Photoaffinity Labeling of Wild-type and Mutant Forms of the Yeast V-ATPase A Subunit by 2-Azido-[32P]ADP*

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Molecular modeling studies have previously suggested the possible presence of four aromatic residues (Phe\textsuperscript{452}, Tyr\textsuperscript{353}, Tyr\textsuperscript{358}, and Phe\textsuperscript{363}) near the adenine binding pocket of the catalytic site on the yeast V-ATPase A subunit (MacLeod, K. J., Vasilyeva, E., Baleja, J. D., and Forgac, M. (1998) J. Biol. Chem. 273, 150–156). To test the proximity of these aromatic residues to the adenine ring, the yeast V-ATPase containing wild-type and mutant forms of the A subunit was reacted with 2-azido-[\textsuperscript{32P}]ADP, a photoaffinity analog that stably modifies tyrosine but not phenylalanine residues. Mutant forms of the A subunit were constructed in which the two endogenous tyrosine residues were replaced with phenylalanine and in which a single tyrosine was introduced at each of the four positions. Strong ATP-protectable labeling of the A subunit was observed for the wild-type and the mutant containing tyrosine at 532, significant ATP-protectable labeling was observed for the mutants containing tyrosine at positions 452 and 538, and only very weak labeling was observed for the mutants containing tyrosine at 535 or in which all four residues were phenylalanine. These results suggest that Tyr\textsuperscript{353} and possibly Phe\textsuperscript{452} and Tyr\textsuperscript{358} are in close proximity to the adenine ring of ATP bound to the A subunit. In addition, the effects of mutations at Phe\textsuperscript{452}, Tyr\textsuperscript{352}, Tyr\textsuperscript{358}, and Glu\textsuperscript{356} on dissociation of the peripheral V\textsubscript{1} integral V\textsubscript{0} domains both in vivo and in vitro were examined. The results suggest that in vivo dissociation requires catalytic activity while in vitro dissociation requires nucleotide binding to the catalytic site.

The vacuolar proton-translocating ATPases (or V-ATPases)\textsuperscript{1} are a family of ATP-dependent proton pumps responsible for acidification of intracellular compartments in eukaryotic cells (1–6). The V-ATPases function in a variety of cellular processes, including protein processing and degradation, receptor-mediated endocytosis, intracellular membrane traffic, and coupled transport (1–6). For certain specialized cells, including renal intercalated cells (7), macrophages and neutrophils (8), tumor cells (9), and osteoclasts (10), the V-ATPase has been identified at the plasma membrane and acidifies the extracellular space. In yeast, acidification of the central vacuole by the V-ATPase serves to activate protein degradation and to drive the uptake of solutes such as Ca\textsuperscript{2+} and amino acids for storage (5).

The V-ATPases are composed of two domains, a peripheral, cytoplasmic 570-kDa V\textsubscript{1} domain responsible for nucleotide binding and hydrolysis, and a membrane integral 260-kDa V\textsubscript{0} domain responsible for proton translocation across the membrane (1–6). The V\textsubscript{1} domain contains eight different subunits (subunits A–H, with molecular masses 100–17 kDa) while the V\textsubscript{0} domain contains five different subunits (subunits a, d, e, c, and e', with molecular masses from 100 to 17 kDa). The nucleotide binding subunits have been identified as the A and B subunits, which are believed to form a hexameric structure containing three copies of each subunit (11).

Previous studies have demonstrated that the catalytic sites reside on the A subunits and that a second class of sites, termed “noncatalytic” sites, reside on the B subunits, giving a total of six nucleotide binding sites per complex (12–15). The A and B subunits of the V-ATPase are approximately 25% identical with the β and α subunits of the F-ATPase, respectively (16, 17). The F-ATPases function in ATP synthesis in mitochondria, chloroplasts, and bacteria (18–20). The x-ray crystal structures of F\textsubscript{1} indicate that the nucleotide binding sites are located at the interface of the β and α subunits, with the catalytic sites residing primarily on the β subunits and the noncatalytic sites residing primarily on the α subunits (21, 22).

Although mutagenesis studies of the yeast V-ATPase have begun to identify residues important for catalytic activity (23–26), the structure of the nucleotide binding sites on the V-ATPase has yet to be determined. One approach to characterizing the structure of nucleotide binding sites is the use of photoaffinity analogs. 2-Azido-[\textsuperscript{32P}]ATP has been used to label both the catalytic and noncatalytic nucleotide binding sites of the F-ATPase (27–29), as well as the nucleotide binding sites of the bovine coated vesicle V-ATPase (13). The reactive nitrene moiety generated upon photoactivation of 2-azido-ATP has been shown to have a strong preference for reaction with tyrosine residues (over, for example, phenylalanine residues). Thus, 2-azido-[\textsuperscript{32P}]ATP reacts with Tyr\textsuperscript{368} at the catalytic site of the bovine mitochondrial F\textsubscript{1} αβ subunit (27), but substitution of phenylalanine at the corresponding position in the Escherichia coli β subunit prevents the formation of a stable adduct.\textsuperscript{2}

In addition, when 2-azido-[\textsuperscript{32P}]ATP is loaded at the noncatalytic sites of F\textsubscript{1}, modification of Tyr\textsuperscript{354} is observed (28, 29). Replacement of this tyrosine residue with phenylalanine eliminates labeling of the β subunit and shifts the label to the α subunit (29). When Argo\textsuperscript{335} at the noncatalytic site is changed to tyrosine, significant labeling of the α subunit is observed. This shift of labeling to α is not observed on substitution of

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¶The abbreviations used are: V-ATPase, vacuolar proton-translocating adenosine triphosphatase; F-ATPase, F-type ATP synthase; YEPD, yeast extract-peptone-dextrose; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis, WT, wild-type; Tricine, N-tris(hydroxymethyl)methylglycine.
phenylalanine at position 365 (29). The x-ray crystal structures of F₀ have confirmed the proximity of these aromatic residues to ATP bound at the catalytic and noncatalytic sites (21, 22). These results suggest that reaction with 2-azido-[32P]ATP can be used to identify tyrosine residues in close proximity to bound nucleotides.

Molecular modeling studies using the x-ray coordinates of F₀; the sequence homology between the α, β, A, and B subunits; and energy minimization programs have predicted the presence of four aromatic residues (Phe52, Tyr352, Tyr355, and Phe538) near the adenine binding pocket at the catalytic site on the yeast V-ATPase A subunit (26). Mutagenesis studies have indicated that alteration of these residues leads to significant changes in activity or affinity for ATP (26), but it is possible that these changes are the consequence of conformational changes rather than direct effects at the nucleotide binding site. To further test the proximity of these aromatic residues to the ATP-binding site, we have carried out photochemical labeling studies of the wild-type and mutant forms of the A subunit using 2-azido-[32P]ADP. Mutants in which all four residues were phenylalanine or in which single tyrosine residues were introduced were tested.

We also wished to determine the role of the nucleotide binding sites in controlling dissociation of the V₁ and V₀ domains. It has previously been shown that nucleotide binding activates dissociation of the V₁ and V₀ domains in vitro (30–32). In addition, reversible dissociation of V₁ and V₀ domains has been observed in vivo in yeast (33) and insects (34). We have therefore addressed the role of nucleotide binding and activity in regulating in vivo and in vitro dissociation of the V-ATPase complex.

EXPERIMENTAL PROCEDURES

Materials and Strains—Yzymolase 100T was obtained from Sekagaku America, Inc. Concanamycin A was obtained from Fluka Chemical Corp. Tran'^'S-S label (a mixture of [35S]methionine and [35S]cysteine) and [32P]ADP, were purchased from ICN Biomedical. Leupeptin, aprotinin, and pepstatin were all purchased from Roche Molecular Biochemicals. ATP and most other chemicals were purchased from Difco. Zwittergent 3-14 was purchased from Calbiochem. ATP and 32Pi were purchased from ICN Biomedical. Leupeptin, aprotinin, and 2-mercaptoethanol, were purchased from Sigma.

Yeast strain SF838::5αa vma1Δ-8 (MATa, leu2-3, 112, ura3-52, ade6, vma1Δ::LEU2), was used for integrations and subsequent biochemical characterization was a kind gift from Dr. Patricia Kane (Department of New England Biolabs. ATP and most other chemicals were purchased from Sigma.

Yeast strain SF838::5αa vma1Δ-8 (MATa, leu2-3, 112, ura3-52, ade6, vma1Δ::LEU2), was used for integrations and subsequent biochemical characterization was a kind gift from Dr. Patricia Kane (Department of Biochemistry and Molecular Biology, State University of New York, Syracuse). The plasmid pPK17–7, containing VMA1 lacking the entire yeast-derived endonuclease (VDE) subcloned into the yeast integration vector YIp5 (New England Biolabs) using the ATP-binding site, we have carried out photochemical labeling studies of the wild-type and mutant forms of the A subunit using 2-azido-[32P]ADP. Mutants in which all four residues were phenylalanine or in which single tyrosine residues were introduced were tested.

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**Transformation**—Wild-type VMA1 in YIp5 (WT-VMA1) as a positive control, or vma1 mutant Yip5 plasmids were linearized with ApoI to target the integration of the constructs to the URA3 locus. Yeast cells SF838::5αa vma1Δ-8 were transformed with the linearized constructs using the lithium acetate method (36). The transformants were then grown on YEPd plating plates at pH 3.6 (37). Chromosomal DNA was isolated from the transformed yeast cells and the VMA1 gene was amplified by a polymerase chain reaction. The presence of the site-directed mutations was confirmed by sequencing the polymerase chain reaction products. Growth phenotypes of the mutants were assessed on YEPd plates buffered with 50 mM KH₂PO₄ and 50 mM sucrose. Wild-type VMA1 was cultured overnight in 1 liter of YEPd pH 5.5 to log phase. Vacuoles were isolated from the different strains as described previously (39). For purification of the V-ATPase, vacuolar membranes were washed three times with 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA, and solubilized in buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 0.5 mM phenylmethylsulfonfyl fluoride, and 2 mM dithiothreitol) with 0.5% Zwittergent 3-14, and the V-ATPase was isolated by glycerol density gradient sedimentation on 20–50% glycerol gradients as described previously (40).

**Synthesis of 2-Azido-[32P]ADP—2-azido-AMP was synthesized as described previously (41) and 2-azido-[32P]ADP by 3-phosphoglycerate kinase and adenylate kinase (42). 2-Azido-AMP (3 μmol) were incubated in the following reaction overnight at room temperature at pH 7.7: 50 mM Tricine, pH 8.0, 100 μM EDTA, 2 mM MgCl₂, 10 mM K₂HPO₄, 25 μM ADP, 200 μM NAD, 40 mM glyceraldehyde-3-phosphate, 15 mM pyruvate, 5 mM β-mercaptoethanol, 47 μM glyceraldehyde-3-phosphate dehydrogenase, 20 μg/ml lactate dehydrogenase, 20 μg/ml adenylate kinase, 27 μg/ml 3-phosphoglycerate kinase, and 500 μCi of [32P]. The phosphorylating enzymes were removed using a Centricron 10 concentrator (Amicon) for 1.5 h at 4 °C. 2-Azidoadenine nucleotides were separated by anion exchange using an Accell Plus QMA anion exchange column (Waters Corp.). Concentrations were determined by measuring the absorbance at 271 and 310 nm using the extinction coefficients of 10 and 7 mM⁻¹ cm⁻¹, respectively. The peak fractions containing 2-azido-[32P]ATP were lyophilized and dissolved in buffer containing 50 mM Tricine, pH 8.0, 100 μM EDTA, 2 mM MgCl₂, 10 mM K₂HPO₄, 2-Azido-[32P]ATP was converted to 2-azido-[32P]ADP by addition of glucose to a final concentration of 5 mM followed by addition of 20 units of hexokinase. The reaction was incubated at 25 °C for 3 h in the dark, and the hexokinase was removed using a Centricron 10 concentrator.

**Labeling of the V-ATPase by 2-Azido-[32P]ADP—V-ATPase purified on 20–50% glycerol gradients was concentrated 10–15-fold using Centricron 30 concentrators (Amicon) to a final concentration of 10–20 μg of protein/100 μl. Protein was incubated with 120 μM 2-azido-[32P]ADP and 6 mM MgCl₂, in the presence or absence of 5 mM ATP, for 20 min at 4 °C in the dark followed by irradiation of the sample with a ultraviolet lamp (9UVGL-25 Mineralight 254/366) at short wavelength at a distance of 1 cm at 4 °C for 20 min. Laemmli sample buffer was added to the samples followed by SDS-PAGE on a 10% acrylamide gel. The gel was incubated in 30% methanol and 7.5% acetic acid, dried, and autoradiography performed.

**Metabolic Labeling and Immunoprecipitation of the V-ATPase—** Yeast strains WT-VMA1 and vma1 mutants were grown in SD-methio-nine-free medium overnight, converted to spheroplasts, and metabolically labeled with Tran³²S-S label (50 μCi/S × 10⁶ spheroplasts) for 60 min at 30 °C. At the end of the incubation, unlabeled methionine and cysteine were added to a final concentration of 0.33 mg/ml each. For the chase, spheroplasts were pelleted and resuspended in YEP media containing 1.2 m sorbitol alone, or YEP media with 1.2 m sorbitol and 2% dextrose and incubated for 30 min at 30 °C. Spheroplasts were pelleted, washed, and lysed in solubilization buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10% glycerol) with 1% C₄₆₇₅₀, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 5 μg/ml aprotenin, 1 μg/ml pepstatin). The V-ATPase was cross-linked using dithiobis-succinimidylpropionate and immunoprecipitated using the monoclonal antibody 6B1-F5 against the yeast vacuole (Molecular Probes, Inc.), and protein A–Sepharose. Phosphoamino acid analysis of the immunoprecipitates were subjected to SDS-PAGE on a 12% acrylamide gel, fixed in 30% methanol and 7.5% acetic acid for 1 h, incubated in Enlightening solution (NEN Life Science Products) for 30 min, dried, and autoradiography performed.

**In Vitro Dissociation of the V-ATPase—** Vacuolar vesicles were washed three times with 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA, and...
resuspended in buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10% glycerol). 30–50 μg of washed vacuoles were then incubated with or without 5 mM ATP and 6 mM MgCl₂ for 10 min on ice. Potassium iodide was added to the vacuoles to a final concentration 300 mM, and the vesicles were incubated on ice for 60 min. Membranes were pelleted at 100,000 × g for 90 min, and supernatants were precipitated with 12% trichloroacetic acid for 30 min on ice. The precipitated proteins were solubilized in 50 mM Tris-HCl, pH 6.8, 8% urea, 5% SDS, 1 mM EDTA, 5% β-mercaptoethanol at 70 °C for 10 min, and subjected to SDS-PAGE using a 12% acrylamide gel. Dissociation of V₁ was determined by Western blot analysis using the monoclonal antibody 8B1-F3 against the yeast V-ATPase A subunit (Molecular Probes, Inc.). Following SDS-PAGE, samples were transferred to nitrocellulose, and probed with the monoclonal antibody 8B1-F3, followed by horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Immunoblots were developed using a chemiluminescent detection method following the manufacturer’s protocol from Kirkegaard and Perry Laboratories.

Other Procedures—ATPase activity was measured using a coupled spectrophotometric assay (39) in the absence or presence of 1 μM concanamycin A, a specific inhibitor of the V-ATPase (43). SDS-PAGE was carried out as described by Laemmli (44), and silver staining was performed by the method of Oakley et al. (45). Protein concentrations were determined by Lowry assay (46), and determinations on purified enzyme included initial protein precipitation with 10% trichloroacetic acid.

RESULTS

Kinetic Analysis of vma1 Mutants—Site-directed mutations of the VMA1 cDNA encoding the yeast V-ATPase A subunit were constructed as described under “Experimental Procedures.” The mutant vma1 cDNAs were subcloned into the yeast integration vector YIp5 and expressed in a vma1Δ strain in which the VMA1 gene was deleted. The aromatic residues suggested from molecular modeling studies to be in proximity to the adenine binding pocket at the catalytic site on the A subunit are Phe452, Tyr532, Tyr535, and Phe 538. The following nomenclature was employed to simplify discussion of tyrosine and phenylalanine mutants at these positions. Tyr refers to the double mutant in which the two endogenous tyrosine residues have been replaced by phenylalanine (Y532F/Y535F). Y452 refers to the triple mutant (F452Y/Y532F/Y535F) containing a tyrosine at position 452 but phenylalanines at the other three positions. Y335 refers to the single mutant (Y532F) containing a tyrosine at position 535 but phenylalanines at the other three positions. Y532 refers to the single mutant (Y535F) containing a tyrosine at position 532 but phenylalanines at the other three positions. Finally, Y538 refers to the triple mutant (Y532F/Y535F/Y538F) containing a tyrosine at position 538 but phenylalanines at the other three positions. All other mutations (i.e. Y532S, Y535S, etc.) refer to constructs containing only the indicated amino acid substitution.

Deletion of genes encoding subunits of the V-ATPase results in a conditional lethal phenotype (35, 47, 48), which is also observed for vma mutants possessing less than approximately 20% wild-type V-ATPase activity (24). Strains bearing these mutations are able to grow on medium buffered to acidic pH (5.0–5.5) but are unable to grow at neutral pH (7.5) or in neutral medium containing 50 mM CaCl₂. Of the mutants tested, three showed altered growth at neutral pH. As previously reported, E286Q and F452A were both unable to grow at neutral pH (25, 26), while the mutant Y538S showed slower growth at neutral pH. In addition, based on Western blot analysis of whole cell lysates and isolated vacuoles using the monoclonal antibody 8B1-F3 against the yeast V-ATPase A subunit, all of the vma1 mutants tested showed normal levels of protein expression and V-ATPase assembly (data not shown).

Table I summarizes the kinetic analysis of the vma1 mutants. Vacuoles were isolated from the wild-type WT-VMA1 and vma1 mutant strains and then solubilized and the V-ATPases purified by glycerol density gradient sedimentation as described under “Experimental Procedures.” ATPase activities of the purified enzymes were measured over a range of ATP concentrations from 25 μM to 2.5 mM ATP, while the MgCl₂ concentration was maintained at 1 mM above the ATP concentration. Kₘ and Vₘₐₓ values were calculated from double reciprocal plots of ATP concentration versus ATPase activity expressed in micromoles/min/mg of protein.

| VMA1 mutant       | Kₘ (μM ATP) | Vₘₐₓ (μmol/min/mg) |
|--------------------|-------------|---------------------|
| WT-VMA1            | 0.70        | 2.5                 |
| Tyr (Y532F/Y535F)  | 0.14        | 0.90                |
| Y452 (F452Y/Y532F/Y535F) | 0.14    | 0.96                |
| Y535 (Y532F)       | 0.50        | 2.7                 |
| Y532 (Y535F)       | 0.13        | 0.95                |
| Y538 (Y532F/Y535F/Y538F) | 0.11    | 2.1                 |
| E286Q              | 0           |                      |
| F452A              | 0           |                      |
| Y532S              | >5          |                      |
| Y535S              | >5          |                      |

| 2-Azido-[³²P]ADP Labeling of the Yeast V-ATPase |

ATPase activities were measured on enzymes purified from WT-VMA1 and vma1 mutant strains as described under “Experimental Procedures.” ATPase activities were measured over a range of ATP concentrations from 25 μM to 2.5 mM ATP, while the MgCl₂ concentration was maintained at 1 mM above the ATP concentration. Kinetic analysis of WT-VMA1 and the vma1 mutants

Table I

| VMA1 mutant | Kₘ (μM ATP) | Vₘₐₓ (μmol/min/mg) |
|-------------|-------------|---------------------|
| WT-VMA1     | 0.70        | 2.5                 |
| Tyr (Y532F/Y535F) | 0.14    | 0.90                |
| Y452 (F452Y/Y532F/Y535F) | 0.14    | 0.96                |
| Y535 (Y532F) | 0.50        | 2.7                 |
| Y532 (Y535F) | 0.13        | 0.95                |
| Y538 (Y532F/Y535F/Y538F) | 0.11    | 2.1                 |
| E286Q       | 0           |                      |
| F452A       | 0           |                      |
| Y532S       | >5          |                      |
| Y535S       | >5          |                      |
2-Azido-[32P]ADP Labeling of the Yeast V-ATPase

**FIG. 1. 2-Azido-[32P]ADP labeling of the yeast V-ATPase A subunit.** Yeast V-ATPase (20 μg of protein) purified on 20–50% glycerol gradients was incubated with 120 μM 2-azido-[32P]ADP and 6 mM MgCl2, in the absence or presence of 5 mM ATP, for 20 min at 4 °C in the dark followed by irradiation of the sample with an ultraviolet lamp (UVG-25 Mineralight 254/366) at short wavelength at a distance of 1 cm at 4 °C for 20 min. Samples were then denatured in Laemmli sample buffer, separated by SDS-PAGE on a 10% acrylamide gel, the gel washed and dried, and autoradiography performed, as described under "Experimental Procedures." Panel A shows the labeling pattern of the A subunit for the wild-type WT-VMA1 and vma1 mutants — Tyr, Y452, Y532, and Y538 in the absence (lanes 1, 3, 5, 7, 9, and 11) or presence (lanes 2, 4, 6, 8, 10, and 12) of 5 mM ATP. Panel B shows the labeling of vma1 mutants E286Q, F452A, Y532S, and Y535S in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of 5 mM ATP. See Table I for the definition of the mutant strains.

very strong ATP-protectable labeling, Y452 and Y538 showed somewhat less but still very significant labeling with substantial ATP protection, and Y535 showed only relatively weak labeling, with little protection by ATP. It should be noted that the limited amounts of purified V-ATPase that can be isolated from yeast vacuoles makes it infeasible to purify and identify the radiolabeled peptides, as had previously been done for the bovine V-ATPase A subunit (13). Nevertheless, the very low level of ATP-protectable labeling observed for the Tyr mutant (despite its high affinity for ATP) makes it likely that the radiolabel is attached to the introduced tyrosine residues in the Y452, Y532, and Y538 mutants.

Labeling by 2-azido-[32P]ADP was also used to assess the ability of other mutants to bind nucleotides. As shown in Fig. 1B, E286Q showed even stronger ATP-protectable labeling by 2-azido-[32P]ADP than the wild-type enzyme. The Y535S mutant also showed good ATP-protectable labeling, whereas both Y532S and F452A showed considerably reduced labeling. These results indicate that the E286Q mutant is competent to bind nucleotides, whereas the F452A mutant binds nucleotides only poorly.

*In Vitro Dissociation of the V-ATPase—*It has previously been shown that mutation of aromatic residues postulated to be present at the catalytic site of the V-ATPases, including the A subunit residues Phe452, Tyr452, and Phe535, have significant effects on V-ATPase activity or affinity for ATP (26). Thus, substitution of alanine at position 452 completely eliminates ATP hydrolytic activity while substitution of serine at position 532 causes a greater than 7-fold increase in K_m for ATP (26). In the present study it was shown that replacement of Tyr452 by serine also causes a significant increase in K_m for ATP. These results suggested the involvement of these residues in nucleotide binding at the catalytic site, but it is possible that the effects observed on activity and affinity are due to conformational changes induced by the mutations introduced.

To attempt to determine the proximity of these aromatic residues to the adenine ring of the bound nucleotide, photochemical labeling by 2-azido-[32P]ADP was employed. Because 2-azido-adenine nucleotides show selectivity for reaction with tyrosine residues but not phenylalanine residues (29), we compared the reaction of mutant forms of the A subunit containing substitutions at these four aromatic residues. Significant labeling by 2-azido-[32P]ADP was observed for tyrosines at positions 452, 532, and 538, with little labeling observed at position 535 or for the mutant containing four phenylalanine residues. The labeling observed for this latter mutant may be due to reaction...
of 2-azido-[32P]ADP with other residues at the catalytic (or noncatalytic) sites. The absence of reaction of Tyr535 with 2-azido-[32P]ADP should not be taken as proof that this residue is not present at the catalytic site, since it is possible that the reactive nitrene is not oriented in such a way as to readily react with this residue. The stronger labeling observed for the Tyr522 mutant than for the wild-type enzyme (which contains tyrosine residues at both positions 532 and 535) may be due to the higher affinity of the Y532 mutant for nucleotides.

The results described above suggest that at least residues Phe452, Tyr532, and Phe538 are near the adenine ring of bound ATP. In support of this, previous labeling studies of the bovine V-ATPase have revealed that the A subunit is modified by 2-azido-[32P]ADP within a 12-kDa V8 fragment, which begins at residue 511 and which includes Tyr525 and Tyr532 (13). These residues correspond to Tyr532 and Tyr535, respectively, in the yeast V-ATPase A subunit. In the mitochondrial F-ATPase β subunit, the A subunit residues Phe452, Tyr532, and Tyr535 correspond to Tyr345, Phe348, and Phe349, respectively (17, 47, 52). The x-ray crystal structures of F1 show residues at both positions 532 and 535) may be due to the fact that dissociation of V1 and V0 is maximal (51). These results argue that changes in the intracellular ATP concentration are probably not the primary signal responsible for controlling the assembly state of the V-ATPase, but do not rule out the possible involvement of the nucleotide binding sites on the V-ATPase in this process. Parra and Kane (51) have demonstrated that V-ATPase complexes inactivated by mutations in the V0 domain show reduced dissociation in vivo in response to glucose deprivation, but the nucleotide binding properties of these enzymes have not been investigated. In the present study we observe that the catalytically inactive mutants E286Q and F452A show no dissociation of V1 and V0 in response to glucose deprivation, despite the fact that E286Q appears to be competent to bind nucleotides, based upon 2-azido-[32P]ADP labeling. These results suggest that in vivo dissociation of V1 and V0, unlike in vitro dissociation, is primarily dependent upon catalytic activity of the V-ATPase complex rather than on nucleotide binding. Interestingly, both the Y532S and Y535S mutants show in vivo dissociation in response to glucose deprivation. Because both of these mutants have a Kd for ATP greater than 5 mM (as compared with a Kd of 0.7 mM for the wild-type enzyme), it is likely that under intracellular conditions both of these enzymes will be turning over more slowly than the wild-type enzyme. Nevertheless, even under these conditions, in vivo dissociation is observed, suggesting that a very high rate of turnover may not be required for dissociation. It is possible that, in order for the enzyme to dissociate in vivo, it must pass through a conformational state which is only achieved during catalysis. The demonstration that the homologous F-ATPases undergo rotational motion during the catalytic cycle (54–56) suggests that some intermediate state reached during rotation of the complex may be required for dissociation.

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