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Expression, refolding and crystallization of *Aquifex aeolicus* elongation factor P

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Elongation factor P is a universally conserved protein stimulating peptidyltransferase activity during protein synthesis. The factor is sensitive to classical inhibitors of the ribosomal peptidyltransferase activity and is possibly involved in alignment of the substrate tRNAs in the catalytic centre of 70S ribosomes. Elongation factor P from the thermophilic *Aquifex aeolicus* was overexpressed as a soluble protein in *Escherichia coli* and crystallized. A fast generally applicable refolding protocol was developed to improve crystal quality and circumvent strong binding of oligonucleotides to the protein. Diffraction data collected to 2.7 Å resolution present twinning.

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1. Introduction

Ribosomes facilitate protein synthesis in all living organisms. In bacteria, the 30S ribosomal subunit is primed with the messenger RNA (mRNA) and the initiation factors IF1, IF2 and IF3 assure recruitment of the initiator transfer RNA (tRNA) to the 30S peptidyltransfer site (P site), base pairing with the start codon of the mRNA. Upon formation of the initiation complex the 50S ribosomal subunit associates, forming the active 70S ribosome. Based on mRNA codon matching, aminoacyl-charged tRNAs are recruited from the surroundings to the ribosomal acceptor site (A site). In the peptidyltransferase centre on the 50S ribosomal subunit, the peptidyl group on the P-site tRNA is transferred to the aminoacyl group of the A-site tRNA. Cycles of peptidyl transfer and translocation of peptidyl-tRNA from the A site to the P site extend the peptidyl product. A stop codon on the messenger terminates the process and the peptide product is released.

The ribosomal peptidyltransferase centre consists entirely of ribosomal RNA (rRNA) and peptidyl transfer (PT) is believed to be catalyzed exclusively by rRNA (Nissen *et al.*, 2000). *In vitro* experiments show that elongation factor P (EF-P) stimulates peptidyl transfer on the 70S ribosome (Ganoza *et al.*, 1985). The catalytic properties of EF-P are seriously affected by PT inhibitors binding in the peptidyl transferase cavity on the 50S ribosomal subunit (Schlunzen *et al.*, 2001), but remain insensitive to antibiotics that affect translocation and occupation of the ribosomal A site (Aoki *et al.*, 1997). The detailed molecular role of EF-P in stimulation of PT activity is still unclear. However, EF-P is most likely to not be directly involved in transport and binding of the substrates of the peptidyltransfer reaction, but may in certain cases modulate the alignment of substrates or indirectly optimize the peptidyltransferase centre for catalysis (Ganoza & Aoki, 2000).

Crystallization of *E. coli* EF-P has been reported by others, but without any note of diffraction data or crystallographic parameters (Aoki *et al.*, 1997). Here, we report the crystallization and preliminary crystallographic study of the 21.8 kDa EF-P from the thermophilic organism *A. aeolicus* as well as a fast refolding protocol generally applicable to His_6-tagged recombinant proteins.

2. Materials and methods

2.1. Cloning, expression and purification

The *efp* gene was amplified by PCR using genomic *A. aeolicus* DNA template, *pfu*Turbo DNA polymerase (Stratagene), an upstream primer 5′-GCGGCAGCCATATGGCGACG-GAGATAGACAT-3′ and a downstream primer 5′-GCTCGAGTGCGGCCGCTCATT-TTGCTTCTTTAACCCTCTC-3′. The primers introduce unique leading *Nde*I and tailing *Not*I cleavage sites. 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 vector (Novagen) to produce the pHisEfp plasmid expressing EF-P with an N-terminal His_6 tag. The construct was confirmed by two-directional sequencing (ABI Prism 377, Version 2.1.1). The pHisEfp plasmid was transformed into Epicurian Coli BL21-CodonPlus(DE3)-RIL competent cells (Stratagene) and grown in LB medium containing ampicillin and kanamycin (34 and 100 µg ml^{-1}, respectively). After 3–4 h, protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of

1 m*M* and incubation continued for 3 h at 310 K. Fermentation was performed either in a 4 l Chemap fermentor or in standard shake flasks. 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 in start buffer (20 m*M* Tris, 150 m*M* NaCl, 0.2 m*M* phenylmethylsulfonyl fluoride, 50 mg l^{-1} DNAse I pH 7.5 at 293 K) by either 5 mg ml^{-1} lysozyme or mechanical stress in an X-press (AB Biox). Cell debris was removed by centrifugation (40 000*g*, 30 min) and the supernatant applied to a cobalt metal-affinity column (TALON, Clontech) at 277 K. The 10 ml column was flushed with 300 ml wash buffer (20 m*M* Tris–HCl, 150 m*M* NaCl, 10 m*M* imidazole pH 7.5 at 293 K) and His_6-tagged protein eluted in the same buffer but with 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, 50 m*M* NaCl, 0.5 m*M* DTT pH 8.0). The effect of the presence of the additional His_6 tag on crystallization was tested by proteolytic removal using thrombin (Sigma).

2.2. Refolding

Purification prior to refolding followed the procedure described above. Native ammonium sulfate precipitated protein obtained after affinity chromatography was dissolved at a concentration of 1 mg ml^{-1} in denaturing buffer (5 m*M* β-mercaptoethanol, 20 m*M* Tris–HCl, 150 m*M* NaCl, 8 *M* urea pH 7.5 at 293 K) and applied at 2 ml min^{-1} to 5 ml cobalt resin (TALON) packed in an empty HiTrap column (Pharmacia) equilibrated in the same buffer. All operations were carried out at 277 K. The column was washed with 150 ml (4 ml min^{-1}) denaturing buffer containing 10 m*M* imidazole. Refolding was achieved on the column by a rapid change to renaturation buffer (20 m*M* HEPES, 100 m*M* NaCl, 5 m*M* β-mercaptoethanol pH 7.5 at 293 K) and a volume of 150 ml (6 ml min^{-1}) was passed through the resin before elution with renaturation buffer containing 50 m*M* imidazole (4 ml min^{-1}). As previously, the protein was precipitated with 70% ammonium sulfate, redissolved in buffer (5 m*M* Tris–HCl, 50 m*M* NaCl, 0.5 m*M* DTT pH 8.0) and subjected to Superdex 200 size-exclusion chromatography.

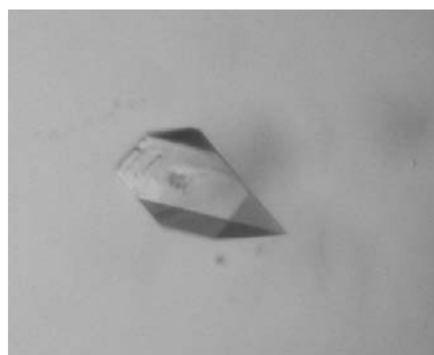

Figure 1
Bipyramidal crystal of selenomethionine-derivatized EF-P.

2.3. Crystallization and data collection

The sample obtained directly from gel filtration was concentrated to 2–4 mg ml^{-1} by Microcon centrifugation (Amicon). The hanging-drop vapour-diffusion technique was used in the screening of suitable crystallization conditions by mixing equal volumes of protein and reservoir solution. Both commercial crystal screens (Hampton Research) and systematic grid-screening approaches were used (Cox & Weber, 1988). Diffracting crystals of insufficient quality were obtained under high-salt conditions: 1.7–1.8 *M* ammonium sulfate, 40–112 m*M* Tris base and 10 m*M* $MgCl_2$. Separation of the protein into a distinct phase was a common feature observed under experimental conditions of low ionic strength and with various polyethylene glycol (PEG) precipitants. However, we identified one grid-screening experiment with PEG 4000 as precipitant which contained a promising phase-separated precipitate. In this case, the crystallization solution contained 17% PEG 4000, 10 m*M* $MgCl_2$, 100 m*M* MES and 65 m*M* Tris base. Crystal growth was obtained in similar experiments with lower precipitant concentration by streak seeding directly after mixing the drops (Stura & Wilson, 1991). Crystals were reproducibly produced from experiments with reservoirs containing 5% 2-propanol, 10 m*M* $MgCl_2$, 4 m*M* DTT, 100 m*M* Tris base, 45 m*M* acetic acid, 10% PEG 400 and 10.5% PEG 4000. Seeds were initially obtained from the PEG 4000 grid-screening experiment and subsequently from crystals grown under the optimized conditions just mentioned. Rapid seeding before actual equilibration of the drops was adopted as a methodological modification, as supersaturation was achieved immediately after mixing protein and reservoir solutions; this was indicated by initial formation of a white precipitate which transformed within 1 h to a clear drop with phase separation. Spontaneous nucleation was consistently prevented in the oily phase-separated crystallization drops and seeding was required in all further experiments. Phase separation was in this case not an effect of mixing PEG and salt-containing solutions in certain ratios and EF-P was clearly collected in a distinct phase, potentially revealing a hydrophobic property of the protein. Crystals appeared to initiate growth from inside the protein phase, which was slowly consumed upon continued growth.

Synchrotron radiation was used in all diffraction experiments with the EF-P crystals (BL711, MaxLab, Lund, Sweden; BW7A, DESY, Hamburg, Germany; station 9.5, SRS, Daresbury, England). Crystals were either transferred to Paratone-N or placed briefly in a cryobuffer containing 10 m*M* $MgCl_2$, 4 m*M* DTT, 100 m*M* Tris base, 45 m*M* acetic acid, 20% PEG 400 and 10.5% PEG 4000 before flash-freezing in liquid nitrogen.

3. Results and discussion

3.1. Refolding and crystallization

The procedure described in this paper allowed us to obtain crystals of EF-P from *A. aeolicus* suitable for crystallographic examination (Fig. 1). However, a persistent difficulty in the present study has been the production of well diffracting isomorphous crystals. Unfortunately, this situation is independent of whether or not the protein carries an N-terminal His_6-tag extension. Initially, crystals of native non-refolded protein were produced and analysed. An obstructing feature of the mother liquor was noticed as a syrup-like stickiness during loop-mounting of crystals directly from the crystallization drop. The mechanical stress imposed under this manipulation resulted in high crystal mosaicity. Examination of the protein used in crystallization revealed a possible explanation for this unusual sticky behaviour. In various preparations the ratio OD_{260}/OD_{280} was between 0.8 and 0.9, which indicated that the sample could be contaminated with remaining oligonucleotides despite the previous purification steps. Several attempts to separate protein-bound oligonucleotides by inclusion of ion-exchange chromatography in the purification were unsuccessful. Considering the ribosomal context of EF-P function, it is a reasonable suggestion that it is prone to interact even non-specifically with oligonucleotides and a refolding protocol was developed to clear samples from the

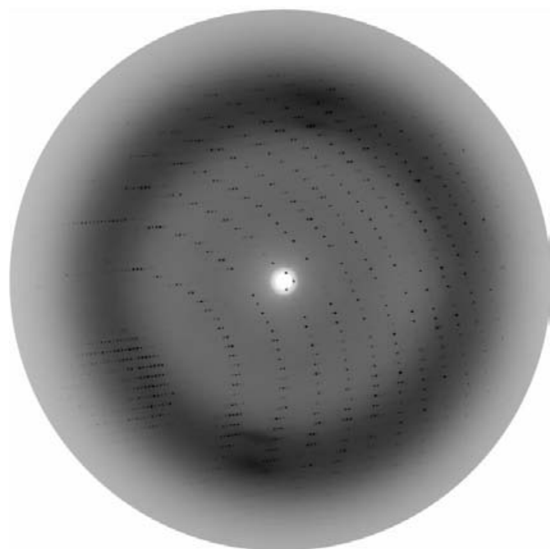

Figure 2
Native crystal diffraction image. The resolution extends to 2.7 Å.

Table 1
Data-collection and reduction statistics for the merohedrally twinned data set.

Values in parentheses are for the outer resolution shell.

Space group	$P4_{1(3)}$
Unit-cell parameters (Å)	$a = b = 71.24$, $c = 185.88$
Resolution limits (Å)	50.0–2.7 (2.8–2.7)
Total No. of observations	115927
No. of unique reflections	23159
Completeness (%)	93.2 (96.0)
Average $I/\sigma(I)$	11.0 (3.0)
$R_{\rm merge}$	0.044 (0.315)

contamination. Denaturation of the protein with a high concentration of urea was effective in lowering the OD_{260}/OD_{280} ratio and the viscosity of the mother liquor upon crystallization. The refolding procedure described here uses an affinity matrix to allow a simple and rapid change of buffers and limits the sample volumes to be handled and typically concentrated later on. This refolding protocol works well with both native and selenomethionine-substituted EF-P.

3.2. Twinning

All data processing was performed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Several data sets were collected from tetragonal crystals that belong to space group $P4_{1(3)}22$ with typical unit-cell parameters $a = b = 70.5$, $c = 195–213$ Å. The diffraction from the crystals is very sensitive to mechanical stress or changes in solvent conditions and typically extends to 3.7–4.2 Å (Fig. 2), with no notable difference between native and SeMet crystals. The estimated solvent content is around 70%, with one molecule per asymmetric unit. However, one native crystal diffracted to 2.7 Å and had unit-cell parameters $a = b = 71.3$, $c = 185.9$ Å. The crystal had apparent 422 point-group symmetry, a situation which requires special attention to possible twinning problems. All data collected on EF-P crystals were checked at the twin server (Yeates, 1997) and only the 2.7 Å diffraction data appeared to suffer from the crystallographic complication of twinning. The twin fraction was estimated to be above 45%. The crystal is best described as a perfect merohedral twin with true space group $P4_{1(3)}$. Data statistics for the twinned data are summarized in Table 1.

3.3. Structure determination

The sequence of EF-P contains five methionine residues. We have produced crystals of selenomethionine-substituted protein and confirmed proper incorporation by mass spectrometry. Despite the limited resolution that can be achieved reproducibly, our current approach to structure determination of EF-P is to collect SeMet MAD data. Recent examples have shown that MAD experiments with limited resolution and weak data are possible (Kunishima *et al.*, 2000). After initial structure determination at low resolution, it is highly plausible that refinement can be extended to 2.7 Å using the twinned data (Chandra *et al.*, 1999). Attempts to use structural models of the partially homologous protein eIF-5A in molecular replacement have all been unsuccessful.

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