Glutamate 779, an Intramembrane Carboxyl, Is Essential for Monovalent Cation Binding by the Na,K-ATPase*

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Incubation of purified renal Na,K-ATPase with the fluorescent carboxyl-selective reagent, 4-(diazoaethyl)-7-(diethylamino)-coumarin (DEAC), results in enzyme inactivation via disruption of the monovalent cation binding sites and loss of K⁺ and Na⁺ binding capacity. Modification of 1 or 2 carboxyl residues in the α-subunit in a K⁺ or Na⁺-preventable manner leaves the ATP binding altered, and the enzyme is still able to undergo the major conformational transitions (Argüello, J. M., and Kaplan, J. H. (1991) J. Biol. Chem. 266, 14627–14635). Labeled α-subunits of Na,K-ATPase were isolated by gel electrophoresis and digested using V₈ protease. The digests contained two bands which were fluoroscence labeled in a cation-protectable fashion. Four peptides were identified in these bands. Peptides I (apparent molecular mass ~17 kDa), II (~15 kDa), and IIIa (~5 kDa) start at Glu₇⁷⁹ while Peptide IIIb (~5 kDa) starts at Glu³⁶¹. Subsequent proteolysis of peptides IIIa and IIIb with thermolysin followed by electrophoresis revealed a single smaller fluorescent peptide which passed through 3-kDa cut-off membrane filters but was retained by 1-kDa cut-off filters. N-terminal sequence analysis of this peptide gave the sequence Leu-Thr-Ser-Asn-Ile-Pro-Glu-Ile-Thr-Pro-Phe-Leu. The length of this peptide was also examined in labeling experiments with single smaller fluorescent peptide which passed through a 3-kDa cut-off membrane filter. N-terminal sequence analysis of this peptide gave the sequence Leu-Thr-Ser-Asn-Ile-Pro-Glu-Ile-Thr-Pro-Phe-Leu. The length of this peptide was also examined in labeling experiments with cysteine-reactive probes which indicated that the peptide did not extend to the next carboxyl-containing amino acid residue in the α-subunit sequence. (Asp₀⁴₀) The site of attachment of DEAC is thus Glu₇⁷⁹, an intramembrane carboxyl residue present in all known sequences of the α-subunit isoforms of the Na,K-ATPase. This glutamate is essential for Na⁺ and K⁺ binding and active transport by the sodium pump. Its location in the major cytoplasmic loop of the enzyme is transmitted to intramembrane cation sites during the reaction cycle.

The Na,K-ATPase (EC 3.6.1.37) actively transports Na⁺ and K⁺ across the plasma membrane of eukaryotic cells. This enzyme together with the gastric H,K-ATPase, the sarcoplasmic reticulum Ca-ATPase, and the plasma membrane Ca-ATPase are members of a specialized class of ion pumps, termed P-type ATPases due to the phosphorylated intermediate formation in their reaction cycle. The P-type ATPases share a high homology in the amino acid sequence of their catalytic subunits (1–11, for review see Ref. 12) as well as a common catalytic mechanism (13–15). A large amount of work has been done to describe the relation between hydrolytic and transport cycles of the Na,K-ATPase (14, 16). However, little detail is known about the molecular mechanism which couples active cation transport to ATP hydrolysis.

The primary structure of the α- and β-subunits in their different known isoforms have been determined (1–5, 11, 12, 17–21). The glycosylated β-subunit (M, 35,000 for the protein component) has one transmembrane segment and most of its mass in the extracellular space (21, 22). The α-subunit (M, 112,000) is considered to be the catalytic subunit. Models for the α-subunit structure, containing 7, 8, or 10 transmembrane segments with a large central cytoplasmic loop, have been proposed. The cytoplasmic loop is probably primarily involved in ATP hydrolysis, while the required structure for the cation binding and occlusion seems to be restricted to the transmembrane segments of the enzyme (23–25).

Chemical modification studies have been performed in an attempt to localize functional domains of the enzyme and to identify the particular amino acids involved in the binding of various physiological ligands (for review, see Ref. 26). Amino acid residues within the cytoplasmic loop have been associated with the ATP-binding domain, including lysines, tyrosines, cysteines, and aspartates (for review, see Refs. 24 and 26). In contrast, little is known about the putative cation binding sites (24, 25). A plausible early hypothesis was that carboxyl-bearing amino acids, in or near the membrane, might be involved in cation charge neutralization and coordination at sites in the transport pathway. In the case of the Na,K-ATPase, this idea obtained some support from the well known monovalent cation-protectable inactivation of the enzyme by carbodiimides (27–30). However, complexities in the chemistry of the modification of proteins by carbodiimides (cross-linking and rearrangement subsequent to carboxyl modification) make it difficult to confidently associate the enzyme inactivation with covalent incorporation of carbodiimide and therefore to locate the modified and essential carboxyl residues involved in cation binding (for discussion, see Refs. 26, 32, and 33). Recently, Goldshleger et al. (34) have reported the Rb⁺-protectable modification by N,N'-dicyclohexylcarbodiimide (DCCD)¹ of Glu⁹⁶³ in the α-subunit of the Na,K-ATPase. However, their interpretation that Glu³⁶¹ is an essential part of the cation binding site (34–36) is rendered unlikely by the results of mutagenesis of α-subunit residues expressed in HeLa cells where Glu³⁶¹ has been replaced and Na,K-ATPase activity is still observed (37).

We have overcome the problems inherent to the chemistry of carbodiimides by using 4-(diazoaethyl)-7-(diethylamino)-coumarin (DEAC) (38, 39). This is a stable fluorescent derivative of

¹ The abbreviations used are: DCCD, N,N'-dicyclohexylcarbodiimide; DEAC, 4-(diazoaethyl)-7-(diethylamino)-coumarin; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; Tricine, N-Tris(hydroxymethyl)-methylglycine; PVDF, polyvinylidene fluoride; DMSM, 2,5-dimethoxy-stilbene-4'-maleimide.
diazomethane which reacts specifically with carboxylic residues to yield fluorescent esters (40). Treatment of the isolated Na,K-ATPase with DEAC results in enzyme inactivation due to the loss of cation binding capacity (38, 39). The inactivation rate is dramatically increased when the enzyme is treated with DEAC under conditions which produce enzyme phosphorylation (presence of Pi and Mg^2+); but most importantly, inactivation can be almost completely prevented by K^+ and by Na^+, with a lower apparent affinity. Gel electrophoresis of modified protein reveals intense fluorescence labeling of the α-subunit, which is substantially reduced if treatment with DEAC is performed in the presence of K^+ ions. The extent of inactivation is linearly related to the amount of K^+-protectable DEAC incorporation, and Na^+-protected enzymes were isolated by SDS-PAGE (46) in the presence of Na^+.

The modified enzyme is unable to occlude K^+ or Na^+ ions, but exhibits normal levels of high affinity ATP binding, and is able to undergo the catalytic cycle in the absence of Na^+ and in the presence of Mg^2+ and ATP, with a rate linearly related to the amount of K^'-protectable DEAC incorporated. The rate is dramatically increased when the enzyme is treated with DEAC, and the Ki (for K^+ protection) is identified as G~u~~~ in the fifth transmembrane segment.

These results indicate that (i) there are 1 or 2 carboxyl residues which are essential for K^+ and Na^+-binding and occlusion. (ii) These residues are probably part of the monovalent cation binding domain and occlusion site in the Na,K-ATPase α-subunit (38, 39). We describe here the identification of Glu^{79} as the carboxyl residue modified by DEAC which is protectable by both K^+ and Na^+ and propose its involvement in the binding and occlusion of both cations in the Na,K-ATPase. A preliminary report of some of our data has been presented (41).

**EXPERIMENTAL PROCEDURES**

**Materials—**ATP, bovine serum albumin (BSA), and thermolysin (Bacillus thermoproteolyticus neutral protease, EC 3.4.24.2) were from Sigma. DEAC was from Molecular Probes. Endoproteinase Glu-C (Protease V8, EC 3.4.21.9) was from Boehringer Mannheim. Acrylamide, bis(acrylamide), and urea were from Bio-Rad. The protein molecular weight standards were from Amersham International plc.

**Enzyme Isolation—**Na,K-ATPase was purified from canine kidney outer medulla according to Jorgensen (42) with the modifications of Liang and Winter (43). After ultracentrifugation, the enzyme was washed with 25 mM imidazole, 1 mM EDTA, pH 7.5 (buffer C), before storing at 5 °C. Protein concentration was determined by the method of Lowry et al. (44) using BSA as standard. The Na,K-ATPase activity of the enzyme used in these studies was about 17-23 pmol of P_i mg"^{-1} min"^{-1}, assayed as described below.

**Enzyme Treatment with DEAC—**The enzyme, usually 10-20 mg (0.5 mg/ml), was treated in 50 mM imidazole, pH 6.5, 1 mM EDTA, 3 mM MgCl_2, 3 mM P_i, 250 μM DEAC, 10% dimethyl sulfoxide during 2 h at 37 °C (38). The modified protein obtained using this procedure retained 10% of its initial activity and will be referred to as DEAC-enzyme in this paper. 100 mM NaCl was included in the treatment media when it was desired to obtain K^+-protected enzyme (90% active); likewise, 100 mM NaCl was present in the media when Na^+-protected enzyme (75% active) was prepared. The reaction was stopped by dilution in buffer C and ultracentrifugation or by dilution (1:10) in the ATPase assay medium; both methods gave the same results.

**Na,K-ATPase Activity Assay—**The assay medium (mm): EGTA, 0.5; NaCl, 130; KCl, 20; MgCl_2, 3; ATP, 3; and imidazole, 50, pH 7.2, 0.3 mM MgCl_2, 0.5 μM ATP, 0.5 μM enzyme protein. The assay was performed at 37 °C for 15 min and the P_i released determined (45). The Na,K-ATPase activity was calculated from the difference between the ATP hydrolysis measured in the absence and in the presence of 0.5 mM ouabain.

**α-Subunit Isolation—**The α-subunits from DEAC-enzyme, K^+-protected, and Na^+-protected enzymes were isolated by SDS-PAGE (46) in 7.5% acrylamide gels. Large gels were used because of our preparative scale requirements (0.225 x 10 x 10 cm). They were loaded in a single gel-wide well with 2.5 mg of ATPase protein and run 1 h at 100 and 4 h at 200 V. After electrophoresis, DEAC-labeled subunits (as well as the DEAC-labeled peptides in later gels) were detected by their fluorescence emission on illumination with a hand-held long wavelength (360 nm) UV lamp and photographed using TRIX film and a yellow filter. Photographic negatives were scanned in a densitometer when comparative quantification was required. The bands corresponding to α-subunit bands were cut in 1-2 mm pieces, homogenized with 5 volumes of 0.1% SDS, 0.1 mM NH_4HCO_3, pH 8.5, in a glass-Teflon homogenizer. The gels were extracted during 5-8 h, and the gel pieces were kept in suspension with a rotating mixer. The elution was repeated twice, and the eluates were concentrated using Centricon 30 ultrafiltration devices (Amicon, Grace Co.). α-Subunits were then precipitated with 9 volumes of methanol at -20 °C overnight.

The isolated α-subunits were washed twice with resuspension with 1 ml of 0.1% SDS, 0.1 mM NH_4HCO_3, pH 8.5, and precipitated with 9 volumes of methanol at -20 °C for 8-10 h. Finally, the protein was resuspended in 0.05% SDS, 20 mM phosphate buffer, pH 7. This procedure yielded 0.3-0.4 mg of α-subunit/mg of initial Na,K-ATPase. Protein concentration in isolated α-subunit (as well as in later purified peptides) was determined according to Bradford (47).

**Peptide Isolation—**The general strategy for the isolation of DEAC-labeled peptides is shown in Scheme 1.

Purified α-subunits were cleaved with protease V8 (1:20, w/w) in 0.05% SDS, 20 mM phosphate buffer, pH 7, 6 h, 37 °C. Then, more protease V8 was added bringing the final protease/protein ratio to 1:10 (w/w) and the proteolysis continued overnight at room temperature. The resulting digests were separated by SDS-PAGE either in 15% polyacrylamide (Tricine-urea) gels (46) or in 16.5% polyacrylamide (Tricine-urea) gels (48).

Tricine-urea SDS-PAGE of V8-treated α-subunit (50 μg/well) was performed in 16.5% polyacrylamide gels, including 6 μm urea in the separating portion of the gel (0.75 mm thick, 18.5% T, 6% C separating, 10 cm; 10% T, 3% C spacers, 1.5 cm; 4% T, 3% C stacking, 1 cm). The gels were aged overnight, prern (3, 25 mA) with "gel buffer" (diluted 1:3) containing 0.1 mM thioglycollate in the cathode chamber (49), the samples were run (25 mA, 16-20 h) with thioglycollate (0.1 mM) added to the running cathode buffer. After electrophoresis the gels were photographed and scanned as indicated above (α-subunit isolation). Two DEAC-labeled cation-protectable bands with approximate apparent molecular masses of 15-17 kDa (peptides I and II) and 5-6 kDa (peptides IIIa and b) were observed in these gels. Peptides I and II which generally appeared as a broad band sometimes resolved as a doublet were better separated in Tris-glycine gels (see below). The band corre-
120A phenylthiohydantoin analyzer. Automated Edman degradation was done using the manufacturer's reaction vessel cycle PRO-1, with modifications including the use of a higher reaction temperature (49 °C) and a 30% increase in the delivery time of trimethylamine vapors. In particular, for sequencing of peptide IV, the PVDF membrane was positioned on top of a Polybrene-conditioned filter disc (50). This procedure has been found necessary for similarly small decapetides (50).

Labeling of Peptide IV and Peptides IIIa and b with 2,5-Dimethoxy-stilbene-4'-maleimide (DMSM)—To probe the length of peptide IV, double-labeling experiments were designed. DEAC-labeled peptides IIIa and IIIb and peptide IV were treated with 20 μM DMSM, a fluorescent cystein-reactive reagent in pH 2.5, respectively. At the higher pH both chromophores fluoresce; at pH 8.2, at room temperature, in the dark, during 1 h. The fragments were then run in a non-reducing Tricine-urea gel. Peptide-bound DEAC and DMSM chromophores were detected by their fluorescence emission after equilibrating the gel for 20 min either in 0.1 M Tris, pH 8.2, or in 10% acetic acid, pH 2.5, respectively. At the higher pH both chromophores fluoresce; at the lower pH only DMSM is fluorescent.

RESULTS

In our previous studies (38, 39), we described the inactivation of Na,K-ATPase upon chemical modification by DEAC. The characteristics of the reaction as well as those of the resulting DEAC-enzyme led us to propose that DEAC modifies 1 or 2 carboxyl residues at the cation binding site of the enzyme. We then set out to localize the residues and to determine at the same time if Na+ and K+ were indeed preventing modification of Na,K-ATPase upon chemical modification by DEAC. The enzyme (50 μg/lane) was modified as indicated under "Experimental Procedures." The fluorescent bands (lanes A1, A3) in the unstained gel were visualized by illumination with a hand-held UV lamp. Lanes B1, B3 show the Coomassie Brilliant Blue staining of the gel. The positions of molecular weight markers, run in the same gel, are indicated.

α-subunits. Fig. 2 shows the patterns resulting from extensive proteolysis of DEAC-treated (lanes A1 and B1), Na+-protected (lanes A2 and B2), Na+-protected (lanes A2 and B3), and K+-protected (lanes A3 and B3) α-subunits with V8 protease. The Coomassie Brilliant Blue staining of the gel (Fig. 2B) shows an identical proteolytic pattern for the three α-subunits, this allows direct comparison of the fluorescent patterns in terms of the extent of DEAC labeling and cation protection of specific peptides. The extent of fluorescence labeling of the proteolysis fragments indicates that the mixture can be resolved into both cation-protectable and non-protectable peptides. The unprotected peptides are obviously not associated with enzyme inactivation. When Na+-protected and K+-protected α-subunits are compared, a very similar distribution of DEAC labeling is observed (compare A2 and A3 in Fig. 2). This supports the idea that both cations protect the same carboxyl residue against DEAC modification. Inspection of the fluorescent labeling pattern (in Fig. 2) of digested α-subunit from DEAC-enzyme (lane A1) compared with any of the two α-subunits from protected enzymes (lane A2 and A3) reveals the presence of two DEAC-labeled cation-protectable bands, to-
Peptides in undigested α-subunits when protected and unprotected estrogen α-subunits maintains the same relationships observed protease treated n-subunits from DEAC-enzyme (A, and B1), 44 cent bands and &). The tr-subunits Na+-protected enzyme (A, and isolated from Tricine gels, yielded inconsistent results. The associated with any particular band. The fragments with an apparent molecular mass of 15–17 kDa ran as a broad band in the Tricine gels, and in some samples they appeared as a doublet. The Coomassie staining of the gel shows very little protein associated with this particular fluorescent band, and the sequencing of peptides in this band, positions as the only peptide (peptide IV) in this fluorescent band. When this peptide was sequenced using standard procedures with PVDF membranes as the only immobilizing matrix, the yields of amino acids dropped quickly after the first cycle, and it was impossible to sequence this peptide with confidence beyond 5 or 6 residues (Table I). This drop in yield after the first cycle is characteristic of short peptides sequenced on PVDF. Our results agree with those obtained earlier when sequencing model decapeptides (see Table 9, Ref. 50). Inclusion of Polybrene discs under the PVDF membrane (50) significantly increased the yield in each cycle (Table I). Consequently, we were able to sequence peptide IV. Since Glu779 is the only carboxyl residue in this DEAC-modified peptide, it is thus identified as the target of DEAC inactivation. The presence of Glu779 during the sequencing is due to the hydrolysis of the DEAC-carboxyl ester under the acidic sequencing conditions. The extensive hydrolysis of glutamatic and aspartic esters under these conditions is well known (51, 52).

The possibility that peptide IV extends beyond Leu784 and contains another carboxyl residue which is modified by DEAC is unlikely. The next carboxyl residue in the Na,K-ATPase sequence is Asp804. To show that peptide IV does not reach Asp804, we took advantage of (i) the fact that Cys892 is the only thiol group between Leu779 and Asp804, and (ii) the pH sensitivity of DEAC (DEAC is not fluorescent at low pH). We treated the DEAC-labeled peptide IV with the sulfhydryl reagent DMSM. We then ran this DMSM-treated DEAC-labeled peptide IV in a non-reducing Tricine gel and lowered the pH of the gel to eliminate the fluorescence due to DEAC (Fig. 5). Under this condition, peptide IV was not fluorescent indicating that it did not

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3 R. A. Farley and R. W. Mercer, personal communications.
The peptides were isolated and sequenced as indicated under "Experimental Procedures." The picomole yield of each amino acid is indicated in parentheses. Sequencing of peptides I and II was stopped after 10 cycles. Sequencing of peptides IIIa and IIIb was halted after seven cycles. Peptide IV on PVDF support was sequenced for 10 cycles, and no amino acid was detected beyond cycle 8. Peptide IV on PVDF and Polybrene was sequenced reported earlier and ascribed to problems related to the sequencing chemistry on PVDF membranes (61).

| Cycle | Peptide I | Peptide II | Peptide IIIa | Peptide IIIb | Peptide IV and PVDF | Peptide IV, PVDF, and Polybrene |
|-------|-----------|------------|--------------|--------------|---------------------|---------------------------------|
| 1     | Gly (50)  | Gly (51)   | Gly (40)     | Leu (52)     | Leu (60)            |
| 2     | Arg (43)  | Arg (37)   | Arg (38)     | Phe (61)     | Thr (14)            |
| 3     | Leu (32)  | Leu (27)   | Leu (41)     | Glu (68)     | Ser (5)             |
| 4     | Ile (41)  | Ile (30)   | Ile (24)     | Phe (50)     | Asn (10)            |
| 5     | Phe (20)  | Phe (17)   | Phe (19)     | Asp (51)     | Ile (6)             |
| 6     | Asp (11)  | Asp (11)   | Asp (21)     | Thr (14)     | Pro (6)             |
| 7     | Asn (12)  | Asn (11)   | Asn (12)     | Asp (40)     | Glu (2)             |
| 8     | Leu (12)  | Leu (12)   | Leu (12)     | Ile (2)      | Ile (22)            |
| 9     | Lys (6)   | Lys (5)    | Lys (6)      | Ile (12)     | Thr (12)            |
| 10    | Lys (5)   | Lys (6)    | Lys (5)      | Thr (12)     | Pro (8)             |
| 11    |           |            |              |              | Phe (8)             |
| 12    |           |            |              |              | Leu (20)            |

Assigned starting position Gly1758 Gly1758 Gly1758 Gly1781 Leu1773 Leu1773

![Fig. 4. SDS-PAGE (Tricine-urea-16.5% polyacrylamide) of peptides IIIa and IIIb (lane A) and thermolysin-treated peptides IIIa and IIIb (lane B).](image)

Peptides IIIa & IIIb

Pep tide IV

![Fig. 5. SDS-PAGE (Tricine-urea-16.5% polyacrylamide) of DMSM-treated DEAC-labeled peptide IV and peptides IIIa and b (indicated as DEAC and DMSM).](image)

Deac-labeled peptides were not treated with DMSM were run as controls (indicated as DEAC). The pH values indicated are those of the media in which the gels were incubated for 20 min prior to visualization. DMSM and DEAC fluorescence was visualized by illumination with a hand-held UV lamp.

We have previously described the inactivation of the Na,K-ATPase by DEAC (38, 39). We presented evidence showing that the modification of 1 or 2 carboxyl residues in the α-subunit of enzyme was responsible for the removal of the Na⁺ and K⁺ binding capabilities of the enzyme with its consequent inactivation. The effects of the modification were limited to the cation binding site because DEAC-inactivated enzyme was able to bind ATP with high affinity and undergo E₁ ↔ E₂ conformational transitions. The characteristics of the inactivation and the protection by Na⁺ or K⁺, together with those of the DEAC-modified enzyme, strongly suggested the direct involvement of the modified carboxyl groups in the cation binding and occlusion. We have now identified Glu779 in the α-subunit as the amino acid modified by DEAC in a monovalent cation-protectable manner. This result, together with our previous findings, suggests that Glu779 which is localized in the putative fifth transmembrane segment of the protein (see Fig. 6) is part of the cation binding site in the Na,K-ATPase.

**DISCUSSION**

We have previously described the inactivation of the Na,K-ATPase by DEAC (38, 39). We presented evidence showing that the modification of 1 or 2 carboxyl residues in the α-subunit of enzyme was responsible for the removal of the Na⁺ and K⁺ binding capabilities of the enzyme with its consequent inactivation. The effects of the modification were limited to the cation binding site because DEAC-inactivated enzyme was able to bind ATP with high affinity and undergo E₁ ↔ E₂ conformational transitions. The characteristics of the inactivation and the protection by Na⁺ or K⁺, together with those of the DEAC-modified enzyme, strongly suggested the direct involvement of the modified carboxyl groups in the cation binding and occlusion. We have now identified Glu779 in the α-subunit as the amino acid modified by DEAC in a monovalent cation-protectable manner. This result, together with our previous findings, suggests that Glu779 which is localized in the putative fifth transmembrane segment of the protein (see Fig. 6) is part of the cation binding site in the Na,K-ATPase.

**Isolation and Sequencing of Peptides—**In order to design a strategy for localization of the DEAC-labeled carboxyl residues, the probe characteristics and the properties of the likely labeled fragments had to be considered. (i) Free DEAC or DEAC degradation products (38) partition into the membrane; if they are not effectively removed they produce high fluorescence backgrounds during peptide isolation. (ii) DEAC is not fluorescent at pH <6. (iii) The labeled carboxyl residues might be located in or near transmembrane segments which are extremely hydrophobic and therefore difficult to isolate and purify using high performance liquid chromatography (HPLC). (iv) The lactone ring of the DEAC molecule is unstable at pH
reach Aspso4, and, thus, G\textsubscript{u~~~} is the amino acid modified by DEAC. However, peptides I and II, extending perhaps to Glu93 and Glu92, respectively, have a number of additional carboxyl residues that might also be modified by DEAC or protected by Na\textsuperscript{+} or K\textsuperscript{+}, in addition to Glu770. At this time, this issue is not fully resolved, but all our results point to Glu770 as the only DEAC-labeled cation-protectable residue. Our previous studies (38) suggested that the inactivation was likely caused by a single residue.

Monovalent Cation Protection—We have shown that Na\textsuperscript{+} is slightly less efficient than K\textsuperscript{+} in protecting enzyme against inactivation by DEAC (38, 39) and DEAC labeling (Fig. 1); furthermore, after modification the loss of Na\textsuperscript{+} binding (21% of control) was also slightly smaller than the loss of K\textsuperscript{+} binding (9% of control) (38, 39). Consequently, an important point to be addressed in the localization studies was whether or not Na\textsuperscript{+} protects the labeling of the same carboxyls as K\textsuperscript{+} and, as seemed to be indicated by our previous results, Na\textsuperscript{+} and K\textsuperscript{+} both utilize this carboxyl in their interaction with the enzyme. There were no detectable differences in the labeling pattern of proteolytic digests of α-subunits coming from Na\textsuperscript{+}- or K\textsuperscript{+}-protected enzymes. This indicates that Na\textsuperscript{+} and K\textsuperscript{+} protect the same peptides against modification with DEAC. Furthermore, since we have observed a single residue modified by DEAC, Glu779, it is protectable by both cations. This supports the idea that residues involved in Na\textsuperscript{+} binding and transport are also involved in K\textsuperscript{+} binding and transport. The lack of sensitivity of phosphoenzyme to K\textsuperscript{+} ions, we reported earlier (38, 39), also suggests that K\textsuperscript{+} binding to the phosphoenzyme involves the same residues.

Significance and Role of Glu779—Some idea of the functional significance of a particular amino acid residue may be provided by its conservation in different isoforms of the enzyme, among various species, and by its presence in the sequences of structurally and functionally related enzymes. Glutamate residues corresponding to Glu779 are conserved in all the sequences we examined for Na,K-ATPase, gastric H,K-ATPase, and the sarco(endo)plasmic reticum Ca-ATPase, but it is not present in other P-type ATPases such as plasma membrane Ca pump, yeast H pump, or Escherichia coli K pump. For comparison, Table II shows an alignment of partial sequences surrounding Glu779 in the Na,K-ATPase (different isoforms and various species) and highly homologous areas from the gastric H,K-ATPase, and the sarco(endo)plasmic reticum Ca-ATPase. It is clear that this particular carboxyl is in a highly conserved region of the Na,K-ATPase and also the H,K-ATPase. In all of the pumps which possess a residue corresponding to Glu779, it appears in the fifth transmembrane segment of models based on hydroathy analysis. The sarcoplastic reticum calcium pump also has this residue, although the surrounding sequence shows little identity with the corresponding Na,K-ATPase primary structure. The different residues surrounding Glu779(Glu771 in the rabbit SERCA2) compared with the Na,K-ATPase sequence may explain our observation that DEAC inhibits the sarcoplasmic reticum Ca-ATPase to only a limited extent, and this inactivation is not protectable by Ca\textsuperscript{2+} ions. 4 However, Glu779 in the Ca\textsuperscript{2+} pump (SERCA2) has been the target of mutagenesis studies, and the products of those mutations (Glu771→Asp or Glu771→Gln and Glu771→Asn) are unable to support Ca\textsuperscript{2+}-dependent functions of the enzyme (53, 54). The conservation shown in Table II together with this latter finding supports the idea that Glu779 may play an important role in cation binding in the Na\textsuperscript{+} pump. It is interesting to note that Glu863 previously identified from chemical modification studies to be at the K binding (35, 36) site (but see Ref. 37), is not conserved in the α-subunit sequences of Drosophila (3), Artemia (4), or Hydra (5). Chemical modification of carboxyl residues in the Na,K-ATPase has been previously performed using carbodiimides (27-34). Goldshleger et al. (34) have shown, under conditions of very low inactivation (necessary to reduce high nonspecific labelings), Rb\textsuperscript{+}-preventable DCCD labeling of Glu850 and other

4 J. M. Argüello and J. H. Kaplan, unpublished results.
(unidentified) carboxyl residues together with Rb+-preventable cross-linking and concluded that Glu$^{563}$ was at the cation binding site. However, recent data from mutagenesis studies of heterologously expressed enzyme in HeLa cells have shown that neither Glu$^{563}$ nor its neighbor Glu$^{564}$ are essential for enzyme activity and hence monovalent cation binding (37). Using a different experimental system where a large proportion of the extramembranous portions of the α-subunit have been removed proteolytically to produce so-called "19 kDa membranes," Karlish et al. (35) also found monovalent cation-preventable incorporation of DCCD in an electrophoresis band containing the transmembrane fragments M1 + M2 and M3 + M4 (see Fig. 6) and in the 19-kDa carboxyl end of the α-subunit starting at Asn$^{630}.$ It is interesting that the peptide starting at Gly$^{729} \ (M_r = 8,000)$ which contains the Glu$^{729}$ was not modified by DCCD (35). Furthermore, the peptides labeled with DEAC (I, II, and IIIa) do not coincide with any of the residues labeled with DCCD. Our identification of Glu$^{729}$ as the target for DEAC inactivation emphasizes the importance of those transmembrane segments in the native enzyme and in post-tryptic residues which form part of the cation occlusion site but are not contained in the C-terminal 19-kDa fragment.

**Cation Pump Mechanism—Analysis of the primary structure of the α-subunit of the Na,K-ATPase using one of several available forms of hydrophathy analysis has led to models for its topological arrangement in the membrane containing either 7, 8, or 10 transmembrane segments (1, 23). Subsequent studies using antibodies or selective proteolytic cleavage demonstrate the cytoplasmic location of the carboxyl terminus (55-57) have reduced the possibilities to models containing either 7 or 10 transmembrane segments. Fig. 6 shows one such model containing 10 transmembrane segments; this is based on a composite of studies on the Na,K-ATPase and alignments with sequence from other P-type ATPases. Glu$^{779}$, the residue we have identified and postulate is directly involved in monovalent cation binding, is close to the middle of the fifth transmembrane segment. In other models with eight transmembrane segments, this residue is at the beginning (the cytoplasmic side) of the fifth transmembrane segment. This segment is directly connected to the major cytoplasmic loop which contains the phosphorylation domain, including Asp$^{566}$ and the ATP-binding domain. The involvement of Glu$^{779}$ in cation binding makes clear how binding of ATP (or phosphorylation) could affect cation binding and vice versa. Alterations in the cytoplasmic loop conformation would directly transmit to transmembrane segment 5 and thus to Glu$^{779}$ and the cation binding site. Thus, the antagonistic effects of K+ on high affinity ATP binding, and the ATP- or Pγ-stimulated deoclusion of K+ ions are readily explained. These types of interactions could also account for the observed effects of Na+ or K+ ions on the reactivity of lysine 501 toward isothiocyanate reagents (58-60) and the increased reactivity of Glu$^{779}$ toward DEAC when enzyme is phosphorylated (38).**

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