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5′-p-Fluorosulfonylbenzoyladenosine as an ATP Site Affinity Probe for Na^+,K^+-ATPase

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We have investigated the suitability of 5′-p-fluorosulfonylbenzoyladenosine (FSBA) as an ATP site affinity probe for the canine kidney Na^+,K^+-ATPase. The purified enzyme is slowly inactivated by this compound in suitable buffers, losing about half of its activity over a two-hour period. The rate of inactivation is more rapid in 0.1 M KCl than in 0.1 M NaCl. Low concentrations of ATP protect the enzyme against inactivation, with half-maximal effects at 4 μM ATP in 0.1 M NaCl and 350 μM ATP in 0.1 M KCl. ADP also protects against FSBA inhibition, but AMP is ineffective when present at 100 μM levels. This pattern is consistent with the previously described nucleotide specificity of the Na^+, K^+-ATPase. Addition of protective amounts of ATP after inactivation has occurred does not restore enzyme activity, indicating that inhibition is irreversible.

Measurement of the concentration-dependence of FSBA inactivation suggests an apparent K_d for binding of this compound well above 1 mM, the solubility limit of the analog. This finding is reinforced by the failure of 1 mM FSBA to compete effectively with ATP for the high-affinity ATP site of the enzyme. Nevertheless, attachment of the analog to this site is indicated by its ability to prevent [^{3}H]-ADP binding in proportion to the number of sites it has inactivated. Studies with [^{3}H]-FSBA show that about 1 mole of the analog attaches specifically to the α subunit per mole of enzyme inactivated. A similar amount of nonspecific labeling also occurs with negligible effect on enzyme activity. These findings suggest that FSBA may be useful in probing the topography of the high-affinity ATP binding site of the Na^+,K^+-ATPase and related enzymes.

Key words: 5′-p-fluorosulfonylbenzoyladenosine, ATP binding site, affinity probe, Na^+, K^+-ATPase, substrate analog, dog kidney, canine kidney, catalytic subunit

The Na^+,K^+-transport ATPase is one of a growing class of membrane enzymes known to utilize an ATP-driven phosphorylation-dephosphorylation cycle to carry out vectorial pumping of cations. Information on the ATP binding site of this enzyme is therefore of general interest. Only a few previous studies have aimed at attaching an affinity-labeling analog of ATP specifically to the active site region of this enzyme. This paper reports results from our laboratory indicating that 5′-p-fluorosulfonylbenzoyladenosine (FSBA) can be used successfully for this purpose with canine kidney Na^+,K^+-ATPase.

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Haley and Hoffman first showed that erythrocyte membranes could be photolabeled with 8-azido-ATP [1]. One of the labeled polypeptides had a mobility on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) corresponding to that of the catalytic subunits of Na^+,K^+-ATPase and Ca^{+2}, Mg^{+2}-ATPase. Subsequently, Patzelt-Wenczler and co-workers designed ATP site probes based on the premise that an active site sulfhydryl group interacts with the 6-amino group of adenine during normal ATP binding [2, 3]. These 6-mercaptopurine-based nucleotides showed ATP site specificity with beef brain Na^+,K^+-ATPase and, more recently, with the pig kidney enzyme [4]. The dinitrophenylthioinosine triphosphate analog specifically inhibits the beef brain enzyme [2] but not that from dog kidney, where only a nonspecific inactivation is seen [5]. A similar nonspecific inhibition occurs with the mercaptopurine disulfide analog of ATP using the Na^+,K^+-ATPase from rainbow trout gills [6].

Because there was some question of whether an affinity analog of ATP suitable for structural studies on the dog kidney enzyme could be devised, we investigated the effects of FSBA as a candidate for use with this enzyme. This compound is easily synthesized in both labeled and unlabeled forms and does not require photoactivation to react with suitable functional groups. It has been used successfully with a number of ATP-utilizing enzymes [7–11], despite the lack of the charged 5′-triphosphate residue. The results of this study indicate that it appears to be a suitable ATP site probe for canine kidney Na^+,K^+-ATPase.

METHODS

Purification and Assay of Na^+,K^+-ATPase

Na^+,K^+-ATPase was purified from the outer medulla of dog kidney and assayed for protein content and activity as previously described [12, 13]. The resulting enzyme preparation shows only the Na^+,K^+-ATPase α and β subunit bands on SDS-PAGE at loads as high as 160 μg per 5 mm disc gel. A small amount of staining material does not penetrate the gel. Some enzyme assays were also carried out using a coupled enzyme assay, as described by Josephson and Cantley [14], with slight modification. Both assay methods yielded the same results within experimental error.

Reaction of ATPase With FSBA

Reaction of purified enzyme with FSBA was carried out at 37°C in 10 mM barbital buffer, pH 8, containing 1 mM EDTA and other salts and ligands as indicated. Usually 0.5 mM FSBA was used, since 1 mM FSBA solutions were slightly turbid, indicating precipitation of the analog. Early studies used methanolic stock solutions of FSBA, freshly prepared, to initiate the reaction. Later studies used stock solutions of FSBA in dimethylsulfoxide (DMSO). These solutions are stable for longer periods if kept anhydrous. Similar results were obtained with both approaches. Appropriate controls corrected for the slight amount of inactivation of the enzyme by solvent alone. Up to 8% methanol and 6% DMSO can be added to the enzyme without significant inhibition. The inhibition produced by higher concentrations of DMSO is reversible [15].

Measurement of FSBA Breakdown in Aqueous Buffer

Hydrolysis of FSBA with release of fluoride was followed with a calibrated fluoride-specific electrode in 10 mM barbital, pH 8, containing 0.1 M NaCl and 10^{-4} M fluoride. Potential measurements were made with a Heath Recording Electrometer (model EU-20-31). The product formed is presumably the sulfonate; it yields adenosine on saponification, indicating that the ester linkage is intact.

Measurement of [^{3}H] ADP Binding to Na^+,K^+-ATPase

Enzyme (500 μg/ml) was incubated at 0° for 2 hours in 100 μl of a medium containing 25 mM Tris, pH 7.4, 1 mM EDTA, 100 mM NaCl, and [^{3}H]-ADP at various concentrations. A 10 μl aliquot of each sample was taken to determine the specific radioactivity by liquid scintillation counting. The remainder was placed in the chamber of an MRA Corporation ultrafiltration cell and a portion filtered under pressure through Amicon XM-50 ultrafiltration membranes. Ten microliters of the ultrafiltrate was then counted, and the amount of ADP bound was determined by difference. The percentage of total ADP bound in the experiments reported here ranged between 17% and 35%.

Measurement of Apparent Stoichiometry of Reaction of [^{3}H]-FSBA with Na^+,K^+-ATPase

[^{3}H]-FSBA (113,700 dpm/nmole, 0.5 mM initial concentration) was incubated at 37°C with Na^+, K^+-ATPase (0.8–1.0 mg/ml) in 10 mM barbital, pH 8, 1 mM EDTA, 0.1 M NaCl. At zero time and 2 hours, aliquots were removed for assay of enzyme activity and application to 7% SDS-polyacrylamide gels.

In two experiments, the enzyme was sedimented at this point for 100 minutes at 36,000 rpm in a Beckman 40 rotor. The pellet was then resuspended in 2% SDS for application to the SDS-polyacrylamide gels. This procedure markedly lowered the background radioactivity on the gel, thereby yielding a more accurate value for the stoichiometry of labeling. Control samples were run without labeling and stained to determine the position of α and β subunits. Electrophoresis was carried out as previously described [16] for twice the length of time required for the tracking dye to reach the bottom of the gel. The gels were then frozen and sliced into 2 mm pieces and placed in scintillation counting vials. Five-tenths of one milliliter of 9:1 NCS:H_2O (Amersham/Searle Corporation) was added, and the vials were capped and placed in a 50°C oven for 2 hours. After cooling, 10 ml of Biofluor (New England Nuclear Corporation) was added, and the samples were stored at room temperature in the dark overnight. Repeated counting ensured that chemiluminescence had declined to undetectable levels.

Synthesis of FSBA

FSBA was synthesized by the method of Colman and co-workers [17]. This method was scaled down from 3 nmoles to 17 μmoles to synthesize [^{3}H]-FSBA from 2-[^{3}H]-adenosine. Total reaction volume was 50 μl, and the acylation was carried out in a 300-μl Pierce Reacti-vial. The amount of acyl chloride was increased to 59 μmoles to ensure complete acylation. Purity of the labeled product was monitored by thin-layer chromatography on EM Laboratories F-254 silica gel plates. Solvent systems were absolute methanol and methyl ethyl ketone:acetone:water (60:20:15). The labeled compound was dissolved in dry DMSO and stored at room temperature in a desiccator over KOH. Its spectral properties correspond to those reported previously for the compound dissolved in absolute ethanol, provided that traces of water are present. Spectra of FSBA and [^{3}H]-FSBA determined using ethanol stored over molecular sieves are quite different. Aqueous solutions of FSBA yield spectra like those in ethanol:water mixtures.

RESULTS

The purified Na^+,K^+-ATPase is known to exist in two different conformational states, depending on whether Na^+ or K^+ is the predominant monovalent cation in the medium. These alternative conformational states show different products of trypsinolysis [18, 19], and the affinities of the adenine nucleotide binding sites differ [20, 21]. Similar effects are seen when FSBA reacts with the enzyme. With a single addition of 0.5 mM FSBA in

buffered 0.1 M NaCl, about half the activity is lost over 2 hours. Periodic additions of more FSBA result in essentially complete inactivation of the remaining enzyme (results not shown). Low concentrations of ATP protect the enzyme against this loss of activity. Carrying out the same experiment in 0.1 M KCl yields similar results, except that the inactivation is somewhat more rapid. In addition, higher levels of ATP are required to protect the enzyme activity in KCl medium, consistent with the previously reported nucleotide site affinities of the two conformers. Figure 1 summarizes these results.

An additional indication that protection against inactivation by FSBA is the result of interaction at the high-affinity nucleotide binding site is shown in Figure 2. While ATP and ADP both protect the enzyme activity, AMP does not, a result consistent with the known adenine nucleotide specificity of this enzyme [22].

Inactivation by FSBA appears to be an irreversible process. Figure 3 shows that addition of protective amounts of ATP after some inhibition by FSBA has occurred does not restore the activity already lost. Instead, further loss of activity is prevented. These results are consistent with the behavior to be expected from an ATP-site affinity probe, but they are not sufficient, by themselves, to establish only that interpretation.
Since the rate of inactivation of Na^+,K^+-ATPase appears to decrease with time (Figs. 2 and 3), breakdown of FSBA to an inactive derivative must be occurring. In fact, nonenzymatic hydrolysis of FSBA with release of fluoride can be measured with a fluoride-specific electrode (Fig. 4). The apparent rate constants for fluoride release are 0.0127 per minute at 37°C and 0.0029 per minute at 25°C. Addition of primary amines such as Tris buffer accelerates the rate of fluoride release about 30-fold (data not shown). Such buffers were therefore avoided.

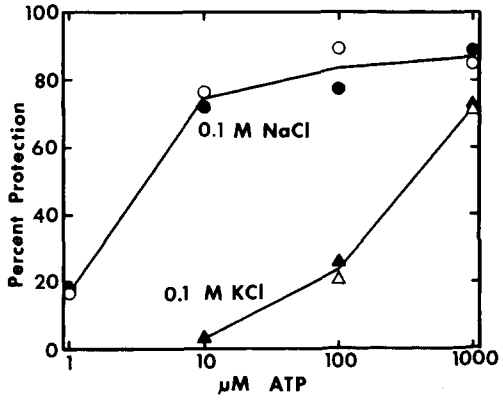

Fig. 1. Comparison of relative protective effects of ATP against FSBA inactivation in 0.1 M NaCl and 0.1 M KCl. Purified enzyme (100 μg protein/ml) was incubated with 0.5 mM FSBA in 0.1 M NaCl or 0.1 M KCl as described under Methods. ATP was added to some samples at the concentrations indicated. Aliquots were removed at 1 and 2 hours, diluted tenfold, and assayed for Na^+,K^+-ATPase activity by measuring phosphate release. Duplicate assays were performed with and without 1 mM ouabain, and the results were averaged. Individual values in such assays rarely differ by more than 5%. Symbols: ○△, after 1 hour incubation; ●▲, after 2 hours incubation.

Measurements of the effect of FSBA concentration on enzyme inactivation rate may provide an estimate of the apparent affinity of the enzyme for this analog. Such measurements require, however, that the FSBA concentration be held essentially constant. The nonenzymatic breakdown shown in Figure 4 complicates such studies. To solve this problem, inactivation of enzyme was carried out at several concentrations of FSBA, with periodic additions of more analog in an attempt to hold the concentration within ± 15% of its initial value. (The schedule and amounts of these additions were based on the rate constants calculated from Figure 4). The time-course showed the inactivation to be first-order within experimental error (results not shown). A control experiment showed that 1 mM NaF has no effect on the enzyme under these conditions. Figure 5 shows the apparent rate constants for inactivation plotted as a function of FSBA concentration. It is apparent that little evidence of saturation is seen at 1 mM FSBA, the solubility limit of this compound. Furthermore, 1 mM solutions are slightly turbid, thereby raising doubts as to the actual concentration of FSBA in these particular samples.

In another attempt to measure the affinity of FSBA for the ATP binding site, a coupled enzyme assay was used to measure hydrolysis of very low concentrations of ATP in the presence and absence of added FSBA. Figure 6 shows that 1.1 mM FSBA is unable to compete with 3–10 μM ATP being hydrolyzed on the high-affinity ATP binding site under conditions where the low-affinity regulatory site of the enzyme is essentially nonfunctional [23–25]. Only noncompetitive inhibition occuring during the assay at 37°C is seen. The apparent K_m's for ATP calculated from Figure 6

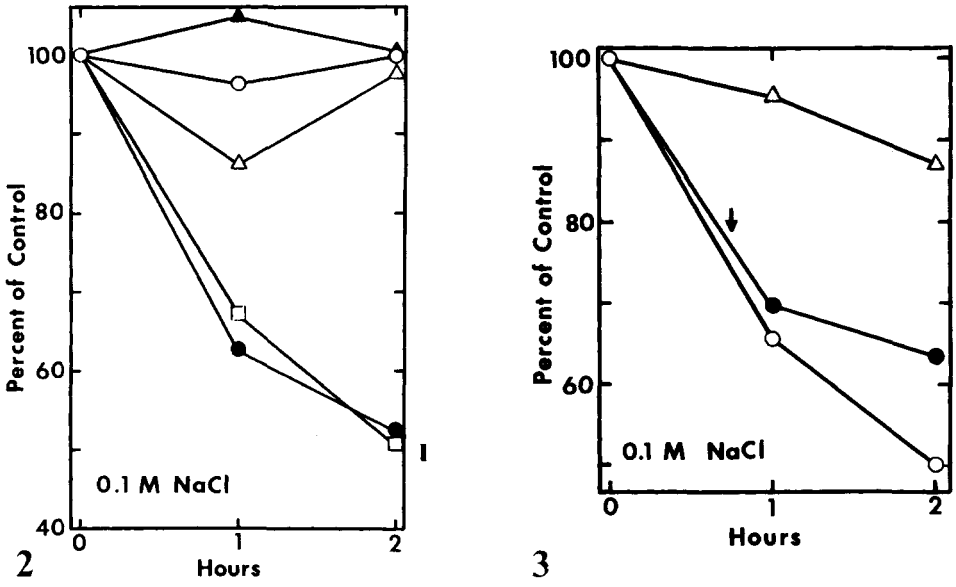

Fig. 2. Adenine nucleotide specificity for protection against FSBA. Conditions were as in Figure 1. Symbols: ○, methanol solvent alone (no FSBA): ●, FSBA alone; △, FSBA + 100 μM ATP ▲, FSBA + 100 μM ADP; □, FSBA + 100 μM AMP.

Fig. 3. Irreversibility of activity loss caused by FSBA. Enzyme was treated with FSBA as described in Figure 1 and Methods. Symbols: ○, FSBA alone; ●, FSBA + 1 mM ATP, which was added after 45 minutes inactivation (arrow); △, FSBA + 1 mM ATP (added at zero time).

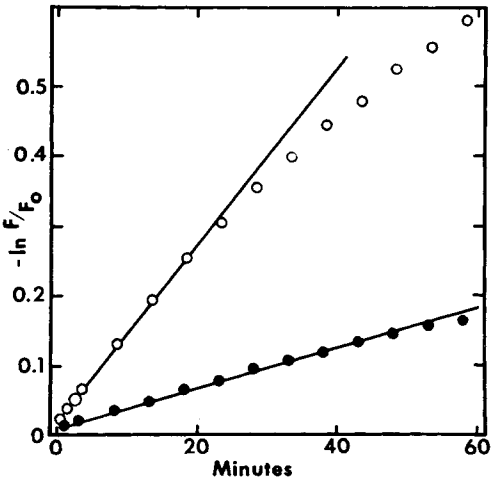

Fig. 4. Release of fluoride from FSBA in aqueous barbital buffer. Initial FSBA concentration was 1 mM. Data are plotted in terms of the fraction of FSBA remaining at the time indicated. Symbols: ○, T = 37°C; ●, T = 25°C.

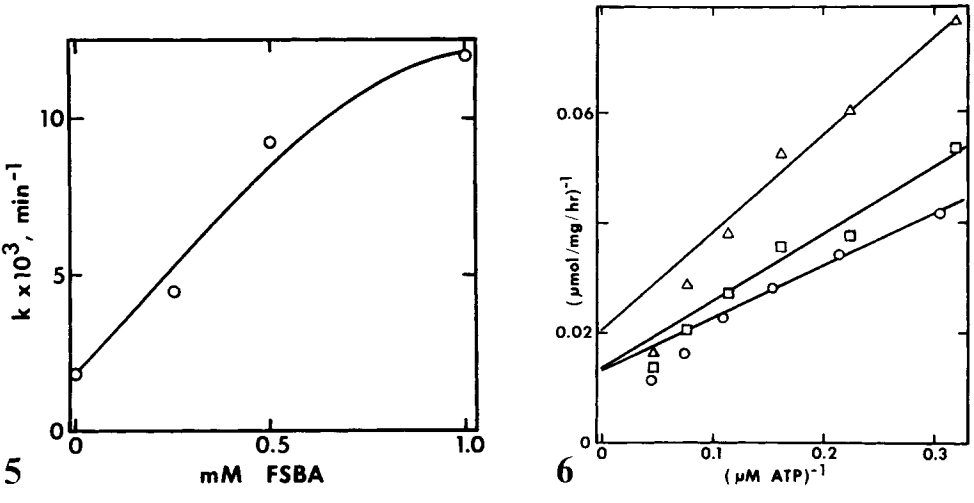

Fig. 5. Concentration-dependence of the rate of inactivation of Na^+,K^+-ATPase by FSBA. Incubation conditions were as described in Methods. Concentrations were maintained essentially constant as described in the text. Rate constants were calculated from time-course data after logarithmic transformation.

Fig. 6. Failure of FSBA to compete with ATP for the high-affinity ATP hydrolysis site of Na^+,K^+-ATPase. A coupled assay was used to measure ATP hydrolysis as described under Methods. Independent experiments established that the coupling enzymes were not limiting under the conditions of each experiment. The ATPase concentration was 8.8 μg protein/ml. Lines were fitted by linear regression using data from the four lowest ATP concentrations. Note that at higher concentrations of ATP the influence of the low-affinity regulatory site becomes apparent. Symbols: ○, control: □, DMSO solvent only; △, + 1.1 mM FSBA.

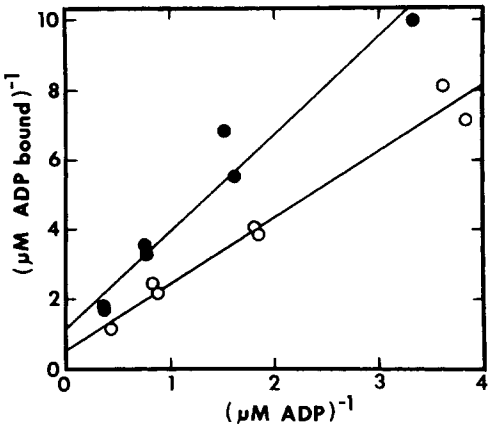

Fig. 7. Failure of [^{3}H]-ADP to bind to FSBA-inhibited Na^+,K^+-ATPase. Enzyme (1 mg protein/ml) was incubated at 37°C with 1 mM FSBA for 2 hours, as described in Methods. Aliquots were removed for assay of activity and measurement of ADP binding as described in Methods. At the time of the binding measurement, 44% of the enzyme activity remained in the FSBA-treated samples. Lines were fitted by linear regression. Control values (open circles): K_d = 3.4 μM; B_{max} = 3.6 nmoles/mg. FSBA-treated (filled circles): K_d = 2.4 μM; B_{max} = 1.7 nmoles/mg (47% of control).

are 7.2 μM in the control, 8.7 μM with DMSO alone, and 8.6 μM with 0.5 mM FSBA added in DMSO carrier. Assuming that a 20% increase in K_m would be detectable in these experiments, these results indicate that the apparent dissociation constant for FSBA occupying the high-affinity ATP site of the enzyme exceeds 5 mM.

Despite the failure to demonstrate competition between FSBA and ATP for the high-affinity binding site, there is nevertheless evidence that FSBA occupies that site. Figure 7 shows that when FSBA inactivates individual Na^+,K^+-ATPase units, an equivalent number of nucleotide-binding sites are also inactivated. There is a decrease in binding capacity proportional to the loss of enzyme activity without a significant change in the apparent affinity of the remaining sites. This finding implies that occupation of the high-affinity sites by covalent attachment of the analog renders them unavailable for ATP or ADP binding.

Any attempt to isolate and carry out structural studies on analog-labeled polypeptides requires that unique sites be labeled. It is conceivable that FSBA might form covalent adducts nonspecifically, with a large number of residues on the enzyme but without any significant effects on enzyme activity. Therefore, it was necessary to determine the stoichiometry of reaction of FSBA with the enzyme. This was carried out with [^{3}H]-FSBA as described in Methods, using SDS-PAGE to isolate the labeled catalytic subunit. The results are shown in Table I and Figure 8. In some experiments (1 and 2 of Table I), SDS was added to the [^{3}H]-FSBA labeling reaction mixture, and suitable aliquots were applied directly to SDS polyacrylamide gels. Some ATP-preventable labeling is detected in the α-subunit band of such gels, as well as some nonspecific, time-dependent labeling. However, a large background labeling obscured any counts that might be present in the β-subunit. Furthermore, estimation of nonspecific labeling of α required subtraction of a sizable gel blank of adsorbed nucleotide analog. Therefore, labeled protein was sedimented and the soluble radioactive analog largely removed prior to SDS-PAGE (experiments 3 and 4 of Table I). Such gels showed much lower gel blanks, as shown in

TABLE I. Apparent Stoichiometry of Labeling of α-Subunits*

Experiment		Activity lost (%)	dpm in α	pmoles bound	pmoles bound (ATP-inhibitable)	pmoles bound per pmole enzyme inactivated[a]
1 (99.9 μg/gel)						
no ATP	a	47.1	15,820	139.2	65.3	0.74
	b		21,496	189.1	115.2	1.31
100 μM ATP	a	–	7,039	61.9	–	
	b		9,763	85.9		
2 (81.5 μg/gel)						
no ATP	a	44.9	12,402	109.1	54.8	0.77
	b		13,600	119.7	65.4	0.92
1 mM ATP	a	–	5,530	48.7	–	–
	b		6,830	59.9		
3 (163 μg/gel)						
no ATP	a	41.3	28,944	254.7	56.9	0.53
	b		35,994	316.5	118.7	1.10
1 mM ATP	a	–	23,313	205.1	–	–
	b		21,619	190.5		
4 (125 μg/gel)						
no ATP	a	40.4	24,621	216.6	88.0	1.06
	b		24,530	215.8	87.2	1.05
1 mM ATP	a	–	14,732	129.6	–	–
	b		14,501	127.6		
						average = 0.94 ± 0.24

*Enzyme was incubated with 0.5 mM [^{3}H]-FSBA (113,700 dpm/nmole) as described under Methods. Aliquots were taken for assay of enzyme activity remaining and for SDS-PAGE. In experiments 3 and 4, samples of the reaction mixture were mixed with 4 volumes of 1 mM dithiothreitol and centrifuged as described under Methods prior to SDS-PAGE. Gels were subsequently sliced, digested, and counted as described under Methods.

[a]Assumes 105 pmole enzyme / (μmol/min) based on ouabain binding [12].

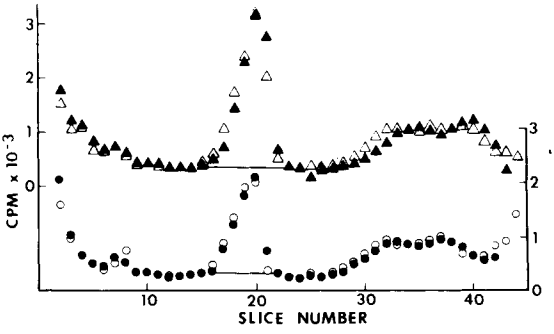

Fig. 8. Labeling of catalytic subunit of Na^+,K^+-ATPase by [^{3}H]-FSBA. Incubation was carried out with 0.5 mM [^{3}H]-FSBA as described in Methods. Aliquots were taken for measurement of enzyme activity and for application to duplicate 7% SDS gels after centrifuging, as described in Methods. Symbols: △▲, duplicate gels of samples 2 hours with FSBA; ○●, 2 hours with FSBA plus 1 mM ATP. The scale on the ordinate is labeled on the left for the upper curves and on the right for the lower ones. These results are analyzed in Table I as experiment 4. The α subunit generally runs in slices 16–22 and the β-subunit in slices 29–34, as determined by stained gels run in parallel.

Figure 8. The heavy labeling at the top of the gel, where very little protein is found, may represent some sort of reaction of FSBA with the gel matrix. A small amount of ATP-insensitive labeling can be seen in the β-subunit when the experiment is done in this fashion.

It is clear that both methods of carrying out the labeling experiment yield an ATP-protectable covalent attachment of analog to the enzyme with a stoichiometry of about 1 mole per mole enzyme inactivated. This is the value expected for an ATP site affinity probe that attaches only to the single high-affinity site seen in each enzyme unit. In addition, there is a slightly larger amount of nonspecific (ATP-insensitive) labeling observed. Whether the nonspecifically attached analog is located at a single site or is spread among a variety of attachment sites remains to be determined. Fragmentation studies will undoubtedly yield information on this point.

DISCUSSION

The evidence presented here supports strongly the interpretation that FSBA is an ATP site affinity probe for canine kidney Na^+,K^+-ATPase. This analog inactivates the enzyme, yet occupation of the high-affinity ATP binding sites by nucleotide protects against this inactivation. The specificity of protection corresponds to the known specificity of this site, and the relative affinities shift with conformation, as previously described [20, 22]. An unexplained observation is the apparent increase in rate of inactivation of the enzyme by FSBA in KCl medium, a condition that greatly decreases the affinity of ATP for the site. Apparently, the functional group with which FSBA reacts becomes more reactive in this conformation, offsetting the poorer affinity of the binding site. Alternatively, the two conformations of the enzyme might control the tightness of nucleotide binding by an effect on the part of the site that binds the triphosphate moiety of ATP. Such an effect might be relatively unimportant for an analog like FSBA, which has no charged groups in this part of the molecule. Such an explanation is also consistent with the relatively poor affinity of FSBA seen with this enzyme, which might depend solely on the adenosine-binding portion of the ATP site. It is known that the di- and triphosphate binding area on the enzyme contains at least one arginine residue and that this residue appears to interact with the α,β-phosphates [26, 27].

The kinetics of inactivation are consistent with FSBA reacting at a single site. Although it is conceivable that FSBA might react with either high or low-affinity ATP binding sites indiscriminately, the complete protective effect of very low ATP concentrations tends to rule this out, especially if the two types of ATP sites alternate as both catalytic and regulatory sites, as postulated by others [23–25, 28]. The fact that the ATP-sensitive binding of [^{3}H]-FSBA to the α subunit corresponds to a stoichiometry of about one mole of analog per mole of enzyme inactivated also supports this view.

Future studies will require isolation of labeled α subunits and determination of the location of both ATP-protectable and nonspecific labeling by FSBA. It is hoped that such studies will define further the nature of the interaction of adenine nucleotides with the high-affinity ATP binding site.

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