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ScienceDirect
Protein Expression and Purification 53 (2007) 378–383
Protein Expression & Purification
www.elsevier.com/locate/yprep

Cloning, purification, and nucleotide-binding traits of the catalytic subunit A of the V_1V_O ATPase from *Aedes albopictus*

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Received 1 December 2006, and in revised form 11 January 2007
Available online 21 January 2007

Abstract

The Asian tiger mosquito, *Aedes albopictus*, is commonly infected by the gregarine parasite *Ascogregarina taiwanensis*, which develops extracellularly in the midgut of infected larvae. The intracellular trophozoites are usually confined within a parasitophorous vacuole, whose acidification is generated and controlled by the V_1V_O ATPase. This proton pump is driven by ATP hydrolysis, catalyzed inside the major subunit A. The subunit A encoding gene of the *Aedes albopictus* V_1V_O ATPase was cloned in pET9d1-His_3 and the recombinant protein, expressed in the *Escherichia coli* Rosetta™ 2 (DE3) strain, purified by immobilized metal affinity- and ion-exchange chromatography. The purified protein was soluble and properly folded. Analysis of secondary structure by circular dichroism spectroscopy showed that subunit A comprises 43% α-helix, 25% β-sheet and 40% random coil content. The ability of subunit A of eukaryotic V-ATPases to bind ATP and/or ADP is demonstrated by photoaffinity labeling and fluorescence correlation spectroscopy (FCS). Quantitation of the FCS data indicates that the ADP-analogues bind slightly weaker to subunit A than the ATP-analogues. Tryptophan fluorescence quenching of subunit A after binding of different nucleotides provides evidence for secondary structural alterations in this subunit caused by nucleotide-binding.

Keywords: Vacuolar-type ATPase; V_1V_O ATPase; V_1 ATPase; Subunit A; Photoaffinity labeling; Fluorescence correlation spectroscopy; Mosquito

Vacuolar ATPases (V-ATPases)[1] are present in every eukaryotic cell where they are responsible for acidification of lysosomes, the Golgi apparatus, and endosomes; these enzymes are involved in protein sorting, pH and calcium homeostasis, and zymogen activation [1–3]. The V-ATPases are composed of a water-soluble V_1 ATPase and an integral membrane subcomplex, V_O. ATP is hydrolyzed on the V_1-headpiece consisting of an A_3:B_3 hexamer, and the energy released during that process is transmitted to the membrane-bound V_O domain, to drive ion translocation. This energy-coupling occurs via the so-called stalk structure, an assembly of the V_1 and V_O subunits C–H and *a*, respectively, that forms the functional and structural interface. The proposed subunit stoichiometry of V_1 is A_3:B_3:C_1:D_1:E_1:F_1:G_2:H_x. The V_O complex can be subdivided into two parts that move relative to each other, the peripheral stalk and the proton-translocating ring. The V_O part of the stator consists of subunit *a* [4,5]. The proton-translocating ring is composed of the subunits $c_{4\text{-}5}$:c'_1:c''_1, each having multiple transmembrane domains and are termed proteolipids because of their hydrophobic nature [6]. The fifth V_O subunit, subunit *d*, is predicted to be a hydrophilic peripheral membrane protein [7]. V-ATPases have a structure similar to F-ATPases and several of their subunits probably evolved from common ancestors

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[1] *Abbreviations used:* V-ATPases, vacuolar ATPases; AMP-PNP, 5′ adenylylimidodiphosphate; BSA, bovine serum albumin; FCS, fluorescence correlation spectroscopy; IPTG, isopropyl (thio)-β-D-galactoside; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate, Tris; Tris-(hydroxymethyl)aminomethane.

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doi:10.1016/j.pep.2007.01.009

[3,5]. A comparison of independently identified structures of the eukaryotic V_1 ATPase [8,9] with the F_1 ATPase [10] indicates mushroom-shaped enzymes, composed of a headpiece, which is formed by the hexagonally arranged nucleotide-binding subunits A and B of V_1 and the corresponding α and β subunits of F_1, and one stalk, comprised of the V_1 subunits, C–H, and the F_1 subunits γ–ε (mitochondrial nomenclature [10]). Structural comparison of the V_1 and F_1 headpieces indicates different shapes and lengths of the catalytic subunit A and β of the V_1- and F_1 ATPase, respectively, predicted to be caused by the so-called non-homologous region, an insertion of 90 amino-acid residues near the N-terminus of subunit A [8,9].

Understanding the structural and functional roles of the nucleotide-binding subunits of V-ATPases is essential, because they may impart to V-ATPases the characteristics that distinguish them from the F-ATPases, including their activity as dedicated ion pumps rather than ion-driven ATP synthases, and their susceptibility to multiple forms of regulation *in vivo* [3,13,14]. We have turned our attention to examination to the catalytic subunit A of the eukaryotic V-ATPase of the mosquito *Aedes albopictus*, and describe its expression in *Escherichia coli* cells. The high purity of the soluble protein enabled us to analyze the nucleotide-binding traits of this protein by photoaffinity labeling and fluorescence correlation spectroscopy.

Materials and methods

Bacterial strains, plasmids and media

ProofStart™ DNA Polymerase and Ni^{2+}-NTA-chromatography resin were received from Qiagen (Hilden, Germany); restriction enzymes were purchased from Fermentas (St. Leon-Rot, Germany). The expression vector pET9d1-His_3 was provided by S.M. Bailer, (Universitätsklinikum Homburg/Saar, Germany). Chemicals for gel electrophoresis were received from Biorad (USA). Bovine serum albumin was purchased from GERBU Biochemicals (Heidelberg, Germany). All other chemicals were at least of analytical grade and received from Amersham Bioscience (Buckinghamshire, UK), BD (Franklin Lakes, USA), BIOMOL (Hamburg, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Sigma–Aldrich (Deisenhofen, Germany), or Serva (Heidelberg, Germany).

Cloning of the subunit A gene of the V_1V_O ATPase from Aedes albopictus

To amplify the region coding for subunit A ($_{54}$L-D_{624}) of the V-ATPase from *Aedes albopictus*, oligonucleotide primers 5′-GTGAGTCCATGGGTCTGGAGGGTGACA-3′ (forward primer) and 5′-CGGGAGCTCTTAATCTTCC AGGTTGC-3′ (reverse primer), incorporating *Nco*I and *Sac*I, restriction sites, respectively (underlined), were designed. The multicopy vector pET30a-V-ATPase-A-Aal [11] coding for the V-ATPase gene A was used as template for the polymerase chain reaction (PCR). Following digestion with *Nco*I and *Sac*I, the PCR product was ligated into the pET9d1-His_3 vector. The recombinant plasmid was transformed into *E. coli* DH5α and the transformants were confirmed by DNA sequencing. The pET9d1-His_3 vector containing the A gene as an insert was then transformed into Rosetta™ 2 (DE3) cells (Novagen) and grown on Luria–Bertoni (LB) agar-plates, containing 30 mg/ml kanamycin- and 34 mg/ml chloramphenicol.

Overexpression and purification of subunit A

To express the N-terminal His_3-tagged subunit A, liquid cultures were shaken in LB medium containing kanamycin (30 mg/ml) and chloramphenicol (34 mg/ml) for about 2.5 h at 37 °C, 180 rpm, until an absorbance at 600 nm of 0.7–0.9 was reached. To induce production of His_3-A, the culture was supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Following incubation for another 4 h at 37 °C, the cells were harvested by centrifugation at $8000 \times g$ for 8 min at 4 °C. Cells expressing subunit A were lysed on ice by sonication for 4×1 min in buffer A (50 mM Tris/HCl (pH 8.5), 150 mM NaCl, 1 mM DTT and 4 mM Pefabloc SC (BIOMOL)). The lysate was cleared by centrifugation at $109\,000 \times g$ for 30 min at 6 °C, the supernatant was passed through a filter (0.45 mm pore size (Millipore)) and supplemented with Ni^{2+}-NTA resin. The His-tagged subunit A was allowed to bind to the matrix for 120 min rotating at 4 °C, and eluted with an imidazole gradient (0–150 mM) in buffer A, respectively. Fractions containing subunit A were identified by SDS–PAGE and concentrated using Centriplus 30 kDa concentrators (Millipore). The concentrated solution was applied on an ion-exchange column (MonoQ, 1 ml, Amersham Biosciences), equilibrated in 50 mM Tris/HCl (pH 8.5), 100 mM NaCl and 1 mM DTT. The protein was purified using a step gradient with Buffer B (50 mM Tris/HCl (pH 8.5), 1 mM NaCl and 1 mM DTT) and Buffer C (50 mM Tris/HCl (pH 8.5), 1 M NaCl and 1 mM DTT). The purity of the protein sample was analyzed by SDS–PAGE [12]. The SDS-gels were stained with Coomassie Brilliant Blue R250. Protein concentrations were determined by the bicinchonic acid assay (BCA; Pierce, Rockford, IL, USA). Western blot analyses were performed as described [13]. The production and purification of Anti-A antibodies has recently been described [11]. ATPase activity, which is stimulated by Mg^{2+}, was performed in the presence of an ATP-regenerating system as described by Lötscher et al. [14].

MALDI-TOF analysis

A solution (20 μl) of purified A was desalted with a C18ZipTip (Millipore) and diluted in 70% acetonitrile containing 1% formic acid before injection directly into a matrix assisted laser desorption-ionisation/time-of-flight