HETEROLOGOUS EXPRESSION AND BIOCHEMICAL CHARACTERIZATION OF THE V-ATPase SUBUNIT d

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Abstract:

The role of the V-ATPase subunit d for structural and functional coupling $V_1$ and $V_o$ subunits within the yeast V-ATPase complex is under study in our lab. Because high-resolution structures of the yeast V-ATPase subunit d are not available, the crystal structure of its homologue from the archeabacteria *Thermus thermophilus* at 1.95 Å resolution (PDB ID code IR5Z) served as structural model for this study. We are performing complementation studies aimed to establish structure-function conservation analysis of this subunit. Heterologous complementation of *T. thermophilus* subunit d in yeast would be further supported by mutagenesis analyses. Overall, atpC is a decent model for yeast subunit d. Our study shows that atpC is similar enough to structurally replace d, however, functionality was not observed with heterologous expression.

Introduction:

V-ATPases acidify intracellular compartments such as yeast vacuoles and human lysosomes by pumping protons. V-ATPases can be found on plasma membranes as well, where they play important physiological roles in renal acidification, tumor metastasis, and bone resorption. Proton transport by yeast V-ATPases is coupled to ATP hydrolysis and it has been found to drive other transport processes at the membrane, including calcium and amino acids by generating a membrane potential. In the archeabacteria, *Thermus thermophilus*, V-ATPases at the plasma membrane are responsible for ATP synthesis, rather than hydrolysis.

V-ATPases consist of a rotor made of four to six different subunits that moves relative to a stator made up of seven different subunits. The rotation of the enzyme is
either generated by ATP hydrolysis which causes pumping of a proton against the gradient or it is the means by which energy is harnessed to synthesize the high energy ATP molecule from ADP and Pi. The rotor and stator consist of both membrane bound and peripheral subunits which are identified as \( V_1 \) and \( V_0 \) subunit respectively, therefore; the entire complex consists of two sub-complexes \( V_1 \) and \( V_0 \). \( V_0 \) is the membrane bound domain that forms the pore for the proton transportation. \( V_1 \) is the peripheral complex attached to \( V_0 \) which has the sites for ATP binding and hydrolysis. Studies of the yeast vacuolar ATPase has shown that eight genes encode for proteins that compromise the peripheral \( V_1 \) sub-complex and five genes encode for the membrane bound \( V_0 \) sub-complex. Vma6 or subunit d appears to be part of the rotor and is particularly interesting because it is the only subunit of the membrane integrated \( V_0 \) complex that is peripherally located to the membrane. It is also known that subunit d of \( V_0 \) interacts with subunit F of \( V_1 \). This makes it conceivable that subunit d may play a role in the interaction between the two domains.

The \( V_1V_0 \) complex is important because when active, it couples \( H^+ \) pumping with ATP hydrolysis. In yeast, this action is controllable via reversible disassembly and mediated by glucose (energy) availability. A further understanding of the significance of subunit d’s role in assembly and function of V-ATPases is desired to better understand V-ATPase regulation.

Two species of single celled organisms were used in this study. *Thermus thermophilus*, a prokaryotic bacterium, grows optimally at temperatures between 70°C and 75°C. The second organism is yeast, *Saccharomyces cerevisiae*, which is an eukaryotic organism that grows optimally at 30°C. Unlike eukaryotic V-ATPases which primarily
hydrolyze ATP, the *thermophilus* V-ATPase complex works as an ATP synthase *in vivo*. Although their overall structure is very similar, the synthase and the hydrolase rotate in opposite directions. *Thermophilus* was selected for the study because the crystal structure of its atpC subunit, the equivalent of subunit d in yeast, has been recently solved. Since electron microscopy reveals a common structure the two enzymes, we examined whether atpC and subunit d also share a similar structure.

Amino acid sequence alignment analysis reveals relatively low identity between atpC and subunit d which share only 18% identity. Similarly, atpF and yeast subunit F share only 17% identity. Although the two enzymes are functionally different and their subunit sequence identity is low, we studied whether similarities in the structure of the V$_1$V$_0$ complex structure and location of the subunit d (atpC) within were significant enough to support genetic complementation. If atpC complements the growth phenotype of yeast vma6Δ mutants (subunit d deletion strain), then we can conclude that the function and structure of subunits d and atpC are equivalent in both organisms. If complementation were observed, the available structure from *thermophilus* could be used to design new mutations in the yeast subunit d.

**Experimental:**

**Cloning:**

Cloning strategy to express atpC in yeast. – Quik-change mutagenesis was used to introduce the unique restriction enzyme sites for *AflIII* (CTTAAG) and *MluI* (ACGCGT) immediately upstream of the start codon and immediately downstream of the stop codon, respectively, in the yeast *VMA6* gene cloned in the pRS316 vector. The ORF for the atpC
gene of *T. thermophilus* (a generous gift from Dr. Yokoyama, Japan) was PCR amplified with oligonucleotides containing the *AflII* (forward) and *MluI* (reverse) restriction enzyme sites. The *VMA6 ORF* was removed by restriction digestion with *AflII* and *MluI*. Upon restriction digestion with *AflII* and *MluI*, the *atpC ORF* was ligated into the plasmid lacking the *VMA6 ORF*. The recombinant plasmid had the following structure: *VMA6promoter-atpC ORF-VMA6terminator*. DNA sequencing confirmed proper insertion of *atpC* into the plasmid.

**Epitope Tagging:**

A novel *BspE I* site was introduced within the yeast plasmid used for expressing *atpC* and *atpF*. This site was introduced by Quikchange site-directed mutagenesis. The restriction site was introduced in two places (3'- and 5'- end) in the sequence to allow for amino- and carboxyl- end expression of the tag in each of the proteins (*atpC* and *atpF*). Also, a DNA fragment coding for 3HA flanked by a *BspE I* site at each end was PCR amplified. The PCR product was purified by phenol extraction and digested with *BspE I*. *BspE I* is a sticky end cutter and the digested plasmid and digested PCR product were used to set up a ligation reaction to insert the 3HA sequence. Transformations into competent *E.coli* cells were preformed and colony PCR screening was used to check for insertion of the tag sequence. The primers M13-20 and M13-RSP2 (see figure IV) were used to amplify a portion of the plasmid that would include the gene and a tag, if one were inserted. The amplified segment was visualized on a gel and a shift upward in the distance the band traveled indicated an insertion of the tag (figure V). The plasmid DNA of the colonies containing insertions was prepared and PCR reactions were used to confirm
correct insertion of the tag. It should also be noted that the yeast \textit{vma6} and \textit{vma7} genes were tagged with a 3HA epitope in the same manner. This served as a control to ensure that the HA tag would not affect \textit{V}_1\textit{V}_0 assembly and function.

\textit{Phenotype Analysis:}

\textit{Complementation analysis of} \textit{T. thermophilus} \textit{genes in yeast}. – Plasmids containing the HA tagged (and non-tagged) genes were used to transform the corresponding yeast deletion strains (\textit{vma6\Delta}, \textit{vma7 \Delta}). Cells were grown in liquid culture and ten-fold serial dilutions of the cells were spotted onto the SD-Ura plates at pH 5, 7.5, and 7.5 + \textit{CaCl}_2 and incubated at 30°C for 72 hours. Yeast cells expressing wild-type \textit{VMA6} (and \textit{VMA7}) have active V-ATPases and grow at all three conditions. Mutant cells with inactive V-ATPases grow at pH 5, but will fail to grow at either pH 7.5 or pH 7.5 +\textit{CaCl}_2.

\textit{Whole Cell Total Protein Extract:}

Yeast cells were grown to mid-log phase in liquid cultures overnight. Cells were converted to spheroplasts by zymolase treatment and lysed at 50°C by addition of cracking buffer containing SDS, urea, and β-mercaptoethanol (1) Proteins in the cell lysates were separated by SDS-PAGE and V-ATPase subunits detected by Western blots analysis using antibodies against subunits A, B, d, and E.

\textit{Preparation of Vacuolar Membranes:}

Cells were converted to spheroplasts by zymolase addition, lysed, and vacuoles isolated by two ficoll density gradients (1). Vacuolar membrane vesicles were prepared by
diluting the vacuoles in a 10 mM Mes-Tris pH 6.9, 5mM MgCl2, 5% glycerol solution. Vacuoles (10 μg) were analyzed by SDS-PAGE and Western Blots using antibodies against subunits a, A, B, d, D, and E. Western blots showed some V1V0 complexes assembled at the membrane.

Activity Assays:

Concanamycin A-sensitive ATPase activity was measured spectrophotometrically at 37°C using a coupled enzymatic assay (1). Protein concentration was determined by the Lowry method.

Results & Discussion:

Gene Cloning and Epitope Tagging:

Insertion of atpC and atpF were confirmed by sequencing and visualization on agarose gels. Figure I (see addendum) shows the digestion of the gene VMA6 out of the cloning plasmid, the PCR product used for insertion of atpC into the digested plasmid, and the digestion of atpC cloned into the yeast plasmid. This gel confirmed the insertion of atpC into the yeast plasmid pRS316. Accordingly, gels showed a small shift of about 100 bp for atpC since it is slightly smaller than VMA6.

In order to visualize atpC and atpF in Western blots, addition of an epitope tag was required because the yeast specific specific antibodies against subunit d and F do not recognize thermophius subunits. Epitope tagging of VMA6 (and VMA7) was preformed to ensure that the HA tag could be used because the tag would only be useful if it did not affect the protein’s structure or function. Figure II shows the strategy and directionality of the primers used in PCR reactions that revealed the orientation of the tag. A the correct
orientation of the tag was important for proper protein translation, correct aa sequence for both the tag and the gene in study. Figures IIIa and IIIb show agarose gels of the PCR reactions used to check the orientation of the insertion of the HA tag. In IIIa, the WT was shown as a comparison. Of the two colonies, #3 showed the correct orientation and #8 showed the incorrect orientation. In Figure IIIb, The WT was also shown as a comparison. Both of the colonies (C-tag and N-tag) shown in this figure showed the correct orientation of the tag. Figure IV confirmed the successful tagging of the VMA6 gene. Phenotype analysis also showed that the HA tag did not affect cell growth. Cells expressing tagged VMA6 grow as well as untagged strain (see Figure V).

Growth Phenotype:

The phenotype analysis of the atpF and atpC hybrids showed that atpF and atpC cannot complement vma6Δ phenotype. Lack of growth at pH 7.5 and pH 7.5 + CaCl₂ indicated that there is a significant reduction of V-ATPase function when yeast cells express atpF and atpC instead of VMA6 and VMA7 respectively (See Figure III). The conditionally lethal phenotype exhibited by the hybrids indicates that less than 20% of the V-ATPases are functional. Even though this analysis did not reveal genetic complementation at all, a more detailed protein analysis was required determine whether atpF and atpC supported assembly of the V₁V₀ at the vacuolar membrane.

Whole Cell Lysates:

Total protein extracts from yeast cells were prepared by whole cell lyses. Western blots and SDS-PAGE showed normal expression and stability of the V-ATPase subunits in
cells expressing the *thermophilus* subunits (see Figure VI). This reveals that atpC is not so
different from yeast subunit d and the expression of the other V-ATPase subunits is not
affected. It also indicates that atpC is similar enough to the yeast subunit to support some
assembly of the V-ATPases; although, atpF supports less assembly.

*Analysis of Vacuolar Membranes:*

Western blot analysis of the yeast vacuolar membranes (see figure VII) showed that
atpC supports assembly of complexes when compared to vma6Δ and yeast cells expressing
the gene VMA6. The amount of assembled is not as much as the observed for the wild-
type yeast as indicated by reduced levels of V₁ and V₀ subunits at the membrane. However,
the level of assembly in atpC is significant when compared to the deletion strain. The
subunit d deletion strain (vma6Δ) does not have any of the V₀ or V₁ subunits and the atpC
membranes shows a substantial amount of both V₁ and V₀ subunits. atpF also supported
some structural complementation since V₁ assembly was observed at the membrane
although the level of assembly was less than atpC. This study was complemented with a
functional assay and ATPase activity was compared to the yeast wild-type. As expected
from the vma mutant growth phenotype detected (figure IV) V₁V₀ complexes were
inactive (figure VII). These assays showed that the hybrid V-ATPases containing atpC and
atpF, although assembled, are not functional(atpC and atpF exhibited less than 10% of the
activity). Our result supports the growth phenotype of these hybrids cells which exhibited
a conditional lethality.

*Future Directions:*
Overall, atpC is a decent model for yeast subunit d. Our study shows that atpC is similar enough to structurally replace d. This is encouraging, but more analysis are necessary since V-ATPases with atpC or atpF are not active. It is important to understand why atpC and atpF cannot functionally complement the \emph{vma6}Δ (and \emph{vma7}Δ), respectively. Future studies addressing this issue would allow a better determination as to whether atpC can be used as a model for mutagenesis.
References:


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Figure I. This 1% agarose gel shows the appropriate shift downward of the band representing the smaller atpC protein. These fragments were obtained from Afl II/Mlu I digestions of the appropriate plasmids and PCR product.
Figure II. Illustrates the PCR approach used to check the orientation of the tag. The novel BspE I site is shown where the HA tag was inserted. Depending on the primers used and the orientation of our HA tag, different PCR products can be obtained. From this figure, we can determine which size bands for which primer set indicates a correct orientation.
Figure IIIa This agarose gel shows the PCR reactions for atpF tagging where Reaction A: M13-20/M13-RSP Reaction B: M13-20/3HA-Reverse. If correct orientation anticipated Product – 500 bp. Reaction C: 3HA-Reverse/M13-RSP. If incorrect orientation, anticipated product – 700 bp.
Figure IIIb This agarose gel shows the PCR reactions for atpF tagging where Reaction A: M13-20/M13-RSP (Positive Control) Reaction B: M13-20/3HA-Reverse If correct orientation, anticipated Product – 900 bp Reaction C: 3HA-Reverse/M13-RSP If incorrect orientation, anticipated product – 1500 bp. Note that colony #3 has the correct orientation.
**Figure IV.** Western blot of the tagged *Vma6* subunit. Whole Cell lysates were preformed as described under materials and methods. Blots were incubated with anti-HA monoclonal antibodies and alkaline phosphate – conjugated secondary antibodies. Lines containing pRS316 and pRS416 are negative controls (plasmid alone). atpC and atpC (multicopy) contain the gene atpC cloned in a CEN plasmid (pRS316) and a multicopy (2μ) plasmid (pRS426) respectively. N-3HA-*vma6*, *vma6*-C3HA, and *vma6*C-3HA, correspond to cells expressing the yeast vma6 gene tagged at the amino end (N-) or carboxyl end (C-) with the three (3HA) or six (6HA) copies of the HA tag.
Figure V. This phenotype analysis reveals that atpC and atpF cannot complement growth of the deletion strains. When compared to the wildtype growth, atpF and atpC can be assumed to have a significant reduction in ATPase function. Note that although the figure does not show the growth phenotype of the VMA6 tagged cells, the growth was found to be the same as the VMA7 tagged cells. Plasmids and strain names are the same as in figure IV.

Complementation analysis of \textit{vma7A} with \textit{atpF} and \textit{vma6A} with \textit{atpC}
Figure VI. Total Protein Analysis by Western blot. The VMA6 was compared to atpC and the VMA6 tags. When compared to VMA6, atpC looks very similar except that subunit d is not visible because the yeast anti-d will not allow for detection of atpC. The protein from the tagged VMA6 looks identical to the untagged VMA6 supporting growth phenotype data that indicates that the tag does not have an affect of the cells.

V-ATPase specific antibodies  *=V_o subunit
anti-a, anti-A, anti-B, anti-d, anti-D, anti-E
Figure VII. Western blot analysis of yeast vacuolar membrane from vma6Δ cells expressing VMA6, atpC, and atpF in the pRS316 plasmid. Results show that atpF and atpC support some assembly of the V-ATPase complex. Band intensity reflects how much protein is present (each well contained 10 ug of vacuolar protein). The wild-type has the most intense bands indicating the most assembly. atpC has less assembly than the wild-type, but more than atpF. Activity assays however indicate no functional complementation.