 side chain to both the backbone and side chain of Ser377, the first residue beyond β_{16} in the b_5R domain, and the side chain of Glu207 (in β_4 of the CS domain). There is also a cation- π stacking interaction between the side chain of Arg205 (CS strand β_4) and Tyr274 (the first residue before the start of β_{10} in b_5R). The side-chain interactions are conserved in mammalian Ncb5or proteins, most notably the salt bridge between Asp198 and Arg276 and the cation- π stacking between Arg205 and residue 274 (Tyr, Phe or His in all known examples).

Three crystal structures have been reported of intermolecular protein complexes in which one component has a CS domain. The most recently reported complex is between the TPR2 domain of human RPAP3 and the CS domain of human PIH1K1 (PDB entry 6gxz; Henri *et al.*, 2018). Approximately 30 residues at the C-terminus of the RPAP3 protein wrap around the CS domain of PIH1K1, engaging in β -structure with strands β_3 and β_6 on one edge of the β -sandwich and strand β_1 on the other edge. Eleven intervening residues apparently pass over the three-stranded face of the β -sandwich, but their electron density could not be located, suggesting that they are disordered and do not engage

in specific interactions. Two subsequent α -helices pack against the four-stranded face of the β -sandwich. The corresponding

Figure 3

(a, b) Comparison of the structures of the human Ncb5or b_5R domain NADP⁺ complex (a) and human Cyb5R3 (b) (PDB entry 1umk). (c) Amino-acid sequence alignment of the b_5R domain of Ncb5or with human Cyb5R3 and *P. polycephalum* b_5R (produced using ClustalW; McWilliam *et al.*, 2013) followed by manual adjustments on the basis of structural alignments. The numbering shown is for full-length Ncb5or. Amino-acid identity and similarity are indicated in yellow and green, respectively. The underlined and italicized residues in the human Cyb5R3 sequence represent the FAD adenine recognition pocket and the three-stranded β -sheet between the NAD and FAD subdomains, respectively.

Figure 3 (continued)

(d) Global view showing the location of the loop insert relative to the domains in CS-*b*₅R-NAD. (e) Enlarged view depicting the hydrophobic and hydrogen-bonding interactions between the loop insert and α 2/ α 3 residues in CS-*b*₅R-NAD.

face of the CS domain in the structures reported here is not involved in any interactions.

The two other reported structures involve the CS domain of cress Sgt1: one is a binary complex with barley Hsp90 (PDB entry 2jki; Zhang *et al.*, 2008) and the other is a related ternary complex that includes the CHORD domain of cress Rar1 (PDB entry 2xcm; Zhang *et al.*, 2010). In both structures the edge of a β -sheet and a helix of Hsp90 pack against the four-stranded face of the Sgt1 CS domain. In the ternary complex, the Rar1 CHORD domain interacts with the side chains of 11 residues on the three-stranded face of the Sgt1 CS domain (blue in Supplementary Fig. S5; Zhang *et al.*, 2010). The corresponding residues in the Ncb5or CS domain are shown in red in Supplementary Fig. S5. The side chains of three of these Ncb5or CS-domain residues (Asp198 in β 3; Arg205 and Glu207 in β 4) contribute to recognition of the *b*₅R domain. The side chain of a fourth residue (His218 in β 5) forms a hydrogen bond to the backbone α -CO group of Lys260, one of five residues in the loop separating β 8 and β 9. The side chains of the eight other residues in this group project into solvent and thus could be involved in additional protein-protein interactions.

Finally, NMR data-driven docking studies have been performed with the CS domain of yeast Shq1 and the nucleolar protein Cbf5 (dyskerin in humans), which are involved in the assembly of H/ACA ribonucleoprotein particles (Singh *et al.*, 2015). The results indicate that a Cbf5 surface helix binds along the edge of the Shq1 CS domain β -sandwich defined by strands β 3 and β 6. Notably, the crystal structure of yeast Shq1 (PDB entry 3eud; Singh *et al.*, 2009) features similar intermolecular interactions, with a short helix in the loop between strands β 2 and β 3 of each molecule nestled into

the space between β 3 and β 6 of a neighboring molecule, and the side chains of residues Asn23 and Arg25 forming hydrogen bonds to the backbone of a residue in each strand. Moreover, the crystal structure of the human Shq1 CS domain (PDB entry 4pbd; Singh *et al.*, 2015) features a novel domain-swap interaction, with the 6 $\times$ His tag residues in each molecule aligned in an antiparallel manner with β 3 in another molecule. Together with the results presented here, these results suggest that the edges of β 3 and β 6 in the CS fold represent 'hot spots' for protein-protein interactions.

3.5. NAD⁺ and NADP⁺ bind with the nicotinamide ring in the active site

Electron density for FAD, NAD⁺ and NADP⁺ was well defined for the majority of the atoms in these molecules [Fig. 5(a) and Supplementary Fig. S6(a)]. The least well

Figure 4
Interactions between the CS (blue) and *b*₅R (yellow) domains in CS-*b*₅R-NAD.

defined atoms make up the adenine ring of FAD, which can be attributed to the absence of the recognition pocket characteristic of CybR3 proteins (see above). The isoalloxazine ring of FAD is essentially flat in each structure, with butterfly bend angles, defined as the angle between the planes of the pyrimidine and benzene six-membered rings, of 179.5° (NAD⁺ complex) and 179.3° (NADP⁺ complex). The nicotinamide rings of NAD⁺/NADP⁺ are positioned above the FAD isoalloxazine ring in each complex at distances of 3.8 and 3.7 Å, respectively, from the centroids of the mean planes defined by the aromatic atoms of the FAD isoalloxazine ring and the NAD⁺/NADP⁺ nicotinamide ring. Likewise, the angle between the mean planes of the nicotinamide and flavin rings are similar, at 17.2° for the NAD⁺ complex [Fig. 5(b)] and 20.8° for the NADP⁺ complex [Supplementary Fig. S6(b)]. The sulfhydryl side chain of Cys493, which is invariant among *b*₅R family members, resides at a distance of 3.3 Å from the C3N atom of NAD⁺/NADP⁺. It has been proposed that this residue may stabilize the positively charged nicotinamide ring of NAD⁺/NADP⁺ when it is poised above the FAD isoalloxazine ring, via charge-transfer interactions (Yamada *et al.*, 2013).

Cyb5R proteins, including human, rat and pig Cyb5R3 and the *P. polycephalum* protein, crystallize with the NADH-binding site unoccupied (Bando *et al.*, 2004; Yamada *et al.*,

2013; Bewley *et al.*, 2001; Kim *et al.*, 2007). In a structure of a NAD⁺ complex of rat Cyb5R3 obtained by crystal soaking (PDB entry 1ib0; Bewley *et al.*, 2001), the nicotinamide ring of NAD⁺ was found to reside outside its binding pocket (Supplementary Fig. S7). On the other hand, the structure of a reduced pig Cyb5R3 complex (PDB entry 3w2g) revealed NADH to be productively bound, with its nicotinamide ring in a virtually identical orientation relative to the flavin ring to that observed for NAD⁺/NADP⁺ in the structures reported here (Yamada *et al.*, 2013). Time-resolved crystallography studies of this complex showed that as NADH became oxidized to NAD⁺ the electron density for the nicotinamide ring became increasingly ambiguous, suggesting that it moves out of the active site. A few structures of ferredoxin NADP⁺ reductase (FNR) have been obtained with NADP⁺ productively bound (for example PDB entries 2bsa and 1qfy), but only when the conserved C-terminal Tyr residue has been mutated (Deng *et al.*, 1999; Tejero *et al.*, 2005). In the structure of wild-type FNR the side chain of this Tyr residue π -stacks with the isoalloxazine ring of FAD, a striking difference in comparison to Ncb5or and Cyb5R3 that may reflect regulation of NADP⁺, which serves as a substrate rather than as a spent reducing agent (Tejero *et al.*, 2005).

The structural data reported here suggest that Ncb5or may be considerably slower to release NAD⁺/NADP⁺ in comparison to Cyb5R3 and other single-domain Cyb5R proteins, a speculation that is strengthened by the observation described in Section 2.3 that NAD⁺- and/or NADP⁺-bound forms of Ncb5or persist through multiple rounds of purification. While there is no evidence to suggest that NAD⁺ or NADP⁺ serves as a substrate in Ncb5or, with electron flow proceeding in reverse, as occurs in FNR, our data suggest fundamental mechanistic differences in the electron-transfer reaction mediated by Ncb5or in comparison to the Cyb5R3/*b*₅ system.

3.6. Insight into the ability of Ncb5or to utilize both NAD⁺ and NADP⁺

In the NADP⁺ complex of CS-*b*₅R, the side chain of the conserved Ncb5or residue Ser457 engages in hydrogen bonding to the 2'-phosphate and 3'-hydroxyl groups of the adenine nucleotide [Supplementary Fig. S8(a)]. The hydrogen bond to the 3'-hydroxyl group is also present in the NAD⁺ complex [Supplementary Fig. S8(b)]. The side chain of the conserved Ncb5or residue His469 provides an additional hydrogen bond (or salt bridge) to the phosphate group of NADP⁺ in CS-*b*₅R-NADP and maintains the same orientation in the CS-*b*₅R-NAD structure.

The residues corresponding to Ser457 and His469 in Cyb5R3 proteins are the conserved Asp239 and Phe251. In the crystal structure of the rat Cyb5R3-NAD⁺ complex, the Asp239 side chain engages in a hydrogen bond to the 3'-OH group of the adenosine ribose ring, and the phenyl side chain of Phe251 is oriented parallel to the adenine ring of NAD⁺ in an apparent π -stacking interaction [Supplementary Fig. S8(b)]. The 2'-OH group is directed towards the protein, in a manner that would not accommodate a phosphate group.

Figure 5
Interaction between FAD and NAD⁺ in CS-*b*₅R-NAD. (a) Electron-density maps ($F_o - F_c$ polder OMIT; Adams *et al.*, 2010; Liebschner *et al.*, 2017) for FAD (green) and NAD⁺ (coral). (b) The angle between the mean planes of the FAD (flavin) and NAD⁺ (nicotinamide) rings.

The presence of two conserved residues in Ncb5or that can form specific interactions with the NADP⁺ phosphate group suggest potentially stronger binding of NADPH over NADH by the protein, but the structures nonetheless provide a structural basis for the ability of the protein to effectively recognize and utilize both reducing agents. In contrast, Cyb5R3 exhibits specificity for NADH, as demonstrated in earlier studies on methemoglobin reductase (Passon & Hultquist, 1972; Hsieh & Jaffé, 1971).

3.7. Implications for interactions between the *b*₅ and *b*₅R domains

It is important to note that the structures reported here do not necessarily reflect the actual arrangement of the CS and *b*₅R domains of Ncb5or during docking and reduction of the heme-containing *b*₅ domain, but they do allow some speculation about this process. As suggested by modeling studies of the Cyb5R3/Cyb5A system, efficient electron transfer requires close approach of the heme to the edge of the flavin ring (Asada *et al.*, 2009). The orientation of the CS domain relative to the *b*₅R domain in the structures reported here limits the possible roles that it could play in recognition of the *b*₅ domain. For example, the four-stranded face of the core CS domain would completely preclude contact of the *b*₅ domain with the *b*₅R domain. The same can be said of the five solvent-exposed polar residues illustrated in Supplementary Fig. S5, the side chains of which are at a distance of at least 29 Å from the flavin edge and are oriented away from the *b*₅R domain.

More likely to be involved in *b*₅ domain recognition is the secondary structure that expands the Ncb5or CS fold in comparison to other structurally characterized proteins with CS domains. As noted in Section 3.4 and illustrated in Fig. 6, the type I β -turn that follows β ₉ converges on the space occupied by two of the four Lys residues in Cyb5R3 previously identified as being involved in the electrostatic recognition of Cyb5A. Such a role would be consistent with previous kinetic

studies in our laboratory, in which we compared electron transfer from Cyb5R3, the individual *b*₅R domain of Ncb5or, and CS-*b*₅R to either Cyb5A or the individual *b*₅ domain of Ncb5or. Surprisingly, the Ncb5or *b*₅R domain reduced Cyb5A more efficiently than its cognate *b*₅ domain. However, replacing it with CS-*b*₅R led to selective reduction of its cognate *b*₅ domain (Deng *et al.*, 2010).

The actual nature of the interactions between the *b*₅ domain and the other two domains of the protein will continue to be the focus of investigations in our laboratory. One of the key challenges to be conquered in these efforts will be the demonstrated resistance of the full-length protein to the formation of crystals. Even if crystallization ultimately proves to be possible, the resultant structures may not necessarily reveal the nature of the three domains during heme reduction, as illustrated by the example of sulfite oxidase proteins from animals (Kisker *et al.*, 1997).

4. Summary and conclusions

High-resolution crystal structures have been obtained for the naturally fused CS and *b*₅R domains of Ncb5or with FAD and NAD⁺ or NADP⁺ bound. NAD⁺ and NADP⁺ bind with their nicotinamide rings in the active site, the first such examples in a native *b*₅R family member. This finding, together with the observation that the NAD⁺ complex survives multiple rounds of purification, suggests unusually strong binding of the spent reducing agent and perhaps a fundamentally different mechanism for electron shuttling from reducing agent to heme in comparison to the Cyb5R3/Cyb5A system. The structures also provide insight into the ability of Ncb5or to recognize and utilize both NADH and NADPH, which distinguishes this protein from Cyb5R3 and other members of the *b*₅R family. The structures further reveal that the CS domain plays two key structural roles: (i) by limiting the conformational flexibility of the polypeptide separating it from the *b*₅R domain via hydrogen-bonding interactions along the 'sticky' edge of

its β -sandwich structure and (ii) by engaging in a conserved network of backbone and side-chain interactions with residues in the core *b*₅R fold. These interactions place a β -turn structure in the region of space that is occupied in Cyb5R3 proteins by two of the four lysine residues that are known to be involved in Cyb5A recognition, providing insight into the results of previously reported kinetic studies from our laboratories suggesting that the CS domain is involved in the specificity of the interactions between the *b*₅R and *b*₅ domains. The presence of two C-terminal residues that are not present in other *b*₅R family members that

Figure 6
Comparison of the environments around the reactive edge of the FAD flavin ring in (a) CS-*b*₅R and (b) rat Cyb5R3 (PDB entry 1ib0). The FAD molecules are rendered as yellow spheres and highlighted residues are in cyan with solvent-accessible surface rendering.

interact with the flavin edge further suggest mechanistic differences in Ncb5or in comparison to the Cyb5A and Cyb5R3 system. Finally, structural and sequence alignments show that the b_5R domain of Ncb5or is more similar to monomeric b_5R proteins from plants, fungi and protists such as *P. polycephalum* than to mammalian Cyb5R3 proteins, suggesting that Ncb5or has an ancient heritage.

5. Related literature

The following references are cited in the supporting information for this article: Bagnris *et al.* (2009), Laganowsky *et al.* (2010), Lee *et al.* (2004), Lu *et al.* (1995), Mani *et al.* (2016) and Zheng *et al.* (2011).

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