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Effects of an ATP site affinity analog on some conformational and enzymatic properties of the canine kidney ($Na^+ + K^+$)-ATPase

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We have shown previously that the canine kidney Na^+,K^+ pump (($Na^+ + K^+$)-ATPase) reacts with the ATP affinity analog *p*-fluorosulfonylbenzoyladenosine (FSBA). At 20°C, we find the time-course of this reaction to be that predicted for a first-order reaction accompanied by competing solvolysis of the reagent. The FSBA-inactivated ($Na^+ + K^+$)-ATPase retains the ability to move between the E_1 and E_2 conformations that predominate in Na^+ and K^+ medium, respectively. Therefore, FSBA reaction with the enzyme does not interfere significantly with either its alkali metal cation binding or its conformational freedom. The ability of ATP to influence the enzyme's conformation by binding to the high-affinity nucleotide site is decreased, however, in proportion to the degree of inhibition of enzyme activity by FSBA. In addition, the ability of the enzyme to shift from the E_1 to the E_2 conformation through the ($ATP + Na^+$)-dependent phosphorylation cycle is inhibited by FSBA treatment, as shown by the decreased ability of these substrates to stimulate the K^+-dependent *p*-nitrophenylphosphatase activity. Both of these effects are consistent with specific reaction of FSBA with the ATP binding site of the enzyme. An additional effect of FSBA treatment is that it causes loss of *p*-nitrophenylphosphatase activity, but to a lesser extent than ($Na^+ + K^+$)-ATPase or Na^+-ATPase activity. Binding of *p*-nitrophenylphosphate to the enzyme is apparently unaffected by FSBA treatment, since the K_m for *p*-nitrophenylphosphate is not changed.

Introduction

The mammalian Na^+,K^+-transport pump driven by ATP (($Na^+ + K^+$)-ATPase) belongs to a family of transport ATPases that share certain structural and mechanistic features. (The family of transport ATPases includes the ($Na^+ + K^+$)-ATPase, the sarcoplasmic reticulum Ca^{2+}-ATPase, the plasma membrane Ca^{2+}-ATPase (such as that found in the erythrocyte), the gastric oxyntic cell ($K^+ + H^+$)-ATPase, fungal and plant H^+-ATPases and possibly the Kdp transport system of bacteria such as *E. coli.*) Each member of this class binds ATP with high affinity (K_d less than 1 micromolar) preliminary to the cation-controlled phosphorylation of an aspartyl residue of the catalytic subunit. The resulting conformational change evoked by this phosphorylation, together with a subsequent cation-controlled dephosphorylation, is harnessed by the catalytic subunit structure to drive cation translocation across the membrane [1–3]. In the case of the ($Na^+ + K^+$)-ATPase, the phosphorylation reaction is controlled by Na^+, while the dephosphorylation is controlled by K^+. Many investigators believe that the transport of the corresponding cations accompanies each of these steps [4].

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Abbreviations: FSBA, *p*-fluorosulfonylbenzoyladenosine; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine.

Because each member of the transport ATPase family uses a similar phosphorylation/dephosphorylation reaction sequence, it is reasonable to presume that the family shares a similar three-dimensional topography (and hence a similar peptide sequence) in the ATP binding site. One might therefore design affinity probes for the ATP site of one member of the class that would be broadly applicable to the other members. This paper describes studies of the interaction of one such candidate probe, *p*-fluorosulfonylbenzoyladenosine (FSBA) with the $(Na^+ + K^+)$-ATPase. Previous work from this laboratory [5] has shown FSBA to react specifically in the ATP-binding site of the $(Na^+ + K^+)$-ATPase. (Preliminary studies in this laboratory indicate that this compound also reacts in the ATP binding site of the sarcoplasmic reticulum Ca^{2+}-ATPase.) The results in this paper describe the effects of FSBA on some additional functions of the $(Na^+ + K^+)$-ATPase: the Na^+- and K^+-dependent conformational transitions and the so-called 'partial' reactions.

Methods

Purification and assay of $(Na^+ + K^+)$-ATPase

$(Na^+ + K^+)$-ATPase was purified from the outer medulla of canine kidney as described previously [6]. The resulting enzyme preparation contains the α and β subunits of $(Na^+ + K^+)$-ATPase with very little contamination by other polypeptides, as judged by SDS-polyacrylamide gel electrophoresis [7]. $(Na^+ + K^+)$-ATPase activity was measured as described previously [6] using a coupled enzyme assay [8]. Na^+-ATPase activity was measured in similar fashion, except that K^+ was omitted from the assay medium and the ATP concentration was 20 μM. ADP-ATP exchange activity was measured as described earlier [9]. K^+-dependent *p*-nitrophenylphosphatase activity was measured spectrophotometrically using a millimolar extinction coefficient of 12.2 at 410 nm for *p*-nitrophenol (pH 7.4) [10]. Protein was measured by the Lowry procedure [11].

Reaction of $(Na^+ + K^+)$-ATPase with FSBA

Reaction of purified $(Na^+ + K^+)$-ATPase with FSBA was carried out in capped 1.5 ml conical plastic tubes in 10 mM Bicine buffer (pH 8.5) containing 1 mM EDTA and other salts and ligands as indicated. The temperature was 20°C unless noted otherwise. FSBA was added as a stock solution (usually 0.1 or 0.2 M) in dimethylsulfoxide to produce the final concentration indicated. These stock solutions are stable over long periods of time when stored in a desiccator at room tempreature.

Measurement of tryptophan fluorescence changes of $(Na^+ + K^+)$-ATPase preparations

The intrinsic tryptophan fluorescence of the $(Na^+ + K^+)$-ATPase was measured using either a Farrand Mark I spectrofluorometer with output to a Honeywell Electronik 194 recorder or a Perkin-Elmer MPF-66 with computer-assisted data acquisition. The sensitivity of the Farrand system for detection of small changes in fluorescence was increased considerably by utilizing the range extension feature of the recorder, allowing one to display the top 5–10% of the signal. The Perkin-Elmer software carried out this manipulation directly on the stored data. The excitation wavelength was 295 nm and the emission wavelength was 325 nm [12]. The temperature of the cuvette chamber was maintained at 20°C with the aid of a specially designed jacket. The contents of the cuvette were stirred continuously during the measurement with the aid of a small magnetic stirring bar. Fluorescence changes were corrected subsequently for the small dilutions caused by additions of concentrated stock solutions during the measurement. Changes in intrinsic tryptophan fluorescence are reported as a percentage of the total fluorescence.

Results

Optimal conditions for treatment of $(Na^+ + K^+)$-ATPase with FSBA

Earlier studies using FSBA as a nucleotide site-specific reagent for $(Na^+ + K^+)$-ATPase were carried out at 37°C [5]. Under these conditions, some 'nonspecific' inactivation of the $(Na^+ + K^+)$-ATPase was always observed (defined as not protectable by saturating concentrations of ATP). We have found that this nonspecific inhibition can be minimized by reducing the incubation temperature to 20°C without significantly affecting the

extent of specific inactivation of the $(Na^+ + K^+)$-ATPase (Fig. 1). Some of the results described below suggest that incomplete protection by ATP during long incubations may result in some catalytic site inactivation being included in the 'nonspecific' inhibition.

Kinetics of inactivation of $(Na^+ + K^+)$-ATPase by FSBA

Previous studies were carried out in barbital buffer to reduce the reaction of the reagent with the buffer itself [5]. The present work was done using 10 mM Bicine (pH 8.5) which also reacts poorly with FSBA. Fig. 2 shows the time-course of inactivation of approx. 1.4 μM $(Na^+ + K^+)$-ATPase by 1 mM FSBA. (Enzyme concentration is estimated from its specific activity and the protein concentration using the relationship between [^{3}H]ouabain binding and enzyme activity determined in Ref. 6.) A second addition of an equivalent amount of FSBA was made after the first 3 h of incubation with FSBA. The time-course is nonlinear because of gradual depletion of the reagent by solvolysis. Also shown in Fig. 2 is the nonspecific inactivation rate (open triangles) measured in the presence of 5 mM ATP.

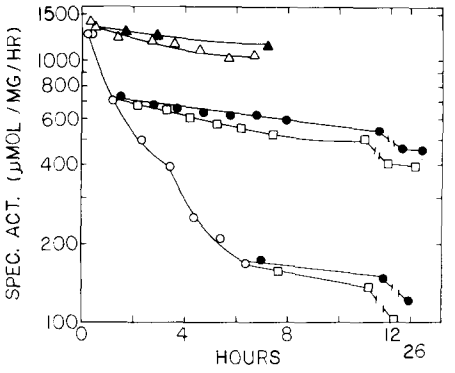

Fig. 2. Kinetics of inactivation of $(Na^+ + K^+)$-ATPase by FSBA in the presence and absence of Tris buffer and dithiothreitol. Two tubes (A and B) were prepared containing $(Na^+ + K^+)$-ATPase (565 μg/ml) in the medium described in Fig. 1. Tube B contained, in addition, 5 mM ATP. At zero time, FSBA was added to a final concentration of 1 mM. At 182 min, a second 1 mM addition of FSBA was made. 30-μl aliquots were withdrawn from each tube into 500 μl 0.25 M Tris, pH 7.6 at 25°C, placed on ice, and assayed as soon as possible for $(Na^+ + K^+)$-ATPase activity. Duplicates of the aliquots taken at 1 h and 6 h were also placed in 500 μl ice-cold 0.25 M Tris/1 mM dithiothreitol. The 1 and 6 h samples were assayed, repeatedly, over the next 25 h. ○, tube A; △, tube B; □, 1- and 6-h aliquots placed in Tris only; ●, 1- and 6-h aliquots placed in Tris/dithiothreitol; ▲, tube B samples placed in Tris/dithiothreitol. Note that activity is plotted on a logarithmic scale.

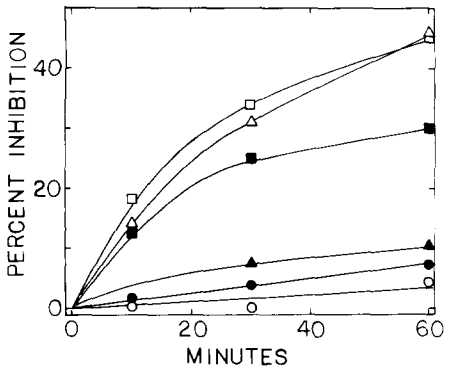

Fig. 1. Effect of temperature on inhibition of $(Na^+ + K^+)$-ATPase by FSBA. FSBA (2 mM final concentration) was added to $(Na^+ + K^+)$-ATPase (565 μg/ml) in a medium containing 10 mM Bicine/0.1 M KCl/1 mM EDTA (pH 8.5). 30-μl aliquots were withdrawn at the times indicated into 500 μl 1 mM dithiothreitol and placed on ice. These samples were assayed for $(Na^+ + K^+)$-ATPase activity as described under Methods. Circles, 0°C; triangles, 20°C; squares, 37°C. Filled symbols represent 'nonspecific' inhibition (FSBA + 1 mM ATP). Open symbols represent specific inhibition (FSBA alone, after subtraction of nonspecific inhibition).

Purdie and Heggie [13] have derived kinetic equations for the reaction of an inhibitor with an enzyme accompanied by solvolysis of the inhibitor. Since there is no significant effect of these concentrations of the membranous enzyme preparation on solvolytic decomposition of FSBA, the equation describing loss of enzyme activity should be [13]:

$$\ln\frac{E}{E_0} = \frac{k_2}{k_1}\ln\frac{\left(K_I + I_0 e^{-k_1 t}\right)}{\left(K_I + I_0\right)}$$

where k_1 is the rate constant for solvolysis, k_2 is that for reaction of the inhibitor with the enzyme, I_0 is the initial concentration of FSBA and K_I is the equilibrium constant for dissociation of the inhibitor from the inhibition site on the enzyme. (Studies on the rate of FSBA solvolysis using a fluoride-sensitive electrode show no detectable change in rate of fluoride release upon addition of

enzyme in the concentrations used here (results not shown).) Fitting the results of two separate experiments like that of Fig. 2 to this equation yields the results shown in Fig. 3. The data fit reasonably well to the expected straight line. For the first addition of FSBA, the calculated value of k_1 is 0.42 h^{-1}, while the corresponding value for the second addition is also 0.42 h^{-1} (open symbols). The corresponding values for the second experiment (filled symbols) are 0.33 and 0.54 h^{-1}, respectively. The calculated value of k_2 for each experiment is lower for the second addition than the first, as reflected in the change in slopes (dashed line). The K_I values calculated for the four lines average 0.165 M ± 0.023 S.D., reflecting the low affinity of this reagent as previously described [5].

Effect of Tris buffer and dithiothreitol on FSBA inhibition of ($Na^+ + K^+$)-ATPase

Fig. 2 also shows the effect of adding a primary amine (Tris buffer) on the rate of FSBA inactivation of the ($Na^+ + K^+$)-ATPase. Reaction of FSBA with the enzyme is rapidly terminated because the reagent reacts with the buffer (open squares). A separate aliquot of reaction mixture was stopped with Tris buffer containing dithiothreitol (filled circles). No reactivation of enzyme activity is seen, suggesting that sulfhydryl groups are not involved in the derivatization of the enzyme by FSBA.

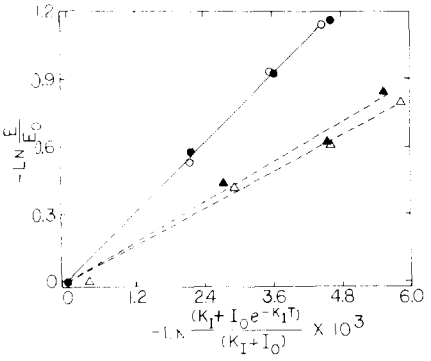

Fig. 3. Time-course of inactivation of ($Na^+ + K^+$)-ATPase by FSBA fitted to the equation of Purdie and Heggie [13]. Data from two different experiments similar to that of Fig. 2 were fitted to equation *a* of Purdie and Heggie [13] using the MLAB modeling system available from the National Institutes of Health for DEC10 computers [21]. Using the value of k_1 obtained from the fit for the first addition of FSBA, the [FSBA] at the time of the second FSBA addition could be estimated, enabling a fit of the data for times greater than 3 h. Open symbols, experiment 1; closed symbols, experiment 2. Circles, first addition of FSBA; triangles, second addition. Equation *a* was plotted in negative form on both axes so that the axes would be positive.

Na^+- and K^+-induced conformational changes in the FSBA-treated ($Na^+ + K^+$)-ATPase

The native ($Na^+ + K^+$)-ATPase shows two major conformations, E_1 or E_2, depending on whether Na^+ or K^+, respectively, is the predomi-

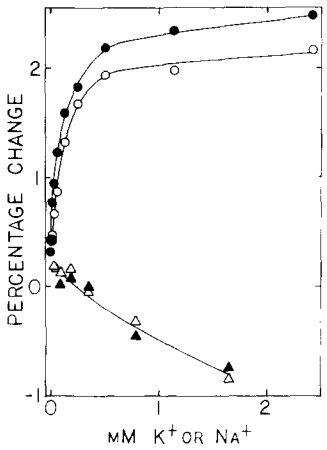

Fig. 4. Effects of K^+ and Na^+ addition on the intrinsic tryptophan fluorescence of FSBA-treated ($Na^+ + K^+$)-ATPase. Enzyme at 600 μg/ml was incubated with 1 mM FSBA as described in Fig. 1, except that the [KCl] was 0.5 mM. After 3 h, a second 1 mM addition of FSBA was made and the reaction continued for a total of 6 h. 480-μl aliquots were taken at zero time and 6 h into 20 mM Tris (pH 7.6)/1 mM dithiothreitol, and stored overnight in the refrigerator. Samples of this material were taken the next day for fluorescence measurements at 30 μg/ml ($Na^+ + K^+$)-ATPase. Additions of K^+ were made in a buffer containing 30 mM Tris/1 mM EDTA (pH 7.6). Additions of Na^+ were made in a buffer with a lower Tris concentration (1.7 mM) because high concentrations of Tris are known to induce the E_1 conformation of the ($Na^+ + K^+$)-ATPase [19]. Open symbols, control enzyme; filled symbols, FSBA-treated enzyme (63% specific inhibition, 85% total inhibition). Circles, K^+ addition; triangles, Na^+ addition. The small linear decrease in fluorescence upon Na^+ addition was subsequently found to be due to the screening effect of a small amount of ultraviolet absorbing material in the stock NaCl solution.

nant alkali metal cation in the medium. A shift from one conformation to the other can be detected in several ways, one of which is to measure a previously described difference in the intrinsic tryptophan fluorescence of the two conformers [12]. Fig. 4 shows a plot of the percentage change in tryptophan fluorescence of native versus FSBA-modified $(Na^+ + K^+)$-ATPase. It is clear that covalent attachment of the reagent to the enzyme does not significantly affect its ability to undergo the K^+-induced conformational transition. It is also clear that the enzyme is predominantly in the E_1 conformation in the buffer used in this experiment, since Na^+ addition produces no marked fluorescence decrease [14]. This finding indicates that the reagent does not inactivate the alkali metal ion binding sites responsible for stabilizing the two detectable conformations, nor does it alter the relative distribution between E_1 and E_2 in the absence of alkali metal cations.

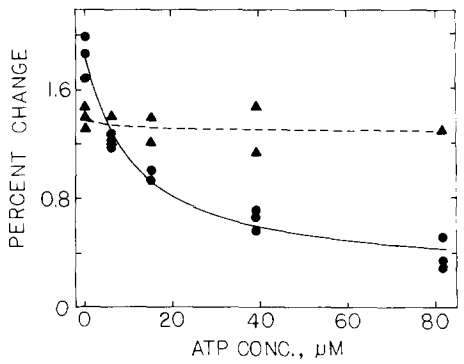

Fig. 5. Effect of FSBA treatment of $(Na^+ + K^+)$-ATPase on the ability of ATP to prevent the K^+-induced fluorescence increase. Enzyme (600 μg/ml) in 10 mM Bicine/1 mM EDTA (pH 8.5) was sampled into an equal volume of 1 mM dithiothreitol for a zero time control. The remainder was incubated with 1 mM FSBA as shown in Fig. 1. At 3 h intervals, an equal amount of FSBA was added until the enzyme had been exposed to a total of 3 μmol/ml over a period of 9 h. At this point, aliquots of the reaction mixture were taken into dithiothreitol as at zero time. Aliquots of all of these samples were then assayed for enzyme activity and used for fluorescence measurements in a medium containing 100 mM Tris/1 mM EDTA/0–82 μM ATP (pH 7.6) and 30 μg/ml $(Na^+ + K^+)$-ATPase. The fluorescence increase upon addition of 1 mM KCl is plotted for each ATP concentration. Circles, control enzyme; triangles, FSBA-treated enzyme. Specific inhibition was 78%, total inhibition 91% under these conditions.

Beauge and Glynn [15] have used tryptophan fluorescence measurements to show that addition of low concentrations of ATP to the enzyme, in the absence of Mg^{2+}, can prevent the K^+-induced transition to the E_2 form. This effect is presumably due to ATP stabilization of the E_1 conformation. Fig. 5 shows that when the $(Na^+ + K^+)$-ATPase is inhibited more than 90% by FSBA, this effect of ATP is also inhibited in proportion. The 13% nonspecific inhibition observed in this experiment is also reflected in a slight decrease in the ability of K^+ to induce the conformational transition (y-intercept). Taken together, these results further strengthen the conclusion of the previous study [5] that FSBA is a nucleotide site-specific probe for this enzyme.

Effect of FSBA treatment of $(Na^+ + K^+)$-ATPase on some of its 'partial' activities

Treatment of the $(Na^+ + K^+)$-ATPase with FSBA inhibits its Na^+-dependent ADP/ATP exchange activity in proportion to the loss in overall $(Na^+ + K^+)$-ATPase activity (Table I). Low concentrations of ATP present during the FSBA treatment protect against this inhibition, this being consistent with FSBA reacting at the high affinity (catalytic) ATP site of the enzyme.

Additional evidence supporting the concept that FSBA blocks the catalytic ATP-binding site of the $(Na^+ + K^+)$-ATPase is shown in Fig. 6. The Mg^{2+}- and K^+-dependent p-nitrophenylphosphatase activity of the $(Na^+ + K^+)$-ATPase has been shown

TABLE I

EFFECT OF FSBA TREATMENT ON THE ADP-ATP EXCHANGE ACTIVITY OF THE $(Na^+ + K^+)$-ATPase

Each sample was incubated for 1 h in 0.1 M NaCl after a single addition of FSBA or solvent only (control).

Additions	ATPase activity		ADP-ATP exchange	
	$\mu mol \cdot mg^{-1} \cdot h^{-1}$	%	$\mu mol \cdot mg^{-1} \cdot h^{-1}$	%
Control	960	100	41	100
0.5 mM FSBA	582	61	23	57
0.5 mM FSBA + 10 μM ATP	895	93	45	109
100 μM ATP	995	104	41	103
1 mM ATP	973	101	39	91

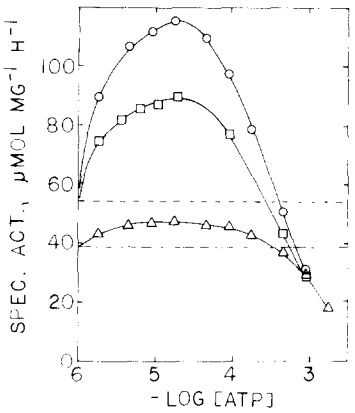

Fig. 6. Effect of FSBA treatment of $(Na^+ + K^+)$-ATPase on the ability of ATP to stimulate *p*-nitrophenylphosphatase activity in the presence of Na^+. $(Na^+ + K^+)$-ATPase (2 mg/ml) was treated with 1 mM FSBA in the medium used in Fig. 1 for 4 h. A second 1 mM addition of FSBA was followed by 16 h incubation. Samples (20 μl) were taken at zero time and 20 h and assayed for $(Na^+ + K^+)$-ATPase and *p*-nitrophenylphosphatase activities as described in Methods. The concentrations of Na^+ and K^+ were 75 mM and 15 mM, respectively, during the *p*-nitrophenylphosphatase assay. 3 mM phospho*enol*pyruvate and excess pyruvate kinase were also included in the *p*-nitrophenylphosphatase assay medium to prevent depletion of ATP at the lower concentrations used. ○, control enzyme; □, enzyme treated with FSBA + 1 mM ATP (nonspecific inhibition); △, FSBA-treated enzyme (52% specific inhibition, 88% total inhibition). The short-dashed line is the *p*-nitrophenylphosphatase activity of control enzyme assayed in the absence of ATP; the long-dashed line is the corresponding activity for FSBA-treated enzyme. ATP concentrations are in molar units. The results suggest that total inhibition of activity more closely reflects active site function than the calculated specific inhibition.

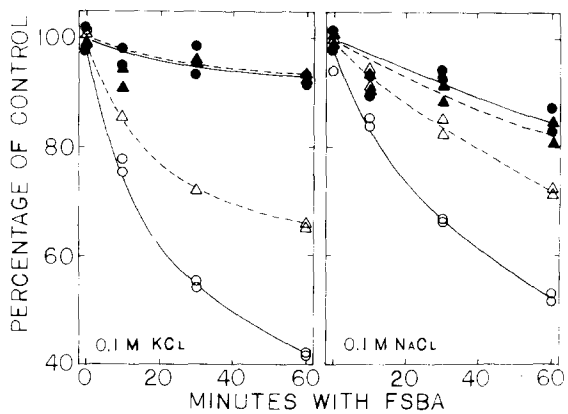

Fig. 7. Disproportionate inhibition of *p*-nitrophenylphosphatase and $(Na^+ + K^+)$-ATPase activities in FSBA-treated enzyme preparations. $(Na^+ + K^+)$-ATPase (1.3 mg/ml) was treated with 1.5 mM FSBA in the medium of Fig. 1, except that 0.1 M NaCl replaced KCl in the right-hand graph. 30-μl aliquots were taken at the times indicated into 500 μl 1 mM dithiothreitol and placed on ice until they could be assayed for both activities. Circles, $(Na^+ + K^+)$-ATPase activity; triangles, *p*-nitrophenylphosphatase activity; open symbols, FSBA alone; filled symbols, FSBA + ATP (5 mM in the left-hand figure, 20 μM in the right-hand figure).

previously to be stimulated by low concentrations of ATP in the presence of Na^+ [16]. This effect involves the use of the normal phosphorylation cycle of the enzyme to shift the distribution of the E_1 and E_2 conformers in favor of the E_2 form, which catalyzes the *p*-nitrophenylphosphatase activity [17]. This interpretation is consistent with the observation that the β,γ-methylene analog of ATP does not stimulate *p*-nitrophenylphosphatase activity under these conditions (results not shown). Fig. 6 shows the ATP-dependence of *p*-nitrophenylphosphatase activity in control and FSBA-treated $(Na^+ + K^+)$-ATPase. It is clear that the ability of ATP to stimulate *p*-nitrophenylphosphatase activity under these conditions is

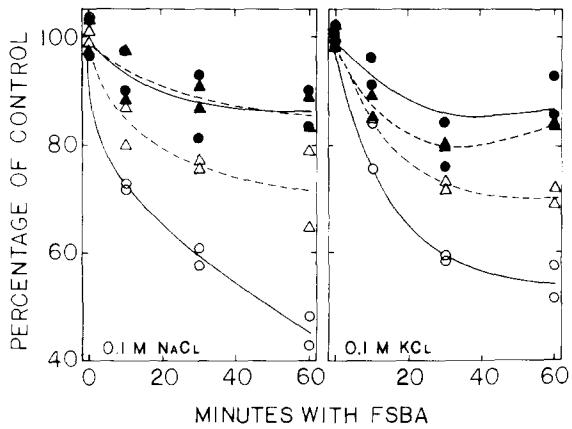

Fig. 8. Disproportionate inhibition of *p*-nitrophenylphosphatase and Na^+-ATPase activities in FSBA-treated enzyme preparations. $(Na^+ + K^+)$-ATPase was treated with FSBA as described in Fig. 7. 40-μl aliquots were taken into 100 μl 10 mM dithiothreitol at the times indicated. These samples were placed on ice until assayed for *p*-nitrophenylphosphatase and Na^+-ATPase activity. The latter assay used 20 μM ATP and omitted KCl from the standard $(Na^+ + K^+)$-ATPase assay medium. Symbols are as in Fig. 7, except that the [ATP] during FSBA treatment (filled symbols) was 5 mM in each case.

abolished in proportion to the extent of inhibition of $(Na^+ + K^+)$-ATPase activity.

Disproportionate inhibition of $(Na^+ + K^+)$-ATPase and p-nitrophenylphosphatase activities

Although the ATP stimulation of *p*-nitrophenylphosphatase activity, which involves the phosphorylation cycle of the enzyme, is inhibited in proportion to the extent of inhibition of $(Na^+ + K^+)$-ATPase activity, overall K^+-dependent *p*-nitrophenylphosphatase activity in the absence of Na^+ and ATP is not. Fig. 7 shows that the *p*-nitrophenylphosphatase is inhibited to a lesser extent than $(Na^+ + K^+)$-ATPase during the course of inactivation, whether the inactivation by FSBA is carried out in Na^+- or K^+-containing medium. Furthermore, this effect does not involve the need for a conformational transition during the $(Na^+ + K^+)$-ATPase cycle because the Na^+-ATPase activity of conformer E_1, measured at low ATP concentration and in the absence of K^+, is also inactivated more rapidly than the *p*-nitrophenylphosphatase activity (Fig. 8). The different degrees of inhibition observed must therefore provide information on the topology of the *p*-nitrophenylphosphate site relative to the ATP site on the enzyme. It is clear, however, that FSBA reaction with the active site does not affect the apparent K_m for *p*-nitrophenylphosphate of the enzyme activity surviving this treatment (Fig. 9).

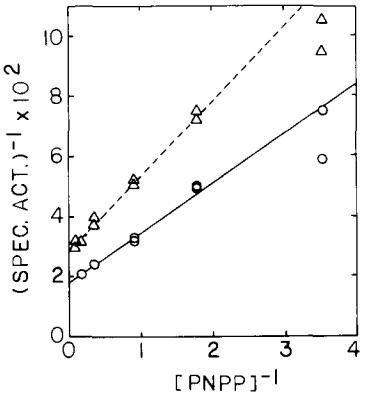

Fig. 9. Effect of FSBA treatment on K_m for *p*-nitrophenylphosphate (PNPP) of the surviving *p*-nitrophenylphosphatase activity. Enzyme (1.3 mg/ml) was treated for 1 h with 1.5 mM FSBA in the medium of Fig. 1. 30-μl aliquots were taken into 500 μl 1 mM dithiothreitol at zero time and after 1 h, placed on ice, and subsequently assayed for *p*-nitrophenylphosphatase activity. There was a 37% decrease in V_{max} with no significant change in apparent K_m. The K_m values calculated from these results are 0.92 mM (control) and 0.87 mM (FSBA-treated). *p*-Nitrophenylphosphate concentrations are in millimolar units. Two additional pairs of data points at even lower concentrations of *p*-nitrophenylphosphate than shown here fell on the fitted lines, but are not shown since they are well below K_m.

Discussion

The results presented above, together with those published earlier [5], clearly demonstrate that FSBA reacts with the high-affinity ATP binding (catalytic) site of the $(Na^+ + K^+)$-ATPase. The covalent complex formed between the enzyme and the nucleotide analog is stable to dithiothreitol, suggesting that a thiol-sulfonate derivative is not involved [18]. The fact that the effects seen in Figs. 5 and 6 correlate better with total inhibition of enzyme activity than with specific inhibition indicates that protection by ATP is not complete and suggests that under some conditions 'non-specific' inhibition includes some active site derivatization by FSBA. The slow rate of inactivation by FSBA is consistent with its low affinity for the $(Na^+ + K^+)$-ATPase, as previously described [5].

The value of approx. 0.4–0.5 h^{-1} for the rate of solvolysis of FSBA in Bicine buffer at 20°C, calculated from the enzyme inactivation kinetics, is consistent with the previously published value [5] of 0.76 h^{-1} measured with a fluoride electrode at 37°C in barbital buffer. This agreement increases our confidence that we are really measuring the first-order rate constant for enzyme inactivation by the differences of the slopes of the lines in Fig. 3. It is not clear why a second addition of FSBA reacts more slowly with the remaining enzyme, unless the rate of reaction of the second subunit of each $(Na^+ + K^+)$-ATPase dimer is reduced when the first subunit has already been derivatized. Competition from the sulfonate solvolysis product is not likely to be the cause, however, since such competition should introduce curvilinearity (concave downward) into the plot. It is possible that fluoride, the other product of the solvolysis of reagent, might complex the unreacted $(Na^+ + K^+)$-ATPase resulting

in lowered reactivity, but this should also result in some curvilinearity. A remaining possibility is that there are two populations of enzyme molecules in the preparation, with intrinsically different reactivities with the probe. If this is so, they are unlikely to be the E_1 and E_2 conformers themselves, because the incubation with FSBA is carried out under conditions where one or the other conformer predominates [12].

It is clear that specific attachment of FSBA to the enzyme does not noticeably affect K^+ binding to the $(Na^+ + K^+)$-ATPase, as reflected by the normal shift in the enzyme conformation from E_1 to E_2 when K^+ is added. This observation indicates that the enzyme's gross conformational flexibility is also not impaired during inhibition of overall $(Na^+ + K^+)$-ATPase activity by FSBA. The ability of ATP binding to the high-affinity site to alter the conformer distribution is, however, inhibited in proportion to overall derivatization of the enzyme. This inhibition is also reflected in the decreased ability of ATP to generate the E_2 phosphatase via the phosphorylation cycle, as well as to participate in the ADP-ATP exchange phenomenon.

The inability of FSBA to inhibit the *p*-nitrophenylphosphatase activity to the same extent as the $(Na^+ + K^+)$-ATPase activity must indicate that the active site for *p*-nitrophenylphosphate is somewhat different from that for ATP, yet close enough for its catalytic activity to be affected by FSBA's derivatization of the ATP binding site. High concentrations of ATP or Na^+ presumably inhibit *p*-nitrophenylphosphatase activity because these ligands shift the enzyme to the E_1 conformer, which lacks *p*-nitrophenylphosphatase activity. If reaction with FSBA 'froze' the enzyme into a roughly equal mixture of E_1 and E_2, one might obtain the results seen in Figs. 7 and 8. However, the results of Fig. 4 show that the FSBA-treated enzyme is able to equilibrate freely between E_1 and E_2. Therefore, the incomplete inhibition of the *p*-nitrophenylphosphatase activity is apparently not a consequence of inhibition of conformational change.

The most probable explanation is that FSBA reaction with the catalytic site of the E_1 or E_2 monomer leaves part of the catalytic site or another site on the enzyme still capable of *p*-nitrophenylphosphatase activity, albeit with reduced activity. Recent work has shown that the dephosphorylation step in the $(Na^+ + K^+)$-ATPase mechanism is inhibited by the antibiotic duramycin without effect on the *p*-nitrophenylphosphatase activity [19]. In addition, monoclonal antibodies have been found that are specific for the ATP binding site of the $(Na^+ + K^+)$-ATPase, but do not inhibit *p*-nitrophenylphosphatase activity [20]. These findings taken together raise the possibility that the *p*-nitrophenylphosphatase site is either only partially overlapping with the ATP binding site or not physically contiguous with that site (though influenced by derivatization of the ATP site). The loss of *p*-nitrophenylphosphatase activity observed with FSBA must involve an effect on the catalytic mechanism itself, rather than *p*-nitrophenylphosphate binding, since the apparent affinity of the surviving *p*-nitrophenylphosphatase activity for substrate (as reflected in its K_m value) is unchanged within the error of the methodology. This separate site explanation is compatible with the current view that the catalytic subunits of the dimeric enzyme act independently of one another.

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