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Expression, purification, crystallization and preliminary X-ray diffraction analysis of the soluble domain of PPA0092, a putative nitrite reductase from *Propionibacterium acnes*

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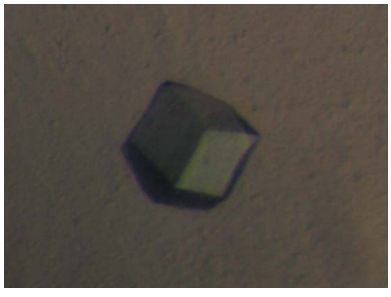
The soluble domain (residues 483–913) of PPA0092, a putative copper-containing nitrite reductase from *Propionibacterium acnes* KPA171202, has been overexpressed in *Escherichia coli*. The purified recombinant protein was crystallized using the hanging-drop vapour-diffusion method. X-ray diffraction data were collected and processed to a maximum resolution of 2.4 Å. The crystal belonged to space group $P2_13$, with unit-cell parameters $a = b = c = 108.63$ Å. Preliminary diffraction data show that one molecule is present in the asymmetric unit; this corresponds to a V_M of 2.1 Å^3 Da^{-1}.

1. Introduction

Propionibacterium acnes is a major inhabitant of adult human skin, where it resides within sebaceous follicles, usually as a harmless commensal, although it has been implicated in acne vulgaris formation. Details of the involvement of *P. acnes* in acne, the most common skin disease, which affects up to 80% of all adolescents in the United States, are still obscure. Several mechanisms have been proposed to account for its role in the disease (Holland *et al.*, 1998; Ingham, 1999; Farrar *et al.*, 2000; Jappe *et al.*, 2002; Koreck *et al.*, 2003). Firstly, damage to host tissues and cells might be accomplished by bacterial enzymes with degradative properties, such as lipases. Secondly, immunogenic factors of *P. acnes* such as surface determinants or heat-shock proteins (HSPs) might trigger inflammation. Other diseases are also associated with *P. acnes*, including corneal ulcers, endocarditis, sarcoidosis, cholesterol gallstones, allergic alveolitis, pulmonary angitis and synovitis, acne, pustulosis, hyperostosis and osteitis (SAPHO) syndrome (Yamada *et al.*, 2002). It has recently been reported that the entire genome sequence of this bacterium encodes 2333 putative genes (Brüggemann *et al.*, 2004). It is expected that these genomic data will provide a basis for finding alternative targets for the therapy of acne and other diseases associated with *P. acnes*.

According to domain-organization analysis with the multi-copper protein (MCP) motif, an open reading frame (ORF) coding for an MCP has been found in the *P. acnes* genome (Nakamura & Go, 2005). The ORF has been annotated as a hypothetical Cu-oxidase domain-containing protein PPA0092. The deduced amino-acid sequence (a total of 913 amino-acid residues) exhibits significant similarities to those of copper-containing nitrite reductases (CuNIRs; 27% identity with *Hyphomicrobium denitrificans* CuNIR, 25% identity with *Neisseria gonorrhoeae* CuNIR and 22% identity with *Achromobacter xylosoxidans* CuNIR; Fig. 1). The PPA0092 protein is composed of at least three domains: a transmembrane region (residues 1–412) consisting of ten α-helices, a cupredoxin-like domain (residues 483–610) and a typical CuNIR-homologue region (residues 647–913). In general, a typical CuNIR is an MCP containing type 1 and type 2 Cu sites and catalyzes the reduction of NO_2^- to NO in the denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) of the bacterial nitrogen cycle (Averill, 1996; Zumft, 1997; Suzuki *et al.*, 1999). One-electron reduction of NO_2^- to NO is a key step in the denitrification process as this is the first step that leads to the gaseous products NO, N_2O or N_2. Moreover, other enzymes involved in denitrification have also been found in the *P. acnes* genome: nitrate reductase (PPA507-511) and nitric oxide reductase subunit B (PPA1975). These findings strongly



suggest that this bacterium possesses systems that are involved in anaerobic respiration such as denitrification.

Recently, the structure of CuNIR from the methylotrophic denitrifying bacterium *H. denitrificans* (HdNIR) has been reported, establishing the existence of a new family of CuNIRs in which an additional cupredoxin domain is located at the N-terminus (Nojiri *et al.*, 2007). The overall structure of HdNIR reveals a trigonal prism-shaped molecule in which a monomer consisting of 447 residues and three Cu atoms is organized into a unique hexamer (*i.e.* a tightly associated dimer of trimers). Three interfaces are formed between the two trimeric C-terminal CuNIR regions in HdNIR by an interaction between the additional cupredoxin domains. Interestingly, analysis of the PPA0092 amino-acid sequence also indicates the presence of an additional cupredoxin-like domain in the region sandwiched between the putative N-terminal transmembrane region and the C-terminal CuNIR-homologue region (Fig. 1). The type 1 Cu-binding motif C-X_n-H-X_m-M (Dennison *et al.*, 1994) in the additional cupredoxin-like domain is easily assigned to almost the same position as that in HdNIR (Fig. 1). Therefore, it is suggested that the protein PPA0092 has a molecular architecture similar to the hexameric structure of the HdNIR molecule. The primary sequence homology between PPA0092 and HdNIR implies a possible relation of their functions, which needs to be proved by structure-based functional interpretation.

Here, we describe the cloning, expression, purification, crystallization and preliminary X-ray analysis of the soluble domain (residues 483–913) of PPA0092, which includes the additional cupredoxin-like domain and the CuNIR-homologue region.

2. Materials and methods

2.1. Cloning, expression and purification

The gene encoding the soluble domain (PaNIRsol) from residues 483 to 913, including the additional cupredoxin domain and the CuNIR-homologue region of the protein PPA0092, was amplified by PCR using the genomic DNA of *P. acnes* KPA171202 (No. DSM 16379) as a template. Primers for amplification were designed based on the genomic sequence data (gene ID NC_006085). *Stu*I and *Hin*dIII restriction-enzyme sites (bold) were incorporated into the sequences of the forward and reverse primers 5′-TT**AGGCCT**-GCGGTCGGTCACCATCTCGACCA-3′ and 5′-GG**AAGCTT**AT-GTCGTCACCTCAATGAATCCTTTAG-3′, respectively. The product DNA fragment that was cloned into an expression vector contained an N-terminal hexahistidine (His_6) fusion tag (pQE30Xa, Qiagen). After the resulting plasmid DNA had been digested with *Eco*RI and *Hin*dIII, the DNA fragment including the gene encoding His_6-PaNIRsol was inserted into the T7 expression vector pET23c

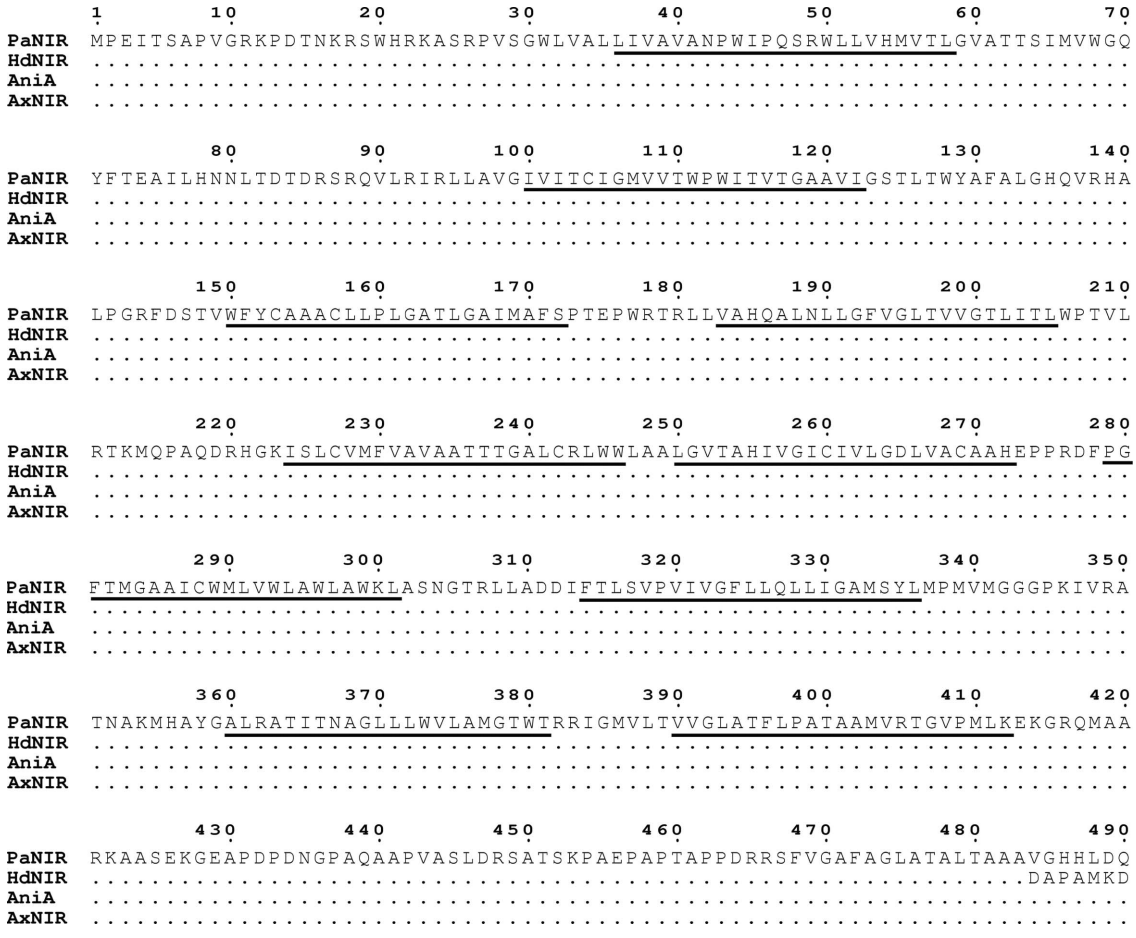


Figure 1
Sequence comparison between PPA0092 and selected CuNIRs. Abbreviations and accession numbers are as follows: PaNIR, PPA0092 from *P. acnes* strain KPA171202 (gi:50841496); HdNIR, CuNIR from *H. denitrificans* strain A3151 (gi:21623659); AniA, CuNIR from *N. gonorrhoeae* strain FA1090 (gi:59717368); AxNIR, CuNIR from *A. xylosoxidans* subsp. *xylosoxidans* strain GIFU1051 (gi:3721763). *ClustalW* was used to perform sequence alignment (Thompson *et al.*, 1994) and the figure was generated using the program *ESPript* (Gouet *et al.*, 1999). Diamonds, type 1 Cu ligands; squares, type 2 Cu ligands. The deduced transmembrane α-helix regions are underlined.

(Novagen) by digestion with *Xba*I and *Hin*dIII. The presence of the inserted gene was confirmed by DNA-sequence analysis.

His_6-PaNIRsol was expressed in *Escherichia coli* JM109 (DE3) cells (Promega). Incubation of the transformed cells at 303 K was continued until the $OD_{600\,nm}$ reached 0.5. Subsequently, the temperature was shifted to 295 K. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (Nacalai Tesque) to a final concentration of 0.1 m*M*. After overnight incubation, the cells were harvested by centrifugation for 10 min at 6800*g*. Bacterial pellets were resuspended in cold lysis buffer (40 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 10 m*M* imidazole) containing 1 m*M* phenylmethanesulfonyl fluoride. The suspension was sonicated and debris was removed by centrifugation at 23 500*g* for 30 min at 277 K. The supernatant was loaded onto an Ni–NTA agarose column (Qiagen) pre-equilibrated with the same buffer. After washing the column with 500 ml wash buffer (40 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 20 m*M* imidazole), the His_6-PaNIRsol was eluted with 300 ml elution buffer (40 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 250 m*M* imidazole). The fractions were desalted by dialysis with 40 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl for 12 h at 277 K and then dialyzed again with the same buffer containing 1 m*M* $CuSO_4$ for 12 h at 277 K to reconstitute the Cu-binding sites. Ammonium sulfate was added to the sample to a final concentration of 20% saturation. After the removal of precipitate by centrifugation, the supernatant was applied onto a Phenyl-Sepharose column (GE Healthcare) pre-equilibrated with 40 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl and 20% saturated ammonium sulfate. The His_6-PaNIRsol protein was eluted with 40 m*M* Tris–HCl pH 8.0 containing 200 m*M* NaCl. The sample fractions were collected and dialyzed with acetate buffer (10 m*M* sodium acetate pH 5.5). After dialysis, the sample was applied onto a HPLC Resource S column (GE Healthcare). The protein was eluted with a linear gradient of 10 to 400 m*M* sodium acetate buffer pH 5.5 containing 1 *M* NaCl. The fractions were collected and dialyzed with 50 m*M* sodium acetate pH 5.5. The purified His_6-PaNIRsol protein solution was concentrated with a Centriprep-YM30 (Millipore) and stored at 193 K. The results of each purification step were monitored by 12.5% SDS–PAGE and the purity of the purified His_6-PaNIRsol was estimated to be >95%.

2.2. Crystallization

Crystallization trials for the His_6-PaNIRsol protein were performed using the hanging-drop vapour-diffusion method at 277 K and the crystallization conditions were screened using Crystal Screens I and II and PEG/Ion Screen from Hampton Research. Each drop was composed of 1 µl His_6-PaNIRsol protein solution (20 mg ml^{-1}) and 1 µl reservoir solution and was equilibrated against 300 µl reservoir solution.

2.3. Data collection

Crystals for synchrotron data collection were first soaked in cryoprotectant (20% 2-methyl-2,4-pentanediol in crystallization solution) by overnight dialysis using dialysis buttons (Hampton Research) and then flash-cooled by immersion in liquid nitrogen. The

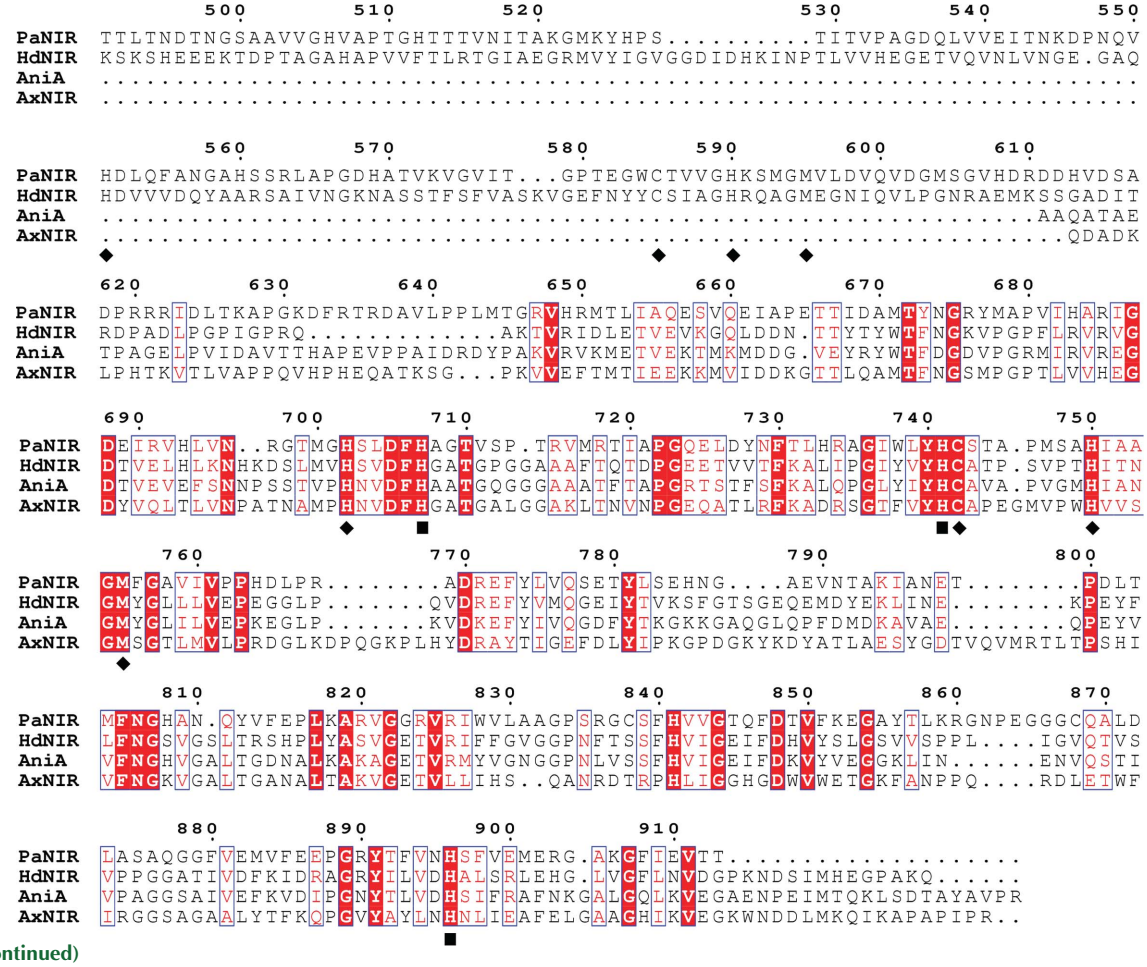


Figure 1 (continued)

data set was collected using a Bruker DIP-6040 imaging-plate detector on beamline 44XU at SPring-8 (Japan Synchrotron Radiation Research Institute), revealing a diffraction pattern to 2.4 Å resolution (Fig. 2). The crystal was maintained at 100 K during data collection. The crystal-to-detector distance was set to 400 mm. A total of 40 images were collected with 1° oscillations and an exposure time of 1 s per image. The *HKL*-2000 program suite (Otwinowski & Minor, 1997) was used to reduce, integrate and scale the collected data. Crystallographic statistics of the data are summarized in Table 1.

3. Results and discussion

Preliminary needle-shaped crystals were initially obtained in several conditions within two weeks, *e.g.* Crystal Screen I condition No. 13 [0.2 *M* trisodium citrate, 0.1 *M* Tris–HCl pH 8.5, 30%(*v*/*v*) PEG 400] and Crystal Screen II condition No. 22 [0.1 *M* MES pH 6.5, 12%(*w*/*v*) PEG 20 000]. Several rounds of optimization of the initial crystallization condition were set up to give optimally sized crystals. X-ray diffraction-quality crystals were grown in hanging drops composed of 1 µl protein solution (20 mg ml^{-1}) and 1 µl reservoir solution containing 0.1 *M* MES–NaOH pH 6.5, 13%(*w*/*v*) PEG 20 000 and 75 m*M* glycine. The drops were equilibrated against 300 µl reservoir solution. Crystals suitable for X-ray data collection appeared within two weeks. The average dimensions of the His$_6$-PaNIRsol crystals were 75 × 75 × 75 µm (Fig. 3).

Table 1
Crystal parameters and data-collection statistics for His$_6$-PaNIRsol.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.9
Resolution (Å)	19.8–2.40 (2.49–2.40)
Space group	$P2_13$
Unit-cell parameters (Å)	$a = b = c = 108.63$
$R_{\rm merge}$†	0.079 (0.600)
$\langle I/\sigma(I)\rangle$	23.5 (3.1)
No. of observations	246038
No. of unique reflections	16947 (1662)
Completeness (%)	100 (100)
Redundancy	5.0 (4.9)
Mosaicity (°)	0.47

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all i observations of reflection hkl.

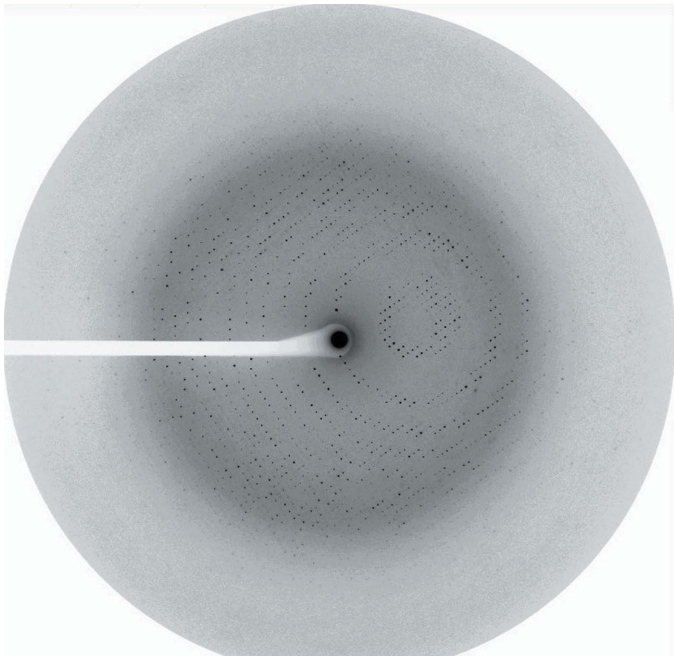


Figure 2
Snapshot of the diffraction pattern of the His$_6$-PaNIRsol crystals. The pattern displays a maximum resolution of 2.4 Å and the crystals belonged to space group $P2_13$.

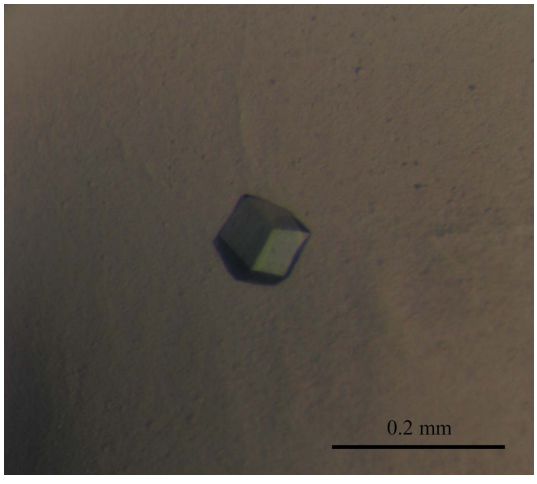


Figure 3
Crystal of His$_6$-PaNIRsol. The crystals have approximate dimensions of 75 × 75 × 75 µm.

The crystal belonged to the primitive cubic space group $P2_13$, with unit-cell parameters $a = b = c = 108.63$ Å. Based upon the expected molecular weight of His$_6$-PaNIRsol (49 534 Da), the Matthews coefficient $V_{\rm M}$ (Matthews, 1968) is calculated to be 2.1 Å^3 Da^{-1}, with an estimated solvent content of 42.0%. These results suggest the presence of one molecule of His$_6$-PaNIRsol per asymmetric unit. It has been found that CuNIRs exist as a trimer when they are biologically active (PDB codes: 1oe1, Ellis *et al.*, 2003; 2bw4, Antonyuk *et al.*, 2005; 1kbw, Boulanger & Murphy, 2002; 1mzy, unpublished work; 1as7, Murphy *et al.*, 1997 and 1bq5, Inoue *et al.*, 1998). The program *MOLREP* (Murshudov *et al.*, 1997) as implemented in the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) confirmed that the His$_6$-PaNIRsol molecule is indeed a trimer composed of three monomeric subunits related by a crystallographic threefold axis. *MOLREP* was used to determine the structure solution of His$_6$-PaNIRsol by molecular replacement using the truncated coordinates of the C-terminal region (residues 163–445) of *H. denitrificans* CuNIR (PDB code 2dv6) as a search model. These findings strongly support the designation of the PPA0092 protein as a CuNIR. The structure-based functional interpretation will provide us with new information about this unique type of CuNIR protein with an additional cupredoxin domain at the N-terminus. Further crystallographic refinement and molecular-structure analysis are in progress.

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