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Published in:
Biochimica et Biophysica Acta-Bioenergetics

DOI:
10.1016/j.bbabio.2005.02.001

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2005

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Interaction between subunit C (Vma5p) of the yeast vacuolar ATPase and the stalk of the C-depleted V₁ ATPase from *Manduca sexta* midgut

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Received 30 November 2004; received in revised form 26 January 2005; accepted 2 February 2005

Available online 16 March 2005

Abstract

Projection maps of a V₁−Vma5p hybrid complex, composed of subunit C (Vma5p) of *Saccharomyces cerevisiae* V-ATPase and the C-depleted V₁ from *Manduca sexta*, were determined from single particle electron microscopy. V₁−Vma5p consists of a headpiece and an elongated wedgelike stalk with a 2.1×3.0 nm protuberance and a 9.5×7.5 globular domain, interpreted to include Vma5p. The interaction face of Vma5p in V₁ was explored by chemical modification experiments.

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Keywords: V-ATPase; V₁Vₒ ATPase; V₁ ATPase; Vma5p; Reversible disassembly; Electron microscopy; *Manduca sexta; Saccharomyces cerevisiae*

1. Introduction

Eukaryotic V-type (V₁Vₒ) ATPases are membrane bound protein complexes. They are involved in the acidification of intracellular compartments and the generation of an electrochemical gradient required for secondary ion transport processes, which are tightly coupled to the hydrolysis of ATP [1,2]. V-ATPases have two structural and functional parts, a peripheral V₁ complex, whose catalytic part faces the cytosol, and a membrane bound, ion conducting Vₒ part. The enzyme V₁ consists of eight subunits A–H, whereas the Vₒ domain is composed of the four different subunits a, c, d and e [1,2]. Both the catalytic A₃B₃-headpiece and the Vₒ moiety are connected via a so-called stalk region consisting of three [3] or four stalks [4], formed by subunits C–H and d [2].

A characteristic feature of the eukaryotic V₁Vₒ ATPase is the regulation by reversible disassembly of the V₁ and Vₒ subcomplexes [5,6], resulting in the decrease of Mg²⁺-dependent ATPase activity and proton pumping across the membrane. Reassembly of both domains restores these activities. It was shown that subunits C and H are important for the inhibition of the Mg²⁺-dependent ATPase activity of dissociated V₁ complexes [7]. The high-resolution structure of the H subunit [8] and data on the gross structure of the V₁Vₒ ATPase complexes suggest that in the intact enzyme this subunit is involved in the formation of the peripheral stalk region [3], despite the fact that a rearrangement within the disassembled V₁ is possible [9]. In contrast, the position of the C subunit of the eukaryotic V₁Vₒ ATPase is more difficult to assign. Electron microscopy studies of the disassembled V₁ complex from tobacco hornworm *Manduca sexta* have shown that subunit C can be easily lost from the V₁ subcomplex [10], although it is essential for the reassembly of the functional V₁Vₒ [11]. Previously, the structure of the C subunit (Vma5p) from the yeast V₁Vₒ ATPase has been studied by small angle X-ray scattering, revealing that the hydrated Vma5p has an elongated boot-shaped structure with a maximum size of 12.5 nm [12]. A recent 1.75 Å map from X-ray diffraction studies of Vma5p [13]...
confirms this feature and shows that this subunit consists of three distinct domains: an upper head domain, composed by the amino acids 166 to 263, a large globular foot, consisting of the N- and C-termini, and an elongated neck domain, which connects the head and foot region (Fig. 1).

An intriguing result was the formation of a stable and ATPase active hybrid complex composed of Vma5p and V1 from M. sexta, lacking subunit C [12]. Here we report the structural characterization of this hybrid complex by single particle electron microscopy and a biochemical approach.

2. Materials and methods

2.1. Purification of the V1(-C) ATPase from M. sexta and assembly with subunit C (Vma5p)

Tobacco hornworms were reared as described in [14]. The Manduca eggs were a generous gift of Prof. Trenczek, University of Giessen, Germany. The V1(-C) ATPase from M. sexta and subunit C (Vma5p) of the Saccharomyces cerevisiae V1VO ATPase was isolated according to Rizzo et al. [14] and Armbröster et al. [12], respectively. To remove 2-mercaptoethanol, the protein was dialyzed in a QuixSep Micro Dialyzer (Roth, Germany) for 6 h against a degassed buffer containing 20 mM Tris/HCl, pH 8.1 and 150 mM NaCl. ATPase activity was measured as described previously [15].

2.2. Labeling of subunit C by TMR or MMN

Subunit C (Vma5p) was selectively labeled at the Cys340 with 30 μM tetramethylrhodamine-5-maleimide (TMR) for 10 min in 20 mM Tris/HCl (pH 7.5) and 150 mM NaCl (buffer A). Monomaleimidonanogold (MMN; NanoProbes, Inc.) was added to the subunit C solution and incubated overnight at 6°C. The excess of TMR or MMN was removed by size-exclusion chromatography using a Superdex 75 HR 10/30 column (Amersham Biotech). The assembly experiment of the TMR- or MMN-labeled C subunit with V1(-C) ATPase has been done as described above.

2.3. Electron microscopy and image analysis

Immediately after elution, the fractions of peak I were applied on freshly glow-discharged carbon-coated copper grids and fixed for electron microscopy by negative staining with 2% uranyl acetate. Images were recorded with a Gatan 4 K slow-scan CCD camera on a Philips CM20FE electron microscope at a calibrated magnification 87,800 ×. A pixel size of 30 Å corresponded to a size of 3.4 Å at the specimen level. A total of 30,000 single particle projections was extracted from the images using the “Boxer” program from EMAN software package [16]. Projections were analysed with GRIP software on a PC cluster. In order to avoid reference bias during alignment the following procedure was used: Images were treated with a double self-correlation function (DSCF) [17]. Transformed data were subjected to a Multivariate statistical analysis (MSA) and hierarchical ascendant classification (HAC) in a minimal variance mode [18]. On the basis of the classification, an initial file was divided into groups representing different classes of the projections. Non-reference alignment [19] was used to align images within each group. The procedure was repeated up to 10 times, until threshold values for a mean rotation and shift of 0.1° and 0.05 pixels, respectively, were reached. Further, conditional sums of the best-aligned single particle projections from homogeneous classes were used for a multireference alignment on a whole data set, followed by MSA and HAC. Alternatively, a multireference alignment to the two-dimensional projections of the M. sexta V1 ATPase model [9] was used.

3. Results and discussion

The ATP-hydrolytic active hybrid complex V1–Vma5p complex has been formed by overnight incubation of the V1(-C) complex from M. sexta together with subunit C (Vma5p) from yeast at high concentrations (Fig. 2). Size-exclusion chromatography of this mixture resulted in an elution diagram with three main peaks I–III, comprising the complexes V1–Vma5p, V1(-C) and Vma5p, respectively.
(Fig. 2A, B). A specific ATPase activity of 2.6±0.1 units/ml was measured for the V1–Vma5p hybrid complex of peak I.

Electron microscopy images of reconstituted V1(-C)–Vma5p complexes showed a large number of projections differing in their shape and size. A multireference alignment of this data set to re-projections of a three-dimensional model of *M. sexta* V1 ATPase [9] did not yield statistically homogeneous classes. To deal with this problem, MSA and HAC on the DSCF treated images were used to sort the projections. Final image analysis showed that projections could be divided into three distinct groups of classes. The first group of projections (about 3500 particles, 16% of the data set) show a large wedgelike density, extending from the V1 headpiece (Fig. 3, panels A–D). In the best projections this extension, with a total length of about 10.5 nm, is formed by a globular mass of about 9.5 in width and 7.5 nm in height, which is connected to the V1 headpiece by a narrow linker density of about 2.1 in diameter and 3.0 nm in length. The second group represents top views, showing all of the details of the hexagonal arrangement of the major subunits A and B with a seventh mass in the center (Fig. 3, panels E, J). The third group has a consistent smaller stalk region (Fig. 3, panels F–H), comparable to 2-D projection of the *M. sexta* V1(-C) complex (panel I). This implies that the third group represents the side views of the V1 complex without the Vma5p attached in the stalk region. This was also confirmed by the high correlation coefficients obtained after alignment of the class averages from the third group to the projections of the 3-D model of *M. sexta* [9] (data not shown). A total length of the stalk elements in the hybrid complex is about 10.5 nm, which exceeds the length of the central stalk in V1 complex lacking subunit C by 4.5 nm (Fig. 3F–H) [10]. Thus these data are in agreement with the value of 11 nm determined for the hydrated V1 ATPase.
using SAXS [20]. Unfortunately the resolution in all side-view classes was rather low, despite the high number of projections processed. This could be a result of the instability of the hybrid complexes due to dilution for electron microscopy [10] or the structural flexibility of Vma5p, as described for the recently determined Vma5p crystal forms of Vma5p [13]. Nevertheless the EM data suggest that within the hybrid complex Vma5p most likely is arranged with its long axis parallel to the stalk direction, as shown in Fig. 1. The overall length of the stalk and structure of Vma5p indicate that this subunit might span the full stalk, thereby linking the catalytic A2B3 domain via its head region to the VO domain via the foot region. The foot region of Vma5p with about 5 nm in width and 4.5 nm in height, proposed to be oriented to the membrane domain [12,13], would fit in the density of the globular stalk domain of the V1–Vma5p complex (Fig. 3).

Site-directed mutagenesis revealed that the C-terminal region of Vma5p might be important for the stable assembly of V1 and VO [21]. In order to determine whether the C-terminus is in close contact to the V1 stalk domain, Vma5p was labeled by monomaleimidonanogold (MMN) at the single cysteine residue (Cys340) of the C-terminal region (Fig. 1). By comparison, the binding of MMN at Cys340 resulted in a faster elution of the labeled Vma5p–MMN complex in the size-exclusion chromatography than the result obtained when Vma5p was incubated with the C-depleted V1 ATPase and subsequently applied onto a gel filtration column no hybrid-complex could be formed (Fig. 2A). The same result was obtained when Vma5p was labeled with smaller maleimides like tetramethylrhodamine (TMR; Fig. 2B, lane 5) and N-ethylmaleimide (NEM) (data not shown), implying that the region at Cys340 may be involved in the interaction of Vma5p with the C-depleted V1 ATPase. As mentioned above, the dimension and shape of the foot domain of the Vma5p molecule [12,13] imply that this domain may be oriented to the membrane domain [12,13]. In line with this is the fact that the bottom of the foot domain shows a hydrophobic surface, [13] and it has been argued that the detachment of the V1 and VO sections following cold treatment [22] may be caused by the weak interactions of the lower foot domain and the membrane sector. Since the binding of different maleimides to Vma5p prevents this subunit from interaction with C-depleted V1, we propose that the point of the foot in which Cys340 is located forms at least partially the surface for binding to the stalk region of V1. From recent studies using cross-linkers it has been shown that subunit C is in close neighborhood to subunit E [23], the latter of which is in close proximity to the stalk subunit D [23,24], and in contact with subunit C of the VO part [24]. Taken together, these data imply that the foot domain of subunit C may be involved in the interaction of the stalk region in V1 and the membrane-embedded VO part.

Unfortunately, at the present resolution it is not possible to determine in the conformation of subunit C changes upon binding to the hybrid complex. In this context it should be mentioned that the 2-D projections (Fig. 2) show the presence of a single compact stalk in the cytosolic V1, as shown in the three-dimensional models of the V1 ATPase with and without subunit C of M. sexta [10,20]. Such structural composition of an A2:B3 hexamer and one stalk domain of the soluble V1 is consistent with a hexameric arrangement of the major nucleotide-binding subunits and a single compact stalk domain in the closely related A1 and F1 ATPases as shown by X-ray solution scattering (reviewed in [25]) and X-ray crystallography [26]. Based on these structural data the question arises whether the peripheral stalk regions in the V1:VO holoenzyme, which are proposed to be formed in part by subunits C and H, and the V1 complex might become more compact following dissociation, with the peripheral stalks collapsing into a single stalk in the free V1 ATPase as recently proposed [9].

Acknowledgments

We thank Drs. Gert Oostergetel and Wilko Keegstra (University of Groningen) for their help in EM data recording and analysis. We are grateful to Andrea Armbrüster and Ünal Coskun (Universität des Saarlandes) for technical support and discussion. This research was supported by a grant of the Dutch Scientific foundation NWO/CW to E. J. B. and Deutsche Forschungsgemeinschaft to G. G. (GR 1475/9-1 and GR 1475/9-2).

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