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Interaction between subunit C (Vma5p) of the yeast vacuolar ATPase and the stalk of the C-depleted V_1 ATPase from *Manduca sexta* midgut

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Abstract

Projection maps of a V_1–Vma5p hybrid complex, composed of subunit C (Vma5p) of *Saccharomyces cerevisiae* V-ATPase and the C-depleted V_1 from *Manduca sexta*, were determined from single particle electron microscopy. V_1–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 V_1 was explored by chemical modification experiments.

Keywords: V-ATPase; V_1V_O ATPase; V_1 ATPase; Vma5p; Reversible disassembly; Electron microscopy; *Manduca sexta*; *Saccharomyces cerevisiae*

1. Introduction

Eukaryotic V-type (V_1Vo) 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 V_1 complex, whose catalytic part faces the cytosol, and a membrane bound, ion conducting V_O part. The enzyme V_1 consists of eight subunits A–H, whereas the V_O domain is composed of the four different subunits a, c, d and e [1,2]. Both the catalytic A_3B_3-headpiece and the V_O 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 V_1V_O ATPase is the regulation by reversible disassembly of the V_1 and V_O subcomplexes [5,6], resulting in the decrease of Mg^{2+}-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 Mg^{2+}-dependent ATPase activity of dissociated V_1 complexes [7]. The high-resolution structure of the H subunit [8] and data on the gross structure of the V_1V_O 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 V_1 is possible [9]. In contrast, the position of the C subunit of the eukaryotic V_1V_O ATPase is more difficult to assign. Electron microscopy studies of the disassembled V_1 complex from tobacco hornworm *Manduca sexta* have shown that subunit C can be easily lost from the V_1 subcomplex [10], although it is essential for the reassembly of the functional V_1V_O [11]. Previously, the structure of the C subunit (Vma5p) from the yeast V_1V_O 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]

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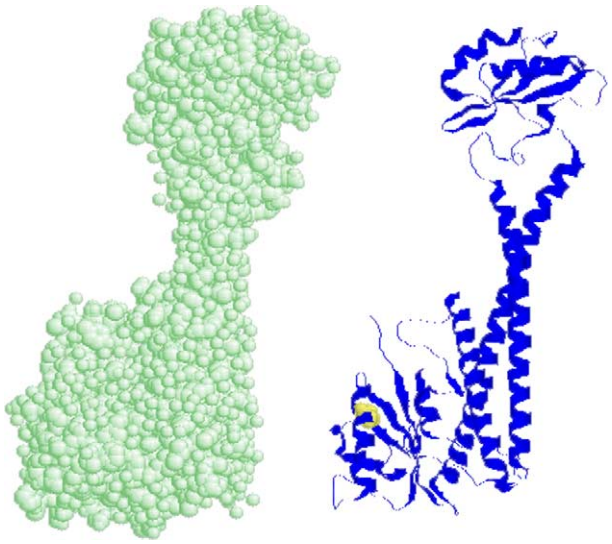

Fig. 1. Structure of subunit C (Vma5p). The atomic model derived from the X-ray coordinates kindly provided by Prof. N. Nelson, Tel Aviv University [13]. The ribbon diagram (right) shows the Cys_{340} (yellow) located at the foot point of the boot-shaped molecule.

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 V_1 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 V_1(-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 V_1(-C) ATPase from *M. sexta* and subunit C (Vma5p) of the *Saccharomyces cerevisiae* V_1V_O ATPase was isolated according to Rizzo et al. [14] and Armbrüster 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 using a 10 kDa Spectra/Por dialysis membrane (Spectrum Laboratories, Canada). The protein was mixed with subunit C (Vma5p) overnight on a sample rotator at 4 °C. The incubated mixture was applied on a Sephacryl S-300 HR column with 20 mM Tris–HCl (pH 8.1), 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 Cys_{340} 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 V_1(-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 CM20FEG electron microscope at a calibrated magnification 87,800×. A pixel size of 30 μm 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* V_1 ATPase model [9] was used.

3. Results and discussion

The ATP-hydrolytic active hybrid complex V_1–Vma5p complex has been formed by overnight incubation of the V_1(-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 V_1–Vma5p, V_1(-C) and Vma5p, respectively

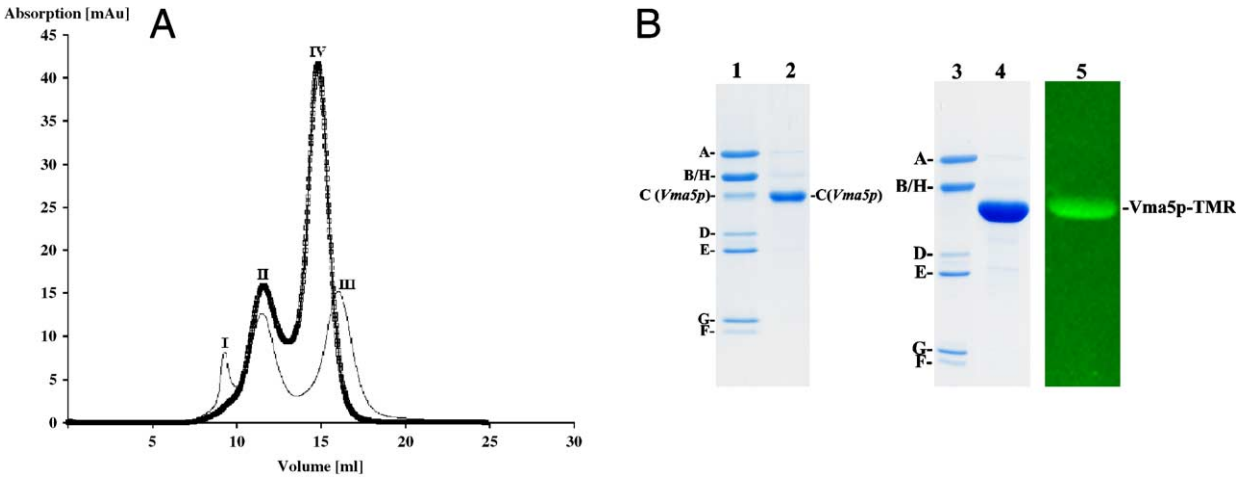

Fig. 2. Elution profile and electrophoretic analysis of the assembled V_1–Vma5p complex. (A) V_1(-C) ATPase from *M. sexta* and Vma5p were incubated overnight and applied onto a Sephacryl S-300 HR column with 20 mM Tris–HCl (pH 8.1), 150 mM NaCl. The elution diagram of this sample (solid line) contains the three peaks, labeled I–III. The fractions of peaks I, II and III were pooled and subjected to a gradient SDS-PAGE (B). Lane 1 of this gel shows that peak I comprises the subunits of V_1(-C) plus subunit C (Vma5p) in stoichiometric amounts. Peak II contains V_1(-C) (lane 3), and subunit C (lane 2) elutes as peak III [12]. In a second experiment the labeled Vma5p-MMN and V_1(-C) ATPase were incubated overnight and injected onto a Sephacryl S-300 HR column as described under Materials and methods. Only peaks II and IV were observed in the elution profile (?), consisting of V_1(-C) (lane 3) and the marked Vma5p-MMN, respectively (lane 4). Note, because of the successful labeling of Vma5p-MMN, the marked subunit (peak IV) elutes faster. Lane 5, Vma5p was incubated for 30 min with tetramethylrhodamine (TMR). The TMR-labeled protein on the single Cys_{340} residue was analyzed by SDS-PAGE.

(Fig. 2A, B). A specific ATPase activity of 2.6±0.1 units/ml was measured for the V_1–Vma5p hybrid complex of peak I.

Electron microscopy images of reconstituted V_1(-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* V_1 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 V_1 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 V_1 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* V_1(-C) complex (panel I). This implies that the third group represents the side views of the V_1 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 V_1 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 V_1 ATPase

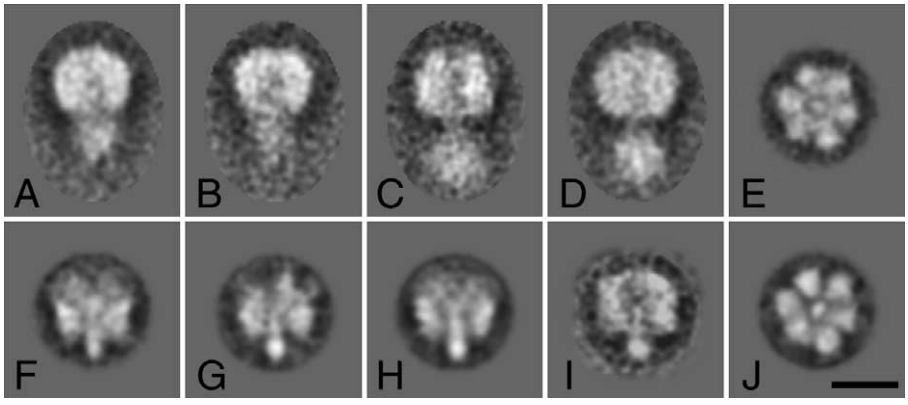

Fig. 3. Classes of the V_1–Vma5p hybrid complex (panels A–E, J), disassembled V_1 complexes from *M. sexta*, lacking subunit C (panels F–H). For comparison, a two-dimensional projection of isolated V_1(-C) ATPase from *M. sexta* has been added in panel I. The bar is 10 nm.

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 A_3B_3 domain via its head region to the V_O 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 V_1–Vma5p complex (Fig. 3).

Site-directed mutagenesis revealed that the C-terminal region of Vma5p might be important for the stable assembly of V_1 and V_O [21]. In order to determine whether the C-terminus is in close contact to the V_1 stalk domain, Vma5p was labeled by monomaleimidonanogold (MMN) at the single cysteine residue (Cys_{340}) of the C-terminal region (Fig. 1). By comparison, the binding of MMN at Cys_{340} resulted in a faster elution of the labeled Vma5p–MMN complex in the size-exclusion chromatography than the unlabeled protein (Fig. 2A, peaks II and IV). When Vma5p–MMN was incubated with the C-depleted V_1 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 Cys_{340} may be involved in the interaction of Vma5p with the C-depleted V_1 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 V_1 and V_O 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 V_1, we propose that the point of the foot in which Cys_{340} is located forms at least partially the surface for binding to the stalk region of V_1. 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 V_O 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 V_1 and the membrane-embedded V_O 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 V_1, as shown in the three-dimensional models of the V_1 ATPase with and without subunit C of *M. sexta* [10,20]. Such structural composition of an A_3:B_3 hexamer and one stalk domain of the soluble V_1 is consistent with a hexameric arrangement of the major nucleotide-binding subunits and a single compact stalk domain in the closely related A_1 and F_1 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 V_1V_O holoenzyme, which are proposed to be formed in part by subunits C and H, and the V_1 complex might become more compact following dissociation, with the peripheral stalks collapsing into a single stalk in the free V_1 ATPase as recently proposed [9].

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