Three-dimensional reconstruction of the valyl-tRNA synthetase/elongation factor-1H complex and localization of the δ subunit

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Abstract Eukaryotic valyl-tRNA synthetase (ValRS) and the heavy form of elongation factor 1 (EF-1H) are isolated as a stable high molecular mass complex that catalyzes consecutive steps in protein biosynthesis – aminoacylation of tRNA and its transfer to elongation factor. Herein is the first three-dimensional structure of the particle as calculated from electron microscopic images of negatively stained samples of the human ValRS/EF-1H complex. The ca. 12 × 8 nm particle has two distinct domains and each appears to have twofold symmetry. Bound antibodies place two δ subunits near the particle's center. These data support a dimeric head-to-head arrangement of particle components.

Keywords: Aminoacyl-tRNA synthetase; Elongation factor 1; Three-dimensional reconstruction; Protein biosynthesis; Multiprotein complex; Antibody

1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) are a family of enzymes involved in covalent coupling of amino acids with their matching tRNAs [1]. First an aminoacyl adenylate is formed using ATP then the activated amino acid is transferred to the 3′-adenosine of the cognate tRNA. In addition to their role in protein biosynthesis, aaRSs have been found to have alternate activities [2,3]. Examples are transcriptional and translational regulation, synthesis of dinucleotide polyphosphate signaling molecules, tRNA processing, as well as action as cytokines. A feature that is characteristic of several of these enzymes from multicellular eukaryotes is their ability to form large stable complexes [4,5]. To date, two types of aaRS complexes have been described: the "core" multisynthetase complex and the valyl-tRNA synthetase/elongation factor-1H complex (ValRS/EF-1H). These are likely part of highly organized protein biosynthetic machinery in which close association of aaRSs and other protein synthesis factors aids in maximizing speed and accuracy of this essential biological process.

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Abbreviations: aaRS, aminoacyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; EF-1H, four subunit form of elongation factor 1; aa-tRNA, aminoacyl-tRNA

Significant advances have been made in characterization of the multisynthetase complex [6]. This particle contains 11 polypeptides with molecular masses ranging from 18 to 150 kDa. There are nine aaRS activities and three auxiliary proteins (p18, p38 and p43). The three-dimensional structure of the ca. 1.2×10^6 Da complex has been determined by computational microscopy. It is an asymmetric particle V-shaped particle with several openings into the deep central cleft [7]. The internal topography of proteins in the complex has been studied by genetic and biochemical methods [8–12]. The resulting two-dimensional models arrange the components in either two or three domains [11,12]. Recently, a number of relative locations of proteins in the context of the three-dimensional structure of the multisynthetase complex have been accomplished using tRNAs and labeled proteins as structural probes [13].

In contrast, the ValRS/EF-1H complex has been well-studied biochemically, but not structurally. It has been reported that this assembly contains two copies each of ValRS and the "heavy form" of EF-1H and has an overall mass of ca. 700 kDa [14–18]. EF-1H is composed of four subunits: α, β, γ and δ. The normal stoichiometry is one copy of each, although an extra copy of α can be added. When the actual masses of the components as provided by the human protein database (ValRS, 140 kDa; EF-1α, 50 kDa; EF-1β, 24 kDa; EF-1γ, 50 kDa, EF-1δ, 31 kDa) are added, the total mass of the complex in the normal stoichiometry is ca. 600 kDa. This would increase to ca. 700 kDa if an additional copy of the α subunit is included in each EF-1H assembly. Another nomenclature for EF-1H subunits is extant [19] in which the EF-1α, β, γ, δ subunits are named eEF1A, eEF1B$_\alpha$, eEF1B$_\gamma$ and eEF1B$_\beta$, respectively. We have used the traditional nomenclature in order to be consistent with most of the prior studies that have proposed two-dimensional models of the protein arrangements within ValRS/EF-1H complex or of EF-1H alone.

Although the molecular mechanism is not yet known, the biological role of the ValRS/EF-1H complex can be inferred to be a means of facilitating delivery of charged tRNA to the ribosome. That is, ValRS and EF1 couple two consecutive steps of protein biosynthesis. EF-1α forms a ternary complex with aminoacyl-tRNA (aa-tRNA) and GTP to deliver charged tRNAs to the A-site of the ribosome for protein synthesis. The β, γ and δ subunits recycle inactive EF-1α-GDP to the active GTP-bound form by stimulating guanine nucleotide exchange. The GDP/GTP exchange activity is reported to be in the conserved C-terminal domains of β and δ [20,21]. Additionally, EF-1H co-migrates with ribosomes in sucrose gradient centrifugation experiments. This suggests that EF-1βγδ is in close

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proximity to the ribosome and so any free EF-1α/GDP released from the ribosome could immediately react with EF-1βγδ before diffusing into the cytosol [22].

In this study, the first three-dimensional structural information about the ValRS/EF-1H complex is presented. By electron microscopic visualization of bound antibodies, the δ subunit has been localized. This study provides additional evidence that the particle contains two copies of a ValRS/EF-1H protomer and suggests that they are in a head-to-head arrangement. These data form a basis for understanding the functional interaction of ValRS and EF-1H.

2. Materials and methods

2.1. Analytical methods

aaRS activity was measured by the incorporation of [^{14}C]-amino acids into purified *Escherichia coli* tRNA^{val} (Subriden RNA) [23]. Protein concentrations were determined using the Pierce Coomassie Blue protein assay. SDS–PAGE used 10% acrylamide gels prepared according to Laemmli [24]. The protein bands were visualized by silver staining reagents (Bio-Rad) [13]. Mass standards were commercially prepared (Bio-Rad). For immunoblots, proteins were transferred to nitrocellulose (0.2 μm, Schleicher & Schuell). Non-specific protein binding was blocked with 5% non-fat dry milk. A 1:4000 dilution of mouse anti-EF-1α (Upstate USA Inc.) was used as the primary antibody and a 1:25 000 dilution of HRP-conjugated rabbit anti-mouse immunoglobulin (Pierce) was used as the secondary antibody. Detection was performed using the SuperSignal West Pico Chemiluminescent system (Pierce). Gel-filtration HPLC was performed using a 300 × 4.6 mm Bio-Sep-SEC-S 4000 Peek column (Phenomenex) in HPLC buffer (25 mM HEPES, 100 mM NaCl, pH 7.2) with a flow rate of 3.5 ml/min.

2.2. Cell culture and cell-free extract

Human erythroleukemia K562 cells (ATCC #CCL-243) were maintained in Isocoves media or Complete Serum Free media (Mediatech, Inc.) with addition of 5% FBS and 0.5% antibiotic–antimycotic (Invitrogen Corporation). Cells were grown at 37 °C to a concentration of 1.0–3.0 × 10^6 cells/ml, harvested by centrifugation at 1500 × *g* for 20 min and stored as pellets at −80 °C until needed. For lysis, 40-g cells were thawed on ice in 100 ml hypotonic buffer (10 mM HEPES, pH 7.2) which also contained one protease inhibitor cocktail tablet (Roche) per 50 ml. Three additional protease inhibitors were added just prior to use. These were *N*-α-(*p*-toluene sulfonyl)-L-arginine methyl ester (TAME), phenyl methyl sulfonyl fluoride (PMSF) and di-isopropyl fluorophosphate (DIFP) at 1 mM final concentration. After 12 strokes in a Dounce homogenizer, completeness of cell lysis was checked with light microscopy. Cell lysate was then cleared by centrifugation for 20 min at 16 000 × *g*.

2.3. Isolation of ValRS/EF-1H complex

High molecular mass material was extracted from cell lysate by polyethylene glycol (PEG) fractionation. Specifically, 40% PEG 8000 in column buffer (50 mM HEPES, pH 7.2, 5 mM magnesium acetate, 0.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol and the combination of protease inhibitors listed above) was added to lysate to give a final concentration of 5%. After incubation on ice for 60 min, precipitate was collected by centrifugation 20 min at 16 000 × g. This was dissolved in column buffer in preparation for two successive ion exchange chromatography steps. These were a 10 ml column of S-agarose (Bio-Rad) and a 5 ml column of Q-agarose (Bio-Rad). Elution from both columns used a sodium chloride gradient of 100–700 mM. Fractions with high ValRS enzyme activity were combined and stored in aliquots at −20 °C. Immunoblot detection using EF-1α antibody, SDS–PAGE gel patterns and ValRS enzyme activities were used to detect the complex during the purification.

2.4. Electron microscopy

Electron micrographs for three-dimensional reconstruction were obtained of fractions from gel filtration HPLC that were adsorbed onto thin carbon films and stained with either 1% aqueous uranyl acetate or methylamine vanadate (Nanovan™, Nanoprobes, Yaphank, NY) as previously described [23]. Excess antibody was removed from samples that were reacted with rabbit anti-EF-1δ antibodies (Novus Biological Inc.) using gel-filtration HPLC and the fractions were prepared for electron microscopy as above. Electron micrographs were obtained with a LEO912AB transmission electron microscope at 100 kV with absolute magnifications of 63 000. Micrographs were digitized on a flatbed scanner to give a pixel size of 3.2 Å on the image scale.

2.5. Image analysis

The SPIDER/WEB software package was used for all computations [25]. For all reconstructions, images were interactively selected and then aligned using a reference-free algorithm. Calculation of the primary reference structure was from tilt pairs (0°, −54.9°) of micrographs taken with minimum dose focusing. Tilt pairs are used to determine the three Eulerian angles that properly orient the image views for a de novo structure calculation. Uranyl acetate was used as the negative stain at this initial stage in order to maximize image contrast which aided particle selection. After translational and rotational alignment, 560 images were classified using a K-means grouping algorithm. Reconstructions were calculated from appropriately populated classes. Several rounds of merging were done using angles determined by three-dimensional orientation search. The resulting primary reference was refined with 5560 untilted images using projection mapping at 10° intervals.

Final refinement of the structure used 11 206 untilted images of sample negatively stained with methylamine vanadate. Although images have relatively low contrast, additional structural details are typically preserved [7,13]. Angles were again obtained by projection mapping to the primary reference. Care was taken to use the appropriate parameters and number of images in each projection class to prevent introduction of artifacts due to overrepresentation of particular frequencies or views [26]. Resolution limits were determined from the 50% cutoff of the Fourier shell coefficient between reconstructions of half data sets. Thresholds for surface representation were calculated using mass values of 600 and 700 kDa and a partial specific volume of 0.72. Surface representations were created using IRIS EXPLORER (Numerical Algorithms Group, Downers Grove, IL).

3. Results

3.1. Intact ValRS/EF-1H complex was purified to near homogeneity

Gel-filtration HPLC was used as a final purification step for ValRS/EF-1H complex. As shown in Fig. 1A, the main protein peak elutes as high molecular mass material. This provided evidence that the intact dimeric particle had been isolated. To verify that it contained all of the expected components, SDS–PAGE, aminoacylation activity assay and immunoblotting with anti-EF-1α antibody were used to further analyze fractions from the HPLC column. Fig. 1B shows the electrophoretic band patterns of HPLC fractions 24 and 26, which correspond to elution times of 9–10 min. A strong band is seen at the position of 140 kDa. This is the expected mass of ValRS. The two bands with apparent mass of 50 kDa are consistent with the presence of EF-1α and 1γ. The lower two bands of approximately 30 kDa indicate the presence of EF-1β and δ [15,16]. Other protein bands are also visible, e.g., at ca. 200 kDa, which indicates that the complex is not completely purified. However, the amounts of contaminating proteins vary among preparations (data not shown) while the relative ratios of the ValRS/EF-1H complex components are consistent. Additional confirmation of the presence of ValRS was obtained by assaying the fractions for ability to incorporate [^{14}C] valine into tRNA^{val}. As seen in Fig. 1C, enzyme activity peaked at fraction 26. This fraction also contains the peak of EF-1α as determined by immunoblot analysis (Fig. 1D).