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ORIGINAL ARTICLE

Conservation of the Enzyme–Coenzyme Interfaces in FAD and NADP Binding Adrenodoxin Reductase—A Ubiquitous Enzyme

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Abstract

FAD and NAD(P) together represent an ideal pair for coupled redox reactions in their capacity to accept two electrons and their redox potentials. Enzymes that bind both NAD(P) and FAD represent large superfamilies that fulfill essential roles in numerous metabolic pathways. Adrenodoxin reductase (AdxR) shares Rossmann fold features with some of these superfamilies but remains in a group of its own in the absence of sequence homology. This article documents the phylogenetic distribution of AdxR by examining whole genome databases for Metazoa, Plantae, Fungi, and Protista, and determines the conserved structural features of AdxR. Scanning these databases showed that most organisms have a single gene coding for an AdxR ortholog. The sequence identity between AdxR orthologs is correlated with the phylogenetic distance among metazoan species. The NADP binding site of all AdxR orthologs showed a modified Rossmann fold motif with a GxGxxA consensus instead of the classical GxGxxG at the edge of the first $\beta\alpha$-fold. To examine the hypothesis that enzyme–coenzyme interfaces represent the conserved regions of AdxR, the residues interfacing FAD and NADP were identified and compared with multiple-sequence alignment results. Most conserved residues were indeed found at sites that surround the interfacing residues between the enzyme and the two coenzymes. In contrast to protein–protein interaction hot-spots that may appear in isolated patches, in AdxR the conserved regions show strict preservation of the overall structure. This structure maintains the precise positioning of the two coenzymes for optimal electron transfer between NADP and FAD without electron leakage to other acceptors.

Keywords Enzyme evolution · Rossmann fold · Consensus sequence · Flavoprotein · FDXR

Abbreviations
AdxR Adrenodoxin reductase

Introduction

The coenzymes NAD, NADP, and FAD are involved in many metabolic pathways. In the Enzyme Database, most of the enzymes that are dependent on these coenzymes appear in the class of oxidoreductases (EC 1). There are also enzymes in other classes (transferases, hydrolases, lyases, isomerases, and ligases) that are dependent on these coenzymes. Analyses of the sequences and structures of these enzymes have revealed that these enzymes can be grouped into several, structurally unrelated, superfamilies (Dym and Eisenberg 2001; Ojha et al. 2007; Aliverti et al. 2008).

One of the largest superfamily of enzymes that bind both NAD(P) and FAD has been called flavoproteins with "two dinucleotide binding domains" (Ojha et al. 2007). The name "dinucleotide" is based on the structure of both NAD(P) and FAD that can be viewed as two connected nucleotides (Fig. 1) (Hanukoglu 2015). The enzymes in this group bind both NAD(P) and FAD and catalyze the transfer of a hydride ion ($H^- = H^+ + 2e^-$) from NAD(P)H to FAD forming FADH2 (You 1985) according to the following reaction:

$$NAD(P)H + FAD + H^+ \rightarrow NAD(P)^+ + FADH_2.$$

NAD(P) and FAD together represent an ideal pair for electron transfer in coupled redox reactions. Both NAD(P) and FAD can be reduced by accepting two electrons. The reduction potential of FAD is higher than that of NAD(P)

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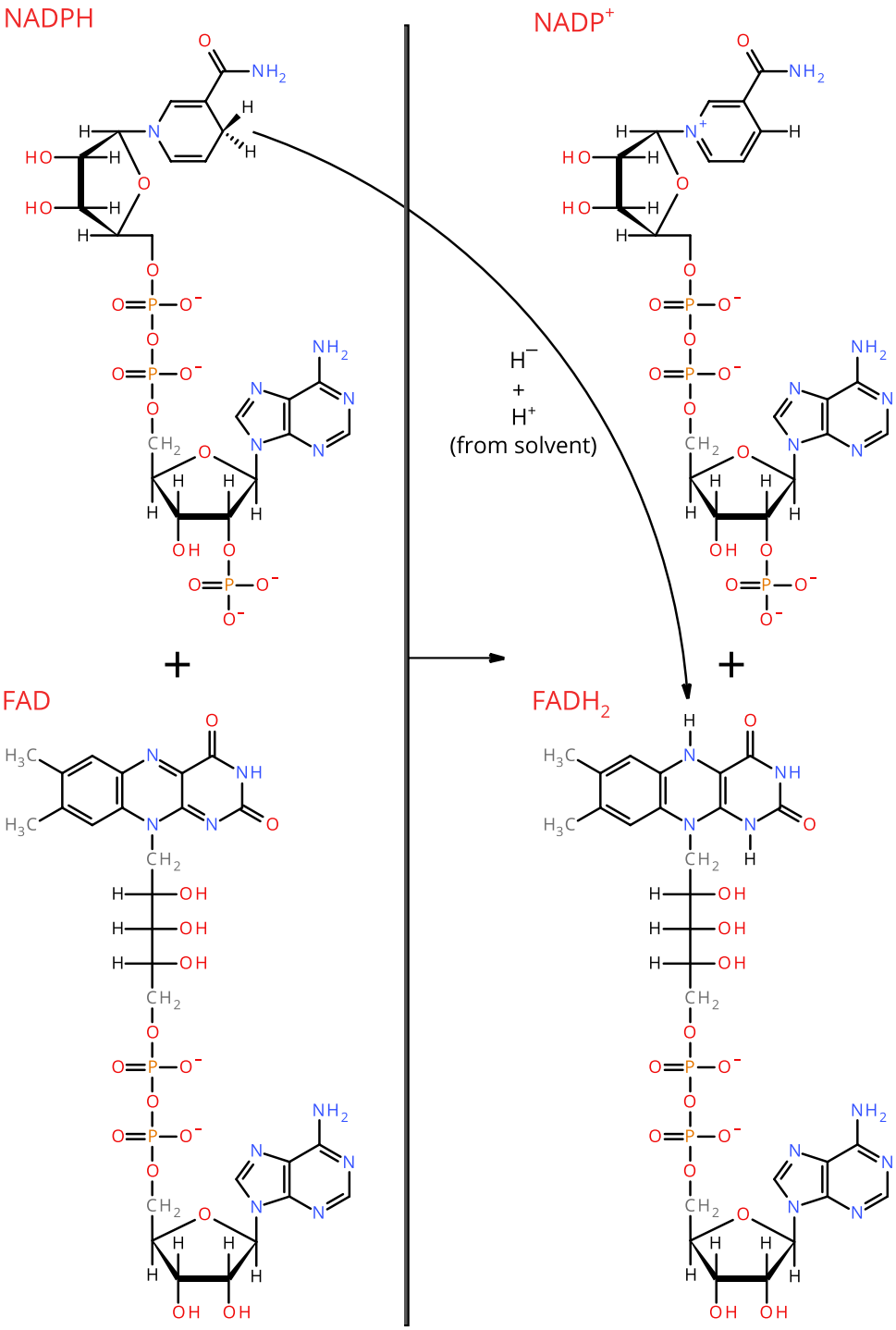

Fig. 1 Reduction of FAD by NADPH. The nicotinamide group of NADPH transfers a hydride ion (H^-) to the isoalloxazine ring of the FAD forming $FADH^-$. After reaction with an additional H^+ from the solvent, $FADH^-$ becomes FADH2

(Table 1). Thus, the tendency of the reaction is towards the reduction of FAD by the transfer of a hydride ion from NAD(P)H to the isoalloxazine ring of the FAD that can accept two electrons (Fig. 1).

Depending on the substrate specificity of the enzyme, reduced FAD (FADH2) can transfer its newly acquired electrons to a two-electron acceptor, such as a disulfide group, or to an external single electron acceptor such as a heme

Table 1 Standard reduction potentials of NAD, NADP, and FAD [*Source* (Voet and Voet 2004)]

Half-reaction	E°′ (V)
$FAD + 2H^+ + 2e^- \rightarrow$ FADH2 (enzyme bound)	− 0.040
$FAD + 2H^+ + 2e^- \rightarrow$ FADH2 (free coenzyme)	− 0.219
$NAD^+ + 2H^+ + 2e^- \rightarrow$ NADH	− 0.315
$NADP^+ + 2H^+ + 2e^- \rightarrow$ NADPH	− 0.320

or a protein with an iron–sulfur cluster (Ojha et al. 2007). Thus, in contrast to NAD(P)H that donates two electrons in one step as a hydride ion, FADH2 can transfer a single electron, remaining relatively stable as a semiquinone, $FADH^{\cdot}$. After the transfer of the second electron, fully oxidized FAD becomes ready for another cycle of redox reactions.

The Role of Adrenodoxin Reductase in the Mitochondrial P450 Systems

Adrenodoxin reductase (AdxR) that is the subject of this article is a prime representative of the flavoproteins with two dinucleotide binding domains. AdxR functions as the first enzyme in the mitochondrial cytochrome P450 systems that are located on the matrix side of the inner mitochondrial membrane (Hanukoglu et al. 1990). AdxR was first identified in the bovine adrenal cortex mitochondria by Omura et al. (Omura et al. 1966) and purified by Chu and Kimura (Chu and Kimura 1973).

Mitochondrial P450 type enzymes catalyze essential steps in the biosynthesis of steroid hormones (CYP11A, CYP11B1, CYP11B2), bile acids (CYP27A), and vitamin D derivatives (CYP24A, CYP27B) (Hanukoglu 1992; Omura 2006; Pikuleva and Waterman 2013). These enzymes catalyze monooxygenase type hydroxylation reactions that depend on molecular oxygen (O_2) and NADPH as a source of two electrons (Hanukoglu 1996). In these reactions, one atom of molecular O_2 is incorporated into the OH group added to the substrate, while the second oxygen atom is reduced to water:

$$\text{Substrate-H} + \text{NADPH} + \text{H}^+ + \text{O}_2 \rightarrow \text{Substrate-OH} + \text{NADP}^+ + \text{H}_2\text{O}.$$

The two electrons donated by NADPH are transferred to P450 via a chain of two electron transfer proteins, AdxR and adrenodoxin (Hanukoglu and Jefcoate 1980; Hanukoglu 1996; Müller et al. 2001b; Hannemann et al. 2007; Ewen et al. 2011). NADPH binds to AdxR and transfers two electrons to the FAD that is tightly bound to AdxR (Lambeth and Kamin 1976). These two electrons are then transferred from FADH2 (one at a time) to adrenodoxin, a [2Fe–2S] ferredoxin type iron–sulfur protein that can be reduced with only a single electron. Thus, electrons are transferred in the following order:

$$\text{NADPH} \rightarrow \text{FAD-adrenodoxin reductase} \rightarrow \text{adrenodoxin} \rightarrow \text{P450}.$$

The full reaction catalyzed by AdxR is

$$\text{NADPH} + 2\ \text{adrenodoxin}_{ox} \rightarrow \text{NADP}^+ + \text{H}^+ + 2\ \text{adrenodoxin}_{red},$$

where the subscripts "ox" and "red" refer to the oxidized and one electron reduced states of adrenodoxin.

The first cDNA of AdxR was cloned by Hanukoglu et al. (1987). In mammals, AdxR is encoded by a single gene (Hanukoglu et al. 1987; Solish et al. 1988). Hence, all the mitochondrial P450 systems are dependent on the same AdxR that is expressed in all tissues that have mitochondrial P450 systems. The highest concentration of AdxR is found in the adrenal cortex, where the ratios of AdxR, adrenodoxin, and P450 are 1:3:8 (Hanukoglu and Hanukoglu 1986).

The two-iron, two-sulfur [2Fe–2S] protein of this system was isolated and characterized prior to AdxR (Suzuki and Kimura 1965). Because of the biochemical characteristics it shared with the photosynthetic ferredoxin (characteristics such as [2Fe–2S] cofactor, absorption spectra, and molecular weight), its name was coined as "adrenodoxin" combining the words "adrenal" and "ferredoxin" (Kimura and Suzuki 1965).

Enzyme and Gene Nomenclature of Adrenodoxin Reductase

In the IUBMB Enzyme Nomenclature, the code for AdxR is EC 1.18.1.6 and its "accepted name" is "adrenodoxin-$NADP^+$ reductase." Its other names include "adrenodoxin reductase, AdR, NADPH:adrenal ferredoxin oxidoreductase, NADPH-adrenodoxin reductase" (http://www.sbcs.qmul.ac.uk/iubmb/enzyme/EC1/18/1/6.html).

In the HUGO (human) Gene Nomenclature, the approved symbol for the gene is FDXR representing "ferredoxin reductase," with recognized synonyms "adrenodoxin reductase," and "adrenodoxin-NADP(+) reductase," and the corresponding symbol ADXR (https://www.genenames.org/).

While the human genes are symbolized with all uppercase letters, the mouse and rat gene nomenclatures require a capital letter as the first character, followed by all lowercase letters. Therefore, the symbol for the rodent genes that are orthologs of the human gene is "*Fdxr.*"

The name "ferredoxin reductase" was assigned to AdxR as a carryover from the initial name of the electron acceptor adrenodoxin that resembled plant ferredoxin from a biochemical/functional view as noted above. Yet, the name "ferredoxin reductase" is a misnomer and its assignment to AdxR is misleading because studies that have compared the sequences and structures of AdxR and plant ferredoxin reductase have concluded unequivocally that adrenodoxin reductase shares no homology with the plant ferredoxin reductase (Hanukoglu 1996; Ziegler and Schulz 2000). Therefore, the name adrenodoxin reductase will be used throughout this article, accompanied by the symbol ADXR for the human gene, and AdxR for the protein.

The Structure of Adrenodoxin Reductase

The first protein sequence of AdxR did not show any homology to any other protein sequence known at that time (Hanukoglu and Gutfinger 1989). However, both the FAD and NADP binding sites of AdxR were identified by searching for modified versions of the Rossmann fold consensus sequence, secondary structural analyses, and protein sequence database screenings (Hanukoglu and Gutfinger 1989). The structure and function of these sequences were verified by the first crystal structure of adrenodoxin reductase (Ziegler et al. 1999).

The NADP binding consensus sequence identified in the first AdxR sequence differed from the traditional Rossmann fold consensus sequence by the substitution of an alanine for a glycine in the $\beta\alpha$ fold (Hanukoglu and Gutfinger 1989). Site-directed mutagenesis of the $\beta\alpha$-fold of a structural homolog, glutathione reductase, showed that a single mutation of the Ala179 to Gly decreased the Km for NADH 40-fold as compared to the wild-type enzyme, attesting to the importance of this residue in conferring coenzyme specificity (Scrutton et al. 1990). However, various superfamilies of NAD(P) enzymes exhibit different features that determine coenzyme specificity (e.g., Sharkey et al. 2012; González-Segura et al. 2015).

The crystal structures of AdxR with FAD (1CJC) and with FAD and NADP (1E1L) revealed that AdxR can be viewed as a protein with two domains that separately accommodate FAD and NADP (Fig. 2) (Ziegler et al. 1999; Ziegler and Schulz 2000). Both the FAD and NADP domains include a β-sheet of the Rossmann fold type with five strands in a parallel orientation (Fig. 2). Figure 3 shows the surface structure of adrenodoxin reductase with the two coenzymes bound to the enzyme. It can be seen that FAD is tightly embedded in the protein with only the isoalloxazine part exposed for electron acceptance from NADPH (Fig. 3c). In contrast, NADP is located in a freely solvent accessible niche (Fig. 3b).

The two coenzymes of AdxR are presented in a stick format in Fig. 4. Both FAD and NADP are positioned in an extended conformation within their domains. The two coenzymes are juxtaposed head to head, at the junction between the domains with precise alignment of the atoms involved in electron transfer from the nicotinamide of NADP to the isoalloxazine ring of the FAD. The hydride ion is transferred from the C4 atom of the nicotinamide to the N5 atom of isoalloxazine (You 1985). The distance between these two atoms is 3.2 Å (inset of Fig. 4). In the space-filling model, it can be seen that the two atoms are juxtaposed.

The close proximity of the electron donating and accepting atoms is expected to minimize the leakage of electrons from NADPH to other acceptors, such as O_2, to avoid producing oxy-radicals. Indeed, our previous studies showed

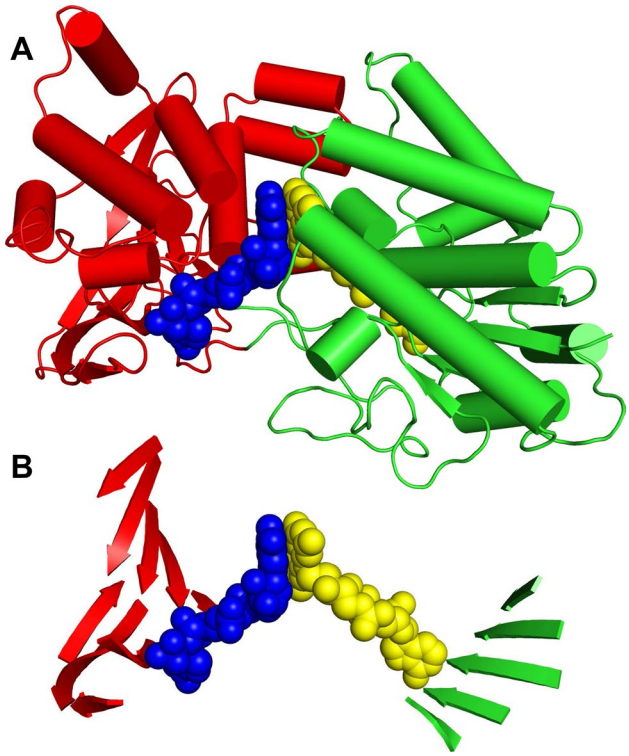

Fig. 2 **a** FAD and NADP domains of bovine adrenodoxin reductase based on PDB 1E1L (Ziegler and Schulz 2000). Secondary structures in the FAD domain (residues 1–108, and 330–460) are green, and in the NADP domain (residues 109–329) are red colored. Helices are shown as cylinders. FAD (yellow) and NADP (blue) are shown in CPK format. **b** FAD (yellow) and NADP (blue) in the same orientation as in **a** with helices and loops hidden. Only the beta sheets are displayed. The β-sheet in the FAD domain and the upper β-sheet in the NADP domain are part of the two Rossmann folds that host these two dinucleotides (Hanukoglu 2015). This and the following molecular model images were produced using PyMOL Molecular Graphics System, Version 1.7 Schrödinger, LLC. (Color figure online)

that adrenodoxin reductase reduced by NADPH hardly shows electron leakage in the absence of adrenodoxin (Hanukoglu et al. 1993; Hanukoglu 2006).

The electrons on FAD are transferred to the single electron acceptor 2Fe–2S protein adrenodoxin (Lambeth et al. 1976). In this electron transfer reaction, adrenodoxin acts a substrate of AdxR (Hanukoglu and Jefcoate 1980; Hanukoglu et al. 1981). The interaction between AdxR and adrenodoxin is mainly dependent on ionic interactions and apparently hydrophobic interactions do not play a role in binding (Hanukoglu et al. 1981). Site-directed mutagenesis and structural cross-linking studies identified four positively charged residues (Lys27, Arg211, Arg240, and Arg244 in mature bovine AdxR) as the sites of salt-bridge formation between AdxR and acidic residues Asp27, Asp76, and Asp79 of the electron acceptor adrenodoxin (Table 2) (Brandt and Vickery 1993; Müller et al. 2001b).

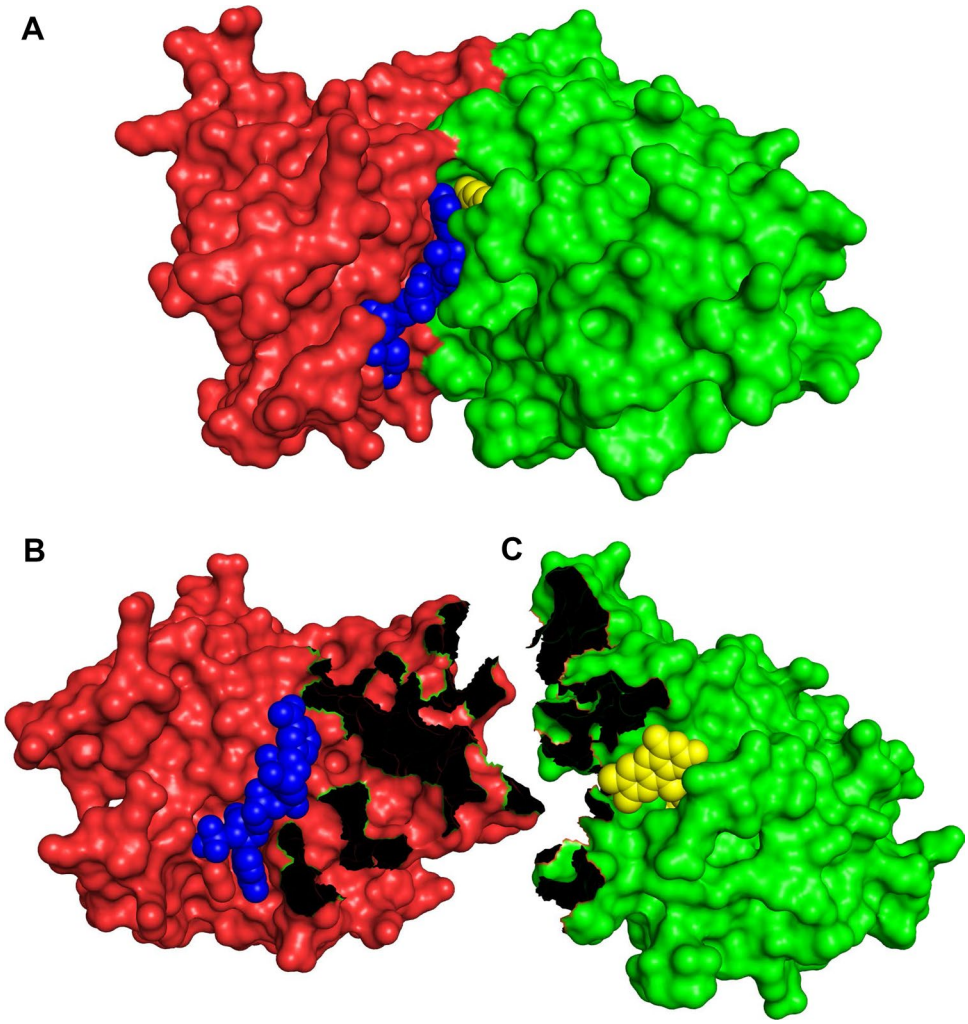

Fig. 3 **a** The surface structure of adrenodoxin reductase based on PDB 1E1L. NADP and FAD domains are red and green colored as described in Fig. 2. The ligands, FAD (yellow) and NADP (blue), are shown in CPK format. **b** The surface of the NADP domain only. Relative to the view in **a**, the surface model was rotated to maximize the visibility of NADP. Note that the entire molecule of NADP is visible and that NADP is located at a freely solvent accessible site. **c** The surface of the FAD domain only. Note that only the isoalloxazine portion of FAD (yellow CPK) is visible. The ADP portion of FAD is invisible as it is buried inside the FAD domain. (Color figure online)

Phylogenetic Distribution of Adrenodoxin Reductase

The multitude of genome sequences that have accumulated during the past decade revealed that adrenodoxin reductase is found in both eukaryotes and prokaryotes (Bossi et al. 2002; Ewen et al. 2008). To determine the spectrum of the phylogenetic distribution of AdxR, the Ensembl genome databases were scanned, because these databases include nearly complete genome sequences and not just isolated protein and gene sequences.

ADXR Gene in Metazoans

A search of the Ensembl genome database of vertebrates (http://www.ensembl.org/) for the ADXR gene orthologs showed that 58 vertebrate species have a single gene for ADXR orthologous to human ADXR gene with the following species distribution: 7 reptiles and birds, 33 placental mammals, 3 marsupials, 1 of each Platypus, Xenopus, and coelacanth, 11 ray-finned fishes, and 1 lamprey. In six species (alpaca, hedgehog, microbat, shrew, sloth, and tarsier) an ADXR gene has not been identified yet. The search of the invertebrate Metazoan Ensembl database (https://metazoa.ensembl.org/) using ADXR of sea urchin (*Strongylocentrotus purpuratus*) yielded 60 species with ADXR orthologs. In 7 species, no orthologs were identified.

For the assessment of sequence conservation, AdxR protein sequences were aligned using CLUSTALW program (Chenna et al. 2003). Among primates, the sequence identity between AdxR from different species varied between 93 and 99%. Among metazoa, the lowest sequence identity was observed between primate and insect sequences in the range of 37–45%. We selected 45 complete AdxR sequences for the construction of the gene tree (Fig. 5). The gene tree shows that the sequence identity between ADXR sequences reflects the phylogenetic distance among metazoan species. For example, the sequences from birds and lizard appear in the same cluster that marks the Sauropsida clade; ray-finned fishes

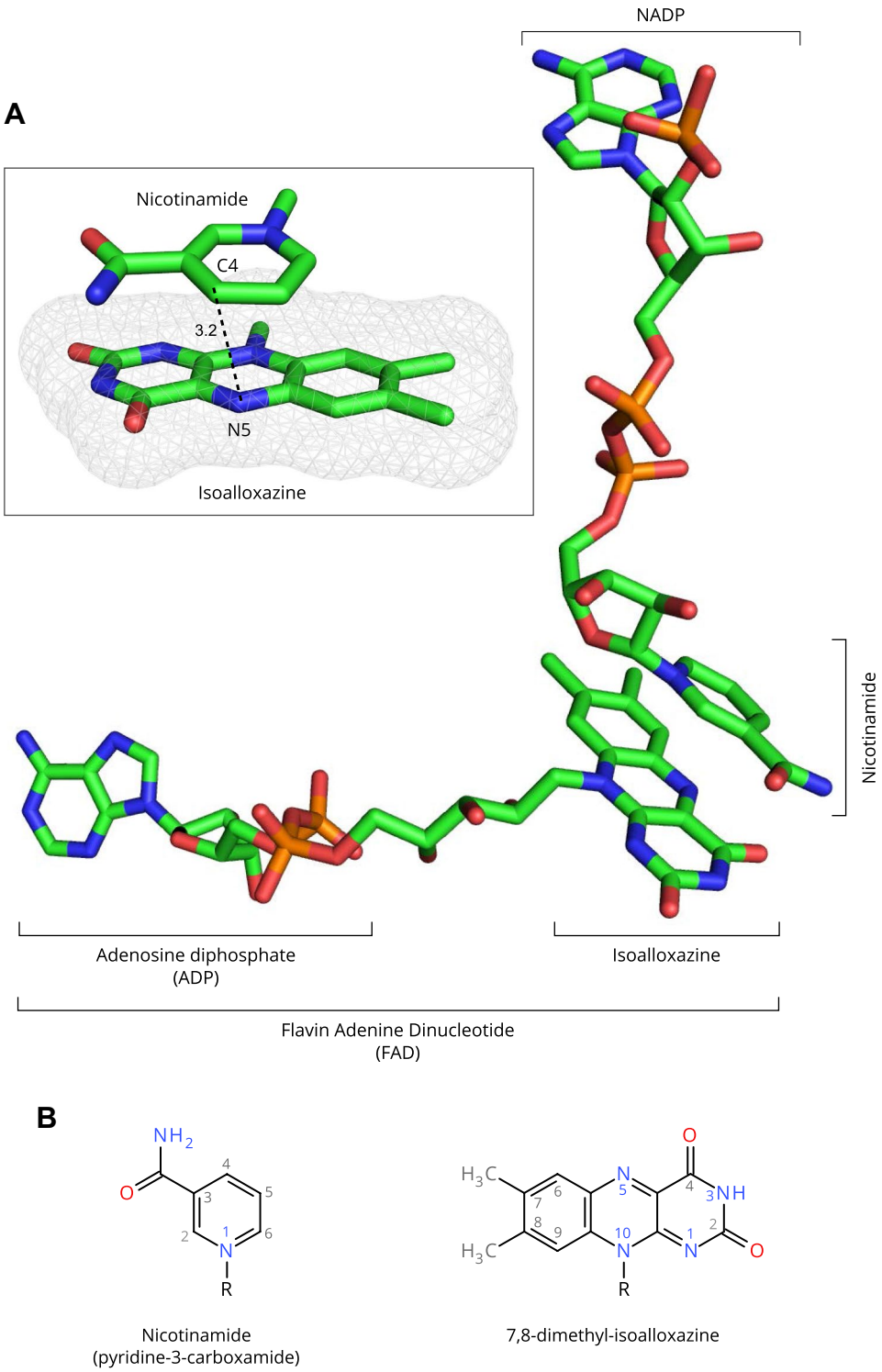

Fig. 4 **a** Proximity of NADP and FAD in adrenodoxin reductase (1E1L). The ligands are shown in stick format. Colors of the atoms: C—green; N—blue; O—red; P—orange. The magnified view in the inset shows the distance between C4 atom of nicotinamide of NADP (donor of electrons) and N5 atom of isoalloxazine (acceptor of the electrons) with wireframe surface representation. **b** Numbering convention of the atoms in nicotinamide and isoalloxazine rings based on the IUPAC-IUB recommendations (IUPAC-IUB 1966). (Color figure online)

appear clustered in a clade that matches Actinopterygii; and Insecta appear in a distant clade separate from the other Metazoa (Fig. 5). These results indicate that all the sequences derive from a common origin.

ADXR Gene Among Plants, Protists, and Prokaryotes

The search of the Plant Ensembl database (https://plants.ensembl.org/) using *Arabidopsis thaliana* MFDR yielded 46 species with ADXR orthologs and 2 species where no ortholog could be identified. The sequence identity between

Table 2 Adrenodoxin reductase residues identified as the adrenodoxin binding sites

Bovine	Human	Reference
Lys27 (Lys59)*		Müller et al. (2001a, b)
Arg211 (Arg243)		Müller et al. (2001b)
Arg240 (Arg272)	Arg239 (Arg271)	Brandt and Vickery (1993); Müller et al. (2001b)
Arg244 (Arg276)	Arg243 (Arg275)	Brandt and Vickery (1993); Müller et al. (2001b)

*The numbers refer to the mature sequence, and those in parentheses refer to the full sequence

plant and metazoan sequences was in the range of 34–40%, and the sequence identity between various plant species was 61–89% (data not shown).

The search of the Protista (single-celled, protozoa) Ensembl database (https://protists.ensembl.org/) using *Dictyostelium discoideum* FDXR yielded 99 species with ADXR orthologs and 16 species where no ortholog could be identified. *D. discoideum* FDXR shares with those of other species between 26 and 32% sequence identity.

The prokaryotic homologs of the fission yeast *Schizosaccharomyces pombe* ADXR (UniProt ID: O59710) were searched in the UniProt database using BLAST program. This yielded hundreds of sequences that showed 34–40% sequence identity with the *S. pompe* sequence.

Numbering of AdxR Sequence

Among all metazoa examined, the length of the full unprocessed ADXR protein was similar and ranged between 466 and 506 amino acids. Among mammals, the sequence length ranged from 489 to 496 residues. In eukaryotes, about 30 residues at the N-terminus serve as the mitochondria-targeting sequence that is cleaved during transfer into mitochondria (Omura 2006). Amino acid sequencing of the bovine AdxR determined the N-terminal sequence of the processed enzyme as STQEQTPQICVVGSGPAG (Hanukoglu et al. 1987). Studies on site-directed mutagenesis of AdxR reported sequence numbering starting with the first residue of the mature enzyme (Ser33 of bovine and Ser34 of human AdxR) (Brandt and Vickery 1993). The same convention of numbering of amino acid sequences will be followed in this article.

Conserved Sequence Regions

To visualize the conserved regions of AdxR from diverse species, the sequences from 60 species (selected to represent mainly the major clades noted above of metazoa, plants, and a few protists) were aligned for comparison (for the full list, see the Supplementary Table in the website). Only sequences that were marked or assumed as complete were included in the analyses.

The conservation of a sequence segment indicates its structural importance. To identify structural features that may dictate sequence conservation, I plotted positions of highly conserved residues (line marked "Cons." in Fig. 6) in parallel with the following positions: (1) residues at the interface between FAD and AdxR, (2) residues at the interface between NADP and AdxR, (3) inaccessible residues, and (4) adrenodoxin binding residues. All of these positions are shown in parallel in Fig. 6.

In the alignment of all eukaryotic sequences, the first conserved sequence was observed at the beginning of the FAD binding Rossmann fold (Figs. 6, 7). The N-terminal sequence that precedes this motif showed no conservation across eukaryotes. However, among mammalian sequences and within a few additional clades, there was a significant clade-specific conservation of the amino-terminal sequences.

It can be seen that dense regions of sequence conservation appear around residues that are at the interface between FAD and NADP (Fig. 6). Yet, the extent of sequence conservation is much broader than the positions of interface residues (Fig. 6). Details of the correspondence between the conserved residues and the residues at the enzyme–coenzyme interface are presented below for the FAD and NADP binding domains separately.

FAD Binding Domain

The FAD binding domain starts at the N-terminus (residues 1–108, and 330–460) (Ziegler et al. 1999). The first β-strand of the FAD binding $\beta\alpha\beta$-fold starts at Gln8 of the bovine AdxR mature sequence (Figs. 6, 7). The structures of the FAD binding domain cores of AdxR in both NADP-free (1CJC) and NADP-bound forms (1E1L) are superimposable with an RMSD of 0.3 Å (Ziegler et al. 1999; Ziegler and Schulz 2000). Thus, apparently NADPH binding does not modify FAD binding.

The most conserved regions in the FAD binding domain is the first $\beta\alpha$ fold that marks the start of the Rossmann fold (Fig. 7). This is followed by two highly conserved α-helices (h2 and h3–h4) (Fig. 7).

In the crystal structures of AdxR, the total number of the residues at the interface between FAD and AdxR is 37 (PISA interface analysis of 1E1L). Thirty-one of these residues at the interface are conserved in > 90% of the AdxR sequences. Nine of the 37 residues participate in hydrogen bonding to FAD (Table 3) and these are all conserved. A cluster of four interface residues (Tyr37, Glu38, Lys39, and Gln40) do not appear to be located at a conserved site (Fig. 6). Yet, with Blosum 35 scoring (counting Asp and

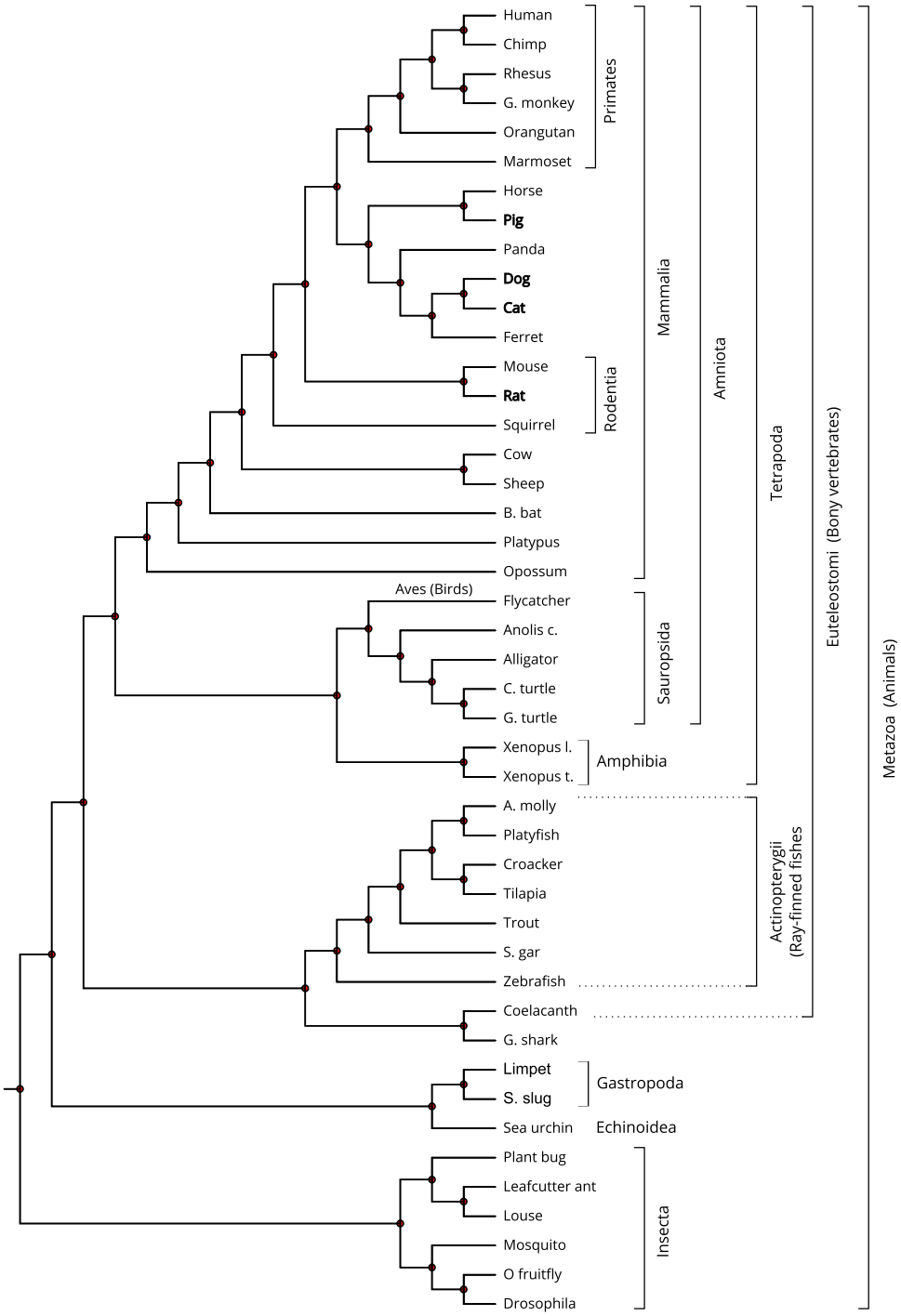

Fig. 5 The Gene tree for adrenodoxin reductase in Metazoan species. The protein sequences were taken from the Uniprot database entries listed in the Supplementary spreadsheet 1. The sequences were aligned using ClustalW. The phylogenetic distances were calculated using BLOSUM62 with Jalview (version 2.9.0b2) (Waterhouse et al. 2009)

Glu, and Arg and Lys as matched pairs) two residues at this patch share > 90% identity. Thus, overall, all residues at the interface with FAD are highly conserved.

NADP Binding Domain

The NADP binding domain extends from Ala109 to Ser329 (Ziegler and Schulz 2000). The first β-strand of the $\beta\alpha$-fold

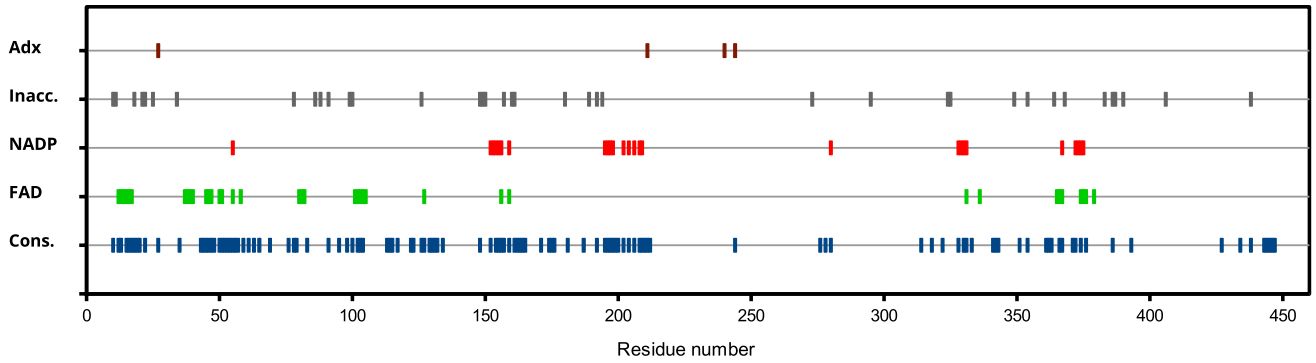

Fig. 6 Comparison of the conserved residues and the residues that are located at the coenzyme binding sites. Legend to line labels—"Cons.": Positions that are identical in > 90% of the sequences compared from 60 species. "FAD" and "NADP": Positions of the residues that are at the enzyme interface with the FAD and NADP coenzymes, respectively, determined using the PISA web server for the exploration of macromolecular interfaces located at PDBe (Krissinel and Henrick 2007). "Inacc.": Residues that are solvent inaccessible (determined by software at PDBe Pisa server by analysis of PDB ID 1E1L). "Adx": Residues determined to bind to the electron acceptor adrenodoxin (see Table 2). The sequence numbering follows the bovine mature AdxR sequence. Percent sequence identities between aligned sequences were determined using GeneDoc (Nicholas and Deerfield 1997)

starts at Thr147 as identified by (Hanukoglu and Gutfinger 1989; Ziegler and Schulz 2000).

Similar to the FAD binding site, the most conserved regions in the NADP binding domain is the first $\beta\alpha$ fold that marks the start of the second Rossmann fold (Fig. 8). This is followed by several highly conserved α-helices (h9, h11, and h12) (Fig. 8).

In the crystal structure of AdxR, the total number of the residues at the interface between NADP and AdxR is 26 (PISA interface analysis of 1E1L). Twenty-five of these residues at the interface are conserved in > 90% of the AdxR sequences (Fig. 6). Seven of these 26 residues participate in hydrogen bonding to NADP (Table 3) and these are all conserved.

A most noteworthy finding is that, in all species, the NADP binding site consensus sequence differs from that of FAD with the presence of an alanine instead of the glycine as observed for the first time in bovine AdxR (Hanukoglu and Gutfinger 1989). Thus, for the NADP binding site of AdxR orthologs, the traditional GxGxxG motif of Rossmann fold is modified to GxGxxA without an exception (Fig. 9).

Another difference of the NADP binding site from the classical $\beta\alpha\beta$ type Rossmann fold is that after the $\beta\alpha$-fold there are two helices (h9 and h10 in Fig. 8). This region is conserved in all sequences (Fig. 8).

Adrenodoxin Binding Residues

As noted above, positively charged residues (Lys27, Arg211, Arg240, and Arg244 in mature bovine AdxR) were identified as sites of adrenodoxin binding (Table 2). Three of these AdxR residues are strictly conserved; the fourth (Arg240) is identical in only ~ 60% of the sequences. Residues homologous to these are also conserved in the FprA, the *M. tuberculosis* homolog of AdxR (Bossi et al. 2002), that may be functional with the P450 systems in *M. tuberculosis* (Ouellet et al. 2010).

Implications for the Rossmann Fold Diversity

The FAD binding domain of AdxR is a prime example of the ADP binding $\beta\alpha\beta$-fold (Wierenga et al. 1986; Hanukoglu 2015).This domain starts with a $\beta\alpha\beta$-fold and carries the classic consensus sequence G-x-G-x-x-G that has been characterized in many FAD and NAD binding dehydrogenases (Wierenga et al. 1986; Ojha et al. 2007).

The NADP binding site of AdxR differs from the "classic" ADP binding $\beta\alpha\beta$-fold, noted above, by the presence of two additional helices before the second β-strand (Fig. 8), and a consensus sequence that has an alanine instead of the third glycine in the common consensus sequence (Fig. 9). The strict conservation of the sequences in this region following the first β-strand provides strong evidence that the structural model of bovine AdxR applies to all orthologs of AdxR, and that the Gly-Ala difference in the FAD vs. NADP binding sites is of structural importance as initially predicted (Hanukoglu and Gutfinger 1989).

The difference of the NADP binding $\beta\alpha$-fold from that observed in FAD raises a question whether these two motifs originated from a common ancestral structure. As noted previously, in both FAD and NADP binding sites the consensus

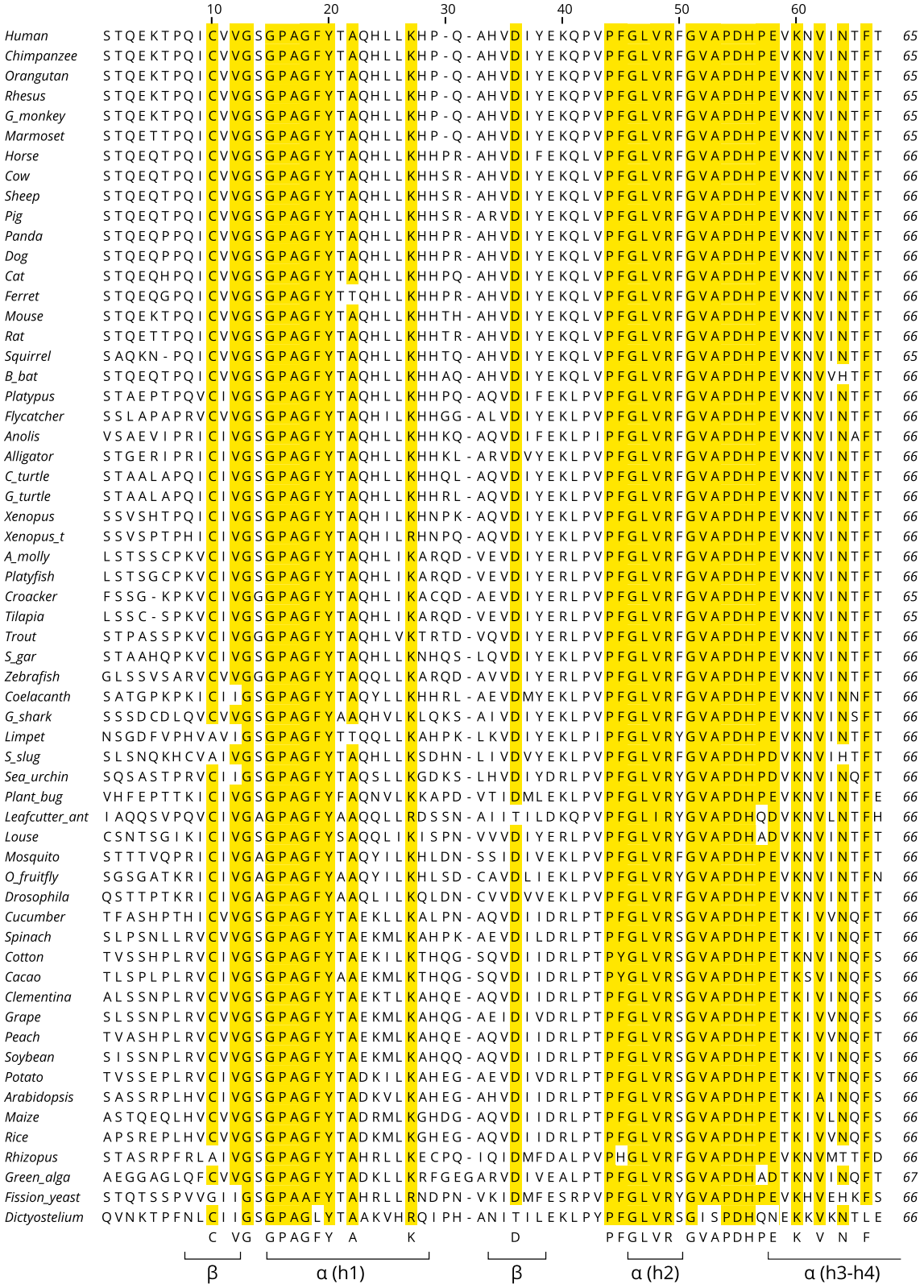

Fig. 7 The FAD binding site of adrenodoxin reductase from 60 eukaryotic species. The protein sequences were aligned as described in the legend of Fig. 2. Residues that are conserved in at least 95% of the sequences are highlighted with a yellow background. The Greek letters α and β at the bottom of the aligned sequences mark the positions of the α-helices and β-strands as identified in the bovine adrenodoxin reductase crystal structure (PDB ID: 1E1L). The number of each helix is shown in parentheses. The UniProt code numbers of the sequences are listed in the Supplementary spreadsheet 1, together with the full species names. (Color figure online)

Table 3 Amino acids that participate in the formation of hydrogen bonds with FAD and NADP in bovine adrenodoxin reductase

	FAD	NADP
1	Ala17	Gln153
2	Lys39	Asn155
3	Leu46	Val156
4	His55	Arg197
5	Val82	Arg198
6	Trp367	Glu209
7	Gly374	Gly 374
8	Ile376	–
9	Thr379	–

AA numbering is based on the bovine mature sequence. The residues were identified by the PDBe PISA server analysis of PDB ID 1E1L (Ziegler and Schulz 2000)

sequence Gly-x-Gly is in contact with the two phosphates of ADP at a common orientation (Hanukoglu 2015). Thus, it is likely that this motif evolved from an original ADP binding motif. As seen in Fig. 2, the NADP domain includes a β-sheet similar to the FAD binding domain. Apparently, during the evolution of the NAD(P) binding enzyme families, additional structural elements may have been inserted between the initial $\beta\alpha$-fold and the remaining β-strands of the sheet as seen in other NAD(P) binding enzymes (Hanukoglu 2015).

In many (if not most) articles, the Rossmann fold is described as a $\beta\alpha\beta$-fold. In view of the highly conserved NAD(P) binding domains that do not match this description, it would be more appropriate to note that the Rossmann fold represents an ADP binding fold, with a common denominator of a $\beta\alpha$-fold at the start of a β-sheet, and that there may be a variable number of structures in between the β-strands.

Unconserved Central Region

A perusal of Fig. 6 shows that a central region in AdxR sequence extending from Met213 to Pro313 (MIQLP … TRAVP, in the bovine mature sequence) is not conserved across all species. This region includes insertions and deletions in various species. But, it also includes two residues where adrenodoxin binds (Fig. 6).

An additional remarkable aspect in the conservation of the AdxR structure is the location of the major unconserved region that extends over a range of ~ 100 residues nearly in the center of the sequence (residues 213–313 in Fig. 6). This region shows relatively great diversity across clades, including insertions and deletions of up to 10 residues (sequences not shown).

In the structure of AdxR, the unconserved region appears at the edge of the enzyme, far removed from the FAD and NADP binding sites (Fig. 10). It is noteworthy that two of the electron acceptor binding residues (Arg240 and Arg244) appear in this region. Therefore, one possibility is that this region has co-evolved to suit itself for binding different electron accepting partners in different clades. Nonetheless, the far removed structural location of this region at the edge of the enzyme again emphasizes that a major structural change has not taken place (or has not survived) in the conserved enzyme-FAD–NADP interface detailed above.

Conclusions

The phylogenetic screening summarized above indicated that AdxR is an enzyme that is widely distributed in both eukaryotic and prokaryotic organisms. The minimum ~ 30% sequence identity observed between sequences from distant species represents a high degree of sequence conservation specifically in the FAD and NADP binding regions of the enzymes. These two regions both have a Rossmann fold structure starting with a $\beta\alpha$-fold that is considered as one of the most ancient and widespread motifs (Ma et al. 2008; Hanukoglu 2015).

The data shown in Fig. 6 support the hypothesis that the conserved residues are located at sites that surround the interface between the enzyme and the two coenzymes. The subject of the conservation of residues at protein–protein interfaces has been extensively investigated (e.g., Halperin et al. 2004; Guharoy and Chakrabarti 2010). These studies revealed that conserved residues are located in clusters, as the so-called hot-spots, on protein–protein interfaces (Guharoy and Chakrabarti 2010). In the present case, the strict conservation of the AdxR sequence over a wide region around the residues at enzyme–coenzyme interface (Fig. 6) shows that the conservation is not limited to patches around the AdxR–FAD and AdxR–NADP interfaces.

The extended conservation of the sequence suggests that the overall structures of the enzymes have been conserved throughout the evolution of the metazoan species to maintain a precise positioning of the two coenzymes for optimal electron transfer between the electron donor NADP and the acceptor FAD. The conservation of the eons-long perfected structure should provide a selective advantage, both for efficient electron transfer and minimization of electron leakage to prevent free radical formation during electron transfer.

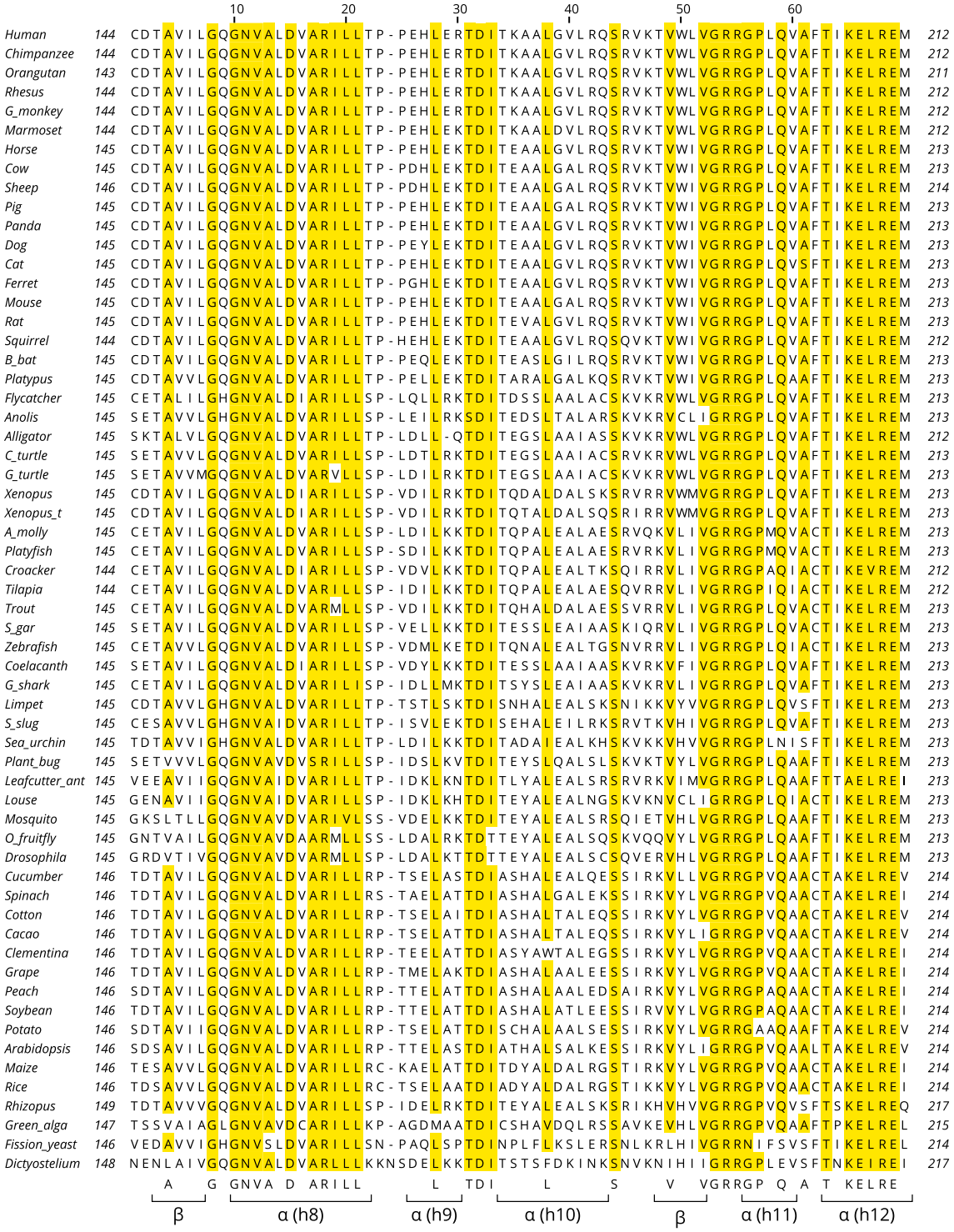

Fig. 8 The NADP binding site of adrenodoxin reductase from 60 eukaryotic species (the full species names are listed in the Supplementary Table). The protein sequences were aligned as described in the legend of Fig. 5. Residues that are conserved in at least 95% of the sequences are highlighted with a yellow background. The Greek letters α and β at the bottom of the aligned sequences mark the positions of the α-helices and β-strands as identified in the bovine adrenodoxin reductase crystal structure (PDB ID: 1E1L). The number of each helix is shown in parentheses. The UniProt code numbers of the sequences are listed in the Supplementary spreadsheet 1, together with the full species names. (Color figure online)

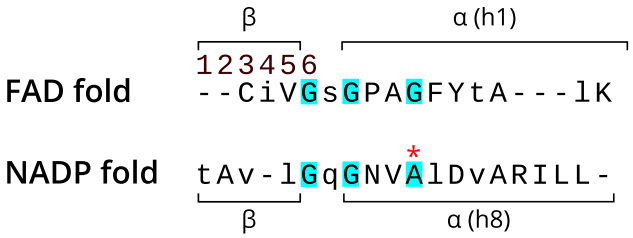

Fig. 9 Consensus sequences for the FAD and NADP binding $\beta\alpha$-fold for the eukaryotic adrenodoxin reductase sequences based on comparisons in Figs. 7, 8. The glycines in the GxGxxG motif (G represents Gly and x any residue) are colored in cyan. The exceptional Ala in the NADP binding motif is marked with a red asterisk. The upper case letters mark residues that are conserved in > 95 of the sequences. The lower case letters (i, l, s, t, and v represent Ile, Leu, Ser, Thr, and Val) mark residues that are 95% conserved when Blosum 35 similarity group is enabled. (Color figure online)

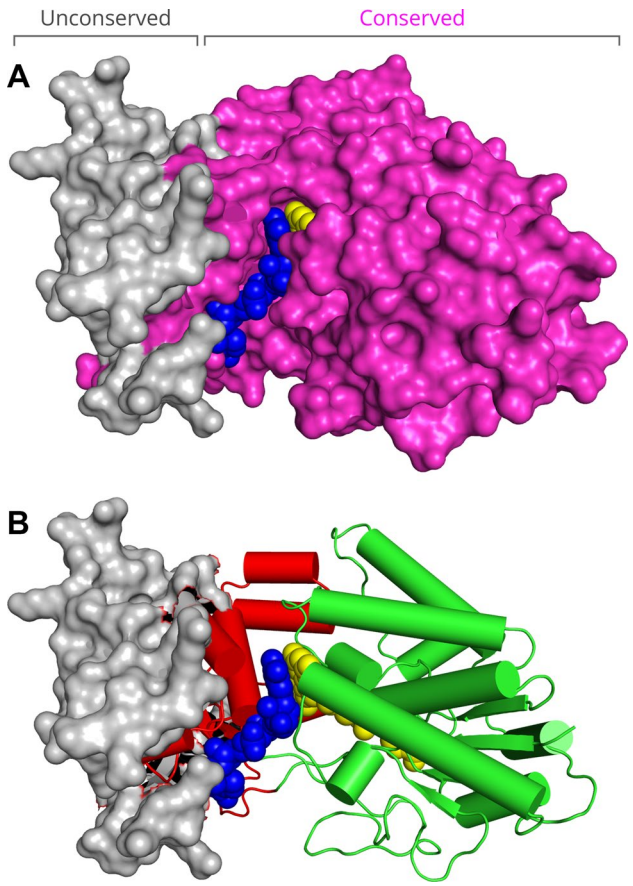

Fig. 10 The location of the unconserved region of adrenodoxin reductase in the overall structure of the enzyme. **a** The surface of the enzyme with unconserved and conserved regions colored gray and magenta, respectively. **b** The surface of the unconserved region (gray colored) extending from Met213 to Pro313 (sequence range: MIQLP…TRAVP in the bovine mature sequence) is shown superimposed on the cartoon model of the enzyme with FAD (yellow) and NADP (blue) in CPK form. Note that the unconserved region that appears at the center of the sequence (see the range of residues from position 213–313 in Fig. 6), defines the gray colored surface at the edge of the enzyme structure, far from the enzyme–coenzyme interface points. (Color figure online)

References

Aliverti A, Pandini V, Pennati A et al (2008) Structural and functional diversity of ferredoxin-NADP + reductases. Arch Biochem Biophys 474:283–291. https://doi.org/10.1016/j.abb.2008.02.014

Bossi RT, Aliverti A, Raimondi D et al (2002) A covalent modification of NADP + revealed by the atomic resolution structure of FprA, a *Mycobacterium tuberculosis* oxidoreductase. Biochemistry 41:8807–8818

Brandt ME, Vickery LE (1993) Charge pair interactions stabilizing ferredoxin-ferredoxin reductase complexes. Identification by complementary site-specific mutations. J Biol Chem 268:17126–17130

Chenna R, Sugawara H, Koike T et al (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31:3497–3500

Chu JW, Kimura T (1973) Studies on adrenal steroid hydroxylases. Molecular and catalytic properties of adrenodoxin reductase (a flavoprotein). J Biol Chem 248:2089–2094

Dym O, Eisenberg D (2001) Sequence-structure analysis of FAD-containing proteins. Protein Sci 10:1712–1728. https://doi.org/10.1110/ps.12801

Ewen KM, Schiffler B, Uhlmann-Schiffler H et al (2008) The endogenous adrenodoxin reductase-like flavoprotein arh1 supports heterologous cytochrome P450-dependent substrate conversions in Schizosaccharomyces pombe. FEMS Yeast Res 8:432–441. https://doi.org/10.1111/j.1567-1364.2008.00360.x

Ewen KM, Kleser M, Bernhardt R (2011) Adrenodoxin: the archetype of vertebrate-type [2Fe–2S] cluster ferredoxins. Biochim Biophys Acta 1814:111–125. https://doi.org/10.1016/j.bbapap.2010.06.003

González-Segura L, Riveros-Rosas H, Julián-Sánchez A, Muñoz-Clares RA (2015) Residues that influence coenzyme preference in the aldehyde dehydrogenases. Chem Biol Interact 234:59–74. https://doi.org/10.1016/j.cbi.2014.12.039

Guharoy M, Chakrabarti P (2010) Conserved residue clusters at protein–protein interfaces and their use in binding site identification. BMC Bioinform 11:286. https://doi.org/10.1186/1471-2105-11-286

Halperin I, Wolfson H, Nussinov R (2004) Protein–protein interactions; coupling of structurally conserved residues and of hot spots across interfaces. Implications for docking. Structure 12:1027–1038

Hannemann F, Bichet A, Ewen KM, Bernhardt R (2007) Cytochrome P450 systems–biological variations of electron transport chains. Biochim Biophys Acta 1770:330–344. https://doi.org/10.1016/j.bbagen.2006.07.017

Hanukoglu I (1992) Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis. J Steroid Biochem Mol Biol 43:779–804. https://doi.org/10.1016/0960-0760(92)90307-5

Hanukoglu I (1996) Electron transfer proteins of cytochrome P450 systems. Adv Mol Cell Biol 14:29–56. https://doi.org/10.1016/S1569-2558(08)60339-2

Hanukoglu I (2006) Antioxidant protective mechanisms against reactive oxygen species (ROS) generated by mitochondrial P450 systems in steroidogenic cells. Drug Metab Rev 38:171–196. https://doi.org/10.1080/03602530600570040

Hanukoglu I (2015) Proteopedia: Rossmann fold: a beta-alpha-beta fold at dinucleotide binding sites. Biochem Mol Biol Educ 43:206–209. https://doi.org/10.1002/bmb.20849

Hanukoglu I, Gutfinger T (1989) cDNA sequence of adrenodoxin reductase. Identification of NADP-binding sites in oxidoreductases. Eur J Biochem 180:479–484. https://doi.org/10.1111/j.1432-1033.1989.tb14671.x

Hanukoglu I, Hanukoglu Z (1986) Stoichiometry of mitochondrial cytochromes P-450, adrenodoxin and adrenodoxin reductase in

adrenal cortex and corpus luteum. Implications for membrane organization and gene regulation. Eur J Biochem 157:27–31. https://doi.org/10.1111/j.1432-1033.1986.tb09633.x

Hanukoglu I, Jefcoate CR (1980) Mitochondrial cytochrome P-450scc. Mechanism of electron transport by adrenodoxin. J Biol Chem 255:3057–3061

Hanukoglu I, Privalle CT, Jefcoate CR (1981) Mechanisms of ionic activation of adrenal mitochondrial cytochromes P-450scc and P-45011 beta. J Biol Chem 256:4329–4335

Hanukoglu I, Gutfinger T, Haniu M, Shively JE (1987) Isolation of a cDNA for adrenodoxin reductase (ferredoxin-NADP + reductase). Implications for mitochondrial cytochrome P-450 systems. Eur J Biochem 169:449–455. https://doi.org/10.1111/j.1432-1033.1987.tb13632.x

Hanukoglu I, Suh BS, Himmelhoch S, Amsterdam A (1990) Induction and mitochondrial localization of cytochrome P450scc system enzymes in normal and transformed ovarian granulosa cells. J Cell Biol 111:1373–1381. https://doi.org/10.1083/jcb.111.4.1373

Hanukoglu I, Rapoport R, Weiner L, Sklan D (1993) Electron leakage from the mitochondrial NADPH-adrenodoxin reductase-adrenodoxin-P450scc (cholesterol side chain cleavage) system. Arch Biochem Biophys 305:489–498. https://doi.org/10.1006/abbi.1993.1452

IUPAC-IUB (1966) IUPAC-IUB commission on biochemical nomenclature. Tentative rules. Trivial Names of miscellaneous compounds of importance in biochemistry. J Biol Chem 241:2987–2994

Kimura T, Suzuki K (1965) Enzymatic reduction of non-heme iron protein (adrenodoxin) by reduced nicotinamide adenine dinucleotide phosphate. Biochem Biophys Res Commun 20:373–379. https://doi.org/10.1016/0006-291X(65)90585-1

Krissinel E, Henrick K (2007) Inference of macromolecular assemblies from crystalline state. J Mol Biol 372:774–797. https://doi.org/10.1016/j.jmb.2007.05.022

Lambeth JD, Kamin H (1976) Adrenodoxin reductase. Properties of the complexes of reduced enzyme with NADP+ and NADPH. J Biol Chem 251:4299–4306

Lambeth JD, McCaslin DR, Kamin H (1976) Adrenodoxin reductase-adrenodoxin complex. J Biol Chem 251:7545–7550

Ma B-G, Chen L, Ji H-F et al (2008) Characters of very ancient proteins. Biochem Biophys Res Commun 366:607–611. https://doi.org/10.1016/j.bbrc.2007.12.014

Müller EC, Lapko A, Otto A et al (2001a) Covalently crosslinked complexes of bovine adrenodoxin with adrenodoxin reductase and cytochrome P450scc. Mass spectrometry and Edman degradation of complexes of the steroidogenic hydroxylase system. Eur J Biochem 268:1837–1843

Müller JJ, Lapko A, Bourenkov G et al (2001b) Adrenodoxin reductase-adrenodoxin complex structure suggests electron transfer path in steroid biosynthesis. J Biol Chem 276:2786–2789. https://doi.org/10.1074/jbc.M008501200

Nicholas KB, Deerfield DWII. (1997) GeneDoc: analysis and visualization of genetic variation. EMBnewNews 4:14

Ojha S, Meng EC, Babbitt PC (2007) Evolution of function in the "two dinucleotide binding domains" flavoproteins. PLoS Comput Biol 3:e121. https://doi.org/10.1371/journal.pcbi.0030121

Omura T (2006) Mitochondrial P450s. Chem Biol Interact 163:86–93. https://doi.org/10.1016/j.cbi.2006.06.008

Omura T, Sanders E, Estabrook RW et al (1966) Isolation from adrenal cortex of a nonheme iron protein and a flavoprotein functional as a reduced triphosphopyridine nucleotide-cytochrome P-450 reductase. Arch Biochem Biophys 117:660–673. https://doi.org/10.1016/0003-9861(66)90108-1

Ouellet H, Johnston JB, Ortiz de Montellano PR (2010) The Mycobacterium tuberculosis cytochrome P450 system. Arch Biochem Biophys 493:82–95. https://doi.org/10.1016/j.abb.2009.07.011

Pikuleva IA, Waterman MR (2013) Cytochromes P450: roles in diseases. J Biol Chem 288:17091–17098. https://doi.org/10.1074/jbc.R112.431916

Scrutton NS, Berry A, Perham RN (1990) Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. Nature 343:38–43. https://doi.org/10.1038/343038a0

Sharkey MA, Gori A, Capone M, Engel PC (2012) Reversal of the extreme coenzyme selectivity of *Clostridium symbiosum* glutamate dehydrogenase. FEBS J 279:3003–3009. https://doi.org/10.1111/j.1742-4658.2012.08681.x

Solish SB, Picado-Leonard J, Morel Y et al (1988) Human adrenodoxin reductase: two mRNAs encoded by a single gene on chromosome 17cen----q25 are expressed in steroidogenic tissues. Proc Natl Acad Sci USA 85:7104–7108. https://doi.org/10.1073/pnas.85.19.7104

Suzuki K, Kimura T (1965) An iron protein as a component of steroid 11-beta-hydroxylase complex. Biochem Biophys Res Commun 19:340–345. https://doi.org/10.1016/0006-291X(65)90465-1

Voet D, Voet JG (2004) Biochemistry, 3rd edn. Wiley, Hoboken

Waterhouse AM, Procter JB, Martin DMA et al (2009) Jalview Version 2–a multiple sequence alignment editor and analysis workbench. Bioinformatics 25:1189–1191. https://doi.org/10.1093/bioinformatics/btp033

Wierenga RK, Terpstra P, Hol WG (1986) Prediction of the occurrence of the ADP-binding beta alpha beta-fold in proteins, using an amino acid sequence fingerprint. J Mol Biol 187:101–107

You KS (1985) Stereospecificity for nicotinamide nucleotides in enzymatic and chemical hydride transfer reactions. CRC Crit Rev Biochem 17:313–451. https://doi.org/10.3109/10409238509113625

Ziegler GA, Schulz GE (2000) Crystal structures of adrenodoxin reductase in complex with NADP+ and NADPH suggesting a mechanism for the electron transfer of an enzyme family. Biochemistry 39:10986–10995

Ziegler GA, Vonrhein C, Hanukoglu I, Schulz GE (1999) The structure of adrenodoxin reductase of mitochondrial P450 systems: electron transfer for steroid biosynthesis. J Mol Biol 289:981–990. https://doi.org/10.1006/jmbi.1999.2807