3'-O-(4-Benzoyl)benzoyladenosine 5'-Triphosphate Inhibits Activity of the Vacuolar (H⁺)-ATPase from Bovine Brain Clathrin-coated Vesicles by Modification of a Rapidly Exchangeable, Noncatalytic Nucleotide Binding Site on the B Subunit*

(Received for publication, January 22, 1996, and in revised form, March 17, 1996)

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It was previously observed that the B subunit of the tonoplast V-ATPase is modified by the photoactivated nucleotide analog 3′-O-(4-benzoyl)benzoyladenosine 5′-triphosphate (BzATP) (Manolson, M. F., Rea, P. A., and Poole, R. J. (1985) J. Biol. Chem. 260, 12273–12279). We have further characterized the nucleotide binding sites on the V-ATPase and the interaction between BzATP and the B subunit. We observe that the V-ATPase isolated from bovine clathrin-coated vesicles possesses approximately 1 mol of endogenous, tightly bound ATP/mol of V-ATPase complex. BzATP is not a substrate for the V-ATPase, but does act as a noncovalent inhibitor in the absence of irradiation, changing the kinetic characteristics of ATP hydrolysis. Irradiation of the V-ATPase in the presence of [3H]BzATP results primarily in modification of the 58-kDa B subunit, with complete inhibition of V-ATPase activity occurring upon modification of one B subunit per V-ATPase complex. Inhibition occurs as the result of modification of a rapidly (t1/2 < 2 min) exchangeable site, and yet this site does not correspond to a catalytic site, as indicated by the effects of cysteine-modifying reagents which react with Cys254 located at the catalytic sites on the A subunit. Thus, the noncatalytic nucleotide binding site modified by BzATP appears to be rapidly exchangeable. The site of [3H]BzATP modification of the B subunit was localized to the region Ile264 to Gln275, which from the x-ray crystal structure of the homologous F-ATPase subunit is within 10 Å of the ribose ring of ATP bound to the noncatalytic nucleotide binding site. Thus, despite the absence of a glycine-rich loop region in the B subunit, these data are consistent with a similar overall folding pattern for the V-ATPase B subunit and the F-ATPase α subunit.

The vacuolar H⁺-ATPases (or V-ATPases)1 acidify intracellular compartments in eukaryotic cells and play an important role in a variety of cellular processes, including receptor-mediated endocytosis, intracellular membrane traffic, protein processing and degradation, and coupled transport of small molecules (for reviews, see Refs. 1–9). V-ATPases in the plasma membrane of certain specialized cells also function in such processes as renal acidification (5), cytoplasmic neutralization (10), bone resorption (11), and tumor metastasis (12).

V-ATPases are composed of two functional domains (1–9). The peripheral V₃ domain, which for the V-ATPase from bovine coated vesicles contains subunits of molecular mass 73 (subunit A), 58 (subunit B), 40 (subunit C), 34 (subunit D), and 33 (subunit E) kDa (13), is a 500-kDa complex that has the structure A3B3CDE (14) and possesses all of the nucleotide binding sites on the V-ATPase complex. The integral V₅ domain, which contains subunits of molecular mass 100, 38, 19, and 17 (subunit c) kDa, is a 250-kDa complex that has the structure 100:38:19:17 (14) and is responsible for proton translocation across the membrane.

Although still somewhat limited, information has begun to emerge concerning the nucleotide binding sites on the V-ATPases. Sequence homology (15–22) indicates that the A and B subunits of the V-ATPases are related to the α and β subunits of the F-ATPases, which function in ATP synthesis in mitochondria, chloroplast, and bacteria (23–27). These proteins are thus all derived from a common, ancestral nucleotide-binding protein. Covalent modification by ATP-protectable inhibitors suggests that the A subunit possesses the catalytic nucleotide binding sites (for references, see Ref. 1), although the B subunit has also been shown to participate in nucleotide binding (28, 29).

To discuss the nucleotide binding sites of the V-ATPase in more detail, it is first necessary to review what is known concerning these sites on the F-ATPases. The F-ATPases possess six nucleotide binding sites on the F₃ domain, three on the β subunits, and three on the α subunits (23–27). Chemical modification and mutagenesis studies indicate that the catalytic sites are located on the β subunits, while the noncatalytic sites are located on the α subunits. The catalytic sites show rapid nucleotide exchange while the noncatalytic sites exchange nucleotides only very slowly. The recent x-ray crystal structure of the bovine heart mitochondrial F₃ shows the nucleotide binding sites at the interface of the α and β subunits, with the three catalytic sites located primarily on the β subunits and the three noncatalytic nucleotide binding sites located primarily on the α subunits (30). The function of the noncatalytic sites remains somewhat uncertain, although results from several laboratories suggest that they may play a role in regulation (31, 32) or assembly process (33, 34) of the F-ATPase complex. Other studies, however, suggest that the noncatalytic nucleotide binding sites do not need to be occupied in order for catalysis to occur (35).

Several studies have focused on the nucleotide binding sites of the V-ATPases. Covalent modification and peptide sequenc-
ing have identified Cys254, located in the glycine-rich loop region of the A subunit, as the residue responsible for sensitivity of the V-ATPases to sulfhydryl reagents (36). The F-ATPase crystal structure indicates that the glycine-rich loop is in close proximity to the triphosphates of ATP (30). Moreover, Cys254 was shown by disulfide bond formation to be within 5–6 Å of Cys532 located in the C-terminal domain of the same A subunit (37). Because the corresponding positions in the F-ATPase—V-ATPase—

V-ATPase—
tation with trichloroacetic acid as described (39). ATPase activity was typically 7–8 times greater than that of the F-ATPase activity previously (13). The specific activity of the purified V-ATPase was measured and isolated by glycerol density gradient sedimentation as described previously (13).

A similar overall folding pattern for the V-ATPase A and F-ATPase subunits are also separated by 5–6 Å (30), these results suggest a similar overall folding pattern for the F-ATPase A and F-ATPase B subunits. Labeling studies employing 2-azido-ATP support this suggestion (29).

Less is currently known concerning the nature and function of the nucleotide binding sites located on the V-ATPase A subunit. The B subunit does not possess a region homologous to the glycine-rich loop (16, 18, 19, 22), suggesting that the nucleotide binding site located on the B subunit is noncatalytic. Site-directed mutagenesis of the yeast V-ATPase B subunit (38) indicated that partial (20–50%) losses in activity resulted from modification of residues in either the region corresponding to the glycine-rich loop or the adenine binding pocket. By contrast, nearly complete loss of activity was observed upon mutation of residues postulated to be contributed by the B subunit to the catalytic nucleotide binding sites on the A subunit.

Previous studies have demonstrated that the tonoplast B subunit is covalently modified by 3-O-(4-benzoyl)benzoyl adenosine 5′-triphosphate (BzATP) (28). In the current study we have further characterized the nucleotide binding sites on the V-ATPase and the nature and function of the nucleotide binding site on the B subunit that interacts with BzATP.

EXPERIMENTAL PROCEDURES

Materials—Calf brains were obtained from a local slaughterhouse. C2H5OH, trypsin, V8 protease, activated charcoal, and ATP were purchased from Sigma. [3H]BzATP was synthesized from [3-3H]4-benzoylbenzoic acid (15.0 Ci/mmol) as described (42). [γ-32P]ATP was synthesized from [γ-32P]ATP (25 Ci/mmol) (42). Labeling of the V-ATPase for peptide isolation and sequencing was carried out using [3H]BzATP prepared from [3-3H]4-benzoylbenzoic acid. Since the radioactivity is on the phenol moiety of the peptide due to cleavage of the 3′-ether linkage between the benzophenone moiety and the ribose ring of ATP.

Hydrolysis of [γ-32P]BzATP—Hydrolysis of [γ-32P]BzATP and [γ-32P]ATP were determined by a modified inorganic 32P, release method (43). The specific activity of [γ-32P]ATP was 1,000 cpm/nmol and [γ-32P]BzATP was 860 cpm/nmol. The hydrolysis incubation mixture contained 0.1 mg purified V-ATPase, 1 mM [γ-32P]ATP or [γ-32P]BzATP, 2 mM MgSO4, 0.05% C12E9, 20 μg of phosphatidylcholine/ml, and 20 μg of phosphatidylserine/ml. As a control, samples without protein were used. After 10-min incubation at 37 °C, the samples were then analyzed by trichloroacetic acid precipitation and counted as described previously (36). The eluted protein was dialyzed using 25-kDa molecular weight cutoff tubing against 150 mM NaCl and 0.02% SDS for 2 days at 4°C and then against 0.02% SDS for 1 day at 4°C to remove excess SDS from the peptide due to cleavage of the 3′-ether bond.

Modification of the V-ATPase A Subunit Cysteine 254 by Cystine—Cystine-modified V-ATPase was prepared as described (37), with some modifications. Stripped vesicles were suspended in 1 mM EDTA, 10% glycerol, and then against 0.02% SDS for 1 day at 4°C to remove excess SDS. The cystine modified V-ATPase was then solubilized and treated with activated charcoal suspended in 10 mM Na2PO4, followed by removal of the activated charcoal by sedimentation for 1 min at 10,000 × g. An aliquot of supernatant was subjected to liquid scintillation counting with correction for 32P, present in controls lacking the V-ATPase.

Labeling of the V-ATPase by [3H]BzATP—Labeling of the V-ATPase A subunit in the continuous presence of [3H]BzATP was carried out as follows. Purified V-ATPase (10–100 g of protein) was added to a mixture of luciferin-luciferase (44). The gel was then dried and autoradiography performed. For some experiments an IS-1000 Digital Imaging System (Alpha Innotech Corp.) was used to quantitate label incorporation.

Modification of the V-ATPase A Subunit Cysteine 254 by BzATP—The V-ATPase A subunit was covalently modified with BzATP (28). Stripped vesicles were suspended in 1 mM EDTA, 10% glycerol, and then against 0.02% SDS for 1 day at 4°C to remove excess SDS. The cystine-modified V-ATPase was then solubilized and treated with activated charcoal suspended in 10 mM Na2PO4, followed by removal of the activated charcoal by sedimentation for 1 min at 10,000 × g. An aliquot of supernatant was subjected to liquid scintillation counting with correction for 32P, present in controls lacking the V-ATPase.

Labeling of the V-ATPase B Subunit—Labeling of the V-ATPase B subunit was carried out as follows. Purified V-ATPase (10–100 g of protein) was added to a mixture of luciferin-luciferase (44). The gel was then dried and autoradiography performed. For some experiments an IS-1000 Digital Imaging System (Alpha Innotech Corp.) was used to quantitate label incorporation.

Modification of the V-ATPase B Subunit Cysteine 254 by Cystine—Cystine-modified V-ATPase was prepared as described (37), with some modifications. Stripped vesicles were suspended in 1 mM EDTA, 10% glycerol, and then against 0.02% SDS for 1 day at 4°C to remove excess SDS. The cystine-modified V-ATPase was then solubilized and treated with activated charcoal suspended in 10 mM Na2PO4, followed by removal of the activated charcoal by sedimentation for 1 min at 10,000 × g. An aliquot of supernatant was subjected to liquid scintillation counting with correction for 32P, present in controls lacking the V-ATPase.

Labeling of the V-ATPase by [3H]BzATP—Labeling of the V-ATPase B subunit in the continuous presence of [3H]BzATP was carried out as follows. Purified V-ATPase (10–100 g of protein) was added to a mixture of luciferin-luciferase (44). The gel was then dried and autoradiography performed. For some experiments an IS-1000 Digital Imaging System (Alpha Innotech Corp.) was used to quantitate label incorporation.

Modification of the V-ATPase B Subunit Cysteine 254 by BzATP—The V-ATPase B subunit was covalently modified with BzATP (28). Stripped vesicles were suspended in 1 mM EDTA, 10% glycerol, and then against 0.02% SDS for 1 day at 4°C to remove excess SDS. The cystine-modified V-ATPase was then solubilized and treated with activated charcoal suspended in 10 mM Na2PO4, followed by removal of the activated charcoal by sedimentation for 1 min at 10,000 × g. An aliquot of supernatant was subjected to liquid scintillation counting with correction for 32P, present in controls lacking the V-ATPase.

Labeling of the V-ATPase by [3H]BzATP—Labeling of the V-ATPase B subunit in the continuous presence of [3H]BzATP was carried out as follows. Purified V-ATPase (10–100 g of protein) was added to a mixture of luciferin-luciferase (44). The gel was then dried and autoradiography performed. For some experiments an IS-1000 Digital Imaging System (Alpha Innotech Corp.) was used to quantitate label incorporation. The cystine-modified V-ATPase was then solubilized and treated with activated charcoal suspended in 10 mM Na2PO4, followed by removal of the activated charcoal by sedimentation for 1 min at 10,000 × g. An aliquot of supernatant was subjected to liquid scintillation counting with correction for 32P, present in controls lacking the V-ATPase.

Labeling of the V-ATPase B subunit was carried out as follows. Purified V-ATPase (10–100 g of protein) was added to a mixture of luciferin-luciferase (44). The gel was then dried and autoradiography performed. For some experiments an IS-1000 Digital Imaging System (Alpha Innotech Corp.) was used to quantitate label incorporation.
and salt. The samples were lyophilized to decrease the volume to 2 ml, and KCl was added to a final concentration 100 mM to precipitate the remaining SDS. To half of the sample (1 ml), EDTA and KH₂PO₄ (pH 7.8) were added to final concentrations of 2 and 50 mM, respectively. V₈ protease was then added to give a protease:protein ratio of 1:40, estimating the protein concentration from the absorbance at 210 nm relative to bovine serum albumin standards. To the remaining 1 ml was added CaCl₂ and NH₄HCO₃ (pH 7.3) to final concentrations of 10 and 100 mM, respectively. Trypsin was then added at a 1:40 ratio to protein. The samples were incubated at room temperature with rotation for 2–3 days, with a second, equal aliquot of protease added on day 2. The samples were then dialyzed against water for 2 days using 1-kD cutoff tubing to remove salt. The sample was then concentrated to less than 100 µl by lyophilization and separated by SDS-PAGE using a 20% acrylamide gel modified as follows for high salt samples. The separating gel contained 16 ml of 30% acrylamide, 0.8% bisacrylamide, 6 ml of 3 M Tris-HCl (pH 8.8), 240 µl of 10% SDS, 240 µl of 0.2 M EDTA, 12 µl of TEMED, 1.4 ml of H₂O, and 120 µl of 20% ammonium persulfate. The stacking gel contained 1.5 ml of 30% acrylamide, 0.8% bisacrylamide, 1.12 ml of 0.5 M Tris (pH 6.8), 90 µl of 10% SDS, 4.2 µl of TEMED, 6.15 ml of H₂O, 45 µl of 20% ammonium persulfate. The running buffer contained 6 g of Tris, 14.4 g of glycine, and 1 g of SDS in water to 1 liter. After electrophoresis, a portion of the gel was sliced, and the position of the [³²P]labeled peptide was identified using the same procedure as described above. The remaining, uncut portion of the gel was transferred to Immobilon (Millipore) at 4°C by electrophoresis at 70 V for 1 h. The portion of the Immobilon corresponding to the peak of radioactivity was cut out and subjected to amino acid sequence analysis using an Applied Biosystems gas-phase sequencer.

RESULTS

Determination of Endogenous Nucleotides Bound to the V-ATPase—To determine the level of endogenous nucleotides bound to the V-ATPase, the purified enzyme was extracted with HClO₄ and the extracted nucleotides quantitated using the luciferin-luciferase system as described under “Experimental Procedures.” We observed 0.72 ± 0.11 mol of ATP/mol of V-ATPase complex. This value assumes that the V-ATPase is 100% pure as isolated and may therefore be closer to a stoichiometry of 1:1 given the presence of low levels of contaminating proteins in the preparation. To determine whether any significant amount of endogenous ADP is also present, the samples were preincubated with an ATP-regenerating system and the amount of inhibition observed at each time point relative to the inhibition observed somewhat complicated.

Photolabeling of the V-ATPase by [³²P]BzATP—Fig. 2 shows the result of UV irradiation of the purified coated vesicle V-ATPase in the presence of [³²P]BzATP. [³²P]BzATP labels mainly the 58-kDa B subunit, with some label associated with the 73-kDa A subunit, the 100-kDa subunit, the 33-kDa subunit, and a 50-kDa polypeptide, which corresponds to the 50-kDa subunit of the AP-2 adaptor complex (46, 47). Quantiﬁcation of incorporation using an Alpha Innotech Digital Imaging System indicated that labeling of the B subunit was at least seven to eight times greater than that of any other polypeptide in the preparation. [³²P]BzATP labeling of the B subunit was dependent upon Mg²⁺ (Fig. 2A) and was partially prevented by ATP (Fig. 2B).

Fig. 2C shows the effect of irradiation of the V-ATPase in the presence of BzATP on ATPase activity. As can be seen, UV irradiation alone caused only slight inhibition of activity, and this was prevented in the presence of ATP. UV irradiation in the presence of BzATP, on the other hand, caused signiﬁcantly greater inhibition. As with labeling by [³²P]BzATP, this inhibition was concentration-dependent, dependent upon Mg²⁺, and was partially protected by ATP. The concentration dependence of labeling and inhibition are more thoroughly documented in Fig. 3, with 50% inhibition observed upon irradiation of the V-ATPase for 20 min in the presence of 70 µM BzATP.

Time Course of Labeling and Inhibition of the V-ATPase by [³²P]BzATP—The time course of labeling and inhibition of the V-ATPase with 100 µM [³²P]BzATP was determined and a t½ for inactivation of 8–10 min was observed (data not shown). Fig. 4A shows the quantitation of the amount of radioactivity incorporated into the A, B, and 33-kDa subunits as a function of time using the Alpha Innotech Digital Imaging System. To facilitate comparison of the curves, all values shown in Fig. 4A have been normalized to 100% at 20 min. Also shown in Fig. 4A is the amount of inhibition observed at each time point relative to the inhibition observed at 20 min (defined as 100%). The actual
inhibition observed at 20 min in the presence of 100 μM of [3H]BzATP was 65% relative to the activity of the nonirradiated sample. A scan can be seen from Fig. 4A, inhibition of activity does not correlate with incorporation of [3H]BzATP into the 33-kDa subunit. Although incorporation into both the A and B subunits correlates with inhibition, the much lower incorporation observed with the A subunit makes incorporation into the B subunit the most likely cause of inhibition.

To further evaluate this relationship, the data were replotted as shown in Fig. 4B. As can be seen, complete inhibition is predicted to occur at approximately 1.3 mol of [3H]BzATP incorporated into the B subunit/mol of V-ATPase complex. Because incorporation into the A subunit is 7–8-fold lower, there is insufficient A subunit labeling to account for the observed inhibition of V-ATPase activity. Moreover, the results indicate that modification of only one of the three copies of the B subunit per V-ATPase complex is sufficient to completely inhibit activity.

Rapidly and Slowly Exchangeable Nucleotide Binding Sites—It was shown recently that the V-ATPase possesses both rapidly (t1/2, 2 min) and slowly (t1/2, 2 min) exchangeable nucleotide binding sites (29). To determine which type of nucleotide binding site was responsible for [3H]BzATP inhibition, the following experiments were carried out. The purified V-ATPase was first incubated with [3H]BzATP in the presence of Mg2+, followed by removal of unbound nucleotide using a Sephadex spin column. The sample was then incubated with ATP and Mg2+ for 2 min to remove label from rapidly exchangeable sites, unbound nucleotides were again removed by gel filtration, and the V-ATPase was irradiated followed by assay of ATPase activity or SDS-PAGE and autoradiography. The enzyme should thus be labeled by [3H]BzATP only at slowly exchangeable sites under these conditions. To label the V-ATPase at rapidly exchangeable sites, the same experiment is carried out, except that ATP is employed during the first incubation and [3H]BzATP is used in the second incubation. As can be seen from Fig. 5, significant incorporation of [3H]BzATP was observed only under conditions designed to label rapidly exchangeable sites (lane 2). Moreover, the inhibition observed following modification of rapidly exchangeable sites was 45% as compared with only 8% upon modification of slowly exchangeable sites. Thus, BzATP appears to inhibit the V-ATPase principally through modification of a rapidly exchangeable site.

Role of the Catalytic and Noncatalytic Sites in [3H]BzATP Binding to the V-ATPase—Previous studies (38, 29) have suggested that, by analogy with the α subunit of the F-ATPase, the...
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Fig. 5. Labeling of the V-ATPase by [3H]BzATP at rapidly and slowly exchangeable sites. To label the V-ATPase at slowly exchangeable sites, 0.2 mg of purified V-ATPase was first incubated with 100 μM [3H]BzATP and 1 mM MgSO4 in a final volume 200 μl for 1 h at 4°C. Unbound [3H]ATP was removed using a Sephadex G-25 centrifuge column as described in the legend to Fig. 4. The sample was then incubated with 0.5 mM ATP and 1 mM MgSO4 for 2 min. Unbound nucleotides were removed using a second centrifuge column under the same conditions, and the V-ATPase was irradiated followed by assay of ATPase activity (2 μl aliquot) or SDS-PAGE on a 12.5% acrylamide gel and autoradiography (80 μl aliquot) (lane 1). To label the V-ATPase at rapidly exchangeable sites, 0.2 mg of purified V-ATPase was incubated with 0.5 mM ATP and 1 mM MgSO4 for 1 h, followed by removal of unbound nucleotide and incubation with 100 μM [3H]BzATP and 1 mM MgSO4 for 2 min. Following a second spin column to remove unbound nucleotide, the V-ATPase was photolyzed and analyzed as described above (lane 2). ATPase activity after labeling of slowly exchangeable sites was 78%, while activity after labeling of rapidly exchangeable sites was 41% relative to control samples incubated in the absence of nucleotides and without irradiation, but which had been passed through the same gel filtration columns used to remove unbound nucleotides without interfering with nucleotide binding (36, 37, 48). We thus used selective modification of Cys254 with NEM or cystine to determine whether inhibition and labeling by [3H]BzATP resulted from modification of catalytic or noncatalytic nucleotide binding sites on the V-ATPase. As shown in Fig. 6A, modification of Cys254 with NEM did not significantly reduce [3H]BzATP labeling of the B subunit, indicating that [3H]BzATP was not modifying catalytic nucleotide binding sites. We have previously demonstrated that NEM quantitatively modifies the catalytic nucleotide binding sites on the A subunit (36). Interestingly, reduction of the oxidized enzyme (in which a disulfide bond is formed between Cys254 and Cys532 of the A subunit) results in a significant increase in labeling of the B subunit (Fig. 6B). This same increase in labeling is observed upon reduction of cystine inactivated enzyme (Fig. 6C). These results suggest that the noncatalytic site on the B subunit is sensitive to changes that occur in the oxidation state of cysteine residues located at the catalytic site on the A subunit. It is also interesting to note in Fig. 6A that NEM modification actually increased [3H]BzATP labeling of the A subunit (the band immediately above the 58-kDa B subunit in A). The reason for this is uncertain, but it is possible that NEM modification of the catalytic site on the A subunit alters residues contributed by the A subunit to the noncatalytic sites on B in such a way as to make them more reactive toward [3H]BzATP.

Isolation and Sequencing of [3H]BzATP-labeled Peptides—To determine the site of [3H]BzATP labeling on the B subunit, the labeled polypeptides were first separated by SDS-PAGE followed by electroelution. The electroeluted B subunit was then subjected to proteolysis and the peptides isolated by a second round of SDS-PAGE on a 20% acrylamide gel. A portion of the gel was sliced and used for quantitation of radioactivity, while the remainder was transferred to Immobilon. Tryptic cleavage of the labeled B subunit gave a single labeled peptide of approximately 18–20 kDa, beginning at the residue Ile164. V8 protease cleavage of the labeled B subunit gave a peptide of approximately 5 kDa starting at Asp126 and ending at either Glu167, Glu168, or Gln171. The overlap of these two peptides localizes the site of [3H]BzATP labeling to the region between residues 164 and 171 of the B subunit, which has the sequence IYPE(EMIQ). The peptide sequenced in each case was the major peak of radioactivity on the gel, possessing at least 10-fold more radioactivity than any other labeled band. The
incubation with 20 mM dithiothreitol for 30 min at room temperature.

At 4°C to remove 2-mercaptoethanol employed during reconstitution.

An aliquot with 20 mM dithiothreitol as described above prior to labeling (27). These tightly bound nucleotides remain associated with ing upon the conditions employed to isolate the enzyme (49, 24, 50). Release of tightly bound nucleotides is also enhanced by treatment with 20 mM dithiothreitol as described under “Experimental Procedures” and that of cystine-modified enzyme was 8%. Dithiothreitol restored activity approximately half of the observed maximal velocity at either the high or low affinity ATP sites (each of which contributes competitively to the enzyme, with $K_m$ values in the experimental conditions of 125 and 500 μM ATP and corresponding $V_{max}$ values of 3.8 and 8.1 μmol of ATP/min/mg of protein, respectively. The presence of BzATP (100 μM) changed the kinetics of ATP hydrolysis such that a single $K_m$ of 310 μM and $V_{max}$ of 4.5 μmol of ATP/min/mg of protein were now observed. It is possible that BzATP inhibits either the high or low affinity ATP sites (each of which contribute approximately half of the observed maximal velocity at saturating ATP concentrations) and simply changes the affinity for ATP of the remaining site. Nevertheless, it is clear that BzATP is not simply a competitive inhibitor of the V-ATPase, since the original maximal velocity cannot be achieved in the presence of BzATP, even at saturating ATP. This behavior is somewhat different than for the tonoplast V-ATPase (28), where it was suggested that BzATP was “somewhat like” a competitive inhibitor given the fact that the same maximal velocity was approached even in the presence of the inhibitor at sufficiently high ATP concentrations.

These results are very different than those obtained for the F-ATPases. In that case, BzATP acts as a substrate and a competitive inhibitor for the mitochondrial F-ATPase, with a specific activity 10–200-fold less than for ATP (42). In addition, BzATP binds predominantly to the β subunit, which possesses the catalytic nucleotide binding sites (42, 56–58).

A plot of the incorporation of $[^{3}H]$ BzATP into the β subunit versus activity indicates that complete inhibition is achieved upon modification of approximately 1 mol of B subunit/mol of V-ATPase complex. The amount of the radioactivity incorpo-
V-ATPase activity due to covalent modification by $[3H]$BzATP modification of Cys254 of the A subunit with cystine or on change nucleotides only very slowly. This site is therefore qualitatively different from the noncatalytic site. Taken together with the data described above, these results indicate that modification of the B subunit by BzATP is not occurring upon oxidation or cystine modification inhibited BzATP labeling. We have demonstrated previously that cystine modification of Cys$^{254}$ or disulfide bond formation between Cys$^{254}$ and Cys$^{322}$ at the catalytic sites is able to block ATPase activity without inhibition of nucleotide binding at these sites (36, 37). By contrast, modification of Cys$^{254}$ with NEM blocks both ATPase activity and nucleotide binding to these sites.

When the effects of these reagents on BzATP labeling were tested, it was found that NEM had no effect on BzATP modification of the B subunit, whereas oxidation or cystine modification inhibited BzATP labeling. These results clearly indicate that modification of the B subunit by BzATP is not occurring through occupancy of a catalytic site, but rather a noncatalytic site. Taken together with the data described above, these results indicate that the V-ATPase possess a rapidly exchangeable, noncatalytic nucleotide binding site on the B subunit whose modification by BzATP results in inhibition of activity. This site is therefore qualitatively different from the noncatalytic nucleotide binding sites on the F-ATPase, which exchange nucleotides only very slowly.

The decrease in labeling of the B subunit sites observed upon modification of Cys$^{254}$ of the A subunit with cystine or on oxidation also indicates that nucleotide binding to the noncatalytic sites is sensitive to changes which occur at the catalytic sites of this important as we have suggested previously that disulfide bond formation between Cys$^{254}$ and Cys$^{322}$ at the catalytic sites serves as a mechanism of regulation of V-ATPase activity in vivo (48, 37). This cross-talk between the A and B subunit suggest that, while bearing no sequence similarity to the noncatalytic subunit, is not conserved in the V-ATPase B subunit (16, 18, 19, 22). The corresponding region in the bovine subunit (SAAGLPHN beginning at Ser$^{174}$) is almost perfectly conserved in the yeast B subunit, but bears no similarity to the GX$^{369}$GKT sequence of the α subunit. Mutagenesis studies have indicated that the glycin-rich loop sequence of the α subunit is crucial for activity of the E. coli F-ATPase (34). Mutagenesis studies of the corresponding region in the yeast V-ATPase B subunit suggest that, while bearing no sequence similarity to the α subunit, changes in this region do result in marked, if less dramatic, decreases in ATPase activity and proton pumping (38). Thus, results from both the previous and current studies suggest that changes (either through mutagenesis or chemical modification) in the noncatalytic nucleotide binding sites on the B subunit result in changes in activity of the V-ATPase complex.

Acknowledgments—We thank Dr. Peter Coleman, Boston Biomedical Institute, for his generous advice on the synthesis and use of BzATP.

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J. Biol. Chem. 1996, 271:12775-12782.
doi: 10.1074/jbc.271.22.12775

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