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N-Acetylimidazole Inactivates Renal Na,K-ATPase by Disrupting ATP Binding to the Catalytic Site†

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ABSTRACT: Treatment of renal Na,K-ATPase with *N*-acetylimidazole (NAI) results in loss of Na,K-ATPase activity. The inactivation kinetics can be described by a model in which two classes of sites are acetylated by NAI. The class I sites are rapidly reacting, the acetylation is prevented by the presence of ATP ($K_{0.5} \simeq 8\ \mu M$), and the inactivation is reversed by incubation with hydroxylamine. These data suggest that the class I sites are tyrosine residues at the ATP binding site. The second class of sites are more slowly reacting, not protected by ATP, nor reversed by hydroxylamine treatment. These are probably lysine residues elsewhere in the protein. The associated K-stimulated *p*-nitrophenylphosphatase activity is inactivated by acetylation of the class II sites only; thus the tyrosine residues associated with ATP binding to the catalytic center are not essential for phosphatase activity. Inactivated enzyme no longer has high-affinity ATP binding associated with the catalytic site, although low-affinity ATP effects (inhibition of phosphatase and deocclusion of Rb) are still present. The inactivated enzyme can still be phosphorylated by P_i, occlude Rb^+ ions, and undergo the major conformational transitions between the E_1 Na and E_2 K forms of the enzyme. Thus acetylation of the Na,K-ATPase by NAI inhibits high-affinity ATP binding to the catalytic center and produces inactivation.

The Na,K-ATPase (EC 3.6.1.3) is the plasma membrane enzyme that performs the transmembrane-coupled active transport of Na^+ and K^+ ions (Glynn, 1985; Kaplan, 1985; Norby, 1983). The biochemical activities catalyzed by the enzyme and their involvement in the associated transport reactions have been the focus of much work (Cantley, 1981; Kaplan, 1983). The enzyme subunit composition (Jorgensen, 1983, 1974b) and the primary sequence of the α subunit from several sources including torpedo (Kawakami et al., 1985),

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sheep kidney (Shull et al., 1985), and rat (Schneider et al., 1985) have recently been determined. There is, however, little detailed information available on which specific regions of the protein are essential for binding of the various ligands or on which residues are specifically involved in function.

One approach toward obtaining this information is the use of chemical reagents to modify the protein at particular regions or residues. Affinity labeling can be employed where the structural similarity of a reagent to a ligand favors the covalent labeling reaction at the ligand binding site. Another approach is to use a group or amino acid specific reagent in the expectation that specific residues at catalytic centers or ligand binding sites are more reactive toward the reagent than others or, if as reactive, that they are specifically protected from the reagent by the ligand. This approach has enabled a number of candidate residues in the primary sequence to be identified as important in the binding sites of various ligands or in the different conformational changes. The information obtained until now from a chemical approach in the study of Na,K-ATPase structure–function relations has recently been reviewed (Pedemonte & Kaplan, 1990).

Early work on the sodium pump reported that acetylation of tyrosine residues of red cell Na,K-ATPase by *N*-acetylimidazole (NAI)[1] produced inactivation of the enzyme (Masiak & D'Angelo, 1973, 1975). The inactivation was prevented by the simultaneous presence of ATP and these workers suggested that an important tyrosine residue was present at the nucleotide binding domain. Later work with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), a potential tyrosine-modifying agent, also described ATP-protectable inactivation of eel electric organ Na,K-ATPase (Cantley et al., 1978). Part of the inactivation was reversed by 2-mercaptoethanol, suggesting the involvement of both tyrosine and cysteine residues. However, subsequent work by Grosse et al. (1978) obtained evidence only for modification of thiols by NBD-Cl. Since the red cell Na,K-ATPase is of extremely low specific activity [about 0.03% of the purified renal enzyme, Kaplan and Kenney (1985)] and conclusions from recent work were controversial with respect to tyrosine involvement at the nucleotide binding site, we decided to investigate the effects of NAI using purified renal Na,K-ATPase. We describe here the effects of NAI treatment on the properties of renal Na,K-ATPase and provide evidence for ATP-protectable inactivation of the enzyme. The loss of enzymatic ATPase activity is due to removal of the high-affinity ATP binding capacity. A preliminary report of some of these data has been presented (Argüello & Kaplan, 1989).

Materials and Methods

Materials. FITC, ATP, *p*-NPP, BSA, AMP, ADP, NH_2OH, and trypsin (type XI) were from Sigma Chemical Co. [^{32}P]P_i, [^{86}Rb]RbCl, and [^{3}H]ADP were from Amersham. Acrylamide, bis(acrylamide), and the protein molecular weight standards were from Bio-Rad Labs. All other chemicals were of the highest quality available.

Enzyme Isolation. Na,K-ATPase was purified from dog kidney according to Jorgensen (1974a) with the modifications of Liang and Winter (1976). After ultracentrifugation, the enzyme was washed with 25 mM imidazole and 1 mM EDTA, pH (20 °C) 7.5 (buffer C), before storing at 5 °C. Protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as standard. The Na,K-ATPase activity of the enzyme used in these studies was about 15 μmol of P_i mg^{-1} min^{-1}, assayed as described below.

Treatment with NAI. NAI was synthesized by acetylation of imidazole in a large excess of acetic anhydride at room temperature for 2 h. The remaining acetic anhydride was then removed by rotary evaporation and the resulting NAI quantified by its UV absorption at 235–255 nm (Stadtman & White, 1953). The yield was greater than 95% and stock solutions of NAI in dimethyl sulfoxide (DMSO) were stored at −20 °C in a desiccator. Unless specified, the enzyme (0.1–0.5 mg/mL) was treated in 50 mM sodium borate buffer (16 mM Na^+), pH (20 °C) 7.5, and 2 mM EDTA, for 1 h at 0 °C. NAI in DMSO was added at the desired concentration. The final concentration of DMSO was lower than 5% in the treatment medium and less than 0.1% in the Na,K-ATPase assay medium. The reaction was stopped by dilution in buffer C and centrifugation or by dilution (1:40) in the ATPase assay medium; both methods gave the same results. Determination of NAI stability was performed by incubating NAI in the treatment medium (50 mM sodium borate buffer, pH (20 °C) 7.5, and 2 mM EDTA) at 0 °C and measuring NAI concentrations by UV absorption (235–255 nm) at different times. The $t_{1/2}$ for hydrolytic breakdown was 3 h. The NAI stability in water was very dependent on the temperature and on the buffer characteristics (Riordan et al., 1965; Connellan & Shaw, 1970).

Assays. The assay medium for Na,K-ATPase activity was (mM) EGTA, 0.5; NaCl, 130; KCl, 20; $MgCl_2$, 3; ATP, 3; and imidazole, 50, pH (20 °C) 7.2, and 0.3 mg/mL BSA and approximately 0.5 μg/mL enzyme protein. The assay was performed at 37 °C for 15 min and the P_i released determined by the method of Brotherus et al. (1981). The Na,K-ATPase activity was calculated from the difference between the ATP hydrolysis measured in the absence and in the presence of 5×10^{-4} M ouabain.

The *p*-nitrophenylphosphatase (*p*-NPPase) activity assay medium was (mM) EGTA, 0.5; $MgCl_2$, 5; KCl, 20; tris(*p*-nitrophenyl) phosphate (*p*-NPP), 3; and imidazole, 50, pH (20 °C) 7.2, and 0.3 mg/mL BSA and about 1.5 μg/mL enzyme protein. The assay was performed at 37 °C for 10 min and it was stopped with 1 volume of 0.2 M NaOH, 2.5% sodium dodecyl sulfate (SDS), and 4 mM EDTA. The *p*-nitrophenol produced was measured at 410 nm (Drapeau & Blostein, 1980). The activity was estimated from the difference between the *p*-NPP hydrolysis measured in the absence and the presence of 5×10^{-4} M ouabain.

ADP binding was determined following the method of Robinson (1980) in a medium containing (mM) Hepes/imidazole, 30, pH (20 °C) 7.2; Na–EDTA, 0.1; NaCl, 5; and [^{3}H]ADP, 0.01, and 0.5 mg/mL enzyme protein. The suspension was shaken at 0 °C during 30 s and centrifuged at 400000*g* for 5 min. The pellet was resuspended in 0.5 M NaOH and the radioactivity and protein were determined. Radioactivity bound to the enzyme in the presence of 0.5 mM ATP was subtracted from the experimental values as a correction for nonspecific binding.

Phosphorylation with P_i was performed as described by Askari and Huang (1982) in a medium containing (mM) $MgCl_2$, 1; [^{32}P]P_i, 0.4; histidine, 15; Tris, 25, pH (20 °C) 7.2; and ouabain, 1 (when indicated), and 0.5 mg/mL enzyme. After 10 min at 0 °C or 4 min at 37 °C with ouabain, the reaction was stopped with 9 volumes of 5.5% trichloroacetic

[1] Abbreviations: NAI, *N*-acetylimidazole; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; P_i, inorganic phosphate; *p*-NPPase, *p*-nitrophenylphosphatase; *p*-NPP, tris(*p*-nitrophenyl) phosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

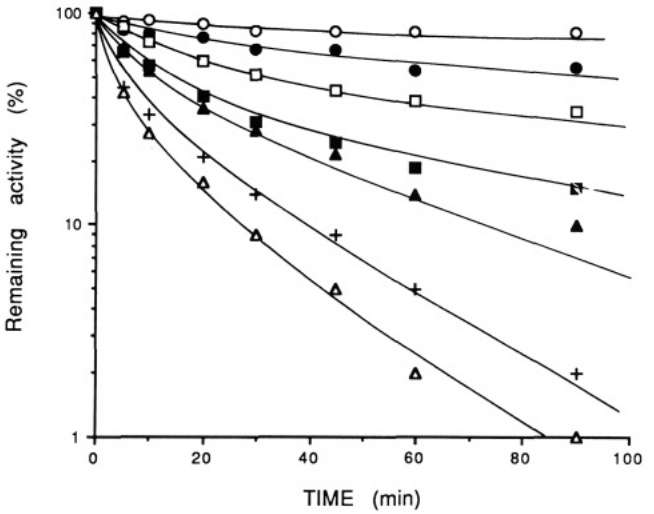


FIGURE 1: Time course of Na,K-ATPase inactivation by NAI. 0.05 mg/mL enzyme was incubated during 60 min at 0 °C, with the following NAI concentrations (mM): 1 (○), 5 (●), 10 (□), 25 (■), 50 (▲), 100 (+), and 200 (△). At the indicated times, aliquots were assayed for Na,K-ATPase activity assay. The points are the mean of four experiments performed with duplicate samples. The continuous lines through the data were drawn according to eq 4 (see text). $\alpha = 0.48$.

acid and 3 mM P_i. The samples were filtered through Millipore filters (pore size 0.45 μm); the filters were washed with the same acid medium and counted in a scintillation counter.

Rb^+ binding was measured in a medium containing (mM) Hepes/imidazole, 30, pH (20 °C) 7.2; Tris–EDTA, 0.1; and [^{86}Rb]RbCl, 0.2, and 0.4 mg/mL enzyme. The samples were incubated for 5 min at room temperature and then centrifuged at 400000*g* for 5 min. The pellets were resuspended in 0.5 M NaOH and the radioactivity and protein were determined. When the effects of ATP on Rb^+ binding were measured, 3 mM ATP was present in the media.

FITC treatment of the enzyme was performed at room temperature, for 30 min, in a medium containing (mM) Tris-HCl, 50, pH (20 °C) 9.0; EDTA, 1; NaCl, 80; and FITC, 0.004, and 1 mg/mL enzyme. The excess FITC was removed by diluting the sample with 1 mg/mL BSA in buffer C and centrifuging at 400000*g* for 5 min. The pellet was washed twice by centrifugation and resuspension in buffer C and finally resuspended in buffer C.

SDS–PAGE was carried according to Laemmli (1970) in 7.5% acrylamide gels. Protein bands were observed by staining the gels with Coomassie brilliant blue. FITC fluorescence was visualized in unstained gels by illumination with a long-wavelength (360 nm) UV lamp and photographed by using TRI-X film and a yellow filter.

Trypsin treatment of the enzyme was performed as described by Jorgensen (1975) in the presence of 40 mM NaCl plus 1 mM ADP or 40 mM KCl. Control enzyme was treated with a 75:1 (μg/μg) enzyme:trypsin ratio and NAI-treated enzyme with a 200:1 (μg/μg) enzyme:trypsin ratio.

Putative O-acetylation of tyrosine residues was reversed by treating 0.1 mg/mL NAI-modified enzyme (25 mM NAI, 0 °C, 60 min) with 1 M NH_2OH, pH (20 °C) 10.0, during 30 min (Balls & Wood, 1956). Control enzyme was subjected to the same treatment and its activity was not reduced by more than 15%.

RESULTS

Treatment of the enzyme with NAI resulted in the inhibition of the Na,K-ATPase activity and, to a lesser extent, the *p*-NPPase activity (Figures 1 and 3). These activities were not

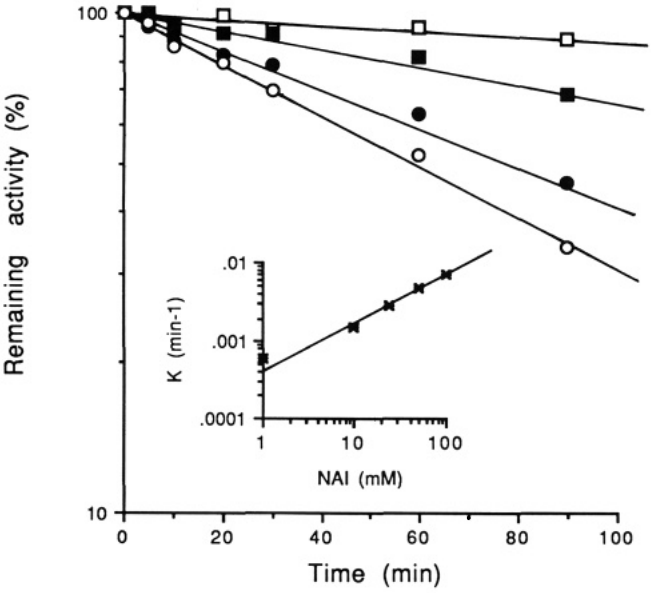


FIGURE 2: Time course of the *p*-nitrophenylphosphatase inactivation by NAI. 0.15 mg/mL enzyme was incubated during 60 min at 0 °C, with the following NAI concentrations (mM): 10 (□), 25 (■), 50 (●), and 100 (○). At the indicated times, aliquots were taken for *p*-NPPase determination. The points are the mean of three experiments performed with duplicate samples. The continuous lines through the data were drawn according to eq 6 (see text). Inset: Logarithmic plot of the apparent first-order rate constant of inactivation ($k_2'' = k_2[I]^n$) versus NAI concentration. The line was drawn with the values 2.1×10^{-4} min^{-1} for k_2 and 0.76 for n.

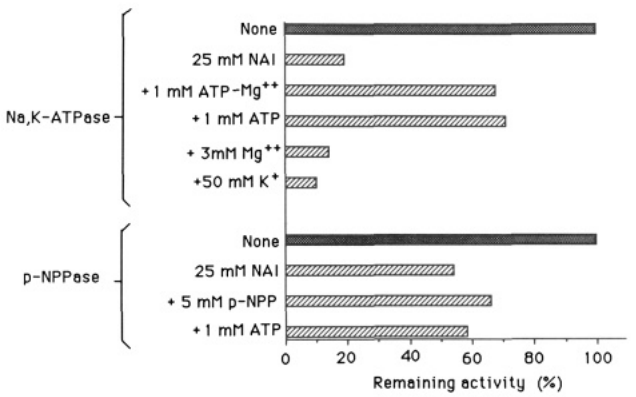


FIGURE 3: Effect of some physiological ligands on NAI inactivation of the Na,K-ATPase. The enzyme was incubated in 50 mM borate–Na^+, 2 mM EDTA, pH (20 °C) 7.5, 60 min at 0 °C, with 25 mM NAI and the specific ligand. When *p*-NPP protection of *p*-NPPase inactivation was tested, 50 mM borate–K, 2 mM EDTA, pH (20 °C) 7.5, 60 min, 0 °C, was used. The Na,K-ATPase activity or *p*-NPPase activity was measured after the treatment. Values are the mean of four experiments performed in duplicate.

recovered after washing the enzyme several times (by centrifugation and resuspension) with buffer C. 2% 2-mercaptoethanol also did not reverse the inhibition (not shown).

Kinetics of Inactivation. The time course of the Na,K-ATPase inactivation at various NAI concentrations is shown in Figure 1. The I_{50} value for NAI after 60 min of incubation at 0 °C was 4.5 mM (see also Figure 4) but high NAI concentrations were required to inactivate completely the enzyme. At these high concentrations NAI might be acetylating numerous other residues; we therefore treated the enzyme with 25 mM NAI for 60 min (which yields enzyme that is about 80% inactivated) for most of our experiments. The nonlinearity of the curves in Figure 1 suggests that either different classes of residues are being modified or NAI is decomposing

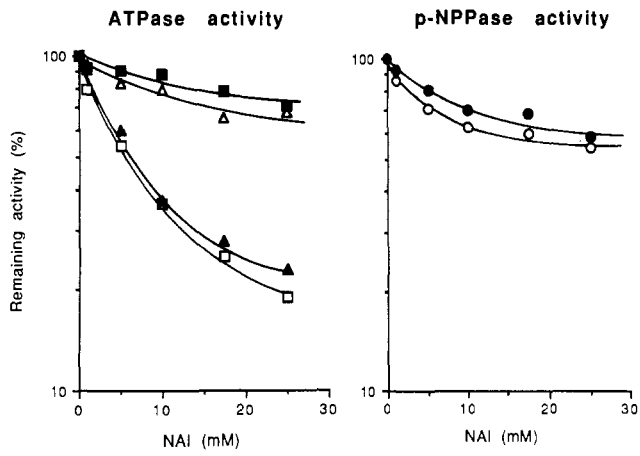
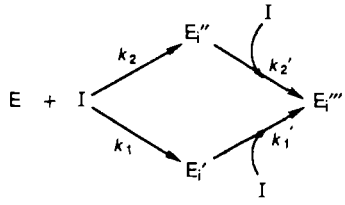


FIGURE 4: Effect of adenine nucleotides on NAI inactivation of the Na,K-ATPase and *p*-NPPase. Enzyme was incubated in presence of 25 mM NAI or 25 mM NAI plus 1 mM adenine nucleotide; after 60 min at 0 °C, Na,K-ATPase and *p*-NPPase activities were determined. Na,K-ATPase: NAI (□); NAI + ATP (□); NAI + ADP (△); NAI + AMP (▲). *p*-NPPase: NAI (○); NAI + ATP (●). Points are the mean of three experiments performed in duplicate.

Scheme I



during the treatment. This latter possibility was examined, under the conditions of the studies in Figure 1. Our results indicated that 50% of the initial NAI in solution decomposed in 2.8 h ($k = 4.2 \times 10^{-3}$ min^{-1}). Such a rate is too low to account for the curvature of the data in Figure 1. The curvature can, however, be accounted for by a two-step inactivation process in which NAI independently acetylates two sites on the enzyme. This reaction can be described by Scheme I, where I is NAI, which acetylates the enzyme E to produce E_i' or E_i'', depending on which site has been modified. According to this model, E_i' and E_i'' are assumed to have partially inhibited activities (Ray & Koshland, 1961). Then either of these inhibited forms of the enzyme can react with a second NAI molecule at the other site to give E_i''', which is fully inhibited.

The formation rate of the final inactivated enzyme is

$$\frac{d[E_i'''']}{dt} = -\left(\frac{d[E_i']}{dt} + \frac{d[E_i'']}{dt}\right) \quad (1)$$

$$-\frac{d[E]}{dt} = \frac{d[E_i']}{dt} + \frac{d[E_i'']}{dt} \quad (2)$$

$$-\frac{d[E]}{dt} = k_1[E][I] + k_2[E][I] \quad (3)$$

Upon integration the fraction of enzyme remaining active at time t is given by

$$[E]/[E_0] = \alpha e^{-k_1''t} + (1 - \alpha)e^{-k_2''t} \quad (4)$$

where $k_1'' = k_1[I]$ and $k_2'' = k_2[I]$. By graphical analysis the values of k_1'', k_2'', and α can be obtained. The lines in Figure 1 were drawn from this equation and a very good fit was obtained. This analysis does not take into account the decomposition of NAI during the enzyme inactivation. However, the maximum change in [NAI] during this period is about 30%, and considering the quality of the fit obtained in Figure 1, we have assumed that the influence of NAI decomposition on the kinetics of inactivation is minimal.

It is possible from these data to estimate the average order of the reaction ("n") with respect to NAI for each of these independent acetylation sites. If it is assumed that there are n molecules of NAI that react with any single kind of site, then

$$k'' = k[I]^n \quad (5)$$

and

$$\log k'' = \log k + n \log [I] \quad (6)$$

From this equation linear relationships were obtained between the logarithms of the obtained constants k_1'' and k_2'' and log [NAI] (not shown). The slopes were close to unity, 0.85, for the slower reacting site ($k_2 = 7.6 \times 10^{-4}$ min^{-1}) and 1.07 for the faster one ($k_1 = 3.9 \times 10^{-5}$ min^{-1}), consistent with the acetylation of only one site of each class producing the associated inactivation.

Figure 2 shows the time course of the *p*-NPPase inactivation with a range of NAI concentrations. The I_{50} for NAI after 60 min of incubation at 0 °C was 26 mM (see also Figure 4). The logarithmic plot of the remaining *p*-NPPase activity versus time gave a linear relationship at all NAI concentrations tested (Figure 2). If we assume that the acetylation reaction producing E_i' (the rapidly reacting site) does not affect the *p*-NPPase activity, then *p*-NPPase will be inactivated following this reaction:

$$E + I \xrightarrow[k_2]{} E_i''$$

In this case, $[E] = [E_0]e^{-k_2''t}$, where $k_2'' = k_2[I]$. The rate constants calculated from these exponentials (Figure 2) were similar to those of the more slowly reacting site obtained from the ATPase inactivation data. The insert in Figure 2 shows the linear relationship between log k_2'' and log [NAI] with a slope $n = 0.76$. The fact that the slope is close to unity is consistent with the modification of only one site (or a single class of sites) for inactivation of the *p*-NPPase. Furthermore, the values for the rate constant for *p*-NPPase inactivation from the intercept of the insert in Figure 2 agree quite well with the intercept value (7.6×10^{-4} min^{-1}) of the slower reacting site (k_2) determined from the Na,K-ATPase inactivation. Thus the model that accounts quite well for the inactivation kinetics of Na,K-ATPase activity also fits the *p*-NPPase inactivation if one of the two classes of sites involved in Na,K-ATPase inactivation (the rapidly inactivated site) is not essential for *p*-NPPase activity.

Ligand Effects on Inactivation. Having established the kinetics of inactivation of the Na,K-ATPase by NAI, it was of interest to see if any of the physiological ligands of the sodium pump affected the inactivation kinetics. In Figure 3, the protective effects of the various ligands are shown. ATP or Mg–ATP were able to reduce (or slow) the inactivation, while Mg^{2+} alone was without effect. K^+, at high concentrations (50 mM) sufficient to transfer the enzyme fully into an E_2 (K) form (Glynn, 1985; Jorgensen, 1975), was also without effect. The effects of ATP and *p*-NPP on the inactivation of *p*-NPPase activity were also examined and only a small protective effect by *p*-NPP was seen. This suggests that the ATP-protectable, NAI-sensitive residue that is important for ATPase activity is not involved in phosphatase activity. Because the effects of Na^+ or Mg^{2+}–P_i could not be studied in the 50 mM sodium borate (16 mM Na^+) buffer, the effects of Mg^{2+}–P_i or Na^+ were examined by treating the enzyme with NAI in *N*-methyglucamine–borate buffer.[2] Under these

Table I: ADP Binding and P_i Phosphorylation of NAI-Treated Enzyme[a]

	ATPase activity (%)	ADP binding [10 μM] (nmol/mg)	phosphorylation (nmol/mg)	
			P_i	P_i + ouabain
control	100	2.26 ± 0.16	1.09 ± 0.09	1.90 ± 0.12
25 mM NAI	21 ± 2	0.70 ± 0.16 (30%)[b]	0.85 ± 0.13	1.82 ± 0.09
25 mM NAI + 1 mM ATP	65 ± 4	1.54 ± 0.25 (68%)	0.81 ± 0.11	1.93 ± 0.11

[a] The inactivation of the enzyme by NAI, ADP binding, and P_i phosphorylation was performed as indicated under Materials and Methods. Values shown are the mean ± SE of four experiments performed in triplicate. [b] Percentage with respect to the control.

Table II: Rb^+ Binding to NAI-Treated Enzyme[a]

	Rb^+ binding (nmol/mg)			(occluded Rb^+)/[P_i binding (+ouabain)][b]
	none	+50 μM ATP	+3 mM ATP	occluded Rb^+
control	8.38 ± 1.04	6.18 ± 1.37	3.75 ± 0.69	4.63	2.43
25 mM NAI	9.98 ± 2.36	7.72 ± 1.23	4.91 ± 0.70	5.07	2.74
25 mM NAI + 1 mM ATP	8.21 ± 1.22	6.85 ± 1.1	3.71 ± 0.68	4.57	2.38

[a] The inactivation of the enzyme and Rb^+ binding were performed as indicated under Materials and Methods. Values shown are the mean ± SE of four experiments performed in triplicate. NAI enzyme had 20–24% of the initial Na,K-ATPase activity, while ATP-protected, NAI-treated enzyme had 64–70% of the initial activity. [b] Ratio indicates the number of Rb^+ occluded by each enzyme molecule.

conditions, neither 3 mM Mg^{2+}–P_i nor 150 mM Na^+ had a protective action (not shown).

Figure 4 shows the effects of adenine nucleotides on NAI inactivation of Na,K-ATPase and *p*-NPPase. Nucleotides that bind to the enzyme with high affinity (ATP and ADP) prevented the Na,K-ATPase inactivation by NAI, while AMP, which does not bind to the enzyme, had no effect. The $K_{0.5}$ for ATP protection of ATPase was about 8 μM (25 mM NAI, 60 min, 0 °C) (Figure 5). Since the protection seems to be mediated by the binding of adenine nucleotide to the enzyme, these results may reflect the acetylation of a residue that is close to or at the high-affinity ATP binding site. In spite of the effectiveness of ATP in protecting the Na,K-ATPase activity from inactivation by NAI, it only slightly reduced the extent of *p*-NPPase inactivation. This suggests that ATP prevents reaction of only the more rapidly reacting of the two classes of NAI acetylating sites. In accordance with this, when the time course of the enzyme inactivation in the presence of ATP was studied (data not shown), single-exponential kinetics ($k = 9.5 \times 10^{-3}$ min^{-1}, treatment with 25 mM NAI + 1 mM ATP) were obtained. This inactivation constant is very similar to the value (12.9×10^{-3} min^{-1}) for k_2'' (the more slowly acetylated site) obtained for the enzyme treated with the same NAI concentration in the absence of ATP.

Properties of the Inactivated Enzyme. The relationship between the NAI acetylating site and nucleotide binding site of the enzyme was also evident in the determination of high-affinity [^{3}H]ADP binding. Table I shows the values of ADP binding and the phosphorylation level from inorganic phosphate of control and NAI-inactivated enzyme. NAI treatment diminished ADP binding to the same extent as the Na,K-ATPase activity. Even though NAI treatment modified the binding of ATP, it did not affect the phosphorylation site since phosphorylation by P_i was not altered (Table I).

It is known that ATP, acting with low affinity on an E_2 form of the enzyme, is able to stimulate the deocclusion of Rb^+ (Glynn & Richards, 1985; Glynn, 1985; Forbush, 1987) and also to inhibit *p*-NPPase activity (Davis & Robinson, 1988; Huang et al., 1985). The data in Table II show that Rb^+ occlusion was not altered by NAI treatment and also that ATP can bind, with low affinity, to the enzyme and release occluded Rb^+ ions. That NAI did not alter this low-affinity ATP effect was confirmed when ATP inhibition of *p*-NPPase activity of

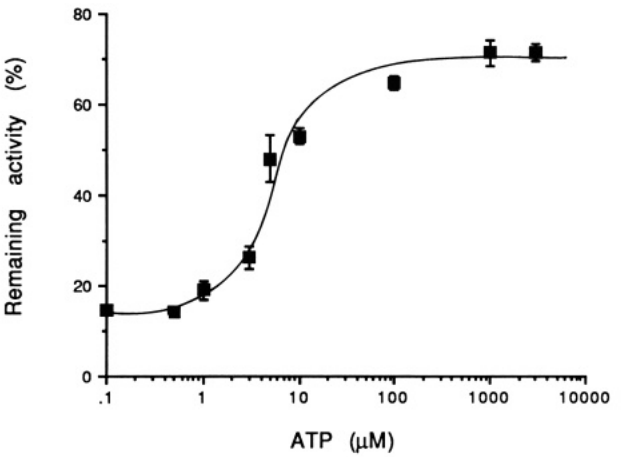


FIGURE 5: Effect of ATP on the Na,K-ATPase inactivation by NAI. The enzyme (0.05 mg/mL) was incubated during 60 min at 0 °C with 25 mM NAI plus the indicated ATP concentration. Na,K-ATPase was measured after incubation. Points are the mean ± SE of three experiments.

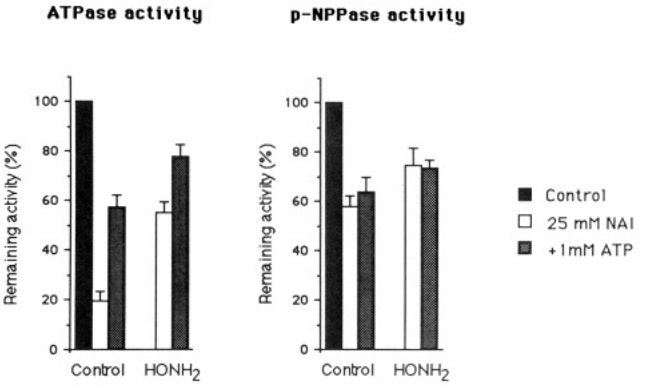


FIGURE 6: O-Deacetylation of NAI-treated enzyme by NH_2OH. 0.1 mg/mL enzyme was treated with 25 mM NAI or 25 mM NAI + 1 mM ATP during 60 min at 0 °C. Aliquots were taken for ATPase and *p*-NPPase assays. The samples were then treated with 1 M NH_2OH, pH 10.0, during 30 min at room temperature. After this treatment, ATPase and *p*-NPPase activities were measured again. The bars indicated the mean ± SE of four experiments.

NAI-treated enzyme was studied. In this situation, ATP ($I_{50} \simeq 1$ mM) was able to inhibit the *p*-NPPase activity in the same way as in the control, NAI-treated, and ATP-protected enzymes (data not shown).

O-Acetyl (esters) groups resulting from acetylation of tyrosine residues may be removed by treatment with NH_2OH

[2] In *N*-methylglucamine–borate buffer the half-time for decomposition of NAI was 2 h.

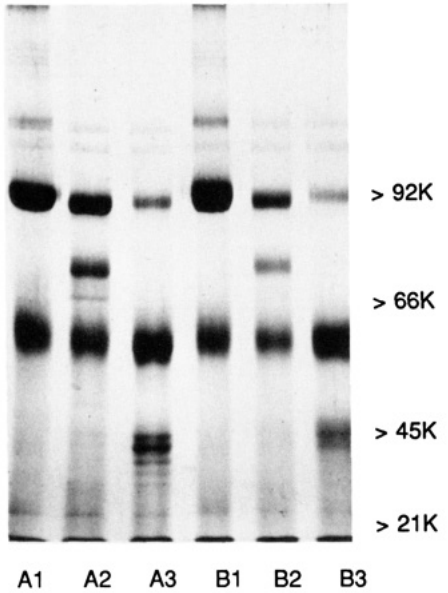


FIGURE 7: SDS–PAGE of control (A1–3) and NAI-treated (B1–3) enzymes after trypsin treatment. NAI enzyme was prepared, treated with trypsin in the presence of either Na^+ plus ADP (2) or K^+ (3), and subjected to SDS–PAGE as indicated under Materials and Methods. Control and NAI-treated enzymes not treated with trypsin were run in lines A1 and B1, respectively. The positions of molecular weight markers, run in the same gel, are indicated.

(Balls & Wood, 1956; Simpson et al., 1963) while N-acetylated residues (amides) from acetylation of lysines are resistant to NH_2OH. When NAI-treated enzyme was subjected to treatment with NH_2OH, a partial recovery of the Na,K-ATPase was observed (Figure 6). In the case of the *p*-NPPase inactivation, almost no recovery was obtained. Thus the *p*-NPPase inactivation is due either to acetylation of inaccessible tyrosines or to acetylation yielding hydroxylamine-insensitive amides with lysine residues (Lumbdlad & Noyes, 1985; Perlmann, 1966; Connellan & Shaw, 1970). The ATPase inactivation occurs via hydroxylamine-sensitive and -insensitive residues. This behavior of the NAI-treated enzyme with respect to the deacetylation with NH_2OH is consistent with our model based on the time course of the inactivations (see Scheme I). That is, only one amino acid seems to be involved in the *p*-NPPase inactivation (evidently not NH_2OH sensitive) and more than one residue is involved in the inhibition of the ATPase activity.

It has been observed that the presence of Na^+ plus ADP stabilizes an E_1 form of the enzyme while K^+ stabilizes an E_2 form [see Glynn (1985)]. We were interested in whether the NAI-treated enzyme was capable of undergoing the conformational transitions between these two enzyme forms. Trypsin treatment of the enzyme produces different electrophoretic patterns dependent on the protein conformation (Jorgensen, 1974a). Control and NAI-treated enzyme were treated with trypsin in the presence of Na^+ plus ADP (E_1 form) or K^+ (E_2 form) and run on SDS–PAGE (Figure 7). The NAI-treated enzyme was more sensitive to the trypsin treatment, undergoing greater degradation than the control enzyme at the same ATPase protein:trypsin ratio. In order to obtain comparable levels of degradation, NAI enzyme was treated with trypsin in a 200:1 ratio while control enzyme was treated in a 75:1 ratio. Under these conditions there were no major differences between control and NAI enzyme, indicating that the NAI enzyme is still able to undergo the conversion between the major E_1 and E_2 forms.

It is known that FITC binds to Lys-501, inactivates the Na^+,K^+-ATPase, and prevents high-affinity ATP binding to

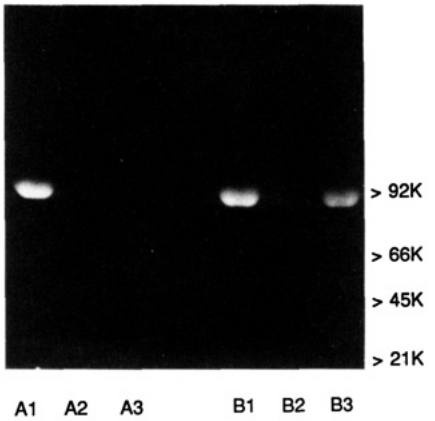


FIGURE 8: Incorporation of FITC into control (A1–3) and NAI-treated (B1–3) enzymes. After the acetylation with 25 mM NAI, during 60 min at 0 °C, the enzymes were treated with 4 μM FITC (1), 4 μM FITC + 3 mM ATP (2), or 4 μM FITC + 50 μM ATP (3) and subjected to SDS–PAGE as indicated under Materials and Methods. The fluorescent bands in unstained gels were visualized by illumination with a long-wavelength UV lamp.

the enzyme (Karlish, 1980; Farley et al., 1984; Kirley et al., 1984). Since this inactivation is prevented by ATP [$K_{0.5}$ = 8 μM; Sen et al. (1981)], it has been assumed that Lys-501 is associated with the ATP binding site. Recent studies have shown the ATP-sensitive modification of other lysine residues (Lys-480 and Lys-766), in addition to Lys-501, by FITC (Xu, 1989). However, the modification of Lys-501 accounted for at least 50% of the fluorescence associated with the Na,K-ATPase after FITC treatment (Xu, 1989). Since NAI might be acetylating a Lys residue in the ATP high-affinity binding site as suggested perhaps by the incomplete deacetylation by NH_2OH (Figure 6), FITC binding to the NAI-treated enzyme was studied (Figure 8). NAI-treated enzyme was still able to bind FITC, making it unlikely that acetylation of Lys-501 occurred with NAI treatment. We also examined the prevention of FITC binding by ATP (3 mM and 50 μM) with native and NAI-treated enzyme. Three millimolar and 50 μM ATP were able to completely prevent FITC binding to control enzyme. In contrast, with enzyme inactivated by treatment with NAI, 3 mM ATP, but not 50 μM ATP, completely prevented FITC binding.

Discussion

The treatment of dog kidney Na,K-ATPase with NAI produces inhibition of the catalytic activities of the enzyme. The inhibition cannot be removed by washing or diluting the enzyme, suggesting that NAI irreversibly inactivates the ATPase by acetylation of one or more of its amino acids.

Kinetics of Inactivation. The time course of the Na,K-ATPase inactivation shows biphasic kinetics (Figure 1). This type of kinetics could be accounted for by either of two models: (i) a two-state, single-site model (Fritzsch, 1985) or (ii) a two-site model (Ray & Koshland, 1961). A two-state model could be considered since either of two interconvertible conformations of the Na,K-ATPase, such as E_1 and E_2 or a dimer–monomer equilibrium, might yield forms in which a single site of modification could have different reactivities in each state. However, this model could not account for our other observations, such as the partial recovery of ATPase activity following NH_2OH treatment (Figure 6). Consequently, a model with at least two different classes of acetylation sites can better describe the overall characteristics of NAI interactions with the Na,K-ATPase. The characteristics of these sites as described by our findings are summarized in Table III. In contrast, *p*-NPPase inactivation follows single-expo-

Table III: Summary of the Properties of Two Classes of Sites on Na,K-ATPase That React with NAI

	acetylated residues	
	class I	class II
reaction rate	fast	slow
inactivation of ATPase	+	+
inactivation of *p*-NPPase	-	+
ATP protectable	+	-
NH_2OH sensitive	+	-

nential kinetics pointing to the involvement of only one (kind of) residue. Although we refer to a residue of class I or class II, we have not measured acetylation stoichiometry and related this quantitatively to inactivation. Thus, we cannot be sure whether there is one or more residue(s) of each type involved in the inactivation kinetics.

The reaction between NAI and the enzyme could be described by a model (Scheme I) that assumes the independent acetylation of two classes of amino acids with two different velocities. The modification of the more rapidly acetylated site (E_i') results in a loss of ATPase activity without any effect on the *p*-NPPase activity (class I, Table III), while reaction with the slower site (E_i'') reduces both the ATPase and *p*-NPPase activities (class II, Table III). This model is supported by (i) the very good fit of the inactivation time course curves (Figures 1 and 2), (ii) the similarity between the *p*-NPPase inactivation rate constants and the rate constants for the second phase in the ATPase inactivation, and (iii) the reaction order near unity obtained for each of the reactions proposed. The presence of ATP during the reaction of the enzyme with NAI partially prevents the inactivation of the ATPase activity but has no effect on the inactivation of the *p*-NPPase activity. This suggests that one of the sites involved in ATPase inactivation is protected by ATP while the other is not. The site that is not protected by ATP is also the site involved in *p*-NPPase inactivation (class II). The inactivation time course of the enzyme in the presence of ATP shows single-exponential kinetics with a rate constant similar to that of the slowly reacting site (k_2). These observations lead to the conclusion that ATP blocks the reaction with NAI at the more rapidly acetylated site (class I).

Nature of Acetylated Residues. Masiak and D'Angelo (1975) have reported inactivation of red cell Na,K-ATPase by NAI. This inactivation was prevented by ATP. The authors proposed that NAI was acetylating a tyrosine residue since the activity could be recovered by treatment with NH_2OH. Cantley et al. (1978), treating eel electric organ Na,K-ATPase with NBD-Cl, a potential tyrosine-modifying reagent, described an ATP-protectable inactivation of the enzyme. However, part of the inactivation was reversed by 2-mercaptoethanol, indicating the involvement of thiol moieties of cysteines as well as tyrosine residues. Later studies also with NBD-Cl, by Grosse et al. (1978), found only modification of thiol groups by this reagent. Consequently, it is not clear whether or not there is an ATP-protectable tyrosine residue on the enzyme, associated with the ATP binding site. NH_2OH is a deacetylating reagent for *O*-acetyl products (esters) but not *N*-acetyl products (amides) (Balls & Wood, 1956; Simpson et al., 1963). The reaction of NH_2OH with NAI-treated enzyme produced a partial recovery of the Na,K-ATPase activity and it did not reverse the inhibition of the *p*-NPPase activity. These results could be explained by the presence of *O*-acetyl and *N*-acetyl residues or by two classes of *O*-acetyl residues (see Table III). Either of these possibilities agrees with our two-site model for the reaction of NAI with the enzyme. Two classes of sites (class I and class II, in Table III) are involved in the ATPase inactivation by NAI, while modification of only one population of residues affects the *p*-NPPase activity (class II). In this case, the amino acid that influences only the ATPase (class I) would be a tyrosine, since the NAI enzyme ATPase activity was recovered after the NH_2OH treatment, reaching a value similar to that of the ATP-protected enzyme (Figure 6). The result that NH_2OH treatment did not significantly affect ATPase activity of the ATP-protected enzyme or the *p*-NPPase activities also supports the model suggesting that (i) in the ATP-protected enzyme only one kind of site is modified, (ii) this site is the same that affects the *p*-NPPase activities, and (iii) this residue would be a lysine or a tyrosine not accessible to NH_2OH (class II, Table III). Masiak and D'Angelo (1975) report almost complete ATP protection and NH_2OH sensitivity of acetylation. The difference between their work and the current study may be explained by the acetylation of a second non-ATP-protectable amino acid in the present work that inactivates the ATPase activity and the *p*-NPPase activity. The appearance of this second acetylatable amino acid may be due to the different source of enzyme used or the different treatment conditions employed in these two studies.

Nucleotide Effects and Enzyme Inactivation. It is known that ATP interacts with the Na,K-ATPase enzyme with two different apparent affinities. The high-affinity binding ($K_d \simeq 0.1$ μM) is associated with the phosphorylation of the E_1 form of the enzyme by ATP at the catalytic site, while the low-affinity ATP binding ($K_m \simeq 100$ μM) produces the deocclusion of K^+ (or Rb^+) and accelerates the $E_2 \rightarrow E_1$ conformational change [see Glynn (1985)]. We have observed that ATP prevents the inactivation of the Na,K-ATPase activity by NAI with a $K_{0.5} \simeq 8$ μM. In addition, acetylation inhibited high-affinity ADP binding (Table I) to the same extent that it inhibited Na,K-ATPase. These results suggest that a residue in the high-affinity ATP binding site is acetylated by NAI, which prevents or greatly reduces the high affinity binding. However, the modification of the Na,K-ATPase by NAI did not affect Rb^+ deocclusion by ATP nor the inhibition of the *p*-NPPase by ATP. This indicates that ATP can still bind to the NAI enzyme with low affinity. This could be explained by the following: (i) there are two independent ATP binding sites and NAI affects the high-affinity site but not the low-affinity one and (ii) there is only one ATP binding site and the acetylated tyrosine is moved out of the ATP binding pocket when the enzyme changes from the E_1 to the E_2 conformation. It is interesting to compare our findings with NAI to those obtained with FITC (Karlish, 1980; Sen et al., 1981), H_2DIDS (Pedemonte & Kaplan, 1988), or EPC (Pedemonte & Kaplan, 1986). These compounds all produce an ATP-preventable inactivation of both Na,K-ATPase and *p*-NPPase activities. Consequently, the sites being modified by these reagents are evidently important for both enzymatic activities. In contrast, NAI modifies an amino acid residue (class I) that is only associated with loss of ATPase activity and not required for *p*-NPPase activity.

Properties of the Modified Enzyme. It has been observed that chemical modification of Na,K-ATPase can lock the enzyme into one of the two major dephosphoenzyme forms. For instance, treatment with H_2DIDS produced an E_2-like form (Pedemonte & Kaplan, 1988). After treatment of the Na,K-ATPase with NAI, the enzyme is still capable of undergoing the major dephosphoenzyme transition between the E_1 or E_2 forms, as demonstrated by our tryptic digestion results (Figure 7). Most of the other enzyme functions are not affected, including the occlusion of K^+ ions, the release of the

occluded ions by ATP (with low affinity) and phosphorylation from P_i. In fact, the loss of ATPase activity in the modified enzyme seems to be correlated well with loss of high-affinity ATP binding. The properties of the NAI-treated enzyme can thus be best explained by the disruption of the high-affinity ATP binding region identified with E_1 forms of the enzyme. This result could also be caused by an indirect conformationally coupled effect, where the modification locks the enzyme into an E_2 form, which does not display high-affinity ATP binding. This explanation seems unlikely in the present work since the acetylated enzyme exhibits largely unaltered E_2-like properties (phosphorylation from P_i, occlusion of Rb^+, release of occlusion by ATP) and can be converted to an E_1 form by Na^+, as shown by trypsin degradation.

In conclusion, reaction of Na,K-ATPase with NAI involves two classes of amino acids. One is a rapidly reacting tyrosine, and its ATP-protectable acetylation is responsible for the loss of ATPase activity (without affecting the *p*-NPPase activity). The other amino acid might be a lysine (or a less accessible tyrosine) that is not ATP protectable and its acetylation reduces the ATPase and predominantly the *p*-NPPase activity. The modified enzyme is able to interconvert between E_1 and E_2 forms and bind ATP with low affinity, but it has reduced ATP binding to the high-affinity site. The protection of the tyrosine by ATP, together with the inhibition of the ATP high-affinity binding, suggests the presence of this tyrosine group in the high-affinity ATP binding site and its absence when ATP binds with a low apparent affinity. The tyrosine residue that is important for the catalysis of ATP hydrolysis does not seem to be required for phosphatase activity.

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