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Crystallization and preliminary crystallographic data of the PAS domain of the NifL protein from *Azotobacter vinelandii*

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The *Azotobacter vinelandii* NifL protein is a redox-sensing flavoprotein which inhibits the activity of the nitrogen-specific transcriptional activator NifA. The N-terminal PAS domain has been overexpressed in *Escherichia coli* and crystallized by the hanging-drop vapour-diffusion method. The crystal belongs to the rhombohedral space group $R32$, with unit-cell parameters $a = b = 65.0$, $c = 157.3$ Å, and has one molecule in the asymmetric unit. Native data were collected to 3.0 Å on the BW7B synchrotron beamline at the EMBL Hamburg Outstation.

1. Introduction

PAS domains[1] are ubiquitous motifs present in bacteria, archaea and eukarya (Zhulin *et al.*, 1997). Unlike most other sensory domains, PAS domains are located in the cytosol (Taylor & Zhulin, 1999) and include histidine (Alex & Simon, 1994) and serine/threonine kinases (Ponting & Aravind, 1997), chemoreceptors and photoreceptors for taxis and tropism (Sprenger *et al.*, 1993), circadian clock proteins (Kay, 1997; Schibler, 1998) and voltage-activated ion channels (Warmke & Ganetzky, 1994), cyclic nucleotide phosphodiesterases (Soderling *et al.*, 1998), as well as regulators of responses to hypoxia (Jiang *et al.*, 1996) and embryological development of the central nervous system (Nambu *et al.*, 1991). The specificity of a PAS domain for detecting input signals is partly determined by the cofactor associated with the PAS domain. Four PAS-domain protein structures are known; the bacterial blue-light photoreceptor PYP (Borgstahl *et al.*, 1995; Genick *et al.*, 1997), the haem-binding domain of the rhizobial oxygen sensor FixL (Gong *et al.*, 1998; Miyatake *et al.*, 2000), the N-terminal domain of the human ether-a-go-go-related gene potassium channel HERG (Cabral *et al.*, 1998) and the FMN containing phototropin module of *Adiantum* PHY3 (Crosson & Moffat, 2001). To date, no crystal structures of PAS domains are known which contain FAD as cofactor. The *A. vinelandii* NifL protein is an FAD-containing redox-sensing protein which inhibits the activity of the nitrogen-specific transcriptional activator NifA in response to molecular oxygen and fixed nitrogen (Dixon, 1998). The N-terminal PAS domain serves as the flavin redox-sensing domain (Söderbäck *et al.*, 1998). In order to understand the different mechanisms by which PAS domains work, detailed information about their cofactor-binding regions is required.

[1] PAS domains are found in sensor proteins and are named after homology between the *Drosophila* period protein (**P**ER), the aryl hydrocarbon receptor nuclear translocator protein (**A**RNT) and the *Drosophila* single-minded protein (**S**IM). For proteins containing this domain, see http://smart.embl-heidelberg.de/browse.shtml.

2. Protein production and crystallization

The amino-terminal PAS domain of *A. vinelandii* NifL, containing an N-terminal histidine tag with an adjacent thrombin cleavage site, was overexpressed in the *E. coli* strain BL21 (DE3) pLysS and purified as described previously (Hefti *et al.*, 2001). For the production of selenomethionyl-labelled protein, *E. coli* strain BL21 (DE3) pLysS was first grown on LB plates supplemented with 50 µg ml^{-1} kanamycin (Sigma). Single colonies were transferred to 5 ml prewarmed LB medium (310 K) containing 50 µg ml^{-1} kanamycin. After overnight growth, 500 µl of medium was transferred to a 2 l flask containing 500 ml prewarmed non-sterile M9 medium (Sambrook *et al.*, 1989). This M9 minimal medium was supplemented with 50 mg l^{-1} of all 20 amino acids (in which methionine was replaced with selenomethionine), 100 mg l^{-1} kanamycin and 0.6%(*w*/*v*) glucose. Cells were grown with vigorous shaking (300 rev min^{-1}) for 15 h at 310 K, before protein expression was induced with 1 m*M* IPTG. After 6 h, the cells were harvested and the protein purified as described previously (Hefti *et al.*, 2001), resulting in 9 mg pure protein from a 4 l culture. Prior to crystallization, the freeze-dried protein was dissolved in double-distilled deionized water and the protein concentration was determined using Bradford's assay. Initial crystallization trials were carried out using the hanging-drop vapour-diffusion method on Linbro plates. A

hanging drop (2 µl) prepared by mixing a 1:l ratio of the protein solution and the reservoir solution was placed on a siliconized coverslip over 0.6 ml of the reservoir solution. Initial screening for crystallization conditions was performed with the sparse-matrix screens (Jancarik & Kim, 1991) Crystal Screen and Crystal Screen II (Hampton Research) and with Wizard I and II (Emerald Biostructures) using protein concentrations of 6–8 mg ml^{-1} at 293 K. The protein was in 50 m*M* Tris buffer pH 7.0 and 100 m*M* NaCl. Initial crystal growth was observed with a bufferless Crystal Screen II condition, namely with 2.0 *M* ammonium sulfate and 5%(*v*/*v*) 2-propanol as precipitants. Because crystals were observed in conjunction with amorphous precipitate, subsequent crystallization experiments were carried out to try to optimize the conditions, including pH, salt and precipitant concentration as well as protein concentration. Protein crystals always formed after initial light precipitate (Fig. 1), appearing after 3–4 d, with no further change after 7 d. Crystal formation seems to be insensitive to pH, with growth occurring over the pH range 5.5–7.0. The best conditions were found to be 1.8 *M* ammonium sulfate and 4.5% 2-propanol. Small crystals of the selenomethionine-containing protein formed after 12 d under the same conditions, except for the addition of 10 m*M* β-mercaptoethanol to the well solution, but dissolved again after a further 5 d. No new crystal growth was subsequently observed.

3. X-ray analysis

Diffraction data to 3 Å resolution were collected at 100 K with synchrotron radiation (λ = 0.8424 Å) at the BW7B beamline at the EMBL Hamburg Outstation using a MAR345 image-plate detector. A total of 104 images were recorded with an oscillation angle of 0.5° and an exposure time of 4 min per image. The crystal-to-detector distance was set to 300 mm. Prior to data collection, a crystal was equilibrated for a few seconds in a cryoprotectant solution prepared by mixing a 1:1 ratio of well solution and 50%(*v*/*v*) glycerol, then directly mounted in a CryoLoop (Hampton Research) and flash-cooled in the cold nitrogen stream. The intensity data were processed and scaled with the *HKL* package (Otwinowski & Minor, 1997). The crystals are rhombohedral, with unit-cell parameters a = 65.0, c = 157.3 Å. With one molecule in the asymmetric unit, the Matthews coefficient is 2.01 Å Da^{-1} (Matthews, 1968). Data-collection details are given in Table 1 and a typical crystal is shown in Fig. 1. Crystal structure determination by molecular replacement is still in progress.

Table 1
Data-collection statistics for NifL PAS domain crystal.

Values in parentheses are for the highest resolution shell.

X-ray source	EMBL Hamburg, BW7B
Wavelength (Å)	0.8424
Temperature (K)	100
Resolution (Å)	27.4–3.0 (3.11–3.0)
Space group	*R*32
Unit-cell parameters (Å)	a = 65.0, c = 157.3
Total observations	7285
Independent reflections	2654
Completeness (%)	97.0 (97.4)
$I/\sigma(I)$	18.2 (2.7)
R_{sym} (%)	5.5 (40.3)
Mosaicity (°)	0.8

4. Mass-spectrometry studies

Prior to crystallization, the mass of the purified PAS-domain protein was checked with MALDI-TOF to verify the correctness of the thrombin cleavage. The determined mass (15 898 Da) was in excellent agreement with the expected theoretical mass (15 897.5 Da). Protein crystals were washed in well solution and dissolved in the MALDI-TOF matrix solution. Mass spectra were recorded with a low signal-to-noise ratio. The estimated mass was approximately 16000 ± 150 Da, indicating that the initial crystals contained thrombin-cleaved protein. Selenomethionyl-labelled protein contains two Se atoms (15 991 Da), as expected (theoretical mass 15 991.3 Da) from the number of methionines in the protein sequence. The selenium incorporation was over 95% efficient as estimated from the peak heights.

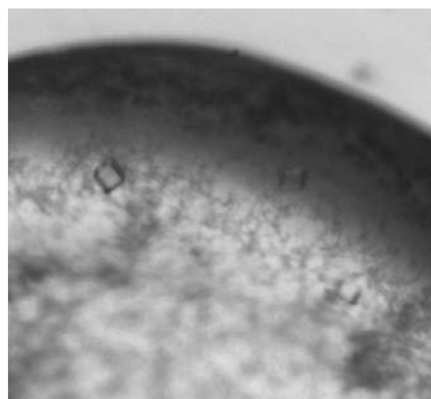

Figure 1
Crystals of NifL PAS domain protein containing the FAD cofactor grown by the hanging-drop method. The average dimensions of these crystals were 0.1 × 0.1 × 0.1 mm.

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