Involvement of the Nonhomologous Region of Subunit A of the Yeast V-ATPase in Coupling and in Vivo Dissociation*

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The catalytic nucleotide binding subunit (subunit A) of the vacuolar proton-translocating ATPase (or V-ATPase) is homologous to the β-subunit of the F-ATPase but contains a 90-amino acid insert not present in the β-subunit, termed the nonhomologous region. We previously demonstrated that mutations in this region lead to changes in coupling of proton transport and ATPase activity and to inhibition of in vivo dissociation of the V-ATPase complex, an important regulatory mechanism (Shao, E., Nishi T., Kawasaki-Nishi, S., and Forgac, M. (2003) J. Biol. Chem. 278, 12985–12991). Measurement of the ATP dependence of coupling for the wild type and mutant proteins demonstrates that the coupling differences are observed at ATP concentrations up to 1 mM. A decrease in coupling efficiency is observed at higher ATP concentrations for the wild type and mutant V-ATPases. Immunoprecipitation of an epitope-tagged nonhomologous region from cell lysates indicates that this region is able to bind to the integral V0 domain in the absence of the remainder of the A subunit, an interaction confirmed by immunoprecipitation of V0. Interaction between the nonhomologous region and V0 is reduced upon incubation of cells in the absence of glucose, suggesting that the nonhomologous region may act as a trigger to activate in vivo dissociation. Immunoprecipitation suggests that the epitope tag on the nonhomologous region becomes less accessible upon glucose withdrawal, possibly due to binding to another cellular target. In vivo dissociation of the V-ATPase in response to glucose removal is also blocked by chloroquine, a weak base that neutralizes the acidic pH of the vacuole. The results suggest that the dependence of in vivo dissociation of the V-ATPase on catalytic activity may be due to neutralization of the yeast vacuole, which in turn blocks glucose-dependent dissociation.

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1 The abbreviations used are: V-ATPase, vacuolar proton-translocating adenosine 5-triphosphatase; F-ATPase, F1F0 ATP synthase; MES, 4-morpholineethanesulfonic acid; Cs2Es2, polyoxyethylene 9-lauryl ether; DSP, dithiobis(succinimidyl propionate); HA, influenza hemagglutinin; NHR, nonhomologous region of subunit A; YPD, yeast extract-peptone-dextrose; YEP, yeast extract-peptone.
100T was purchased from Sekagaku America, Inc. Protease inhibitors and the monoclonal antibodies against the HA epitope, 3F10, were purchased from Roche Applied Science. Protein A-Sepharose, Protein G-agarose, ATP, and other chemicals and reagents were purchased from Sigma-Aldrich, Roche Applied Science, and Kirkegaard & Perry Laboratories. Mouse monoclonal antibodies 8B1-F3 against subunit A, 13D11-B2 against subunit B, and 10D7 against Vph1p were purchased from Protein A-Sepharose, Protein G-agarose, and Kirkegaard & Perry Laboratories. The chemiluminescent substrate for horseradish peroxidase was developed using a chemiluminescent detection method from Kirkegaard & Perry Laboratories.

Protein concentrations were determined by Lowry assay (35). Yeast strains expressing the VMA1 gene bearing mutations within the nonhomologous region were constructed as previously described (29). Plasmid p426-NH:H/A was generated to study the HA-tagged nonhomologous region of Vma1p. Plasmid Construction and Transformation—Both SpeI and BamHI restriction enzyme sites were introduced into the nucleic acid sequence of the nonhomologous region of the VMA1 gene using the polymerase chain reaction. The sequence of the nonhomologous region of the VMA1 gene was amplified from the restriction enzyme sites of Yip5-VMA1 and used as primers containing the restriction enzyme sites SpeI and BamHI and then cloned into the 2-μm plasmid, p426, using the SpeI and BamHI sites (p426-NHR). The sequence of the cloned nonhomologous region of the VMA1 gene was confirmed by DNA sequencing using an automated sequencer from Applied Biosystems. The oligonucleotides used for amplification of the nonhomologous region of the VMA1 gene were derived from the restriction enzyme sites of Yip5-VMA1 and forward (SpeI), 5’-GGGACTAGTAAACAATGCCGGGAAAGTTTCAAGT-3’, reverse (BamHI), 5’-CCGGATCCAGGATGACCGGAGAATGTTTGAATGTGG-3’. The three tandem repeats of the nine-amino-acid HA epitope (YPDYVPDYA) were ligated into the BamHI and KpnI sites of p426-NH:H/A. This resulted in insertion of three tandem repeated HA tags after amino acid residue Pro233 of the nonhomologous region of Vma1p. Yeast strain SF838–5Aalpha vma1Δ 8 were transformed using the lithium acetate method with p426-NHR::HA and p426 vector alone (32). The transformants were selected on Ura–plates as described previously (33). Growth phenotypes of the transformants were assessed on YPD plates buffered with 50 mM KH2PO4 or 50 mM succinic acid to elicit V-ATPase pH-dependent proton transport.

Isolation of Vacuolar Membrane Vesicles—Vacuolar membrane vesicles were isolated using a modified protocol described by Uchida et al. (34). The yeast integrated with wild type plasmid (Yip5-VMA1), the nonhomologous mutants, or the vector Yip5 alone were cultured overnight in 1 liter of YPD (pH 5.5) to log phase. Cells were pelleted, washed once with water, and resuspended in 100 ml of 10 mM dithiothreitol and 100 mM Tris-HCl, pH 9.4. After incubation at 30 °C for 15 min, cells were pelleted again; washed once with 100 ml of YPD medium containing 0.7 m sorbitol, 2 mM dithiothreitol, 100 mM MES-Tris, pH 7.5, and 2 mg of Zymolase 100T; and incubated at 30 °C with gentle shaking for 60 min. The resulting spheroplasts were osmotically lysed, and the vacuoles were isolated by flotation on two consecutive ficoll gradients. Protein concentrations were determined by Lowry assay (35).

Immunoblot Analysis—Yeast were grown to log phase at 30 °C in Ura–selective medium and whole cell lysates were prepared using 50 mM Tris-HCl, pH 6.8, 8 mM urea, 5% SDS, 1 mM EDTA, and 5% β-mercaptoethanol, as described previously (36). Samples were subjected to SDS-PAGE and transferred to nitrocellulose. The expression of the nonhomologous region of subunit A was detected by Western blotting using monoclonal antibodies to peroxidase-conjugated moieties directed against HA, whereas subunits B and D were detected using the monoclonal antibody 8B1-F3 directed against subunit A and the monoclonal antibody 13D11 against subunit B, respectively, followed by horseradish peroxidase-conjugated secondary antibodies. Immunoblots were developed using a chemiluminescent detection method from Kirkegaard & Perry Laboratories.

In Vivo Interaction between the Nonhomologous Region and the V0 Domain—Dissociation and reassembly of the V-ATPase in response to glucose depletion and glucose readdition were measured as described previously (37) with some modification. The yeast cells containing the integrated wild type plasmid (Yip5-VMA1) or the vma1Δ strain expressing the HA-tagged nonhomologous region of Vma1p or the vector alone were grown to log phase in Ura–selective medium. The cells were converted to spheroplasts by treatment with Zymolase 100T and incubated in YEP medium with or without 2% glucose for 15 min at 30 °C. An aliquot of the spheroplasts incubated in the absence of glucose was subsequently incubated in the presence of 2% glucose for an additional 15 min. Spheroplasts were pelleted and lysed in phosphate-buffered saline containing 1% C12E8, protease inhibitors (2 μg/ml aprotinin, 0.7 μg/ml pepstatin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride), and 1 mM dithiobis(succinimidyl propionate). The V0 domain was immunoprecipitated using the monoclonal antibody 10D7-A7 against Vph1p and protein A-Sepharose, whereas the HA-tagged nonhomologous region was followed by the monoclonal antibody 3F10 against HA and protein G-agarose. Where indicated, the V0 domain and intact V0Vs were immunoprecipitated using the monoclonal antibody 8B1-F3 against subunit A and protein A-Sepharose. Samples were then separated on 4–20% gradient gels and transferred to nitrocellulose. Western blotting was performed using the monoclonal antibody 3F10 against HA to detect the nonhomologous region as well as the HA epitope tag and the monoclonal antibody 10D7 against Vph1p to detect the V0 domain, followed by a horseradish peroxidase-conjugated secondary antibody. To detect V0Vs subunits, monoclonal antibody 8B1-F3 against subunit A or 13D11 against subunit B or rabbit polyclonal antibody against subunits C, D, E, F, G, or H was employed as primary antibody in Western blot analysis.

Biochemical Characterization—ATPase activity was measured using a coupled spectrophotometric method as described previously (38) with some modification. Isolated vacuoles were incubated in ATPase assay buffer (50 mM NaCl, 20 mM HEPES-NaOH, pH 7.0, 0.2 mM EGTA, 10% glycerol, 1 mM MgCl2, 1.5 mM phosphono pyruvate, 0.35 mM NADH, 20 units/ml pyruvate kinase, and 10 units/ml lactate dehydrogenase) with 0.1% Me2SO or 1 mM C12E8, and the coupled spectrophotometric method was performed as described previously (37). Protein samples were precipitated with an equal volume of 20% trichloroacetic acid, washed with cold acetone, and resuspended in 50 mM Tris-HCl, pH 6.8, 8 mM urea, 5% SDS, 1 mM EDTA, and 5% β-mercaptoethanol. Samples were then subjected to SDS-PAGE and transferred to nitrocellulose. Blots were incubated with antibodies against either the HA epitope tag or subunits C, D, E, F, G, or H. Secondary antibodies were goat anti-mouse IgG and goat anti-rabbit IgG, and chemiluminescence was visualized using a chemiluminescent detection method as described previously (37). Immunoblots were developed using a chemiluminescent detection method from Kirkegaard & Perry Laboratories.

Quinacrine Staining—Vacuolar accumulation of quinacrine was examined as described previously with slight modifications (40, 41). Approximately 4–5 × 105 log phase yeast cells were harvested, resuspended in 500 μl of YPD buffer with 50 mM NaH2PO4 (pH 7.6). Approximately 200 μm quinacrine or in 500 μl of YPD buffer with 50 mM NaH2PO4 (pH 7.6) containing 200 μM chloroquine, and incubated with shaking at room temperature for 5 min. Cells treated with chloroquine were then sedinted and resuspended in 500 μl of YPD buffer with 50 mM NaH2PO4 (pH 7.6) containing 200 μM chloroquine, and incubated with shaking at room temperature for 5 min. Cells were subjected to SDS-PAGE and visualized on a Zeiss Axiovert fluorescence microscope. Cells were viewed under Nomarski optics to observe cell morphology and in fluorescence mode using a fluorescein filter with a 40× objective to observe quinacrine staining.

Chloroquine Treatment and in Vivo Dissociation of the V-ATPase in Response to Glucose Depletion—Dissociation and reassembly of the V-ATPase in response to glucose depletion and glucose readdition were measured as described previously with some modifications (37). Yeast containing integrated wild type Vma1p were grown in YPD, pH 5.5, overnight to an absorbance at 600 nm of 0.1. The cells were converted
to spheroplasts by treatment with Zymolase 100T and incubated in YEP medium with or without 2% glucose in the presence or in the absence of 200 μM chloroquine for 15 min at 30 °C. Spheroplasts were pelleted and lysed in phosphate-buffered saline containing 1% C12E9 (polyoxyethylene 9-lauryl ether), protease inhibitors (2 μg/ml aprotinin, 0.7 μg/ml pepstatin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride), and 1 mM dithiobis(succinimidyl propionate). The V-ATPase complexes were immunoprecipitated using antibody 13D11 against the B subunit and protein A-Sepharose followed by separation on 8% acrylamide gels and transfer to nitrocellulose. Western blotting was then performed using the antibody 8B1-F3 against the A subunit and antibody 13D11 against the B subunit to detect the V1 domain and antibody 10D7-A7 against Vph1p to detect the V0 domain followed by a horse-radish peroxidase-conjugated secondary antibody. Dissociation of the V-ATPase complex is reflected as a reduction in the a subunit immunoprecipitated using the antibody directed against subunit B of the V1 domain.

RESULTS AND DISCUSSION

ATP Dependence of the Coupling Efficiency of Proton Transport and ATP Hydrolysis for Wild Type and Mutant Forms of Vma1p Containing Mutations in the Nonhomologous Region—We had previously demonstrated that mutations in the nonhomologous region could result in changes of the coupling of proton transport and ATP hydrolysis by the V-ATPases (29). In
Also consistent with previous results (29), at 0.5 mM ATP the cP233V mutants are less tightly coupled relative to the wild type P217V mutant is more tightly coupled and the P223V and bovine coated vesicle V-ATPase (45) and 310

tions of ATP. Fig. 1

We therefore wished to determine whether the changes in ATP leading to a progressive uncoupling of these activities (42).

function of ATP concentration, with higher concentrations of transport and ATPase activity by the V-ATPase varied as a

clathrin-coated vesicles that the tightness of coupling of proton transport activities of ATP. It is therefore possible that the

apparent decrease in coupling efficiency at high ATP concentrations is due to inhibition of proton transport by ADP that either accumulates during the course of the assay or is added as a contaminant with ATP. To address this question, pyruvate kinase and phosphoenolpyruvate were added to the proton transport assay to ensure that any ADP that was generated was quickly reconverted to ATP. The levels of pyruvate kinase and phosphoenolpyruvate were shown to be adequate to completely convert any ADP present to ATP using the coupled spectrophotometric assay used to measure ATPase activity (data not shown). As seen in Fig. 1d, the ATP dependence of proton transport of the wild type and mutant forms of the V-ATPase is similar to that observed in the absence of the regenerating system (Fig. 1b), indicating that the decreased coupling efficiency observed at higher ATP concentrations cannot be accounted for on the basis of ADP inhibition of proton transport.

Association between the Nonhomologous Region of the Subunit A and the V0 Domain of the V-ATPase—In order to identify V-ATPase subunits with which the nonhomologous region of the A subunit was able to interact, an HA-tagged form of this domain was expressed in cells disrupted in the VMA1 gene. As shown in Fig. 2, Western blot of whole cell lysates demonstrated the stable expression of this domain. Disruption of genes encoding subunits of the V-ATPase leads to a characteristic vma− phenotype in which cells are unable to grow at neutral pH (46, 47). As expected, expression of the nonhomo-

logous region in the absence of the remainder of the A subunit was not able to rescue the vma− phenotype. Interestingly, co-expression of the HA-tagged nonhomologous region and an A subunit construct lacking this region led to a wild type phenotype (data not shown). This result suggests that the nonhomologous region is able to fold into a stable domain that is able to bind to the remainder of the A subunit and promote assembly of at least a partially functional V-ATPase complex.

Immunoprecipitation of the nonhomologous region from cells expressing only this domain using an anti-HA antibody followed by Western blot analysis revealed co-precipitation of both subunit a and subunit d of the V0 domain (Fig. 3a) but no co-precipitation of V1 subunits (Fig. 3b). This result suggests that the nonhomologous region is able to directly bind to the V0

FIG. 2. Western blot analysis of the expression level of the HA-tagged nonhomologous region of Vma1p. Whole cell lysates were prepared from the vma13 strain expressing wild type Vma1p (WT), the vector alone (vector) or the HA-tagged nonhomologous region of Vma1p (NHR). Samples were subjected to SDS-PAGE on a 4–20% acrylamide gradient gel and transferred to nitrocellulose. Western blott-
ing was then performed using the monoclonal antibody 8B1 against subunit A, the monoclonal antibody 13D11 against subunit B, or the monoclonal antibody 3F10 against the HA epitope, as described under “Experimental Procedures.” Also shown are the growth phenotypes of the cells on YPD plates buffered to pH 7.5.
domain. In support of this interpretation, immunoprecipitation of the V0 domain using the monoclonal antibody 10D7 directed against subunit a is visible in either the immunoprecipitation left panel or right panel. The proteins were separated by SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed using the monoclonal antibody 10D7 against subunit a, a rabbit polyclonal antibody against subunit d, or the monoclonal antibody 3F10 against the HA epitope, as described under “Experimental Procedures.”

A

| anti-a | WT | NHR | anti-a | WT | NHR |
|-------|----|-----|-------|----|-----|
|       |    |     |       |    |     |
| anti-d |    |     |       |    |     |
|       |    |     |       |    |     |
| anti-HA |    |     |       |    |     |

B

| anti-A | WT | NHR | anti-A | WT | NHR |
|-------|----|-----|-------|----|-----|
|       |    |     |       |    |     |
| anti-B |    |     |       |    |     |
|       |    |     |       |    |     |
| anti-C |    |     |       |    |     |
|       |    |     |       |    |     |
| anti-D |    |     |       |    |     |
|       |    |     |       |    |     |
| anti-E |    |     |       |    |     |
|       |    |     |       |    |     |
| anti-G |    |     |       |    |     |
|       |    |     |       |    |     |
| anti-H |    |     |       |    |     |
|       |    |     |       |    |     |
| anti-HA |    |     |       |    |     |

FIG. 3. Interaction between the nonhomologous region of Vma1p and the V0 domain of the V-ATPase. a, the vma1Δ strain expressing the wild type (WT) Vma1p, the vector alone (vector), or the HA-tagged nonhomologous region of Vma1p (NHR) were grown overnight and converted to spheroplasts. The spheroplasts were lysed in phosphate-buffered saline containing 1% C12E9, protease inhibitors, and 1 mM DSP. The nonhomologous region was immunoprecipitated (IP) using the monoclonal antibody 3F10 against the HA epitope (left panel), whereas the V0 domain was immunoprecipitated using the monoclonal antibody 10D7 against subunit a (right panel). The proteins were separated by SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed using the monoclonal antibody 10D7 against subunit a, a rabbit polyclonal antibody against subunit d, or the monoclonal antibody 3F10 against the HA epitope, as described under “Experimental Procedures.” b, the experiment was performed as described in a except that the vector alone was not included, immunoprecipitation was performed using the antibody against HA (left panel) and the monoclonal antibody SB1-F3 against subunit A (right panel), and Western blotting was performed using the monoclonal antibody 3F10 against the HA epitope or monoclonal antibodies SB1-F3 against subunit A or 13D11 against subunit B or rabbit polyclonal antibodies against subunit C, D, E, G, or H. Although Western blotting was also attempted using a rabbit polyclonal antibody against subunit F, no immunoreactive band was visible in either the immunoprecipitation performed using the antibody against HA or the antibody against subunit A, possibly due to loss of subunit F from the immunoprecipitate below the level detectable using the anti-F subunit antibody.

FIG. 4. Glucose dependence of the interaction between the nonhomologous region of Vma1p and the V0 domain of the V-ATPase. The vma1Δ strain expressing the wild type (WT) Vma1p, the vector alone (vector) or the HA-tagged nonhomologous region of the Vma1p (NHR) were grown overnight and converted to spheroplasts. The spheroplasts were incubated for 15 min in the presence (+D) or absence (−D) of 2% glucose and then lysed in phosphate-buffered saline containing 1% C12E9, protease inhibitors, and 1 mM DSP. The V0 domain was immunoprecipitated (IP) using the monoclonal antibody 10D7 against subunit a (left panel), whereas the nonhomologous region was immunoprecipitated using the monoclonal antibody 3F10 against the HA epitope (right panel). The proteins were separated by SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed using the monoclonal antibody 10D7 against subunit a, a rabbit polyclonal antibody against subunit d, or the monoclonal antibody 3F10 against the HA epitope, as described under “Experimental Procedures.”

FIG. 5. Expression level of the nonhomologous region does not change in response to glucose depletion. The vma1Δ strain expressing the wild type (WT) Vma1p, the vector alone (vector), or the HA-tagged nonhomologous region of the Vma1p (NHR) were grown overnight, converted to spheroplasts, and incubated for 15 min in the presence (+D) or absence (−D) of 2% glucose. Whole cell lysates were prepared, and Western blot analysis was performed using the monoclonal antibodies 10D7 against subunit a, SB1-F3 against subunit A, 13D11 against subunit B, 3F10 against the HA epitope tag, and a polyclonal antibody against subunit d as described under “Experimental Procedures.”
Role of the V-ATPase A Subunit Nonhomologous Region

V0 domain, with this domain adopting a more extended conformation observed in the intact V-ATPase as compared with the isolated V1 and V0 domains. This is because a marked change in the position of the N-terminal domain of subunit a is associated with the soluble domain of subunit a. This is because a marked change in the position of the N-terminal domain of subunit a is associated with the soluble domain of subunit a.

FIG. 4. Immunoprecipitation of the V0 domain remains to be determined. Future experiments will be carried out to determine whether mutations in the nonhomologous region that block in vivo dissociation (29) are also able to prevent glucose-dependent dissociation of the nonhomologous region from V0.

Immunoprecipitation was next performed using the anti-HA monoclonal antibody. Surprisingly, although decreased co-precipitation of the V0 subunits was observed upon glucose depletion, a decrease in the amount of the nonhomologous region immunoprecipitated was also observed (Fig. 4, right panel). This decrease in immunoprecipitation of the HA-tagged nonhomologous region in the absence of glucose could be due either to decreased levels of this domain in the cell (due, for example, to increased degradation) or to the occlusion of the epitope tag (by binding of the nonhomologous region to another protein). To distinguish these possibilities, the levels of the nonhomologous region present in whole cell lysates following glucose depletion were determined by Western blot. As can be seen in Fig. 5, the level of the nonhomologous region present in the whole cell lysate was unchanged following glucose depletion. This result suggests that the decreased immunoprecipitation of the nonhomologous region by the anti-HA antibody upon glucose removal (Fig. 4) is due to occlusion of the antibody binding site rather than to increased degradation of this domain in the absence of glucose. As with interaction of the nonhomologous region with the V0 domain, the decrease in the HA-tagged nonhomologous region immunoprecipitated with the anti-HA antibody observed upon glucose removal is reversed upon glucose readDITION (data not shown). The identity of the protein to which the nonhomologous region binds upon its release from the V0 domain remains to be determined.

Effect of Vacular Neutralization on in Vivo Glucose-dependent Dissociation of the V-ATPase—In yeast, dissociation of the V1 and V0 domains in response to glucose depletion has been shown to require catalytic activity of the V-ATPase (16, 58). Consistent with this, the R219K mutation in the nonhomologous region that inhibits >90% of both proton transport and ATPase activity in isolated vacuolar membranes results in a block in glucose-dependent dissociation (29). This dependence of in vivo dissociation on V-ATPase activity could be explained in at least two possible ways. First, the V-ATPase may need to adopt a particular conformational state in order for dissociation...
to occur, and by blocking activity the V-ATPase is prevented from adopting this critical conformation. Alternatively, there may exist a pH sensor that senses the pH of the compartment lumen and prevents dissociation of the V-ATPase if the luminal pH is too alkaline. This sensor might either be part of the V-ATPase itself or a separate transmembrane protein. Thus, if the V-ATPase is inactive, the luminal pH becomes high, and dissociation of the V-ATPase is blocked.

To distinguish between these two possibilities, the pH of the vacuolar compartment needs to be increased without directly inhibiting V-ATPase activity. To accomplish this, cells were treated with the weak base chloroquine, which has previously been shown to lead to neutralization of intracellular compartments (59). As can be seen in Fig. 6, treatment of wild type yeast cells with chloroquine leads to the loss of staining of cells with the fluorescence dye quinacrine, which accumulates in acidic compartments in cells (40). This loss of quinacrine staining is also observed upon disruption of the VMA1 gene in yeast (Fig. 6), consistent with the expected neutralization of the vacuolar compartment in this strain. To assess the effect of chloroquine treatment on in vivo dissociation of the V-ATPase, spheroplasts were incubated in the absence or presence of chloroquine and the absence or presence of glucose, followed by detergent solubilization, immunoprecipitation of the V0 domain using a monoclonal antibody against subunit B, and Western blotting using antibodies against subunits A and B of the V0 domain and subunit a of the V1 domain. Dissociation appears as a decrease in the amount of subunit a immunoprecipitated using an antibody against subunit B (37). As can be seen in Fig. 7, chloroquine treatment blocks in vivo dissociation of the V-ATPase in response to glucose depletion. To test whether chloroquine inhibition of in vivo dissociation was due to a partial inhibitory effect on V-ATPase activity, the effect of chloroquine on V-ATPase activity in isolated vacuolar membranes was measured. An assay of wild type vacuolar membranes in the presence of 200 \( \mu \)M chloroquine reduced concanamycin-sensitive ATPase activity to 81 ± 2% of control levels. Initial results suggested a larger inhibitory effect, but this was shown to be due to absorbance of chloroquine at 341 nm, the wavelength at which the coupled spectrophotometric assay is performed. This absorbance effect was eliminated by measuring the decrease in NADH absorbance at 360 nm instead of 341 nm, at which wavelength interference due to absorbance of light by chloroquine is negligible. Because 80% of control activity is far in excess of what is required to allow in vivo dissociation of the V-ATPase to occur (16, 58), chloroquine does not appear to block glucose-dependent dissociation of \( V1V0 \) by directly inhibiting V-ATPase activity.

These results suggest that in vivo dissociation of the V-ATPase complex is sensitive to the luminal pH, such that if the luminal pH is too alkaline, dissociation of the V-ATPase is blocked. Such a mechanism makes sense as a way to prevent intracellular compartments from becoming too alkaline, where severe secondary effects, such as accumulation of undegraded proteins, may occur. Because the pH of the Golgi is more alkaline than that of compartments such as the lysosome (60), this pH dependence of in vivo dissociation may also account for why V-ATPase complexes containing Stv1p (which normally resides in the Golgi) show no dissociation when localized to the Golgi but dissociate normally when targeted to the vacuole (37). At odds with this idea is the observation that Stv1p-containing complexes have much lower proton transport activity than Vph1p-containing complexes and hence might not be expected to sufficiently acidify the vacuolar compartment to allow dissociation to occur (37). However, Vma8p mutants possessing as little as 6% of wild type proton transport activity (in vitro) are still competent for glucose-dependent dissociation (61), so the exact level of activity required for dissociation remains uncertain.

CONCLUSIONS

In summary, we have shown that the coupling differences resulting from changes in the nonhomologous region are largely preserved over a range of ATP concentrations, although partial uncoupling is observed at higher ATP. The nonhomologous region is shown to bind to the \( V0 \) domain, and this interaction is observed to be glucose-dependent, suggesting that changes in the interaction between the nonhomologous region and \( V0 \) may trigger in vivo dissociation of the complex. Finally, in vivo dissociation of the V-ATPase is shown to be sensitive to the luminal pH of the compartment, although the identity of the pH sensor controlling dissociation remains to be determined.

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REFERENCES

1. Nishi, T., and Forgac, M. (2002) Nat. Rev. Mol. Cell. Biol. 3, 94–103
2. Forgac, M. (1999) J. Biol. Chem. 274, 12951–12954
3. Graham, L. A., Powell, B., and Stevens, T. H. (2000) J. Exp. Biol. 203, 61–70
4. Kane, P. M., and Parra, R. J. (2000) J. Biol. Chem. 275, 81–87
5. Bowman, E. J., and Bowman, B. J. (2000) J. Biol. Chem. 275, 97–106
6. Nelson, N., Perzov, N., Cohen, A., Hagai, K., Padler, V., and Nelson, H. (2000) J. Biol. Chem. 275, 89–95
7. Futai, M., Oka, T., Sun-Wada, G., Moriyama, Y., Kanazawa, H., and Wada, Y. (2000) J. Biol. Chem. 275, 107–116
8. Sue, H., Li, X., and Palmgren, M. G. (1999) Plant Cell 11, 677–690
9. Brown, D., and Breiten, S. (1999) J. Exp. Biol. 203, 137–145
10. Li, Y.-P., Chen, W., Liang, Y., Li, E., and Stashenko, P. (1999) Nat. Genet. 23, 447–451
11. Nanda, A., Brumell, J. H., Nordström, T., Kjeldsen, L., Sengbøl, H., Borregaard, N., Rotstein, O. D., and Grinstein, S. (1996) J. Biol. Chem. 271, 15963–15970
12. Wieczorek, H., Gruber, G., Harvey, W. R., Huss, M., Merzendorfer, H., and Zeiske, W. (2000) J. Biol. Chem. 275, 127–135
13. Ludwig, J., Kerscher, S., Brandt, U., Pfeiffer, K., Getlawi, F., Apps, D., and Schagger, H. (1998) J. Biol. Chem. 273, 10939–10947
14. Merzendorfer, H., Huss, M., Schmidt, R., Harvey, W. R., and Wieczorek, H. (1999) J. Biol. Chem. 274, 17372–17378
15. Sambade, M., and Kane, P. M. (2004) J. Biol. Chem. 279, 17361–17365
16. MacLeod, K. J., Sambongi, Y., and Wada, Y. (2000) Biochim. Biophys. Acta 1498, 276–288
17. Zinniak, L., Dittrich, P., Gogarten, J. P., Kibak, H., and Tait, L. (1988) J. Biol. Chem. 263, 9102–9112
18. Bowman, E. J., Teeny, K., and Bowman, B. (1988) J. Biol. Chem. 263, 13994–14001
19. Hirata, R., Ohsumi, Y., Nakano, A., Kawasaki, H., Suzuki, K., and Anzaku, Y. (1990) J. Biol. Chem. 265, 6726–6733
20. Paupoko, K., Kumasato, C., Adachi, I., and Forgac, M. (1991) J. Biol. Chem. 266, 24564–24572
21. Bowman, B. J., Allen, R., Wechsler, M. A., and Bowman, E. J. (1988) J. Biol. Chem. 263, 14002–14007
22. Shaq, E., Nishi, T., Kawasaki-Nishi, S., and Forgac, M. (2003) J. Biol. Chem. 278, 1295–1299
23. Kane, P. M. (1995) J. Biol. Chem. 270, 17025–17032
24. Sumner, J. P., Dow, J. A., Early, F. G., Klein, U., Jager, D., and Wieczorek, H. (1995) J. Biol. Chem. 270, 5649–5653
25. Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Nucleic Acids Res. 20, 1425
26. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1992) Currentprotocols in Molecular Biology, John Wiley & Sons, Inc., New York
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19236–19244
37. Kawasaki-Nishi, S., Nishi, T., and Forgac, M. (2001) J. Biol. Chem. 276, 17941–17948
38. Forgac, M., Cantley, L., Wiedenmann, B., Altstiel, L., and Branton, D. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 1300–1303
39. Feng, Y., and Forgac, M. (1992) J. Biol. Chem. 267, 5817–5822
40. Roberts, C. J., Raymond, C. K., Yamashiro, C. T., and Stevens, T. H. (1991) Methods Enzymol. 194, 644–661
41. Liu, Q., Leng, X., Newman, P. R., Vasilyeva, E., Kane, P. M., and Forgac, M. (1997) J. Biol. Chem. 272, 11750–11756
42. Arata, H., Pink, S., and Forgac, M. (1989) Biochemistry 28, 3075–3082
43. Nelson, N. (1992) J. Bioenerg. Biomembr. 24, 407–414
44. Canonaco, F., Schlattner, U., Prueti, P. S., Wallimann, T., and Sauer, U. (2002) J. Biol. Chem. 277, 31303–31309
45. Vasilyeva, E., and Forgac, M. (1998) J. Biol. Chem. 273, 23823–23829
46. Kane, P. M., Yamashiro, C. T., Wolczyk, D. F., Neff, N., Goebl, M., and Stevens, T. H. (1992) Science 250, 651–657
47. Nelson, H., and Nelson, N. (1999) Proc. Natl. Acad. Sci. U. S. A. 87, 3503–3507
48. Tomashok, J. J, Garrison, B. S., and Kionsky, D. J. (1997) J. Biol. Chem. 272, 16618–16623
49. Xu, T., Vasilyeva, E., and Forgac, M. (1999) J. Biol. Chem. 274, 28909–28915
50. Arata, Y., Ballein, J. D., and Forgac, M. (2002) Biochemistry 41, 11301–11307
51. Leng, X. H., Nishi, T., and Forgac, M. (1999) J. Biol. Chem. 274, 14655–14661
52. Landolt-Marticorena, C., Williams, K. M., Correa, J., Chen, W., and Manolson, M. E. (2000) J. Biol. Chem. 275, 15449–15457
53. Arata, Y., Ballein, J. D., and Forgac, M. (2002) J. Biol. Chem. 277, 3357–3363
54. Zhang, Z., Charsky, C., Kane, P. M., Wilkens, S. (2003) J. Biol. Chem. 278, 47299–47306
55. Wilkens, S., Vasilyeva, E., and Forgac, M. (1999) J. Biol. Chem. 274, 31804–31810
56. Wilkens, S., and Forgac, M. (2001) J. Biol. Chem. 276, 44064–44068
57. Wilkens, S., Inoue, T., and Forgac, M. (2004) J. Biol. Chem. 279, 41942–41949
58. Parra, K. J., and Kane, P. M. (1998) Mol. Cell. Biol. 18, 7064–7074
59. Emerson, L. R., Nau, M. E., Martin, R. K., Kyle, D. E., Vahey, M., and Wirth, D. F. (2002) J. Microbiology 46, 787–796
60. Schapirio, F. B., and Grinstein, S. (2000) J. Biol. Chem. 275, 21025–21032
61. Xu, T., and Forgac, M. (2000) J. Biol. Chem. 275, 22075–22081
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