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Bacterial (CYP101) and mitochondrial P450 systems—how comparable are they?

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Abstract

The bacterial CYP101 system and mitochondrial P450 systems show high similarity. Both systems contain the same protein components, a FAD containing reductase, a ferredoxin of the [2Fe2S] type, and a cytochrome P450. At a first glance they seem to be comparable but there are considerable differences among both proteins. Thus, the ferredoxin components of the two systems display significant structural homology but cannot substitute for each other in functional assays. Going into more detail, pronounced differences between the two systems that affect their biological functions are found.

Keywords: Putidaredoxin; CYP101; Adrenodoxin; CYP11A1; Protein–protein interaction

I met Gunny for the first time at the second meeting of Cytochromes P450: Biochemistry and Biophysics, held in Eberswalde/GDR (East Germany) in 1977. At that meeting Gunny offered my former supervisor, Klaus Ruckpaul, to invite young scientists to come to his lab. Because of political difficulties with scientific collaboration between Eastern and Western countries, it took a few years until it was possible to send young scientists from the former East Germany to the United States. From November 1983 to June 1984 I stayed in Urbana in Gunny's lab working on the heterologous reconstitution of P450 activities between the P450cam and P450lin systems and the microsomal P450LM2 (CYP2B4) system. It was an impressive and memorable stay, starting with ambitious discussions on different (not only scientific) topics and starting also a long-lasting friendship between Gunny and me. Although I had difficulties in understanding Gunny (I though that this was due to my bad English before I realized that others had the same difficulties), from the first minute I was impressed by his enthusiasm, his open-minded and original approach to scientific problems, and his broad interest in music, literature, and wine. I admire him as a cosmopolite, as a researcher who added several fields to our understanding of biological problems, and as a friend with whom I always like to share a good meal with a very good bottle of wine after a nice concert.

The bacterial P450cam (CYP101) system is part of the catabolic pathway of *Pseudomonas putida* for the degradation of camphor, which can be used as a carbon source. It was first shown by Rheinwald and Gunsalus that the genetic information for the proteins involved in camphor degradation is located on a plasmid [1]. The reaction catalyzed by CYP101 is the hydroxylation of camphor, yielding 5-*exo*-hydroxycamphor (Fig. 1, top). To perform this reaction, CYP101 receives reduction equivalents from NADH via an FAD-containing putidaredoxin reductase (PdR) and a [2Fe-2S] cluster-containing ferredoxin, putidaredoxin (Pdx). All components of the CYP101 system (i.e., PdR, Pdx, and CYP101) are soluble and have therefore been readily available for detailed studies on their biochemical and biophysical properties. Due to its catabolic nature the reaction is fast compared to anabolic reactions such as the production of steroid hormones in the adrenal mitochondria of mammals. The components of the mammalian mitochondrial steroid hydroxylating P450 systems (CYP11A1, CYP11B1, CYP11B2) are identical to the CYP101 system at a first glance (Fig. 1, bottom).

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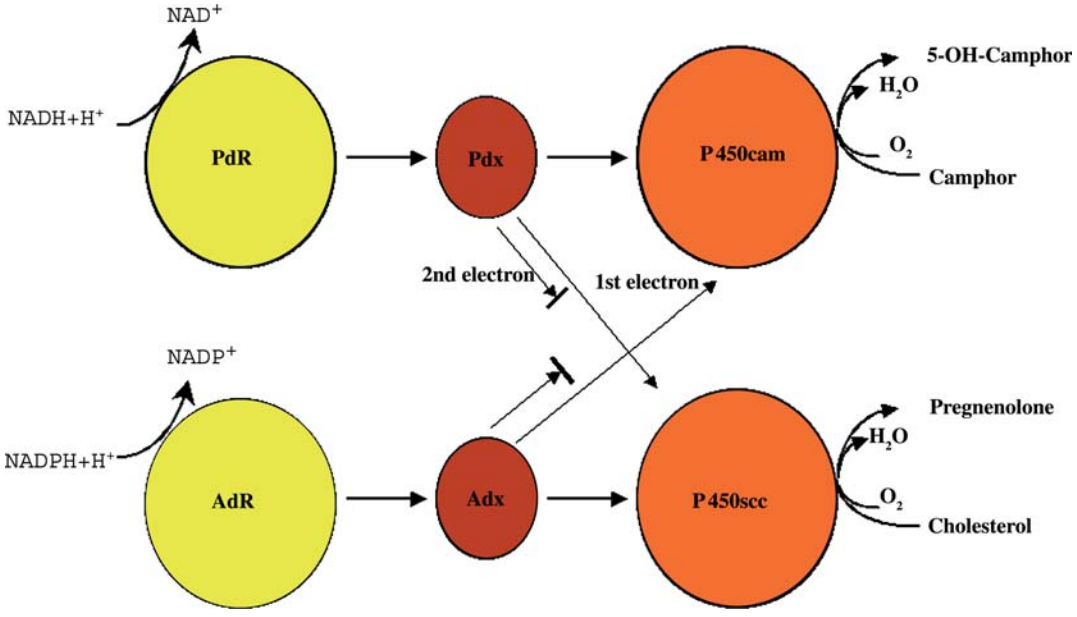

Fig. 1. Schematic presentation of the soluble bacterial CYP101 system and of the mammalian mitochondrial CYP11A1 system. The possibility of transferring the first electron to the heterologous P450 is indicated by arrows.

For the hydroxylation of their corresponding substrates, these membrane-bound P450s receive reduction equivalents from NADPH via a membrane-associated adrenodoxin reductase (AdR) and a soluble [2Fe-2S] cluster-containing ferredoxin, adrenodoxin (Adx).

Although the two systems resemble each other strongly, the ferredoxins cannot substitute for each other in the respective P450 substrate conversion assays [2]. However, the transfer of the first electron to the heterologous P450 (i.e., CYP101 or CYP11A1) is mediated by putidaredoxin as well as by adrenodoxin [3] (Fig. 1). Nevertheless, it could be shown that, in general, heterologous reconstitution systems can be functionally active. Interestingly, such a system, containing PdR, Pdx, and the microsomal P450 CYP2B4, leads to productive substrate conversion [4,5], even though microsomal P450 systems lack a ferredoxin compound.

Over the last decades, studies on the structure–function relationships of vertebrate-type ferredoxins were mainly performed with Pdx and Adx due to the good availability of these proteins in their purified forms, and yielded plenty of information about the molecular details of their biological activities. Significantly, these ferredoxin components turned out to be a defining point for the different velocities of the fast bacterial and the slow mammalian P450 system. The reaction rates observed for the different systems are in the range of 3000 per minute in the case of CYP101 [6,7] and 100 per minute for CYP11A1 [8,9], the latter of which catalyzes the first and rate-limiting step in steroid hormone biosynthesis (i.e., the conversion of cholesterol to pregnenolone). In particular, the technique of heterologous overexpression of proteins opened the way to investigate the function of single amino acids in Pdx and Adx [10–17]. A comparison of the primary structures of both ferredoxins (Fig. 2) reveals a similarity of about 40% with 34 identical amino acids and 18 conservative substitutions. The overall length of putidaredoxin is 106 residues, whereas mature adrenodoxin (i.e., after cleavage of its mitochondrial targeting sequence) consists of 128 amino acids. The [2Fe-2S] cluster-coordinating residues of the two ferredoxins (four cysteines in both proteins) are found in the same relative positions of the aligned primary structures. The geometry of the cluster region is similar in both proteins as documented by EPR studies (Fig. 3) and X-ray crystallographic structures (Fig. 4). Pdx and Adx belong to the group of [2Fe-2S] proteins with planar cluster geometry.

Important information on the function of a protein can be drawn from its three-dimensional structure. The tertiary structures of both ferredoxins have been elucidated recently (Fig. 4). The overall topology of Adx and

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Pdx   1      MSKVVYVSHDGTRRELDVADGVSLMQAAVSNGI-YDIVGDCGGSA     44
             ...|.:::.||.........|.||:...|.|.: .|..|.||.|:.
Adx   1 SSSEDKITVHFINRDGETLTTKGKIGDSLLDVVVQNNLDIDGFGACEGTL     50

Pdx   45 SCATCHVYVNEAFTDKVPAANEREIGMLECVTAELKPNSRLCCQIIMTPE    94
         :|:|||:...:...:|:.|..:.|..||: :...|...|||.|||.:|..
Adx   51 ACSTCHLIFEQHIFEKLEAITDEENDMLD-LAYGLTDRSRLGCQICLTKA    99

Pdx   95 LDGIVVDVPDRQW                      107
         :|.:.|.|||..
Adx  100 MDNMTVRVPDAVSDARESIDMGMNSSKIE     128
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Fig. 2. Alignment of the primary structures of Adx and Pdx. The four cysteine ligands of the iron sulfur cluster are indicated in green. Identities (34 aa) are marked with a connecting line, and conservative substitutions (18 aa) are labeled with double dots. The C-terminal Trp residue and its homologous position in Adx (S112) is labeled in red.

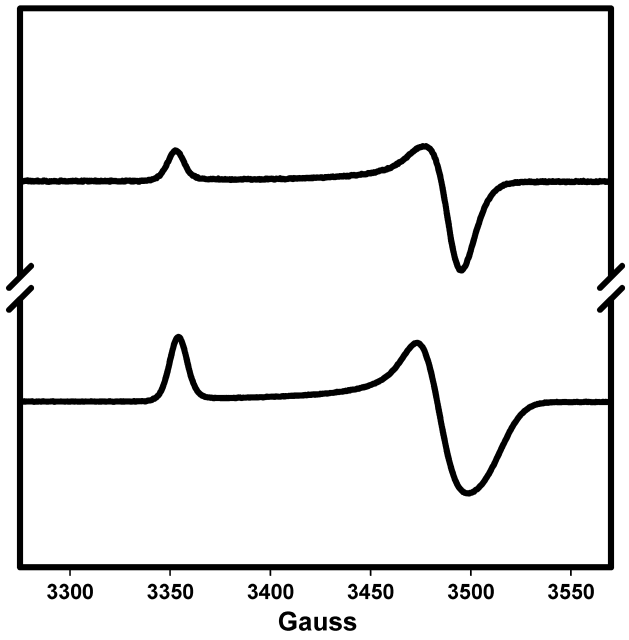

Fig. 3. EPR spectra of Adx (top) and Pdx (bottom). These structures clearly reflect the conformational similarity between the two ferredoxins. The values are $g_1 = 2.024$ and $g_2 = 1.937$ for Adx and $g_1 = 2.019$ and $g_2 = 1.935$ for Pdx.

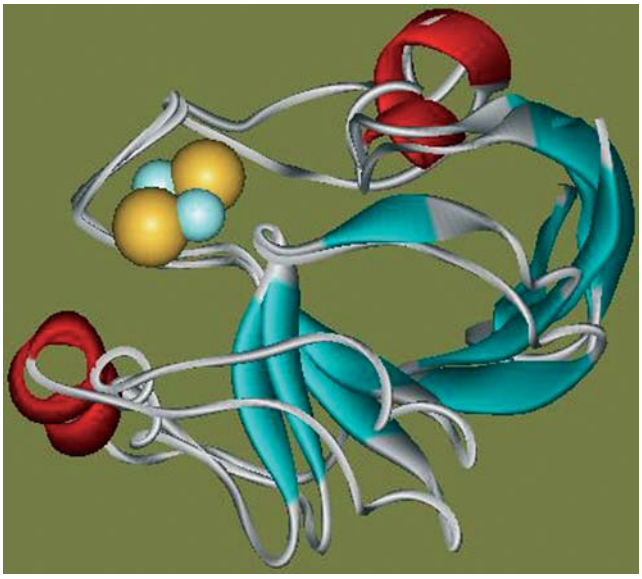

Fig. 4. 3D structures of Adx (top) [19] and Pdx (bottom) [18]. The relationship between the two structures is obvious.

Pdx is similar and both are typical examples for vertebrate-type ferredoxins [18,19]. The putidaredoxin structure has been solved by NMR [18]. For adrenodoxin, the NMR structure and the crystal structures of the truncated as well as of the wild type protein have been obtained [19–21]. Furthermore, the crystal structure of adrenodoxin reductase [22,23] and the structure of a complex of adrenodoxin reductase and adrenodoxin have become available recently [24]. Very recently, the solutions of the crystal structures of putidaredoxin reductase and also of putidaredoxin have been reported [25]. For CYP101 crystal structures with different ligands are available (for a detailed list, search http://www.ncbi.nlm.nih.gov/). In addition, for the very first time, the detailed redox cycle of a cytochrome P450 could be solved using crystal structures of the oxygenated CYP101 intermediate of this cycle [26]. The tertiary structure of any mitochondrial P450, on the other hand, remains to be elucidated because they are membrane-bound proteins including the associated problems to acquire material suitable for crystallization. Nevertheless, models of mitochondrial P450s are available and can be used for computational protein–protein interaction studies [27–29].

The *Pseudomonas* CYP101 system is also an excellent model for understanding ferredoxin-redox partner interactions in hydroxylating-type systems. A wealth of kinetic data on the interaction between Pdx and CYP101 have been obtained, for example, early studies of the redox dynamics between Pdx and CYP101 [6], the reduction kinetics of CYP101 by Pdx [7], and the electron transfer from Pdx to CYP101 at very low temperatures [30]. Furthermore, studies on proton coupling [31], molecular recognition [32], and site-directed mutagenesis of the CYP101 system have been performed [17].

As already mentioned, the three-dimensional structures of Pdx and its electron acceptor, CYP101, are well known. The interaction sites in the mitochondrial P450 system seem to be partially different and are partially overlapping compared with those in the *Pseudomonas* system. For both systems, the ionic character of the interaction between the respective ferredoxin and cytochrome P450 has been shown [7,8,33]. The structure-based model for CYP101–Pdx interactions presented by Pochapsky et al. [34], however, indicated that none of the acidic residues of Pdx homologous to those implicated in Adx binding to CYP11A1 and AdR are involved in ionic intermolecular interactions. Instead, three salt bridges have been proposed in this model: R112 (CYP101)/D38 (Pdx), R109 (CYP101)/D34 (Pdx), and R79 (CYP101)/W106 (Pdx). Mutagenesis studies of the Pdx residues D34, D38, and W106 confirmed the involvement of these residues in binding of CYP101, whereas mutation of C73 affects the interaction with PdR [17,35]. For CYP11A1 binding, on the contrary, the homologous residues D39 and D41 of Adx are of minor importance [36]. Rather, the negatively charged "interaction domain" located between residues 72 and 79 of Adx could be shown to be important for the recognition of the redox partners CYP11A1 and AdR [37,38]. Corresponding positively charged amino acids could be identified on the surfaces of AdR and CYP11A1 [39–41], confirming that the protein–protein

interaction in the mitochondrial P450 system is mainly depending on electrostatic forces. Early work of Hintz and Peterson [7] indicated an effect of the ionic strength on the interaction of Pdx and CYP101 but similar importance of a negatively charged interaction domain seems not to apply in the case of Pdx [34]. The interaction domain of Adx is coupled to the [2Fe-2S] cluster via the conserved H56 of Adx, as well as the residues Y82 and S88. These residues could be shown to be involved in a network of hydrogen bonds that mediate between the interaction domain of Adx [42,43] and the cluster. Particularly the histidine residue in position 56 of Adx, which corresponds to histidine residue in position 49 of Pdx, was very recently demonstrated to be critical for redox dynamics [43,44]. Lately, site-directed mutagenesis studies on the cluster binding loop of adrenodoxin (aa 47–aa 51) have been performed by mutating amino acid T49 [45]. Mutants T49A, T49S, T49L, and T49Y and a deletion mutant, T49Δ, were generated to investigate the function of the hydroxyl group and the spatial structure of this residue. It could be shown that T49 modulates the redox potential in Adx and that the region around T49 (i.e., the cluster binding loop) is a new interaction site for the physiological redox partners AdR and CYP11A1. In addition, the participation of T49 in electron transfer from Adx to CYP11A1 was detected by variations of the apparent rate constants of all T49 mutants as well as by calculation of coupling rates [45]. On the Adx molecule, a small region with high coupling rates was identified containing a loop (aa 47–aa 54) that covers the iron sulfur cluster of Adx. The importance of this region in Pdx was investigated by Shimada et al. [46]. They demonstrated that mutation of the homologous position S44 in Pdx causes the same spectral changes of Pdx as the formation of the ternary complex Pdx/CYP101/CO, which indicates that this residue functions as an interface together with the residues D34, D38, and S42 [35]. Moreover, amino acids D38 and C39 of Pdx have been predicted to be involved in the electron transfer from Pdx to CYP101 [47].

Besides the ionic interactions discussed above, the C-terminal tryptophan residue of Pdx, W106, was shown to be extremely important for high-affinity binding to CYP101 [16,17,48]. Site-directed mutagenesis studies in which W106 was replaced by other amino acids, or even deleted, confirmed the importance of this aromatic residue for the binding affinity in the Pdx/CYP101 system (Fig. 5). The free energy values for the binding of different Pdx species to CYP101 presented in Fig. 5 clearly reflect the necessity of at least an aromatic residue in position 106 of Pdx. A possible region for aromatic–aromatic interaction between the Pdx residue W106 and CYP101 is postulated to be near the residues Y78 and H352 at the proximal surface of the cytochrome [34]. It was also shown that W106 affects the interaction with PdR.

A comparison of the primary structures of the two ferredoxin components of the respective P450 systems reveals a major difference in the C-terminal part of these proteins. Adx has an extended C-terminus, but does not contain an aromatic residue in this region that could fulfill a role similar to W106 in Pdx. Serine in position 112 of bovine adrenodoxin aligns to the last amino acid in putidaredoxin, tryptophan in position 106. In order to mimic the Pdx structure at the Adx C-terminus, truncation to 112 residues and introduction of a tryptophan into the C-terminal position of Adx were performed [9]. The mutant Adx protein (S112W) did not mediate heterologous transfer of the second electron to CYP101, which is not unexpected taking into account the differences in the ionic interaction sites presented above. The binding to CYP11A1, however, was signifi-

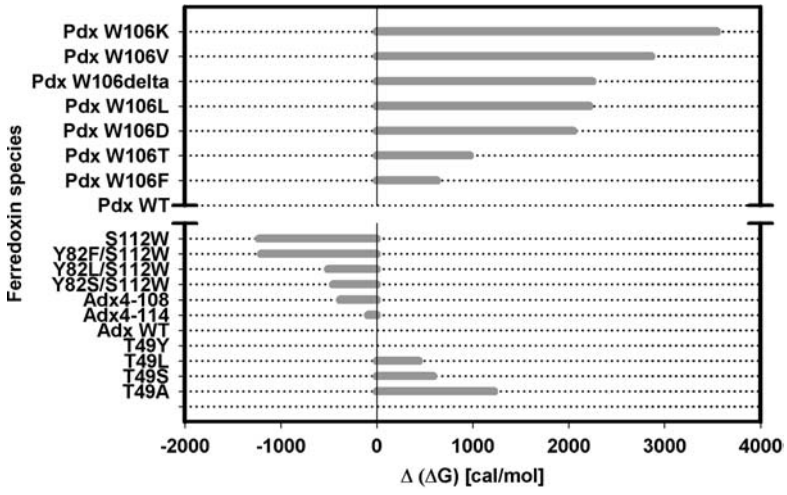

Fig. 5. Binding of different Adx species to CYP11A1 and of various Pdx species to CYP101. Differences in the apparent free energy of ferredoxin mutants in binding to their respective P450s are plotted. $\Delta(\Delta G)$ values were calculated according to [37]. References for the ferredoxin species are Adx T49 species [44], all other Adx species [9], and Pdx species [17].

cantly improved upon introduction of a Trp in position 112 of Adx as shown in Fig. 5. According to the dramatic change in free energy, an aromatic residue in position 112 and position 82 leads to an enhanced binding of Adx to CYP11A1. With the physiological electron acceptor CYP11A1 a 75-fold (with mutant Adx S112W/Y82F even a 100-fold) increase in the efficiency of cholesterol conversion has been detected due to stronger binding and increased k_{cat} rates. Thus, it was possible to significantly improve the efficiency of a mitochondrial P450 system by making Adx (Adx S112W) more similar to Pdx.

Taken together, the bacterial and mitochondrial P450 systems show a high degree of similarity but also significant differences that affect their biological functions. It is still not yet understood why the second electron cannot be transferred in the corresponding heterologous system, although Adx and Pdx are very close to each other in structure. The detailed knowledge acquired by studying both systems will help solve this question and lead to better understanding of redox-dependent enzyme systems.

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