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Inhibition of ion pump ATPase activity by 3′-*O*-(4-benzoyl)benzoyl-ATP (BzATP): assessment of BzATP as an active site-directed probe

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The interaction of 3′-*O*-(4-benzoyl)benzoyl-ATP (BzATP) with the renal ($Na^+ + K^+$)-ATPase, the sarcoplasmic reticulum Ca-transport ATPase, and the gastric ($H^+ + K^+$)-ATPase has been investigated in order to determine whether BzATP is a suitable probe for the labeling and identification of a peptide from the ATP binding sites of these ion pumps. After ultraviolet irradiation BzATP inhibited the enzymatic hydrolysis of ATP by each of the ion pumps, and also was covalently incorporated into the 100 000 dalton polypeptides of each protein. The presence of excess ATP in the reaction solution did not prevent either the inactivation of ATPase activity or the labeling of the catalytic polypeptides by BzATP. Prior modification of the ATPases with fluorescein-5′-isothiocyanate (FITC), however, prevented much of the labeling of the 100 000 dalton polypeptides by BzATP. BzATP competitively inhibited the high-affinity binding of ATP to the ion pumps, but ATP did not block the high-affinity binding of BzATP by the enzymes. BzATP binds to the membrane-bound ATPases at a high-affinity site with a K_d of 0.8–1.2 μM and a B_{max} of 2–3 nmol/mg, and also binds to at least one low-affinity, high-capacity site on the membranes. HPLC separation of the soluble peptides from a tryptic digest of BzATP-labeled ($Na^+ + K^+$)-ATPase revealed the presence of several labeled peptides, none of which was protected by either ATP or FITC. Although BzATP can displace ATP from a high-affinity binding site on the ion pumps, it appears, therefore, that inactivation of enzymatic activity is the result of reactions between BzATP and the proteins at locations outside this site. Thus, it is concluded from these experiments that BzATP is not likely to be a useful probe for the ATP binding sites on the ion transport ATPases.

Introduction

Proteins that catalyze the hydrolysis of ATP and couple the energy released by this process to the translocation of ions across cell membranes are called ion pumps. The ($Na^+ + K^+$)-ATPase, the sarcoplasmic reticulum Ca-transport ATPase, and the gastric ($H^+ + K^+$)-ATPase are the best characterized ion pumps among those that have been identified in animal cell membranes, and several similarities have been observed for the reaction mechanisms and structures of these three proteins. All three, for example, contain a polypeptide of about 100 000 daltons that is transiently phosphorylated during turnover by the γ-phosphate moiety of ATP. The amino acid sequences around the phosphorylated sites in ($Na^+ + K^+$)-ATPase and Ca-transport ATPase are the same [1,21,22], and the amino acid sequences of fluorescein-labeled peptides from the adenine ring-binding region of the ATP binding sites of each protein are also nearly identical [2]. The phosphorylation of each of these 100 000 dalton polypeptides by ATP is an essential step in the en-

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zymatic coupling of ATP hydrolysis to ion translocation, and although the mechanisms whereby these proteins couple the energy released from ATP hydrolysis to ion translocation are unknown, the kinetic models for the transport activity of any one pump are nearly indistinguishable from the models of the others. The similarities in the structures and the kinetics of these three ion pumps have been interpreted to indicate that $(Na^+ + K^+)$-ATPase, Ca-transport ATPase and $(H^+ + K^+)$-ATPase have been derived from a common ancestral gene [3], and also suggest that the ATP binding sites on all of the ion pumps are structurally similar.

One approach to the investigation of the structures of the ion pumps is to identify the amino acids that are involved in the functions of the pumps, and to determine the locations of these amino acids within the protein structure. This approach involves the use of chemical probes that can specifically label these amino acids and can also serve as markers for peptides during subsequent purification procedures. Using this technique it has been shown that fluorescein-5′-isothiocyanate (FITC) labels a lysine residue within the ATP binding sites of $(Na^+ + K^+)$-ATPase, Ca-transport ATPase and $(H^+ + K^+)$-ATPase [2]. The amino acid sequences around this lysine are nearly identical among the ion pumps, but are very different from the amino acid sequences around an essential lysine in the ATP binding sites of other ATP-binding proteins that are not ion pumps. This suggests that ATP binding sites associated with proteins of different functions are not identical.

In this report the probe 3′-*O*(4-benzoyl)benzoyl-ATP (BzATP), a photoactivated derivative of ATP, is evaluated as a potential probe for amino acids found in the ATP binding sites of the ion pumps. BzATP has previously been shown to label the ATP binding sites of mitochondrial F_1-ATPase [4], chloroplast coupling factor 1 [5] and myosin [6], enzymes that also bind and hydrolyze ATP but do not form phosphorylated intermediates during turnover. BzATP has also been found to label the 100 000 dalton polypeptide of the sarcoplasmic reticulum Ca-transport ATPase [7]. The amino acid labeled by BzATP was inferred to be at the ATP binding site because of a stoichiometric relationship between the incorporation of BzATP into the protein and inhibition of activity. Only partial reduction of the labeling of the protein by BzATP was observed in the presence of high concentrations of ATP, however, and two non-overlapping tryptic peptides from the Ca-transport ATPase polypeptide were also labeled by BzATP. These data could be explained by reactions of BzATP outside the ATP binding site on Ca-transport ATPase. In order to resolve this ambiguity and to determine whether BzATP is a suitable probe for the ATP binding sites of the ion transport ATPases, the interaction of BzATP with $(Na^+ + K^+)$-ATPase, Ca-transport ATPase and $(H^+ + K^+)$-ATPase has been examined in the experiments described in this report. The results of this investigation indicate that although BzATP inhibits all of the ion pumps, the reactions of the probe at sites other than the ATP binding site make BzATP unsuitable as an active site probe for these proteins.

Experimental procedures

$(Na^+ + K^+)$-ATPase [8], sarcoplasmic reticulum Ca-transport ATPase [9] and gastric $(H^+ + K^+)$-ATPase [10] were purified as described in those reports. BzATP and [^{3}H]BzATP were synthesized and purified according to Williams and Coleman [4]. [α-^{32}P]BzATP was synthesized from [α-^{32}P]ATP (ICN) and purified on a μBondapak C_{18} HPLC column. Solvent A was 0.1 M triethylamine/formic acid (pH 6.8), and solvent B was 0.1 M triethylamine/formic acid (pH 6.8) in ethanol. A linear gradient of 0–100% solvent B was used. An extinction coefficient of $4.1 \cdot 10^4$ $cm^{-1} \cdot M^{-1}$ at 261 nm was used to determine the concentration of BzATP [6]. The product was characterized by TLC, HPLC, ^{1}H-NMR and absorbance spectroscopy, and was identical to authentic BzATP generously provided to us by Mahmood and Yount [6].

Inhibition of ion pump ATPase activity by BzATP was performed at 4°C by irradiation at 366 nm of a solution of the protein (0.2 mg/ml) and BzATP (0–1000 μM) in 25 mM imidazole-HCl, 1 mM Na_2EDTA, pH 7.4 $(Na^+ + K^+)$-ATPase, or 0.25 M sucrose 10 mM Tris-HCl, pH 8.0 (Ca-transport ATPase), or 20 mM Tris-HCl, 3

mM Na_2 EDTA, pH 7.4 ($(H^+ + K^+)$-ATPase) using a Brinkman Desaga apparatus at a distance of 4.5 cm. The samples were shielded with Pyrex glass to filter out wavelengths below 300 nm, and were continually mixed on an aliquot mixer during the irradiation. ATPase activities were measured either by the release of inorganic phosphate [11] or in a continuous assay that couples ATP hydrolysis to the oxidation of β-NADH [12]. The incorporation of radiolabeled BzATP into protein was measured after SDS-polyacrylamide gel electrophoresis [13] either by densitometric scanning of an autoradiogram and electronic integration of peak area using a Shimadzu CR-3A integrator, or by liquid scintillation counting [6].

Equilibrium binding of [^{3}H]ATP or [α-^{32}P]BzATP to $(Na^+ + K^+)$-ATPase was performed according to Jørgensen [14], and binding to Ca-transport ATPase was done as described by Shoshan and MacLennan [15].

Results and Discussion

In the absence of irradiation, BzATP had no effect on the hydrolysis of ATP that is catalyzed by any of the ion pumps. Upon irradiation, however, the enzymatic activity of each protein was inhibited by BzATP in a concentration-dependent manner. Fig. 1 illustrates the effect of different concentrationsof BzATP on the ATPase activity of each ion pump after irradiation for 30 min. (Na^+K^+)-ATPase, Ca-transport ATPase and $(H^+ + K^+)$-ATPase were all inhibited by BzATP under these conditions, and 50% of the enzymatic activity was inhibited by 100–250 μM BzATP in each case. Less than 15% of the ATPase activity was lost by irradiation in the absence of BzATP. Fig. 1 also shows that inhibition of the ion pumps by BzATP was not prevented by ATP. When 2 mM ATP were included in the irradiation buffer with BzATP, less than 10% of the ATPase activity of each ion pump was protected from inactivation. Furthermore, a concentration-dependent inhibition of activity was observed for all of the ion pumps when they were irradiated in the presence of benzoylbenzoic acid, the photoactivated moiety of BzATP. The concentration dependence of inhibition by benzoylbenzoic acid was nearly identical to the concentration dependence of inhibition

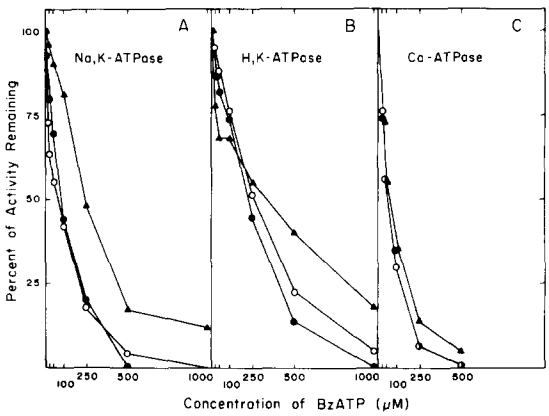

Fig. 1. Inactivation of ion pump ATPase activity by BzATP. 0.2 mg/ml of $(Na^+ + K^+)$-ATPase (A), $(H^+ + K^+)$-ATPase (B), or Ca-transport ATPase (C) were irradiated with the indicated concentrations of BzATP for 30 min at 4°C, as described in Experimental procedures. Irradiation was performed in the absence of ATP (●) or in the presence of 2 mM ATP (○). Inactivation of enzymatic activity by irradiation of the ATPases with the indicated concentrations of benzoylbenzoic acid instead of BzATP are shown (▲). Irradiation of enzymes in the absence of BzATP inactivated less than 15% of enzymatic activity.

by BzATP. These observations suggest that inactivation of the ion pumps by BzATP may be due to reactions with the proteins at sites other than the ATP binding sites.

Inhibition of ATPase activity was associated with the incorporation of BzATP into the 100 000 dalton polypeptides of each ion pump. The incorporation of BzATP into the polypeptides did not saturate, however, when up to 400 μM BzATP were present. The extent of labeling of the polypeptides was nearly the same in either the presence or absence of 2 mM ATP, consistent with the inability of ATP to protect the pumps against inactivation by BzATP. When the ATP binding sites of the ion pumps were blocked by reaction with FITC [16], however, some of the labeling of the polypeptides was prevented. In different experiments, between 20 and 50% of the labeling of the $(Na^+ + K^+)$-ATPase by BzATP was prevented when the reaction with FITC inhibited 90% of the enzymatic activity of $(Na^+ + K^+)$-ATPase and completely abolished the high-affinity binding of ATP to the enzyme. These results indicate that the site of ATP hydrolysis on $(Na^+ + K^+)$-ATPase was completely blocked by

the reaction with FITC. Similar results were obtained for $(H^+ + K^+)$-ATPase and Ca-transport ATPase, except that maximum protection from BzATP labeling by FITC was occasionally greater for these pumps than for $(Na^+ + K^+)$-ATPase. In order to determine whether BzATP was reacting with the ion pumps at an ATP binding site that could be sterically blocked by FITC, either FITC-labeled $(Na^+ + K^+)$-ATPase or unlabeled $(Na^+ + K^+)$-ATPase was labeled by [α-^{32}P]BzATP, and the BzATP-labeled proteins were cleaved by trypsin. The peptides that were released from the membrane were separated by HPLC, and were monitored by simultaneous measurement of absorbance at 214 nm for peptides and fluorescence at 520 nm (excitation = 495 nm) for fluorescein. Fractions were collected and analyzed for the presence of ^{32}P. As the chromatograms shown in Fig. 2 indicate, several ^{32}P-labeled peptides were observed in each sample, but the FITC reaction did not prevent the labeling of any of the soluble peptides. In several experiments, the amount of ^{32}P incorporated into the soluble peptides was greater for the FITC-labeled enzyme than for the unmodified enzyme. The radioactivity found in the peptides of the pellet fraction after centrifugation could not be reliably quantitated due to the large excess of radioactivity associated with the membrane lipids.

As illustrated in Fig. 3a, BzATP competitively inhibits the high-affinity binding of ATP to the $(Na^+ + K^+)$-ATPase. The $(Na^+ + K^+)$-ATPase has a B_{max} for ATP of 3.3 nmol/mg, and a K_d of 0.5–1.0 μM. The K_d for inhibition of ATP binding by BzATP is 12 μM. BzATP also serves as a substrate for $(Na^+ + K^+)$-ATPase (K_m = 10 mM, V_{max} = 0.6 μmol/min per mg protein), and the hydrolysis of BzATP by $(Na^+ + K^+)$-ATPase is inhibited by ouabain. BzATP also competitively inhibits the high-affinity binding of ATP to Ca-transport ATPase (data not shown) and serves as a substrate for Ca-transport ATPase [7]. These results indicate that BzATP can occupy the sites on these enzymes where ATP is hydrolyzed. At least two different binding sites for BzATP in these samples, however, can be inferred from the equilibrium binding of [α-^{32}P]BzATP to $(Na^+ + K^+)$-ATPase (Fig. 3b). One population of sites has a K_d of 0.8–1.2 μM and a B_{max} of 2–3 nmol/mg protein. Although the characteristics of BzATP binding to this site are similar to the binding of ATP to the $(Na^+ + K^+)$-ATPase, the inset in Fig. 3b indicates that the binding of BzATP to this site is not affected by the presence of 2 mM ATP. In addition to binding at this high-affinity site, BzATP binds to another location on $(Na^+ + K^+)$-ATPase with low affinity and an undetermined but very high B_{max}.

The results obtained in these experiments indi-

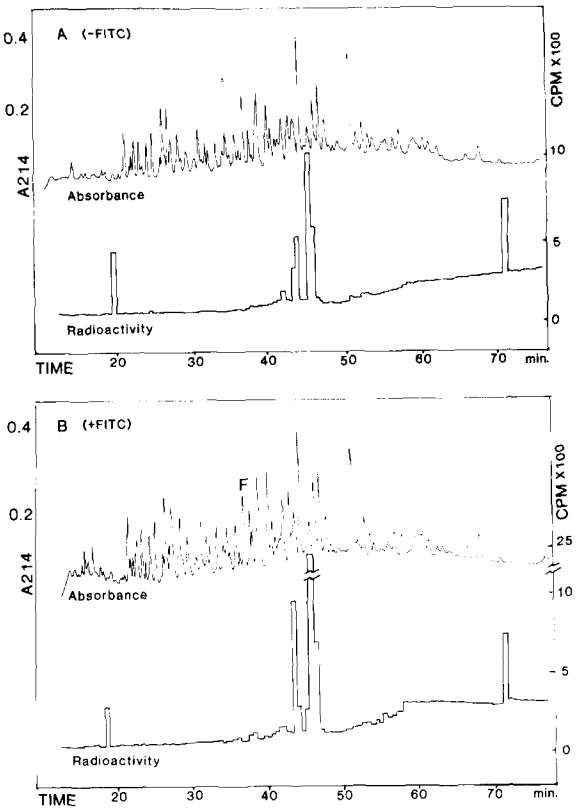

Fig. 2. HPLC separation of peptides from a tryptic digest of BzATP-labeled $(Na^+ + K^+)$-ATPase. 3 mg of $(Na^+ + K^+)$-ATPase (A) or FITC-modified $(Na^+ + K^+)$-ATPase [16] (B) were labeled with 100 μM [α-^{32}P]BzATP and were then digested with 0.3 mg of trypsin for 4 h at 37 °C. The reaction was stopped by the addition of 1 mg of soybean trypsin inhibitor and the membranes were separated from the supernatant by centrifugation at 225000 × g for 90 min. The supernatants were lyophilized and applied to the C_{18} HPLC column as described before [16]. The eluant was monitored simultaneously for fluorescein fluorescence and for peptide absorbance. Fractions were collected at 30-s intervals and the content of ^{32}P was measured by scintillation counting. The peak that is labeled by FITC is indicated by F.

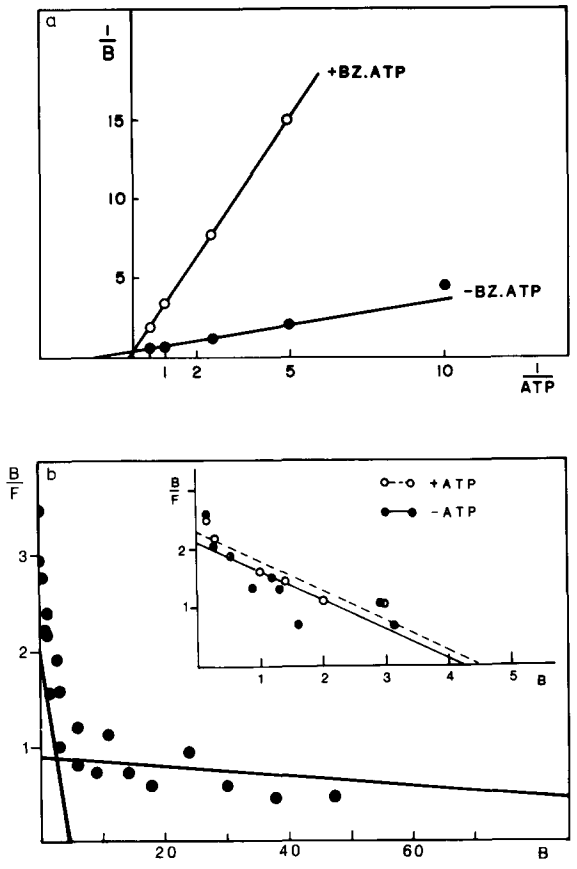

Fig. 3. Equilibrium binding of ATP and BzATP to $(Na^+ + K^+)$-ATPase. (a), 50 μg of $(Na^+ + K^+)$-ATPase were incubated with 0.1–2.0 μM [α-^{32}P]ATP in 25 mM imidazole-HCl (pH 7.4) for 5 min in the absence of BzATP (●) or the presence of 100 μM BzATP (○). The reaction suspension was centrifuged at 300000 × g for 60 min and the supernatants and pellets were separated. The reciprocal of the amount of ATP bound (mg/nmol) is plotted on the ordinate and the reciprocal of the initial ATP concentration (1/μM) is plotted on the abscissa. (b), 50 μg of $(Na^+ + K^+)$-ATPase were incubated with 0.1–500 μM [α-^{32}P]BzATP as in (a). The data are plotted according to Scatchard [23], and the lines through the data points are the best fits for a two-site model using a non-linear least-squares method. The inset shows the binding of BzATP to the high-affinity sites in the absence of ATP (●) and in the presence of 2 mM ATP (○) after subtraction of the contribution of the low-affinity binding component from the data points.

cate that, although BzATP can occupy the sites on the ion pumps $(Na^+ + K^+)$-ATPase, $(H^+ + K^+)$-ATPase, and Ca-transport ATPase where ATP is hydrolyzed, additional sites for BzATP binding to these ion pumps are found outside of this ATP binding site. Since ATP does not prevent photochemical inactivation of the ion pumps by BzATP, the reaction of BzATP with the proteins at these other sites is likely to be the cause of enzyme inactivation. Reaction at these other locations may be a result of the presence of BzATP in the lipid membrane around the protein before irradiation. The inactivation of the ion pumps by benzoylbenzoic acid and the presence of low-affinity, high-capacity binding sites for BzATP on $(Na^+ + K^+)$-ATPase are consistent with this interpretation, as is the observation that after irradiation of the ATPases with [α-^{32}P]BzATP and separation of the protein on SDS-polyacrylamide gels, most of the radioactivity associated with the samples is found in the gels at the position of the lipids. Non-specific labeling of soluble myosin subfragment 1 by BzATP has been observed [6], and the incorporation of BzATP into several proteins of submitochondrial particle membranes has also been reported [4]. Soluble mitochondrial F_1-ATPase [4] and chloroplast coupling factor 1 [5], however, could be labeled specifically at their nucleotide binding sites.

The inactivation of enzymatic activity and the labeling of the 100000 dalton polypeptides in the presence of ATP support the suggestion that BzATP reacts outside of the ATP binding sites on the ion pumps. The prevention of labeling by FITC modification, however, may indicate a reaction at an ATP binding site. FITC has previously been shown to react at a lysine residue found in the high-affinity ATP binding sites of the ion pumps [2,16,19,20]. The observations made here may be consistent with a two-site model in which BzATP and ATP compete for a high-affinity ATP binding site, but labeling of the polypeptides is due to reaction at a second site that has high affinity for BzATP but a low affinity for ATP. FITC modification of the high-affinity ATP binding site may prevent the reaction of BzATP with the protein at the second site by an indirect mechanism.

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