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Crystallization of a stringent response factor from *Aquifex aeolicus*

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The crystallization of a key enzyme from *Aquifex aeolicus* with suggested bifunctional activity, acting as an exopolyphosphatase and a guanosine pentaphosphate phosphohydrolase, is reported. Native data were collected to below 2 Å resolution from an orthorhombic crystal with unit-cell parameters a = 50.8, b = 70.3, c = 90.9 Å. Methionine residues were introduced by mutation and deliberate oxidation of the protein allowed us to produce additional crystal forms with reproducible diffraction ability and increased phasing potential. This is the first report on the crystallization of a member of the Ppx/GppA phosphatase family.

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1. Introduction

Bacteria have developed systems to cope with sudden changes in the environment. Accumulation of inorganic polyphosphate (polyP) and guanosine tetraphosphate (ppGpp) can be observed in response to amino-acid depletion and represent the action of a system which coordinates protein synthesis, transcription and protein degradation (Gottesman & Maurizi, 2001; Kuroda *et al.*, 2001). Nutritional downshift activates the ribosome-bound protein stringent factor (SF or RelA) in response to an increased amount of uncharged tRNA in the ribosomal acceptor site (A-site) (Ogawa & Sy, 1977). Once activated, SF catalyzes an ATP to GTP 3′- pyrophosphoryl group transfer reaction forming guanosine pentaphosphate (pppGpp; Gropp *et al.*, 2001). In Gram-negative bacteria, the reverse reaction is facilitated in a ribosome-independent manner by the sequence-related hydrolase spoT. Furthermore, SpoT promotes pppGpp synthesis upon carbon or phosphate starvation (Mittenhuber, 2001). Gram-positive bacteria have collected both activities in only one enzyme, named Rel. Conversion of pppGpp to the biologically effective ppGpp, known as ‘magic spot’, is regulated by the gppA gene product (GPP) in *Escherichia coli*. *In vitro*, it has been shown that GPP has dual activity and can work as an exopolyphosphatase on polyP substrates (Keasling *et al.*, 1993). Furthermore, GPP is homologous to the *E. coli* exopolyphosphatase PPX. The *A. aeolicus* genome has been fully sequenced and it appears that both functions are contained in a single 35.5 kDa protein encoded by the *ppx* gene (Deckert *et al.*, 1998). Increased levels of ppGpp are known to affect the level of PPX activity. A consequence of reduced PPX activity is a dramatic shift in the equilibrium between polyP breakdown and accumulation catalysed by polynucleotide kinase, PPK (Gottesman & Maurizi, 2001; Kuroda *et al.*, 2001). Subsequently, association of polyP with the Lon protease controls free ribosomal protein degradation as observed in bacteria during starvation. Thus, the *ppx* gene product plays a central role in this fascinating regulatory system. Here, we report the crystallization and preliminary crystallographic study of GPP/PPX from the thermophilic organism *A. aeolicus* as well as providing evidence suggesting deliberate protein oxidation to be a useful technique to enhance the success of crystallization.

2. Materials and methods

2.1. Cloning, expression and purification

The *ppx* gene was amplified by PCR using genomic *A. aeolicus* DNA, *pfu*Turbo DNA polymerase (Stratagene) and two primers containing unique restriction sites for *Nde*I and *Not*I: (i) 5′-GCGGCAGCCATATGTCTTTA-GATAATAAACCAATTATG-3′ and (ii) 5′-GCTCGAGTGCGGCCGCTTAGGAATGA-TTTTCCTTTAATACTTC-3′. The amplified fragment was digested with *Nde*I and *Not*I, purified and joined by T4 ligase (Promega) to *Nde*I/*Not*I-digested pET28a plasmid (Novagen) to produce the pAQppx expression construct coding for PPX with an N-terminal His_6 tag. The inserted gene was checked by DNA sequencing (MWG Biotech AG). A double mutant (V82M and V306M) was constructed by PCR using primers with *Eco*RI and *Not*I restriction sites, respectively: (iii) 5′-GCGGCAGCGAATTCAAGGTAGAACG-GATGAAGG-3′ and (iv) 5′-GCTCGAGT-

GCGGCCGCTTAGGAATGATTTTCCT-TTAACATTTCAC-3′. The PCR product was inserted into *Eco*RI/*Not*I-digested pAQppx vector, providing the pAQppx2m plasmid. A third mutation (C138M) was introduced in a similar manner with primers (iv) and (v) 5′-GCGGCAGCCTTTAA-AGCCCGAAGGAGAGGTTATGGTAG-3′ with a *Dra*I site, yielding the pAQppx3m construct. Plasmids were transformed into Rosetta (DE3) competent cells (Novagen) and grown in LB medium containing chloramphenicol and kanamycin (34 and 100 μg ml^{-1}, respectively). After 3–4 h, expression of both wild-type and mutant protein was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 m*M* and growth continued for 3 h at 310 K.

The production of selenomethionine-substituted protein followed a published procedure (Van Duyne *et al.*, 1993). Cells were harvested by centrifugation and stored at 193 K before disruption by sonication in the starting buffer (20 m*M* Tris–HCl, 150 m*M* NaCl, 0.2 m*M* PMSF, 50 mg l^{-1} DNAse I, 5 m*M* β-mercaptoethanol pH 8.0 at 293 K) containing 0.25% NP-40. Cell debris was removed by centrifugation (40 000*g*, 30 min) and the supernatant was applied to a cobalt metal-affinity column (Talon, Clontech) at room temperature. The 5 ml column was cleaned with 200 ml wash buffer (20 m*M* Tris–HCl, 150 m*M* NaCl, 10 m*M* imidazole, 5 m*M* β-mercaptoethanol pH 8.0 at 293 K) and the His-tagged protein was eluted in the same buffer with the inclusion of 50 m*M* imidazole and was precipitated by addition of ammonium sulfate to 70% saturation. Dissolved precipitate was loaded onto a Superdex 200 gel-filtration column (Pharmacia) and eluted in a suitable buffer for crystallization (5 m*M* Tris–HCl, 100 m*M* NaCl, 1 m*M* DTT pH 8.0). For production of protein under oxidizing conditions, β-mercaptoethanol and DTT were excluded from the purification and the sample was incubated with 0.1% H_2O_2 for 1 h at room temperature prior to gel filtration. Thrombin was used for proteolytic removal of the N-terminal His tag.

2.2. Crystallization

The sample obtained directly from gel filtration was concentrated to 2–4 mg ml^{-1} by Microcon centrifugation (Amicon Corporation). The hanging-drop vapour-diffusion technique was used to screen for suitable crystallization conditions by the sparse-matrix approach (Jancarik & Kim, 1991) using Crystal Screen I and Crystal Screen II (Hampton Research). Equal volumes of protein and reservoir solution were mixed in the crystallization drop. Diffracting crystals of reduced native hexa-histidine-tagged protein were obtained with a solution consisting of 0.1 *M* HEPES pH 7.5 and 70% MPD. All concentrations mentioned in relation to crystallization refer to the composition of reservoir solutions. Conditions for crystal production of native PPX without tag were optimized to 57% MPD, 0.1 *M* MES, 0.05–0.07 *M* Tris-base, 0.14 *M* KCl, 5 m*M* DTT pH 6.2–6.6. With reducing agent included in the protein sample, crystals of selenomethionine-derivatized triple-mutant protein were obtained under similar conditions. Crystals were also produced with MPD exchanged for 38% polyethylene glycol (PEG) mono-methylether 350 as precipitant. However, substitution with PEG 400 prevented crystal formation and resulted in the appearance of spherulite aggregates only.

Oxidized triple-mutant protein was crystallized under different conditions: 26% PEG 4000, 5% PEG 400, 0.1 *M* Tris-base, 0.06–0.02 *M* acetic acid, 0.15 *M* $MgCl_2$ pH 8–8.5. Further, the protocol mentioned above for the reduced protein did not produce crystals of oxidized PPX. For all crystals produced in this study, crystal appearance and growth was completed within 1–10 d.

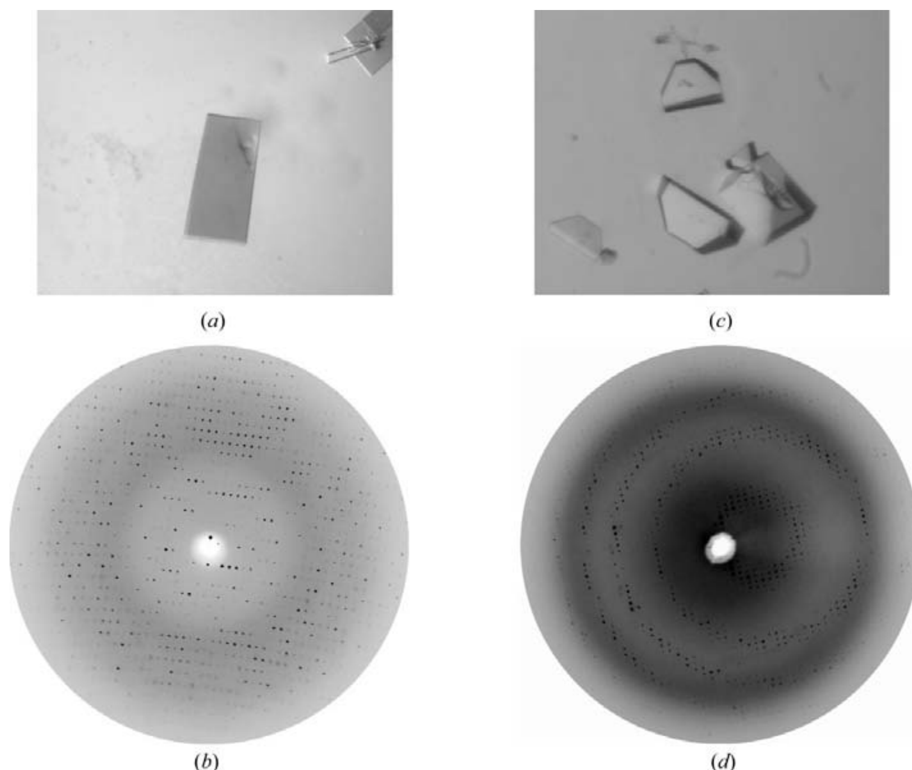

Figure 1
The *A. aeolicus* stringent response factor PPX was crystallized in different forms. (*a*) Fragile crystals of type I have typical dimensions 500 × 200 × 10 μm; the diffraction quality (*b*) is often impaired by high mosaicity. Crystals of type II (*c*) diffract reproducibly to 2.2 Å (*d*).

2.3. Crystallographic data

Synchrotron radiation was used in all preliminary diffraction experiments on the PPX crystals (MaxLab, Lund, BL711, Sweden and ESRF, Grenoble, France). Crystals were in all cases flash-frozen in liquid nitrogen directly from the crystallization drop.

A number of data sets (>20) were collected from orthorhombic crystals grown in solutions of MPD of native as well as heavy-atom-substituted PXX. The crystals belong to space group $P2_12_12_1$, with approximate unit-cell parameters a = 50, b = 70, c = 90 Å. This crystal form is referred to as type I. The estimated solvent content is around 45%, with one molecule per asymmetric unit. Two crystal forms of oxidized triple-mutant protein have been examined in diffraction experiments. Type II are monoclinic $P2_1$ crystals, with unit-cell parameters a = 54.5, b = 83.5, c = 69.8 Å, β = 97.4°. Crystals of type III belong to space group $P2_12_12_1$ and have unit-cell parameters a = 53.0, b = 70.3, c = 78.7 Å.

Table 1
Example of data-collection and reduction statistics for type II crystals.

Values for the outer resolution shell are given in parentheses.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 54.5, b = 83.5, c = 69.8, β = 97.4
Resolution limits (Å)	30.0–2.4 (2.5–2.4)
Total No. of observations	378539
No. of unique reflections	24124
Completeness (%)	99.0 (98.6)
Average $I/\sigma(I)$	23.9 (10.6)
$R_{\rm merge}$	0.057 (0.185)

3. Results and discussion

3.1. Purification and crystallization

In all cases, expression levels exceeded 10 mg of protein per litre of culture. Under reducing conditions, PPX eluted as a monomer in gel filtration. This result was confirmed by dynamic light scattering at 298 K (DynaPro, Protein Solutions Inc.). However, upon oxidation the triple-mutant protein, whether selenomethionine-derivatized or not, behaved in a different manner. Gel filtration produced two distinct peaks corresponding to monomer and dimer. The dimer association was preserved in denaturing SDS–PAGE without reducing agent, but was destroyed by including DTT in the loading buffer. From the PXX sequence, which has two cysteine residues, one of which was mutated (C138M), it can be concluded that dimer formation under oxidizing conditions is a consequence of an intermolecular disulfide cross-link involving Cys289. Despite homogeneous purification, the dimer was never crystallized and the appearance of crystal forms II and III must necessarily have a different explanation (Fig. 1). It is likely that a similar oxidation procedure would create problems if the protein had a large number of potentially surface-exposed cysteine residues.

Use of hydrogen peroxide as a tool to enhance anomalous signals in MAD phasing of selenomethionine-substituted (SeMet) crystals has been reported previously (Thomazeau *et al.*, 2001). In the current study, we have extended this approach to deliberate protein oxidation prior to crystallization, which allowed us to crystallize two new crystal forms. Both forms, types II and III, were grown by spontaneous nucleation under identical conditions and occasionally even appeared in the same drop, with a tendency for type III crystals to form more readily. This could indicate a lack of uniform oxidation. However, the fact that both the SeMet and native version of the triple-mutant protein could be crystallized in forms II and III leads to the suggestion that not only can selenomethionine be oxidized, but also methionine. This might be a useful a tool to crystallize other proteins. There might also be other protein modifications as a result of oxidation (Kim *et al.*, 2001). In the case of PPX, structure determination and refinement is expected to give a definite answer. Although the issue of accidental oxidation has obvious relevance to several industrial applications, it has not been systematically examined by structural biologists.

3.2. Data collection and perspectives

All data processing was performed using either *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) or *MOSFLM* and *SCALA* (Evans, 1993; Leslie, 1992). The program *SOLVE* (Terwilliger & Berendzen, 1999) was used extensively to evaluate heavy-atom derivatives. Several data sets were collected from type I crystals and MIR phasing was attempted. This approach was limited by the highly fragile and variable nature of type I crystals, where the diffraction limit even for native crystals varied over the range 1.85–3.2 Å. The plasmid pAQppx3m was constructed to circumvent this problem and to allow us to use selenomethionine-based SAD or MAD techniques for phasing. The new crystal forms, types II and III, produced from oxidized protein have a common and constant diffraction limit around 2.2 Å (Table 1) and increased physical stability. Data have been collected and structure determination is well in progress.

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