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Further biochemical studies on aminopyrrolnitrin oxygenase (PrnD)

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ABSTRACT

Active site modeling of dimerization interface in combination with site-directed mutagenesis indicates that the electron in the PrnD Rieske oxygenase can be transferred by either of two pathways, one involving Asp183' and the other involving Asn180'. In addition, the overexpression of the *isc* operon involved in the assembly of iron–sulfur clusters increased the catalytic activity of PrnD in *Escherichia coli* by a factor of at least 4.

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Rieske oxygenases are widespread in nature and catalyze a diverse set of oxidation reactions including *cis*-dihydroxylation, mono-hydroxylation, desaturation, sulfoxidation, and O- and N-dealkylation.^{1,2} Previously, we reported for the first time the characterization of a Rieske *N*-oxygenase, aminopyrrolnitrin oxygenase (PrnD) that catalyzes an unusual arylamine oxidation.^{3,4} PrnD was functionally expressed in *Escherichia coli* and the activity of the purified PrnD was reconstituted, which required *in vitro* assembly of the Rieske iron–sulfur cluster into the protein. PrnD is involved in the biosynthesis of the antibiotic pyrrolnitrin that is produced by many Pseudomonads and that has broad-spectrum antifungal activity.⁵ In the proposed biosynthetic pathway of pyrrolnitrin,⁶ PrnD catalyzes the oxidation of the amino group of aminopyrrolnitrin to a nitro group, forming pyrrolnitrin. We reported direct evidence for the involvement of hydroxylamine and nitroso intermediates in the PrnD-catalyzed arylamine oxygenation reaction, substantiating the catalytic mechanism for the conversion of arylamine to arylnitro compounds for the first time.^{3,4} This may be the primary mechanism by which arylamines are oxygenated to give arylnitro compounds in biological processes. We also probed the molecular determinants of the substrate specificity of

PrnD using molecular modeling and mutational analysis.⁷ Of note, although arylamine oxidases seem to be widely distributed and used in a variety of metabolic reactions,⁸ PrnD represents one of only two known examples of arylamine *N*-oxygenases involved in nitro group formation, the other being the AurF involved in aureothin biosynthesis.⁹

It has been suggested that IscS, IscU, and IscA are likely to have housekeeping functions related to the assembly or repair of iron–sulfur cluster-containing proteins.^{10,11} The location and apparent coexpression of *iscSUA* and *hscBA* genes also indicate the possible presence of a macromolecular system that functions in the proper folding of iron–sulfur cluster-containing proteins.¹¹ The PrnD protein has been shown to include an iron–sulfur cluster crucial for electron transfer.³ However, no experimental evidence is yet available regarding the relationship between the availability of iron–sulfur clusters and the oxygenation of arylamine. Electron transfer in PrnD has also never been investigated.

In this study, we investigate the electron transfer mechanism of PrnD by homology modeling and site directed mutagenesis. In addition, we show that arylamine oxygenation can be enhanced by coexpression of genes from the *isc* operon that are required for the assembly of iron–sulfur clusters.

The EPR and optical spectra of the recombinant PrnD protein suggested the presence of an iron–sulfur cluster, which has been proposed to be crucial for catalytic activity.^{3,4} Intermolecular transfers of electrons from the Rieske cluster of one monomer to the active site mononuclear iron in the neighboring monomer are

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required for proposed catalysis of PrnD. However, electron transfer in PrnD has never been thoroughly investigated.

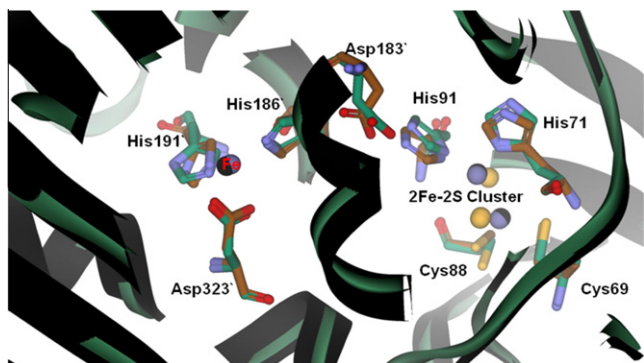


Figure 1. The superposed view of the binary complex of PrnD structure. Amino acid residues of Rieske domain (Cys69, Cys88, His71 and His91) and catalytic domain (Asp183', His186', His191' and Asp323') of PrnD (green color) superimposed on the crystal structure of KshAB (2ZYL) (orange color) have similar orientations. Residues from adjacent subunit molecule are indicated with prime. PrnD, iron and sulfur ions are shown in black and yellow sphere, respectively.

In an attempt to gain an insight into the [2Fe–2S] cluster in PrnD, a dimeric model of PrnD was constructed using the crystal structure of KshAB (3-ketosteroid 9 α -hydroxylase) of *Mycobacterium tuberculosis*, a Rieske oxygenase (PDB ID 2ZYL)¹² as a template. The amino acid sequence of the KshAB α -subunit shares 24% identity with PrnD. Following alignment, the backbone coordinates of the residues in the PrnD were generated with the automated MODELER 9v7 module¹³ within Discovery Studio 2.5 (Accelrys Software Inc., CA, USA) using template-derived restraints in combination with the CHARMM force field to obtain a full objective function. The coordinates for biologically active non-covalent dimers were generated from the appropriate Protein Data Bank files using crystallographic symmetry operators.¹⁴ Of 20 structural models created, the best model (Fig. 1) was selected and validated on the basis of visual inspection for obvious flaws, the score from the Profiles 3-D function, and the ProStat inspection of ϕ and ψ angles (Supplementary data).¹⁵ Optimization of the target function with conjugate gradient and simulated annealing via molecular dynamics led to a model satisfying all the spatial restraints.

PrnD is a two-domain protein that contains a Rieske domain consisting of 12 β strands that bind the 2Fe–2S cluster and a catalytic domain composed of a seven-stranded antiparallel β -sheet flanked by six α -helices and extended loops. Although the sequence identity was only 24%, upon structural overlap, 97% of

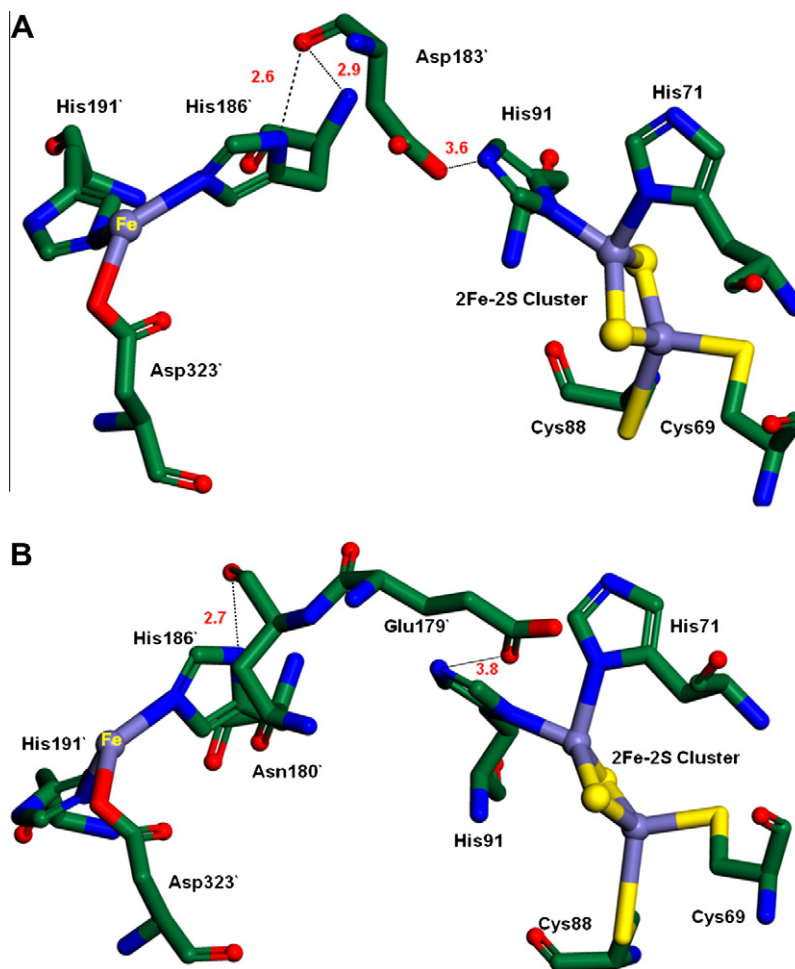


Figure 2. Electron transfer pathways from the Rieske cluster to the nonheme iron. Interaction of the Rieske cluster and the mononuclear Fe center on the neighboring subunits in PrnD. Multiple possible electron transfer pathways are illustrated as black dotted lines, with residues from other monomer at interface indicated with a prime. (A) Asp183' is located between the Rieske center and the catalytic site and is a prime candidate for electron shuttling between the two iron centers. (B) Another possible electron transfer pathway, whereby an electron is transported via potential H-bonds interactions between His91, Glu179', Asn180', and His186'. All amino acid residues and atoms are colored with element colors except carbon shown in green. 2Fe–2S clusters are represented as ball and stick models. Hydrogen atoms were removed to provide clear image. These interactions were predicted using Material Studio 5.51 (Accelrys Software Inc., CA, USA).

the overlapped residues showed the main chain RMSD below 1 Å. Moreover, the RMSD between two structures was 0.9 Å on all C α atoms, but only 0.2 Å on the dimeric interface. In addition, the conserved active site residues at the interface in the PrnD model have similar orientations and locations in the template enzyme, 2ZYL (Fig. 1).

Catalysis in PrnD requires an inter-molecular transfer of electrons from the Rieske cluster of one monomer to the active site mononuclear iron in the neighboring monomer. The potential similarity in the catalytic dimeric interface of the α -subunits of the KshAB and PrnD suggests that there is an inter-subunit transfer of electrons from the Rieske iron–sulfur cluster of one subunit of PrnD to the free iron atom at the catalytic site of a closely adjacent other subunit (Fig. 2). The Rieske cluster and mononuclear iron center of adjacent subunits of PrnD interact via a network of hydrogen bonds over the subunit interface. This includes a conserved Asp183', whose carboxylate bridges the metalcenters by forming hydrogen bonds with the N ϵ 2 atom of His91 (3.6 Å) and the N δ 1 atom of His186' (2.6 Å), one of the iron ligands in the mononuclear center. The N1 atom of His186' is also involved in another H-bond (2.9 Å) with O1 atom of Asp183' (Fig. 2A). This bridging aspartate has been proposed to compose a protonation-dependent electron transfer pathway in several Rieske oxygenases.¹⁶ The interface region of the PrnD also contains another potential H-bond network that could be involved in inter-subunit electron transfer (Fig. 2B). In the alternative H-bond network at the subunit interface, the H-bond network reaches from N ϵ 2 atom of His91 (3.8 Å) to O ϵ 1 atom of Glu179' and O1 atom of Asn180' to N δ 1 atom of His186' (2.7 Å), the iron ligand in the mononuclear center. The residues His91, Glu179', Asn180', and His186' compose a path that may bypass Asp183'. Its elimination by mutation is expected to result in a moderately impaired enzyme in the catalytic activity. Indeed, when Asn180' was replaced by alanine, the resulting N180A pTKXb-PrnD mutant exhibited ~70% activity of the wild type. The homology model, in combination with the site-directed mutagenesis result, suggests that the electron in the PrnD Rieske oxygenase can be transferred by two pathways.

In an attempt to improve the in vivo activity of PrnD, a Rieske 2Fe–2S protein, we decided to construct a strain harboring a plasmid that directed the expression of the *isc* operon genes, which are known to be involved in the assembly of iron–sulfur clusters.¹¹ A segment of the *E. coli* *isc* operon (Fig. 3) comprising the *iscS*, *iscU*, *iscA*, *hscB*, *hscA*, and *fdx* genes¹⁷ was amplified by PCR and was placed in-frame into the chloramphenicol resistance cassette of the low copy plasmid pACYC, which is compatible with plasmids of the Col-E1 group due to its P15A replicon (Fig. 3).

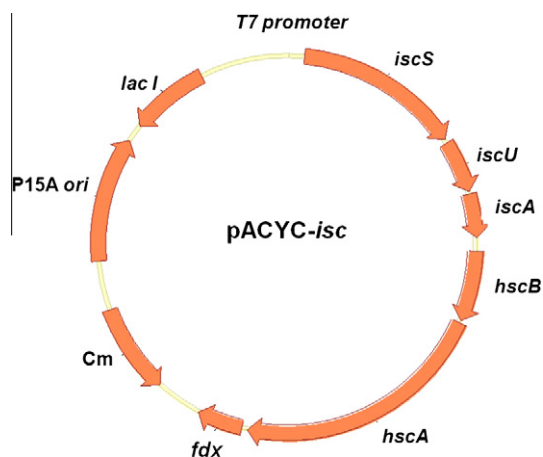


Figure 3. Physical map of expression vector pACYC-isc. Cm, selection marker; P15A ori, origin of replication.

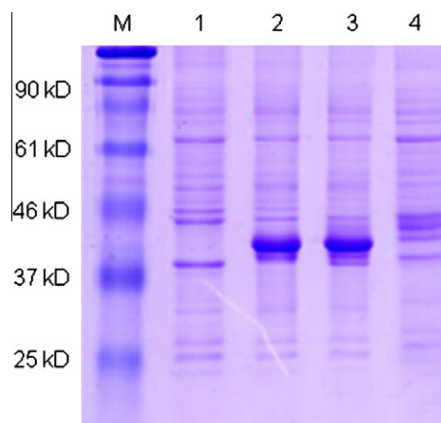


Figure 4. SDS-PAGE of the isolated *E. coli* cell harboring pTKXb-prnD and pACYC-isc. Protein samples (20 μ g) were analyzed on 12% SDS-PAGE gels under denaturing conditions, and stained with Coomassie Brilliant Blue. Lane M—molecular weight markers; lane 1—soluble fraction of induced *E. coli* BL21(DE3)(pTKXb-prnD) cells; lane 2—total fraction of induced *E. coli* BL21(DE3)(pTKXb-prnD) cells; lane 3—total fraction of induced *E. coli* BL21(DE3)(pTKXb-prnD + pACYC-isc) cells; lane 4—soluble fraction of induced *E. coli* BL21(DE3)(pTKXb-prnD + pACYC-isc) cells.

This plasmid construct was transformed into the recombinant *E. coli* strain engineered for the expression of the PrnD protein. Polyacrylamide gel electrophoresis, in conjunction with mass spectrometric protein spot assignment, confirmed that the genes *iscS* and *hscA* were expressed when compared with an *E. coli* strain harboring the vacant pACYC vector without the insert (Fig. 4). Whole cells of the *E. coli* strain expressing the *prnD* gene and the *isc* cluster genes catalyzed the conversion of *p*-aminobenzyl amine into *p*-nitrobenzyl amine at a rate of 42 nmol min^{−1} mg-cell^{−1} under standard conditions. By comparison, whole cells of the *E. coli* strain carrying the plasmid for expression of the recombinant *prnD* gene but not that for the *isc* operon expression had a catalytic activity of only 9.6 nmol min^{−1} mg-cell^{−1}. Thus, the apparent catalytic activity was enhanced by a factor of at least 4.

The genome of *E. coli* is believed to specify approximately 50 iron–sulfur proteins, which are involved in redox reactions as well as in various nonredox processes.¹⁸ To become functionally competent, the iron–sulfur apo-proteins require the incorporation of iron–sulfur clusters, which must be assembled by complex biochemical machinery involving several proteins. *E. coli* uses two different production pipelines, the *isc* and the *suf* operons, for this purpose.¹⁹ The massive overexpression of recombinant iron–sulfur proteins can overwhelm the synthetic capacity of iron–sulfur assembly pipeline.^{20,21} In such cases, the deficit can be compensated by the overexpression of the relevant iron sulfur assembly *isc* proteins.

In conclusion, the electron in the PrnD Rieske [2Fe–2S] oxygenase can be transferred by either of two pathways, one involving Asp183' and the other involving Asn180'. The in vivo activity of the PrnD protein was significantly increased by the coexpression of the *isc* cluster. The catalytic activity of a PrnD expression strain of *E. coli* without expression of *isc* genes was only about 23% of the activity in an *E. coli* strain that also expressed the *isc* genes. This is well in line with the hypothesis that the availability of iron–sulfur clusters is the limiting factor for the formation of fully functional PrnD protein in PrnD-expressing *E. coli* strains.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bmcl.2011.03.087](https://doi.org/10.1016/j.bmcl.2011.03.087).

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