Disulfide bond formation is not involved in cap-binding activity of *Xenopus* translation initiation factor eIF-4E

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Received 1 April 1997; revised version received 30 April 1997

Abstract The eukaryotic initiation factor eIF-4E from *Xenopus laevis* was expressed in *Escherichia coli* and refolded in an active form. To define the cysteine residues forming a disulfide bond in *Xenopus* eIF-4E, each of the 3 cysteine residues was changed to serine by site-directed mutagenesis. Cap-binding activities of the mutant proteins were evaluated by 7-methyl-GTP(m^7GTP)-affinity column chromatography. Even the mutant protein containing no cysteine showed an affinity for m^7GTP. From the above results and the estimation of the sulfhydryl groups by Ellman's assay method, we concluded that a disulfide bond is not involved in the active *Xenopus* eIF-4E.

Key words: Translation initiation factor; eIF-4E; *Xenopus laevis*; Site-directed mutagenesis; Disulfide bond

1. Introduction

Most of the eukaryotic mRNAs have the m^7G-containing cap structure at their 5′ termini [1]. eIF-4E is the only initiation factor thus far demonstrated to interact with the cap as an isolated polypeptide. eIF-4E also binds to eIF-4G which is anchored to ribosome through other initiation factors [2,3]. At the initiation step of translation, it is thought that the protein-protein interaction between eIF-4E and eIF-4G brings the mRNA to the ribosome.

The primary sequence of eIF-4E from a number of species has been determined [4–11]. However little is presently known about the secondary and tertiary structures of eIF-4E. One of the conserved structural features among the mammalian eIF-4Es is the presence of four Cys residues. It is suggested that one pair of the Cys residues forms a disulfide bond [12]. *Xenopus* eIF-4E is highly homologous to the mammalian counterparts whereas it has only 3 cysteine residues [10]. Recent report showed that 2 of the 3 Cys residues are also conserved in *Drosophila* eIF-4E [11].

In this study, to determine whether a disulfide bond was involved in the active *Xenopus* eIF-4E, the mutant proteins, in which Cys residues were replaced by Ser residues, were expressed in *E. coli*, and their structure and cap-binding activity were studied.

2. Materials and methods

2.1. Materials

The cDNA for *Xenopus* eIF-4E was described previously [10]. Restriction enzymes, T4 DNA ligase, *Bca*BEST dideoxy sequencing kit and *Takara Ex Taq* were purchased from Takara Shuzo Co. Ltd. QIAEXII gel extraction kit was from Qiagen. 7-Methyl-GTP Sepharose-4B was from Pharmacia Biotech. 7-Methyl-GTP and L-cysteine were obtained from Sigma Chemical Co. Centriprep-30 was purchased from Amicon. Protein Assay Kit was from Bio-Rad Laboratories.

2.2. Construction of expression vector

The plasmid containing the cDNA for *Xenopus* eIF-4E [10] was used as a template to amplify the coding region. The primers, 5′-TTTGGTACCATGGCGGCCGTGGAACCGGA-3′ (5′ *Nco*I) and 5′-CCTTTGGATCCTCGAGATTGCTTGACGCAGTCTCCT-3′ (3′XB) were used for the amplification to introduce *Nco*I site at the initiation codon and *Bam*HI site at position 50 bp downstream of the termination codon. The PCR reaction was performed for 8 cycles using 0.2 μg of the template and 100 pmol each of the primers. Amplified DNA was cloned into pUC19 vector and sequenced using *Bca*BEST dideoxy sequencing kit on Hitachi automated DNA sequencer WS-10A to confirm the integrity. The DNA was digested with *Nco*I and *Bam*HI. The digested product was separated by electrophoresis in 1% agarose gel followed by recovering with QIAEXII gel extraction kit (QIAGEN). The purified DNA was ligated into pET11d vector (Novagen) that had been linearized with *Nco*I and *Bam*HI to make pETX4E.

Site-directed mutagenesis was performed by the recombinant PCR technique using the above and following primers: 5′-TAATGTCCGGA TCTGACTATTCACTC-3′ (C85S(+)), 5′-GAGTGAATAGTCAGATCCGGACATTA-3′ (C85S(−)), 5′-AGACGCTCATGTCCCTTATTGGAGAG-3′ (C132S(+)), 5′-CTCTCCAATAAGGGACATGAGCGTCT-3′ (C132S(−)), 5′-GCGACGATGTATCTGGCGCAGTTGTA-3′ (C146S(+)), 5′-TACAACTGCGCCAGATACATCGTCGC-3′ (C146S(−)). Fig. 1 shows the strategy of the recombinant PCR.

2.3. Expression, refolding and purification of eIF-4E

E. coli BL21(DE3) transformed with the eIF-4E expression vector pETX4E was cultured in LB medium containing 50 μg/ml ampicillin at 37°C. Expression of eIF-4E was induced with 0.4 mM IPTG when the cells reached a density of 0.5 (A_{600}). The induced cells were incubated for an additional 16 h and harvested by centrifugation. This and all subsequent procedures were carried out at 4°C or on ice. The cells were disrupted by sonication and centrifuged. The expressed protein was recovered as an inclusion body in the pellet fraction.

The method for the refolding of denatured protein was described previously [13]. The pellet containing 2 mg of eIF-4E protein was dissolved in 10 ml of solution A (20 mM HEPES/KOH, pH 7.5, 20 mM dithiothreitol, 1 mM EDTA, 6 M guanidine-hydrochloride) and incubated on ice for 2 h with occasionally shaking. The protein solution was diluted with 100 ml of solution B (20 mM HEPES/KOH, pH 7.5, 1 mM EDTA, 3 mM glutathione, 0.3 mM glutathione disulfide, 1.5 M KOAc) and incubated at 4°C for 12 h. Then the protein solution was diluted with solution C (20 mM HEPES/KOH, pH 7.5, 1 mM EDTA) to achieve a final concentration of 150 mM KOAc and applied to a 0.5 ml 7-methyl-GTP-Sepharose 4B column. The column was washed with 50 ml of solution D (20 mM HEPES/KOH, pH 7.5, 1 mM EDTA, 100 mM KCl). The refolded eIF-4E was eluted with 0.1 mM m^7GTP in solution D and concentrated by Centriprep-30 (Amicon). Protein concentration was determined by the Bradford method using Protein Assay Kit (Bio-Rad).

2.4. Purification of eIF-4E from Xenopus oocytes

The excised ovary was washed with solution A (described in the

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PII S0014-5793(97)00562-0

previous section) and homogenized with a Dounce-type homogenizer in the presence of equal volume of solution A containing 0.5 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, 0.1 mg/ml soybean trypsin inhibitor. The homogenate was centrifuged at 10 000×*g* for 20 min at 4°C. The clear middle layer was collected in a new tube and centrifuged again. Then the clear lysate was centrifuged at 220 000×*g* for 2 h at 4°C. The supernatant was subjected to 7-methyl-GTP-Sepharose 4B column. Native *Xenopus* eIF-4E was eluted with m^7GTP as described in the previous section.

2.5. Circular dichroism measurements

CD measurements were performed on a JASCO J-720 circular dichroism spectrophotometer. The light path length of the cell was 1 mm. The cell was maintained at 20°C using a circulating water bath. The samples were prepared in 20 mM Tris-HCl (pH 7.5) buffer containing 100 mM KCl.

2.6. Assay for free sulfhydril groups

Sample was dissolved in solution E (0.1 M Tris-HCl (pH 8.0), 10 mM EDTA, 6 M guanidine-hydrochloride). Free sulfhydril groups were assayed by Ellman's assay method [14].

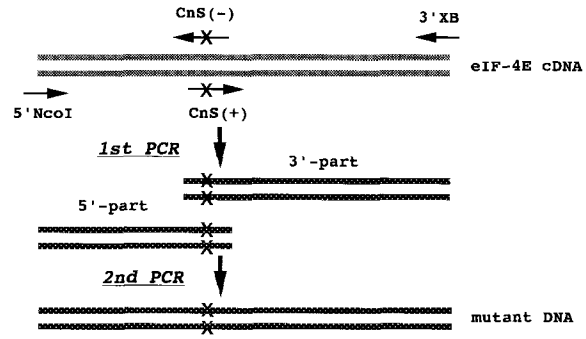

Fig. 1. Strategy of the recombinant PCR. The bold lines are the eIF-4E DNAs. The arrows indicate the PCR primers. Two steps of PCR reactions were performed. First, eIF-4E DNA was amplified dividing into two parts using 5′ *Nco*I and C*n*S(−) primers (*n* indicates 85, 132 or 146) for the 5′ part, C*n*S(+) and 3′ XB primers for the 3′ part. Second, the entire region of the mutant eIF-4E DNA was amplified between 5′ *Nco*I and 3′ XB using the first PCR products as templates. The resultant fragment was cloned into pUC19 and confirmed the sequence.

3. Results and discussion

We wished to determine whether a disulfide bond was present in *Xenopus* eIF-4E. Fig. 2 shows the alignment of the amino acid sequence of eIF-4Es from various species. In the case of mammalian eIF-4Es, 4 residues of cysteine (Cys) are conserved [5–7]. And it is suggested that one pair of Cys residues forms a disulfide bond [12]. *Xenopus* eIF-4E is highly homologous in its amino acid sequence with mammalian counterparts [10]. However, it has only 3 Cys residues (Cys85, Cys132, Cys146). Cys132 and Cys 146 are also conserved in *Drosophila* eIF-4E [11]. So we first assumed that these 2 Cys residues form a disulfide bond.

To facilitate studies on *Xenopus* eIF-4E, we constructed an overexpression system of *Xenopus* eIF-4E in *Escherichia coli*. We have two different cDNAs for *Xenopus* eIF-4Es which encode products of 213 and 231 amino acids [10]. These clones differ by a 54-nt segment, which is present in one copy in the shorter clone, but in two copies in the longer clone. In this paper we used the shorter clone.

The cDNA was placed into an IPTG-inducible *E. coli* expression vector pET-11d and the protein was expressed in *E. coli* BL21(DE3). Analysis by SDS-PAGE and staining with Coomassie Blue shows that the expressed eIF-4E was in the insoluble fraction. The expressed eIF-4E was refolded as described in the materials and methods section and applied to m^7GTP-affinity chromatography followed by eluting with m^7GTP. Analysis of the amino-terminus sequence and the

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Xenopus                                    MAAVE PENTN-PQS- -TEEEK--ET GQEIVSP--- ----------
Human                                      **T** **T*PT*NPP T*****-T*S N**VAN*--- ----------
Rabbit                                     **T** **T*PT*NPP PA****-T*S N**VAN*--- ----------
Mouse                                      **T** **T*PTTNPP PA****-T*S N**VAN*--- ----------
Drosophila  MQSDFHRMKNFANPKSMFKTSAPSTEQGRP* *PTS-AAAPA EAKDV*PK*D P**TGE*AGN TATTTAPAGD

                                                                          85
-----DQYIK HPLQNRWALW FFKNDKSKTW QANLRLISKF DTVEDFWALY NHIQLSSNLM SGCDYSLFKD GIEPMWEDEK
-----EH*** ********** ********** ********** ********** ********** p********* **********
-----EH*** ********** ********** ********** ********** ********** p********* **********
-----EH*** ********** ********** ********** ********** ********** p********* **********
DAVRTEHLY* ***M*V*T** YLE**R**SW EDMQNE*TS* *******S** ***KPP*EIK L*S******K N*R*****AA
                          132                 146                 166
NKRGGRWLIT LNKQQRRNDL DRFWLETLMC LIGESFDEHS DDVCGAVVNV RAKGDKIAIW TTEFENKDAV THIGRVYKER
*N******** *******S** ********I* *******DY* ********** ********** ***C**RE** **********
********** *******S** ********I* *******DY* ********** ********** ***C**RE** **********
********** *******S** ********I* *******DY* ********** ********** ***C**R*** **********
**Q****V** ***SS-KTDL *NL**DV*I* ****AF*N-* *QI*****I*I *G*SN**S** *ADGN*EEAA LE**HKLRDA

LGLPAKVVIG YQSHADTATK SGSTTKNRFV V
**F*P*I*** ********** ********** *
**P*P*I*** ********** ********** *
****P*I*** ********** ********** *
*R*GRNNSLQ **L*K*TMV* Q**NV*SIYT L
```

Fig. 2. Alignment of the amino acid sequences of eIF-4Es from *Xenopus* [10], human [5], rabbit [7], mouse [6] and *Drosophila* [11]. Identical and absent amino acid residues are indicated with asterisks and hyphens, respectively. Cysteine residues are boxed.

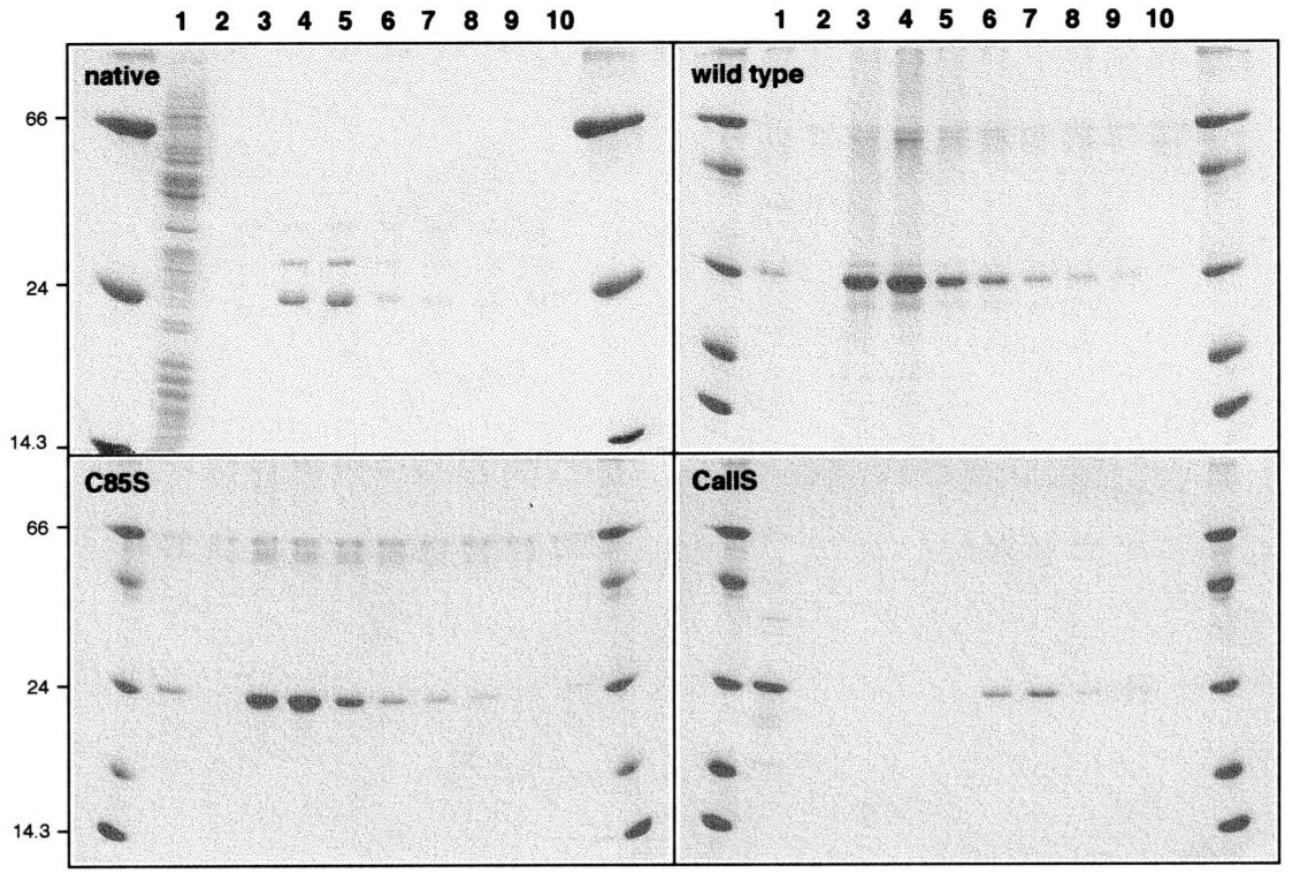

Fig. 3. Cap-binding activities of the native eIF-4E and the recombinant proteins. Each protein was applied to m^7GTP-affinity chromatography. Equal aliquot of the following fraction was subjected to 14% SDS-PAGE. Lanes: 1, flow through; 2, washing step; 3–10, elution with solution D (20 mM HEPES/KOH, pH 7.5, 1 mM EDTA, 100 mM KCl) containing 0.1 mM m^7GTP. The bands in both outsides of the gel in each column are the molecular weight marker proteins. The numbers indicated at left side of the columns are the molecular weight of the marker proteins.

amino-acid composition indicated that the purified protein was *Xenopus* eIF-4E starting with Ala next to the initiation Met (data not shown).

Site-directed mutagenesis was used to convert Cys85 to Ser. The mutant protein was expressed and refolded in the same manner as used in the case of wild-type eIF-4E. Cap-binding activity of the mutant protein was evaluated by m^7GTP-affinity chromatography. The mutant protein was specifically eluted with m^7GTP from the affinity column as shown in Fig. 3. CD spectra of native *Xenopus* eIF-4E, wild-type recombinant eIF-4E and the mutant (C85S) protein were measured (Fig. 4). All spectra showed similar pattern characterized by a minimum near 207 nm and a broad shoulder beginning around 215 nm. At present we do not know the cause of the difference between the recombinant proteins and the native protein in the region of 230–250 nm. We focused on the region of 200–230 nm because the signals derived from typical secondary structures such as α-helix and β-sheet appear in this

Table 1
Estimation of the free SH groups by Ellman's assay method

	No. of Cys	No. of SH group (mol/mol protein)
Native	3	2.9
Wild type	3	3.1
C85S mutant	2	2.4

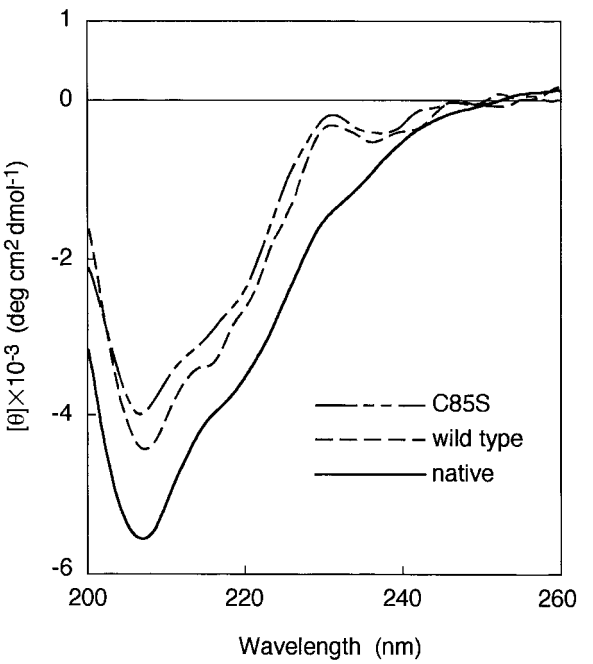

Fig. 4. CD spectra for the native, the wild-type and the C85S mutant eIF-4Es in 20 mM Tris-HCl, pH 7.5, 100 mM KCl.

region. In this region, the spectra resemble that of eIF-4E from Saccharomyces cerevisiae [15]. The difference of [θ] value between native and the recombinant proteins would be caused by the error of the estimation of the amount of native eIF-4E. Native eIF-4E fraction contained the isoform of *Xenopus* eIF-4E (Fig. 3 upper band) [10,16].

The contents of the sulfhydryl groups were assayed by Ellman's assay method [14] for the native, the wild-type and the mutant (C85S) eIF-4Es. As shown in Table 1, the results suggested that all of the Cys residues were presented as sulfhydryls.

Then all the Cys residues were converted to Ser residues to prepare a mutant eIF-4E with no Cys residue, CallS. Actually this mutant protein has a cap-binding activity as shown in Fig. 3. However, the recovery of the cap-binding protein was very low through 3 separate experiments. It is possible that this mutant protein is not refolded efficiently in this condition. Another possibility is that these Cys residues (Cys132, Cys146) are involved in cap-binding pocket.

Consequently, we conclude that a disulfide bond is not formed in *Xenopus* eIF-4E. The contribution of Cys residues to cap-binding activity should be studied in more detail.

Acknowledgements: We are grateful to Professor T. Sakai (Nagoya City University) for his valuable advice on refolding the expressed protein. We also thank K. Tanizawa and T. Hirai for their technical assistance. This work was supported by grant from New Energy and Industrial Technology Development Organization (NEDO).

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