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Ligand-sensitive Interactions among the Transmembrane Helices of Na^+/K^+-ATPase*

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Noune A. Sarvazyan, Alexander Ivanov, Nikolai N. Modyanov, and Amir Askari‡

From the Department of Pharmacology, Medical College of Ohio, Toledo, Ohio 43699-0008

An extensively trypsin-digested Na^+/K^+-ATPase, which retains the ability to bind Na^+, K^+, and ouabain, consists of four fragments of the α-subunit that contain all 10 transmembrane α domains, and the β-subunit, a fraction of which is cleaved at Arg^{142}-Gly^{143}. In previous studies, we solubilized this preparation with a detergent and mapped the relative positions of several transmembrane helices of the subunits by chemical cross-linking. To determine if these detected helix-helix proximities were representative of those existing in the bilayer prior to solubilization, we have now done similar studies on the membrane-bound preparation of the same digested enzyme. After oxidative sulfhydryl cross-linking catalyzed by Cu^{2+}-phenanthroline, two prominent products were identified by their mobilities and the analyses of their N termini. One was a dimer of a 11-kDa α-fragment containing the H_1-H_2 helices and a 22-kDa α-fragment containing the H_7-H_{10} helices. This dimer seemed to be the same as that obtained in the solubilized preparation. The other product was a trimer of the above two α-fragments and that fraction of β whose extracellular domain was cleaved at Arg^{142}-Gly^{143}. This product was different from a similar one of the solubilized preparation in that the latter contained the predominant fraction of β without the extracellular cleavage. The cross-linking reactions of the membrane preparation, but not those of the solubilized one, were hindered specifically by Na^+, K^+, and ouabain. These findings indicate that (*a*) the H_1-H_2 transmembrane helices of α are adjacent to some of its H_7-H_{10} helices both in solubilized and membrane-bound states, (*b*) the alignment of the residues of the single transmembrane helix of β with the interacting H_1-H_2 and H_7-H_{10} helices of α is altered by detergent solubilization and by structural changes in the extracellular domain of β, and (*c*) the three-dimensional packing of the interacting transmembrane helices of α and β are regulated by the specific ligands of the enzyme.

The coupled active transport of Na^+ and K^+ across the plasma membrane of most eucaryotic cells is carried out by Na^+/K^+-ATPase. The enzyme consists of two subunits, α and β, both of which are essential to normal function (1). That some transmembrane domains of the subunits must be involved in cation transport is self-evident, and specific roles of several such domains of the α-subunit in ion binding and transport have been explored (1–6). There is one transmembrane domain in the β-subunit, and the 10-span model of α is favored by most evidence (1).

To define the roles of the intramembrane domains in ion binding and transport, it is clearly necessary to go beyond two-dimensional schemes of folding and obtain information about the three-dimensional packing of the transmembrane domains of Na^+/K^+-ATPase. There is a paucity of such information, however, because it has been difficult to apply high resolution structural methods to hydrophobic helix-bundle proteins such as Na^+/K^+-ATPase. Molecular modeling studies have been helpful (7–9). Limited experimental data on the helix packing of Na^+/K^+-ATPase have resulted from our recent chemical cross-linking studies on enzyme preparations that were subjected to controlled proteolysis and solubilized in a detergent (10). These findings suggested contact between the intramembrane H_1,H_2 helix pair and H_8-H_{10} intramembrane helices of α, and between these N-terminal and C-terminal helices of α and the single intramembrane helix of β (10). The use of detergent-solubilized membrane proteins in chemical cross-linking studies is both necessary and problematic. On the one hand, solubilized preparations must be used to rule out the possibility of cross-linking due to random collisions of proteins and peptides that are highly concentrated within the membrane phase (11–13). On the other hand, once the existence of a stable noncovalent complex is established by the formation of a cross-linked product in a solubilized preparation, it is necessary to ensure that the detected protein-protein interaction is not entirely an artifact of solubilization (11). The purpose of the present experiments was to determine if the helix-helix proximities that we had detected in solubilized preparations (10) were indeed representatives of the interactions within the native membrane.

EXPERIMENTAL PROCEDURES

The purified membrane-bound Na^+/K^+-ATPase of canine kidney medulla, with specific activity of 1000–1600 μmol of ATP hydrolyzed/mg of protein/h, was prepared and assayed as described previously (14). The enzyme was exposed to trypsin (Sigma, Type III-S, bovine pancreas) in the presence of Rb^+ and EDTA at pH 8.5 (10, 15) to remove large portions of the cytoplasmic domains of the α-subunit, and obtain a preparation containing an essentially intact β-subunit and several fragments of the α-subunit (10, 16, 17). This preparation is often called the "19-kDa membranes" because its cation occlusion capacity was originally thought to be due to a C-terminal fragment of α with the apparent molecular mass of 19 kDa (15). It is now evident, however, that several, if not all, peptides of this preparation are involved in its functions (5, 16, 17). The capacity of this digested preparation to occlude Rb^+ was verified before use by previously described procedures (15, 18).

For cross-linking, the digested enzyme was suspended (2 mg/ml) in a solution containing 10 mM Tris-HCl (pH 7.4), 0.25 mM $CuSO_4$, and 1.25 mM *o*-phenanthroline, and incubated at 24 °C for 15 min. These conditions were chosen after preliminary experiments using the approach described previously (13). The reaction was terminated by the addition of EDTA to a final concentration of 30 mM, followed by SDS to a final concentration of 2%. Samples were subjected to electrophoresis on

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‡ To whom correspondence should be addressed: Dept. of Pharmacology, Medical College of Ohio, P. O. Box 10008, Toledo, OH 43699-0008. Tel.: 419-381-4182; Fax: 419-381-2871.

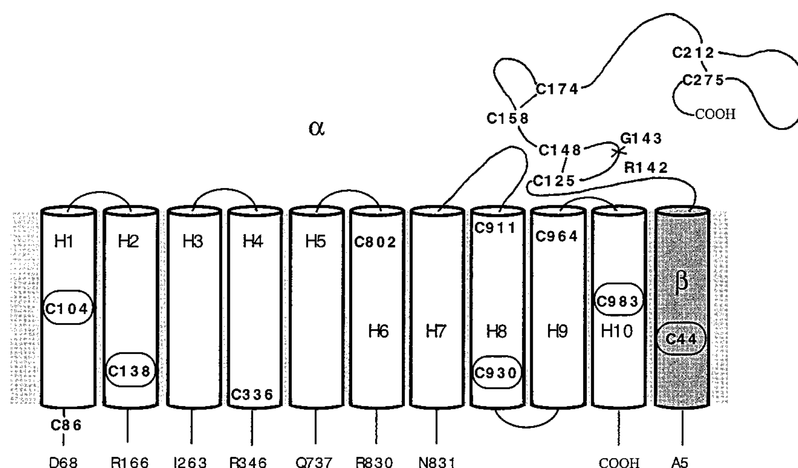

FIG. 1. **Schematic representation of the α-subunit fragments and an essentially intact β-subunit in the extensively trypsin-digested Na^+/K^+-ATPase.** The indicated N termini have been determined experimentally, but most of the indicated C termini of the fragments have been estimated based on apparent molecular masses (10, 16, 17). The *circled* cysteines are those most likely to be involved in cross-linking reactions of the transmembrane helices of α and β. See "Results" for other details.

Tricine[1]/SDS-polyacrylamide gels (10). Unless indicated otherwise, sulfhydryl reagents were omitted from the buffers used for electrophoresis to preserve the cross-linked products. The stained peptide bands were quantified by a scanning densitometer (Bio-Rad). N-terminal analyses of the peptide bands were done as described previously (10).

RESULTS

Our previous conclusions regarding the intramembrane helix-helix interactions of Na^+/K^+-ATPase were based on the identification of cross-linked products obtained from enzyme preparations that were cleaved proteolytically, solubilized in digitonin, and oxidized in the presence of Cu^{2+} (10). The present experiments were initiated with the specific aims of determining (*a*) if the same or similar cross-linked products could be obtained in membrane-bound preparations that were not detergent-solubilized prior to cross-linking, and (*b*) if the functional specificities of the protein-protein interactions could be established by the sensitivities of the cross-linking reactions to ligands specific to Na^+/K^+-ATPase. Based on promising results of preliminary experiments, we focused attention on the cross-linking reactions of the membrane moiety of the trypsin-digested enzyme in the presence of Cu^{2+}-phenanthroline complex.

The trypsin-digested preparation used here consists of four well characterized fragments of the α-subunit (10, 16, 19) that contain the 10 transmembrane helices of the α-subunits, and an essentially intact β-subunit, a fraction of which is cleaved at Arg^{142}-Gly^{143} (16, 19). The schematic structure of this preparation is shown in Fig. 1, and the resolution of its constituent peptides on Tricine/SDS gels is shown in Fig. 2 (*lane 1*).

When the membrane-bound preparation was first exposed to Cu^{2+}-phenanthroline and then resolved on Tricine/SDS gels, several of the original bands were reduced in intensity, and several new bands appeared (Fig. 2, *lane 2*), indicating the cross-linkings of some peptides. The most prominent, and the most consistently observed, cross-linked products were the two designated as bands *a* and *b* in Fig. 2. These bands were excised from gels such as those of *lane 2* of Fig. 2, electroeluted, and subjected to N-terminal analysis. Also analyzed as controls were the materials eluted from the positions corresponding to those of bands *a* and *b* from the gels on which the uncross-linked samples were resolved (Fig. 2, *lane 1*). These analyses (Table I), in conjunction with the apparent molecular mass of band *a*, showed that band *a* was the cross-linked dimer of the 22-kDa C-terminal fragment of α containing the H_7-H_{10} transmembrane helices (Fig. 1), and the 11-kDa N-terminal fragment of α containing the H_1,H_2 helix pair (Fig. 1).

[1] The abbreviation used is: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

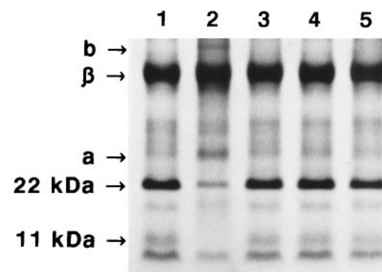

FIG. 2. **Ligand-sensitive cross-linking reactions of the membrane-bound preparation of the trypsin-digested Na^+/K^+-ATPase in the presence of Cu^{2+}-phenanthroline.** The extensively digested preparation was prepared, cross-linked, and resolved on 10% Tricine gels as described under "Experimental Procedures." *Lane 1*, control prior to cross-linking. *Lane 2*, after cross-linking. The other lanes are samples that were cross-linked in the presence of 100 mM NaCl (*lane* 3), 5 mM KCl (*lane 4*), and 10 μM ouabain plus 7.5 mM $MgCl_2$ (*lane 5*).

Four sequences shown in Table I were identified upon N-terminal sequencing of band *b*, indicating that this cross-linked product contains the following four peptides in equimolar stoichiometry: the 11-kDa N-terminal fragment of α, the 22-kDa C-terminal fragment of α, a fragment of β beginning at Ala^5, and a fragment of β beginning at Gly^{143}. That the trypsin-digested enzyme contains β beginning at Ala^5, and that a fraction of this truncated β is also cleaved at Arg^{142}-Gly^{143} has been demonstrated (10, 16, 17, 19). Since the latter cleavage site (Fig. 1) is between Cys^{125} and Cys^{148}, which are connected by a disulfide bridge (1), and since the bridge is expected to remain intact under oxidative cross-linking reaction conditions, we conclude that band *b* is the cross-linked trimer of 11-kDa and 22-kDa fragments of α-subunit and a β-subunit that begins at Ala^5, is cleaved at Arg^{142}-Gly^{143}, but is otherwise intact. Because we have not been able to detect β that is cross-linked either with the 11-kDa fragment alone or with the 22-kDa fragment alone, we suspect that formation of a disulfide bond between the two α-fragments stabilizes a conformation of the α-subunit helix-bundle, which is the mandatory first step to the subsequent direct contact of the Cys^{44} of β with an intramembrane cysteine of the two α-fragments.

The above identifications of 11-kDa and 22-kDa fragments of α in the two most prominent cross-linked products are consistent with the reduced intensities of 11-kDa and 22-kDa bands after exposure to Cu^{2+}-phenanthroline (Fig. 2, *lanes 1* and *2*). Closer examination of these gels shows, however, that other peptides also may have participated in cross-linking reactions. To date, we have been unable to characterize other discrete cross-linked products.

TABLE I
N-terminal sequences of isolated cross-linked products

Products designated as *a* (11,12-dimer) and *b* (11,22,β-trimer) in Fig. 2 (*lane 2*) were sequenced as indicated under "Experimental Procedures." The sequences were followed for 11 cycles and were identical with the indicated fragments of α- and β-subunits of canine kidney Na,K-ATPase. Xaa, amino acid residues that were not identified upon sequencing and correspond to Trp^{11} and Cys^{148} of β-subunit. The yield of each sequence was estimated from the average elevation of residues in cycles 2–11.

Cross-linked product	Sequence	Polypeptide chain region	Yield
			pmol
11,22	^{68}Asp-Gly-Pro-Asn-Ala-Leu-Thr-Pro-Pro-Pro-Thr-	α-Subunit, H1–H2	19.8
	^{831}Asn-Pro-Lys-Thr-Asp-Lys-Leu-Val-Asn-Glu-Arg-	α-Subunit, H7–H10	18.9
11,22,β	^{68}Asp-Gly-Pro-Asn-Ala-Leu-Thr-Pro-Pro-Pro-Thr-	α-Subunit, H1–H2	6.0
	^{831}Asn-Pro-Lys-Thr-Asp-Lys-Leu-Val-Asn-Glu-Arg-	α-Subunit, H7–H10	6.5
	^{5}Ala-Lys-Glu-Glu-Gly-Ser-Xaa-Lys-Lys-Phe-Ile-	β-Subunit, N-terminal half	6.2
	^{143}Gly-Glu-Arg-Lys-Val-Xaa-Arg-Phe-Lys-Leu-Glu-	β-Subunit, C-terminal half	5.6

The specificities of the cross-linking reactions were tested in experiments, a representative of which are shown in Fig. 2. Formations of the cross-linked products, and the disappearances of 11-kDa and 22-kDa α fragments were prevented by Na^+ and K^+ (Fig. 2, *lanes 3* and *4*), but not by the same concentrations of choline and *N*-methylglucamine (data not shown). Incubation in the presence of ouabain and Mg^{2+} also prevented cross-linking (Fig. 1, *lane 5*), but Mg^{2+} alone was not effective (data not shown). When the protective effects of different concentrations of Na^+ and K^+ were quantitated, $K_{0.5}$ values were in the range of 10–20 mM for Na^+ and less than 0.5 mM for K^+ (Figs. 3 and 4). The relative values of these constants are in general agreement with the relative $K_{0.5}$ values for occlusions of Na^+ and Rb^+ by this digested preparation (15), and clearly indicate the specificities of the cation effects on the cross-linking reactions. The concentration of ouabain for half-maximal protection of cross-linking was reduced when Mg^{2+} was present (Fig. 5), indicating that the well established interactive effects of ouabain and Mg^{2+} on the native enzyme (14) continue to exist within the peptide fragments of the digested enzyme.

When the digitonin-solubilized preparation of the digested enzyme was cross-linked in the presence of Cu^{2+} as described previously (10) or in the presence of Cu^{2+}-phenanthroline as in experiments of Fig. 2, no Na^+, K^+, or ouabain effects on the cross-linking reactions of the solubilized preparation were noted (data not shown).

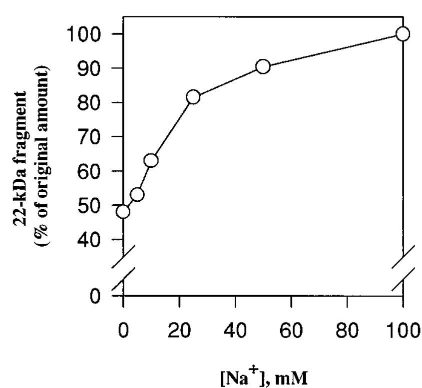

FIG. 3. **Protective effects of varying Na⁺ concentrations on the cross-linking reactions of the 22-kDa α-fragment of Na⁺/K⁺-ATPase.** Experiments were done as in Fig. 2, and the stained bands were quantitated by densitometry.

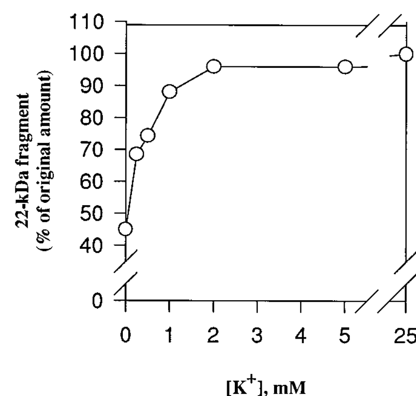

FIG. 4. **Protective effects of varying K⁺ concentrations on the cross-linking reactions of the 22-kDa α-fragment of Na⁺/K⁺-ATPase.** Experiments were done as in Fig. 2, and the stained bands were quantitated by densitometry.

DISCUSSION

The trypsin-digested preparation of the membrane-bound Na^+/K^+-ATPase used here (Fig. 1) has no ATPase activity, but retains the capacity to occlude Na^+ and Rb^+ and to carry out a slow Rb^+/Rb^+ exchange after reconstitution (15). When this preparation is solubilized in digitonin and oxidatively cross-linked, a major product is a dimer of the 11-kDa N-terminal fragment of α and the 22-kDa C-terminal fragment of α (10). We now show (Fig. 2 and Table I) that such a dimer is also a major cross-linked product of the membrane-bound version of this preparation. It has long been recognized that the demonstration of similar cross-linked complexes of proteins in native membranes and in solubilized preparations of the membranes is a good indication of the existence of specific protein associations within the native membranes (11, 13). The present findings and our previous data (10), therefore, establish the proximities of the N-terminal and C-terminal helices of α in the functional membrane. Although the details of the contact domains remain to be mapped, as discussed previously (10), the estimated locations of the sulfhydryl groups of these helices (Fig. 1) suggest that the most likely interacting helices are the H_1,H_{10} pair and the H_2,H_8 pair.

The other prominent cross-linked product of the membrane preparation identified here is a trimer of an essentially intact β and the above two fragments of α (Fig. 2 and Table I). This is also similar to such a trimer that we obtained previously from the detergent-solubilized preparation (10). The two trimers, however, differ significantly in respect to the nature of β that participates in cross-linking. In the solubilized preparation the predominant reactant is the β that begins at Ala^5, but is otherwise intact (10), while the predominant reactant in the membrane preparation is the same N-terminally truncated β that is also cleaved at Arg^{142}-Gly^{143} between two half-cystines (Table I). Since under the conditions we have used to prepare the digested enzyme (*i.e.* at pH 8.5, the Arg^{142}-Gly^{143} cleavage occurs in a small fraction of total β content (16)), we conclude that without this cleavage β does not cross-link with the α-fragments in the membrane-bound preparation. Evidently, some

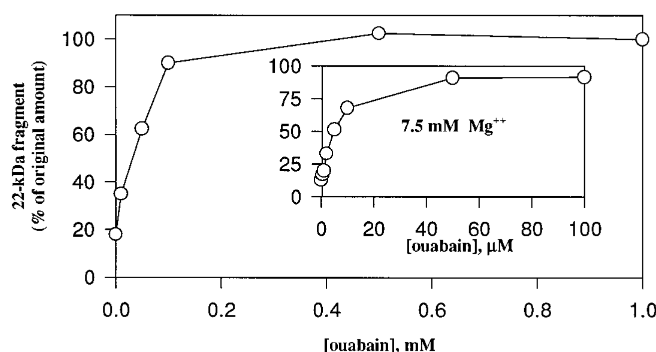

FIG. 5. **Protective effects of varying ouabain concentrations on the cross-linking reactions of the 22-kDa α-fragment of Na^{+}/K^{+}-ATPase.** Experiments were done as in Fig. 2 in the presence of ouabain alone or ouabain plus $MgCl_2$ (*inset*). The stained bands were quantitated by densitometry.

spatial flexibility of the β-subunit chain, induced either by cleavage between the half-cystine residues or by detergent solubilization, is required for close juxtaposition of the single transmembrane cysteine of β with one of the nearby transmembrane cysteines of α to allow the zero-length cross-linking of the two sulfhydryl groups. The five intramembrane sulfhydryl groups available for the formation of the two disulfides of the cross-linked trimer are Cys^{44} of β and cysteines 104, 138, 930, and 983 of α (Fig. 1).

That the area of contact between the transmembrane helix of β and one or more transmembrane helices of α is altered significantly by a cleavage in the extracellular domain of β is pertinent to a number of studies that have provided considerable information on the effects of various structural modifications of the extracellular domains and the transmembrane domain of β on the assembly of the α,β-complex (20–23), and on the functions of the enzyme (17, 21–24). We suggest that, in the interpretation of these findings, the often neglected possibility should be considered that a structural change of an extramembrane domain of β may be transmitted to its transmembrane helix, and then to the interacting helices of α.

A large number of studies on ligand-induced conformational transitions of Na^{+}/K^{+}-ATPase in relation to its function have been conducted over the years (25). Few of these, however, have addressed such structural transitions of the transmembrane domains of the enzyme. Experiments on hydrophobic labeling, followed by proteolysis, have suggested the existence of different Na^{+}- and K^{+}-dependent conformations of the transmembrane domains (8, 26); a fluorescent label attached to a specific transmembrane domain of the α-subunit of the intact enzyme has been shown to respond differently to various ligand-induced states (27); and structural changes of some transmembrane helices, either by chemical modification or by site-directed mutagenesis, have been shown to alter specific ligand interactions with the enzyme (2–6). To our knowledge, these and related previous studies, however, have not provided experimental evidence for ligand-induced interactions among the intramembrane domains. The sensitivities of the cross-linking reactions to Na^{+}, K^{+}, and ouabain demonstrated in the present studies (Figs. 2–5) clearly indicate that the highly specific interactions of these ligands with the extensively digested enzyme regulate the three-dimensional packing of the transmembrane helices. Specifically, our data show that the conformational states induced by the indicated ligands affect the alignments of the residues of H_1,H_2 helix pair relative to those of the H_8-H_{10} helices, and the proximities of these helices with the residues of one transmembrane helix of β. This does not exclude the involvement of other transmembrane domains in ligand-induced conformational changes of the helix-bundle. The use of cysteine-scanning mutagenesis, other cross-linking reagents, and other functionally competent fragments of the enzyme may reveal additional ligand-sensitive transmembrane helix-helix interactions. Such studies are in progress.

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