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Purification and crystallization of the yeast elongation factor eEF2

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Crystals of the *Saccharomyces cerevisiae* elongation factor 2 (eEF2) in complex with GDP were obtained with the vapour-diffusion technique after rapid purification from industrial yeast. The crystals diffract to 2.85 Å and belong to the space group $P2_12_12_1$. A yeast strain expressing a functional histidine-tagged eEF2 as the only form of the protein further allows facilitated purification of the factor for both structural and functional studies.

1. Introduction

Translation of mRNA into protein occurs in three phases: initiation, elongation and termination (reviewed in Merrick & Hershey, 1996). These steps are regulated by a variety of mechanisms with important results for gene expression. In eukarya, protein synthesis begins with the action of a series of soluble initiation factors, which catalyse the positioning of the initiator Met-tRNA$_i$ and the 80S ribosome at the initiator AUG codon of an open reading frame. In the elongation cycle of protein synthesis, a peptide bond is formed between the peptidyl-tRNA in the ribosomal P-site and the aminoacylated tRNA (aa-tRNA) in the A-site. During translocation the peptidyl-tRNA moves to the P-site, the deacylated tRNA moves to the E-site and the mRNA advances by one codon. The elongation cycle continues until the stop codon is encountered by the ribosome, where the newly synthesized polypeptide chain is released from the ribosome by the action of release factors.

The GTP-dependent elongation eukaryotic factor 1A (eEF1A) catalyzes placement of aa-tRNA in the ribosomal A-site prior to peptide-bond formation. In fungi, this reaction is further stimulated by the elongation factor eEF3, which promotes the release of deacylated tRNA from the E-site in an ATP-dependent fashion, thereby increasing the affinity of aa-tRNA for the A-site (Triana-Alonso *et al.*, 1995). The 93 kDa eEF2 catalyzes the translocation reaction through GTP hydrolysis. The sequence identity between the prokaryotic homolog EF-G, such as that from *Thermus thermophilus*, and yeast eEF2 is 30%. EF-G, and most likely eEF2 as well, contains five structural domains (Czworkowski *et al.*, 1994; AEvarsson *et al.*, 1994). The overall shape of EF-G is similar to that of the ternary complex EF-Tu:GTP:aa-tRNA (Nissen *et al.*, 1995), where EF-Tu is the prokaryotic homologue of eEF1A. His699 of eEF2 (yeast) is post-translationally modified to diphthamide and ADP ribosylation of this residue by bacterial toxins inactivates the factor. Mammalian and yeast eEF2 can also be reversibly phosphorylated at Thr57 (yeast), which is located just prior to the switch 1 region that most likely undergoes conformational change between the GDP and GTP states of eEF2. Phosphorylation inactivates the factor by reducing the affinity for GTP, while that of GDP remains unchanged (Dumont-Miscopein *et al.*, 1994).

As a key component in protein synthesis, eEF2 and the factors that post-translationally modify the protein are targets for drug discovery. Sordarins are selective inhibitors of the fungal protein synthesis and although the target has been identified as eEF2, high-affinity binding of sordarins to the factor requires 80S ribosomes (Justice *et al.*, 1998). The sordarins prevent the transition of the ribosome from the pre-eEF2-GTP to post-eEF2-GTP state. From genetic studies, three amino acids within residues 521–524 in yeast are vital for sordarin selectivity (Shastry *et al.*, 2001) and the homologous region in EF-G is located in domain 3 of the protein. However, recent cross-linking experiments between a photoactivatable sordarin derivative and eEF2 map the binding site to the G′′ subdomain of eEF2, far away from the selectivity region in domain 3 (Dominguez & Martin, 2001).

Structural studies of prokaryotic elongation factors have generated key knowledge on the mechanism of protein synthesis. Only recently have these studies been extended to eukaryotes through our structure determination of eEF1A in complex with a catalytical fragment of its nucleotide-exchange factor eEF1Bα to 1.7 Å resolution (Andersen *et al.*, 2000). We also determined the crystal structures of this exchange complex with bound GDP and GDPNP. This allowed for the first time a visualization of intermediates in nucleotide

exchange for any G protein (Andersen *et al.*, 2001). We are now extending these studies to the translocation factor eEF2. We show that the factor can be rapidly purified from industrial yeast and crystallized to give crystals suitable for structure determination. Furthermore, we have also produced a functional histidine affinity-tagged form of eEF2 that allows facilitated purification of the factor, especially of mutants suitable for functional studies.

2. Materials and methods

2.1. Purification of eEF2 from industrial yeast

All operations were performed at 277–279 K. Protein concentrations were measured with the Bradford reagent (Bio-rad). For purification of wild-type untagged eEF2, 0.1 kg of industrial yeast cake (Danisco) was resuspended in 200 ml of buffer *S* (20 m*M* HEPES–NaOH pH 7.2, 10% glycerol, 5 m*M* $MgCl_2$, 1 m*M* DTT) with 300 m*M* KCl (*S*-300). Immediately prior to lysis in a French press, protease inhibitors (1 m*M* PMSF and 3 mg of α_2-macroglobulin) were added. After lysis, the pH of the acidic lysate was readjusted to pH 7 by addition of 1 *M* untitrated Tris, centrifuged at 12 000 rev min^{-1} for 20 min and the resulting supernatant was centrifuged further at 50 000 rev min^{-1} in a Beckman TI60 rotor for 1 h. The high-speed supernatant (200 ml) was diluted with one volume of buffer *S* and dialysed overnight against 1.8 l of buffer *S* containing 0.1 m*M* PMSF. The dialysed lysate was centrifuged at 12 000 rev min^{-1} for 20 min and filtered through a 0.9 µm Versapor filter (Gelman) followed by a 0.45 µm syringe filter (Sartorius). The sample was then loaded onto a 30 ml S-Sepharose (Amersham-Pharmacia) column equilibrated in *S*-30 (buffer *S* containing 30 m*M* KCl). After washing with two to three volumes of *S*-30, the column was eluted overnight with a 240 ml linear gradient from *S*-30 to *S*-150. Pooled fractions were loaded directly onto a 9 ml Source-Q (Amersham-Pharmacia) column equilibrated in buffer *Q* (20 m*M* Tris–HCl pH 7.6, 10% glycerol, 5 m*M* $MgCl_2$, 1 m*M* DTT) with 40 m*M* KCl (*Q*-40) and eluted with a 90 ml linear gradient from *Q*-40 to *Q*-275. Fractions pooled from the Source-Q were diluted with two volumes of buffer *Q* and a maximum of 5 mg was loaded onto a 1 ml Uno-Q (Bio-rad) column. Essentially pure eEF2 was eluted with a 20 ml linear gradient from *Q*-40 to *Q*-200. The identity of the factor was confirmed by N-terminal sequencing of protein from two independent purifications.

2.2. Cloning and expression of histidine-tagged eEF2

In order to facilitate purification, a six-histidine tag was added to the C-terminus of *S. cerevisiae* eEF2. An *Nco*I restriction site (bold) was engineered into the 5′ oligonucleotide at the AUG (5′-CATG**CCATGG**-TTGCTTTCACTGTTG-3′) and six histidine codons were introduced just upstream of a UAA stop codon followed by an *Nco*I restriction enzyme site in the 3′ oligonucleotide (5′-CATG**CCATGG**TTAG-TGATGGTGATGGTGATGCAATTTGT-CGTAATATTCTTGCCAG-3′). The oligonucleotides were used to amplify the yeast eEF2 coding sequence from the *EFT2* gene on plasmid YCpEF2 (Justice *et al.*, 1998). The resulting fragment was digested with *Nco*I and cloned into plasmid pTKB328 (T. Kinzy and L. Valente, unpublished work), which is a CEN-based plasmid containing the yeast *LEU2* selectable marker and which directs expression off the *TEF5* promoter, resulting in constitutive expression. The resulting yeast expression plasmid pTKB612 was transformed into *S. cerevisiae* strain YEFD12h/pURA3-EFT1 (*MATa ade2 lys2 ura3 his3 leu2 trp1 eft1* Δ*:HIS3 eft2* Δ*:TRP1* pURA3-EFT1; Phan *et al.*, 1993) and loss of the wild-type EFT1 URA3 plasmid monitored by growth on 5-fluoroorotic acid (Boeke *et al.*, 1987). The resulting strain expressing only eEF2-6×His (TKY675) was monitored for altered growth at 286, 297, 303 and 310 K as described by Carr-Schmid *et al.* (1999). The same fragment was also cloned into pET11d (Clonetech). The resulting *Escherichia coli* expression plasmid pTKB613 was transformed into BL21 DE3 cells (Clonetech) and expression of eEF2-6×His monitored by Coomassie staining and Western blot analysis. To purify His-tagged eEF2 from yeast, 4 l of TKY675 were grown in YEPD to an A_{600} of 1.5. Cells were harvested by centrifugation, suspended in buffer *A* (50 m*M* potassium phosphate pH 7.6, 300 m*M* KCl, 1 m*M* DTT, 0.2 m*M* PMSF) with 10 m*M* imidazole (*A*-10) and lysed in a French press. After lysis, the pH of the lysate was adjusted to 7.7 with 1 *M* untitrated Tris and clarified by centrifugation at 12 500 rev min^{-1} for 20 min. The resultant supernatant was centrifuged at 50 000 rev min^{-1} in a Sorvall T-865 for 1 h; the resulting supernatant was filtered through a 0.22 µm filter and applied to a 1 ml Ni^{2+}-charged HiTrap Chelating Sepharose HP (Amersham-Pharmacia) column. After washing with five volumes of *A*-20 (buffer *A* containing 20 m*M* imidazole), the protein was eluted with *A*-250. The identity of the protein was confirmed by Western blot.

2.3. Crystallization of eEF2 in complex with GDP and data collection

Prior to crystallization, eEF2 was transferred to crystallization buffer (20 m*M* HEPES–NaOH pH 7.2, 75 m*M* KCl, 5 m*M* $MgCl_2$, 0.5 m*M* EDTA, 0.5 m*M* DTT, 1 m*M* GDP) and concentrated to 4.5 mg ml^{-1}. Crystallization trials were carried out at 277 K using the sitting-drop vapour-diffusion method by mixing 5 µl eEF2-GDP with 5 µl reservoir solution. Initial conditions were established through a crystallization screen (Hampton Research) and bundles of crystals were obtained in experiments with reservoirs containing 0.1 *M* HEPES pH 7.2, 5% ethylene glycol, 3 m*M* DTT, 0.9 m*M* NaN_3 and 7–9% PEG 8000. To obtain single crystals, the crystallization drops were streak-seeded from serial dilutions of crushed crystals 1 d after setup. Crystals appeared after 2–3 d and reached their final size within 3–4 weeks. Prior to data collection, crystals were harvested in stabilization buffer (7.5% PEG 8000, 5% ethylene glycol, 0.1 *M* HEPES–NaOH 7.2, 5 m*M* $MgCl_2$, 0.5 m*M* GDP, 0.5 m*M* DTT), rapidly transferred to cryo-protection buffer (three volumes of stabilization buffer and one volume of glycerol) and immediately flash-frozen in a nitrogen stream at 100 K. Data were collected at both the X-ray diffraction beamline (Elettra, Trieste) and the 711 beamline (Maxlab, Lund) both equipped with a MAR CCD detector. The data were reduced with the *HKL* package (Otwinowski & Minor, 1997).

3. Results and discussion

The described purification procedure for untagged eEF2 typically results in 10–12 mg suitable for crystallization (Fig. 1*a*). The procedure is simple and can be completed in less than 3 d. The most important step is the first cation-exchange column (S-Sepharose), which is very selective but requires that the KCl concentration after dialysis is well below 40 m*M*. If the first step is an anion-exchange column (Q-Sepharose), much less pure eEF2 is obtained (data not shown). Since industrial yeast cake is used, no time is spent on fermentation, which is very convenient for repeated purification of large quantities for structural studies.

The addition of a six-histidine tag to the C-terminus of eEF2 resulted in a protein able to function as the only form of the protein *in vivo* in a strain where both genomic copies of genes expressing eEF2 (*EFT1* and *EFT2*) are deleted (Fig. 2*a*). Analysis of growth of this strain at a variety of temperatures indicates that these cells grow essentially as wild-type cells at 302, 297 or 290 K and show a slight reduction in growth at 310 K (Fig. 2*a* and data not shown). The tagged protein could be purified directly from yeast on a single Ni^{2+}-affinity column (Fig. 2*b*), yielding approximately 0.33 mg of protein per litre of culture. The smaller fragments are degradation products of eEF2 as determined by Western blot analysis (data not shown). While the eEF2 coding sequence in the pET11d-based plasmid pTKB613 was identical to the sequence in the yeast expression vector, no expression in *E. coli* could be obtained under a variety of conditions (data not shown). The resulting yeast strain thus provides an efficient system to affinity purify functional eEF2, especially mutated eEF2 for functional and structural studies.

Single crystals of eEF2 in complex with GDP can reach maximum dimensions of $0.1 \times 0.4 \times 0.4$ mm (Fig. 1*b*), but in practice crystals of this size cannot be soaked in a cryoprotection buffer without cracking. Thin crystals no thicker than 0.02–0.03 mm can be soaked and medium-resolution data collected (Table 1). The self-rotation function and self-Patterson do not indicate the presence of more than one molecule in the asymmetric unit and this leads to a solvent content of 74% in the crystals. To obtain an unbiased experimental electron density, we have grown industrial yeast in a defined medium with selenomethionine (Bushnell *et al.*, 2001); total amino-acid analysis of eEF2 purified from such yeast cells indicate 80% substitution, which is significantly higher than the 65% reported by Bushnell *et al.* (2001). This substitution is likely to be sufficient to obtain experimental phases from anomalous data and we have also obtained small single crystals of selenomethionine-substituted eEF2. In contrast, attempts to determine the structure through molecular replacement with the structure of EF-G have so far not resulted in convincing solutions.

The crystals of eEF2-GDP are suitable for structure determination and such a structure will provide valuable information regarding the translocation process in eukarya and hence a comparison with other kingdoms may be performed. The structure of the prokaryotic homologue EF-G in the GDP state is known, but eEF2 contains large inserts. Furthermore, in a recent cryo-EM reconstruction of the yeast 80S ribosome in complex with eEF2, tRNA and sordarin, the eukaryotic factor appears to interact more extensively with both the ribosome and the P-site tRNA compared with similar reconstructions of 70S ribosomes in complex with EF-G. Domains 3, 4 and 5 of a docked molecule of EF-G representing eEF2 were also reorientated by 25° relative to domains 1 and 2 (Gomez-Lorenzo *et al.*, 2000) compared with the crystallographic structure of EF-G. The orientation of domains 3, 4 and 5 relative to 1 and 2 can also change in response to altered packing in crystals of EF-G (Laurberg *et al.*, 2000). The conformations of EF-G known from the crystal structures appear to be quite different from those observed in cryo-EM reconstructions of complexes between the 70S ribosome and EF-G (Stark *et al.*, 2000). Hence, the crystals of yeast eEF2 in complex with GDP may produce a significantly different conformation compared with that observed in crystals of EF-G in complex with GDP and this may be closer to those observed for both eEF2 and EF-G in complex with the 80S/70S ribosome.

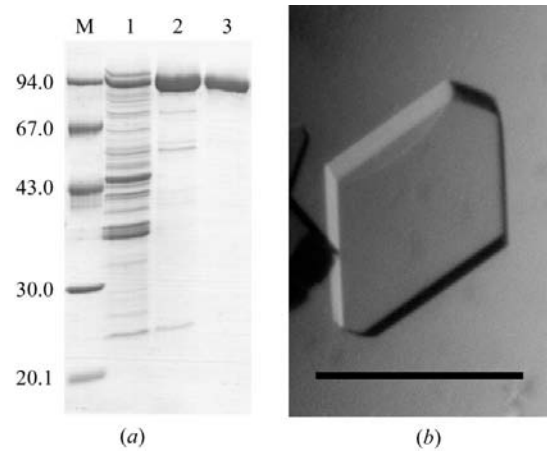

Figure 1
(*a*) Coomassie blue stained 12% SDS gel of pools from eEF2 purification. Lane M, molecular-weight markers in kDa; lane 1, pool from S-Sepharose column; lane 2, pool from Source-Q column; lane 3, pool from Uno-Q column. (*b*) A single crystal of eEF2 in complex with GDP. The scale bar represents 0.25 mm.

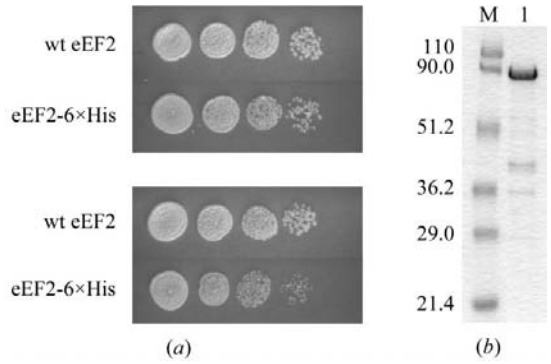

Figure 2
(*a*) Yeast strains YEFD12h/pURA3-EFT1 (WT eEF2) and TKY675 (eEF2-6×His) were grown overnight in YEPD to an A_{600} of 1.0, spotted as tenfold serial dilutions on YEPD medium and incubated for 2 d at either 303 (top panel) or 310 K (bottom panel). (*b*) Coomassie blue stained 10.8% SDS gel of eEF2-6×His. Lane M, molecular-weight markers in kDa; lane 1, pool from Ni-chelating column.

Table 1
Data-collection statistics at the 711 beamline at Maxlab, Lund.

The data were merged from a high-resolution sweep to 2.85 Å which contained overloaded reflections and a low-resolution sweep to 4.0 Å. The wavelength was 1.038 Å. Values in parentheses are for the outer resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters	a = 98.835, b = 114.546, c = 177.312
Unique reflections	47539
Resolution	25–2.85 (2.95–2.85)
Completeness	99.7 (99.4)
Redundancy	5.3 (4.1)
Mean $I/\sigma(I)$	39.1 (5.9)
Reflections with $I/\sigma(I) > 3$ (%)	83 (46)
$R_{\rm merge}$† (%)	5.4 (31.6)

† $R_{\rm merge} = (\sum_h \sum_{j=1,N} |I_h - I_h(j)|/\sum_N I_h)$ for the intensity of reflection h measured N times.

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