Crystallization and preliminary crystallographic analysis of the proline dehydrogenase domain of the multifunctional PutA flavoprotein from *Escherichia coli*

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The PutA flavoprotein from *Escherichia coli* is a multifunctional protein that plays pivotal roles in proline catabolism by functioning as both a membrane-associated bifunctional enzyme and a transcriptional repressor. Peripherally membrane-bound PutA catalyzes the two-step oxidation of proline to glutamate, while cytoplasmic PutA represses the transcription of its own gene and the gene for a proline-transporter protein. X-ray crystallographic studies on PutA have been initiated to determine how the PutA structural scaffold enables it to be both an enzyme and a repressor, and to understand the mechanism by which PutA switches between its enzymatic and DNA-binding functions. To facilitate crystallization, a recombinant protein (PutA669) corresponding to the N-terminal 669 amino-acid residues of the 1320 residues of PutA was engineered. Activity assays demonstrated that PutA669 catalyzes the first step of chemistry performed by PutA, the conversion of proline to Δ1-pyrroline-5-carboxylate. Crystals of PutA669 have been obtained from PEG 3000 buffered at pH 6–7. The crystals occupy an *I*-centered orthorhombic lattice with unit-cell parameters $a = 72.5$, $b = 140.2$, $c = 146.8$ Å; a 2.15 Å data set was collected using a rotating-anode source. Assuming one molecule per asymmetric unit, the Matthews coefficient V_M is 2.5 Å^3 Da^{-1}, with a solvent content of 50%. The structure of PutA669 will be solved by multiple isomorphous replacement.

1. Introduction

PutA (proline utilization A protein) is a multifunctional flavoprotein from *E. coli* that functions as both a membrane-associated proline catabolic enzyme and a transcriptional repressor of two genes that code for proline-utilization proteins. Peripherally membrane-bound PutA catalyzes the two-step oxidation of proline to glutamate (Menzel & Roth, 1981a,b; Brown & Wood, 1992, 1993; Ostrovsky De Spicer & Maloy, 1993; Muro-Pastor *et al.*, 1997), while cytoplasmic PutA regulates proline catabolism at the gene level by repressing the transcription of both its own gene and the gene for the Na$^+$/proline transporter PutP (Menzel & Roth, 1981c; Maloy & Roth, 1983; Ostrovsky De Spicer *et al.*, 1991; Brown & Wood, 1992). Together, PutA and PutP enable bacteria such as *E. coli* and *Salmonella typhimurium* to use proline as the sole source of carbon and nitrogen (Menzel & Roth, 1981c; Wood, 1981).

The first enzymatic step catalyzed by PutA is the oxidation of proline to Δ1-pyrroline-5-carboxylate (P5C), which is coupled to the two-electron reduction of the non-covalently bound FAD (Fig. 1). Electrons from the reduced FAD are subsequently transferred to an acceptor in the electron-transport chain. In the second step of catalysis, glutamic semi-aldehyde, presumably the product of non-enzymatic hydrolysis of P5C, is oxidized to glutamate, reducing NAD$^+$ to NADH (Fig. 1).

Proline availability and the flavin redox state are thought to determine the intracellular location and therefore the function of PutA. When proline is available as a substrate, PutA associates with the membrane, where it performs its enzymatic functions (Brown & Wood, 1993; Muro-Pastor *et al.*, 1997; Surber & Maloy, 1999). However, in the absence of proline, PutA accumulates in the cytoplasm and represses the divergent transcription of the put regulon by binding to the 419 base-pair putP-putA intergenic region (Wood, 1987; Ostrovsky De Spicer *et al.*, 1991; Brown & Wood, 1992; Ostrovsky De Spicer & Maloy, 1993). Since proline reduces the flavin in the first step of catalysis, the FAD redox state is implicated as a regulatory signal for the association of PutA with the membrane. Spectro-electrochemical studies of PutA have shown that the flavin redox state has an almost negligible effect on PutA–DNA interactions (Becker & Thomas, 2001), which suggests the

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hypothesis that changes in PutA affinity for the membrane modulate PutA intracellular location and function.

A detailed understanding of the mechanism by which PutA switches between its functions is limited by the lack of three-dimensional structural information for this protein. *E. coli* PutA is a single polypeptide of 1320 amino-acid residues and purifies as a homodimer with an apparent molecular mass of ~293 kDa (Brown & Wood, 1992). Sequence comparison with proteins of known function has identified the proline dehydrogenase and P5C dehydrogenase domains as residues 340–590 and 650–1130, respectively (Ling *et al.*, 1994). A sequence-based query of the PDB revealed no homologues of PutA or its functional domains suitable for molecular replacement. The PDB contains no structures of proline dehydrogenases from any organism. The only structures related to PutA are those of aldehyde dehydrogenases, but the sequence identity to the P5C dehydrogenase domain of PutA is low (30%).

The current work is part of a research program undertaken to develop a structure-based model for how PutA functions as both a membrane-associated enzyme and a transcriptional repressor. Since crystallization of PutA is challenging owing to its large size, a 'divide-and-conquer' strategy has been designed in which shorter polypeptides that retain one or more of the functions of PutA are engineered and crystallized separately. The data presented in this paper represent our first successful implementation of this strategy. We describe the purification, crystallization and native X-ray data collection for a polypeptide chain corresponding to the first 669 residues of PutA (PutA669). As expected, PutA669 exhibits proline dehydrogenase activity similar to that of full-length PutA and does not exhibit P5C dehydrogenase activity. The anticipated structure of PutA669 will provide the first three-dimensional images of a proline dehydrogenase domain from any organism and will provide a fundamental framework for understanding the molecular mechanism of how PutA switches between its mutually exclusive functions.

2. Methods and results

2.1. Cloning, expression and purification

The pML9 construct created by Ling and Wood was used to generate PutA669 by inserting a *Hin*dIII-*Sma*I 5.7 kbp fragment which contains the putA gene into pT7-6 (Ling *et al.*, 1994). An *Nde*I site was posi-tioned at the translational start codon and an *Eco*RI site was inserted immediately after codon 669 of the putA gene in pML9. The *Nde*I and *Eco*RI sites were introduced using synthetic oligonucleotides and the QuikChange (Stratagene) site-directed mutagenesis kit. The *Nde*I-*Eco*RI fragment containing residues 1–669 was then subcloned into a pET-23b vector carrying a C-terminal hexahistidine tag (Novagen). The resulting construct, pUT669, was introduced into the *E. coli* expression strain BL21(DE3) pLysS. DNA sequencing of pUT699 demon-strated that no mutations were introduced into PutA669 during the QuikChange protocol. PutA669 with the C-terminal 6×His tag was expressed by induction with isopropyl β-D-thiogalactoside (IPTG) when the culture, which had been grown in Terrific broth, reached an OD of approximately 1.0. Induction with IPTG lasted for 3 h at 310 K.

Pelleted *E. coli* cells were resuspended in 1× binding buffer (20 mM Tris pH 7.9 containing 5 mM imidazole, 0.5 M NaCl and 10% glycerol) supplemented with 1 mM FAD and the protease inhibitors phenyl-methanesulfonyl fluoride (0.5 mM), L-1-chloro-3-(4-tosylamido)-7-amino-2-hepta-none–HCl (0.13 mM), L-1-chloro-3-(4-tosyl-amido)-4-phenyl-2-butanone (0.08 mM), ε-amino caproic acid (5 mM) and leupeptin (1 μM). The resuspended cells were disrupted by sonication at 277 K using a pulse sequence of 15 s on, 45 s off (5 min total pulse time). The broken cells were spun at 30 000 rev min^{-1} for 30 min. The resulting supernatant was subsequently added to a Ni^{2+} (NTA) affinity column equilibrated with 1× binding buffer. PutA669 was eluted with 20 mM Tris buffer pH 7.9 containing 0.5 M imidazole, 0.5 M NaCl and 10% glycerol. Fractions exhibiting proline dehy-drogenase activity were then pooled and dialyzed overnight in a 14 kDa cutoff membrane into 70 mM Tris buffer pH 8.1, 10% glycerol, 2 mM EDTA. Because a significant amount of FAD dissociated from PutA669 during the Ni^{2+} (NTA) affinity chromatography step, the pooled protein was incubated with 1 mM FAD overnight at 277 K. Further purification was performed on a high-performance Q-Sepharose column equilibrated with 70 mM Tris pH 8.1 buffer, 10% glycerol and 2 mM EDTA. A 1 1
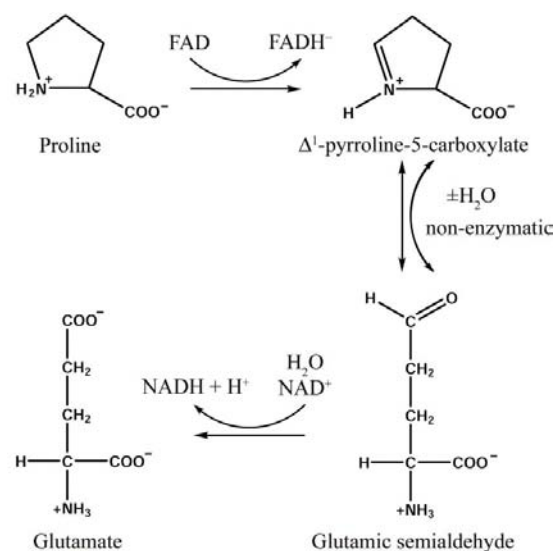

Figure 1
The reactions catalyzed by PutA. PutA669 catalyzes only the first reaction.

Table 1
Data-collection statistics.

Values for the outer resolution shell of data are given in parentheses.

Space group	I222
Unit-cell parameters (Å)	$a = 72.5$, $b = 140.2$, $c = 146.8$
Resolution (Å)	99–2.15 (2.23–2.15)
Total observations	313564
Unique reflections	40753
Completeness (%)	99 (96)
Average $I/\sigma(I)$	28.4 (3.3)
R_{merge}	0.065 (0.466)

gradient of 0–0.5 M KCl in 70 mM Tris pH 8.1 buffer and 10% glycerol eluted PutA669. Fractions exhibiting proline dehydrogenase activity were pooled and dialyzed into 70 mM Tris pH 7.5, 10% glycerol and 5 mM EDTA. PutA669 was concentrated to 16.5 mg ml^{-1} using an Amicon ultrafiltration cell with a 30 kDa molecular-weight cutoff.

The concentration of PutA669 was determined spectrophotometrically using an extinction coefficient of 12 700 M^{-1} cm^{-1} at 452 nm and by the Bradford method using the Bio-Rad reagent with bovine serum albumin as the standard (Brown & Wood, 1992). SDS–PAGE analysis using silver-staining methods demonstrated PutA669 was purified to homogeneity. The predicted molecular weight of PutA669 is 76.1 kDa. The C-terminal hexahistidine tag was not removed prior to crystallization.

2.2. Crystallization

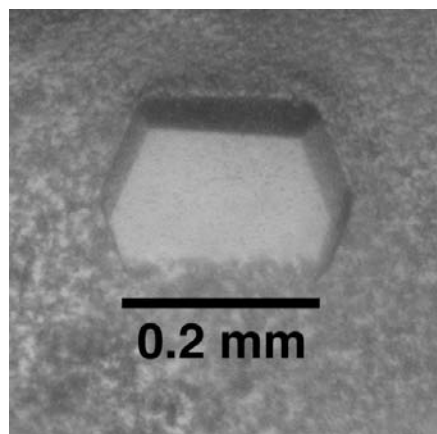All crystallization experiments were performed at 295 K using the hanging-drop

Figure 2
A crystal of PutA669 grown amongst protein precipitation in a hanging drop.

method of vapor diffusion with drops formed by mixing equal volumes of the reservoir and the protein solutions. Hampton Research Crystal Screen and Emerald Biostructures Wizard kits were used to find initial crystallization conditions. Several positive results were obtained from the screens, including small crystals from one reagent and crystalline precipitate from 24 other reagents. The common ingredient of the successful trials was polyethylene glycol (PEG) in the molecular-weight range 400–8000. The most promising condition, which involved PEG 3000 and citrate buffer, was selected for optimization.

Large diffraction-quality crystals of PutA669 were obtained using reservoir solutions of 21–26% PEG 3000, 0.1 M citrate buffer pH 6–7. These crystals typically appeared within 3–14 d as yellow blocks in a field of protein precipitation and grew to a maximum dimension of 0.2–0.3 mm after about one month (Fig. 2). The crystals were cryoprotected by moving them with a Hampton mounting loop to a 5 ml hanging drop containing 24% PEG 3000, 10% PEG 200, 0.1 M citrate buffer pH 6.6. After a 15 min soak, the crystal was picked up with the loop and plunged into liquid nitrogen.

2.3. Data collection and processing

X-ray diffraction data from native crystals were recorded at 173 K with an R-AXIS IV detector coupled to a Rigaku rotating-anode generator equipped with Osmic MaxFlux confocal optics and an X-stream low-temperature system. The data collection consisted of 436 frames with a crystal-to-detector distance of 150 mm, an oscillation angle of 0.5° and an exposure time of 8 min per frame. The data were processed with *HKL* (Otwinowski & Minor, 1997) and the resulting statistics are given in Table 1.

The crystals occupy an *I*-centered orthorhombic lattice with unit-cell parameters $a = 72.5$, $b = 140.2$, $c = 146.8$ Å. Strong diffraction to 2.2 Å resolution was observed, with weaker diffraction extending to 2.0 Å. The high-resolution limit for processing was chosen such that more than half of the reflections in the highest resolution shell of data have $I/\sigma(I) > 2$ (Dauter, 1997), which corresponds to 2.15 Å. The merged data set consists of 313 564 observations of 40 753 unique reflections and is 99% complete to 2.15 Å resolution. The overall R_{merge} on I is 0.065, with an $\langle I/\sigma(I)\rangle$ of 28.4. The average crystal mosaicity obtained from post-refinement is 0.94°. Based on the method of Matthews (1968), one molecule per asymmetric unit is expected, which is consistent with a solvent content of 50%. The structure of PutA669 is being solved by multiple isomorphous replacement using mercury and bromide derivatives.

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