Redox Partners: Function Modulators of Bacterial P450 Enzymes

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The superfamily of cytochrome P450 monooxygenases (P450s) is widespread in all kingdoms of life. Functionally versatile P450s are extensively involved in diverse anabolic and catabolic processes. P450s require electrons to be transferred by redox partners (RPs) for O2 activation and substrate monooxygenation. Unlike monotonic eukaryotic cytochrome P450 reductases, bacterial RP systems are more diverse and complicated. Recent studies have demonstrated that the type, the amount, the combination, and the mode of action of bacterial RPs can affect not only the catalytic rate and product distribution but also the type and selectivity of P450 reactions. These results are leading to a novel opinion that RPs not only function as auxiliary electron transfer proteins but are also important P450 function modulators.

**Cytochrome P450 Monooxygenases**

Cytochrome P450 monooxygenases (see Glossary) (P450s, EC 1.14.x.x) are a superfamily of heme-thiolate proteins ubiquitously distributed in all kingdoms of life [1]. P450s are among the oldest enzyme families, and their common ancestor gene is believed to have existed in the last universal common ancestor [2]. The long evolutionary history (>3 billion years) of P450s has witnessed one of the greatest divergent molecular evolution events in nature [3]: ~300 000 P450 protein sequences are available in the Identical Protein Groups database (https://www.ncbi.nlm.nih.gov/ipg/) and >40 000 P450 sequences with systematic nomenclature (http://drnelson.uthsc.edu/cytochromeP450.html) are available as of December 31, 2019. As the most versatile biocatalysts, P450s catalyze C–H hydroxylation, C=C double bond epoxidation, N- and S-oxidation, O-, N-, and S-dealkylation, aromatic coupling, C–C bond cleavage, and many other common and uncommon reactions towards a vast variety of substrates [4,5]. Thus, functionally versatile P450s extensively participate in human drug/xenobiotic metabolism and hormone anabolism, natural product biosynthesis in plants and microorganisms, diverse microbial catabolic processes, and increasing applications in biotechnology and synthetic biology [6].

The highly diversified P450 superfamily (with the amino acid sequence identity as low as 16% [7]) maintains a well conserved 3D structure fold, the absolutely conserved cysteine axially linked to the heme prosthetic group, and a common catalytic cycle for O2 heterolytic cleavage and the formation of the highly reactive species including the ferryl oxo cation radical (compound I) and the ferric hydroperoxo species (compound 0) for substrate oxidation [8,9] (Figure 1).

**Diversity of P450 RP Systems**

To achieve the reductive activation of inert O2 for substrate monooxygenation, the vast majority of P450s interact with one or more redox partners (RPs) in order to source reducing equivalents from NAD(P)H. In most cases, NAD(P)H→RP(s)→P450→O2 forms an electron transfer (ET) chain; and the ET mediated by RP(s) is often the rate-limiting step in a P450 catalytic cycle [10].

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**Highlights**

Ubiquitous P450s catalyze various oxidative reactions towards an enormous number of substrates. Bacterial P450s in soluble forms represent the most diverse subset with great application value and potential.

In the conventional notion, redox partners are auxiliary proteins influencing electron transfer efficiency and product distribution. Lately, growing evidence has demonstrated that redox partners can endow their paired P450s with novel functionalities and may have more unexplored physiological roles.

Bacterial redox partners can form various combinations to serve different P450s in vivo. This flexibility may be important for the host microorganisms to deal with ever-changing environments.

Selection and engineering of redox partners for an optimized P450-redox partner interaction interface will become an important strategy for the improvement of industrially relevant P450 catalysts.

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P450s can be categorized into different classes according to their cognate RPs [11] (Box 1). The most abundant Class I soluble P450s, including prokaryotic and mitochondrial P450s, require two RP proteins: a small iron–sulfur protein ferredoxin (Fdx) and a ferredoxin reductase (FdR) that contains a flavin adenine dinucleotide (FAD) cofactor. First, the two-electron carrier FdR accepts a hydride from NAD(P)H. Second, FdR reduces Fdx with one electron upon a transient protein–protein interaction. Third, the single electron carrier Fdx transiently contacts P450 to deliver the first electron to the heme iron. Finally, another Fdx shuttles the second electron from FdR to P450 for the O–O bond cleavage [12]. By contrast, the Class II endoplasmic reticulum (ER)-associated P450s employed by eukaryotes (humans, animals, plants, and fungi) recruit a single RP protein to transfer electrons—that is, the membrane-bound cytochrome P450 reductase (CPR) carrying both FAD and flavin mononucleotide (FMN) to form an ET chain [13].

In addition to these two main RP systems, nature has evolved many other uncommon RP systems (Box 1) to serve various specific P450s [11]. In particular, RP and P450 sometimes form a fusion protein to enable an intramolecular ET pathway, resulting in self-sufficiency and often higher ET efficiency [14].

**The Classic Function of RPs in P450 Catalytic Systems**

Typically, RPs are treated as auxiliary ET proteins in P450 catalytic systems. The importance of RPs in regulating and tuning P450 reactions has long been underestimated [15,16]. The ET process takes place in two steps. The first electron can be delivered to the heme-iron of P450 before or after substrate binding. The latter situation is more common, since substrate binding is often
required to lower the redox potential of P450, thus thermodynamically favoring ET from RPs [9]. Upon reduction of the heme-iron from ferric to ferrous, dioxygen binds and then the second ET occurs (Figure 1).

In the case of Class I P450 systems, the single electron carrier Fdx shuttles one electron after the other from FdR to P450. The nature of the ET complex has been the subject of long debates and has been intensively studied in the mammalian mitochondrial system consisting of adrenodoxin (Adx) and adrenodoxin reductase (AdR). Besides the widely accepted electron shuttle mode based on a 1:1:1 ratio of AdR, Adx, and P450, an alternative quaternary complex comprised of a P450, an AdR, and two Adxs was also proposed [17]. However, structural analyses of CYP11A1–Adx and AdR–Adx complexes revealed that the interaction interfaces of Adx with CYP11A1 and AdR highly overlap, indicating that the CYP11A1–Adx–AdR cannot form [18,19].

The variety of ET processes in bacterial P450 systems remains relatively underexplored. On one hand, the structural data on ET complexes are scarce. The only structurally investigated complex is the putidaredoxin (Pdx, i.e., Pseudomonas putida ferredoxin)/P450cam (CYP101A1) complex [20]. Its solution NMR data were in good accordance with previous results on chemical modification, mutagenesis analysis, and biophysical studies underlining the key role of salt bridges, especially the one between Asp38 of Pdx and Arg112 of P450cam, and of the Trp106 residue for protein–protein recognition and complex formation [21]. More detailed structural analyses of this complex by X-ray diffraction and paramagnetic NMR spectroscopy revealed the occurrence of both productive and futile encounters in this complex [22,23]. Strikingly, unlike many other P450s, the open rather than the closed conformation of P450cam was found to be favored by Pdx [20]. In particular, the activity of P450cam strictly requires the pairing with Pdx, indicating highly specific interactions between these two redox proteins. Thus, a significant proportion of knowledge gained from this ‘prototypic’ P450 system cannot be extended to other bacterial P450 systems.

On the other hand, despite more and more functionally characterized bacterial P450s, their native preferred RPs are often unclear unless the P450 gene and the RP gene(s) are localized in the same operon. Compared with their eukaryotic counterparts, usually with one or two CPR(s), the prokaryotic RP systems are more diverse and complicated since bacterial
cells mostly contain multiple Fdxs and FdRs (Box 2). Thus, it has long been the practice to use surrogate RP proteins from heterologous hosts to reconstitute the activities of diverse bacterial P450s [24–26]. The most popular surrogate RPs for bacterial P450s include spinach Fdx/FdR, Adx/AdR, Pdx/putidaredoxin reductase (PdR), and the SelFdx1499/SelFdR0978 from *Synechococcus elongatus* PCC 7942 [27–29]. Interestingly, all of these Fdxs are [2Fe-2S] Fdxs.
It has been shown that different endogenous redox pairs can be used by a common P450 from *Streptomyces coelicolor* A3(2) [24] as well as *Sorangium cellulosum* Sc ce56 [25], giving rise to varying activities. More recently, Zhang *et al.* systematically interrogated whether there are any bacterial P450-preferred RPs. It turned out that the plastidic-type FdR and the [2Fe-2S] Fdx are more likely to endow a bacterial Class I P450 enzyme with higher ET efficiency [26]. This finding will probably enable quick prediction and identification of the optimal RPs for any bacterial P450 of interest.

### Novel Functions of RPs in P450-Catalyzed Reactions

It is well known that different choices and ratios of RPs would alter both P450 catalytic efficiency and product distribution (in multiproduct reactions) [30]. The influence on catalytic efficiency is unsurprising because the second electron transfer step is often the rate-limiting step in a P450 catalytic cycle [10]. Other indications that RPs have more functions in addition to being the ET proteins for P450s include: (i) the RP-induced product profile changes suggest that RPs could tune the site-selectivity of P450 reactions; (ii) as described, Pdx acts as a required function effector for P450cam owing to the very special role of Trp106 [20]; (iii) the presence of cytochrome b5

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**Box 2. Fdxomes and FdRomes of Three Representative Bacteria**

Ferredoxins (Fdxs) belong to simple iron-sulfur proteins, which have only iron-sulfur cluster(s) as prosthetic group(s). According to the chemical composition of the iron-sulfur cluster(s), Fdxs can be classified as [2Fe-2S] (including plant-like, vertebrate-type, and thioredoxin-like), [3/4Fe-4S], [4Fe-4S][3/4Fe-4S], Rieske proteins (specialized [2Fe-2S]), and high-potential iron-sulfur proteins [49] (Figure I). Ferredoxin reductases (FdRs) are classified into plant-type (plastidic type and bacterial-type) and glutathione reductase (GR)-type (AdR-like and oxygenase-coupled NADH-ferredoxin reductase (ONFR)-like) [50]. Interestingly, the plastidic-type FdR and [2Fe-2S] Fdx were found more likely to be optimal RPs for bacterial Class I P450s [26]. Different classes of Fdxs and FdRs are randomly distributed in bacteria, archaea, fungi, plants, and animals.

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**Figure I. Fdxomes and FdRomes of Three Representative Bacteria.** Please see Box 2 text for legend.
trend human CYP17A1 from a 17α-hydroxylase into a 17,20-lyase in steroid biosynthesis [31];
(iv) some genetic defects in human CPR would cause not only changes in drug metabolism but
also the severe Antley–Bixler syndrome [32]; and (v) Fdx1 from S. cellulosum So ce56 was
shown to be involved in Fe-S cluster assembly [33].

Apart from these additional functions, more novel modulating effects of RPs on P450-catalyzed
reactions have been discovered. For example, MycG is a bacterial multifunctional biosynthetic
P450 enzyme responsible for hydroxylation and epoxidation of 16-membered ring macrolide
mycinamicins [34]. Although the cognate RPs of MycG have yet to be elucidated, its physiological
hydroxylation and epoxidation reactions have been reconstituted using either spinach Fdx/FdR or
the fused RhFRED reductase domain. When partnered by RhFRED in separate, strikingly, MycG
was shown to gain new functionality of mediating unnatural demethylation reactions in addition to
native monooxygenations [16] (Figure 2). This finding provided the first evidence that the choice of
‘auxiliary’ RP proteins and their mode of action could enable the new type and selectivity of reac-
tions catalyzed by a bacterial P450.

Mechanistic and Physiological Insights for RPs as Function Modulators of
Bacterial P450s
RPs can affect both the activity and the chemo-, regio-, and stereoselectivity of P450 reactions.
Mechanistically, the function-modulating ability of RPs may stem from alternative binding or allo-
steric effects caused by different Fdxs or reductases that directly interact with the proximal side
of P450s. Since these redox interactions are transient, electronically steered, geometric comple-
mentation dependent, and entropy-driven [26], an interesting possibility is that the negatively
charged Fdxs with variant shapes may be docked by different positively charged areas on the
P450 proximal side, which could induce alternative active-site conformations and change the
substrate orientation in the substrate-binding pocket [16]. It has been demonstrated that different
binding positions of various RPs on CYP106A2 indeed led to (i) the changes in ET ef
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ciencies;
(ii) variations in the distance between substrate and the heme-iron reactive center; (iii) the differ-
ence in the binding strength of RPs; and (iv) the product reorientation, which together gives rise
to multiple products at varying ratios [35].

Physiologically, except for the self-sufficient P450s with a fused Fdx or reductase domain, a bac-
terial P450 would inevitably confront multiple RPs in cells. An interesting hypothesis naturally
arises: different RPs may endow P450s with alternative activities, thus making P450 more versa-
tile in vivo. This versatility improvement may be evolutionarily advantageous for the host microor-
ganisms by detoxifying more xenobiotics and/or synthesizing more secondary metabolites to
adapt to various environments. This may help to explain why bacteria have not evolved P450-
specific RPs and why the natural P450–RP fusions are rare. After all, both P450s and Fdxs are
among the oldest proteins, with the latter even being speculated to be an evolutionary relic of a
prebiotic mineral surface catalyst [36]. These two functionally related classes of ancestral proteins
should have had a sufficiently long time to coevolve. Moreover, as the universal single-electron
carrier [or a currency exchanger since NAD(P)H is considered as a universal energy currency],
in addition to P450s, Fdxs also interact with many other oxidoreductases such as FdRs from pho-
tosystem I, hydrogenases, and nitrogenases [37]. In this context, it would be unfavorable for bac-
teria to allow P450s to monopolize Fdxs through highly specific interactions.

RP Selection and Engineering
Due to the importance of RPs for the catalytic efficiency and function modulation of bacterial
P450s, RP selection and engineering (Box 3) has been attracting increasing attention in recent
years. With regard to activity improvement, Adx/AdR provided a three- to fourfold better
supporting activity than endogenous RPs for the \textit{in vitro} hydroxylation of lauric acid by CYP109D1 from \textit{S. cellulosum} So ce56 [38]. Fdx8/FdR\_B from \textit{S. cellulosum} and especially SynFdx/FNR from \textit{Synechocystis} sp. PCC6803 were reported to be much better RP systems for P450 EpoK in the biotransformation of epothilone D to epothilone B than spinach Fdx/FdR [39]. The
cyanobacterial photosynthesis-related RP proteins SeaFx1499/SeaFdr0978 were recently shown to be optimal for an increasing number of P450s such as CYP-sb21 [29], Rif16 [40], AbmV [41], CreJ [42], TiaP1/TiaP2 [43], MycG, PkC, CYP105AS-1, and P450sca2 [26]. Notably, making a P450–RhFRED self-sufficient fusion has also become a widely accepted strategy for bacterial P450 activity reconstitution [14] since the pioneering work of the Sherman laboratory [44].

RPs can also be used to tune the product profile of multifunctional P450s. For example, the amounts of three oxidative products of 11-deoxycorticosterone (DOC), namely, 1α-OH DOC, C1-C2-ene-DOC, and 1α,14 dihydroxy-DOC, were elegantly controlled by tuning the ratio of CYP260A1:Adx:AdR [45]. This demonstrates that the RP amounts and the availability of reducing equivalents to P450s can tune the formation of various products. The observed differences between in vitro and in vivo product patterns [46,47] might be due to this effect. Sagadin et al. [33] investigated 11 RP combinations for the CYP106A2-mediated conversion of progesterone to 15β-hydroxyprogesterone. The best selectivities but low conversion rates were achieved with Fdp/FdR and Adx(4–108)/BmCPR, respectively. In the reactions of high conversion rates, polyhydroxylated (over-oxidation) products became significant, indicating that it is not necessarily better to use the most efficient RPs for a specific P450 reaction if product selectivity is the major concern [26]. Furthermore, it was shown that site-directed mutagenesis of RPs on the basis of computational modeling can fine tune the selectivity of P450 reactions [48].

Driven by the discovery that the mode of action of RPs can modify the type and selectivity of P450 reactions, the bacterial multifunctional P450 MycG was reacted with mycinamicin I–V in the presence of RhFRED or RhFRED-Fdx (a chimera of RhFRED and spinach Fdx); seven novel mono-or didemethylated mycinamicins were produced [16] (Figure 2). This work demonstrated a new strategy for making ‘unnatural’ natural products through RP engineering for bacterial P450s.

Concluding Remarks and Future Directions
Along with the recent discoveries of new RP functions other than the fundamental ET functionality, we are witnessing the trend of conceptual transformation for RPs from auxiliary ET proteins to functional modulators of P450s. However, the mechanistic details about how RPs modulate P450 functions have yet to be fully understood. It has been shown that slight modifications of the P450–RP interaction interface can affect the substrate binding mode, RP binding strength,

Box 3. RP Selection Strategies for Bacterial P450 Activity Reconstitutions
Selection of appropriate RPs is a prerequisite for bacterial P450 activity reconstitutions. The fast-growing number of RP sequences, deeper understanding on RP structure–function relationships and RP–P450 interactions, and more efficient synthetic biology approaches have been leading to multiple strategies for the more accurate RP selection for studies and applications of bacterial P450s.

i. Traditional use of surrogate RPs such as spinach Fdx/FdR, Pdx/PdR, and Adx/AdR.
ii. Construction of P450–RP fusions to make the self-sufficient P450s. The most frequently used RP domains for this purpose include the reductase domains of P450 BM3 (CYP102A1) from Bacillus megaterium and of P450 Rnf (CYP116B2) from Rhodococcus sp. NCIMB 9784.
iii. Direct use of the RPs whose encoding genes are located in the same operon of the P450 of interest.
iv. Given the availability of whole-genome sequencing data, all RPs can be heterologously expressed and purified. Then, their supporting activities to a specific bacterial P450 can be compared in vitro.
v. Computational structure modeling and molecular docking can be used for prediction of the optimal RPs for bacterial P450s. We envision that machine learning and artificial intelligence will play more important roles in simulating P450–RP interactions and identifying P450-preferred RPs.
vi. Systematic construction of an RP library. For any P450 of interest, the supporting activities of different Fdx/FdR combinations can be screened in a high-throughput manner in vitro.

Outstanding Questions

Why do the photosynthesis-related RPs (e.g., SeaFx1499/SeaFdr0978 and spinach Fdx/FdR) turn out to be very good RPs for bacterial P450s?

In bacterial cells, how does a P450 compete for RPs with other P450s and oxidoreductases? Are there any controlling mechanisms?

What are the detailed mechanisms for the different behaviors of bacterial RPs in terms of protein–protein interactions, electron transfer efficiencies, and product profile tuning?

Can we accurately predict bacterial P450–RP interactions and the consequences thereof in the near future?

Are there any rules to be followed in the biotechnological application of P450s and RPs?
as well as ET efficiency, leading to this way to a changed reactivity and selectivity of P450. Relatively low binding affinities between P450s and RPs that are essential for transient interactions prevent direct observation of the complex structures. Although several structures of the fused or crosslinked P450 and RP have been resolved [19,20,22,23], the artificially fixed conformations might disrupt some essential dynamics involved in the physiological protein–protein interactions. Thus, structural elucidation of a natural P450–RP fusion protein is fairly important. Unfortunately, such a structure for the full length of P450 BM3, P450 RhF, or any other P450–RP natural fusion remains unavailable despite considerable efforts. At this point, the breakthroughs through X-ray crystallography or cryogenic electron microscopy (cryo-EM) technology from structural biologists are excitedly awaited. Progress in this direction will also pave the way for engineering the P450–RP interactions for higher productivities and/or desired selectivities.

Another important challenge is to further understand the P450–RP interactions in the more complicated cellular environments. The modulating activities of bacterial RPs observed in enzymatic assays are still short of direct in vivo evidence. At this point, the in-cell stoichiometry between P450s and RPs, as well as the availability of different potential RPs, could significantly modulate conversion rates and reaction selectivities. Moreover, the RP engineering strategy is waiting to be more practiced in vivo (see Outstanding Questions).

Finally, we suggest that (i) more attention should be paid to the previously underexplored RPs when studying bacterial P450s; (ii) the optimal endogenous RPs for a specific P450 should be identified given the availability of whole-genome sequencing data; (iii) RP engineering could be practiced when a P450’s activity and/or selectivity needs to be improved; (iv) an RP library would be very helpful to identify the optimal RPs; and (v) machine learning and artificial intelligence approaches should be practiced more in the studies on P450–RP interactions.

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