Evidence for Essential Carboxyls in the Cation-binding Domain of the Na,K-ATPase*

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Treatment of isolated canine renal Na,K-ATPase with a stable diazomethane analog, 4-[(diazomethyl)-7-(diethylamino)-coumarin (DEAC), results in enzyme inactivation. The inactivation rate was dramatically increased when the enzyme was treated with DEAC in the presence of ATP and Mg2+ (in imidazole buffer) or K+ and Mg2+, conditions which produce enzyme phosphorylation. Inactivation in the presence of P+ and Mg2+ could be partially prevented by Na+ and almost completely prevented by K+. The quantity of DEAC covalently bound to the Na,K-ATPase was determined spectrophotometrically. The extent of inactivation was linearly related to the amount of K-protectable DEAC incorporation. Complete inactivation of ATPase activity occurred with 2.14 ± 0.18 nmol of DEAC covalently bound/mg of protein. This suggests that only 1 or 2 carboxyl residues/catalytic center (estimated by high affinity Affinity Sephadex) are involved in the modification leading to inactivation. The modified enzyme exhibited normal levels of high affinity [3H]ADP (and hence ATP) binding, thus, the nucleotide-binding domain of the enzyme seems unaffected by the modification. In contrast, under conditions where native enzyme was able to occlude 3.82 nmol of K+ ions/mg of protein, DEAC-modified enzyme occluded only 0.33 nmol of K+ ions. Na+ occlusion by the enzyme (in the presence of oligomycin) was also reduced (by 80%) following treatment with DEAC. Phosphorylation by [32P]inorganic phosphate and Na+-activated phosphorylation of the modified enzyme with [32P]ATP yielded reduced levels of phosphoenzyme (about 36%) compared to native enzyme. The DEAC-modified [32P]phosphoenzyme formed from [32P]ATP was insensitive to the addition of K+ ions, under conditions which led to the rapid hydrolysis of native phosphoenzyme. Gel electrophoresis of modified protein revealed strong fluorescence labeling of the α-subunit, which was substantially reduced if treatment with DEAC was performed in the presence of K+ ions. Partial tryptic digestion and electrophoretic analysis revealed normal degradation patterns in the presence of ADP (E1 form) but the typical patterns, seen with K+ ions (E1K) or Na+ ions (E1Na) in native enzyme, were absent. A typical E1-like tryptic degradation pattern was seen, however, in the presence of vanadate and ouabain, suggesting that the modification does not freeze the enzyme in an E1 conformation and that the enzyme is still able to undergo the E1→E2 conformational transition after modification.

Our results suggest that a small number of carboxyl residues in the sodium pump α-subunit (perhaps one) are essential for K+ and Na+ binding and stabilizing the occluded enzyme cation forms. Esterification of the carboxyl groups by DEAC inactivates the enzyme. The residues are probably part of the monovalent cation-binding domain and occlusion site in the Na,K-ATPase α-subunit.

The Na,K-ATPase (EC 3.6.1.37) is the eukaryotic plasma membrane enzyme which is responsible for the coupled active transport of Na+ and K+ (1–3). Although the Na+ pump has been studied extensively, many questions concerned with the mechanism of ion transport, ATP hydrolysis, and energy transduction remain unanswered. This is in part due to the lack of detailed information about the enzyme structure and the structural changes that it undergoes during the catalytic cycle. In recent years, the enzyme subunit composition (4, 5) and the primary structure of the α- and β-subunits (6–12) have been determined.

Chemical modification studies have provided further information about the structure of the Na,K-ATPase and the specific amino acids involved in the interaction of ligands with the enzyme (for review, see Ref. 13). These amino acids may be modified by affinity labeling where the structural similarity of a reagent to a ligand favors covalent labeling of the ligand-binding site. Another alternative is the use of a chemical group-specific reagent in the expectation that specific residues at the ligand-binding sites are more reactive toward the modifier or that the groups at the ligand site are specifically protected from the reagent by the bound ligand. Several amino acid residues have been implicated at the bind-site following these strategies, i.e. lysines (14–18), tyrosines (19, 20), cysteines (21, 22), and aspartates (23). However, no specific amino acid or identified sequence of the ATPase have been associated with putative monovalent cation-binding sites. Although by analogy with the results of mutagenesis studies in the sarcoplasmic reticulum Ca-ATPase, carboxyl residues in the C-terminal intramembrane helices are candidates (24). The absence of affinity-directed probes toward these sites makes their localization difficult. Consequently, studies have been performed using residue-specific probes, looking for amino acids likely to be involved in the interaction with cations.

It has seemed plausible that anionic carboxyl side chains of aspartate or glutamate residues might be involved in coordination with cations at their binding sites on the protein. The most popular class of reagent for testing this hypothesis has been the carbodiimides. Over the last 20 years, several reports have described monovalent cation-protectable inactivation of the Na,K-ATPase (25–32). The results obtained using these reagents have been complex. The major complication in such

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Cation-site Modification of Na,K-ATPase

Fig. 1. Scheme of the reaction between DEAC (R = Et$_2$N-) or MEOC (R = MeO-) and amino acid carboxyl residues.

R=Et$_2$N; DEAC
R=MeO; MEOC

Glutamyl or aspartyl residue

Modified protein

studies has been the propensity for the initial adduct, obtained with a carboxyl residue and a carbodiimide, to then undergo internal condensation with an endogenous amine producing an isopeptide bond and elimination of the carbodiimide as the diacil urea (for discussion, see Refs. 13, 20, 21). Thus, it is difficult to decide if enzyme inactivation is due to observed covalent incorporation of the carbodiimide or parallel intra-molecular cross-link formation which would not involve incorporation. Although cation-protection against carbodiimide modification of carboxyls has often been observed, it is only recently that a covalent labeling of carboxyl residues has been reported and their identity with cation binding or occlusion sites been claimed (32).

Since there is an inherent ambiguity in using carbodiimides to label carboxyl residues of proteins, we turned our attention to other carbonyl-reactive reagents. Diazomethane is a familiar reagent for the preparation of methyl esters of carboxylic acids. Unfortunately, diazomethane is very unstable in aqueous solution and, although it has been used to modify proteins, it is of little use for stoichiometric labeling and localization studies (see Ref. 33). Recently, Ito and Maruyama (34) introduced more stable fluorescent analogs of diazomethane which react specifically with carboxylic residues to yield fluorescent esters (see Fig. 1). We have investigated the application of two of these reagents, 4-(diazomethyl)7-(methoxy)-coumarin (MEOC) and 4-(diazomethyl)-7-(diethylamino)-coumarin (DEAC), to studies on the essential carboxyl residues of the Na,K-ATPase. Following our previous strategy with carbodiimides (30, 31), we hoped that, in particular, the positively charged reagent (DEAC) might select for carboxyl residues on the protein which are involved in cation binding.

We describe here the effects of DEAC treatment on the properties of the Na,K-ATPase. We provide evidence for the K-protectable DEAC modification of one carbonyl group. The cationification of this carboxyl group inactivates the enzyme by removal of the major monovalent cation binding capacity.

Experimenntal Procedures

Materials—ATP, ADP, 5'-adenylylimidodiphosphate (AMP-PNP), Tris-p-nitrophenylphosphate (p-NPP), Trypsin (Type XI), Proteinase K (Type XI), BSA, bovine serum albumin (BSA), Trypsin (Type XI), and Proteinase K (Type XI) were from Sigma Chemical Company. [3P]inorganic phosphate (P$_i$), [3H][Rb] RbCl, [32P]ATP, and [3H]ADP were from Amersham Corp. DEAC and MEOC were from Molecular Probes. Acrylamide, bis(acrylamide), and the protein molecular weight standards were from Bio-Rad. 4-(Hydroxymethyl)-7-(diethylamino)-coumarin (DEAC-OH) was synthesized following Ito and Maruyama (1983). 4-(Hydroxymethyl)-7-(hydroxyethylamino)-coumarin (DEAC-OMe) was obtained by hydrolyzing DEAC-benzoate with 1 equivalent of LiOH at 25 °C for 90 min and purified by thin layer chromatography (solvent system ethyl acetate:hexane, 1:1, v/v). The use of stronger bases than LiOH (NaOH for example) results in hydrolysis of DEAC-benzoate but also cleavage of the fluorescein coumarin ring structure. All other chemicals were of the highest quality available.

Enzyme Isolation—Na,K-ATPase was purified from canine kidney outer medulla according to Jorgensen (36) with the modifications of Liang and Winter (37). After ultrafiltration, the enzyme was washed with 25 mM imidazole, 1 mM EDTA, pH (20 °C) 7.6 (Buffer C), before storing at 5 °C. Protein concentration was determined by the method of Lowry et al. (38) using BSA as standard. The Na,K-ATPase activity of the enzyme used in these studies was about 17-23 pmol of Pi mg$^{-1}$ min$^{-1}$, assayed as described below.

Enzyme Treatment with DEAC or MEOC—Unless specified, the enzyme (0.1-0.5 mg/ml) was treated in 50 mM imidazole, pH (20 °C) 6.5, 1 mM EDTA, during 2 h at 37 °C. DEAC or MEOC in dimethyl sulfoxide (Me$_2$SO) was added at the desired concentration. The final Me$_2$SO concentration was 10% in the treatment medium and less than 0.25% in the Na,K-ATPase assay medium. In most of the experiments, 3 mM MgCl$_2$ and 5 mM P were included in the DEAC treatment medium, the modified protein obtained using this procedure will be referred to as DEAC-enzyme in this paper. The reaction was stopped by dilution in Buffer C and ultrafiltration or by dilution (1:100) in the ATPase assay medium, and both methods gave the same results.

Determination of DEAC Decomposition Kinetics—DEAC-OH is the hydrolytic breakdown product of DEAC in aqueous media. Determination of DEAC stability was performed by incubating DEAC in the treatment medium (50 mM imidazole, pH (20 °C) 6.5, 1 mM EDTA), at 37 °C for various times and measuring the DEAC remaining by high pressure liquid chromatography. Aliquots of the solution were taken, a known amount of DEAC-benzoate was added as an internal standard, and the solution extracted with ethyl acetate. The organic phases were separated and injected into a high pressure liquid chromatography system (Waters) equipped with a silica column ( Resolve Silica, 5 μm, 5 mm x 100 mm, Radial-Pak, Waters, Millipore Co.), DEAC-benzoate, DEAC, and DEAC-OH were separated using hexane/ethyl acetate (2:1, v/v), 1 ml/min$^{-1}$). A chromatogram showing the resolution of the three components is shown in Fig. 5B.

DEAC Binding Stoichiometry Determinations—Enzyme aliquots (250-500 μg, 9.5 μM) were treated with a range of DEAC concentrations in a medium containing 3 mM MgCl$_2$, 3 mM P, 50 mM imidazole, pH (20 °C) 6.5, 1 mM EDTA, during 2 h at 37 °C. 40 mM KCl was included in the treatment media when it was desired to obtain K-protected enzyme. After treatment, the Na,K-ATPase activity was determined. The samples were then centrifuged (400,000 × g, 10 min, 0 °C), the pellets resuspended in Buffer C, and aliquots were used for protein determination. The samples were then extracted with 5 volumes of cold (–20 °C) acetone and the protein allowed to precipitate at –20 °C during 30 min. The protein was collected by centrifugation (5 min) in an Eppendorf microfuge and the superna-
tant discarded. The protein pellets were washed twice with acetone, and the final pellets were resuspended in 250 mM Tris-acetate, pH (20°C) 7.8, 8 mM urea, 1% SDS. To obtain clear homogenous solutions, the samples were diluted 1:5 (v/v) and treated with proteinase K (1:5, w/w, protease) overnight at room temperature. The absorbance at 280 nm of the resulting solutions was determined. The amount of DEAC was calculated in the samples, and the concentration curve obtained with a range of concentrations of DEAC-benzoxate added to control samples (native Na,K-ATPase) previously processed as indicated above. The DEAC-benzoxate absorbance was linear with concentration in the range examined, 0–10 μM.

**Assay medium for Na,K-ATPase activity** was (mM) EGTA, 0.5; NaCl, 130; KCl, 20; MgCl₂, 3; ATP, 3; and imidazole, 50; pH (20°C) 7.2; 0.3 mg/ml BSA; and approximately 0.5 μg/ml enzyme protein. The assay was performed at 37°C for 15 min and then centrifuged at 400,000 g for 5 min at 0°C. The samples were filtered through Millipore filters (pore size 0.45 μm) and concentrated by centrifugation at 400,000 g for 5 min. The pellet was resuspended in 0.5 mM NaOH and 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 non-specific (or low affinity) binding.

**Phosphorylation with ATP** was carried out in a medium containing (mM) NaCl, 20; EGTA, 0.5; Hepes/imidazole, 75; pH (20°C) 7.2; MgCl₂, 1; [³²P]ATP, 0.02; and protein, 0.5 mg/ml. The reaction was initiated by addition of ATP and stopped after 15 at 0°C with 9 volumes of 5% (v/v) trichloroacetic acid containing 0.5 mM ATP and 2 mM P₃. The samples were filtered through Millipore filters (pore size 0.45 μm); the filters were washed five times with 0.2 M NaOH; and the radioactivity was measured in a liquid scintillation counter. The activity was estimated from the difference between the [³²P] incorporation measured as described and that obtained in a medium containing 0.5 mM ATP. The effects of EDTA, ADP, or K⁺ on phosphoenzyme levels were examined in samples phosphorylated as described above. 5 mM EDTA, 50 mM KCl with 5 mM EDTA, or 1 mM ATP with 5 mM EDTA (final concentrations) were added to the medium after 15 s. After 30 s of incubation, the samples were filtered through Millipore filters (pore size 0.45 μm), and the radioactivity was measured in a liquid scintillation counter. Values were corrected for non-specific binding by using apparent binding measured in the absence of oligomycin as a blank for experimental samples.

**Trypsin treatment** of the enzyme was performed as described by Jorgensen (44) with a 100:1 (w/w) enzyme/trypsin ratio in 20 mM Tris, pH 7.5, and in the presence of 1 mM ADP, 40 mM KCl, 40 mM NaCl, or 1 mM ouabain, 1 mM MgCl₂, 0.1 mM Na₃VO₄. The pH (100 mM) was kept constant by adding in each case the appropriate amount of choline chloride. After 20 min at 37°C, proteolysis was stopped by the addition of 1 volume of Laemmli's sample buffer (45). SDS-PAGE was carried out according to Laemmli (45) in 10% acrylamide gels. Protein bands were observed by staining the gels with Coomasie Brilliant Blue. Proteins labeled with DEAC were detected by their fluorescence emission on illumination with a hand-held long wavelength (580 nm) UV lamp.

**RESULTS**

**Characterization of the Inactivation and Ligand Effects—** The effects of two diethylmethyl coumarins, DEAC and MEOC, on the Na,K-ATPase were initially tested. Treatment of enzyme with DEAC or MEOC, at 37°C during 120 min, led to a partial inactivation of the Na,K-ATPase (Fig. 2). These effects were irreversible, the activity could not be recovered by washing the enzyme (by centrifugation and resuspension in Buffer C). Both compounds, DEAC and MEOC, reduced the Na,K-ATPase activity. Under comparable conditions, the inactivation by DEAC was greater, and only the inactivation produced by DEAC could be significantly reduced by the presence of monovalent cations, Na⁺ or K⁺ (Fig. 2). It was also observed, particularly with DEAC at concentrations higher than 500 μM, that an immediate reversible inhibition (20–30%) of the Na,K-ATPase activity was produced. This inhibition was not altered by any of the enzyme ligands when added to the media, and the original enzyme activity was recovered following dilution. To avoid this small reversible inhibition, the samples were diluted (1:100) prior to any kind of binding or activity determination. At pH 6.5, the greatest protection against inactivation was obtained when K⁺ was present in the treatment media. In experiments performed at higher pH values (up to pH 7.5), the inactivation level did not change, but less or no cation-protective effects were observed (data not shown). Unfortunately, experiments at lower pH values than pH 6.5 could not be performed due to the loss of native enzyme activity during the incubation at 37°C. Our major interest was to study the modification of putative cation-binding sites. Since K-preventable inactiva-

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**FIG. 2. Inactivation of Na,K-ATPase by DEAC or MEOC.** The enzyme was incubated as indicated under "Experimental Procedures" for 120 min at 37°C in presence of 250 μM coumarin (DEAC); 250 μM coumarin, 100 mM KCl (MEOC); 250 μM coumarin, 100 mM NaCl (MEOC); 250 μM coumarin, 3 mM ATP (MEOC). The Na,K-ATPase activity was assayed after the treatment. Values are the mean ± S.E. of three experiments.
tion might be likely associated with inactivation at K⁺-binding sites, we focused our subsequent studies on the modification produced by DEAC at pH 6.5.

The effects of a variety of Na,K-ATPase ligands present in the media during DEAC treatment at pH 6.5 are shown in Fig. 3. Na⁺ ions had only a slight protective effect, while K⁺ more effectively preserved the Na,K-ATPase activity during DEAC treatment (Figs. 2 and 3). The cation protection was not due to (nonspecific) ionic strength effects since choline chloride (100 mM) did not affect the inactivation (not shown). No significant effect was observed when either Mg²⁺ or Pᵢ⁻ were added separately to DEAC treatment media (not shown). Interestingly, however, the simultaneous presence of 3 mM MgCl₂ and 3 mM Pᵢ⁻ greatly increased the inactivation produced by DEAC. Under this latter condition, K⁺ almost completely prevented the modification by DEAC, whereas Na⁺ produced only a partial protection (see Fig. 3). It should be kept in mind that Pᵢ⁻, together with Mg²⁺ phosphorylates the Na,K-ATPase (producing an E₂P form) and that both cations, Na⁺ and K⁺, cause dephosphorylation, producing E₁Na or E₂K forms, respectively (5, 46). The presence of ATP alone had little effect on the inactivation by DEAC (Fig. 2). However, when ATP together with Mg²⁺ were included in the DEAC treatment medium, a significant increment in the inactivation was seen (Fig. 3). These experiments were performed in imidazole buffer, and it has been shown that imidazole has a Na⁺-like effect on the phosphorylation of the Na,K-ATPase by ATP-Mg²⁺ although it is unlikely that imidazole binds to the Na⁺-binding site to produce this effect (47). It is possible that the greater inactivation obtained in presence of ATP and Mg²⁺ was due to phosphorylation of the enzyme in a similar way that the presence of Mg²⁺ plus Pᵢ⁻ resulted in enhanced inactivation. In keeping with this notion, the presence of Mg²⁺ and ADP or Mg²⁺ and AMP-PNP (a non-phosphorylating ATP analog) did not enhance inactivation due to DEAC (see Fig. 3) although both of these nucleotides bind to the ATP site of the Na,K-ATPase. This idea was also supported by the results obtained after treating the Na,K-ATPase with 250 µM DEAC in 50 mM MES/N-methylglucamine, (Na⁺, K⁺), and imidazole-free media. Under these conditions, the inactivation observed in presence of 3 mM ATP, with 3 mM MgCl₂ (remaining Na,K-ATPase activity 17 ± 5% of the control) was not significantly different from that produced by DEAC alone (remaining Na,K-ATPase activity 20 ± 3% of the control).

In Fig. 4A the kinetics of inactivation during enzyme treatment with DEAC are shown. The inactivation rate was greatly increased by the presence of Pᵢ⁻ and Mg²⁺ (Fig. 4B). However, the additional presence of K⁺ removed the enhanced inactivation rate due to the presence of Pᵢ⁻ and Mg²⁺ and presumably enzyme phosphorylation. Therefore, in both the presence (Fig. 4B) and absence (Fig. 4B) of Pᵢ⁻ and Mg²⁺, K⁺ ions provide protection against inactivation. These curves did not fit single exponential kinetics and approached completion long before enzyme was fully inactivated. One explanation for this might be that DEAC was decomposing in aqueous buffers (to produce DEAC-OH) during the course of the inactivation so that the inactivation rate would progressively decrease. To examine this possibility, DEAC stability under the conditions of the enzyme treatment was examined. The data from such studies are shown in Fig. 5. DEAC decomposed in 50 mM imidazole, pH (20 °C) 6.5, 1 mM EDTA at 37 °C in presence of A, 250 µM DEAC alone (□); 250 µM DEAC, 40 mM KC1 (□); 250 µM DEAC, 3 mM MgCl₂, 3 mM Pᵢ⁻ (▲); 250 µM DEAC, 3 mM MgCl₂, 3 mM Pᵢ⁻, 40 mM KCl (▲). At different times, aliquots were taken for Na,K-ATPase activity assay. At 120 min of incubation (arrow) additional DEAC (250 µM final concentration) was supplied into the samples treated in absence of Pᵢ⁻ plus Mg²⁺. The time course of p-NPPase inactivation in samples treated with 250 µM DEAC in presence of 3 mM MgCl₂, 3 mM Pᵢ⁻ is also shown (▲). The values are the mean of three experiments.

![Fig. 3. Effect of the enzyme ligands on DEAC inactivation of the Na,K-ATPase.](image)

The enzyme was incubated in 50 mM imidazole, pH (20 °C) 6.5, 1 mM EDTA for 120 min at 37 °C in the presence of 250 µM DEAC and each ligand before assaying the Na,K-ATPase activity. The values are the mean ± S.E of five experiments. Asterisks indicate a significant difference (p < 0.01) from the activity of enzyme treated with DEAC alone.

![Fig. 4. Time course of Na,K-ATPase and p-NPPase inactivation by DEAC.](image)

The enzyme was incubated in 50 mM imidazole, pH (20 °C) 6.5, 1 mM EDTA at 37 °C in presence of A, 250 µM DEAC alone (□); 250 µM DEAC, 40 mM KC1 (□); 250 µM DEAC, 3 mM MgCl₂, 3 mM Pᵢ⁻ (▲); 250 µM DEAC, 3 mM MgCl₂, 3 mM Pᵢ⁻, 40 mM KCl (▲). At different times, aliquots were taken for Na,K-ATPase activity assay. At 120 min of incubation (arrow) additional DEAC (250 µM final concentration) was supplied into the samples treated in absence of Pᵢ⁻ plus Mg²⁺. The time course of p-NPPase inactivation in samples treated with 250 µM DEAC in presence of 3 mM MgCl₂, 3 mM Pᵢ⁻ is also shown (▲). The values are the mean of three experiments.
abolished in 90 min by treatment with 250–500 μM DEAC in the presence of P, and Mg++.

The extent to which Na⁺ or K⁺ prevent the inactivation of Na,K-ATPase activity by DEAC are shown in Fig. 7. K⁺ was more effective than Na⁺ in preventing the enzyme inactivation. Enzyme treated in the presence of K⁺ retained significantly more activity than that treated in the presence of Na⁺ under comparable conditions, and K⁺ (Kd = 0.6 mM) clearly protected the enzyme at lower concentrations than Na⁺.

Properties of the Modified Enzyme—The preservation of the Na,K-ATPase activity when the enzyme was treated with DEAC in presence of K⁺, suggested that the modification might occur close to a K⁺-binding site. This possibility was strengthened following the determination of [³²P]Rb⁺ binding and occlusion following modification (Table I). Rb⁺ is a frequently used congener of K⁺ in Na,K-ATPase studies. It is known that ATP in the millimolar concentration range (via a low affinity site) releases Rb⁺ which has been occluded by the enzyme, thus, an estimate of the occluded Rb⁺ can be obtained from the difference between Rb⁺ bound in the absence and presence of ATP (5, 48, 49). The DEAC enzyme did not bind significant amounts of Rb⁺ (in the absence or presence of ATP), and thus the amount of occluded Rb⁺ was minimal, while the enzyme that was protected with K⁺ against inactivation by DEAC showed normal levels of Rb⁺ binding and occlusion (Table I).

These results could imply that the inactivation of the Na,K-ATPase might be due solely to the disappearance of the Rb⁺-
occluding capacity of the enzyme, however, the binding of other ligands might also be affected. The determination of [³H]ADP binding indicated that the high affinity ATP-binding domain was not affected by the modification of the enzyme (Table II). Phosphorylation of the modified enzyme in the presence of [³²P]P, and Mg²⁺ yielded about 30% of the phosphorylation level of native enzyme. This effect could be due to a change in the apparent affinity for P; so that modified enzyme might yield comparable phosphorylation levels at higher [P]. That this was not the case can be seen in Table II, although increases in [P] elevated the steady-state phosphoenzyme level for both native and modified enzyme. The presence of 20% MeSO, which has been shown to increase the apparent affinity for P, (50), results in no further increase in phosphorylation level beyond that seen with 2.5 mM P; in both native and modified enzyme. It seems then that the modification has either reduced the rate of phosphorylation from P, or increased the spontaneous hydrolysis rate of the phosphoenzyme. No further experiments were performed to distinguish between these alternatives.

Phosphorylation of the enzyme by [³²P]ATP in the presence of Na⁺ ions also showed reduced levels of phosphorylase following modification (Table III). The ligand sensitivities of the native and modified phosphoenzymes were also studied. It is well known that the sodium pump phosphoenzyme contains both K⁺-sensitive ADP-insensitive (E₁P) and ADP-sensitive K⁺-insensitive (E₂P) components (46, 51). Addition of K⁺ or ADP to native phosphoenzyme resulted in a fall of the phosphorylase level to 2.5 and 40% respectively within 6 s at 0 °C. With modified enzyme these levels were 50% with K⁺ and 56% with ADP. Thus, the DEAC-modified phosphoenzyme was markedly less sensitive to the addition of K⁺ ions than native phosphoenzyme. Bearing in mind that the treated enzyme still contained 10% ATPase activity, which could be due to a residual 10% unmodified enzyme, these figures tend to over-estimate the K⁺ sensitivity of phosphoenzyme following modification by DEAC. Evidently, the lack of K⁺ binding and occlusion in dephosphoenzyme is also reflected in a lack of K⁺ sensitivity of the phosphoenzyme.

Some of our observations of the Na,K-ATPase inactivation kinetics and of the properties of the modified enzyme indicated that the modification might also be altering the Na⁺ binding characteristics of the enzyme. This was suggested by (a) the small, but significant, protection by Na⁺ against inactivation by DEAC and (b) the observation that modified enzyme showed lower levels of Na⁺-activated phosphorylation from ATP than native enzyme although high affinity ADP (hence ATP) binding was the same as native enzyme. The Na⁺ binding capacities of native and DEAC enzyme were then compared. This was done taking advantage of the slow Na⁺ enzyme dissociation kinetics in presence of oligomycin (52, 53). Na⁺ binding to enzyme previously treated with 250 μM DEAC in the presence of 3 mM P, and 3 mM Mg²⁺ (retaining 15–20% Na⁺-ATPase activity) was significantly lower, 1.10 ± 0.40 nmol/mg; n = 3, than the native enzyme, 5.22 ± 0.13 nmol/mg. These data clearly indicate that DEAC treatment has also altered the interactions of Na⁺ ions with the ATPase.

It is known that K⁺ ions stabilize an E₂ form of the enzyme while Na⁺ stabilizes an E₁ conformation (see Ref. 5) and that trypsin treatment of the enzyme yields different electrophoretic patterns depending on the protein conformation (44). Fig. 8 shows the electrophoretic profiles obtained from control and modified enzyme following treatment with trypsin in the presence of different enzyme ligands. The normal E₁ conformation was observed after the treatment in presence of ADP (Fig. 8, compare lane A3 with B3), but the typical K⁺ form and Na⁺ form were not detected in the DEAC-treated enzyme (compare lane A2 and A4 with B2 and B4). It is important (see “Discussion”) to note, however, that when trypsin treatment is performed in the presence of vanadate ions and ouabain, typical E₁-like degradation patterns are observed (lanes A5 and B5 in Fig. 8). Examination of this gel under UV light, prior to the staining with Coomassie, revealed that the DEAC was bound predominantly to the α-subunit of the Na,K-ATPase (lane C1 in Fig. 8). Esterified carboxyl residue(s) were localized in the 78-kDa fragment after proteolysis with trypsin in the presence of ADP (lane C3) and in fragments corresponding to 58 kDa and 41–46 kDa when the DEAC-modified enzyme was treated in an E₂ conformation (lane C5 in Fig. 8).

**Table II**

<table>
<thead>
<tr>
<th>ATPase activity (%)</th>
<th>250 μM DEAC</th>
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<tbody>
<tr>
<td>Native</td>
<td>100</td>
</tr>
<tr>
<td>ADP binding (10 μM)</td>
<td>2.19 ± 0.20</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>2.14 ± 0.33</td>
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<tr>
<td>1 mM P, K⁺</td>
<td>3.20 ± 0.51</td>
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<tr>
<td>2.5 mM P, K⁺</td>
<td>0.66 ± 0.07</td>
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<tr>
<td>1 mM P, plus 20% MeSO</td>
<td>1.13 ± 0.02</td>
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**Table III**

<table>
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<tr>
<th>Native and DEAC enzyme phosphorylation by ATP</th>
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<tr>
<td>Phosphorylation by ATP</td>
</tr>
<tr>
<td>5 mM EDTA</td>
</tr>
<tr>
<td>mm KCl</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>DEAC enzyme</td>
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* Percentage of the maximum phosphorylase level.

**Stoichiometry of Esterification**—Since most DEAC esters have similar extinction coefficients (34), determination of the stoichiometry of esterification was performed utilizing the absorbance peak of DEAC at 390 nm. A direct measurement of the modified protein absorbance or fluorescence was not, however, possible. The lipophilicity of DEAC leads to high levels of DEAC noncovalently associated (via partitioning) with the membranes. Various procedures were attempted to remove noncovalently bound DEAC prior to determination; they included electrophoresis and electrodialysis, dialysis, etc. All of those methods led to significant losses of sample or introduced significant errors into the determination. Although fluorescence measurements were extremely sensitive, their usefulness for quantitation was compromised by a significant blue shift in the emission spectra of DEAC-modified protein compared with model compounds. The shifts were from 5–10 nm and were present in denatured modified α-subunit, extensively proteolyzed α-subunit, etc. We finally settled on cold organic solvent extraction and absorbance measurements on
Cation-site Modification of Na,K-ATPase

**DISCUSSION**

This report describes the esterification by DEAC of a small number (1 or 2) of carboxyl residues in the α-subunit of the Na,K-ATPase which are essential for the binding of monovalent cations to the protein.

**Characteristics of the Reaction between DEAC and the Na,K-ATPase**—In a search for carboxyl residues which might be located at the cation-binding sites of the Na,K-ATPase, the enzyme was treated with 7-substituted-4-diazomethyl coumarins. These compounds react specifically with carboxyl residues, and their fluorescence allows the quantitation and monitoring of the modified amino acids during their localization. Two diazomethyl-coumarins were tested as putative cation site modifiers, DEAC and MEOC. DEAC with its diethylamino group (positively charged at pH 6.5 and below) was a priori a more promising candidate, since the protonated amine might direct the probe toward a “cation-binding site.” DEAC was a more potent inactivator of the enzyme than MEOC (Fig. 2) and significant K⁺-preventable modification was observed with DEAC at pH 6.5.

Under optimum conditions for inactivation (in the presence of Mg²⁺ and P₄), Na⁺ and K⁺ ions when present in the media produced protection against inactivation by DEAC (Fig. 7). However, K⁺ ions were much more effective. It could be argued that the protection by K⁺ was merely a consequence of dephosphorylating phosphoenzyme and producing a less reactive dephosphoenzyme form. If this were the case, comparable protection by Na⁺ and K⁺ would be observed (albeit at significantly higher Na⁺ concentrations). This is not the case (Fig. 7). It is also evident that K⁺ ions are able to protect the enzyme from the (slower) inactivation by DEAC under non-phosphorylating conditions (Figs. 2–4). In contrast, when the enzyme is treated under non-phosphorylating conditions (when it is predominantly in the E₁ form), Na⁺ has very little protective effect (Fig. 3). Overall, it seems most likely that the relative reactivity of the different enzyme forms account for the effects of different ligands. The reactivity or at least...
FIG. 9. Correlation between DEAC binding and inactivation of the Na,K-ATPase. The enzyme was treated with DEAC and processed as indicated under “Experimental Procedures.” Different inactivation levels were achieved treating the enzyme with various DEAC concentration: 250 μM DEAC, 14% remaining activity; 100 μM, 31%; 50 μM, 56% and 25 μM, 80%. The amount of DEAC bound was estimated as the difference between the amount of DEAC in the samples after the treatment in absence and presence of 40 mM KCl. These values are plotted against the activity remaining after the treatment in absence of KCl. The ADP binding level in the samples used in these experiments was 1.5 ± 0.1 nmol of ADP/mg of protein. Values in the graph are the mean ± S.E. of four to five experiments. The continuous line was drawn following the equation \( y = 2.14 - 0.0218x \), \( r^2 = 0.965 \).

the reactivity of the carboxylates which lead to inactivation, is in the order \( E_2 \)P > G, imidazole > E,Na >> E, K. We believe the reason for this lies in the differing reactivity or accessibility of essential carboxy residue(s) (see below) in the various enzyme conformations, rather than different carboxyl residues being targeted under each of the different conditions.

Comparison of DEAC with Carbodiimides—Treatment of the Na,K-ATPase with carbodiimides has produced a variety of sometimes contradictory results (25-32). Although cation protection against inactivation has often been obtained, there is some question as to whether the direct modification of carboxyl groups or the formation of intramolecular cross links are responsible for the inactivation of the enzyme (28, 30, 32). In some conditions, when the enzyme was inactivated by treatment with carbodiimide, no effect on Rb+ binding was observed and a prevention of inactivation was seen if treatment was carried out in the presence of added exogenous nucleophile (30). In contrast, some studies with \( N\),\( N\)-dicyclohexylcarbodiimide have shown a slight increase in inactivation rate with exogenous nucleophile and a resultant loss of Rb+ occlusion capacity (32). The experimental basis for such differences is not known. The results obtained with DEAC in the present work shows that there is at least one carboxyl group in the \( \alpha \)-subunit of the Na,K-ATPase essentially involved in the binding and occlusion of the critical monovalent cations, Na+ and K+. The diazomethane chemistry involved is free of the ambiguity of multiple routes to different products, the diazomethanes merely esterify carboxyl residues.

Significance of the Site of Modification—K+ ions protect the enzyme against inactivation by DEAC and prevent labeling of the enzyme by DEAC. These observations imply that modification of the enzyme by DEAC might involve esterification of one or two carboxyl groups in the K+ binding site, resulting in disruption of K+ enzyme interactions. Several features of our data support this, following modification: (i) Rb+ ions do not bind to and are not occluded by the enzyme; (ii) an \( E_2 \)K tryptic cleavage pattern is not seen; (iii) the phosphoenzyme is not rapidly hydrolyzed on the addition of K+ ions. An alternative explanation for our data might be that following modification, the enzyme is “frozen” in an \( E_2 \) conformation. In this and other protein modification studies, it is important (but difficult) to decide whether a modification which results in a loss of ligand binding is due to interfering with the binding (site-specific) or whether the enzyme is held in a conformation which does not normally bind that ligand (conformation-specific, see Ref. 13 for discussion). Other modifications described previously could be accounted for by such conformational freezing, e.g. \( \text{H}_2\text{DIDS} \), which results in a loss of ATP binding and may lock the enzyme in an \( E_2 \) form (17). If DEAC-modified enzyme were frozen in an \( E_2 \)-like conformation, it would be unable to bind K+ ions, but would still give normal ATP binding. However, several observations make it more likely that we are dealing with a “site-specific” rather than “conformation-specific” effect. It can be seen that an \( E_2 \)-like tryptic digestion pattern is obtained in the presence of vanadate or ouabain and an \( E_2 \)-like pattern with ADP. Thus the modified protein is still able to undergo the \( E_1 \) \( \rightarrow \) \( E_2 \) conformational transition and is not frozen in one form.

Due to the extremely hydrophobic nature of the DEAC molecule, it has been difficult to obtain an accurate estimate of the total number of carboxyl residues which react covalently with DEAC on the Na,K-ATPase. Nevertheless, we have determined that 1.46 ± 0.12 carboxyl residues/high affinity ADP (hence ATP) binding site were K-protectable and esterified when the enzyme was completely inactivated. The most straightforward inference from our data is that there are one or two essential carboxyl residues located in the K+ binding domain of the Na,K-ATPase. More definite information on this may become available when the K-protectable residues are identified following proteolysis and sequencing. Such studies are now underway.

Our data, of course, do not unequivocally prove that modification is occurring at a cation-binding site; however, the most dramatic effect of the modification is the loss of K+ ion and Na+ ion binding and occlusion. While it is difficult to predict the properties of an Na,K-ATPase when it cannot bind monovalent cations, several other aspects of our results are significant. The modification does not result in a non-specific loss of function. The fact that we observe unaltered ATP binding and that the enzyme can undergo the \( E_1 \) \( \rightarrow \) \( E_2 \) conformational transition (see above) suggests that the modification is limited in its effects. It is also significant that phosphoenzyme formed from \([\text{ATP}] \)ATP is still sensitive to the addition of ADP although insensitive to the addition of K+ ions. A consequence of this observation, along with our measured stoichiometry of binding, is that the same carboxyl residue(s) which are critical for K binding in the non-phosphorylated enzyme are also important in K binding and cleavage of the phosphoenzyme intermediate. Since K stimulation of ATP hydrolysis is assumed to be carried out by the K+ ions which are actively transported by the enzyme and the occlusion site is thought to be on the transport pathway, the modified carboxyl residue(s) also seem to be critically important for ion pump transport function. It is the most parsimonious hypothesis that the binding sites at the extracellular side of the sodium pump which take up two K+ ions were just prior to this the sites which unloaded the Na+ ions which had previously been bound at the cytoplasmic side to activate phosphorylation. Although the mechanistic details are not clear, the concatenation of coordinating ligands which bind three Na+ ions rearrange to bind the two K+ ions. Our data...
show that the modification of 1 or 2 carboxyl residues results in the loss of almost all K\(^+\) and Na\(^+\) binding capacity.

REFERENCES