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Binding of translation elongation factors to individual copies of the archaeal ribosomal stalk protein aP1 assembled onto aP0

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ABSTRACT

Ribosomes in all organisms contain oligomeric and flexible proteins called stalks, which are responsible for the recruitment of translational GTPase factors to the ribosome. Archaeal ribosomes have three stalk homodimers $(aP1)_2$ that constitute a heptameric complex with the anchor protein aP0. We investigated the factor binding ability of aP1 proteins assembled onto aP0, by gel-retardation assays. The isolated $aP0(aP1)_2(aP1)_2(aP1)_2$ complex, as well as the form bound to the *Escherichia coli* 50S core, as a hybrid 50S particle, interacted strongly with elongation factor aEF2, but weakly with aEF1A. These interactions were disrupted by a point mutation, F107S, at the C-terminus of aP1. To examine the ability of each copy of aP0-associated aP1 to bind to elongation factors, we constructed aP0·aP1 variant trimers, composed of an aP0 mutant and a single $(aP1)_2$ dimer. Biochemical and quantitative analyses revealed that the resultant three trimers, $aP0(aP1)^{I}_2$, $aP0(aP1)^{II}_2$, and $aP0(aP1)^{III}_2$, individually bound two molecules of aEF2, suggesting that each copy of the aP1 C-terminal region in the aP0·aP1 trimers can bind tightly to aEF2. Interestingly, the unstable binding of aEF1A to each of the three aP0·aP1 trimers was remarkably stabilized in the presence of aEF2. The stability of the aEF1A binding to the stalk complex may be affected by the presence of aEF2 bound to the complex, by an unknown mechanism.

1. Introduction

The ribosomal interactions with two elongation factors are pivotal events for translation elongation. These interactions strictly depend on the presence of multiple copies of a flexible ribosomal protein on the large subunit, the so-called “stalk” [1,2], which is responsible for the recruitment of GTP-bound forms of elongation factors to the ribosome and the accompanying GTP hydrolysis at the sarcin/ricin loop (SRL) region of the large subunit [3,4]. The stalk proteins are designated as L12 (or bL12) in bacteria, P1/P2 in eukaryotes [5], and aP1 in archaea [6]. In all cases, the stalk proteins form dimers through interactions between their N-terminal regions [7], and two or three stalk dimers bind to their respective stalk base proteins: bacterial L10 [2,8], eukaryotic P0 [9], and archaeal aP0 [6]. These bindings occur through interactions between the N-terminal dimerization regions of the stalk and the C-terminal helical regions of the stalk base proteins [2,10]. A comparison of the primary structures indicated a close evolutionary relationship between the archaeal aP1 and eukaryotic P1/P2 stalks [11]. In contrast, there is little structural similarity between the bacterial and archaeal/eukaryotic stalk proteins, despite their functional similarity [7,11].

In the case of bacterial L12, the C-terminal 70 amino acid residues form a globular structure (CTD), which has long been implicated in factor binding [7,12–14]. Our recent biochemical study demonstrated that the isolated archaeal stalk aP1 protein binds to the translation factors, aEF1A and aEF2, in a nucleotide (GTP/GDP)-independent manner, and that three hydrophobic amino acid residues at the C-terminal end of aP1 are responsible for the factor binding [6]. More recently, we determined the crystal structure of the complex between aEF1A and the C-terminal fragment including residues 83–108 of aP1 [15]. This structure revealed that the long extended α-helix of the C-terminal fragment bound to the cleft between domains 1 and 3 of aEF1A. In contrast, an NMR study of the human P1-P2 stalk dimer showed that the C-terminal halves of P1/P2 are unstructured and can extend up to 125 Å away from the dimerization domains [16]. These lines of evidence suggest that,

Abbreviations: aEF1A, archaeal elongation factor 1A; aEF2, archaeal elongation factor 2; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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unlike bacterial L12, the archaeal/eukaryotic stalk does not form a stable domain structure in the C-terminal region, although a helical C-terminal structure is induced by factor binding.

One of the common features shared by the stalk proteins of the three domains of life is their oligomeric state: the bacterial pentameric L10(L12)$_2$(L12)$_2$ [8] or heptameric L10(L12)$_2$(L12)$_2$(L12)$_2$ [2], the archaeal aP0(aP1)$_2$(aP1)$_2$(aP1)$_2$ heptamer [10], and the eukaryotic P0(P1·P2)(P1·P2) pentamer [17]. The functional significance of these oligomeric forms of the stalk has been suggested to be the enhancement of factor recruitment to the ribosome [2,10]. However, it remains unknown whether all of the copies of the stalk in the complex participate equally in binding to translation factors. In the present study, we prepared *Pyrococcus horikoshii* aP1, aP0, and their mutants, and reconstituted various stalk complex variants including complexes composed of aP0 and one of the three aP1 dimers. The experimental results suggested that all copies of the C-terminal region of aP1 associated with aP0 have efficient ability to bind aEF2, and that aEF1A binding to the aP0·aP1complex is stabilized in the presence of aEF2.

2. Materials and methods

2.1. Plasmid construction and protein expression

The gene encoding *P. horikoshii* ribosomal protein aP0 was cloned into the *E. coli* expression vector pET28c (Novagen), as described previously [18]. By site-directed mutagenesis, two (aP1)$_2$ binding sites among the three sites, I, II, and III, in aP0 (see Fig. 2A) were disrupted in three combinations, as described previously [10]. The resultant aP0 mutant genes were used for the expression of aP0 mutants to constitute three aP0·aP1 variant trimers, aP0(aP1)$_2^{\mathrm{I}}$, aP0(aP1)$_2^{\mathrm{II}}$, and aP0(aP1)$_2^{\mathrm{III}}$ (see Fig. 2A). For some experiments, the same mutations were also introduced into an aP0 mutant, aP0(F341S), which lacks the factor binding ability of aP0. The *P. horikoshii* aP1 gene and its point mutant aP1(F107S), lacking the factor binding ability, were cloned into pET28c [6]. *P. horikoshii* elongation factors aEF1A, His-tagged aEF1A, and aEF2 were cloned into pET22b (Novagen) as described previously [6,15], and a Flag-tag was introduced at the C-terminus of aEF2. Individual proteins were expressed by *E. coli* cells.

2.2. Formation and purification of stalk complexes

The expressed aP0, aP1, and their mutants were purified as described [18], except that conditions without urea were used. The aP0·aP1 complexes were reconstituted by mixing isolated aP0 and aP1 samples at a molar ratio of 1:8 (for WT heptamer, see Fig. 2A) or 1:2.4 (for aP0(aP1)$_2^{\mathrm{I}}$, aP0(aP1)$_2^{\mathrm{II}}$, and aP0(aP1)$_2^{\mathrm{III}}$ trimers) and heated at 70 °C for 10 min, as described [4]. *P. horikoshii* aEF1A, aEF2 and Flag-tagged aEF2 were purified as described [6]. His-tagged aEF1A was purified as described [15].

2.3. Ribosomal 50S core and hybrid 50S

The 50S core particles deficient in L10, L11, and L12 were prepared by extraction of the L11-deficient 50S subunit [19] in 50% ethanol, as previously described [20]. The hybrid 50S particle was formed by mixing the *E. coli* 50S core with various reconstituted aP0·aP1 complex samples, together with aL11, as described [18].

2.4. Factor binding and quantitative analyses

The ability of individual variants of the aP0·aP1 stalk complex to bind to the 50S core and the ability of the resultant hybrid 50S particles to access aEF2 or aEF1A were observed by electrophoresis on an acrylamide/agarose composite gel, as described previously [20]. The quantity of elongation factors bound to individual aP0·aP1 variant trimers was analyzed as followed. Each aP0·aP1 variant trimer was mixed with excess amounts of aEF2, incubated at 70 °C for 10 min, and then subjected to polyacrylamide gel electrophoresis under non-denaturing conditions [6]. The band appearing as the complex of aP0·aP1·aEF2 was excised from the gel and incubated in SDS sample solution at 37 °C for 10 min. The sample was subjected to SDS gel electrophoresis, followed by staining with Quick-CBB Plus (Wako). Each stained gel was scanned and converted to a TIFF format [21]. The intensities of the aP0 and aEF2 bands were measured using the image processing program ImageJ, ver. 1.48v (http://rsbweb.nih.gov/ij/). The amounts of the proteins were estimated using the calibration curves for the quantification of aP0 and aEF2, which were made using purified protein samples and analyzed by the same image processing program, as described above.

In some experiments, the abilities of individual aP0·aP1 variant trimers to bind His-tagged aEF1A or Flag-tagged aEF2 were also confirmed by gel electrophoresis under non-denaturing conditions, followed by immunoblotting using anti-His or anti-Flag antibodies.

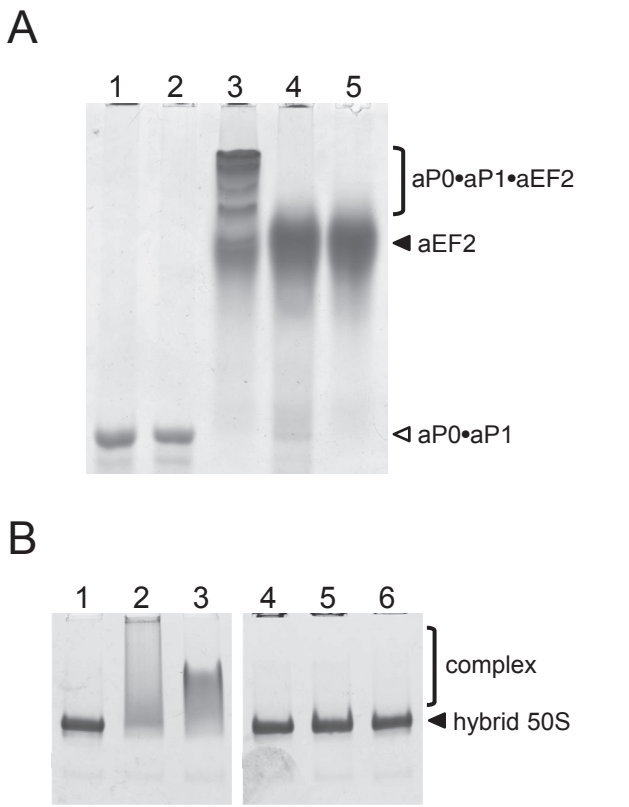

Fig. 1. Binding of elongation factors to the C-terminal regions of aP1 in the aP0(aP1)$_2$(aP1)$_2$(aP1)$_2$ heptamer. (A) aP0 (25 pmol) was mixed with 200 pmol of aP1 (lanes 1, 3) or aP1 mutant (F107S) (lanes 2, 4) and the aP0·aP1 heptameric complexes were reconstituted, as described in the Materials and Methods. The aP0·aP1 complex was incubated in the absence of aEF2 (lanes 1, 2) or the presence of 100 pmol aEF2 (lanes 3, 4). aEF2 was also incubated alone (lane 5). Individual samples were subjected to electrophoresis on a 6% polyacrylamide gel at 12.5 V/cm for 2 h, and the gel was stained with Coomassie Brilliant Blue G-250, as described in the Materials and Methods. (B) The 50S core ribosome (5 pmol) was incubated with 10 pmol of the WT aP0·aP1 heptameric complex (lanes 1–3) or the variant in which all aP1s were replaced with aP1(F107) (lanes 4–6), at 37 °C for 5 min. The hybrid ribosomes were incubated in the absence of aEF1A/aEF2 (lanes 1, 4), or with 100 pmol of aEF1A (lanes 2, 5) or aEF2 (lanes 3, 6), and subjected to acrylamide/agarose composite gel electrophoresis, as described in the Materials and Methods. The gel was stained with 0.2% Azur B.

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