CHAPTER IV

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

IV-1. Introduction

A characteristic feature of the eukaryotic V₁V₀ ATPase is the regulation by reversible disassembly of the V₁ and V₀ subcomplexes (Sumner et al., 1995; Kane, 1995), 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 inhibition of the Mg²⁺-dependent ATPase activity of dissociated V₁ complexes (Curtis & Kane, 2002). The high-resolution structure of the H subunit (Sagermann et al., 2001) and data on the gross structure of the V₁V₀ ATPase complexes suggest that in the intact enzyme this subunit is involved in the formation of the peripheral stalk region (Wilkens et al., 2004), despite the fact that a rearrangement within the disassembled V₁ is possible (Kane & Smardon, 2003). In contrast, the position of the C subunit of the eukaryotic V₁V₀ 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 (Radermacher et al., 2001), although it is essential for the reassembly of the functional V₁V₀ (Vitavska et al., 2003). Previously, the structure of the C subunit (Vma5p) from the yeast V₁V₀ 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 (Armbrüster et al., 2004). A recent 1.75 Å map from X-ray diffraction studies of Vma5p (Drory et al., 2004) 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 (Drory et al., 2004).

An intriguing result was the formation of a stable and ATPase active hybrid complex composed of Vma5p and V₁ from Manduca sexta, lacking subunit C (Armbrüster et al., 2004). Here we report the structural characterization of this hybrid complex by single particle electron microscopy and a biochemical approach.

IV-2. Materials and methods

IV-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 (Rizzo et al., 2003). 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$_1$V$_0$ ATPase was isolated according to Rizzo et al. (Rizzo et al., 2003) and Armbrüster et al., 2004, 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/HC1, 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 (Lötscher et al., 1984).

IV-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/HC1 (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.

IV-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 CM 20 FEG electron microscope at a calibrated magnification x 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 were extracted from the images using the "Boxer" program from EMAN software package (Ludtke et al., 1999). Projections were analyzed 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) (Schatz & van Heel, 1992). Transformed data were subjected to a Multivariate statistical analysis (MSA) and hierarchical ascendant classification (HAC) in a minimal variance mode (van Heel, 1984). On the basis of the classification, an initial file was divided into groups representing different classes of the projections. Non-reference alignment (Penczek et al., 1992) 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 was used.

**IV-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. IV-1). 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. IV-1A, 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 (Radermacher et al., 2001) 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) shows a large wedgelike density, extending from the V₁ headpiece (Fig. IV-2, 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 nm in width and 7.5 nm in height, which is connected to the V₁ headpiece by a narrow linker density of about 2.1 nm 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. IV-2, panels E, J). The third group has a consistent smaller stalk region (Fig. IV-2, panels F-H), comparable to 2-D projection of the \textit{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 \textit{M. sexta} (Radermacher et al., 2001) (data not shown).

**Figure. IV-2. Classes of the \textit{M. sexta} \( V_1 \)-ATPase.** Panels A-E, J, \( V_1 \)-Vma5p hybrid complex. Panels F-H, disassembled \( V_1 \) complexes from \textit{M. sexta}, lacking subunit C. For comparison, a two-dimensional projection of isolated \( V_1 \)(-C) ATPase from \textit{M. sexta} has been added in panel I. The bar is 10 nm.

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. IV-2, panels F-H; Radermacher et al., 2001). Thus these data are in agreement with the value of 11 nm determined for the hydrated \( V_1 \) ATPase using SAXS (Svergun et al., 1998). 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 (Radermacher et al., 2001) or the structural flexibility of Vma5p, as described for the recently determined Vma5p crystal forms of Vma5p (Drory et al., 2004). 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. IV-3, p. 69. 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 \( V_0 \) 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 (Armbrüster et al., 2004; Drory et al., 2004), would fit in the density of the globular stalk domain of the \( V_1 \)Vma5p complex (Fig. IV-2).

Site-directed mutagenesis revealed that the C-terminal region of Vma5p might be important for the stable assembly of \( V_1 \) and \( V_0 \) (Curtis et al., 2002). 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 (Cys340) of the C-terminal region (Fig. IV-3, p. 69). 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 unlabeled protein (Fig. IV-1A, 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. IV-1A). The same result was obtained when Vma5p was labeled with smaller maleimides like tetramethylrhodamine (TMR; Fig. IV-1B, 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 imply that this domain may be oriented to the membrane domain (Armbrüster et al., 2004; Drory et al., 2004). In line with this is the fact that the bottom of the foot domain shows a hydrophobic surface, (Drory et al., 2004) and it has been argued that the detachment of the V₁ and V₀ sections following cold treatment (Moriyama & Nelson, 1989 a) 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 (Xu et al., 1999), the latter of which is in close proximity to the stalk subunit D (Xu et al., 1999; Coskun et al., 2004), and in contact with subunit C of the V₀ part (Coskun et al., 2004). 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 V₀ 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. IV-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 (Svergun et al., 1998; Radermacher et al., 2001). Such structural composition of an A₃B₃ 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 Grüber et al., 2001a) and X-ray crystallography (Gibbons et al., 2000). Based on these structural data the question arises whether the peripheral stalk regions in the V₁V₀ 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 (Kane & Smardon, 2003).