Zinc is required for structural stability of the C-terminus of archaeal translation initiation factor aIF2β

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Abstract aIF2β is an archaeal homolog of eukaryotic translation factor eIF2β necessary for translation initiation and involved in recognition of the initiation codon. In the present study, we demonstrate for the first time zinc binding to the C_2–C_2 zinc finger at the C-terminus of aIF2β. Nuclear magnetic resonance backbone assignments were also determined and the secondary structural elements identified. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: aIF2β; Translation initiation; Zinc finger; RNA binding; Secondary structure; Nuclear magnetic resonance

1. Introduction

The zinc finger is an important structural motif first described in the *Xenopus laevis* transcription factor TFIIIA [1] and other nucleic acid binding proteins such as ADR1 [2], Xfin [3] and SW15 [4]. It is estimated that about 1% of all mammalian genes encode zinc fingers [5]. The zinc finger is a simple structure consisting of a short polypeptide loop folded back on itself with the aid of a metal ion [6]. Zn^{2+} is tetrahedrally coordinated by different combinations of histidine and cysteine.

Archaeal translation initiation factor aIF2 is a heterotrimeric protein consisting of α, β and γ subunits. By analogy to their highly similar (≥48%) eukaryotic counterparts, the function of aIF2 is believed to be the formation of a ternary complex with GTP and aminoacylated initiator methionyl-tRNA (Met-$tRNA_i^{Met}$) which then binds to the small ribosomal subunit [7]. With the aid of other factors, this complex scans from the 5′ end of mRNA to the first initiation codon, GTP is then hydrolyzed and the aIF2-GDP complex is released. In order to start a new cycle of translation initiation, GDP bound to aIF2 must be exchanged for GTP in a reaction catalyzed by the guanine nucleotide exchange factor aIF2B [8,9]. Phosphorylation of aIF2α regulates the exchange rate of GDP to GTP [10]. The γ subunit contains GTP binding motifs as well as sequences that have been involved in tRNA binding [11]. In archaea, the β subunit is the most divergent of the three because it comprises only the C-terminal half of eIF2β which contains a zinc finger domain involved in RNA binding [12]. The structure of eIF2α has been recently reported [13]

The conserved residues in or near the C_2–C_2 finger motif of aIF2β have been subjected to extensive mutational analysis in yeast eIF2β showing that the cysteine residues in this motif are essential for eIF2β function [14]. Elimination of only one of them, alteration of their spacing or the elimination of the complete motif results in inactivation of the protein. Some of the mutations found in yeast eIF2β are the sites of missense mutations that lead to translation initiation at non-AUG codons [14,15]. Some of the mutants also display an intrinsic GTPase activity [16]. Surprisingly, neither binding of zinc nor a functional requirement for zinc has been demonstrated for aIF2β or eIF2β [8,9,17,18]

In the present study, we demonstrate for the first time that zinc is an important factor for the stability of the C_2–C_2 motif at the C-terminus of archaeal IF2β form. This suggests that zinc may play a role in the initiation of translation in both eukaryotes and archaebacteria. In addition, complete nuclear magnetic resonance (NMR) chemical shifts as well as the identification of the secondary structural elements for aIF2β are reported.

2. Materials and methods

2.1. Protein expression and purification

Initiation factor aIF2β from *Methanobacterium thermoautotrophicum* (gene MTH1769) was subcloned into pET15b (Novagen, Madison, WI, USA) and expressed in *Escherichia coli* BL21 as an oligohistidine (His tag) fusion protein of 161 residues. Cells were grown at 37°C to an OD_{600} of 0.8 and induced with 1 mM IPTG. Afterwards, the temperature was reduced to 30°C and the cells were allowed to express the protein for 3 h before harvesting. The media used were either Luria broth (LB) or minimal salts (M9) media containing [^{15}N]ammonium chloride and/or [^{13}C]glucose (Cambridge Isotopes Laboratory, Andover, MA, USA) and 50 μM $ZnCl_2$. aIF2β was purified by heat denaturation of endogenous *E. coli* proteins and affinity chromatography on Ni^{2+}-loaded chelating Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The N-terminal His tag was cleaved from aIF2β by treatment for 24 h at room temperature with thrombin (Amersham Pharmacia Biotech) at 1 U/mg fusion protein. Benzamidine Sepharose was used to remove thrombin.

2.2. Mass spectrometry

ESI/MS analyses were performed utilizing a Perkin Elmer, API III spectrometer. aIF2β was purified as above and dialyzed extensively against 10% acetic acid. For protein grown in M9 medium, 1 Da per

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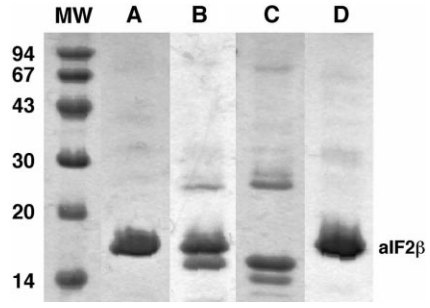

Fig. 1. Proteolytic sensitivity of aIF2β in the absence of Zn^{+2}. A: SDS gel of aIF2β purified from LB culture. The protein migrates as a single band and has the expected size of 16.2 kDa. B: Protein purified from M9 minimal medium revealing a proteolytic fragment about 20 residues smaller than full-length aIF2β. C: Cleavage of the N-terminal His tag demonstrating that the heterogeneity is C-terminal. D: aIF2β purified from M9 minimal medium supplemented with 50 μM $ZnCl_2$ showing the enhanced the stability of aIF2β against endogenous *E. coli* proteases.

nitrogen was subtracted from the measured mass to account for the ^{15}N isotope enrichment.

2.3. Cobalt visible spectrophotometry

Ultraviolet and visible light spectra of cobalt–aIF2β complexes were obtained on a Cary 100 UV-visible spectrophotometer at 20°C. The spectrum was scanned at 600 nm/min from 800 to 200 nm with a 1 cm path length. Data points were collected each 2 nm. The sample was 0.6 mg/ml protein in 50 mM phosphate buffer, 150 mM NaCl, 2 mM dithiothreitol (DTT) at pH 6.0. $CoCl_2$ was added from a 1 mM stock solution to final concentrations of 80, 160 and 240 μM. The protein was grown on LB medium and purified as described. Samples was dialyzed extensively against buffer containing 1 mM EDTA, to remove bound zinc, and dialyzed again against buffer to get rid of EDTA.

2.4. NMR spectroscopy

All NMR spectra were recorded at 310 K on Bruker DRX500 and Varian UNITYplus 800 MHz spectrometers. Chemical shifts were measured to internal sodium 2,2-dimethyl-silapentane-5-sulfonate for ^{1}H and calculated for ^{13}C and ^{15}N, assuming $\gamma^{15}N/\gamma^{1}H = 0.101329118$ and $\gamma^{13}C/\gamma^{15}H = 0.251449530$ [19]. Spectra were processed using GIFA and XWINNMR software (Bruker Biospin) and analyzed with XEASY [20]. The assignments of ^{1}H, ^{15}N and ^{13}C resonances were based on the following experiments: CBCA(CO)NH, HNCACB, HNHA, ^{15}N-edited TOCSY, ^{15}N-edited NOESY and HCCH-TOCSY. The secondary structure of aIF2β was analyzed using proton and carbon chemical shifts [21] and identification of characteristic NOEs. NMR samples were ~1.0 mM protein in 50 mM Bis–Tris buffer, 0.30 M NaCl, 50 μM $ZnCl_2$, 1 mM DTT, 0.02% (w/v) sodium azide at pH 6.0.

3. Results and discussion

The first evidence that zinc is important for the stability of aIF2β came from differences in the size of aIF2β when produced in *E. coli* grown in LB and M9 media. SDS–PAGE of the His tag fusion protein from an LB culture showed the presence of a single band with the expected molecular size of 16.2 kDa (Fig. 1). In contrast, an additional band, smaller by ~20 residues, was observed for protein purified from M9 cultures. This pointed to nutrient deficiency as a probable cause. Cleavage of the N-terminal histidine tag showed the same pattern as the uncleaved form, demonstrating that the degradation was at the C-terminus. As the predicted zinc finger motif for aIF2β is also located at the C-terminus (Fig. 2), this suggested that zinc was possibly the missing nutrient. Supplementation with 50 μM $ZnCl_2$ allowed us to obtain full-length aIF2β from minimal medium. Mass spectrometry revealed cleavage sites at L121 and L120 (Fig. 3). As proteolytic digestion of proteins in the absence of their metal ligand has been observed previously for other metalloproteins [22], we conclude that the absence of zinc causes the C-terminus of aIF2β to unfold, making it susceptible to cleavage by endogenous proteases.

To test the metal binding properties of aIF2β, visible spectra of aIF2β in the presence and absence of Co^{2+} were recorded. Cobalt substitution for zinc has been commonly used to address the coordination environment of structural zinc sites in proteins [23]. Fig. 4 shows the visible spectrum of a cobalt–aIF2β adduct. The spectrum contains an absorbance maximum at 730 nm which is characteristic of tetrahedral coordination of Co^{2+} [24].

The importance of zinc for folding of the C-terminus was clearly seen in ^{15}N–^{1}H HSQC spectra of aIF2β with and without zinc (Fig. 5). Upon addition of $ZnCl_2$ to the protein, several amide resonances shifted away from the overlapped amide resonances between 7.5 and 8.5 ppm in the proton dimension. These spectral changes indicate acquisition of additional folded structure in the presence of zinc. The dependence of zinc binding on the oxidation state of the cysteines was tested by recording ^{15}N–^{1}H HSQC spectra in the absence of DTT. No spectral changes were observed upon addition of zinc to oxidized aIF2β (data not shown). The assignments of

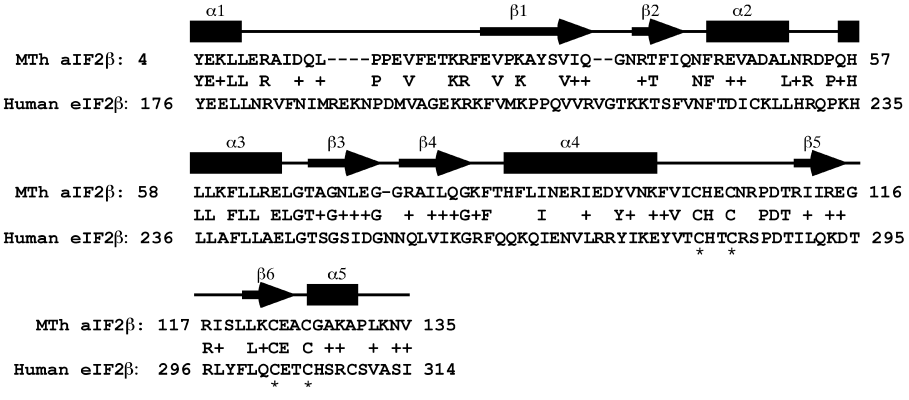

Fig. 2. Predicted secondary structure of *M. thermoautotrophicum* aIF2β and sequence alignment with human eIF2β. The positions of secondary structural elements in aIF2β are shown along with conserved residues and the four cysteines in the C_2–C_2 zinc finger motif (asterisks).

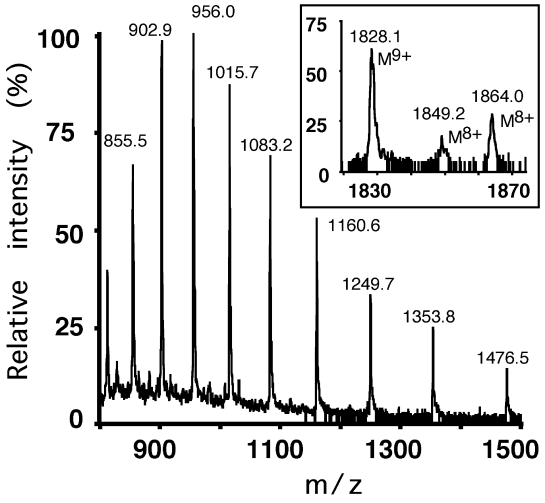

Fig. 3. ESI/MS mass spectrum of aIF2β grown in LB showing a single form of 16234.30 Da. Inset: Spectrum of protein grown in M9 medium (without zinc) showing the presence of fragments cleaved after residues L120 and L121 (M^{8+} peaks at 1849 *m/z* and 1864 *m/z*) in addition to the full-length product (M^{9+} peak at 1828 *m/z*).

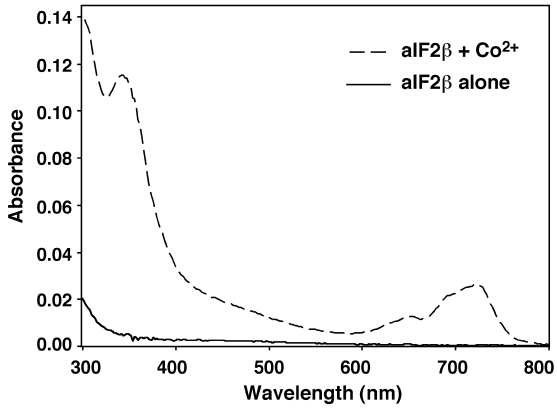

Fig. 4. Visible absorption spectra of aIF2β with and without Co^{2+}. In the absence of $CoCl_2$ aIF2β (45 μM) is colorless (continuous line). Addition of 80 μM $CoCl_2$ led to an absorption peak at 730 nm, which is distinctive of tetrahedrally complexed cobalt (dashed line). Further additions of $CoCl_2$ caused no additional changes in the absorption spectrum.

the backbone resonances of the zinc-bound aIF2β reveal that most of the zinc-shifted peaks are from the putative C-terminal C_2-C_2 zinc binding motif.

Knowledge of the H^{α}, C^{α} and C^{β} chemical shifts was used to determine the secondary structure of aIF2β (Fig. 2). It is an α/β protein with five α-helices and six β-strands. It has been clearly demonstrated for other zinc fingers that Zn^{2+} is tetrahedrally bound [25,26] and that, for C_2–C_2 motifs, the cysteines are responsible for the Zn^{2+} coordination [27]. Based on the secondary structure of aIF2β, the identification of medium-range NOEs and the location of the cysteine residues, it is possible to predict the structure of its zinc finger. Fig. 2 shows that the zinc finger motif consists of a loop containing C102 and C105 followed by a β-hairpin between strands 5 and 6, and a short helix containing C126. This result contrasts with the classical zinc finger motif which is composed of a β-strand and an α-helix. Chemical shift information have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under accession number 5202. Determination of the three-dimensional solution structure of aIF2β is currently in progress in our laboratory and will be described in detail elsewhere.

Zinc is essential for the structural stability of known zinc fingers, so it was surprising that given the high conservation of the cysteines in aIF2β and eIF2β, the importance of this metal for IF2β structure or function has not been previously demonstrated. Several mutagenesis studies have shown the importance of residues at the C-terminus for the function of yeast eIF2β [15]. Especially interesting are the non-conservative substitutions R248T, R253I, R253S and L254P (corresponding to R114, R117 and I118 of the archaeal protein) as they are located at the tip of the zinc finger and allow translation to initiate at UUG codons instead of AUG. This region is strongly conserved. We believe that these residues play an important role in the specific recognition of the Met-$tRNA_i^{Met}$–AUG duplex. If true, the zinc finger would also constitute an important structural element signal for the release of the initiation complex. Determination of the three-dimensional structure of aIF2β should help test this and other models of aIF2β function.

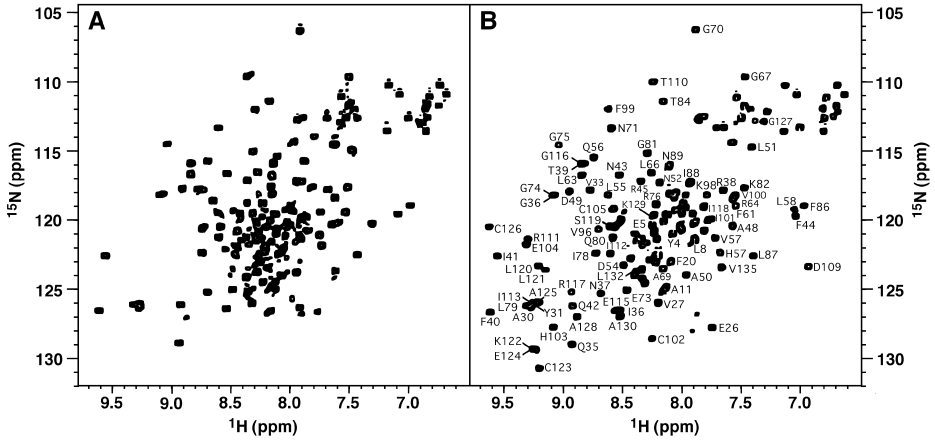

Fig. 5. Zn^{2+} binding to the C-terminus of aIF2β. A: ^{15}N–^{1}H HSQC spectrum in the absence of Zn^{2+}, showing random coil amide chemical shifts or missing signals for most of the C-terminal residues. B: Spectrum in the presence of Zn^{2+}, showing greater dispersion with additional downfield peaks characteristic of β-strands.

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