Customized exogenous ferredoxin functions as an efficient electron carrier

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Abstract

Ferredoxin (Fdx) is regarded as the main electron carrier in biological electron transfer and acts as an electron donor in metabolic pathways of many organisms. Here, we screened a self-sufficient P450-derived reductase PRF with promising NADPH reduction activity and 9OHAD production yield and proved the importance of [2Fe-2S] clusters of Fdx-containing oxidoreductase in transferring electrons in steroidal conversion. The truncated Fdx domain in all oxidoreductases, together with mutagenesis data, further elucidated the indispensable role of [2Fe-2S] clusters in the electron transfer process. By adding the independent plant-type Fdx to the reaction system, the AD conversion rate have been significantly improved. A novel efficient electron transfer pathway of PRF+Fdx+KshA in the reaction system rather than KshAB complex system was proposed based on analysis of protein-protein interactions and redox potential measurement. Adding free Fdx created a new conduit for electrons to travel from reductase to oxygenase. This electron transfer pathway provides new insight for the development of efficient exogenous Fdx as an electron carrier.

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ABSTRACT

Ferredoxin (Fdx) is regarded as the main electron carrier in biological electron transfer and acts as an electron donor in metabolic pathways of many organisms. Here, we screened a self-sufficient P450-derived reductase PRF with promising NADPH reduction activity and 9OHAD production yield and proved the importance of [2Fe-2S] clusters of Fdx-containing oxidoreductase in transferring electrons in steroidal conversion. The truncated Fdx domain in all oxidoreductases, together with mutagenesis data, further elucidated the indispensable role of [2Fe-2S] clusters in the electron transfer process. By adding the independent plant-type Fdx

to the reaction system, the AD conversion rate have been significantly improved. A novel efficient electron transfer pathway of PRF+Fdx+KshA in the reaction system rather than KshAB complex system was proposed based on analysis of protein-protein interactions and redox potential measurement. Adding free Fdx created a new conduit for electrons to travel from reductase to oxygenase. This electron transfer pathway provides new insight for the development of efficient exogenous Fdx as an electron carrier.

KEYWORDS: ferredoxin; electron transfer; electron bifurcation; [2Fe-2S] clusters

INTRODUCTION

Ferredoxin (Fdx) was firstly discovered and isolated from *Clostridium pastoris* (a non-photosynthetic anaerobic bacteria) by Mortenson et al. in 1962 (Mortenson et al., 1962). It is a small metalloprotein with [2Fe-2S] clusters as redox centers (Arnon, 1988). They play a key role as electron carriers in various metabolic processes (Huang et al., 2016). In non-photosynthetic organisms, electron transfer is mediated by NAD(P)H (Arnon, 1965; Goss & Hanke, 2014; Martı́nez-Espinosa et al., 2003). The Fe-S cluster in Fdx physiologically transfers only one electron from NAD(P)H, and the reduced Fdx can drive the reaction more strongly because its redox potential is lower than that of NAD(P)H (Kameya et al., 2020). Fdx has been classified as [2Fe-2S], [4Fe-4S], [3Fe-4S] and [7Fe-8S] (Aono et al., 1992; Imai et al., 2001; Lovgreen et al., 2011; Ricagno et al., 2007), in which [2Fe-2S] clusters are divided into two types: plant-type and Rieske-type. In plant-type Fdxs, the [2Fe-2S] clusters are organized as [2Fe-2S] tetrahedrons, containing two inorganic Fe atoms and sulfurs from four conserved cysteine residues. In Rieske-type Fdxs, they are composed of inorganic iron atoms, two cysteine and histidine residues. Importantly, plant-type Fdx plays a central role in the electron transfer between the photosynthesis chain and various metabolic pathways (Bohme, 1977; Kimata-Ariga et al., 2006; Lee et al., 2011).

Previous research indicated that Fdx and Fdx-NADP⁺-reductase (Fdx/FNR) could form complex during electron transfer process (Bohme, 1977; Hurley et al., 1997; Kimata-Ariga et al., 2006; Lee et al., 2011; Quaranta et al., 2016). The functional and structural properties of Fdx/FNR interaction have been investigated suitable redox partners are needed to carry out effectively electron transfer serving as intermediates in steroidal conversion catalyzed by P450 enzymes (Kimata-Ariga et al., 2007; Quaranta et al., 2016; Ueno et al., 2013). Studies have established the principle of customizing non-physiological Fdx to support similar native P450 activity (Bell et al., 2012). The addition of the [2Fe-2S] binding domain in the N-terminus of KshB (Fdx reductase of 3-ketosteroid-9-hydroxylase) or TDO (toluene 2,3-dioxygenase reductase) was also reported for efficiently strengthening electron transfer by [2Fe-2S] Fdx (Zhu et al., 2020). The electron transfer pathway was proposed from NADH via FAD to the terminal active-site mononuclear iron of the dioxygenase in the reductase and the Fdx protein by elucidating the molecular mechanism of the redox-dependent interaction between NADH-dependent reductase and Rieske-type [2Fe-2S] Fdx (Zhu et al., 2020). The structures of TDO including TDO-R (3EF6) and TDO-F (3DQY), and BphA4-BphA3 complex (FAD-NADH containing Fdx reductase BphA4 and Rieske-type [2Fe-2S] Fdx BphA3 in the electron transfer system of biphenyl dioxygenase BphA) were reported previously (Senda et al., 2007) that the reductase BphA4 receives two electrons from NADH. The reduced BphA4-BphA3 complex then transfers an electron to the [2Fe-2S] clusters of two Fdx molecules through redox-dependent transient interactions. The reduced Fdx transfers the electron to the active center of the oxygenase to support the activation of dioxygen for biphenyl dihydroxylation. Self-sufficient P450 monooxygenases are highly active and convenient systems without partner reductase (Chen et al., 2020). The crystal structure of the self-sufficient P450 enzyme CYP116B46 has indicated that it contains the cofactor FMN to capture electrons from NADPH and RP Fdx, which has most efficient electron transfer. The direction of electron transfer NADPH-FMN-[2Fe-2S] -heme correlates with the domain arrangement, which undoubtedly provides a great help for the screening of redox partners (Zhang et al., 2020). Otherwise, Based on the flavin-based electron bifurcation theory (FBEB), the electron bifurcation phenomenon is formed by the oxidation of NADPH in the oxidoreductase complex (Demmer et al., 2015; Duan et al., 2020; Lubner et al., 2017; Schut et al., 2019; Tokmina-Lukaszewska et al., 2018; Yuly et al., 2019). The FBEB bifurcation reaction forms a highly unstable flavonoid anion semiquinone (ASQ) to reduce Fdx (Kremp et al., 2020; Sucharitakul et al., 2020). The key step is to transfer electrons from a

molecule with a medium reduction potential to one of the lower potentials (thermodynamically unfavorable) by coupling the electrons with the transfer to the high potential acceptor (thermodynamically favorable) (Miller et al., 2019; Muller et al., 2018; Schut et al., 2019). Fdx is the electron carrier requiring for all reactions catalyzed by electron bifurcation enzymes (Huang et al., 2016; Liang et al., 2019).

Here, we screened and characterized the reductases involving plant-type and Rieske-type Fdxs. A promising reductase of partial self-sufficient P450 CYP116B46 (V465-L779) from *Tepidiphilus thermophiles*, named PRF, which contains the flavin mononucleotide (FMN)-binding reductase and [2Fe-2S] clusters was characterized. We added the independent Fdxs to the reaction system instead of KshAB to improve the conversion rate of AD to 9OHAD. A novel electron transfer pathway was proposed using exogenous Fdx as an efficient electron carrier based on analysis of protein-protein interactions and redox potential measurement.

2 MATERIALS AND METHODS

2.1 Plasmids construction, expression and purification of enzymes

The coding sequences for the reductase domain and Fdx (V465-L779) of *Tepidiphilus thermophilus* CYP116B46 (PRF) (WP_055423153.1), *Thermosynechococcus elongatus* BP-1 Fdx (TeFdx) (WP_011056851.1), *Drosophila melanogaster* Fdx1 (DmFdx1) and Fdx2 (DmFdx2) (BI626767.1 and BI168285.1), *Pseudomonas resinovorans* Fdx (PrFdx) (WP_011077882.1), *Pseudomonas sp.* Fdx (PsFdx1) (WP_015014864.1), *Sphingobium yanoikuyae* Fdx (SyFdx) (WP_025548169.1), were synthesized by GENEWZ (Suzhou, China) and cloned into the vector pET28a (+) between the *Nde*I and *Eco*RI restriction sites. KshA (Rieske-type oxygenase of 3-ketosteroid-9-hydroxylase) from *Rhodococcus erythropolis*, KshB (a Fdx reductase of 3-ketosteroid-9-hydroxylase) from *Mycobacterium tuberculosis* H37Rv, TDO_R (toluene 2,3-dioxygenase) from *Pseudomonas putida* were kept in the laboratory. The recombinant plasmid was introduced into *Escherichia coli* strains JM109 and BL21(DE3) as the host organism for gene cloning and protein expression, were purchased from Novagen (Madison, WI, USA). The strains, plasmids, and primers used in this study are listed in Tables S1. Bacteria were grown with shaking in lysogeny broth medium containing 40 µg/mL kanamycin at 37 °C. When the OD₆₀₀ reached 0.6-0.8, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce the overexpression of enzymes at 16 °C for 16-18 h. Then the bacteria were collected by centrifugation at 5,000 × *g* for 15 min at 4 °C, and resuspended in the lysis buffer (20 mM Tris-HCl, 40 mM imidazole, 0.5 M NaCl, 1 mM DTT, pH 7.4), lysozyme can be added to improve cell disruption. After ultrasonic disruption, the supernatant was collected by centrifugation at 40,000 × *g* for 30 min at 4 °C and the His₆-tagged enzymes were trapped on Ni-NTA Superflow resin (Qiagen, Hilden, Germany). The enzymes were eluted by elution buffer (20 mM Tris-HCl, 400 mM imidazole, 0.5 M NaCl, 1 mM DTT, pH 7.4), and dialyzed against 20 mM HEPES, pH 7.4 finally. The fractions were further purified using anion exchange chromatography on a Resource Q column (column volume: 6 mL, flow rate: 4 mL/min, GE Healthcare, Stockholm, Sweden).

2.2 Activity assays

The NADH reduction activity was monitored by following UV absorption at 340 nm using a multimode plate reader (SpectraMax i3x, Molecular Devices, Silicon Valley, CA, USA). The specific operation method can refer to our existing research. The Michaelis-Menten constants (K_m), turnover number (k_{cat}), catalytic efficiencies (k_{cat}/K_m) for each substrate were calculated by the Michaelis-Menten function in the GraphPad Prism (GraphPad Software, San Diego, CA, USA).

2.3 Multienzyme cascade catalysis *in vitro*

The cell-free multienzyme catalysis was performed and mixing with 50 mM PBS (pH 7.4), 200 µM AD dissolved in 2% methanol, 300 µM NAD(P)H, and enzymes (5 µM reductases, 5 µM Fdxs, and 2.5 µM KshA). The HPLC was used to determine the yield of 9OHAD (Zhu et al., 2020). The strains used are listed in Tables S1, plasmids and primers used in this study are listed in Tables S2.

2.4 Site-directed mutagenesis

All variants described in this work were generated using the KOD-Plus Site-Directed Mutagenesis Kit (Toyobo, Japan) and the pET-28a (+) or pCold I vector harboring the wild-type sequence. The oligonucleotides designed to introduce the mutations are depicted in Table S2. All mutations were confirmed by DNA sequencing.

2.5 Spectroscopic absorption

All UV-vis spectroscopic absorption experiments were performed with Agilent Cary 2500 UV-visible Spectrophotometer (Agilent Technologies, Beijing, China) at 25 °C. The enzymes were dialyzed against the same buffer (20 mM HEPES, pH 7.4). The spectral absorbance of 20 mM HEPES buffer was scanned in the range of 300-800 nm as a blank control, and then the UV-vis spectral absorbances of the oxidized state enzymes were measured. 100 mM sodium dithionite was added into the samples until there was no further decrease in absorbance in the 300-800 nm range and the spectrum of the reduced state enzymes was measured.

2.6 Homology modeling

A three-dimensional (3D) homology model were constructed using automodel command in Modeller 9.9.2, with the crystal structure of *Rhodococcus rhodochrous* 3-ketosteroid-9- α -hydroxylase 5 (PDB ID: 4QDC, 75.38% sequence identity with KshA), *Haloarcula marismortui* Fdx (PDB ID: 1DOI, 32.14% sequence identity with KshB-Fdx), human Fdx 2 (PDB ID: 2Y5C, 63.21% sequence identity with DmFdx1), and urate oxidase (PDB ID: 3PLM, 61.74% sequence identity with DmFdx2) as the template. The DOPE assessment scores were used to choose the best model. The model structures were visualized by PyMOL software (<http://www.pymol.org>).

2.7 Protein-protein docking

All protein-protein docking used the website <http://zdock.umassmed.edu/>. The oxygenase-reductase complex was first obtained. The Fdx was then docked to the oxygenase-reductase complex, and obtained the best results by manually analysis the best top 10 results obtained on website <http://zdock.umassmed.edu/>. The subsequent processing and analysis are carried out using the PyMOL software (<http://www.pymol.org>).

2.8 ITC measurement

The affinity of ferredoxin DmFdx2 with oxygenase KshA and reductase PRF was determined by ITC 200 instrument (MicroCal, USA) at 25 °C. Each sample solution was dialyzed against the same buffer (20 mM HEPES, pH 7.4) and degassed under vacuum before calorimetry. The reaction cell and injection syringe were filled with 0.02 mM KshA/PRF and 0.2 mM DmFdx2, respectively. The reaction temperature, stirring speed, and reference power were set at 25 °C, 800 rpm, and 5.00 μ cal/s, respectively. An aliquot comprising 2 μ L of the DmFdx2 solution was added per injection, and each injection cycle lasted for 120 s. The interval between two injections was 60 s to allow for the signal to return to the baseline. The data were analyzed using Origin 7.0 software, and the data were fitted to the Wiseman equation using a one-site binding model. The stoichiometry (n), binding constant (K_D), binding enthalpy (^{H}), and entropy change (^{S}) could be determined. The binding Gibbs energy change, ^{G} , is calculated from the equations $^G = -RT \ln K_D$.

2.9 Redox potential measurement

The electrochemical titration experiments were performed using Mettler T50 Automatic Potentiometric Titrator with an Ag/AgCl electrode at 25 °C. The 25 μ M protein samples were dispersed in Tris-HCl buffer at pH 7.4. Sodium dithionite (100 mM) was used as the reducing agent. All solutions were degassed under vacuum with argon. The plug and play (PnP) burette with chip and the Ag/AgCl electrode were selected. Before the measurement, the burette was washed by pump ultrapure water under automatic cleaning mode program. In setting interface parameter, the titrant of sodium dithionite, the stirrer speed of 50 %, and the sample volume of reducing agent were set to 10 mL. 10 mL protein sample was added to the titration cup. When the potential retained stable, sodium dithionite was titrated. Record the reduction potential and the volume of reducing agent when potential was no longer changed.

2.10 Construction of whole-cell catalysts system

Whole-cell catalysts [*E. coli* BL21 (DE3)] with different combinations of pET28a- and pETDuet-derived vectors bearing genes encoding KshA, PRF, and Fdx were used to convert AD to 9OHAD. To construct strain BLA-PD, the *PRF* gene was amplified, digested, and inserted into MCS1 site of pETDuet between *Eco* RI and *Hin* dIII sites, the gene encoding DmFdx2 was inserted into MCS2, between *Nde* I and *Xho* I, which created pETDuet-PRF-DmFdx2. pETDuet-PRF-DmFdx2 and pET28a harboring *KshA* were co-transformed into *E. coli* BL21(DE3) cells. The similar operation mode was used to construct strain BLA-PP, in which the *PRF* and *PsFdx1* genes were inserted into pETDuet and created pETDuet-PRF-PsFdx1. pETDuet-PRF-PsFdx1 and pET28a harboring *KshA* were co-transformed into *E. coli* BL21(DE3) cells. The control strain of BLA-P was constructed using pET28a-KshA and pCold I-PRF, and co-transformed into *E. coli* BL21(DE3) cells. The constructed strains are listed in Table S1 and plasmids are listed in Table S2.

2.11 Bioconversion of AD to produce 9OHAD

The constructed recombinant strains are listed in Table S1. The plasmid construction and protein expression operation are the same as described above. The cells were harvested by centrifugation at $5,000 \times g$ for 10 min and resuspended in 30 mL 50 mM PBS (pH 7.4). The production of 9OHAD was conducted in reaction mixture containing 50 g/L wet cells, 5 g/L AD dissolved in 2% methanol and 22.8 g/L methyl- β -cyclodextrin, at 35 °C for 5 h (Zhu et al., 2020). The products were determined by HPLC.

3 RESULTS AND DISCUSSION

3.1 Comparison of reductases for hydroxylation reaction toward AD

The reductases of KshB and TDO have been screened previously with AD as the substrate (Zhu et al., 2020). A novel reported reductase of the self-sufficient P450 enzyme CYP116B46 from *Tepidiphilus thermophilus* (named PRF), which contains plant-type [2Fe-2S] cluster, was first mined for bioconversion of AD. We further investigated the catalytic efficiency of PRF in both NAD(P)H reduction activity and 9OHAD reaction system based on research on KshB and TDO (Table 1). By comparing the reduction activity toward NADH, we found that PRF showed higher activity with k_{cat}/K_m of $1.70 \text{ s}^{-1} \mu\text{M}^{-1}$ than TDO ($0.43 \text{ s}^{-1} \mu\text{M}^{-1}$) and KshB ($0.34 \text{ s}^{-1} \mu\text{M}^{-1}$). Furthermore, PRF also showed the higher AD conversion rate of 68.4% than TDO (54.8%) and KshB (42.0%) (Figure 1 and Table S3). Therefore, PRF showed the promising potential as reductase partner of KshA during hydroxylation of steroidal substrates than KshB or TDO.

3.2 Structural analysis of reductase TDO and PRF

We compared the crystal structures of two reductases TDO-R (PDB ID: 3EF6) with PRF (PDB ID: 6LAA). Structural analysis revealed that both TDO and PRF contained NAD(P)H domain, and their flavin binding motifs belonged to different types: FAD-binding and FMN-binding (Figure 2). In reductase TDO, both the FAD-binding and NADH-binding domains were α/β -fold type characteristic of the glutathione reductase (GR) family. They had a central parallel β -sheet, three α -helices on one side of the sheet and a three-stranded β -meander on the other side. FAD captured electrons from NADH and transfers to [2Fe-2S] cluster Fe atom of TDO-Fdx (Friemann et al., 2009). The structural comparison showed that the TDO contained an extra domain (gray part). In previous research, a “swing domain model” of P450BM3 FMN-binding domain operated the back-and-forth motions, which was governed by a flexible hinge domain (Chen et al., 2020). That implied that TDO-Fdx might swing to a favorable position to carry out the effective electron transfer from FAD to [2Fe-2S] cluster (Figure 2a). In PRF, the FMN binding motif was a six-stranded antiparallel β -barrel while the NADPH-binding motif adopted a Rossmann fold containing a partial β -sheet. FMN captured electrons from NADH and transferred the electrons from the methyl group of C8 to Fe atom of [2Fe-2S] cluster (Figure 2b) (Zhang et al., 2020). The truncated [2Fe-2S] clusters of the oxidoreductases (*KshA*, *KshB*, *TDO*, *PRF*) had no longer exhibited catalytic activity toward AD. It indicated that [2Fe-2S] clusters played their irreplaceable role during the hydroxylation reaction of AD (Table 1). Importantly, the structures of [2Fe-2S] clusters in TDO, KshB and PRF are also different, which may cause the completely different catalytic activities and conversion of TDO — is the Rieske — type (Fe ligated by 2 Cys and 2 His), while PRF and KshB are the plant —

type(Feligatedby4Cys).

To further investigate the importance of [2Fe-2S] clusters in reductases, we compared the catalytic activity of various reductases containing two different types of [2Fe-2S] clusters. Besides, we mutated the TDO to MTDO (plant-type). Meanwhile, KshB and PRF were mutated to MKshB and MPRF (Rieske-type), respectively. After mutation, the NADH reduction activity k_{cat}/K_m of MTDO has been increased from $0.43 \text{ s}^{-1} \mu\text{M}^{-1}$ to $0.54 \text{ s}^{-1} \mu\text{M}^{-1}$. KshB showed the same activity ($0.34 \text{ s}^{-1} \mu\text{M}^{-1}$) with MKshB ($0.35 \text{ s}^{-1} \mu\text{M}^{-1}$). Surprisingly, MPRF showed the decreased activity from $1.70 \text{ s}^{-1} \mu\text{M}^{-1}$ to $0.50 \text{ s}^{-1} \mu\text{M}^{-1}$ (Table 1). The data of AD conversion rate of three mutants also showed the same tendency with NADH reduction activity that muted MPRF also decreased the AD conversion rate (Table S3). These results implied that plant-type cluster seems to have higher electron transfer efficiency than Rieske-type cluster.

More importantly, the UV-vis absorption spectra (Figure 3a and 3b) were typical characteristics of flavo-proteins. Plant-type PRF (Figure 3a) and MTDO (Figure 3b) had almost identical UV-vis spectra, with three main absorptions at 378, 447 and 475 nm. In contrast, the wild-type TDO (Rieske-type) also showed the same main absorptions at 378 and 447nm, but a slight bathochromic shift of the band at 475 nm and a relative amplitude of the 530–550 nm shoulder (Figure 3b). This indicated that the modification of TDO (Rieske-type) to MTDO (plant-type) resulted in the slight changes in the prosthetic group environment. These small changes implied that the electronic structure of Rieske-type and plant-type [2Fe-2S] cluster was different and hence of different interactions between the two types of [2Fe-2S] clusters and the protein.

3.3 Screening of free Fdx proteins for enhancement of steroidal conversion

The [2Fe-2S] clusters have been testified to be important for catalytic activities. To verify whether free Fdx functions as electron transfer intermediate, we investigated the effects of two types of free Fdxs (Rieske-type and plant-type) according to the conversion of AD to 9OHAD using multi-enzyme catalytic system in vitro (KshA+PRF) (Figure S1 and Table S4). Six Fdxs genes were screened as candidates namely PsFdx1, SyFdx, PrFdx (Rieske-type Fdxs) and TeFdx, DmFdx1, DmFdx2 (Plant-type Fdxs).

After adding various free Fdxs in the 9OHAD catalytic reaction system containing reductase PRF and oxygenase KshA, the AD conversion rate were determined and the Fdxs candidate enzymes were evaluated. The most outstanding in six Fdxs are Rieske-type PsFdx1 and plant-type DmFdx2 (Figure S1). Therefore, we selected these two Fdxs for further bioconversion of AD to 9OHAD in oxidoreductase system using reductases KshB, TDO and PRF. In particularly, when adding Rieske-type Fdx PsFdx1 into the catalytic reaction system, the AD conversion rates of KshA+PRF+PsFdx1 and KshA+PRF+DmFdx2 were 80.6% and 85.2% (Figure S2a), which was 1.18 and 1.25 folds higher than only using KshA+PRF (68.4%) respectively. The reaction systems of TDO and KshB also showed the similar increase of AD conversion rates when adding two types of Fdx (Figure S2b and S2c). When free Fdx was added to the catalytic reaction system, the electron transfer was effectively promoted due to the mediation of free Fdx among reductase and oxygenase. Furthermore, the plant-type DmFdx2 was more efficient than Rieske-type PsFdx1, when adding these two different kinds of Fdxs to catalytic reaction system.

3.4 Structural analysis of two types of Fdxs

All the six free Fdxs are [2Fe-2S] clusters, which can be divided into plant-type and Rieske-type according to the difference of coordinating amino acids in the [2Fe-2S] cluster (Figure S3). Sequence alignment showed that all the Rieske-type Fdxs have conserved Cys-X1-His-segment-Cys-X2-His motif (Figure S4a). N-terminal portion of the Rieske-type Fdx consists of a Rieske [2Fe-2S] cluster domain, which consists of β strands and loops (Ferraro et al., 2007; Murzin et al., 1995). Four residues in the α subunit, 2His and 2Cys, coordinate the Rieske non-heme iron in [2Fe-2S] cluster. 2His coordinate one iron, while 2Cys coordinate the other (Figure S3a, b, c). At the same time, all the plant-type Fdxs have conservative Cys-X4/X5-Cys-X2-Cys-segment-Cys motif (Figure S4b). The C-terminal portion consists of a plant-type [2Fe-2S] cluster domain, which consists of loops, β strands and α spiral. Four residues of Cys in the α subunit coordinate two irons of non-heme iron cluster [2Fe-2S] (Figure S3d, e, f).

The UV-vis spectroscopic absorption of all oxidized Fdxs showed characteristic absorption spectra in range of 300-800 nm. DmFdx2 (Figure 3c) and PsFdx1 (Figure 3d) showed distinguished different UV-Vis spectra. DmFdx2 showed typical spectrum of plant-type Fdxs with three main absorptions at 330, 415 and 465 nm. After reduction with sodium dithionite, the absorption decreased by 52% at 420 nm and 49% at 465 nm. The UV-vis absorption spectrum of Rieske-type PsFdx1 (Figure 3b) showed bands at 330 and 450 nm, but a relative amplitude of the 550-580 nm shoulder. When reduced with sodium dithionite, the absorbance of PsFdx1 decreased in the entire UV-visible range. These typical features of two types [2Fe-2S] Fdxs indicated that the electronic structure of Rieske-type and plant-type [2Fe-2S] cluster was different.

3.5 Structural analysis of proposed electron transfer pathway

To analyze the electron transfer pathway between free Fdx, reductase and oxygenase, we targeted the reaction system of the KshA (oxygenase), PRF (reductase) and DmFdx2 (Fdx) to investigate the electron transfer pathway using protein-protein docking (Figure S5). Interestingly, the docked structure complex revealed that there were two potential electron transfer pathways existing in the reaction system. Adding free Fdx creates a new conduit for electrons to travel from reductase to oxygenase. In the KshA+PRF catalytic reaction system, the electron transfer pathway is PRF (NADPH-FMN-[2Fe-2S]) - KshA ([2Fe-2S]-Fe). After free Fdx was added to the catalytic reaction system, an auxiliary electron transfer pathway of PRF(NADPH-FMN-[2Fe-2S]) - DmFdx2 ([2Fe-2S]) - KshA ([2Fe-2S]-Fe) might be formed between PRF and DmFdx2, in which the electrons could also be transferred to the [2Fe-2S] of free Fdx, and then were captured by the [2Fe-2S] of the PRF. The physical distance between two [2Fe-2S] clusters of PRF and KshA (20.9Å) was almost the same with the distance between [2Fe-2S] clusters of PRF and DmFdx2 (20.4Å). It seems that electrons have the same probability to be transferred by both pathways (Figure S5). Furthermore, the distance between [2Fe-2S] clusters of DmFdx2 and KshA was only 9.3Å, implied that electrons could be transferred more effectively to KshA. The structural information implied that two electron transfer pathways might be more efficient for improving the AD conversion rate.

3.6 Analysis of protein-protein interactions identified by ITC

To further identify the proposed electron transfer pathway of free Fdx as an electron carrier, the interactions of DmFdx2 with KshA and PRF were measured via thermodynamics ITC experiments. The results of the calorimetric titrations of DmFdx2 to PRF (Figure 4a) and DmFdx2 to KshA (Figure 4b) were shown in Figure 4. The thermodynamic parameters were collected in Table 2. In both cases, an exothermic heat pulse ($\Delta H < 0$) was observed after each injection of DmFdx2 into the aptamer solution KshA and PRF. This indicated that the binding process was an exothermic reaction. However, the interaction of PRF with DmFdx2 was associated with a larger favorable enthalpy ($H = -8KJmol^{-1}$) compared with KshA ($H = -6KJmol^{-1}$). The values of the binding constants and the Gibbs energy changes indicated that the associations were strongly favored. $2.10 \times 10^7 M^{-1}$ is about 1.5 fold greater than that for the PRF - DmFdx2 interaction ($K_D = 1.50 \times 10^7 M^{-1}$). The values of entropy change ($S < 0$) showed that, in both cases, the binding processes were enthalpically driven. The

3.7 Analysis of redox potential and electron bifurcation

To clarify the auxiliary electron transfer pathway between two [2Fe-2S] clusters of reductase PRF and ferredoxin DmFdx2, we performed the measurement of the redox potential (Table 2). The redox potential measurement indicated that the single-pathway potential and the dual-pathway potential are different. Particularly, single-pathway showed the redox potential of PRF (-365mV) and KshA (-346mV). However, DmFdx2 had lower reduction potential (-390mV) and higher reduction ability in dual-pathway. It might be reasonable that the lower the free energy of the final state, the higher would be the electron transfer efficiency. After the PRF captures the two electrons from the FMN, one electron with a lower energy level is directly transferred to the oxygenase KshA, and the other electron with a higher energy level is transferred to reduce DmFdx2 (Figure 5). Compared with the potential difference of PRF (-365mV) and KshA (-346mV), the larger potential difference between PRF (-365mV) and DmFdx2 (-390mV) has more energy and tendency to transfer electrons. Furthermore, the distance between the two [2Fe-2S] clusters of DmFdx2 and oxygenase KshA was only 9.3Å, which seems to transfer electrons more effectively (Figure S5). The analysis of redox

potential and electron bifurcation was consistent with the bioconversion of AD that adding Fdx into the catalytic system might improve conversion rate.

The redox potentials also indicated that PRF (-365 mV) has lower redox potential than KshB (-352 mV). It implied that reductase PRF had stronger redox capacity and higher biotransformation capacity than KshB. In addition, by using free Fdx to titrate the mixed system of reductase and oxygenase, we found that the final titration states showed lower redox potential of PRF+KshA (-94 mV) than KshB+KshA (-80 mV). All these redox potential data explain excellent ability in steroid transformation of PRF+Fdx+KshA reaction system.

3.8 Bioconversion of AD to produce 9OHAD

Whole-cell catalysis systems were constructed in the *E. coli*BL21(DE3) for stable and sustainable production of 9OHAD. We constructed three industrial strains of BLA-PD, BLA-PP and BLA-P. Whole-cell catalytic system without free Fdxs of BLA-P (KshA+PRF-BL21) only showed the yield of 9OHAD with 76.7% in 5 h. However, the whole-cell catalytic system with free Fdxs of BLA-PD (KshA+PRF+DmFdx2-BL21) showed the highest yield of 9OHAD with (95.4%) in 5 h, followed by BLA-PP (KshA+PRF+PsFdx1-BL21) (90.2%). This implied that add free Fdxs, especially plant-type DmFdx2, attributed to increase the electron transfer efficiency in the catalytic system of KshA+PRF. It also implied the importance of the predominant second electron transfer pathway mentioned above.

In comparison, the engineered *Mycobacterium neoaurum* ATCC 25795 reached a 54.5% yield of 9OHAD from 20 g/L phytosterol in 72 h as substrate by modifying key genes related to metabolism to increase cell permeability (Xiong et al., 2020). The *Rhodococcus erythropolis*RG1-UV29 strain reached a 93% yield of 9OHAD from 20 g/L AD in 24 h (Geize et al., 2001). However, the recombinant *E. coli* only reached a 63% yield of 9OHAD from 1 g/L AD in 48 h (Petrusma et al., 2009). Therefore, the constructed whole-cell catalytic system of BLA-PD with free Fdxs exhibited an excellent catalytic ability, and has the potential to be used industrially for efficient 9OHAD production.

In our previous research, Zhu *et al.* constructed *E. coli*BL21(DE3) strain of BLKA-RTM-F, which contained the NADH recycling system and a modified TDO mutant with two Rieske-type [2Fe-2S] clusters at the N-/C-terminus. It exhibited excellent 9OHAD productivity with 5.24 g/L from 5 g/L AD and a yield of 99.3% without by-products.¹⁸ However, BLA-PD (KshA+PRF+DmFdx2-BL21) constructed in the present work showed the highest AD conversion rate of 95.4%, slightly lower than BLKA-RTM-F. It may be due to the construction of NADH recycling system in BLKA-RTM-F, because NADH could be completely consumed in about 16 min. It was thought to be a limiting factor. The future work would focus on the NADH recycling system combined with the current whole-cell catalysts to improve the AD conversion rate and sustainability.

4 CONCLUSIONS

We screened the reductase PRF with high NADPH reduction activity and AD conversion rate. The screened plant-type DmFdx2 could strengthen the biotransformation of AD due to the high electron transfer efficiency. Based on the electron bifurcation theory, we inferred that free Fdx created a new conduit for electrons to travel from reductase to oxygenase. The protein-protein structure docking and redox potential measurement had proven this speculation. The thermodynamic data of ITC also indicated the rationality of the new and efficient electron transfer method using exogenous Fdx as an effective electron carrier. Whole-cell catalytic system with free Fdxs of BLA-PD (KshA+PRF+DmFdx2-BL21) showed a 95.4% yield of 9OHAD indicating the importance of effective electron transfer carrier and a predominant reductase with high activity.

ABBREVIATIONS

9OHAD, 9 α -hydroxy-4-androstene-3,17-dione; AD, 4-androstene-3,17-dione; KshA, Rieske-type oxygenase of 3-ketosteroid-9-hydroxylase; KshB, pant-type reductase of 3-ketosteroid-9-hydroxylase; TDO, toluene 2,3-dioxygenase reductase; PRF, self-sufficient P450 CYP116B46 (V465-L779) from *Tepidiphilus thermophilus*; Fdx, ferredoxin; TeFdx, *Thermosynechococcus elongatus* BP-1 Fdx; DmFdx1, *Drosophila melanogaster* Fdx1;

DmFdx2, *Drosophila melanogaster* Fdx2; PrFdx, *Pseudomonas resinovorans* Fdx; PsFdx1, *Pseudomonas sp.* Fdx; SyFdx, *Sphingobium yanoikuyae* Fdx.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHORS' CONTRIBUTIONS

Zhan Song: Performed the experiments, Data analysis, Writing - original draft. Cancan Wei, Chao Li and Xin Gao: Performed the experiments, Data analysis. Shuhong Mao, Fuping Lu and Hui-Min Qin: Designed the research, Writing - review & editing, Supervision. All authors read and approved the final manuscript.

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Table 1 Kinetic parameters of reductases and ferredoxin toward NADH

Reductase	K_m (μM)	k_{cat} (s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)	k_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)
TDO*	65.37 ± 2.71	65.37 ± 2.71	28.21 ± 0.91	28.21 ± 0.91	0.43
^T DO	/	/	/	/	/
MTDO	63.53 ± 2.95	63.53 ± 2.95	34.27 ± 0.69	34.27 ± 0.69	0.54
KshB*	70.85 ± 4.08	70.85 ± 4.08	23.88 ± 1.06	23.88 ± 1.06	0.34
^K shB	/	/	/	/	/
MKshB	69.94 ± 2.12	69.94 ± 2.12	24.13 ± 0.89	24.13 ± 0.89	0.35
PRF	39.11 ± 1.49	39.11 ± 1.49	66.43 ± 0.34	66.43 ± 0.34	1.70
^P RF	/	/	/	/	/
MPRF	63.12 ± 2.37	63.12 ± 2.37	31.27 ± 1.17	31.27 ± 1.17	0.50

Note: Data are shown as the mean $\pm$ SD from three independent experiments.

* data from Reference *Zhu et al.*

Table 2 Thermodynamic parameters for the interaction with DmFdx2

	n	K_D (M^{-1})	H (KJmol^{-1})	S ($\text{KJmol}^{-1}\text{K}^{-1}$)	$G_{298\text{k}}$ (KJmol^{-1})
PRF	0.75 ± 0.01	$(1.50 \pm 0.10) \times 10^7$	-8 ± 0.82	0.11 ± 0.02	-40.94 ± 1
KshA	0.40 ± 0.02	$(2.10 \pm 0.10) \times 10^7$	-6 ± 0.13	0.12 ± 0.03	-41.81 ± 1

Table 3 Redox potential of reductase and ferredoxin

Enzymes(V1)	Titant(V2)	Volume ratio (V1:V2)	Electric potential (mV)
KshA	$\text{Na}_2\text{S}_2\text{O}_4$	2:1	-346 ± 1

Enzymes(V1)	Titratant(V2)	Volume ratio (V1:V2)	Electric potential (mV)
KshB	$\text{Na}_2\text{S}_2\text{O}_4$	2:1	-352 ± 1
PRF	$\text{Na}_2\text{S}_2\text{O}_4$	2:1	-365 ± 2
DmFdx2	$\text{Na}_2\text{S}_2\text{O}_4$	2:1	-390 ± 1
KshB+KshA	DmFdx2	1:3	-80 ± 3
PRF+KshA	DmFdx2	1:3	-94 ± 1

FIGURE LEGENDS

Figure 1. Conversion rate of AD to 9OHAD. AD conversion rate of various reductases of KshB, PRF, TDO or mutants MKshB, MPRF, MTDO in KshA+reductase catalytic reaction system.

Figure 2. Structural comparison of reductase domain of TDO and PRF. The cartoon and surface representation of the reductase domain of TDO (a) and PRF (b). NADH binding domain is shown in shallow and FAD/FMN binding domain is shown in pink, FAD and FMN are represented by sticks.

Figure 3. UV-vis absorption spectrum of Fdxs and reductase. The UV-vis absorption spectra of the oxidation state and sodium dithionite-reduced are black and red respectively, and shown as (a) reductase PRF, (c)DmFdx2 (plant-type) and (d)PsFdx1 (Reiske-type). (b)Comparison of the UV-vis absorption spectra of wild-type TDO (blue) and mutant MTDO (green).

Figure 4. The affinity measurements between DmFdx2 and PRF/KshA by ITC. The affinity of reductase PRF (a) and oxygenase KshA (b) (0.2 mM DmFdx2 vs 0.02 mM PRF/KshA) to DmFdx2 was determined by ITC 200 in 20 mM HEPES buffer (pH 7.4) at 25 °C. The raw data of the calorimetric titration and the curve fit of the observed heat are displayed in the upper and lower panels, respectively.

Figure 5. Analysis of the proposed electron transfer pathway between oxidoreductase and free Fdx. The overall structures of reductase PRF, free ferredoxin DmFdx2 and oxygenase KshA are shown as surface representation. NADH and FMN are shown as sticks, iron atoms and [2Fe-2S] cluster groups are shown as spheres.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.