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The active site structure of Na^+/K^+-transporting ATPase: Location of the 5′-(*p*-fluorosulfonyl)benzoyladenosine binding site and soluble peptides released by trypsin

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ABSTRACT **When the dog kidney Na^+/K^+-transporting ATPase (EC 3.6.1.37, formerly EC 3.6.1.3) was labeled with an ATP analogue, 5′-(*p*-fluorosulfonyl)benzoyladenosine (FSBA), there was a concomitant loss of ATPase activity. The presence of ATP protected the enzyme from both labeling and inactivation. The ATP-sensitive incorporation of FSBA is associated only with modification of the α subunit from which two labeled tryptic peptides were purified and sequenced. To establish any regions of the enzyme protruding from the membrane, the native Na^+/K^+-transporting ATPase from the electric ray, *Torpedo californica*, was treated with trypsin; and four peptides, which were released into the water phase, were purified and sequenced. A comparison of the peptide sequences with the deduced amino acid sequences of the DNA coding for the α subunit of *T. californica* and sheep kidney reveal the following. (*i*) FSBA-labeled peptides from the dog kidney enzyme are located in the central hydrophilic domain and show almost complete sequence homology with the same region in the α subunit from the electric ray and sheep kidney. Furthermore, the sequence homology of one of the two labeled peptides can be extended to the sarcoplasmic Ca^{2+}-transporting ATPase and B subunit of *Escherichia coli* K^+-transporting ATPase. (*ii*) Three trypsin-exposed peptides are found in the central hydrophilic domain, and one peptide is in the hydrophilic segment near the C terminus of the α subunit. (*iii*) The active center of Na^+/K^+-transporting ATPase is likely to be constructed from at least four different stretches in the primary sequence and, irrespective of the different specificity of cations, the various cation transport ATPases that form phosphorylated enzyme appear to have a common structure at the catalytic site for ATP hydrolysis.**

Na^+/K^+-transporting ATPase (EC 3.6.1.37, formerly EC 3.6.1.3) is an integral membrane enzyme responsible for the active Na^+ and K^+ transport through animal cell membranes. The enzyme consists of a large α subunit (M_r 110,000) and a small β subunit (M_r 40,000–60,000) containing glycosides (1). At least two amino acid residues are essential for the activity of this enzyme, namely, aspartic acid, which is phosphorylated by ATP, and lysine, which is labeled by fluorescein 5′-isothiocyanate (FITC). The sequences around these two amino acid residues are highly homologous among Na^+/K^+-transporting ATPase, Ca^{2+}-transporting ATPase and B subunit of *Escherichia coli* K^+-transporting ATPase (2–7). Cooper and Winter (8) reported that 5′-(*p*-fluorosulfonyl)benzoyladenosine (FSBA), an ATP analogue, labeled the enzyme with concomitant inhibition of the Na^+/K^+-dependent hydrolytic activity of ATP. We also reported that the K^+-dependent *p*-nitrophenylphosphate hydrolytic activity and ouabain binding capacity of the enzyme remained intact even when Na^+/K^+-dependent ATPase activity and Na^+-dependent phosphorylation of the enzyme was significantly inactivated. ATP, but not GTP, CTP, or ADP, protects the enzyme from FSBA inactivation. The incorporation of radioactive FSBA into two peptides was observed (9). Isolation of the two labeled peptides from dog kidney enzyme and their amino acid sequences will be described in this paper. Furthermore, to determine the regions exposed to the aqueous phase, tryptic peptides of the native Na^+/K^+-transporting ATPase of the electric ray were analyzed. Integrating these results and taking into account the primary sequences of the α subunit of the enzyme from the electric ray (10) and sheep kidney (11), a plausible model for the active site structure of Na^+/K^+-transporting ATPase is discussed.

MATERIALS AND METHODS

Materials. ATP, ADP, and other nucleotides were obtained from Boehringer Mannheim. 5′-(*p*-fluorosulfonyl)benzoyl-[^{3}H]adenosine ([^{3}H]FSBA) was a kind gift from W. S. Allison (University of California). 5′-(*p*-fluorosulfonyl)benzoyl[8-^{14}C]adenosine ([^{14}C]FSBA, 40 cpm/pmol), [γ-^{32}P]ATP, and [^{3}H]ouabain were purchased from Amersham. L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin was obtained from Worthington. All other reagents were of the highest quality available. Na^+/K^+-transporting ATPase was purified from the outer medulla of dog kidneys (12) or from the electric organs of *Torpedo californica* (7). Purified enzyme preparations from the kidneys and electric organs had specific activities around 1500 and 400–500 μmol P_i hydrolyzed per mg of protein per hr, respectively.

Labeling of Dog Kidney Na^+/K^+-Transporting ATPase with [^{14}C]FSBA and Isolation of the Labeled Peptides from Its α Subunits. The dog kidney enzyme (27 mg) was incubated at 25°C in 5 ml of 0.5 mM [^{14}C]FSBA (specific activity, 40 cpm/pmol), 50 mM imidazole hydrochloride (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, and 20% (vol/vol) dimethylsulfoxide. The second and third dose of 0.5 mM [^{14}C]FSBA was added at each 30 min interval, and the remaining Na^+/K^+-transporting ATPase activity was assayed. The reaction was stopped after 90 min by addition of 5 mM dithiothreitol, and the labeled enzyme was washed twice with 50 mM sodium acetate (pH 4.0) by centrifugation at 4°C to remove unreacted FSBA. The labeled enzyme was solubilized in a medium containing 50 mM Tris·HCl (pH 6.8) and 2% (wt/vol) $NaDodSO_4$, and solid urea was added to a final concentration of 6 M. The solubilized enzyme was applied to a Bio-Gel A-1.5m column equilibrated with 0.1% $NaDodSO_4$ and 50 mM sodium acetate (pH 4.0) to separate the α subunit and β subunit. The purified α subunit (7.5 mg of protein in 25 ml) was concentrated in a cellulose tube by letting the tube stand

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Abbreviations: FSBA, 5′-(*p*-fluorosulfonyl)benzoyladenosine; FITC, fluorescein isothiocyanate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; PhNCS, phenylthiohydantoin.

in polyvinylpyrrolidone K-30 powder and then dialyzed for 2 days at 4°C against 10 mM sodium acetate (pH 4.0) containing 5 M urea in which particles of an ion-exchange resin, Dowex AG1X-2, were suspended for the purpose of efficient removal of $NaDodSO_4$. Urea was removed from the dialysate through another dialysis against 1 mM HCl. The dialysate containing FSBA-labeled α subunit was digested with TPCK-trypsin (0.35 mg) in a medium containing 100 mM sodium phosphate (pH 7.0) and 1 mM $CaCl_2$. After an 8-hr incubation at room temperature, another 0.35 mg of TPCK-trypsin was added, and the mixture was incubated for an additional 16 hr. Then 40 μl of trifluoroacetic acid (F_3CCOOH) was added to stop the digestion. Purification of the FSBA-labeled peptides was carried out by reverse-phase HPLC (Waters model 204). The solvent gradient was formed by mixing 0.1% F_3CCOOH (solvent A) and 95% acetonitrile containing 0.1% F_3CCOOH (solvent B). The tryptic peptide mixture was applied to a Bio-Rad Hi-Pore C_4 column (4.6 mm × 259 mm) equilibrated with solvent A and was eluted with a linear gradient of solvent B (0–70% solvent B). The eluant was monitored by absorbances at 260 nm and 280 nm and by radioactivity. Two radioactive peak fractions were further purified by HPLC on the same column with an extremely shallow gradient of solvent B (0.05% solvent B per min). The peak of radioactivity coincided well with that of UV absorbance in the second HPLC run.

Isolation of Extra-Membrane Peptides from Electric Ray Na^+/K^+-Transporting ATPase. Na^+/K^+-transporting ATPase was purified from the electric ray and labeled with FITC as described by Ohta *et al.* (7). Without any further treatment including denaturation or subunit separation, the labeled membrane-bound enzyme (10 mg) was digested with TPCK-trypsin in 50 mM imidazole hydrochloride (pH 7.5) and 1 mM $CaCl_2$, at 37°C. TPCK-trypsin (200 μg) was added twice at 1.5-hr intervals. The reaction was stopped by addition of 1 mM phenylmethylsulfonyl fluoride. The centrifuged supernatant containing released peptides was applied on a Bio-Rad Hi-Pore C_4 column equilibrated in 0.1% F_3CCOOH. Elution was carried out with a linear gradient of 10–40% acetonitrile.

Table 1. Effect of Mg^{2+}, Na^+, K^+, and ATP on the inactivation of dog kidney Na^+/K^+-transporting ATPase by FSBA

Ligands added	Residual activity (% of control)
None	100
Na^+	100
K^+	96
$Na^+ + K^+$	100
Mg^{2+}	14
$Mg^{2+} + Na^+$	0
$Mg^{2+} + K^+$	0
$Mg^{2+} + Na^+ + K^+$	0
$ATP + Mg^{2+} + Na^+ + K^+$	100

The added concentrations of Mg^{2+}, Na^+, K^+, and ATP were 5 mM, 100 mM, 10 mM, and 3 mM, respectively. The enzyme was incubated at 25°C in 1 mM FSBA at pH 7.0, and residual activity was assayed after 90 min. The other conditions were the same as described (9).

Peak fractions were further purified by a second HPLC on the same column with a shallower gradient of acetonitrile.

Amino Acid Analysis and Sequencing of Peptides. Samples were hydrolyzed at 160°C for 40 min in evacuated tubes containing 50% F_3CCOOH and 6 M HCl (13), and amino acid compositions were analyzed by the precolumn *o*-phthalaldehyde derivatization technique (14). Cysteine, proline, and tryptophan were not detected by this method. The purified peptides were sequenced on a gas phase sequenator (model 470A; Applied Biosystems, Foster City, CA).

RESULTS

Effect of Mg^{2+}, Na^+, and K^+ on FSBA-Inactivation. The Na^+/K^+-transporting ATPase was not inactivated by FSBA in the absence of Mg^{2+}, and addition of Na^+ and K^+ had no effect (Table 1). When Mg^{2+} was present in the solution, the enzyme was inactivated at a considerable rate, and further addition of Na^+ and K^+, alone or together, resulted in the most efficient inactivation. The enzyme was protected from inactivation by the addition of ATP to the mixture.

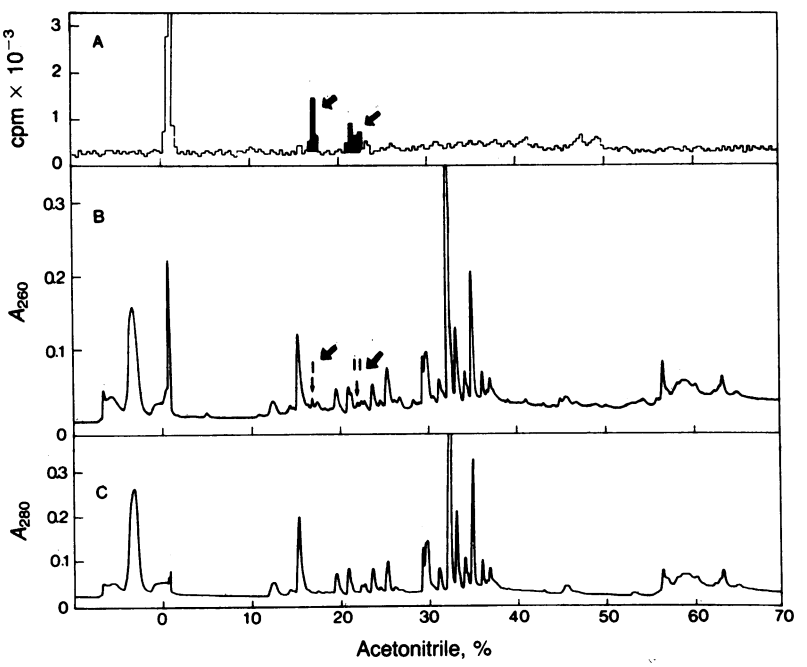

FIG. 1. Reverse-phase HPLC of tryptic peptides derived from [^{14}C]FSBA-labeled α subunit of dog kidney Na^+/K^+-transporting ATPase. The tryptic digest of the labeled α subunit was applied on a Bio-Rad Hi-Pore C_4 column. Radioactivity (*A*) and absorbances at 260 nm (*B*) and 280 nm (*C*) were monitored. Positions of [^{14}C]FSBA-labeled peptides I and II are indicated with shaded histograms and arrows.

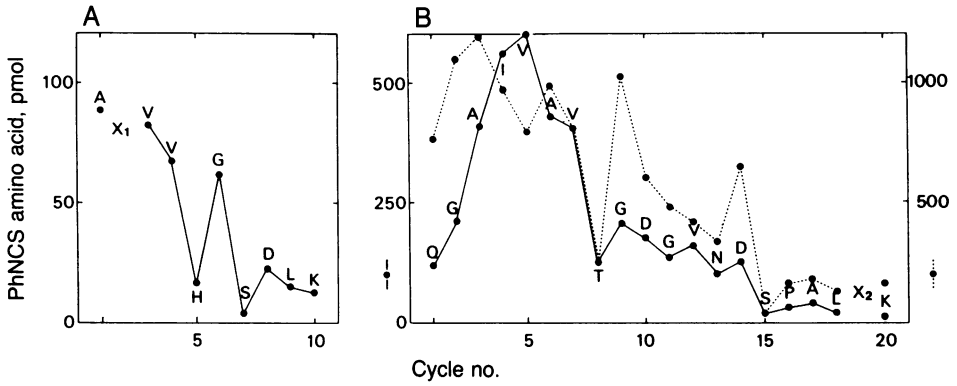

FIG. 2. Recovery of PhNCS-amino acids in sequence analyses of the FSBA-labeled peptides. (*A*) Peptide I labeled with [^{14}C]FSBA. (*B*) Peptide II labeled with [^{14}C]FSBA (solid lines), or with unlabeled FSBA (broken lines).

Isolation and Sequencing of [^{14}C]FSBA-Labeled Peptides. When the labeled α subunit was treated with TPCK-trypsin and analyzed through a Bio-Rad C_4 column (Fig. 1), three significant radioactive peaks were observed. These peaks disappeared when ATP was included in the labeling mixture. The first peak that eluted just after the gradient was applied was [^{14}C]adenosine, a decomposition product of the labeling compound during trypsin treatment. The peak fractions I and II, indicated by arrows in Fig. 1, were separately pooled and further purified on the second HPLC column. Amino acid analyses of the purified peptides confirmed the homogeneity of each peptide, and the amount of peptides estimated from the amino acid analysis showed a good agreement with that calculated from the specific radioactivity. The yield of phenylthiohydantoin-derivatized (PhNCS)-amino acid at each cycle in Edman degradation is shown in Fig. 2. No recognizable amount of PhNCS-amino acid was recovered at the positions of X_1 and X_2. In the case of peptide II, determination of the sequence was repeated using a larger amount of the α subunit labeled with nonradioactive FSBA, which was purified with the same procedures used for the radioactive peptide, to confirm the result obtained from the [^{14}C]FSBA-labeled peptide. The sequence obtained from this nonradioactive peptide was identical to that from the radioactive peptide. All the amino acids present in sequences except X_1, X_2, and proline were found quantitatively by amino acid analyses of peptides I and II. There was no unknown peak on the chart of the amino acid analysis of peptides I and II.

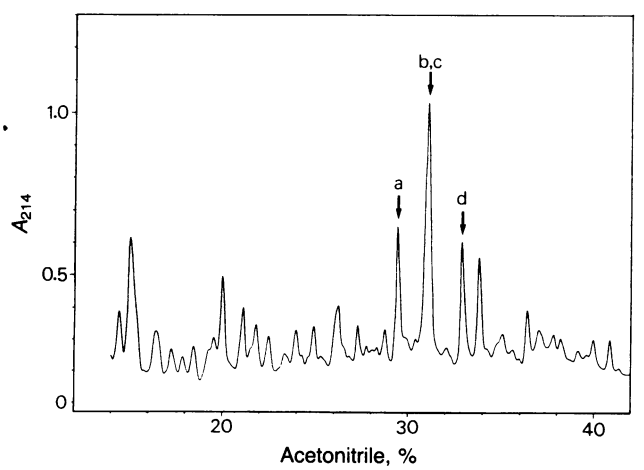

FIG. 3. Elution pattern of the tryptic digest of electric ray Na^+/K^+-transporting ATPase α subunit. The purified enzyme was labeled with FITC, and the supernatant containing water-soluble peptides, released from the FITC-labeled enzyme by trypsin digestion, was subjected to reverse-phase HPLC. Absorbance at 214 nm was monitored.

Identification of Extra-Membrane Peptides of Intact Electric Ray Na^+/K^+-Transporting ATPase. When FITC-labeled electric ray Na^+/K^+-transporting ATPase was treated with trypsin, a relatively small number of peptides were released into the aqueous phase as shown in the HPLC elution diagram of the supernatant fraction (Fig. 3). Because it is reasonable to assume that FITC labeling does not induce a significant change in the gross structure of the Na^+/K^+-transporting ATPase, these peptides appear to be derived from regions that are exposed to the water phase. Three conspicuous peaks were purified by second runs on HPLC. The second peak (denoted as b,c in Fig. 3) was split into two peaks, b and c. The fourth peak (d) was labeled with FITC (7). The sequences corresponding to the four peaks, a to d, were contained in the whole α subunit sequence (10) (Table 2).

DISCUSSION

FSBA has been widely used for the affinity labeling of the ATP-binding site of various enzymes (15). In this report, FSBA is shown to label two different sites on the α subunit of the dog kidney Na^+/K^+-transporting ATPase, and the amino acid sequences of the labeled tryptic peptides were determined. The labeling of both sites was specific, because it was completely abolished when ATP was included in the solution. The inactivation of Na^+/K^+-transporting ATPase by FSBA was dependent on the presence of Mg^{2+} whereas Na^+ and K^+ had no effect on it. However, when Na^+ and K^+, alone or together, were included in addition to Mg^{2+}, further acceleration of the inactivation was observed (Table 1). Lack of a sharp discrimination between Na^+ and K^+ on the inactivation suggests that the regions around the sites modified by FSBA may be more likely to be involved in ATP binding (or ATP hydrolysis) rather than related to subtle discrimination between the ion species. FSBA-labeled enzyme lost Na^+/K^+-dependent ATP hydrolytic activity and

Table 2. Sequences of four peptides released into the supernatant by trypsin digestion of electric ray Na^+/K^+-transporting ATPase

Peptide	Sequence
a	^{671}DLSHENLDDILHYHTEIVF----------[^{691}R]
b	^{386}MTVAHMWFDNQIHEADT----------[^{413}K]
c	^{887}EKWDELWTQDLEDSYGQQWT----------[^{910}R]
d	^{502}YLLVMKGAPE^{512}R

The superscript at the left of each sequence indicates the position of the first amino acid of the peptide in the electric ray whole sequence (10). The arginine or lysine flanking each peptide is indicated in brackets.

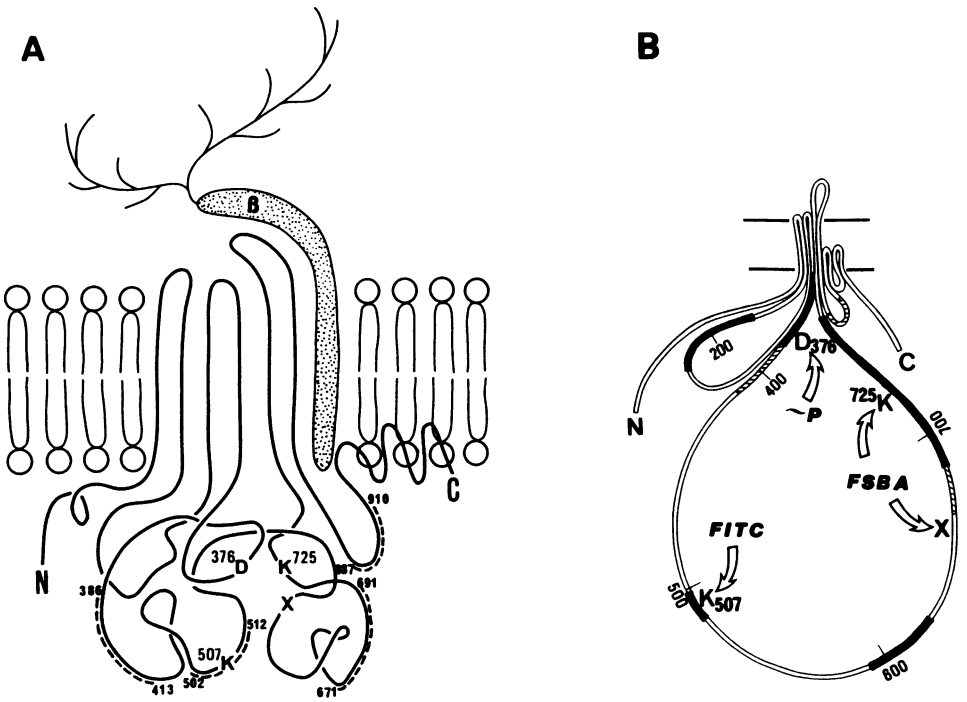

FIG. 4. (*A*) Possible model for Na^+/K^+-transporting ATPase. Numbers on the model indicate positions of the modified amino acid residues allotted on the whole sequence of the α subunit of the Na^+/K^+-transporting ATPase of electric ray (10). Four stretches containing these amino acid residues are likely to be involved in the composition of the active site. The indicated positions of the following amino acids are obtained by comparison of the result of dog kidney enzyme with the electric ray sequence: D^{376}, phosphorylated aspartic acid; K^{507}, lysine that is modified by FITC; X, unidentified amino acid of peptide I that binds FSBA; K^{725}, lysine of peptide II that probably binds FSBA. Peptides that are likely to be exposed at the surface of the enzyme are indicated by broken lines. (*B*) The locations of the trypsin-released peptides (hatched areas), the sites of labeled amino acid residues, and five highly homologous regions (solid areas) among Na^+/K^+-transporting ATPase, Ca^{2+}-transporting ATPase and B subunit of *E. coli* K^+-transporting ATPase. The locations are shown on the amino acid sequence of the α subunit of electric ray Na^+/K^+-transporting ATPase. For the purpose of showing the relative distance and the lengths of regions, each of the out-of-membrane segments, predicted from hydropathy analysis (10), is extended to a loop.

Na^+-dependent phosphorylation of the enzyme by ATP. However, *p*-nitrophenylphosphate hydrolytic activity was resistant to FSBA inactivation (9). FSBA appears to block ATP binding but *p*-nitrophenylphosphate can still reach the active site in different ways. Ouabain binding site is probably apart from ATP binding site, since ouabain binding capacity is preserved for FSBA-labeled enzyme (9).

The labeled amino acid residues were not identified in this study. If the sequences of the two FSBA-modified peptides are conserved in the dog and the sheep kidney and electric ray enzymes (see Fig. 5), then the predicted amino acids in the missing cycles (X_1 or X_2) should be cysteine in the peptide I and lysine in the peptide II. Supposition of lysine as the reactive residue in peptide II is probably valid, since lysine is known to react with FSBA to form a stable derivative of which the PhNCS derivative will behave differently from PhNCS-lysine in the analysis of the products from the Edman degradation. Also its *o*-phthalaldehyde derivative will not be detected in the precolumn method of amino acid analysis used in this study. If X_2 is really cysteine, this cysteine will not be the labeled amino acid residue, since the sulfonylated cysteine would be degraded, and radioactivity would be released from the peptide when dithiothreitol was added to quench the reaction (15). If the possibility of cysteine is excluded, then other potentially reactive residues, such as histidine, serine, and aspartic acid in the sequence, have to be considered as candidates for the labeled residue. If the cysteine in electric ray is replaced by the corresponding amino acid in the corresponding peptide from dog kidney, FSBA may bind to this substituted residue.

The locations of the FSBA-labeled peptides from the dog kidney enzyme and the trypsin-released peptides from the electric ray enzyme are schematically shown in Fig. 4. The sequences corresponding to peptide I and II of the dog kidney enzyme, labeled by FSBA, are found in the electric ray enzyme at amino acids 661–670 and amino acids 707–726 (10), respectively. They are also found in the sheep kidney enzyme at almost the same positions, amino acid 655–664 and amino acid 701–720 (11). The sequences around the two FSBA-binding sites are highly homologous among the Na^+/K^+-transporting ATPases from three sources (Fig. 5). If X_1 is cysteine and X_2 is lysine in the dog enzyme, sequences are all the same except one amino acid substitution in peptide I in electric ray enzyme. So far at least two amino acid residues have been reported to be essential for the activity of Na^+/K^+-transporting ATPase, that is, lysine, which is labeled by FITC (5–7), and aspartic acid, which is phosphorylated with ATP (2, 3, 17). This study identifies two FSBA-labeled peptides that are derived from two regions other than the FITC and phosphorylation sites. The simplest interpretation of these results is that the active center of the Na^+/K^+-transporting ATPase is composed of at least four stretches of peptide chain, that is, a FITC site, a phosphorylation site, and two FSBA sites (Fig. 4*A*). Although they are far apart in the primary sequence, folding of the peptide chain may enable them to comprise the active site in the tertiary structure.

The importance of the two FSBA-labeled regions, especially peptide II, is confirmed by their homology to other cation-transporting ATPases. From a comparison of the entire sequence of the α subunit of electric ray Na^+/K^+-transporting ATPase to that of the rabbit Ca^{2+}-transporting ATPase and the B subunit of *E. coli* K^+-transporting ATPase, Kawakami *et al.* showed the presence of three very homologous regions in addition to the FITC and the phosphorylation site (10). We have found that the region of peptide II represents one of these homologous regions and the location

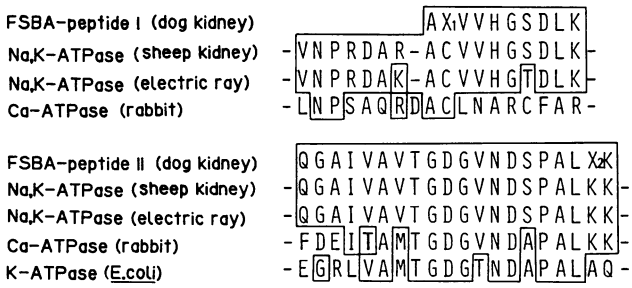

FIG. 5. Homology of FSBA-labeled peptides of dog kidney Na^+/K^+-transporting ATPase (Na,K-ATPase) to other ATPases. The amino acid sequences of the FSBA-labeled peptides I and II are compared with homologous sequences of sheep kidney (11) and electric ray Na^+/K^+-transporting ATPase (Na,K-ATPase) (10), rabbit skeletal sarcoplasmic reticulum Ca^{2+}-transporting ATPase (Ca-ATPase) (16), and B subunit of *E. coli* K^+-transporting ATPase (K-ATPase) (4). For FSBA peptide I, the flanking sequence to the N-terminal side of other enzymes is also shown. Sets of identical residues are enclosed with solid lines. X_1 and X_2 indicate the missing amino acids.

of peptide I is at about the middle of two homologous regions, where homology to B subunit of *E. coli* K^+-transporting ATPase disappears but homology to Ca^{2+}-transporting ATPase still partially remains (Fig. 4*B* and ref. 5).

It is suggested that there are at least six (10) or eight (11) transmembrane segments in the α subunit of Na^+/K^+-transporting ATPase and that the membrane-protruding segment between the fourth and fifth transmembrane segment is as long as 40% of the total sequence. Three trypsin-released water-soluble peptides, peptide a, b, and d in Table 2, were found to belong to this central hydrophilic domain (Fig. 4). The sequence of peptide c was found in the adjacent protein segment protruding from the membrane. Both accessibility to trypsin and facile release into the water phase indicate that these peptides are derived from the exposed surface of the intact enzyme, and thus, the prediction, which was derived from hydropathy analysis (10, 11), is confirmed. Interestingly, one of the peptides, peptide a, is adjacent to the FSBA-labeled peptide I. The peptide b contains the FITC-reactive lysine. All four peptides labeled with FSBA, FITC, and phosphorylation are located in the central hydrophilic domain, and three of them represent the highly homologous regions found among other cation transport ATPases within this domain (Fig. 4*B*). The fourth homologous region around amino acid 600 in this domain has not been characterized yet by labeling study. The fifth conserved sequence is located in the out-of-membrane segment between the second and third transmembrane segment (10). It is likely that ATP hydrolysis occurs in the central hydrophilic domain either with or without the other exposed membrane segment mentioned above in a mechanism common among various cation transport ATPases that form a phosphorylated intermediate. The energy of hydrolysis seems to be transferred to the ion-transporting segment, which has a specific structure for the species of cation to be transported and is presumably embedded in the lipid bilayer.

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