Cysteine Scanning Mutagenesis of the Noncatalytic Nucleotide Binding Site of the Yeast V-ATPase*

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To investigate residues involved in the formation of the noncatalytic nucleotide binding sites of the vacuolar proton-translocating adenosine triphosphatase (V-ATPase), cysteine scanning mutagenesis of the VMA2 gene that encodes the B subunit in yeast was performed. Replacement of the single endogenous cysteine residue at position 188 gave rise to a Cys-less form of the B subunit (Vma2p) which had near wild-type levels of activity and which was used in the construction of 16 single cysteine-containing mutants. The ability of adenine nucleotides to prevent reaction of the introduced cysteine residues with the sulfhydryl reagent 3-[(4-benzoyl)benzoyl]adenosine 5'-triphosphate (BzATP) was evaluated by Western blot. Biotin-maleimide labeling of the purified V-ATPase from the wild-type and the mutants S152C, L178C, N181C, A184C, and T279C was reduced after reaction with the nucleotide analog 3'-O-(4-benzoyl)benzoyladenosine 5'-triphosphate (BzATP). These results suggest the proximity of these residues to the nucleotide binding site on the B subunit. In addition, we have examined the level of endogenous nucleotide bound to the wild-type V-ATPase and to a mutant (the A subunit mutant R483Q) which is postulated to be altered at the noncatalytic site and which displays a marked nonlinearity in ATP hydrolysis (MacLeod, K. J., Vasilyeva, E., Baleja, J. D., and Forgac, M. (1998) J. Biol. Chem. 273, 150–156). The R483Q mutant contained 2.6 mol of ATP/mol of V-ATPase compared with the wild-type enzyme, which contained 0.8 mol of ATP/mol of V-ATPase. These results suggest that binding of additional ATP to the noncatalytic sites may modulate the catalytic activity of the enzyme.

Vacular (H⁺)-ATPases (or V-ATPases)¹ acidify intracellular compartments in eukaryotic cells and therefore play a crucial role in a variety of cellular processes (1–7). V-ATPases in the plasma membrane of certain specialized cells also function in vacuole, an acidic organelle that plays an important role in amino acid and calcium homeostasis and also contains a variety of proteases and other degradative enzymes (6). V-ATPases are composed of two functional domains: the peripheral V₁ domain possesses the nucleotide binding sites and is responsible for ATP hydrolysis, and the integral V₀ domain carries out proton translocation across the membrane (1–7). The V₁ domain is a 570-kDa complex composed of eight different subunits (subunits A–H) of molecular mass 70–14 kDa; the V₀ domain is a 260-kDa complex containing five different subunits (subunits a, d, c, c’ and c”) with molecular masses of 100–17 kDa (1–7).

Both subunits A and B of the V₁ domain participate in nucleotide binding, and both are present in three copies per complex (12). In Saccharomyces cerevisiae, the A and B subunits are encoded by the VMA1 and VMA2 genes, respectively (13–15). Information about the nucleotide binding sites on the V-ATPase, especially that on the B subunit, is limited. The A and B subunits of the V-ATPases are related to the β and α subunits, respectively, of the F-ATPases from mitochondria, chloroplasts, and bacteria (16–21). Based on sequence homology it was proposed that all four subunits are derived from a common ancestral nucleotide-binding protein (22, 23). The x-ray structures of F₁ indicate that the nucleotide binding sites are located at the interface of the α and β subunits, with the catalytic site residing primarily on the β subunit and the noncatalytic site residing primarily on the α subunit (24, 25).

Several lines of evidence suggest that the catalytic nucleotide binding sites of the V-ATPase reside on the A subunits. These include chemical modification by sulfhydryl reagents, such as cystine (26), and nucleotide analogs, such as 2-azido-ATP (27), as well as site-directed mutagenesis of residues critical for catalytic activity or nucleotide binding (28–30). Nevertheless, the B subunit has also been suggested to participate in nucleotide binding. Thus, the B subunit in the intact complex is modified by both BzATP (31, 32) and 2-azido-ATP (27), and the purified, recombinant B subunit can be labeled on irradiation in the presence of [³²P]ATP (33). On the other hand, for the expressed, isolated B subunit of the V-ATPase from Thermos thermophilus, no evidence for binding of nucleotides was obtained (34). A region that is critical for nucleotide binding in the α and β subunits of the F-ATPases as well as the A subunit of the V-ATPases is the glycine-rich loop, or P-loop, sequence (GXXGXXGTV) (35). The x-ray crystal structures of F₁ show that this sequence wraps around the terminal phosphates of bound ATP, with the lysine in position to stabilize the negative charges of the phosphate groups (24, 25). By contrast, the B subunit does not possess a glycine-rich loop sequence, but instead contains a unique sequence that is nevertheless highly conserved among species (23, 36, 37). This suggests that the structure of the nucleotide binding site on the B subunit may differ in significant ways from that of the A, α, and β subunits.

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The abbreviations used are: V-ATPase, vacuolar proton-translocating adenosine triphosphatase; BzATP, 3'-O-(4-benzoyl)benzoyladenosine 5'-triphosphate; biotin-maleimide, 3-(N-maleimidopropionyl)biocytin; ACMA, 9-amino-6-chloro-2-methoxyacridine; AMP-PNP, adenosine 5'-(β,γ-methylene)triphosphate.
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The function of the noncatalytic nucleotide binding site on the V-ATPase is also unclear. Site-directed mutagenesis studies in yeast suggest that alterations at this site result in partial inhibition of V-ATPase activity (30, 38). In addition, nonlinear kinetics of ATP hydrolysis was observed for the V-ATPase isolated from the yeast strain containing an A subunit mutation (R483Q) at this site (30). These results have led to the suggestion that the noncatalytic nucleotide binding sites may play a role in regulating V-ATPase activity.

In the present study we wished to address the structure of the noncatalytic nucleotide binding sites using cysteine scanning mutagenesis. The ability of BzATP to protect unique cysteine residues on the B subunit from reaction with biotin-maleimide was evaluated by Western blot. In addition, we determined the effect of the R483Q mutation in the A subunit on the level of endogenous nucleotide binding by the V-ATPase. These results are discussed in terms of the structure and function of the noncatalytic nucleotide binding sites on the V-ATPase.

EXPERIMENTAL PROCEDURES

Materials and Strains—Zymolase 100T was obtained from Seikagaku America, Inc. Concanamycin A was purchased from Fluka Chemical Corp. 3-N-Maleimidopropionyl-biotin (biotin-maleimide), 9-amino-6-chloro-2-methoxyacridine (ACMA), and anti-yeast vacuolar (H^+) ATPase 60-kDa subunit mouse monoclonal 131D11 antibodies were obtained from Molecular Probes, Inc. The yeast VMA2 deleted strain, SF583–5AV1, was described previously (38).

Construction of Mutants—Various vma2 mutants were constructed using Altered Sites II in vitro mutagenesis systems (Promega) as described previously (38). The mutagenesis oligonucleotides were as follows, with the substitution sites underlined:

| Site | Sequence |
|------|----------|
| T279C | 5'-AACAAGGGTATCTGCCCACCAATCAAC-3' |
| A184C | 5'-GGCTTACAGCGAATTGGCAGAGA-3' |
| E182C | 5'-CACAACGAAATTTGTGCACAAATTTCT-3' |
| N181C | 5'-GGTTTACAGCGAATTGGCAGAGA-3' |
| P179C | 5'-ACCAGTTTCCTGATGTTATGCTGATGCT-3' |
| H365C | 5'-GGTTTACAGCGAATTGGCAGAGA-3' |
| S152C | 5'-GGTTTACAGCGAATTGGCAGAGA-3' |
| P371C | 5'-GGTTTACAGCGAATTGGCAGAGA-3' |
| C188S | 5'-GGTTTACAGCGAATTGGCAGAGA-3' |
| H365C | 5'-GGTTTACAGCGAATTGGCAGAGA-3' |
| P179C | 5'-GGTTTACAGCGAATTGGCAGAGA-3' |
| S152C | 5'-GGTTTACAGCGAATTGGCAGAGA-3' |
| P371C | 5'-GGTTTACAGCGAATTGGCAGAGA-3' |
| C188S | 5'-GGTTTACAGCGAATTGGCAGAGA-3' |

The purified V-ATPase (200 μM containing 5 μg of protein) was incubated in the presence or absence of 400 μM BzATP and 2 mM MgCl₂ for 10 min at room temperature in the dark followed by irradiation of the sample with a UV lamp (UVGL-25 Mineralight 254/366) at long wavelength at a distance of 1 cm on ice for 20 min. 100 μM biotin-maleimide was then added and reaction carried out for 10 min at room temperature. Excess reagent was quenched by the addition of 10 mM dithiothreitol and incubation for 10 min at room temperature and 20 μM at 4 °C. The V-ATPase was then immunoprecipitated with the monoclonal antibody 881-F3 against the yeast A subunit and protein A-Sepharose as described (44), followed by SDS-polyacrylamide gel electrophoresis on a 10% acrylamide gel and transfer to nitrocellulose. The blots were probed with horseradish peroxidase-conjugated Neutra-vidin, which recognizes biotin, and developed using Supersignal ULTRA chemiluminescent detection kit (Pierce).

Determinations of Enzymatically Bound Nucleotides Associated with the V-ATPase Using the Luciferin-Luciferase System—To determine endogenous nucleotides bound to the purified V-ATPase, a neutralized perchloric acid extract of the protein was prepared as described (45) with some modifications. To extract nucleotides, cold perchloric acid (66 μl of a 10% solution (w/v)) was added to 100 μl of purified V-ATPase containing 10–20 μg of protein. The mixture was incubated on ice for 10 min followed by sedimentation for 10 min at 6,000 × g. Samples of supernatant were neutralized with cold 0.1 M Tris and 10% KOH (w/v) to pH 7.0–7.5. ATP present was assayed using the luciferin-luciferase system. Aliquots of the extracted nucleotides (1–2 μl) were added to 100 μl of a mixture of luciferin-luciferase (40 mg/ml) obtained from Sigma. The emitted light was measured using a TD-20/20 Turner bioluminometer containing 200 μM ADP and 200 μM ACMA as described by Peterson (51). Immunoblot analysis of the purified V-ATPase was carried out as described previously (26). SDS-polyacrylamide gel electrophoresis was carried out as modified by Laemmli (50). Protein concentrations were determined following precipitation with trichloroacetic acid by the method of Lowry as modified by Peterson (51). Immuno blot analysis of the purified V-ATPase was carried out as described (52). Blots were developed using a chemiluminescent detection method obtained from Kirkegaard & Perry Laboratories. Quantitation was carried out using an IS-1000 digital imaging system (Alpha Innotech Corporation).

RESULTS

Construction of vma2 Mutants Containing Unique Cysteine Residues—To study the structure of the nucleotide binding site of the B subunit of the V-ATPase we used a cysteine scanning approach. The B subunit of the yeast V-ATPase contains a single cysteine residue at position 188 (15). Replacement of this cysteine with serine gave a V-ATPase with near wild-type levels of activity (Fig. 1). Single cysteine residues were then introduced into the Cys-less form of Vma2p by site-directed mutagenesis. The mutant vma2 ΔDNA2s were subcloned into the yeast shuttle vector pRS316 and expressed in a vma2Δ strain in which the VMA2 gene was deleted. Thus, in each double mutant Cys¹⁸⁸ has been converted to serine and a unique cysteine has been introduced at another position. Among the residues mutated to cysteine were those near the site of modification by BzATP (32) (Ile¹⁴⁵, Ser¹⁵²), amino acid residues within the sequence corresponding to the P-loop (Ser¹⁷⁶-Leu¹⁷⁸-Pro¹⁷⁹-His³⁸⁰-Asn¹⁸¹-Glu¹⁸²-Ala¹⁸⁴) (38), and residues postulated to be at the nucleotide binding site based on the F-ATPase α subunit structure (24, 25) and sequence alignment with the ACMA-dependent fluorescence quenching of ACMA as described previously (53). ATP-dependent fluorescence quenching of ACMA was carried out as described by Laemmli (50). Protein concentrations were determined following precipitation with trichloroacetic acid by the method of Lowry as modified by Peterson (51). Immunoblot analysis of the purified V-ATPase was carried out as described (52). Blots were developed using a chemiluminescent detection method obtained from Kirkegaard & Perry Laboratories. Quantitation was carried out using an IS-1000 digital imaging system (Alpha Innotech Corporation).
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alignment of the α subunit with the B subunit of the V-ATPase (Ser283-Thr279-Thr336-His365-Tyr370-Tyr371-Ala448). Effect of vma2 Mutations on V-ATPase Activity and Proton Transport—Fig. 1A shows the V-ATPase activity and proton transport in vacuoles isolated from the wild-type strain, the C188S (Cys-less) mutant, and each of the double mutants containing unique cysteine residues. All of the vacuoles isolated from the double mutants had activities ranging from 24 to 100% of wild-type levels of activity with the exception of the P371C mutant, which was almost totally inactive. Effects on proton transport generally paralleled effects on ATPase activity, although for some mutants, such as H180C and A448C, a lower proton transport than ATPase activity suggests the possibility of a partial uncoupling. Western blot analysis of whole cell lysates and isolated vacuoles using the monoclonal antibody 13D11 against the yeast B subunit (14) indicated that all of the mutants tested had B subunit levels comparable to the wild-type strain with the exception of P371C, which showed greatly reduced levels of the B subunit in both whole cell lysates and isolated vacuoles (data not shown) and which was not analyzed further.

As a further test of the effects of the mutations on V-ATPase function, the V-ATPase was purified from each mutant by detergent solubilization of isolated vacuoles and separation by glycerol density gradient sedimentation (43). The activities of the purified enzymes isolated from the double mutants (shown in Fig. 1B) range from 35 to 95% of wild-type activity (which had a specific activity of 2.5 μmol of ATP/min/mg of protein). These activities generally paralleled those measured in isolated vacuoles and indicated that assembly of the mutant enzymes was normal. Despite the reduced activity of some of the mutant enzymes, all of the mutants (with the exception of P371C) were used in the subsequent analysis because assembly was normal, and some effects on activity might be expected for mutations occurring at the nucleotide binding sites of the enzyme.

As a further test of the effects of the mutations on V-ATPase function, the V-ATPase was purified from each mutant by detergent solubilization of isolated vacuoles and separation by glycerol density gradient sedimentation (43). The activities of the purified enzymes isolated from the double mutants (shown in Fig. 1B) range from 35 to 95% of wild-type activity (which had a specific activity of 2.5 μmol of ATP/min/mg of protein). These activities generally paralleled those measured in isolated vacuoles and indicated that assembly of the mutant enzymes was normal. Despite the reduced activity of some of the mutant enzymes, all of the mutants (with the exception of P371C) were used in the subsequent analysis because assembly was normal, and some effects on activity might be expected for mutations occurring at the nucleotide binding sites of the enzyme.

Labeling of vma2 Mutants by Biotin-maleimide and Protection by Nucleotides—To identify residues present at the nucleotide binding site on the B subunit, biotin-maleimide was used as a probe to label the single cysteine residues in the B subunit in the presence or absence of nucleotides. Preliminary experiments indicated that both the A subunit and the B subunit were labeled by biotin-maleimide and that although both MgATP and MgADP protected the A subunit from labeling, only very weak protection of the B subunit was observed (data not shown). Nucleotide protection of A subunit labeling by biotin-maleimide was expected on the basis of the previous identification of a highly reactive cysteine residue at the catalytic site whose reaction with sulphydryl reagents is blocked by ATP (26).

The weak protection of B subunit labeling by ATP and ADP suggested that the nucleotide affinity of the noncatalytic sites might not be sufficiently high to offer effective protection of the site from a covalent modification. Alternatively, the noncatalytic sites might already be occupied with endogenous nucleotide and hence not show any difference in labeling in the presence or absence of added nucleotide. To address this latter possibility, we measured the level of endogenous nucleotide bound to the wild-type enzyme using the luciferin-luciferase method. We observed approximately 0.8 (±0.1) mol of ATP bound/mol of V-ATPase, in good agreement with our previous measurement of 0.72 mol of ATP/mol of V-ATPase measured for the enzyme isolated from clathrin-coated vesicles (32). Incubation with an ATP-regenerating system indicated that all of the bound nucleotide was ATP rather than ADP. The fact that the endogenous nucleotide was ATP rather than ADP, despite the presence of Mg2+, suggests that the nucleotide is bound to a noncatalytic site. Because there are three copies of the B subunit/V-ATPase complex (12), these results suggest that at least two of the noncatalytic nucleotide binding sites must be unoccupied on the isolated enzyme.

To probe further the proximity of the introduced cysteine residues to the nucleotide binding site on the B subunit, we employed modification of the V-ATPase by BzATP. We have
shown previously that BzATP is able to modify the B subunit of the V-ATPase from clathrin-coated vesicles and that, based on the effects of sulphydryl reagents on BzATP modification, the site modified corresponds to a noncatalytic site (32). For the present experiments, the purified V-ATPase was first irradiated with UV light in the absence or presence of 400 μM BzATP, and 2 mM MgCl₂ as described under "Experimental Procedures." The samples were then reacted with 100 μM biotin-maleimide for 10 min at room temperature followed by quenching of the excess reagent and immunoprecipitation of the V-ATPase using the monoclonal antibody 8B1-F3 directed against the A subunit as described. The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis on a 10% acrylamide gel, transferred to nitrocellulose, and probed with horseradish peroxidase-conjugated NeutrAvidin. The blot was then developed using a chemiluminescent substrate. For the V-ATPase isolated from the Cys-less mutant, 6 μg of protein was employed, whereas for the E182C mutant 1.5 μg of protein was used.

DISCUSSION

Several lines of evidence suggest that the B subunit of the V-ATPase participates in nucleotide binding. First, there is approximately 25% amino acid identity between the V-ATPase B subunit and the F-ATPase α subunit, which can be seen to form the noncatalytic nucleotide binding sites in the x-ray crystal structures of Fₐ (24, 25). The B subunit in the intact V-ATPase complex is also labeled by both 2-azido-ATP (27) and BzATP (32), and the isolated, recombinant B subunit expressed in Escherichia coli is labeled on irradiation in the presence of [¹²⁵I]ATP (33). This latter result suggests that the B subunit is actually able to form a nucleotide binding site itself rather than...
FIG. 3. Model of the noncatalytic nucleotide binding site of the yeast V-ATPase. Shown is an energy-minimized model of the structure of the noncatalytic nucleotide binding site on the yeast V-ATPase B subunit based upon the x-ray crystal structure of the bovine mitochondrial F$_1$-ATPase (24) and sequence alignment of the B and a subunits (see "Experimental Procedures"). Highlighted are the B subunit residues mutated in the current study and the bound AMP-PNP molecule. The bound Mg$^{2+}$ is shown as a lightly shaded sphere and a bound water molecule as a darkly shaded sphere. The A subunit residues contributing to the noncatalytic site are not highlighted for simplicity. Also not shown are two B subunit residues (Ile$^{145}$ and Ser$^{155}$), which are predicted by the model to be too far from the noncatalytic nucleotide binding site to be shown in this view.

The structure of the nucleotide binding site on the B subunit is more uncertain. This is particularly true given the absence of a consensus P-loop region in the B subunit sequence. The sequence that replaces the P-loop in the B subunit (Ser$^{174}$-Ala-Ser-Gly-Leu-Pro-His-Asn-Glu$^{182}$) is nevertheless perfectly conserved among species (15, 23, 36, 37) and is rich in amino acids (Gly, Asn, Ser, Pro, Asp, and Arg) that are frequently found in random coil conformations or in turns (53). This suggests that the B subunit sequence may allow for a degree of conformational flexibility comparable to the glycine-rich loop sequence. The site of modification of the B subunit by BzATP has also been localized to an eight-amino acid stretch of the bovine V-ATPase B subunit beginning at Ile$^{164}$ and ending at Gln$^{171}$ (32), corresponding to Ile$^{145}$ to Ser$^{152}$ in the yeast B subunit sequence. This is just upstream of the sequence corresponding to the P-loop discussed above.

It is also possible to make predictions about B subunit residues located at the noncatalytic nucleotide binding sites of the V-ATPase based upon sequence alignment of the B and a subunits and the available x-ray crystal structures of mitochondrial F$_1$ (24, 25). These B subunit residues include Thr$^{279}$ (corresponding to mitochondrial a Asp$^{268}$), Ser$^{283}$ (a Lys$^{273}$), Thr$^{336}$ (a Glu$^{328}$), His$^{365}$ (a Phe$^{357}$), Tyr$^{370}$ (a Arg$^{362}$), Pro$^{371}$ (a Pro$^{363}$), and Ala$^{448}$ (a Gln$^{432}$). A model for the noncatalytic site on the B subunit, Fig. 3, was constructed on the basis of sequence alignment of the F and V-ATPase subunits, the coordinates of the bovine mitochondrial F$_1$ (24), and energy minimization. The A subunit is also predicted to contribute residues to the noncatalytic nucleotide binding sites, including Phe$^{373}$ (corresponding to mitochondrial b Phe$^{357}$) and Arg$^{381}$ (b Tyr$^{368}$) (not labeled). An important question is whether these residues actually contribute to the nucleotide binding site on the B subunit.

The effect of mutations at several of these sites has been evaluated, and it is of interest to compare the results with the effect of mutations in the corresponding residues of the a subunit. Replacement of the B subunit residues His$^{180}$ with Gly or Asp or Asn$^{181}$ with Val led to the loss of 30–50% of V-ATPase activity (38), whereas mutation of the corresponding P-loop residues in the a subunit (Lys$^{175}$ to Gly or Thr$^{176}$ to Leu or Val) caused a more dramatic (70–90%) reduction in activity (54). Substitution of Phe or Trp for Lys$^{175}$ actually led to defective assembly of the F-ATPase, leading to the suggestion that the noncatalytic sites play a role in assembly of the complex (54).

Mutation of Tyr$^{370}$ postulated to be present in the adenine binding pocket of the B subunit to Arg, Phe, or Ser also led to modest (20–40%) reductions in activity (38), similar to those observed for replacement of the a subunit residue Arg$^{362}$ by Tyr or Phe (55). These results, of course, do not provide direct evidence that the B subunit residues in question are located at the nucleotide binding site.

To provide more definite information concerning the identity of the residues present at the noncatalytic sites, we have employed a cysteine scanning approach. By replacing the single endogenous cysteine residue in the B subunit and introducing unique cysteine residues at different positions in the protein, it is possible to probe the ability of these sites to be protected by nucleotide. Cysteine residues at the nucleotide binding site would be predicted to be protected from reaction with sulphydral reagents by the presence of nucleotides. In this study, we have employed BzATP as the protecting nucleotide because it provides a stable occupancy of the site and shows the largest protection effect.

Of the 16 cysteine residues introduced into the B subunit, many showed little if any reaction with biotin-maleimide. This includes almost all of the residues postulated to contribute to the adenine binding pocket of the noncatalytic site. This suggests that these residues are simply not accessible to the labeling reagent, which may be too large to reach the residues buried within the site effectively. Six cysteine residues (S152C, L178C, N181C, A184C, Cys$^{188}$, and T279C) showed clear protection effect. Of the 16 cysteine residues introduced into the B subunit, many showed little if any reaction with biotin-maleimide. This includes almost all of the residues postulated to contribute to the adenine binding pocket of the noncatalytic site. This suggests that these residues are simply not accessible to the labeling reagent, which may be too large to reach the residues buried within the site effectively. Six cysteine residues (S152C, L178C, N181C, A184C, Cys$^{188}$, and T279C) showed clear protection from biotin-maleimide labeling following treatment with BzATP. In addition, one cysteine residue (P179C) showed a marked increase in biotin-maleimide labeling with BzATP. This residue also showed enhanced labeling on addition of ATP (data not shown). These results suggest that these residues are likely to be in close proximity to the nucleotide binding site of the B subunit, with the cysteine at position 179 made more accessible (or reactive) as a result of nucleotide binding. Although it cannot be ruled out that these residues are altered in their reactivity toward biotin-maleimide as a result of a nucleotide-induced conformational change in the protein, they suggest that both the region corresponding to the P-loop and the sequence surrounding Thr$^{279}$ form part of the nucleotide binding site on the B subunit.

All of the residues that show a change in reactivity toward biotin-maleimide in the presence of BzATP are predicted from the energy-minimized model (Fig. 3) to be in close proximity to the noncatalytic nucleotide binding site, with the exception of...
Ser<sup>152</sup>. Molecular modeling places Ser<sup>152</sup> approximately 22 Å from the nucleotide bound at this site. This is clearly too far for BzATP to exert a direct steric effect on labeling. On the other hand, Ser<sup>152</sup> is within the octapeptide that is labeled by BzATP (32), and no residues within that peptide are predicted to be closer than about 20 Å to the noncatalytic (or catalytic) site. This suggests that the actual structure of the B subunit may differ somewhat from that predicted from the modeling studies. One possible reason for this difference may be the presence of additional V-ATPase subunits that interact with the A and B subunits in the intact complex but have not been included in the molecular modeling analysis.

Mutations have also been constructed in A subunit residues postulated to be contributed to the noncatalytic site (30). Although these mutations generally have similar modest effects on activity, two mutations (R483Q and R483E) display a marked nonlinearity in the rate of ATP hydrolysis, with an initial lag phase followed by a gradual increase in hydrolysis with time (30). One possible explanation for this result is the loss of an endogenous nucleotide bound at the noncatalytic site which is only gradually refilled upon addition of the enzyme to the assay mixture. This model predicts that the level of endogenous nucleotide bound to the mutant would be reduced relative to the wild-type enzyme. To test this model, we measured the nucleotide bound to the wild-type and mutant forms of the enzyme (2.6 mol of ATP/mol of V-ATPase) than the wild-type enzyme. To test this model, we measured the nucleotide bound to the wild-type and mutant forms of the enzyme and found that the R483Q mutant actually bound more nucleotide (2.6 mol of ATP/mol of V-ATPase) than the wild-type enzyme (0.8 mol of ATP/mol of V-ATPase). These results rule out the positive regulatory role discussed above and instead suggest that nucleotide binding to the noncatalytic sites may have an inhibitory effect on turnover or ATP binding to the catalytic site. Alternatively, the additional nucleotides may be bound at the catalytic sites due to impaired cooperativity. Analysis of additional mutations at the noncatalytic sites and their effects on nucleotide binding will be required to resolve this question.

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