Three-dimensional Organization of the Archaeal A1-ATPase from Methanosarcina mazei Gö1*

Received for publication, December 16, 2003, and in revised form, February 20, 2004

Published, JBC Papers in Press, February 26, 2004, DOI 10.1074/jbc.M313741200

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A modified isolation procedure provides a homogenous A1-ATPase from the archaeon Methanosarcina mazei Gö1, containing the five subunits in stoichiometric amounts of A₃B₃C:D:F: A₁, obtained in this way was characterized by three-dimensional electron microscopy of single particles, resulting in the first three-dimensional reconstruction of an A₁-ATPase at a resolution of 3.2 nm. The A₁ consists of a headpiece of 10.2 nm in diameter and 10.8 nm in height, formed by the six elongated subunits A₃ and B₃. At the bottom of the A₃B₃ complex, a stalk of 3.0 nm in length can be seen. The A₃B₃ domain surrounds a large cavity that extends throughout the length of the A₃B₃ barrel. A part of the stalk penetrates inside this cavity and is displaced toward an A-B-A triplet. To investigate further the topology of the stalk subunits C–F in A₁, cross-linking has been carried out by using dithiobis[sulfosuccinimidylpropionate] (DSP) and 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (EDC). In experiments where DSP was added the cross-linked products B-F, A-D, A-B-D, and A-B-D were formed. Subunits B-F, A-D, A-B-D, and A-B-C-D could be cross-linked by EDC. The subunit-subunit interaction in the presence of DSP was also studied as a function of nucleotide binding, demonstrating movements of subunits C, D, and F during ATP cleavage. Finally, the three-dimensional organization of this A₁ complex is discussed in terms of the relationship to the F₁- and V₁-ATPases at a resolution of 3.2 nm.

Methanogenic, halophilic, and thermophilic Archaea synthesize ATP by means of ion gradient-driven phosphorylation. Although it was speculated for some time, due to the lack of in-depth information, that the ATP synthases of Archaea may be either F₅F₄ ∼ or V₅V₄-like enzymes, it is now clear that they evolved as a separate class of ATPases/ATP synthases, the A₁-ATPase/synthases (1–4). This class of enzymes is different from F₅F₄ or V₅V₄-ATPases by function, subunit composition, regulation, and structure (1). The A₁-ATPase has at least nine subunits (A₃B₃C:D:F:H:IK), but the actual subunit stoichiometry, especially regarding the proteolipid subunits K in A₁-ATPases, is different in various organisms (12, 6, 4 or, as suggested by genomic data, only 1 (5)). As suggested by its bipartite name, the A₁A₀-ATPase is composed of a water-soluble A₁-ATPase and an integral membrane subcomplex, A₀. ATP is synthesized or hydrolyzed on the A₁ headpiece, consisting of an A₃B₃ domain, and the energy provided for or released during that process is transmitted to the membrane-bound A₀ domain (1). The energy coupling between the two active domains occurs via the so-called stalk part, an assembly proposed to be composed by the subunits C, D, and F (2). The archaeal A₁A₀-ATPase/synthase is regarded as a chimeric protein in which the membrane domain is closely related to F₁F₄-ATP synthases but the catalytic subunits closely to V₁V₀-ATPases (3, 4).

The A₁-ATPase from Methanosarcina mazei Gö1 is made up of at least the five different subunits A–D and F with apparent molecular masses of 65, 54, 41, 28, and 9 kDa (6). The enzyme, as shown by small angle x-ray scattering data, consists of an ~10-nm long headpiece and an 8.5-nm high and 6.0-nm diameter stalk (7). A comparison of the central stalk of this A₁ complex with bacterial F₁- and V₁-ATPase indicates different lengths of the stalk domain (7–9). The prevailing view is, however, that ATP synthesis/hydrolysis in the A₁ headpiece is coupled to ion flow in A₀ through rotational movements of the central stalk subunit(s) as demonstrated for the F₁ (reviewed in Refs. 10 and 11) and the Thermus thermophilus A₁V₁-ATPase (12). Note, the so-called V₁V₀-ATPase from T. thermophilus, which also synthesizes ATP, is of archaeal origin (13, 14); therefore, the ATPase headpiece will be considered A₁/V₁-ATPase throughout this work.

Here we describe a modified isolation procedure of the A₁-ATPase from M. mazei Gö1, which facilitates the first three-dimensional reconstruction of this enzyme by using tmt pairs of negatively stained molecules. The structure adds to the emerging picture of A₁ in which three copies of A and B subunits are arranged as a hexagonal barrel, enclosing a large cavity in which a shaft is asymmetrically located. This three-dimensional model allows comparison with structural models of related F₁- and V₁-ATPase determined by crystallography (15) and electron microscopy (16, 17). Furthermore, insights into the topology and subunit-subunit interactions of the A₁-ATPase as a function of nucleotide binding are observed using different cross-link reagents.

** Experimental Procedures

* This work was supported by Grant GR 1475/9-1, 9-2 from the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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according to Lemker et al. (6) with the following differences. (i) The ammonium sulfate-precipitated enzyme was resolved in 10 ml of TMDG buffer (50 mM Tris/HCl, pH 7.5, 5 mM MgSO₄, 10% (w/v) glycerol, 1 mM 1,4-dithioerythritol) and dialyzed overnight against TMDG buffer using a 300-kDa Spectra/Por Dialysis Membrane (Spectrum Laboratories, Canada), in order to remove ammonium sulfate. (ii) The further isolation of protein was done by ion-exchange chromatography (Resource™ Q (6 ml), Amersham Biosciences) by using a step gradient with Buffer A (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM DTT) and Buffer B (50 mM Tris/HCl, pH 7.5, 1000 mM NaCl, 1 mM DTT). ATPase-containing peaks were pooled, concentrated separately on Centriprep 50-kDa and Centricon 100-kDa concentrators (Millipore), and applied on a Sephacryl™ S-300 HR column (10/30, Amersham Biosciences). The A₁ complex eluted in one peak containing the five subunits A–D and F. The purity and homogeneity of the protein sample were analyzed by native-PAGE (19) and SDS-PAGE (20). SDS gels were stained with Coomassie Brilliant Blue G-250. Protein concentrations were determined by the BCA assay (Pierce). ATPase activity was measured as described previously (7).

Electron Microscopy and Two-dimensional Image Analysis—For electron microscopy the protein was diluted in 20 mM Tris/HCl, pH 7.5, and 150 mM NaCl to 20–40 μg/ml. The sample was applied to 400 mesh carbon-coated copper grids and stained with uranyl acetate. Micrographs were recorded at a calibrated magnification of ×58,300 on a Philips CM 120 electron microscope under low electron dose conditions (10 e⁻/Å²). The three-dimensional reconstruction followed the random conical reconstruction technique of Radermacher (21) implemented in the SPIDER software package (22). Ten pairs of micrographs, the first image in each pair recorded at 53° and the second at 0° tilt angle, were scanned on a flat-bed SCAI (Zeiss) microdensitometer with 7 μm pixel size, which was subsequently reduced by binning to a pixel size of 21 μm, corresponding to 3.6 Å on the scale of the specimen. A total of 7851 pairs of images of single A₁-ATPase molecules were selected. The criteria used for this selection were that the A₁ molecules should be clearly visible and separated from neighboring particles in both the tilted and untitled versions. The alignment procedures, correction of the contrast transfer function, determination of the resolution, and the final three-dimensional reconstruction have been made by the use of the same procedures as described by Radermacher et al. (17). Pattern recognition was carried out using a neural network algorithm (23) as implemented in XMIPP (24). A network of 13 x 13 nodes was used, and 25 nodes evenly spaced were selected as references (Fig. 3A) for alignment of the images by multireference alignment. Reconstructions were calculated from the images belonging to each node. The average class resulting from the first node, which also represented the largest number of particles (Fig. 3A), was chosen for final three-dimensional reconstruction of the enzyme. For representation the reconstruction was low pass filtered to 3.2 nm.

Cross-link Formation in the A₁ Complex—The A₁ complex (1 mg/ml), diluted in HEPES buffer (20 mM HEPES and 100 mM NaCl, pH 7.0),

1 The abbreviations used are: DTT, dithiothreitol; DSP, dithiobis[sulfo succinimidylpropionate]; EDC, 1-ethyl-3-(dimethylaminopropyl)carbodiimide; MgAMP-PNP, Mg⁻⁵'-adenylylimidodiphosphate.

Fig. 1. Chromatographic purification of A₁-ATPase from M. mazei Gö1. A, ion-exchange chromatography on Resource™ Q in Tris-HCl buffer. After sample injection, an isocratic elution of 5 column volumes with Buffer A (Tris/HCl, pH 7.5, 150 mM NaCl, and 1 mM DTT) and a flow rate of 6.0 ml/min was employed, followed by a gradient program 0–10% Buffer B (Tris/HCl, pH 7.5, 1 M NaCl, and 1 mM DTT (—)). 5 μl of the collected and concentrated samples of peaks I, II, and III were applied on an SDS gel (see inset). B, after ion-exchange chromatography, the sample of peak III was applied onto a Sephacryl™ S-300 HR column, and the isolated A₁ complex was subjected to SDS-PAGE and stained with Coomassie Blue R-250. C, immunoblot of the same A₁ sample with polyclonal rabbit antisera directed against the subunits A–D and F of the A₁-ATPase.

Fig. 2. Electron micrographs of negatively stained A₁-ATPase from M. mazei Gö1 at tilt angles of 0 (A) and 53° (B). The line (B) indicates the tilt angle. Bar represents 50 nm.
was incubated with 4 mM nucleotide and MgCl$_2$ respectively, for 5 min. Cross-linking was induced by addition of 50 $\mu$m dithiobis(sulfosuccinimidylpropionate) (DSP, Pierce) resolved in Me$_2$SO on a sample rotator (Neolab) at 7 ºC for 2 h. The cross-linking reaction was stopped by quenching the DSP cross-linking reaction with the addition of 130 mM Tris/HCl, pH 7.5, and incubation for 10 min at 4 ºC. Samples were dissolved in DTT-free dissociation buffer and applied to an SDS-PAGE as described above. For the use of 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (EDC) as a cross-linker, A$_1$-ATPase was incubated with 5 mM EDC for 30 min on a sample rotator at room temperature. The reaction was stopped by addition of Laemmli buffer (63 mM Tris/HCl, pH 6, 10% glycerin, 2% SDS, and 0.01% bromphenol blue). The subunits involved in cross-linking were identified by Western blotting, using antisera against subunit A–D and F.

**RESULTS**

Isolation and Characterization of the A$_1$-ATPase—The A$_1$-ATPase has been purified by modification of an earlier method (6). The main differences from the earlier procedure are that the gel filtration column BioPrepSE1000/17 and the anion-exchange column were replaced. In the modified procedure a 6-ml Resource$^\text{TM}$ Q column has been used to remove contaminating proteins (Fig. 1A). In the final step the enzyme eluted from a size-exclusion column (Sephacryl$^\text{TM}$ S-300 HR column) in a single peak. The enzyme contains the five subunits A–D and F as identified by use of antisera against these polypeptides (Fig. 1, B and C). The molar ratio of the five subunits is A$_3$:B$_3$:C:D:F (Fig. 1B), based on a quantitation of the staining intensity of the five bands of these subunits, indicating the improvement of this new isolation protocol, when compared with a previous preparation in which subunit C is present in substoichiometric amounts (6). The contaminating bands running above subunit A are identified as *E. coli* DnaK and Grp E chaperons (data not shown). Small angle x-ray scattering patterns from solutions of the purified A$_1$-ATPase were recorded and processed. Comparison with the scattering from the reference solutions of bovine serum albumin yields a molecular mass of 445 ± 10 kDa, in agreement with a molar ratio of A$_3$:B$_3$:C:D:F and apparent molecular masses of 65, 54, 41, 28, and 9 kDa. This indicates that the two proteins above subunit A do not form a complex with the A$_1$-ATPase. ATPase activity of the A$_1$ complex in the presence of MgCl$_2$ was determined to be 6 ± 0.5 μmol/(min·mg).

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\*2 G. Grüber, U. Coskun, and M. H. J. Koch, unpublished data.

**FIG. 3.** Visual representation of a self-organizing map of the 7851 particles at 0° (A) and averages of 486 (B1) and 176 (B2) images at 0°. Bar represents 10 nm.

**FIG. 4.** Three-dimensional structure of the A$_1$-ATPase. Surface representation (A1–A3) of the three-dimensional reconstruction of the A$_1$-ATPase. The particle is represented as rotating around an axis parallel to the specimen support. B, Fourier ring correlation curve (solid line) and noise correlation reference curve (FRC$^*$). CI and C2, summation image of contour slices (23–40) of the three-dimensional structure (A1) overlaid on slices 37 (C1) and 41 (C2), respectively, showing the closer connection of the stalk to the major subunits A and B. Slices 37 and 41 correspond to the planes (––) and (– – –) in the surface representation (A1), respectively.
Electron Microscopy and Three-dimensional Reconstruction—Electron micrographs of the same field of negatively stained A1-ATPase taken at tilt angles of 0 and 53° (Fig. 2, A and B) show a homogenous distribution of this enzyme. A total of 7851 pairs of particles from 10 tilted/untilted micrographs were selected, and a self-organizing map of these particles was calculated by using a neural network approach to obtain the variations present in the data (Fig. 3A). In the upper left corner (number 1) a pseudohexagonal view of the particle can be seen with a seventh mass to the left of a hole. This feature strongly resembles the overall average (Fig. 3B1). The projection in the middle (Fig. 3A, number 5) shows this seventh mass in the center of the hexameric densities. Most of the variations seen may be caused by a rocking behavior of the molecule around its preferred orientation or by small tilts of the enzyme perpendicular to its hexagonal axis, sufficient to displace the central mass without apparent distortion of the outer hexagon.

The two-dimensional average at a resolution of 2.8 nm and calculated from 486 particles 0° micrographs shows a pseudohexagonal arrangement of six protein densities with an overall diameter of ~11.0 nm (Fig. 3B1). The center of the particle shows a seventh mass, which would be consistent with the presence of a stalk. The final three-dimensional reconstruction (Fig. 4A) has been calculated from the tilt images that correspond to the 0° images used for this two-dimensional average. The resolution was determined to be 3.2 nm by the Fourier shell correlation criterion (21, 26) with a threshold of five times the noise correlation value (Fig. 4B). The surface representation of the A1-ATPase displays some well known features of six elongated lobes, ~3.2 and 2.8 nm in diameter and 7.5 and 5.0 nm in length, respectively, which are parallel to the 3-fold axis (Fig. 4, A1 and A3). These lobes presumably represent the alternating three copies each of the major subunits A and B of the A1 headpiece. The A3B3 hexagon encloses a core of ~2.3 nm in the middle of the shaft, which narrows to ~0.6 nm at the top of the A1 headpiece (Fig. 4A2). Inside this cavity a seventh mass penetrates offset to the central axis of the cavity as shown by cross-sections in Fig. 4C. At the bottom of the A1 complex a “stalk” with a length of about 3.0 nm and a maximum diameter of 4.0 nm can be seen, which runs slightly offset to the hexamer (Fig. 4A2). On the opposite site of the hexagonal barrel extensions can be observed (Fig. 4, A1 and A3). These extensions are consistent with the three-dimensional reconstruction of the closely related V1-ATPase, where elongated features in a crown-like fashion can be seen at the very top of the V1 domain (16, 17).

Cross-linking of A1 Subunits Induced by DSP and EDC—Intersubunit cross-linking is a useful method for establishing the relative position of subunits (16, 27). The first cross-linking reagent employed was the homobifunctional N-hydroxysuccinim-
imide-ester DSP containing a 1.2-nm linker arm. Treatment of A1 with DSP gave rise to defined new bands (I–IV) and several A-B oligomers at higher molecular mass as observed in SDS-PAGE (Fig. 5A). Product I was recognized by the antibodies against subunit B and F (Fig. 5B). The migration of this product agrees well with the predicted molecular mass of a complex containing one copy each of subunits B and F (63 kDa). Although the intensity of subunit D and subunit C disappears (Fig. 5A, lane 2), the cross-link products II and III were obtained. The products II and III are assembled by an A-B-D and A-D formation, respectively, as determined by Western blotting with antibodies against A, B, and D (Fig. 5B). The cross-link product IV is formed by an A-B-D complex, confirmed by the anti-A, -B, and -D subunit antibody.

The DSP cross-link approach was also used to study possible nucleotide-dependent topology changes in the archaeal A1-ATPase. Fig. 5A illustrates the results of cross-linking of the A1-ATPase with DSP under different nucleotide conditions. When A1 was suspended in MgAMP-PNP, cross-link products resembled the formation in the absence of nucleotides. The presence of MgATP leads to lower cross-link formation, which goes along with a low depletion of subunits C and D. Notably, the cross-link products I and II are formed in marginal amounts. Addition of MgADP + P1 results in cross-link products that resembled the formation in the presence of MgAMP-PNP. Differences are in the higher depletion of subunits C and D and additional strong bands at higher molecular mass. The cross-linking pattern after addition of MgADP is comparable with that of the MgADP + P1-bound enzyme, with slightly differences in the intensities at higher molecular mass. These results rule out that MgATP was completely converted into MgADP or MgADP + P1 before cross-link formation under the conditions used.

The second cross-linking reagent employed was EDC, which results in cross-linking of carboxyl and amino groups. Treatment of A1 with EDC resulted in the formation of four cross-linking products (I–IV). The first cross-linking formation, with an apparent molecular mass of 63 kDa, was recognized by the antibodies against subunits B and F (Fig. 5C). The yield of this product is close to 90%, based on the disappearance of the F subunit. The second and third products were recognized by the antibodies against A and D and A, B, D, respectively. The fourth formation has been identified as a complex consisting of the subunits A, B, C and D.

**DISCUSSION**

The A1-ATPase from *M. mazei Göl* originally purified gives an ATPase active A2:B2:C:D:F complex, with subunit C being sub-stoichiometric (6). The modified method of A1-ATPase purification described here results in a monodisperse preparation, with an enzyme consisting of all five subunits in stoichiometric amounts, which makes it suitable for structural examinations like three-dimensional electron microscopy of single particles. The most striking organizational feature of such isolated A1-ATPase is the hexagonal arrangement of densities seen in projection images of single particles, deduced to be made up by the subunits A and B (Fig. 3). In three dimensions these subunits are elongated structures, interdigitated for most of the height of the hexagon. Three of these masses appear to be slightly larger than the remaining ones, confirming the stoichiometry of A2B2 and the alternating arrangement for the nucleotide-binding subunits in the enzyme. The hexagonal barrel has dimensions of 10.2 nm in diameter and 10.8 nm in height, in agreement of dimensions of 10 × 9.4 nm determined by solution x-ray scattering data of the same enzyme (7). Extensions, visible on the top of the hexagon, are asymmetric, similar
to the top domain of the three-dimensional reconstruction of the V1-ATPase from M. sexta determined at 3.2 (16) and 1.8 nm (17) resolution (Fig. 7). These protuberances of the V1 headpiece have been proposed to belong to the catalytic A subunits (14, 17), which possess the so-called “non-homologous region” in the A subunits, an insert of an 80–90-amino acid long loop, which is similar to the catalytic A subunits in A1 (14). By comparison, this region is not present in the related β subunits of F-ATPases, whose top domain consist of six globular masses, made by a β-barrel domain, containing the N termini of the nucleotide-binding subunits α and β (15) (Fig. 7).

Comparison with the recently determined envelope of the M. mazei G01 A1 in solution (7) yields that the stalk, which protrudes from the opposite side of the A3B3 hexamer, is only partially (35%) solved in our three-dimensional reconstruction (Fig. 6). This might be due to dehydroxylation and/or adsorption of the enzyme to the carbon film. However, the stalk in the three-dimensional model of A1 protrudes from the A3B3 hexamer by about 6.5 nm and is inclined at a 20° angle to the hexamer, similar as shown in the three-dimensional model of F1 (Fig. 7). A key feature of the structural model of A1 is that a part of the stalk penetrates inside a large cavity formed by the A3B3 complex (Fig. 4A2) and is displaced toward an A-B-A triplet (Fig. 4C). Trypsin cleavage and zero length cross-linking of the A1 from M. mazei G01 imply that subunit D is located inside the cavity formed by the A3B3 barrel (28). The large cavity in the center of the A3B3 complex would allow a relative rotation of the central D subunit as demonstrated for the A3B3DF subcomplex of the related T. thermophilus A3V1-ATPase (12). By comparison, the central stalk inside a large cavity of the F1 (formed by the γ subunit) and V1 can be seen inclined to two of the catalytic β (15 βAmp,F-PNP and βEmpty) and A subunits (17), respectively. These features suggest that both modules, a large cavity, formed by the hexameric arrangement of the major subunits, and a shaft, made up by the central stalk subunit, are the structural elements conserved in the three types of ATPases, facilitating rotational movements inside these enzymes.

An important consideration in the construction of a topological model for the multisubunit complex A1-ATPase is the proximity of the stalk subunits B and F. Subunit F, which belongs to the minimal functional A3B3DF complex of A1 (6), can be cross-linked in high yield to the nucleotide-binding subunit B, independent of whether DSP (1.2 nm) or the zero length cross-linker EDC was used. A summary of the cross-links observed is given in Table I. These data indicate that both subunits are in close proximity (Fig. 6). Nucleotide-dependent cross-linking of the enzyme with DSP yields that the B-F formation is reduced in the presence of MgATP, implying that subunit F moves dependent on the nucleotide bound to the enzyme. This observation is important, because subunit F in the related A3V1-ATPase from T. thermophilus has been shown to constitute a rotor shaft together with subunit D (12).

Cross-linking by EDC also revealed an A-B-C-D formation, indicating that subunit C is in close contact to the A3B3D core complex. It is of particular interest that subunit C undergoes significant structural rearrangements depending on nucleotide binding as observed by the depletion of subunit C in the gel when DSP was used as cross-link reagent (Fig. 5A). A similar nucleotide-dependent behavior is shown for subunit D (Fig. 5A) which can be cross-linked to the nucleotide-binding subunits A and B. Recently, CuCl2-mediated disulfide formation indicated that the N and C termini of subunit D are in close proximity with the catalytic A subunit after addition of MgADP or the hydrolyzable MgATP (28). Taken together, the nucleotide-dependent alterations of the stalk subunits C, D, and F described and the close proximity of these subunits to the major subunits A and/or B would provide coupling between the catalytic site events via subunit D into the central CDF stalk domain, and thereby the physical and structural linkage between the A3B3 headpiece and the ion-conducting A1 part.

In summary, the first three-dimensional reconstruction of the A1-ATPase from M. mazei G01 presented provides the structural basis toward a fuller understanding of the mechanistic events occurring in this enzyme (28). Both features, a large cavity surrounded by the A3B3 barrel and the shaft inside this cavity and presumably formed by subunit D, are similar to the structural elements in the related F- and V-ATPases, in which they facilitate the rotational movements inside these complexes. The composition of the various cross-link products of the subunits in A1 provide an organization of the subunits in the ATP-hydrolyzing enzyme.

Acknowledgments—We thank A. Armbriëster and C. Hohn (Universitat des Saarlandes) for skilled technical assistance. We are grateful to Dr. G. Kohring (Universitat des Saarlandes) for help with the 50-liter fermentor.

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