Structural studies of *Saccharomyces cerevisiae* 
$V_1$-ATPase in the stationary phase of yeast cell culture

Jana Tuhman-Mushkin

A thesis submitted in conformity with the requirements for the degree of Master of Science
Biochemistry Department
University of Toronto

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2012

Abstract

Vacuolar-type ATPases (V-ATPases) are ubiquitous membrane-bound protein complexes present in the endo-membrane system of all eukaryotic cells. In eukaryotic cells, the reversible dissociation of the V$_1$ and V$_o$ regions is an essential mechanism for regulating V-ATPase activity. Therefore, knowledge of the structure of the dissociated V$_1$-ATPase is necessary for understanding the regulation of V-ATPase activity. In this thesis, I showed that by introducing a 3xFLAG tag at the C terminus of different V$_1$-ATPase subunits, highly purified V$_1$-ATPase complex could be isolated. Electron cryomicroscopy (cryo-EM) was used for initial analysis of the intact V$_1$-ATPase. In addition to the intact complex, partial V$_1$-ATPase subcomplexes with different subunit compositions were isolated from yeast cells in late log phase. All of the isolated subcomplexes were found to contain the major V$_1$-ATPase subunits A and B, but differed in the peripheral stalk subunit composition.
Acknowledgment

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List of Abbreviations

2D  two - dimensional
3D  three – dimensional
xg  times the force of gravity
ATP adenosine 5’-triphosphate
ADP adenosine 5’-diphosphate
AscI restriction enzyme
BSA bovine serum albumin
C. albicans Candida albicans
C-terminus carboxyl-terminus
Cu copper
cryo-EM electron cryomicroscopy
DNA deoxyribonucleic acid
DMSO dimethyl sulfoxide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDM</td>
<td>n-dodecyl beta-D-maltoside</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EB</td>
<td>expansion buffer</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>F₁</td>
<td>soluble region of F-type ATP synthase</td>
</tr>
<tr>
<td>F₀</td>
<td>membrane-bound region of F-type ATP synthase</td>
</tr>
<tr>
<td>FEG</td>
<td>field emission gun</td>
</tr>
<tr>
<td>FLAG</td>
<td>polypeptide protein tag</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster Resonance Energy Transfer/ Fluorescence Resonance Energy Transfer</td>
</tr>
<tr>
<td><em>in vivo</em></td>
<td>within the living</td>
</tr>
<tr>
<td>LiOAc</td>
<td>lithium acetate</td>
</tr>
<tr>
<td>LB</td>
<td>liquid media Luria-Bertani</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>NEB</td>
<td>New England Biolaboratories</td>
</tr>
</tbody>
</table>
Native-PAGE native, non-denaturing polyacrylamide gel electrophoresis

PCR polymerase chain reaction

Pa pascal pressure unit, $10^5$ bar

PAGE polyacrylamide gel electrophoresis

*P. shermani* *Propionic shermani*

*Pac1* restriction enzyme

PEG polyethylene glycol

RT room temperature

RAVE regulator of the ATPase of vacuolar and endosomal membranes

Rh rhodium

rpm revolutions per minute

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

SD-URA synthetic defined dropout, uracyl marker selection

SDS sodium dodecyl sulphate

SOC super optimal broth medium with glucose

*S. cerevisiae* *Saccharomyces cerevisiae*
TEM transmission electron microscope

Tris tris (hydroxymethyl) aminomethane

TBE tris, borate, EDTA

TE tris, EDTA

TE/LiOAc tris, EDTA, lithium acetate

UV ultraviolet light

URA3 gene that encodes orotidine 5-phosphate decarboxylase

v/v volume/volume

V-ATPase vacuolar ATPase

V₁-ATPase soluble region of vacuolar ATPase

V₀-ATPase membrane-bound region of vacuolar ATPase

w/v weight/volume

YPD yeast extract peptone dextrose

YEPD yeast extract, peptone 2%, dextrose medium
CHAPTER 1. Introduction

1.1 THE ROLE OF V-ATPases IN CELLULAR PROCESSES

The regulation of intracellular pH plays a crucial role in cellular homeostasis and survival. Many biological processes, such as protein trafficking, protein degradation and transport of small molecules are sensitive to pH changes (Schwartz 1990; Fuller et al 1989). Different cellular compartments have specific pHs necessary for their function. Protein trafficking and processing are disrupted when the luminal pH of one or more compartments is changed (Carnell and Moore 1994; Missiaen et al 2004). The secretory proteins that are transferred to different compartments undergo several maturation steps due to decreasing compartmental pH (Kim et al 1998; Demaurex et al 1998). The disruption of the pH gradient from cis- Golgi to the trans- Golgi membranes impedes processing of secreted proteins and posttranslational modification (Carnell 2004). In addition, the modification of the pH of secretory compartments has also been found in tumors and papillomas (Weisz 2003; Kellokumpu et al 2002). The mechanisms that regulate the specific pH of different compartments remain unclear. However, it is well established that vacuolar-ATPases (V-ATPases) play an important role in these pH regulatory processes (Nelson and Harvey 1999).

V-ATPases are ubiquitous membrane-bound protein complexes that comprise a family of ATP-driven proton pumps (Boekema et al 1999, Wilknes et al 1999; Wilknes et all ). V-ATPases were first identified in vacuolar membranes of fungi and plants (Bowman and Bowman 1982; Ward and Lai 1992), leading to the somewhat misleading name of vacuolar ATPases. V-ATPases generate an electrochemical gradient across membranes by coupling ion
transport with adenosine triphosphate (ATP) hydrolysis. Isolation of eukaryotic V-ATPase from the yeast *Saccharomyces cerevisiae* (Ohsumi and Anraku 1985) provided detailed insight into its structural and biochemical properties (Al-Awqati 1986; Nelson 1990). Currently, it is well established that V-ATPases are present in the endo-membrane system of all eukaryotic cells (for example, vacuoles, lysosomes and endosomes) and play an essential role in the acidification of intracellular space (Nelson and Harvey 1999; Jefferies et al 2008; Chumacher and Krebs 2010). V-ATPases are also present in the plasma membrane of some specialized cells (Forgac 1999; Rojas et al 2006) and have been implicated in a large number of health related issues, including osteoporosis, and tumour invasion and metastasis (Yuan et al 2010; Toei et al 2010).

1.1.1 Functions of V-ATPases in Normal Cell Processes

V-ATPase activity is essential for acidification of vacuoles, lysosomes, endosomes and the Golgi apparatus (Nishi and Forgac 2002). This acidification affects diverse processes including protein sorting and degradation, overall ion homeostasis, and protection of cells from oxidative stress (Kakinuma et al 1981; Moriyam et al 1992). In Figure 1.1 various roles of V-ATPases have been shown. For example, in the sorting endosome, V-ATPases acidify the endosome, which allows the release of internalized ligands, such as transferin or low density lipoprotein, from their receptors (Johnson et al 1993). The released ligand is delivered by endosomal carrier vesicles from sorting endosomes to later endosomal compartments, a process that requires the low pH established by V-ATPase (Gu and Gruenberg 2000). Also, the acidification of the lysosome by V-ATPases is responsible for the degradation of proteins (Nishi and Forgac 2002). Finally, the acidification of secretory vesicles by V-ATPases is
required for the transport of neurotransmitters into the vesicles and is therefore necessary for endocrine function.

The localization of V-ATPases to the plasma membrane is important in cell-specific functions such as renal acidification, sperm maturation, and dissolution and resorption of bone minerals (Chatterjee et al, 1992). Figure 1.1 B shows a summary of the normal processes in which V-ATPases at the plasma membrane are involved. For instance, in the proximal tubule cells of the kidney, V-ATPases are involved in the regulation of acid-base balance of the organism by controlling the proton secretion and absorption of bicarbonate (Brown and Breton 2000). V-ATPases, in the apical membrane of α--intercalated cells, secrete acid into the urine (Wagner et al 2004). Plasma membrane V-ATPases in osteoclasts play an essential role in bone resorption by creating the low pH necessary to dissolve the inorganic bone material and to degrade the organic bone matrix (Toyomura et al 2003). In the apical membrane of clear cells in the epididymus, V-ATPases preserve the acidic pH of seminal fluid, a property crucial for the maturation and storage of sperm.
Figure 1.1. A summary of cell processes in which V-ATPases are involved. (A) In endomembrane systems, acidification of the endosome by the V-ATPase allows for the release of ligands and the recycling of receptors. Budding of endosomal carrier vesicles and multivesicular bodies is also dependent on the acidic environment. Lysosomal proteins are synthesized in the trans -Golgi network and are trafficked to the lysosome. V-ATPases are necessary for the secretion of neurotransmitters and other molecules by secretory vesicles. Acidification of the late endosome allows for the release of the lysosomal proteins. (B) In the apical membrane of the renal alpha-intercalated cells, V-ATPases secrete protons into the lumen of collecting ducts. In the plasma membrane of osteoclasts, V-ATPases are involved in bone resorption. In epididymal clear cells, plasma membrane V-ATPases decrease the pH of the lumen, a process that is required for sperm maturation (figure adapted from Jefferies et al 2008).
1.1.2 Functions of V-ATPases in Disease Processes

V-ATPases have been implicated in various disease processes. Defective V-ATPase activity in the plasma membrane of osteoclasts leads to osteopetrosis, which is characterized by developmental defects resulting from the inability to degrade and remodel bone (Kartner et al 2010). Increasing evidence suggests that V-ATPases are targeted to the plasma membrane at a high concentration in many types of metastatic cancer cells (Forgac 2007). Such studies have established that plasma membrane V-ATPases cause extracellular acidification. An acidic tumour extracellular microenvironment can contribute to degradation of the surrounding tissue, which leads to cancer invasion and metastasis. Recently, V-ATPase was found in high concentrations in breast cancer cells and inhibition of V-ATPase activity was shown to decrease cancer invasion and, consequently, may point the way to improved disease therapy (Hinton et al 2009).

1.2 OVERALL STRUCTURE AND MECHANISM OF THE ROTARY MOTOR OF V-ATPase

There is evidence that the structure of V-ATPases may differ from species to species (Perzov et al 2001). A complete picture of the molecular structure of this enzyme is now emerging, but important features, especially the structure of the peripheral stators, are still the subject of ongoing investigations. Table 1 describes the nomenclature of different V-ATPase subunits.

The vacuolar-ATPases are evolutionarily related to F-type ATPase synthases (Cross and Müller 2004). This close relationship was shown by sequence homology between subunits (Mandel et al 1988; Manolson et al 1988; Nelson and Nelson 1989). The structure
and ATP-driven proton pumping mechanism of V-ATPase is similar to that of the F-type ATP synthase. Both enzymes are found in eukaryotic cells, but F-type ATP synthases are localized to mitochondria and chloroplasts, whereas V-ATPases are found in most intracellular membranes.

Table 1. Subunit nomenclature and function for V-ATPase in comparison to F-type ATPase synthases (see Figure 1.2).

<table>
<thead>
<tr>
<th>Subunit name in V₁-ATPase</th>
<th>Molecular weight (kDa)</th>
<th>Yeast gene subunit name</th>
<th>F-type ATPase synthase homologue</th>
<th>Subunit Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>70</td>
<td>VMA1</td>
<td>B</td>
<td>Catalytic site regulation</td>
</tr>
<tr>
<td>B</td>
<td>60</td>
<td>VMA2</td>
<td>A</td>
<td>Non-catalytic</td>
</tr>
<tr>
<td>C</td>
<td>45</td>
<td>VMA5</td>
<td>-</td>
<td>Peripheral stalk</td>
</tr>
<tr>
<td>D</td>
<td>30</td>
<td>VMA8</td>
<td>Γ</td>
<td>Central stalk</td>
</tr>
<tr>
<td>E</td>
<td>27</td>
<td>VMA4</td>
<td></td>
<td>Peripheral stalk</td>
</tr>
<tr>
<td>F</td>
<td>14</td>
<td>VMA7</td>
<td>E</td>
<td>Central stalk</td>
</tr>
<tr>
<td>G</td>
<td>12</td>
<td>VMA10</td>
<td></td>
<td>Peripheral stalk</td>
</tr>
<tr>
<td>H</td>
<td>55</td>
<td>VMA13</td>
<td></td>
<td>Peripheral stalk</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subunit name in Vo-ATPase</th>
<th>Molecular weight (kDa)</th>
<th>Yeast gene subunit name</th>
<th>F-type ATPase synthase homologue</th>
<th>Subunit Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>110</td>
<td>VPH1(vacuole) STV1(golge)</td>
<td>a</td>
<td>H⁺ transport,targeting</td>
</tr>
<tr>
<td>d</td>
<td>40</td>
<td>VMA6</td>
<td>-</td>
<td>Central stalk</td>
</tr>
<tr>
<td>e</td>
<td>10</td>
<td>VMA9</td>
<td>-</td>
<td>Unknown</td>
</tr>
<tr>
<td>c</td>
<td>17</td>
<td>VMA3</td>
<td>c</td>
<td>H⁺ transport</td>
</tr>
<tr>
<td>c'</td>
<td>17</td>
<td>VMA11</td>
<td>c</td>
<td>H⁺ transport</td>
</tr>
<tr>
<td>c''</td>
<td>21</td>
<td>VMA16</td>
<td>c</td>
<td>H⁺ transport</td>
</tr>
</tbody>
</table>
The F- and the V-type ATPases are composed of a membrane-bound $F_o/V_o$ region, which contains the proton translocation channel, a central connecting stalk, and a soluble $F_i/V_1$ region that contains the catalytic sites. All these enzymes have one or more peripheral stalks that connect the $V_1/F_1$ region to the $V_o/F_o$ region. Despite their similarities, these two types of ATPases differ in the number and structure of their peripheral stalks. The exact arrangement of the peripheral stalks in V-type ATPases is still unclear (Figure 1.2) (Li and Zhang 2004).

The eukaryotic V-ATPases are ~900 kDa protein complexes (Forgac et al. 2007). The soluble $V_1$ region is a ~600 – 650 kDa complex and is composed of eight different subunits (A, B, C, D, E, F, G, H) in a stoichiometry of $A_3B_3CDE_3FG_3H$, as determined by quantitative amino acid analysis (Arai et al. 1988; Xu et al. 1999). The $V_o$ region is a ~260 kDa membrane-integral complex containing six different subunits (a, d, e, c, c', c'') with a stoichiometry that is probably $ac_4c'c''de$ (Arai et al. 1988; Powell et al. 2000).

The core of the $V_1$ region is composed of a hexamer of alternating A and B subunits (Zhang et al. 2003). Binding and hydrolysis of ATP occurs at three of the six interfaces between the A and B subunits. Catalytic ATP binding sites are located primarily in the A subunits, whereas the B subunits contain nucleotide binding sites with a modulatory role. The $V_o$ region includes a ring of proteolipid subunits (c, c' and c'') that are adjacent to the a and e subunits (Wilkens and Forgac 2001). The multiple peripheral stalks are composed of subunits C, E, G, H and serve as stators preventing the rotation of the rotor $A_3B_3$ subcomplex relative to $V_o$ during ATP hydrolysis (Figure 1.2 A).
Figure 1.2. V-ATPase structure and comparison with F-type ATP synthases: (A) The $V_1$ region of V-ATPases consists of the A, B, C, D, E, F, G, and H subunits, whereas the $V_0$ region consists of the a subunit, c-ring (subunits c, c', and c''), d subunit, and e subunit. The rotor part of the proton-pumping machine is formed by the c-ring, the d subunit, and the D and F subunits, which are coloured blue, whereas the ATP-hydrolysing $A_B$ motor is shown in green and red. The E/G peripheral stalk structures are shown in yellow and orange and the a, C, and H subunits of the membrane proximal base structure are shown in gray. (B) A schematic representation of F-type ATP synthase shown for comparison. Subunits that are homologous to V-ATPase subunits are shown in the same colour (figure adapted from Boesen and Nissen, 2009).
1.2.1 Mechanisms of Regulation of the V-ATPase Activity

Since V-ATPases are involved in a multitude of physiological processes (see above), their activity and localization is tightly controlled and regulated by different intracellular mechanisms. The localization of V-ATPases is regulated in specialized cells, such as epidermal clear cells, renal collecting duct cells, and intercalated cells, through the utilization of specific subunit isoforms (Toei et al 2010). Studies in yeast show that reversible formation of a disulfide bond between cysteine residues in the catalytic site of the A subunit of the V$_1$ region can prevent hydrolysis of ATP (Forgac, 1999). For example, approximately half of V-ATPases in native clathrin-coated vesicles exists in this disulfide-bonded non-catalytic state (Feng and Forgac 1992).

In addition to the regulatory mechanism described above, in eukaryotic cells, the reversible dissociation of the V$_1$ and V$_o$ regions regulates V-ATPase complex activity (Kane 1995). Once dissociated, the V$_1$ region loses its ATPase activity while the isolated V$_o$ region does not allow protons to cross the membrane (Saroussi 2009). It has recently been shown that this process occurs during conditions such as glucose depletion, basic extracellular pH, exposure to heavy metals and a variety of drugs (Nishi and Forgac 2002). Several proteins and protein complexes are involved in the dissociation and re-association of the V$_1$ and V$_o$ regions. For instance, in the yeast, S. cerevisiae, dissociation is driven by the interaction of the V-ATPase with aldolase (Lu et al 2001), whereas the assembly between V$_1$ and V$_o$ is promoted by the RAVE complex (Figure 1.3). The RAVE complex is composed of three proteins Rav1p, Rav2p, and Skp1, and was shown to bind to subunits E, G and C of the V$_1$ region (Smardon et al 2002). The dissociation has been best characterized in yeast, where it occurs rapidly, on the order of a few minutes, reversibly and without the synthesis of new proteins (Smardon and Kane 2007). It was also shown that the C-subunit of the V$_1$ region,
which is part of the peripheral stalk, is released from V₁-ATPase upon the dissociation of the V₁ region from the V₀ region and represents another regulatory mechanism. However, there are still many unanswered questions about the dissociation process, such as, does the V₁-ATPase remain intact after disassembly from the V₀ region?

Figure 1.3. Regulation of V-ATPase activity by reversible dissociation: V-ATPase complexes reversibly dissociate in vivo into the V₁ region and V₀ region. This process occurs in response to environmental changes such as glucose depletion, addition of salts, and pH changes. The dissociation is also driven by interaction of the intact complex with aldolase. The assembly of the V₁ and V₀ regions in yeast is promoted by the RAVE complex, which is composed of Skp1, Rav1p and Rav2p (figure adapted from Toie et al 2010).
1.3 ELECTRON MICROSCOPY AND IMAGE ANALYSIS

1.3.1 Techniques in structural biology

The major techniques used to study protein structures are X-ray crystallography, nuclear magnetic resonance spectroscopy (NMR) and electron microscopy (EM). The field of X-ray crystallography is the oldest and most established of these techniques. It can provide structural information about proteins with atomic or near-atomic resolution. NMR has been utilized to determine structural information on proteins and also provide insight into dynamic properties of biomolecules in solution. However, NMR is usually unable to determine the structures of large biological complexes. The structures of these complexes can be determined by electron microscopy. With new developments in both hardware and software, new possibilities have opened in the field of electron microscopy in the determination of large biomacromolecular structures at high resolution. Electron microscopy has become a well-established technique to obtain structural information about large complexes at a resolution between 10–20 Å (Van Heel et al, 2000; Lau and Rubinstein 2010). Recently, an all atom model of viral particles with icosahedral symmetry was determined to a resolution of 3.3 Å (Zhang et al 2010).

1.3.2 Electron microscopy

The transmission electron microscope functions on the same basic principles as the transmission light microscope, but uses electrons instead of photons. Both in a light and in an electron microscope, images are produced by the transmission of the beam through the specimen. However, the diffraction limit of lenses limit the resolution of a light microscope to approximately half the wavelength of the light used (400 – 600 nm), whereas the wavelength
(0.0037 nm) makes it possible to obtain a higher resolution interaction with an electron microscope than with a light microscope.

The contrast in images is proportional to the cross-section of interaction between the electron beam and the specimen. This cross section is determined by the average atomic number of the atoms in the specimen. Low contrast is a major disadvantage in the imaging of biological specimens, which mainly consist of atoms with low atomic numbers (carbon, nitrogen, oxygen, and hydrogen). However, it is possible to image biological molecules with higher contrast by surrounding the molecules with materials of high atomic number during specimen preparation (see below).

Biological samples are radiation sensitive, and are damaged by illumination with the high-energy electron beam. The radiation dose experienced by the specimen during imaging can cause structural damage, such as covalent bond breakage, mass loss and shrinkage. In order to reduce specimen damage, minimal exposure times to the electron beam are required (Glaeser and Taylor 1978). Therefore, a low exposure in the ranges between 10 - 100 electrons Å² is typically used to image biological samples (Amos et al 1982). However, imaging with such a low exposure causes a low signal-to-noise ratio in images and requires computational processing in order to calculate average images from a number of images of different complexes. Negative staining and electron cryomicroscopy followed by computational single particle image analysis are used to obtain and resolve structural information on biological molecules with high resolution (Frank 2009).
1.3.3 Negative Stain Electron Microscopy

The negative stain effect was first unintentionally discovered in a study in which particles were stained with phosphotungstic acid (Hall 1955). Later, the same effect with tobacco mosaic virus was also observed through an experiment to intentionally create this effect and was named "negative staining" (Brenner et al. 1959).

In negative staining, biological samples adsorbed to carbon support films are visualized with an electron microscope after surrounding the material with a thin amorphous film of a heavy metal solution, such as methylamine tungstate, sodium/potassium phosphotungstate or uranyl acetate (Figure 1.4). The higher density of the stain as opposed to the low density of the biological specimen causes the negative appearance of the image, which appears light against the darker background of the stain. However, this method does not allow for the high-resolution determination of a structure due to a number of technical limitations. The negative staining procedure can generate artifacts, where the stain layer is too thick, or too thin so that the biological material is only partly covered by stain. Flattening and stretching of specimens may also occur during grid preparation (Glaeser et al 1991). Nevertheless, negative staining remains a widely used and rapid, qualitative method for determining lower-resolution structures.
1.3.4 Electron Cryomicroscopy

Electron cryomicroscopy (cryo-EM) allows biological molecules to be imaged after immobilization in vitreous ice in their native conformation, thus potentially mimicking the protein’s native environment and structure. This approach allows for obtaining structures with better resolution compared to imaging in negative stain (Rubinstein 2007). However, specimens embedded in ice have very low contrast and require significantly more computational data processing in order to obtain averages from a large number of images.

Figure 1.4. Negative stain and cryo grid preparation: (A) Three macromolecules in different orientations. (B) Negative stain specimen preparation (left) and recorded images (right). Macromolecules are adsorbed onto the carbon substrate. The negative stain method may flatten particles during drying. (C) Cryo-EM specimen preparation (left) and recorded images (right). Macromolecules are rapidly frozen to generate an amorphous ice, which preserves native or near-native structure (figure adapted from Steven and Belnap, 2005)
1.3.5 Image analysis

Single Particle Analysis is a computational image processing technique used to calculate 3D structures from electron microscopy images of biological specimens. In order to improve the low signal-to-noise ratio of the recorded images, several thousand single particle images are required. Single particle determination begins with selection of the single particle images from electron micrographs. Then the computer programs employing multivariate statistical analysis are used to group similar particle images together into multiple sets (Thuman-Commike 2001). Figure 1.5 summarizes all these steps into a computational single particle image analysis.

Figure 1.5. Single particles image analysis process: (A) Objective. (B) Micrograph with three unique views of the objective (side, front, and top views). (C) Selected single particle images from the micrograph. (D) Aligned particle images to common views. (E) Classified particle images. (F) Determined orientations (side, front, and top views) (Figure adapted from Thuman-Commike 2001).
1.4 RATIONALE AND OBJECTIVES

There is evidence to suggest that the structure of V-ATPase may differ from species to species and the number and design of the V-ATPase peripheral stators are still unclear. Recent electron microscopy studies of the prokaryotic V-ATPases from *Thermus thermophilus* showed two peripheral stalks (E₂G₂) (Lau and Rubinstein 2010) whereas three peripheral stalks (E₃G₃) were found in the eukaryotic yeast *Saccharomyces cerevisiae* V-ATPase (Zhang et al 2008; Benlekbir et al, manuscript in preparation).

The reversible dissociation of the V₁ and V₀ regions is an essential mechanism for regulating V-ATPase activity. This mechanism is a rapid and effective way to reduce or increase proton pumping. A complete picture of the molecular structure of the intact complex is now emerging but important features, especially the structure of the dissociated V₁-ATPase are still unknown. There are still many unanswered questions about the distinct structural details of V₁-ATPase. Does the dissociated V₁ region have the same structure as when it is part of the intact V-ATPase complex? Do all of the subunits still have the same structure in dissociated V₁ region or do they change conformation to stop ATP hydrolysis? Do some of the subunits of V₁-ATPase play a role in the dissociation and inhibition processes?

The overall goal of the current study is to determine the structure of dissociated *Saccharomyces cerevisiae* V₁-ATPase in the late stationary phase of yeast culture by using electron microscopy. The thesis is comprised of three major tasks:

1) To determine the structure of dissociated V₁-ATPase.
2) To identify the sub-complexes of V₁-ATPase in cells.
3) To investigate the behavior of peripheral stalk subunits in dissociation.
CHAPTER 2. Materials and methods

2.1 MATERIALS

2.1.1 Chemicals

All reagents were purchased from Bioshop, unless specified otherwise. De-ionised water was purified with a Milli-Q Academic purification system (Millipore) and all aqueous solutions were prepared with Milli-Q purified water.

2.1.2 Reagents and kits used for molecular biology

All enzymes, buffers and reagents for polymerase chain reaction (PCR) in this study were purchased from Fermentas, unless otherwise noted. The QIAEX II Gel extraction Kit (Qiagen) was used for DNA extraction from agarose gels, and the QIAquick PCR purification Kit (Qiagen) for PCR product purification.

2.1.3 Bacterial and Yeast growth media

Bacterial growth liquid media Luria-Bertani (LB) contained 1 % (w/v) peptone, 0.5 % (w/v) yeast extract, and 1 % (w/v) NaCl. Plates of LB were made from 1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl and 2 % (w/v) bacto agar. LB selective plates were prepared with a suitable antibiotic (Ampicillin).

Yeast growth liquid YPD media consisted of 2 % (w/v) peptone, 1 % (w/v) yeast extract and 2 % (w/v) D-glucose. Plates of YPD contained 2 % (w/v) peptone, 1 % (w/v) yeast extract, 2 % (w/v) D-glucose and 2 % (w/v) bacto agar. Synthetic defined dropout Ura marker selection, (SD-URA) plates contained 0.67 % (w/v) yeast nitrogen base, 0.2 % (w/v) amino acid mixture minus uracil, 2 % (w/v) glucose, and 2 % (w/v) bacto agar.
2.1.4 Vectors, bacterial strains and yeast strains

pMK159, a gift from M. Kampmann (Rockefeller University, New York, NY), was used as the starting plasmid for 3xFLAG vector construction. pMK159 encodes an 8 kDa biotinylation signal sequence from \textit{P. shermanii} followed by the \textit{C. albicans URA3} marker (See Figure 2.1 for more details). A description of the 3xFLAG vector is given in Results Chapter 3.1.

\textit{E. coli DH5α} competent cells were used for the isolation of recombinant plasmid DNA.

The \textit{Saccharomyces cerevisiae} strains MM93 (aka BJ2168) - \textit{MATa leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2} and MM940 -\textit{MATa Δvma2 kan leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2,} were kindly provided by Dr. Morris Manolson (Department of Biochemistry, University of Toronto). The MM93 was used as the background strain for constructing new yeast strains. The MM940 -\textit{MATa Δvma2 kan leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2} was used as the negative control strain for yeast growth assay.
Figure 2.1. Plasmid pMK159 map and the sequences of the biotinylation signal, Ura3 marker and restriction sites.
2.2 STANDARD BIOCHEMICAL TECHNIQUES

2.2.1 Denaturing polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) was employed to separate proteins. Protein samples (100 μl) were mixed with 30 μl of 10xSDS gel loading buffer (0.5 M tris-HCl [pH 6.8], 4.4 % [w/v] SDS, 20 % [w/v] glycerol, 2 % [w/v] 2-mercaptoethanol, and 0.04 % [w/v] bromophenol blue). Approximately, 50 μl of each sample was loaded on the gel, composed of 12 to 15 % (w/v) polyacrylamide (as indicated) for the resolving gel and 4 % (w/v) polyacrylamide for the stacking gel. The gel electrophoresis apparatus was assembled and filled with running buffer (25 mM Tris [pH 8.3], 192 mM glycine, 0.1 % [w/v] SDS). The gel was run at 180 volts for 50 minutes, or until the dye front reached the bottom of the gel.

2.2.2 SDS-PAGE Coomassie staining

Coomassie staining was used for qualitative analysis of gels after protein purification. The SDS-PAGE gels were stained for up to two hours with staining solution (0.2 % [w/v] Coomassie brilliant blue, 50 % [v/v] methanol and 7 % [v/v] acetic acid). Polyacrylamide gels were destained in gel destain solution (20 % [v/v] methanol, and 7 % [v/v] acetic acid) until unbound stain was removed.

2.2.3 SDS-PAGE silver staining

Silver staining was used to detect proteins at low concentrations. The SDS-PAGE gels were fixed for 40 minutes in 50 % (v/v) methanol and 10 % (v/v) acetic acid. Then, they were incubated in 20 % (v/v) ethanol for a further 10 minutes and washed for five minutes in water. Gels were reduced with 0.02 % (w/v) sodium thiosulfate solution for 1 minute and washed two times with water for 30 second. Gels were then incubated for 30 minutes in 0.2 % (w/v)
silver nitrate. After one wash for 20 seconds in water, proteins were detected with a developing solution consisting of 3.0 % (w/v) sodium carbonate, 0.05 % (v/v) formaldehyde and 0.01 % (w/v) sodium thiosulfate. Development was stopped after reaching the desired intensity of staining by a solution containing 1 % (v/v) acetic.

2.2.4 Native polyacrylamide gel electrophoresis (PAGE)

Non denaturing (native) gels were prepared as described above, except that no SDS or reducing agent was added to the gel, sample or buffer. Gels were run at 80 volts for 2 hours at 4 ºC, or until the dye front reached the bottom of the gel. Native gels were stained with Coomassie blue and destained as described above.

2.2.5 DNA agarose gel electrophoresis

Agarose gel electrophoresis was used to analyze products from polymerase chain reactions (PCR). 1 % (w/v) agarose was dissolved in TBE buffer (45 mM tris-borate [pH 7.5], 1 mM EDTA) and heated in a microwave oven. The gel was allowed to cool and 5 μl of safe view stain was added. The gel electrophoresis tank was assembled and filled with TBE buffer. DNA samples (30 μl) were mixed with 5 μl of 6x loading buffer (10 mM Tris-HCl [pH 7.6], 0.03 % [w/v] bromophenol blue, 0.03 % [w/v] xylene cyanol FF, 60 % [v/v] glycerol, 60 mM EDTA). 25 μl of each sample was loaded onto the gel and run at 100 volts for 45 minutes.

DNA was visualized on the gel with 300 nm of ultraviolet light (UV) and photographed. DNA was eluted from agarose gels while keeping the UV light at low intensity. Single bands from agarose gel were cut out using a sharp sterile scalpel and collected in a sterile microcentrifuge tube. A gel extraction kit (Qiagen) was used to elute the DNA from the gel according to the manufacturer's instructions.
2.2.6 Chromatography

Size exclusion liquid chromatography was performed with AKTA chromatography systems (GE Healthcare), with a Superose 6 gel filtration column. Before loading on the chromatography system, all samples and buffers were filtered and the column pre-equilibrated with the appropriate buffer.

2.3 PLASMID AND VECTOR CONSTRUCTION

2.3.1 DNA restriction enzyme digestion

Restriction enzymes were selected based on the sequenced maps of the pMK159 plasmid (Figure 2.1). DNA digestions were prepared in 2 ml microtubes. In the total 30 μl volume was 3.5 μl of circular DNA (pMK159), 1 μl of each restriction enzyme (PacI and AscI), 5 μl of 10xNEBuffer (50 mM potassium acetate, 20 mM tris-acetate [pH 7.9], 10 mM magnesium acetate 1 mM dithiothreitol), and 0.5 μl of bovine serum albumin (BSA). The restriction reaction was incubated at 37 °C for 1 hour. DNA was analyzed by agarose gel electrophoresis and purified by QIAquick Gel Extraction kit according to the manufacturer's instructions.
2.3.2 Oligonucleotides annealing

Phosphorylated oligonucleotides encoding a 3xFLAG tag with the restriction enzyme sites (AscI and PacI) were designed and synthesized by Invitrogen (See Table 2.1). Both of the complementary oligonucleotides were resuspended at the same molar concentration using an annealing buffer (10 mM tris [pH 7.5], 50 mM NaCl, 1 mM EDTA) and equal volumes of both complementary oligos were mixed in a microfuge tube. The annealing reaction was placed in a heatblock at 95 °C for 5 minutes and afterwards cooled at room temperature (RT) for 60 minutes.

Table 2.1 Complementary oligonucleotides. Oligonucleotides encoding a 3xFLAG tag (pink) with the restriction enzyme sites PacI and AscI (green). Oligonucleotides were synthesized by Invitrogen.

<table>
<thead>
<tr>
<th>Coding strain</th>
<th>Non-coding strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAAAGACTACAAAGACCATGACGGTGATTATAAAGATGATGATCGATTACAAGGATGACGTGACAAAGTGAGG</td>
<td>CGCGCTCCTTGTGCTCATTCTTGGATATCTCGATATCATGATCTTTTATAATCCGTCA TGGTCTTTTGATGTCATTAAAT</td>
</tr>
</tbody>
</table>
2.3.3 DNA ligation

Ligations were performed with a vector-to-insert ratio of ~1:1, ~1:3 and ~1:8 with T4 DNA ligase (Invitrogen) according to the manufacturer's instructions. The reactions were prepared with the following reagents: vector DNA, 3xFLAG tag insert, T4 DNA ligase and ligation buffer (50 mM tris-HCl [pH7.5] 10 mM MgCl₂, 1 mM ATP, 10 mM dithiothreitol). Ligation reactions were incubated at room temperature for 1 hour and then heated at 65 °C for 10 minutes.

2.3.4 Bacterial transformation and plasmid amplification

Plasmid DNA was used to transform competent *E. coli* DH5α cells. The cells were kept on ice for 30 minutes and heat shocked for 15 seconds at 42 °C. For recovery, SOC medium (0.5 % [w/v] yeast extract, 2 % [w/v] tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was added and the cells were incubated in 37 °C for 1 hour. Afterwards, cells were spread on LB-Amp plates, containing Ampicillin and incubated at 37 °C over night. Plasmid isolation was performed with Qiagen miniprep kits according to the manufacturer's protocol.
2.4 YEAST STRAIN CONSTRUCTION AND CONFIRMATION

2.4.1 PCR of linear DNA for yeast transformation

The yeast strains used in this study were engineered to encode a 3xFLAG tag sequence C-terminal to different V$_1$-ATPase subunits. PCR was used to generate a DNA fragment for homologous recombination. All fragments consisted of a 5′ region homologous to the 3′ end of the gene of interest and 3′ region homologous to the genomic DNA downstream of the gene of interest (for more details see Table 2.2). The central sequence of the DNA fragment encoded the 3xFLAG tag sequence followed by the $URA_3$ auxotrophic marker from Candida albicans. The template DNA for the PCR was the plasmid pJT1 (see Results Chapter 3.1 for more details), which encoded 3xFLAG sequence followed by the $C. albicans URA_3$ marker.

The PCR reaction was carried out as follows: 1 minute at 94 °C, 30 cycles of 94 °C for 1 minute, 52 °C for 1 minute and 68 °C for 2.5 minutes, and 7 minutes incubation at 68 °C.
**Table 2.2. PCR Primers.** Forward primers contained 50 bp from the 3' end of the gene of interest (yellow) and 20 bp from the 3xFLAG sequence (green). Reverse primers contained the reverse complement of 50 bp downstream from the gene of interest (orange) and the reverse complement of 25 bp from the gene of interest (blue). All primers were synthesized by Invitrogen.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primers</th>
<th>Reverse primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>VMA1</td>
<td>TCGAAAAATTTGTTGACACTATGCAAGAAAGATTTGCTGAATCTACCGATGACTACAAAG</td>
<td>GAAGAAAAGACATCTAACAAATACCAGAAGATAATGCTACATATATC</td>
</tr>
<tr>
<td>VMA4</td>
<td>CCATCAGATTTGGAATTGTATGTCCTTCCAAGACAAGAAAGTTTTTGTAGATGATATAATCAATTTAGCTACAAAG</td>
<td>TCTGTTAGGAGTGTATATGT</td>
</tr>
<tr>
<td>VMA5</td>
<td>CTCTTGTGCACACAGAGTATGAACCATTGTGATGATATAATCAATTTAGCTACAAAG</td>
<td>TCTGTTAGGAGTGTATATGT</td>
</tr>
<tr>
<td>VMA7</td>
<td>ACCCTGAAAAAGGACTCTGATTTGAGAGTCAGAAAGATTGAAGAGTGCTGGTACGATGACTACAAAG</td>
<td>TCTACAGGCTGGAATATCCAATTTAGCTACAAAG</td>
</tr>
<tr>
<td>VMA13</td>
<td>AATACGAGGCCTCAGCCACAGCAAGATATGCTACATATATC</td>
<td>TCTACAGGCTGGAATATCCAATTTAGCTACAAAG</td>
</tr>
</tbody>
</table>

Reverse primers:
2.4.2 Yeast Transformation

The *Saccharomyces cerevisiae* strain MM93 was streaked onto a YPD agar plate. One yeast colony was selected and inoculated in 50 ml liquid YPD medium and incubated at 30 °C over night, with shaking at 250 rpm. When the yeast culture reached an OD$_{600}$ of 1.0 (+/- 0.2), it was centrifuged at 1 000 x g for 3 minutes. The yeast pellet was resuspended in 10 ml of sterile water and centrifuged at 1 000 x g for 3 minutes. The pellet was then washed with 1 ml of sterile water, transferred to a 1.5 ml sterile microfuge tube and centrifuged at 1 000 x g for 3 minutes. The supernatant was removed and the pellet was resuspended in 1 ml sterile TE/LiOAc buffer (10 mM tris-HCl, 1 mM EDTA, 100 mM LiOAc, [pH 7.5]) and centrifuged at 1 000 x g for 3 minutes. The supernatant was removed and the pellet was resuspended in 0.25 ml TE/LiOAc buffer. 50 μl of yeast cells were mixed with 50 μl of transforming DNA (PCR product, see above) and 5 μl of single stranded salmon sperm carrier DNA at 10 mg/ml (Invitrogen). Single stranded DNA was boiled for 5 minutes and chilled on ice for 5 minutes before it was used in the transformation mix. 300 μl of sterile solution (40% [w/v] PEG 4000 in TE/LiOAc buffer) were added, the contents were mixed thoroughly and incubated at 30 °C for 1 hour with occasional gentle shaking (every 10 minutes). Afterwards, 40 μl of DMSO was added to the reaction tube and mixed. Yeast cells were heat shocked at 42 °C for 15 minutes and then centrifuged at 1 000 x g for 10 seconds. The supernatant was removed and the pellet was resuspended in 1 ml TE buffer. The suspension was centrifuged at 1 000 x g for 10 seconds. The supernatant was removed and the pellet was resuspended in 250 μl of TE, plated on selective media (SD-Ura) and incubated at 30 °C for 3-4 days to recover transformants.
2.4.3 Yeast Chromosomal DNA Isolation

Transformed yeast was inoculated into 5 ml of liquid YPD and grown over night at 30 °C with shaking at 250 rpm. The cells were centrifuged at 4 000 x g for 3 minutes. The pellet was resuspended in 1 ml water and the suspension was transferred to 2 ml screw top tubes and centrifuged at 4 000 x g for 3 minutes. The supernatant was removed, the pellet was resuspended in 200 μl of the lysis buffer (2 % [w/v] Triton X-100, 1 % [w/v] SDS, 100 mM NaCl, 10 mM tris HCl, 1 mM EDTA [pH 8]), to which 200 μl of glass beads along with 200 μl TE and 200 μl of phenol/chloroform, pH 8.0 were added. The suspension was mixed for 3 minutes by vortexing. The mixture was centrifuged at 13 000 x g for 5 minutes. The top aqueous phase was transferred to fresh microfuge tubes, mixed with 1 ml of 100 % ethanol by inversion, after which the suspension was centrifuged at 13 000 x g for 3 minutes. The supernatant was removed and the pellet was washed with 1 ml 70 % (w/v) ethanol and centrifuged at 13 000 x g for 3 minutes. The supernatant was discarded and the pellet was dried in a speedvac. The resulting chromosomal DNA pellet was resuspended in 250 μl of EB buffer (Qiagen). Chromosomal DNA was used for PCR with gene specific primers to confirm construction of yeast strains (see below).
2.4.4 Confirmation of transformed fragments

The confirmation of strain construction was done by PCR with the primers listed in Table 2.3. The PCR was carried out as follows; 2 minutes at 94 °C, 30 cycles of 94 °C for 30 seconds, 52 °C for 1 minute and 72 °C for 2 minutes, and 7 minutes incubation at 72 °C using genomic DNA from the yeast.

Table 2.3. Primers for confirming strain construction. Primers were synthesized by Invitrogen.

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Forward primers</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>VMA1</td>
<td>ACTGCTGACGTTAAGCATG</td>
<td>AGGTGACGGATCGGTGA</td>
</tr>
<tr>
<td>VMA4</td>
<td>GTGGTCTCAATGCAAGAC</td>
<td></td>
</tr>
<tr>
<td>VMA5</td>
<td>GCCACCTCATTGAACATCA</td>
<td></td>
</tr>
<tr>
<td>VMA7</td>
<td>GACTCCCTACCAATGCCG</td>
<td></td>
</tr>
<tr>
<td>VMA13</td>
<td>CAAGACTGGCGGCAAAG</td>
<td></td>
</tr>
</tbody>
</table>
2.4.5 Yeast growth assay

To monitor viability of yeast strains growth assays were performed. Each yeast strain was inoculated into 5 ml of liquid YPD and grown over night at 30 °C with shaking at 250 rpm. Cultures were adjusted to OD_{600} of 1 by dilution with fresh media and serially diluted in water by factors: 10^0, 10^{-1}, 10^{-2}, 10^{-3}. 1 μl of each dilution was spotted on YPD plate buffered to pH 5.5 and supplemented with 50mM CaCl_2. Growth was recorded after 3 days of incubation at 30 °C.

2.4.6 Purification of 3xFLAG Tagged Yeast V_1-ATPase

Over night cultures were transferred into two liters of YPD and again grown over night until they reached OD_{600} of ~ 5. Cells were harvested by centrifugation at 4 000 x g for 15 minutes. The pellet was resuspended in the lysis buffer at 1 g/ml and passed through a homogenizer 5 times at 30 kPa. The homogenate was centrifuged at 3 000 x g for 10 minutes to remove cell debris. Supernatant was decanted into centrifuge tubes and centrifuged at 110 000 x g (Beckmann, 70Ti rotor) for 30 minutes to clarify the cell extract. Again, the supernatant was decanted and then loaded onto a 0.5 ml anti-3xFLAG M2-sepharose column (Sigma) under gravity flow at 4 °C. The column was washed with 10 column volumes of tris buffered saline (TBS; 50 mM tris HCl, 150 mM NaCl, [pH 7.4]). Finally, bound protein was eluted by competition with three times one-column volumes of 150 μg/ml 3xFLAG peptide (Sigma) in TBS.
2.5 SPECIMEN PREPARATION FOR ELECTRON MICROSCOPY

2.5.1 Preparation of continuous carbon film coated EM grids

Continuous carbon film coated EM grids were used for negative stain electron microscopy studies. A thick layer of carbon ~100 Å was evaporated onto a freshly cleaved mica surface. 400 mesh copper/rhodium grids were then arranged onto 3 pieces of filter paper (Whatman No 1) on a metal support in a bath filled with water. The carbon film was floated onto the surface of the water and the water was gently removed allowing the carbon film to rest on the grids. The grids were air-dried and then were used for negative staining of proteins.

2.5.2 Preparation of holey carbon film coated EM grids

Holey carbon film coated EM grids were used for electron cryomicroscopy. 20 drops of 50 % (v/v) glycerol in water was added to a formvar solution (0.5 % [w/v] formvar in chloroform), mixed and sonicated using a probe sonicator for 5 to 6 minutes at maximum power (50 % duty cycle). Glass slides were wiped clean, dipped in formvar solution for approximately 5 seconds, and left to dry. A pyrex dish was filled with water and the formvar film was floated onto the water surface. 400 mesh grids (Maxtaform HR26 Cu/Rh) were placed Rh side down (Cu side visible) on the floating film. Parafilm backing paper was placed on the formvar film covered grids and immediately lifted, flipped, and set on a piece of filter paper to dry.

The parafilm backing paper with formvar film covered grids was transferred to a glass petri dish and wet with methanol and covered for 10 minutes. The lid was then removed and the methanol allowed to evaporate in the fumehood. A thick layer of carbon was evaporated onto the formvar film. The formvar was dissolved by adding chloroform to the filter paper
under the grids, covered for 15 minutes and then dried. Rinsing with chlorform was repeated 5 times.

2.5.3 Negative staining of protein particles

Continuous carbon film coated grids were glow discharged in air in order to make the carbon surface hydrophilic. 5 μl of sample in the final buffer used during purification was placed on the carbon surface and allowed to adsorb to the grids for 1 minute. The grids were then washed 3 times in 40 μl of water. Between rinses, water was removed from the grids by touching the edge of the grids to a piece of filter paper (Whatman No. 1). The grids were stained by washing with 40 μl of 2 % (w/v) uranyl acetate (Electron Microscopy Sciences, Hatfield) solution. Excess stain solution was removed with filter paper and the grids were air-dried.

2.5.4 Preparation of protein particles in amorphous ice

Carbon coated holey grids were washed with acetone and 5 μl of the sample was applied to grids using the automated Vitrobot system (FEI) under controlled conditions: 4 °C with 100 % humidity. The sample was incubated for 30 seconds and excess buffer was removed by blotting with a filter paper (Whatman No. 595) for 13 second. The grids were immediately plunged into liquid ethane cooled by liquid nitrogen. For long-term storage, grids were stored in a grid box in liquid nitrogen.
2.6 ELECTRON MICROSCOPY

Transmission electron microscopy images were recorded with an exposure of \( \sim 12 \) electrons per \( \AA^2 \) with a Tecnai F20 electron microscope (FEI Company) operating at 200 kV with a magnification of 50,000 x. Images were taken with an exposure time of 1 second and a defocus between 1.0 \( \mu \)m and 4.5 \( \mu \)m. Images were recorded on Kodak SO-163 film. Micrographs were developed with Kodak D19 developer and digitized with an Intergraph PhotoScan densitometer (Intergraph).

2.7 IMAGE ANALYSIS

Single particle images were selected interactively with Ximdisp (Smith 1999). Prior to further analysis, images were bandpass filtered with an Imagic-like filter and a soft circular mask was applied to the images. Alignment of particle images and multivariate data analysis were carried out with the SPIDER software package (Frank et al. 1996).
CHAPTER 3. Results

In this study, the endogenous V$_1$-ATPase from *S. cerevisiae* was tagged with 3xFLAG tag. The protein was purified by affinity chromatography and several EM studies were performed.

3.1 3xFLAG VECTOR CONSTRUCTION

The pMK159 plasmid was used as a template for constructing the vector pJT1, which was designed to carry the DNA sequence for the 3xFLAG tag followed by the *URA3* selection marker. pMK159 contained a biotinylation signal sequence flanked by *PacI* and *AscI* restriction sites and the *URA3* selectable marker (Figure 3.1). To insert the 3xFLAG tag sequence into the plasmid vector, ‘sticky ends’ were produced both on the vector and the insert. Based on the plasmid sequence map (see Figure 2.1 in Chapter 2: Materials and Methods), *PacI* and *AscI* were employed to cut out the biotinylation signal sequence from the plasmid. Control reactions including vector + *PacI*, vector + *AscI* and vector alone. All of the digests were analyzed by DNA agarose gel electrophoresis and gave the expected band lengths. Vector with each one of the restriction enzymes showed a single band characteristic of a linearized plasmid, whereas vector alone showed a slower migrating band characteristic of a circular plasmid fragment. Digesting the vector with both *PacI* and *AscI* produced two bands belonging to the digested vector and the biotinylation signal sequence.
The cut vector was extracted from the gel and used for subsequent ligation reactions. The 3xFLAG tag sequence was designed to contain PacI and Ascl restriction sites (for more details see Materials and Methods Chapter 2: 2.3). The construct was synthesized by Invitrogen and ligated into the cut vector using DNA ligase. The insertion was verified by sequencing the resulting vector and henceforth it is referred to as pJT1.

3.2 Vma1 - 3xFLAG STRAIN CONSTRUCTION

Vma1 subunit (A) of the V1-ATPase was tagged with a C-terminal 3xFLAG tag in the background S. cerevisiae strain MM93 (Table 3.1), in order to allow purification of the complex with a high yield. The 3xFLAG tag followed by the URA3 selection marker in pJT1 (see Figure 3.1) was amplified by PCR using forward primers carrying 50 bases of sequence upstream of the stop codon of the gene of interest and the first 20 bases of the 3xFLAG-tag sequence. The reverse primers consisted of the reverse complement of the 50 bases downstream of (and excluding) the stop codon of the gene of interest and 24 bases of the reverse complement of pJT1 downstream of the URA3 sequence. The PCR product was confirmed by agarose gel electrophoresis. Yeast cells were transformed with the PCR product using the Li/Ac method. The linear PCR product was integrated by homologous recombination and transformants were recovered on selective media minus uracil (SD-URA). Correct integration of the DNA fragment was confirmed by PCR using primers 400 bp upstream of the integration site and inside the URA3 sequence.
Figure 3.1 Plasmid and yeast strain construction. The pMK159 vector containing the biotinylation sequences and the URA3 selectable marker, was digested by PacI and Ascl restriction enzymes. The 3xFLAG tag sequence followed by PacI and Ascl restriction sites was ligated into the cut vector. The ligated vector was named pJT1 and was used to generate DNA fragments for homologous recombination into the yeast genome.
3.3 PURIFICATION AND ISOLATION OF INTACT $V_1$-ATPase VIA Vma1 - 3xFLAG

Vma1 – 3xFLAG tagged protein was purified as described in Chapter 2: Materials and Methods 2.4.5. $V_1$-ATPase dissociates from the intact V-ATPase complex into $V_1$ and $V_o$ regions in response to glucose depletion (Kane 2003). To isolate the $V_1$ region, glucose was removed from purification buffers and cells were grown until saturation. An M2-sepharose column was used to purify the complex, which was then analyzed by SDS-PAGE. According to Figure 3.2 all eight subunits of the $V_1$-ATPase (A, B, C, D, E, F, G and H) were present with their expected molecular weights (70, 60, 45, 30, 27, 14, 12 and 55 kDa, respectively). Subunits A, B, E and G are present in multiple copies in the $V_1$-ATPase and thus their bands are more intense than other bands on the SDS-PAGE gel.

Figure 3.2. The purified yeast $V_1$-ATPase sample visualized by 12% SDS-PAGE stained with Coomassie Blue: Lane 1, Molecular mass standard proteins. Lane 2, Purified $V_1$-ATPase shows all eight subunits (A, B, C, D, E, F, G and H) with their expected molecular weights.
The eluate from the M2-sepharose column was concentrated and analyzed by Superose 6 gel filtration chromatography. Protein standards of known molecular weights were used to construct a calibration curve to determine the approximate molecular weights of the eluted complexes. The elution plot shows three distinct peaks corresponding to molecular weights in the ranges: 700 - 650 kDa (Peak 1), 400 – 280 kDa (Peak 2) and 15 - 2 kDa (Peak 3) (Figure 3.3).

**Figure 3.3. Superose 6 size-exclusion chromatography of the V₁-ATPase:** The chromatogram shows three peaks corresponding to molecular weights in the ranges: 700-620 kDa (Peak 1), 400 – 280 kDa (Peak 2) and 15-2 kDa (peak 3).
All eluted fractions were collected and analyzed by SDS-PAGE. Peak 1 showed all eight subunits corresponding to the intact V₁ region, whereas peak 2 showed a partial V₁-ATPase complex, and peak 3 contained 3xFLAG peptide. The partial complex was a surprising observation, because no previous reports in the literature demonstrated its existence in the cell. Previously, other partial V₁-ATPase complexes were obtained in response to detergent and temperature-driven disassembly of V-ATPase (Graf 1996; Rizzo et al 2003; Chaban 2003). To ensure that the complexes were stable, all of the fractions of each peak were collected and gel filtration was applied again on the peaks independently. The peak positions and the magnitudes did not change, indicating that V₁-ATPase and its subcomplex were stable. The stability was also tested by a freeze-thaw cycle and gel filtration chromatography, which showed the same results. These experiments support the notion that this V₁-ATPase partial complex is not an artifact of the experimental conditions used and is a stable complex found in the cell.

**Figure 3.4.** Yeast V₁-ATPase visualized by 12% SDS-PAGE stained with Coomassie Blue after gel filtration chromatography: **Lane 1,** Molecular mass standard proteins. **Peak 1,** The intact V₁-ATPase complex contained all eight subunits (A, B, C, D, E, F, G, and H). **Peak 2,** A partial V₁-ATPase complex containing subunits A, B, E, G, and H.
3.4 \( V_1 \)-ATPase REGION PURIFICATION USING DIFFERENT CELL CULTURE DENSITIES

Because the \( V_1 \) and \( V_o \) regions dissociate from each other due to glucose depletion, the effect of cell culture density on the abundance of the partial complexes was tested. An increase in culture density leads to nutrient depletion. Therefore, culture densities were tested with \( \text{OD}_{600} \) between \( \sim 3 - 5 \) and Superose 6 gel filtration chromatography was used to analyze each sample. These experiments suggested that increasing the culture density decreased the abundance of the intact \( V_1 \) region, but increased the abundance of the partial complex.
3.5 IMAGING OF INTACT V₁-ATPASE BY NEGATIVE STAIN ELECTRON MICROSCOPY

Samples of purified complexes of the intact V₁-ATPase region from the first peak were negatively stained using 2 % uranyl acetate on glow discharged continuous carbon film coated grids. Electron microscopy was performed with a 200 kV electron microscope equipped with a field emission gun (FEG) using low electron exposure conditions. Figure 3.5 shows a representative electron micrograph of the negatively stained yeast V₁-ATPase. The V₁-ATPase is highly purified and monodispersed, as is evident from the presence of homogenous particles in the electron micrograph.

3.5.1 Negative stain image 2D analysis

To obtain characteristic views of the V₁-ATPase, 1500 single particle images were selected manually from micrographs. Particles were then aligned and classified using multivariate statistical analysis as described in Materials and Methods Chapter 2: 2.7. Images were grouped into clusters and averaged to obtain top and side views of the complexes (Figure 3.6). Class averages that appear to show top views show six large densities arranged around a central gap. This symmetry could be due to a six-fold symmetric structure of the A₃B₃ subcomplex. Class averages that appear to show side views seem to show the central stalk. The 2D image analysis results were consistent with previously published data (Wilkens 2003). The peripheral stalk structure features from the negative stain images were not usable. In order to obtain better images, ice embedded specimens were prepared and imaged by electron cryomicroscopy.
Figure 3.5. Electron micrograph of the negatively stained yeast V$_1$-ATPase: On the left is a section from an original electron micrograph of a negatively stained yeast V$_1$-ATPase. The box on the right shows a single particle image extracted from the original micrograph.
Figure 3.6. 2D class averages of yeast $V_1$-ATPase stained with 2 % uranyl acetate: Structural models of a side view (A) and top view (B) of $V_1$-ATPase. Class averages of $V_1$-ATPase after the separation of 1500 particle images into possible (C) side views and (D) top views.
3.6 IMAGING OF INTACT $V_1$-ATPASE IN AMORPHOUS ICE

Electron cryomicroscopy was used to image the purified $V_1$-ATPase embedded in amorphous ice. Images of the $V_1$-ATPase were recorded over holes in the carbon support film. Figure 3.7 shows a section of a representative cryo-EM micrograph of the $V_1$-ATPase in sample buffer. As is visible in the micrograph, many particles appear to be oriented in the “top” or “bottom” orientation.

Figure 3.7. Cryo-EM micrograph of the yeast $V_1$-ATPase: On the left is a section from an original electron cryo-micrograph of yeast $V_1$-ATPase in amorphous ice. The box on the right shows single particles extracted from the original image.
3.6.1 Cryo-EM 2-D image analysis

To obtain characteristic views of the $V_1$-ATPase complex in ice, 20,000 single particle images were selected from micrographs, and aligned and classified as described in Materials and Methods Chapter 2: 2.7.

The resulting class averages show that all the particles were oriented to produce six-fold symmetric top or bottom views (Figure 3.8). The top view 2D averages of the $V_1$-ATPase from negative stain EM (Figure 3.6 D) show very similar features and possess six-fold symmetry. The central cavity is extremely clear in the cryo-EM class averages. From these results it seems that fully purified $V_1$-ATPase dissociates into the six-fold symmetric $A_3B_3$ complex during cryo grid preparation. This breakdown prevents us from analyzing the intact complex and seems to occur only during cryo-EM grid preparation. Attempts were made to prevent the breakdown of $V_1$-ATPase during grid preparation.

Figure 3.8. 2D class averages of yeast $V_1$-ATPase images in amorphous ice: Class averages from 20,000 particle images show that all the particles were oriented to produce six-fold top or bottom views of the partial $A_3B_3$ complex.
3.7 Stabilization of intact V₁-ATPase for cryo-EM

3.7.1 Detergent DDM

One hypothesis is that interaction of the protein with the air-water interface caused the breakdown of the V₁-ATPase intact complex into the A₃B₃ subcomplexes. Therefore the detergent n-dodecyl beta-D-maltoside (DDM) was used in order to reduce interactions with the air-water interface to prevent this artifact. Different concentrations of detergent (0.1 %, 0.15 %, 0.2 %) and cryo-EM grid preparations (blotting time, glow discharge) were tested. However, intact V₁-ATPase was not successfully visualized with any of the conditions tested.

3.7.2 Cold denaturation

Cold denaturation was another tested possibility that could potentially cause a separation between the core (A₃B₃) and the peripheral stalk regions during cryo-grid preparation. In order to test the hypothesis, that cold denaturation may lead to protein destabilization, cryo-EM grid preparation was carried out at room temperature. Thus far, it has been difficult to obtain optimal amorphous ice conditions at room temperature for microscope imaging.

3.7.3 Chemical cross-linking

Chemical cross-linking was used to stabilize the protein complex. Gluteraldehyde was used to cross-link different subunits. Concentration and reaction times were varied and native gel electrophoresis was used to analyze the cross-linked complex. According to Figure 3.9 Part A the concentration of 0.02 % and reaction time of 10 hours were the most optimal conditions that resulted in a single band of an appropriate molecular weight of ~ 650 kDa corresponding to the intact complex. However, after imaging by cryo-EM (Figure 3.9 B) no intact complex was observed. A shorter cross-linking reagent, formaldehyde was also tested.
and similar results were obtained (Figure 3.10). Neither the use of detergent nor cross-linking reagents prevented V$_1$-ATPase break down into the A$_3$B$_3$ complex.

Figure 3.9. Chemical cross-linking of V$_1$-ATPase complex with gluteraldehyde followed by cryo-EM. (A) Native gel electrophoresis stained with Coomassie Blue. The lanes represent optimization of 0.02 % gluteraldehyde reaction times: 0 hours, 2 hours, 6 hours, 10 hours. (B) Cryo-EM images of the cross-linked V$_1$-ATPase.
Figure 3.10. Chemical cross-linking of V₁-ATPase complex with formaldehyde followed by cryo-EM: (A) Native gel electrophoresis stained with Coomassie Blue. The lanes represent optimization of 0.2 % formaldehyde reaction times: 0 hours, 2 hours, 6 hours, 10 hours. (B) Cryo-EM images of the cross-linked V₁-ATPase.
3.8 ISOLATION AND CHARACTERIZATION OF PARTIAL V\textsubscript{1}-ATPASE COMPLEXES IN THE LATE LOG PHASE OF YEAST GROWTH

The intact V\textsubscript{1}-ATPase (Figure 3.1, lane 2) was observed in the purification of the 3xFLAG tagged subunit Vma1 (A) (as described above) despite previous reports that suggested that the C and H subunits dissociate after the V\textsubscript{1} region is released into the cytosol (Boesen and Nissen, 2009). Additionally, a partial complex consisting of A\textsubscript{n}, B\textsubscript{n}, E\textsubscript{n}, G\textsubscript{n} and H was detected, a novel finding not previously reported in the literature. The following experiments were carried out to characterize the partial complexes.

3.8.1 Vma4, 5, 7 and 13 – 3xFLAG yeast strain construction and purification of the corresponding partial complexes

To investigate which V\textsubscript{1} partial complexes are present in the late log phase of yeast cell culture, additional *S. cerevisiae* strains were designed with a 3xFLAG tag fused to the C terminus of each of five V\textsubscript{1} subunits: E (Vma4), C (Vma5), F (Vma7p), H (Vma13) and C(Vma5) (See Table 3.1). For details about strain construction refer to Results Chapter 3: 3.2 and Materials and Methods Chapter 2: 2.4.

V\textsubscript{1}-ATPase was purified and isolated as with Vma1 – 3x FLAG (for more details see Results Chapter: 3.3). Briefly, the cells were allowed to grow until late log phase was reached, which corresponded to an OD\textsubscript{600} ~5.
Table 3.2 Genotype of strains used in this study. The background *S. cerevisiae* strain MM93 was modified to generate four strains in which the 3xFLAG tag was fused to the C terminus of the V₁ subunits: A (Vma1), E (Vma4), C (Vma5), F (Vma7) and H (Vma13).

<table>
<thead>
<tr>
<th>Yeast gene</th>
<th>Background strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vma1 (A)</td>
<td>MM93</td>
<td>ura3-52, prb1-1122, pep4-3, prc1-407, Vm1-3xFLAG –URA3</td>
</tr>
<tr>
<td>Vma4 (E)</td>
<td>MM93</td>
<td>ura3-52, prb1-1122, pep4-3, prc1-407, Vma4-3xFLAG –URA3</td>
</tr>
<tr>
<td>Vma5 (C)</td>
<td>MM93</td>
<td>ura3-52, prb1-1122, pep4-3, prc1-407, Vma5-3xFLAG –URA3</td>
</tr>
<tr>
<td>Vma7 (F)</td>
<td>MM93</td>
<td>ura3-52, prb1-1122, pep4-3, prc1-407, Vma7-3xFLAG –URA3</td>
</tr>
<tr>
<td>Vma13 (H)</td>
<td>MM93</td>
<td>ura3-52, prb1-1122, pep4-3, prc1-407, Vma13-3xFLAG –URA3</td>
</tr>
</tbody>
</table>
To monitor viability of yeast strains, growth essays were performed. YPD plates were buffered at pH 5.5 and contained 50 mM CaCl$_2$. These conditions inhibit growth of strains with defective V-ATPase activity. As expected, the strain MM476, which carries a deletion of the B subunit (Δvma2) was shown not to grow on the plates (Figure 3.11), due to its inability to form a functional V-ATPase complex. Growth comparable to that of the parental Wild-Type strain (WT) was observed for the strains Vma1 – 3x FLAG (A), Vma4 – 3x FLAG (E) and Vma7 – 3x FLAG (F). By contrast Vma5 – 3x FLAG (C) and Vma13 – 3x FLAG (H), were also able to grow, albeit much more slowly than the wild type strain (comparable growth after six days compared to three days for the wild-type). These results indicate that the fusion of the 3x FLAG peptide affected the ability of V-ATPase to function normally for the Vma5 – 3x FLAG and Vma13 – 3x FLAG strains.

![Growth Characteristics of Strains](image)

**Figure 3.11. Growth characteristics of strains.** Growth rate was tested for all strains, and compared to WT (positive control) and ΔB strain (negative control). All strains were adjusted to OD$_{600}$ of ~ 1 and serially diluted by factors $10^0$, $10^1$, $10^2$, $10^3$ and 1μL aliquots of each dilution applied on a YPD plate buffered to pH 5.5 with 50 mM CaCl$_2$. Plate was incubated at 30 °C for 3 days.
3.8.2 Characterization of partial V$_1$-ATPase complexes by gel – filtration chromatography

To resolve the components of the partial complexes found in late log phase, Superose 6 size exclusion chromatography was performed on all purified V$_1$-ATPases. Chromatograms from the V$_1$-ATPase complexes revealed three distinct peaks which overlap over the same range of elution volumes (Figure 3.12). The first peak from the elution profile corresponds to the molecular weight in the range between 700 – 650 kDa, which is consistent with the molecular weight of the intact complex ~ 640 kDa. The second peak corresponds to the range of the molecular mass of ~ 400 – 280 kDa. The third peak corresponds to low molecular weights ~ 2 – 15 kDa and contains 3xFLAG peptide as well as free subunits; however, from the chromatogram of Vma5-3xFLAG, shown in the Figure 3.10 in orange, the third peak could be composed of overlapping multiple peaks.

![Chromatogram from V$_1$-ATPase subcomplexes](image)

**Figure 3.12. Chromatogram from V$_1$-ATPase subcomplexes:** A Superose 6 gel filtration column was used to analyze V$_1$-ATPase complexes tagged with 3xFLAG tags on different subunits: A (Vma1) blue, E (Vma4) yellow, C (Vma5) orange, F (Vma7) green, H (Vma13) brown. Each chromatogram contains three peaks (1 - 3) which were subjected to SDS-PAGE.
3.8.3 Characterization of partial \( V_1 \)-ATPase complexes by SDS-PAGE

To determine the subunit composition of each subcomplex all gel filtration fractions were collected and analyzed by SDS–PAGE. The migration of all the tagged subunits on SDS-PAGE was slower than that of the non-tagged subunits, consistent with their higher molecular weights due to the presence of the 3xFLAG tag (Figure 3.13 – 3.16).

Intact \( V_1 \)-ATPase, containing all \( V_1 \) subunits (A, B, C, D, E, F, G, and H), was found in the first peak in all the tagged protein purifications and, interestingly, also in the purification of C (Vma5) and H (Vma13). The band of subunit C, corresponding to the molecular mass \( \sim 45 \) kDa, appeared in the protein purification of Vma13 (H) (Figure 3.16). In addition to the eight bands corresponding to all the subunits, a ninth protein band was observed (indicated by an asterisk in Figure 3.15 and 3.16) of molecular weight \( \sim 40 \) kDa in the Vma5 (C) and Vma13 (H) purifications. The second peak (Figure 3.12) that was observed in each purification shows subcomplexes containing \( A_n, B_n, G_n, E_n \), but differed in other subunits composition. The subunits that were observed in the E (Vma4) purification were \( A_n, B_n, G_n, E_n \) and subunit H (\( \sim 54 \) kDa) (Figure 3.14), Partial complexes containing Vma5 (C) were resolved and found to contain \( A_n, B_n, G_n, E_n \) along with a high concentration of free subunit C, an additional protein weak band was observed (indicated by an asterisk Figure 3.15). The subunits obtained from the Vma7-3xFLAG strain consisted of \( A_n, B_n, G_n, E_n, D, \) and free F subunit that showed increasing density in late elution fractions (Figure 3.13). Finally, subcomplexes of subunit H (Vma13) contained \( A_n, B_n, D, G_n, E_n \), a weak band with an apparent molecular mass of \( \sim 40 \) kDa, that was also detected in the first peak, and subunit H (Figure 3.16).
Figure 3.13. Yeast V₁-ATPase with F (Vma7) – 3xFLAG visualized by 12% SDS-PAGE stained with Coomassie Blue after the gel filtration column. Lane 1, molecular mass standard. **Intact V₁-ATPase** complex contained all eight subunits (A, B, D, E, F, G, and H). **Partial V₁-ATPase** complexes contain A, B, D, E, F,G and H.
Figure 3.14. Yeast $V_1$-ATPase with E (Vma4) – 3xFLAG visualized by 12% SDS-PAGE stained with Coomassie Blue after the gel filtration column: Lane 1, molecular mass standard. **Intact $V_1$-ATPase** complex contained all eight subunits (A, B, C, D, E, F, G, and H). **Partial $V_1$-ATPase** complexes contained A, B, C, E, G, and H.
Figure 3.15. Yeast V₁-ATPase with C (Vma5) – 3xFLAG visualized by 12% SDS-PAGE stained with Coomassie Blue after the gel filtration column: Lane 1, molecular mass standard. **Intact V₁-ATPase** complex contained all eight subunits (A, B, C, D, E, F, G, and H) and an additional protein band (indicated by an asterisk). **Partial V₁-ATPase** complexes contained the A, B, E, G and free subunit C.
Figure 3.16. Yeast V$_1$-ATPase with H (Vma13) – 3xFLAG visualized by 12% SDS-PAGE stained with Coomassie Blue after the gel filtration column. Lane 1, molecular mass standard. **Intact V$_1$-ATPase** complex contained all eight subunits (A, B, C, D, E, F, G, and H) and an additional protein band (indicated by an asterisk). **Partial V$_1$-ATPase** complexes contain A, B, D, E, G, and H.
CHAPTER 4. Discussion and Conclusion

4.1 ISOLATION OF THE INTACT V₁-ATPase

V-ATPase activity is regulated in cells by dissociation of the soluble V₁ and membrane-bound Vₒ regions of the complex. This process has been characterized mostly in yeast and insect cells, where the dissociation and reassociation processes are rapid and reversible (Jefferies et al 2008). Regulation of V-ATPase activity is an effective way to reduce or increase proton pumping in response to cellular conditions such as glucose depletion, pH changes, and the presence of heavy metals (Kane, 1995; Sumner et al, 1995). As part of this regulatory mechanism, the dissociated V₁ region does not hydrolyze ATP (Gräf et al 1996) and the dissociated Vₒ region does not allow proton translocation through the membrane (Beltran and Nelson, 1992). It is assumed that structural changes in the dissociated V₁-ATPase leads to inhibition of ATP hydrolysis. Therefore, knowledge of the structure of the dissociated V₁-ATPase, as well as the intact V₁Vₒ-ATPase complex, is necessary for understanding the regulation of V-ATPase activity.

In this thesis, I show that by introducing a 3xFLAG tag at the C terminus of different V₁-ATPase subunits, a highly purified V₁-ATPase complex could be isolated. Previous experiments with the V₁-ATPase from yeast have suggested that the V₁-ATPase from glucose-deprived cells may lack subunits C (Vma5) and H (Vma13) (Gräf et al 1996; Svergun et al 1998; Vitavska et al, 2003; Boesen and Nissen 2009). It has therefore been proposed that the C and H subunits play a role in V-ATPase regulation.

In this thesis I demonstrate that the C and H subunits are actually present in the dissociated V₁ complex, at least substoichiometrically, even when yeast cultures are grown to
a high density. Indeed, high density cultures allow for the isolation of a V$_1$-ATPase complex containing all eight subunits (A, B, C, D, E, F, G and H), even though yeast cultures at high density should experience the conditions thought to lead to dissociation of subunits C and H. A V$_1$ complex with eight subunits could be observed even using subunit C (Vma5–3xFLAG) and H (Vma13–3xFLAG) tagged strains for purification. Attempts were made to use the highly purified V$_1$-ATPase in this study for structural analysis by cryo-EM.

4.2 STRUCTURAL STUDIES ON THE INTACT V$_1$-ATPase

In recent years, V$_1$-ATPase structures from several organisms have been published at low resolution. The negatively stained structure of the V$_1$-ATPase from *Manduca sexta* was reported at a resolution of 32 Å (Gruber et al 2000). The model showed the presence of six-fold symmetric densities surrounding a seventh density, likely corresponding to the A$_3$B$_3$D subcomplex. The peripheral subunits were not resolved in this structure. A later model of the *Saccharomyces cerevisiae* V$_1$-ATPase was published at a resolution of 25 Å; however, the peripheral stalk subunits were not visible in this structure either (Zhang et al 2003). Recently, the first cryo-EM structure of V$_1$-ATPase was published (Hildenbrand et al 2010). This model also did not elucidate the peripheral subunits. These studies show that the V$_1$-ATPase complex was disrupted during EM analysis.

For an initial analysis of the intact V$_1$-ATPase, negative stain electron microscopy was used. Images showed highly purified and homogenous protein particles. 2D image analysis was performed with 1500 particle images that were aligned and classified. The class averages showed apparent top views with six large densities arranged around a central density, whereas
apparent side view class averages also seemed to show the central stalk. The six-fold symmetric densities appeared to correspond to the symmetric structure of the A$_3$B$_3$ subcomplex surrounded by the central stalk subunit D. 2D image analysis of the negatively stained single particles was similar to the previously published structures of the V$_1$-ATPase (Gruber et al 2000; Zhang et al 2003).

Cryo-EM was used instead of negative stain in an attempt to prevent disruption of the A$_3$B$_3$CDE$_3$FG$_3$H complex. However, the resulting class averages from cryo-EM showed apparent top and bottom views of the complex with six-fold symmetry only, without the density corresponding to the D subunit. The lack of peripheral and central stalk densities indicated that intact V$_1$-ATPase can break down into the A$_3$B$_3$ complex during cryo-EM grid preparation. In order to prevent this artifact and to stabilize the V$_1$-ATPase structure, various experimental conditions were tested.

It has been shown that interaction with the air-water interface of a cryo-EM specimen grid can affect the structure of proteins (Glaeser et al 1991). Detergents can coat the air-water interface to prevent it from interacting with proteins. To avoid breakdown of the V$_1$-ATPase into the A$_3$B$_3$ subcomplex, an attempt was made to reduce protein interactions with the air-water interface by adding detergent to the sample. However in this experiment, protein particles were not observed in the amorphous ice, probably due to problems in obtaining optimal conditions for freezing the grids.

Another hypothesis tested was that evaporation during cryo-EM grid preparation may have lead to an increased salt concentration in the buffer, which can decrease protein stability in solution. In order to overcome this possible predicament, salts were removed from the protein buffer just prior to cryo-grid preparation by buffer exchange. However, images
obtained from this experiment showed \( V_1 \)-ATPase breakdown into subcomplexes identical to what was observed previously. Therefore it can be argued that the salts were not the cause of the protein instability.

Cold denaturation of the protein was another possibility tested that could potentially cause dissociation of the \( V_1 \)-ATPase into its core (\( A_3B_3 \)) and peripheral stalk regions. Cold denaturation is a phenomenon caused by the temperature-dependent interaction of nonpolar groups of proteins with water molecules. Decreased temperature can lead to increasing order of water molecules, which in turn can enforce nonpolar interaction of water with protein. Therefore, a decrease in temperature can potentially cause significant changes in protein structure and possibly disruption of a protein complex (Privalov 1990). In this study, cryo-EM grid preparation was performed at 4 °C, which is different from the native condition of \( V_1 \)-ATPase, increasing the likelihood of cold denaturation. In order to test the hypothesis that temperature was involved in disruption of the \( V_1 \) complex, cryo-EM grid preparation was carried out at room temperature. However, I was not able to obtain optimal amorphous ice conditions during cryo-EM grid freezing from room temperature.

Chemical cross-linking is widely used in electron microscopy for protein stabilization (Wong and Wong 1992; Prento 1995). Therefore, gluteraldehyde and formaldehyde were used in an attempt to stabilize the protein complex. Even though analysis by native gel electrophoresis revealed cross-linked \( V_1 \)-ATPase, the intact \( V_1 \)-ATPase was not successfully visualized by cryo-EM under any cross-linking conditions tested.

Disruption of the \( V_1 \)-ATPase to the \( A_3B_3 \) subcomplexes was observed previously by other groups and has also prevented them from obtaining the structure of the intact complex by cryo-EM (Zhang et al 2003; Hildenbrand et al 2010). Since the protein complex
consistently breaks down from the intact V₁-ATPase into the A₃B₃ complex during cryo-EM specimen preparation, I was unable to use cryo-EM to determine the structure of the V₁-ATPase. However, by overcoming these barriers it may be possible to use cryo-EM. The future experiments are described in Section 4.4.

4.3 ISOLATION OF PARTIAL V₁-ATPase SUBCOMPLEXES

In addition to the intact complex, partial V₁-ATPase subcomplexes with different subunit compositions were isolated. In the past, similar subcomplexes were observed after detergent and temperature-driven V₁-ATPase disassembly (Graf 1996; Rizzo et al 2003; Chaban 2003). This study shows that these subcomplexes may be extracted from the cytoplasm of yeast cells without chemical or thermal V₁-ATPase disassembly. These partial subcomplexes were isolated from late log phase yeast culture. All of the subcomplexes contained the major V₁-ATPase subunits A and B, but differed in the peripheral stalk subunit composition. Furthermore, I showed that the partial V₁-ATPase subcomplexes were stable by freezing and thawing and gel filtration chromatography.

The presence of partial subcomplexes in the cell may be attributed to the subcomplexes being involved in processes other than their known role in membrane-bound proton-pumping by V-ATPases. Eukaryotic V-ATPase subunits have been shown to interact with other proteins. For example, using a yeast two-hybrid screen it was shown that subunit H interacts with yeast ectoapyrase and acts as an inhibitor of its activity (Zhang et al 2000). Ectoapyrase is a member of an enzyme family that hydrolyzes a wide range of purine and pyrimidine nucleosides in the presence of Ca²⁺ or Mg²⁺ cations (Plesner 1995). It was also reported that subunit C released from the V₁ region is involved in controlling the dynamics of the actin cytoskeleton by interacting with F-actin and G-actin (Vitavska et al 2005).
Another possible explanation for the presence of the partial subcomplexes is that some V-ATPase particles are not completely assembled. This hypothesis is based on the observation that increasing the culture cell density results in increased abundance of the partial complexes. One model of the V-ATPase assembly pathway proposes that the V₁ and V₀ regions can be assembled independently (Tomashek et al 1997). According to this model, V₁ region intermediate subcomplexes are assembled in the cytosol, whereas assembly of the V₀ region occurs in the endoplasmic reticulum (ER). In this study I was able to extract subcomplexes from cells. It can be argued that the partial complexes observed from yeast cells represent these intermediately assembled complexes. Figure 4.1 depicts the possible intermediates of V₁-ATPase assembly.
Figure 4.1 Model of \( V_1\)-ATPase assembly pathway. The model proposes an assembly pathway, comprised of the potential intermediate assembly subcomplexes (the green circles represent intermediate subcomplexes, which were observed in this study).
4.4 FUTURE DIRECTIONS

Several studies have shown that specific cross-linking can link various V₁-ATPase subunits (Arata et al 2001; Inoue and Forgac 2005). In these studies, insertion of cysteine residues, which participate in disulfide bonds were used to directly cross-link V₁-ATPase subunits to each other. In order to prevent the disruption of the V₁-ATPase observed during cryo-EM grid preparation, it could be useful to introduce cysteine residues into subunits to allow for specific cross-link formation. For example, cross-link formation between cysteine residues introduced into the core region subunit B (G106C, A199C, A15C and L45C) and native cysteine residues of the peripheral subunits E and G, could prevent these regions from separating (Arata et al 2002). Previous studies reported that the H and C subunits could be released from the intact V₁-ATPase (Parra et al 2000; Boesen and Nissen 2009) and it would therefore be useful to stabilize the interaction between different subunits, such as E, G and H and C, by introducing cysteine residues into C subunit (A217C and A220C) (Inoue and Forgac 2005). This approach might make it possible to stabilize the V₁-ATPase and determine its intact structure by electron cryomicroscopy.

Before forming the final conclusion about the role of the observed partial subcomplexes in V-ATPase assembly, additional experiments would be necessary. Tandem mass spectrometry might allow for improved analysis of the subunit composition of the subcomplexes. Analytical ultracentrifuge experiments could also precisely determine the molecular weights of each subcomplex. The subunit composition of the subcomplexes could also be estimated from the known molecular masses of the individual V₁-ATPase subunits (molecular masses of the subunits are given in Table 1. Introduction: Chapter 1: 1.2). Furthermore, in vivo studies could provide insight into the involvement of V₁ subunits and their subcomplexes in cellular
processes. For this approach, tagging different subunits with fluorescence probes could be used to determine the subcellular localization of the subunits by *in vivo* Förster Resonance Energy Transfer (FRET) (Seidel et al 2005) using confocal microscopy and spectral imaging.
CHAPTER 5. References

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