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Preparation of a crystallizable mRNA-binding fragment of *Moorella thermoacetica* elongation factor SelB

Maria Selmer,[a]† Reinhard Wilting,[b]‡ Deep Holmlund[a] and Xiao-Dong Su[a]*

[a]Department of Molecular Biophysics, Centre for Chemistry and Chemical Engineering, Lund University, Box 124, S-221 00 Lund, Sweden, and [b]Lehrstuhl für Mikrobiologie der Universität München, Institut für Genetik und Mikrobiologie, Maria-Ward-Strasse 1a, D-80638 München, Germany

† Present address: MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK.
‡ Present address: Novozymes A/S, Krogshøjvej 36, DK-2880 Bagsvaerd, Denmark.

Correspondence e-mail: xiao-dong.su@mbfys.lu.se

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SelB is a bacterial elongation factor required for the decoding of a UGA stop codon together with a specific mRNA hairpin to selenocysteine. In attempts to crystallize *Moorella thermoacetica* SelB, a proteolysis process occurred and crystals of a proteolytic fragment were observed. The crystals, which appeared after a year, contained a C-terminal 30 kDa fragment containing the mRNA-binding domain. This fragment was reproduced through recloning. Crystals diffracting to 2.7 Å were obtained.

1. Introduction

Selenocysteine, the 21st amino acid, occurs in the active site of important enzymes in both prokaryotes and eukaryotes. In bacteria, the selenocysteine is coded by a UGA codon together with a nearby downstream mRNA hairpin (reviewed in Huttenhofer & Böck, 1998). SelB is a specialized elongation factor essential for incorporation of selenocysteine into the growing polypeptide chain (Forchhammer *et al.*, 1989) that specifically recognizes the mRNA hairpin (Baron *et al.*, 1993). By analogy with the elongation factor Tu, SelB delivers the cognate aminoacylated $tRNA^{Sec}$ to the ribosomal A-site.

The N-terminal part of SelB (amino acids 1–342 in *Escherichia coli* SelB) is homologous to elongation factor Tu and binds GTP and selenocysteinyl-$tRNA^{Sec}$, whereas the C-terminal mRNA-binding part does not show significant sequence homology to any other proteins. The mRNA-binding fragment can be minimized to amino acids 472–634 (*E. coli* numbering) and the unique part of SelB has therefore been suggested to consist of two independent domains (Kromayer *et al.*, 1996).

We have initiated the structure determination of SelB to further our understanding of this unique system. In order to obtain a sufficient amount of material for structural studies, SelB from the thermophile *M. thermoacetica* (previously named *Clostridium thermoaceticum*) was cloned (Kromayer *et al.*, 1996). This report describes how a crystallizable fragment of the mRNA-binding domain of SelB was subcloned and purified.

2. Experimental

2.1. Cloning, expression and purification of *M. thermoacetica* SelB

For expression of the full-length *M. thermoacetica selB* gene in *E. coli*, the T7 φ10 promoter/polymerase system was used (Tabor & Richardson, 1985). The working plasmid pCTB71 (Wilting, 1998) was constructed from the previously described plasmid pCTAB1 (Kromayer *et al.*, 1996). For production of SelB, pCTB71 was transformed into *E. coli* strain BL21 (DE3). The transformed cells were grown in LB with antibiotics for 16–20 h at 310 K and harvested at OD_{600} = 1.5. The cells were centrifuged down and lysed by a French press. After resuspension and centrifugation, the supernatant was fractionated by $(NH_4)_2SO_4$ precipitation. The precipitate at 40–50% saturation was solubilized in buffer 1 (30 m*M* potassium phosphate pH 7.5, 3.0 m*M* $MgCl_2$, 2.0 m*M* DTT, 0.5 m*M* EDTA), dialysed and heated to 338 K for 20 min. Denatured proteins were removed by centrifugation at 10 000*g* for 10 min. The supernatant was applied to a Q-Sepharose column (Amersham-Pharmacia) and eluted with a KCl gradient. The fractions containing the protein were pooled and concentrated by precipitation with 70% saturated $(NH_4)_2SO_4$, resuspended and separated on a Superdex 75 gel-filtration column (Amersham-Pharmacia). The fractions containing SelB were pooled and dialysed against buffer 2 [50 m*M* HEPES pH 7.5, 3.0 m*M* $MgCl_2$, 2.0 m*M* DTT, 0.5 m*M* EDTA, 1.0 *M* $(NH_4)_2SO_4$] and separated in a decreasing $(NH_4)_2SO_4$ gradient (1.0–0 *M*) on a phenyl Sepharose FPLC column (Amersham Pharmacia). SelB eluted at 150–100 m*M* $(NH_4)_2SO_4$. For crystallization experiments, the purified protein was precipitated with 70% saturated $(NH_4)_2SO_4$ and stored at 277 K.

2.2. Subcloning, expression and purification of *M. thermoacetica* $SelB_{370–634}$

Plasmid pSelB370 was designed for expression of the sequence of *M. thermoacetica* SelB from Ser370 to the C-terminus fused to an N-terminal His_6 tag with a thrombin cleavage site, as presented in pET28a (Novagen). DNA

coding for SelB 370–634 was amplified by PCR from pCTB71, using the primers 5′-GGGAATTC**CATATG**TCTCTCCAGAGGCGCCTGGA-3′ (*Nde*I restriction site in bold) and 5′-ATAAGAAT**GCGGCCGC**TCAATTACCAACAACTACCCGC-3′ (*Not*I restriction site in bold). The 804 bp *Nde*I/*Not*I double-digested PCR product was ligated into *Nde*I/*Not*I cleaved pET28a and transformed into Novablue cells (Novagen). The plasmid containing an insert of the right size was sequenced to confirm the correctness of the sequence.

E. coli BL21(DE3) cells carrying pSelB370 were grown at 310 K in LB medium supplemented with kanamycin and expression was induced at OD_{600} = 0.8–1.0 by addition of 1.0 m*M* IPTG. 3–4 h after induction, cells were harvested by centrifugation and frozen. Harvested cells were resuspended in buffer 3 (20 m*M* Tris–HCl pH 8.0, 0.5 *M* NaCl) and disrupted by sonication. After centrifugation at 17 000*g* for 20 min at 277 K, the supernatant was applied to a TALON column (Clontech) equilibrated with buffer 3. The column was washed with buffer 3 containing 10 m*M* imidazole and H_6-SelB$_{370–634}$ was eluted with buffer 3 containing 100 m*M* imidazole. The fractions containing H_6-SelB$_{370–634}$ were pooled and precipitated with 60% saturated $(NH_4)_2SO_4$. The pellet was resuspended in buffer 3 after centrifugation, applied to a phenyl HP Sepharose column (Amersham Pharmacia) equilibrated with buffer 4 [20 m*M* Tris–HCl pH 8.0, 1.0 *M* $(NH_4)_2SO_4$] and eluted with a 1.0–0 *M* $(NH_4)_2SO_4$ gradient. The peak fractions from the elution were pooled and concentrated in Centriprep 10 (Millipore) and the buffer was exchanged to 10 m*M* Tris–HCl pH 7.5, 50 m*M* NaCl.

3. Crystallization and X-ray data collection

In trials to optimize 'promising precipitates' obtained in a sparse-matrix screen (Hampton Research), 2 µl of 16 mg ml^{-1} full-length *M. thermoacetica* SelB was mixed in hanging drops with an equal volume of reservoir solution (12% PEG 8000, 0.1 *M* Tris–HCl pH 8.6) and equilibrated against 500 µl reservoir solution. The drops were kept in a cold room. After a year, three block-shaped crystals were found in one drop. The largest crystal had dimensions of 100 × 100 × 300 µm and was picked up and mounted in a quartz capillary. Diffraction data were collected at 298 K using a Rigaku rotating-anode generator and a MAR image-plate detector (MAR Research system). Data were processed using *DENZO* and scaled using *SCALEPACK* (Otwinowski & Minor, 1996).

Initial crystallization conditions for H_6-SelB$_{370–634}$ were found in a screen of pH *versus* concentration of PEG 8000. After optimization of these parameters to yield plate crystals, some metal ions from Additive Screen I (Hampton Research) were used to see if the crystals could be improved further. Addition of yttrium chloride was found to produce nice-looking prismatic shaped crystals (Fig. 1). In the end, reproducible SelB$_{370–634}$ crystals were grown by vapour diffusion in hanging or sitting drops at 293 K. 2 µl of protein at about 10 mg ml^{-1} was mixed with 0.4 µl 100 m*M* YCl_3 and 2 µl reservoir solution [0.1 *M* MES–Tris pH 6.2, 16%(*w*/*v*) PEG 8000] and equilibrated against 500 µl reservoir solution. Within one week, crystals grew as prisms of dimensions 50 × 50 × 200 µm (Fig. 1).

SelB$_{370–634}$ crystals were transferred to cryosolution (14% PEG 8000, 22.5% PEG 400, 90 m*M* MES–Tris pH 6.2) and flash-frozen at 120 K in a stream of boiled-off nitrogen (Oxford Cryosystems Cryostream). Diffraction data were collected on a Rigaku rotating-anode generator equipped with Osmic focusing mirrors and an R-AXIS IV++ detector (Rigaku). Data were processed using the program *XDS* and merged with *XSCALE* (Kabsch, 1988). Statistics are listed in Table 1.

4. N-terminal sequencing and mass spectrometry

For N-terminal sequencing, standard SDS–PAGE to PVDF (polyvinylidene difluoride) membrane blotting was performed. Six N-terminal amino acids were read by the Edman degradation method (BM unit, Lund University). For determination of molecular weight with mass spectrometry, the proteins were dissolved in 50% acetronitrile, 1% formic acid. The samples were analysed on an electrospray ionization mass spectrometer.

Figure 1
Crystals of SelB$_{370–634}$.

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell, 2.85–2.75 Å.

Resolution (Å)	25–2.75
Total observations	34285 (3433)
Unique reflections	7501 (740)
Average redundancy	4.6 (4.6)
R_{sym}† (%)	12.1 (34.8)
Data completeness (%)	98.5 (99.6)
$\langle I/\sigma(I)\rangle$	9.8 (4.2)

† $R_{sym} = \sum|I_{obs} - I_{avg}|/\sum I_{obs}$, where I_{obs} is the observed intensity and I_{avg} is the average intensity of symmetry-related reflections; summation is over all reflections.

Results and discussion

5.1. Full-length SelB preparation

The production of full-length SelB was usually low, presumably owing to its toxicity to the cells. Fortunately, during the expression trials a transformant was identified which produced SelB constitutively in higher yields. This transformant was used for production and purification of full-length SelB. From ten batches of 3 l cultures, 22 mg pure SelB was prepared.

5.2. Identification of a proteolytic fragment from the full-length SelB crystallization trials

In the initial trials to crystallize the full-length SelB, no crystals were observed within two months. However, three crystals were found in one drop after a year of storage in a cold room. One of the crystals diffracted to 4.5 Å on a Rigaku rotating-anode generator. The crystal belonged to an orthorhombic space group, with unit-cell parameters a = 84.8, b = 95.3, c = 93.1 Å.

To investigate the integrity of the protein in these crystals, the best we could do without destroying the crystal drop was to use a neighbouring drop with almost identical conditions for analysis. On an SDS–PAGE gel, SelB was shown to be degraded (data not shown). One band of about 30 kDa seemed to be the major proteolytic product; it was believed that the crystallized protein was this fragment. A protein sample kept in the cold room for the same period of time contained a proteolytic fragment of the same size as seen on the SDS gel (Fig. 2, lane 1). Therefore, identification of the protein fragment was performed on this stored protein sample.

The N-terminal sequencing result of this fragment was SLQR*X*L, agreeing well with the sequence 370–375 of SelB: SLQRRL. This region is located between the EF-Tu homologous domains and the domains

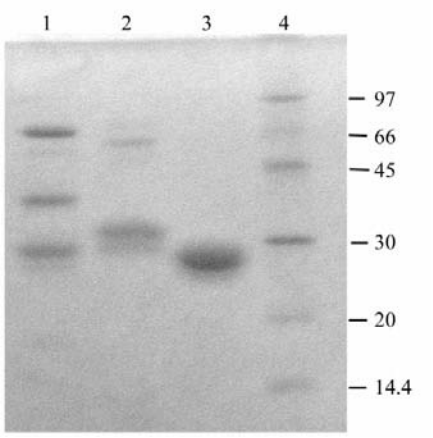

Figure 2
SDS–PAGE analysis of proteolysis of SelB. Lane 1, degraded SelB stored at 277 K; lane 2, freshly purified $SelB_{370–634}$; lane 3, dissolved crystal; lane 4, molecular-weight markers (kDa).

unique for SelB. The calculated molecular weight of this protein fragment from Ser370 to the C-terminal Asn634 is 30.5 kDa.

5.3. Preparation and crystallization of the proteolytic fragment

The fragment from Ser370 to the C-terminus Asn634 was subcloned and the so-called H_6-$SelB_{370–634}$ was then expressed and purified. The yield of purified H_6-$SelB_{370–634}$ from 1 l of culture was about 20 mg. Purified H_6-$SelB_{370–634}$ was used for crystallization without further treatment. The optimized prism-shaped crystals were grown at a different pH compared with the previous crystals, using PEG 8000 as precipitant. Adding yttrium chloride was critical for obtaining crystal growth in the third dimension and incubation in a 293 K incubator significantly improved the crystal quality (Fig. 1). The crystals were dissolved and analysed (Fig. 2, lane 3) on SDS–PAGE together with freshly purified H_6-$SelB_{370–634}$ (Fig. 2, lane 2). The band from the crystal migrated as the same size as the previous proteolytic fragment, but was smaller than the freshly purified protein. This indicated that proteolytic cleavage had again taken place in the H_6-$SelB_{370–634}$ drops.

Using electrospray ionization mass spectrometry, the molecular weights of freshly purified H_6-$SelB_{370–634}$ and 'spontaneously' proteolysed sample were determined to be 32 693 (3) and 30 531 (3) Da, respectively. These values agree well with a calculated molecular weight of 32 696 Da if the N-terminal methionine is lost from H_6-$SelB_{370–634}$ and of 30 533 Da for $SelB_{370–634}$.

5.4. Crystallographic studies

$SelB_{370–634}$ crystals diffracted to at least 2.7 Å at a rotating-anode generator with Osmic mirrors and belonged to space group $P2_12_12_1$. The unit-cell parameters are $a = 38.2$, $b = 67.8$, $c = 105.6$ Å. Assuming one molecule in the asymmetric unit gives a Matthews coefficient of 2.2 $Å^3\ Da^{-1}$ (Matthews, 1968) and a solvent content of 45%. Thus, the new crystals have different unit-cell parameters and improved diffraction power compared with the initial crystals. These results can be explained by the homogeneity achieved by the recloning and purification procedures and the optimization of crystallization conditions.

The determination of the crystal structure was planned to make use of the anomalous signal from yttrium. Since we were unsure whether this signal would give enough phase information, we also prepared selenomethionine-substituted $SelB_{370–634}$ according to previously described methods (Van Duyne *et al.*, 1993) and obtained crystals under very similar conditions. The structure was finally solved by Y/Se two-element MAD (multiwavelength anomalous dispersion) methods. The detailed structure description will be published elsewhere (Selmer & Su, 2002) and the coordinates of the $SelB_{370–634}$ structure have been deposited with the PDB.

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