THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 44, Issue of November 3, pp. 26528–26532, 1995

Printed in U.S.A.

Intersubunit and Intrasubunit Contact Regions of Na^+/K^+-ATPase Revealed by Controlled Proteolysis and Chemical Cross-linking*

(Received for publication, June 25, 1995, and in revised form, August 21, 1995)

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

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

To identify interfaces of α- and β-subunits of Na^+/K^+-ATPase, and contact points between different regions of the same α-subunit, purified kidney enzyme preparations whose α-subunits were subjected to controlled proteolysis in different ways were solubilized with digitonin to disrupt intersubunit α,α-interactions, and oxidatively cross-linked. The following disulfide cross-linked products were identified by gel electrophoresis, staining with specific antibodies, and N-terminal analysis. 1) In the enzyme that was partially cleaved at Arg^{438}-Ala^{439}, the cross-linked products were an α,β-dimer, a dimer of N-terminal and C-terminal α fragments, and a trimer of β and the two α fragments. 2) From an extensively digested enzyme that contained the 22-kDa C-terminal and several smaller fragments of α, two cross-linked products were obtained. One was a dimer of the 22-kDa C-terminal peptide and an 11-kDa N-terminal peptide containing the first two intramembrane helices of α (H_1-H_2). The other was a trimer of β, the 11-kDa, and the 22-kDa peptides. 3) The cross-linked products of a preparation partially cleaved at Leu^{266}-Ala^{267} were an α,β-dimer and a dimer of β and the 83-kDa C-terminal fragment.

Assuming the most likely 10-span model of α, these findings indicate that (*a*) the single intramembrane helix of β is in contact with portions of H_8-H_{10} intramembrane helices of α; and (*b*) there is close contact between N-terminal H_1-H_2 and C-terminal H_8-H_{10} segments of α; with the most probable interacting helices being the H_1,H_{10}-pair and the H_2,H_8-pair.

Na^+/K^+-ATPase is the intrinsic enzyme of the plasma membrane that carries out the coupled active transport of Na^+ and K^+ in most eucaryotic cells. The enzyme consists of two subunits, α and β, that are associated noncovalently in molar stoichiometry of 1:1 (1, 2). The α-subunit, which is often called the catalytic subunit, is phosphorylated and dephosphorylated in the course of ATP hydrolysis, contains a number of residues that have been implicated in nucleotide, ouabain, and cation bindings to the enzyme, and exhibits several characteristic patterns of proteolytic digestion when the enzyme is in different ligand-induced conformational states (3, 4). It has been known for a long time, however, that both subunits are essential to normal function (5, 6), and that the ligand-induced conformational transitions are transmitted across the α,β-interface (7–9). More recent studies have also indicated the role of the β-subunit in the regulation of K^+ binding to the enzyme (10–12), and have provided further evidence for the involvement of both subunits in the enzyme's conformational transitions (13, 14). It is evident, therefore, that identification of the interfaces between the two subunits is necessary for the eventual definition of the structure-function relationship of the enzyme. Since high resolution structures are not available, other approaches must be used for such structural studies. Locations of some α,β-interfaces have been surmised from experiments on the structural requirements for the assembly of the α,β-complex (11, 15–19), and from molecular modeling studies (20–22). In the work presented here, we have used controlled proteolysis and chemical cross-linking techniques to identify some of the contact domains of the two subunits of the purified canine kidney enzyme. Detergent-solubilized preparations were utilized for these studies, because solubilization prevents intersubunit α,α-interactions, but allows continued α,β-associations (23). The results have also provided new information regarding the relative orientations of the intramembrane segments of an α-subunit within the three-dimensional structure of the enzyme. Preliminary accounts of portions of this work have been presented (24, 25).

EXPERIMENTAL PROCEDURES

Purified membrane-bound Na^+/K^+-ATPase of canine kidney medulla, with specific activity in the range of 1000–1600 μmol of ATP hydrolyzed/mg of protein/h, was prepared and assayed as described before (26). Such preparations were subjected to controlled proteolysis by the following well established procedures. 1) The enzyme was exposed to trypsin in the presence of K^+ as described (27) to obtain a preparation in which about half of the α-subunit was cleaved at Arg^{438}-Ala^{439}. 2) The enzyme was exposed to chymotrypsin in the presence of Na^+ (28) to obtain a preparation with partial cleavage of the α-subunit at Leu^{266}-Ala^{267}. 3) Extensive digestion of the enzyme in the presence of trypsin, Rb^+, and EDTA was performed at pH 8.5 according to Karlish *et al.* (29) to remove large portions of the cytoplasmic domains of the α-subunit, and obtain a preparation containing the C-terminal 22-kDa fragment and several smaller fragments of the α-subunit (29, 30). In the three preparations subjected to proteolysis as indicated above, the β-subunit is essentially intact.

Native or digested enzyme preparations were solubilized by mixing a suspension of the enzyme (2 mg/ml) in 10 mM Tris-HCl (pH 7.4) with an equal volume of 6 mg/ml digitonin, stirring for 5 min at 24 °C, and centrifugation in Beckman Airfuge for 30 min at 100,000 × *g*. The clear supernatant containing the solubilized enzyme was collected.

For cross-linking, an aliquot of the above digitonin-solubilized enzyme was mixed with an equal volume of a solution containing 0.25 mM $CuSO_4$, and 10 mM Tris-HCl (pH 7.4), and incubated for 10 min at 24 °C. The reaction was terminated by addition of SDS to a final concentration of 2%, and samples were subjected to electrophoresis either on SDS-polyacrylamide gels (5 or 7.5%) at pH 2.4 (27), or on Tricine[1]/SDS-polyacrylamide gels (31) using 10 or 16.5% gels. These gel systems have

* This work was supported by National Institutes of Health Grant HL-36573 awarded by the National Heart, Lung, and Blood Institute, United States Public Health Service, Department of Health and Human Services. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ 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.

[1] The abbreviations used are: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

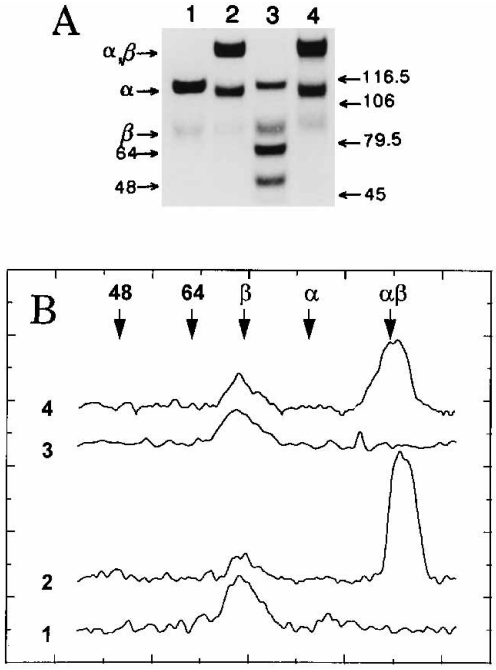

FIG. 1. *A*, Cu^{2+}-induced cross-linking of the digitonin-solubilized Na^+/K^+-ATPase with α cleavage at Arg^{438}-Ala^{439}. Cleavage, cross-linking and electrophoresis on 5% acid gels were done as described under "Experimental Procedures." *Lane 1*, control enzyme without cross-linking. *Lane 2*, cross-linked control enzyme. *Lane 3*, partially cleaved enzyme prior to cross-linking. *Lane 4*, partially cleaved enzyme after cross-linking. Positions of prestained molecular mass markers are indicated by *arrows* on the *right*. *B*, reactivities of the cross-linked products of the native Na^+/K^+-ATPase and the enzyme cleaved at Arg^{438}-Ala^{439} with a β-specific antibody. Gels similar to those of *A* were blotted, stained, and scanned as described under "Experimental Procedures." Lane numbers are the same as those of *A*.

been used before for the resolution of the three digested preparations used here (10, 27–30). Unless indicated otherwise, sulfhydryl reagents were omitted from all buffers used for electrophoresis to preserve the cross-linked products. For Western blot analysis, peptide bands were transferred to nitrocellulose sheets, probed with specific antisera, and detected using appropriate second antibodies conjugated to alkaline phosphatase (32). The blots were either photographed or quantified by a scanning densitometer (Bio-Rad). The scans of multiple lanes presented in the same figure are quantitatively comparable. N-terminal analyses of the peptide bands were done as described before (28). For the smaller peptides resolved on 16.5% Tricine/SDS gels, identical bands from several gels were combined, electroeluted, electrophoresed on a second 10% Tricine/SDS gel, and then transferred to polyvinylidine difluoride membrane for sequencing.

Trypsin (Type III-S, bovine pancreas), α-chymotrypsin (type II, bovine pancreas), and trypsin-chymotrypsin inhibitor (soybean) were obtained from Sigma. Polyclonal antibodies specific for the sequences of sheep kidney α-subunit (33) were provided by Dr. W. J. Ball (University of Cincinnati). These antibodies were against residues 16–30, residues 111–122, and residues 1003–1013. As expected from the comparison of sheep and canine sequences (34, 35), the three antibodies reacted with the canine α-subunit on Western blots. Two additional polyclonal antibodies were obtained by conventional immunization of rabbits using denatured α- and β-subunits of canine kidney Na^+/K^+-ATPase as antigens. These were prepared by resolving sufficient quantities of the purified enzyme on preparative SDS-polyacrylamide gels, and electroelution of the separated subunits.

RESULTS

Previous studies of several laboratories have shown that when detergent-solubilized preparations of Na^+/K^+-ATPase are subjected to oxidative cross-linking catalyzed by Cu^{2+} or a complex of Cu^{2+} and *o*-phenanthroline, the major cross-linked product is the α,β-dimer (1, 2, 8, 23, 36–38). Experiments of Fig. 1*A*, *lanes 1* and *2*, show the results of Cu^{2+}-induced cross-linking of the digitonin-solubilized enzyme. In agreement with previous observations on the digitonin-solubilized enzyme (1), the quantities of α- and β-monomers decreased after cross-linking, a single cross-linked product was formed, and the mobility of the remaining α was altered. That the cross-linked product is indeed an α,β-dimer is indicated not only by its mobility, but also by its reactivities with a β-specific antibody (Fig. 1*B*, *lanes 1* and *2*) and several α-specific antibodies (*e.g.* Fig. 4*C*, *lanes 1* and *2*). In the remaining experiments presented below, enzyme preparations whose α-subunits were subjected to controlled proteolysis in three different ways were similarly solubilized and cross-linked; and the identifications of the cross-linked products were attempted.

Cross-linking of the Enzyme with α-Cleavage at Arg^{438}-Ala^{439}—The native enzyme and the partially cleaved enzyme were solubilized, cross-linked, and subjected to electrophoresis along with control samples that were not exposed to the cross-linking reagent (Fig. 1). The two well characterized products of this α-cleavage, as established before (27, 39), are a 64-kDa C-terminal fragment and a 48-kDa N-terminal fragment (Fig. 1*A*, *lane 3*). Upon cross-linking, these two peptides and a portion of β disappeared, and were converted to products with mobilities similar to those of α and α,β-dimer (Fig. 1*A*, *lane 4*). In experiments of Fig. 1*B*, gels with samples identical to those of Fig. 1*A* were immunostained with the β-specific antibody. The results showed that in the cleaved and cross-linked sample (Fig. 1*B*, *lane 4*), the bands at the position of β and α,β-dimer reacted with the antibody as expected, but the band at the α position did not. This, and the comparison of the intensities of the stained bands of *lanes 3* and *4* of Fig. 1*A*, clearly suggest that cross-linking of the cleaved enzyme resulted in the formation of three products: 1) the α,β-dimer; 2) a dimer of 64-kDa peptide and 48 kDa peptide (48,64-dimer); and 3) a trimer of 48-kDa peptide, 64-kDa peptide, and β (48,64,β-trimer). The formation of 48,64-dimer indicates the continued association of the two α fragments after solubilization. These experiments, however, do not indicate if β associates with the 64-kDa segment of α, or its 48-kDa segment, or both.

Cross-linking of the Enzyme That Is Extensively Digested by Trypsin—Since the ability of this preparation to occlude Rb^+ has been shown to be regulated by β (10), we used the preparation to characterize the association of several identified α fragments (29, 30) with β. When the preparation was solubilized, cross-linked, and subjected to electrophoresis, two major cross-linked products were noted. It was necessary to use two different gel systems to identify these products. Experiments of Fig. 2*A* show the comparison of the native enzyme with the extensively digested enzyme, before and after cross-linking, on 7.5% acid gels. Prior to cross-linking, this digested preparation exhibited only two prominent bands on these gels: the seemingly intact β-subunit, and a peptide with the apparent molecular mass of about 22 kDa (Fig. 2*A*, *lane 3*). Since the latter reacted with the antibody against residues 1003–1013 of α (not shown), we assumed that it was the previously identified C-terminal fragment of α (29). Detection of smaller fragments of α (29) on the 7.5% gel of Fig. 2*A*, *lane 3*, was not expected. Cross-linking of the extensively digested enzyme led to the formation of a product with mobility lower than that of β, concomitant with reductions in the stainings of β and 22-kDa bands (Fig. 2*A*, *lane 4*). There was also a faint band with mobility between those of β and the 22-kDa peptide that is not clearly evident in Fig. 2*A*, *lane 4*. The identify of this will be addressed below. That the prominent product contained β and the C-terminal fragment of α was verified by immunostaining with the β-specific antibody (Fig. 2*B*), and by the reactivity of the product with the antibody against 1003–1013 residues of α (not shown). For further characterization of this cross-linked

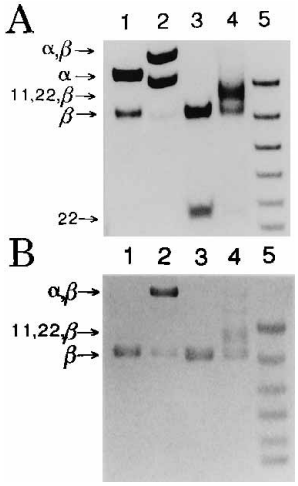

FIG. 2. *A*, cross-linking of the solubilized Na^+/K^+-ATPase whose α is extensively digested. Digestion, cross-linking, and electrophoresis on 7.5% gels were done as described under "Experimental Procedures." *Lane 1*, control enzyme prior to cross-linking. *Lane 2*, control enzyme after cross-linking. *Lane 3*, extensively digested enzyme before cross-linking. *Lane 4*, digested enzyme after cross-linking. *Lane 5*, prestained molecular mass markers (kDa): 106, 80, 49.5, 32, 27.5, 18.5. *B*, reactivities of the cross-linked products of the native and the extensively digested Na^+/K^+-ATPase with a β-specific antibody. Gels similar to those of *A* were probed with the antibody. *Lane 5* contained prestained markers.

product, a band such as that shown in Fig. 2*A*, *lane 4*, was transferred to a polyvinylidine difluoride membrane, and subjected to N-terminal analysis. The results clearly indicated that the cross-linked product contained three peptides. Comparison of the residues found in the first 10 cycles with the known sequences of canine α and β (35, 40), identified the three peptides as β beginning at Ala^5, the 22-kDa C-terminal fragment of α beginning at Asn^{831}, and an N-terminal fragment of α beginning at Asp^{68} (Table I). The latter fragment has been noted before (29) and appears as an 11-kDa peptide when the extensively digested preparation is resolved on Tricine gels (see below). The identical yields of three peptides (Table I) suggested that the cross-linked product contained the three peptides in a molar stoichiometry of 1:1:1; and that the three were derived from the same polypeptide chain.

Since the gels used in the experiments of Fig. 2 were not suitable for the detection of smaller peptides of the extensively digested enzyme, Tricine gels were also used to resolve this preparation before and after cross-linking. Prior to cross-linking, on the lower portions of these gels we noted two prominent bands below the 22-kDa peptide (Fig. 3, *lane 1*). After cross-linking, intensities of the 22-kDa band and one of the other bands decreased, while a product with mobility lower than that of 22-kDa band was formed (Fig. 3, *lane 2*). The product bands from a number of gels were combined, electrophoresed again, and used for further identification. N-terminal analysis (Table I) identified the 22-kDa C-terminal and the 11-kDa N-terminal fragments, in 1:1 ratio, as the major components of the product. As expected from the data of Table I, the cross-linked product reacted with antibodies against 1003–1013 and 111–122 sequences of α on Western blots (not shown).

These findings clearly indicate the proximities of the 22-kDa C-terminal and the 11-kDa N-terminal segments of the α-subunit to each other, and to the β-subunit.

Cross-linking of the Enzyme with α-Cleavage at Leu^{266}-Ala^{267}—Although the properties of this cleaved enzyme have been studied extensively (28, 41), the only product of this cleavage that has been well characterized is the 83-kDa C-terminal fragment of α (28, 39). When the cleaved enzyme was resolved

TABLE I
N-terminal sequences of the cross-linked products of digested Na^+/K^+-ATPase preparations

The products designated as 11,22,β in Fig. 2*a*, *lane 4*; 11,12 in Fig. 3, *lane 2*; and 83,β-dimer in Fig. 4*a*, *lane 4*, were sequenced as indicated under "Experimental Procedures." The yield of each sequence was estimated from the average elevation of the residues in cycles 2 through 10.

Product		Sequence	Yield
			pmol
11,22,β	Found	AKEEGSWKKF	24
	β	A(5)KEEGSWKKF	
	Found	NPKTDKLVNE	22
	22-kDa peptide	N(831)PKTDKLVNE	
	Found	DGPNALLTPPX	23
	11-kDa peptide	D(68)GPNALTPPP	
11,22	Found	NPKTDKLVNE	10
	Found	DGPNALTPPX	10
83,β	Found	ASGLEGGQTP	13
	83-kDa peptide	A(267)SGLEGGQTP	
	Found	ARGKAKEEGS	11
	β	A(1)RGKAKEEGS	

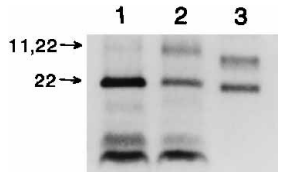

FIG. 3. **Cross-linking of solubilized Na^+/K^+-ATPase whose α is extensively digested, and its resolution on a Tricine gel.** The enzyme was digested and cross-linked as described in the legend to Fig. 2. Samples were resolved on 16.5% Tricine gels. Only the lower portions of the gels are shown. *Lane 1*, digested enzyme prior to cross-linking. *Lane 2*, digested enzyme after cross-linking. *Lane 3*, molecular mass markers (kDa): 27.5, 18.5.

on the gel systems used here, neither the expected 30-kDa N-terminal product of the cleavage, nor any other well defined smaller peptide bands were noted consistently. This suggests that under the conditions of this cleavage, the 30-kDa product of the cleavage is converted to a number of heterogeneous small peptides that are difficult to characterize even on the Tricine gels.

When the enzyme that was partially cleaved in this manner was solubilized and cross-linked, two cross-linked products were detected (Fig. 4*A*, *lane 4*): the expected α,β-dimer, and a band with mobility consistent with that of a dimer of β and the 83-kDa C-terminal fragment of α (83,β-dimer). Note that on these 5% gels used to separate the two cross-linked products, β and the 83-kDa monomer move together as a diffuse band (Fig. 4*A*, *lanes 3* and *4*). The two uncross-linked peptides, however, may easily be separated on 7.5% gels (28).

For further identification of the presumed 83,β-dimer, gels similar to those of Fig. 4*A*, *lane 4*, were immunostained with several antibodies. As expected, the product reacted with the β-specific antibody (Fig. 4*B*) and the antibody against residues 1003–1013 of α (not shown). More importantly, the presumed 83,β-dimer did not react with antibodies against the 111–122 sequence of α (Fig. 4*C*) and the 16–30 sequence of α (not shown); suggesting that it did not include small fragments containing the epitopes of these antibodies. When the presumed 83,β-dimer was subjected to N-terminal analysis, only two amino acid sequences were clearly identified (Table I), confirming its identity as the 83,β-dimer. There was no evidence for the existence of other peptides in the cross-linked product. These findings, in conjunction with the results of the experiments on the enzyme extensively digested by trypsin

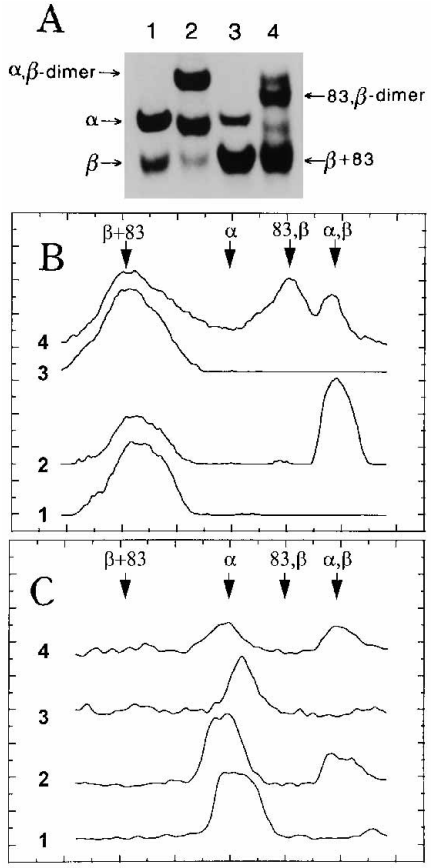

FIG. 4. *A*, cross-linking of solubilized Na^+/K^+-ATPase with α cleavage at Leu^{266}-Ala^{267}. Cleavage, cross-linking, and resolution on 5% gels was done as described under "Experimental Procedures." *Lane 1*, control enzyme before cross-linking. *Lane 2*, control enzyme after cross-linking. *Lane 3*, partially cleaved enzyme before cross-linking. *Lane 4*, partially cleaved enzyme after cross-linking. *B* and *C*, reactivities of the cross-linked products of the native Na^+/K^+-ATPase and the enzyme cleaved at Leu^{266}-Ala^{267} with a β-specific antibody (*B*) and an antibody against sequence 111–122 of α (*C*). Gels similar to those of *A* were blotted, stained, and scanned. Lane numbers are the same as those used in *A*.

(Figs. 2 and 3, and Table I), indicate the direct contact of β with the C-terminal segment of α, and clarify the nature of interactions involved in 11,22,β-trimer formation (see "Discussion").

Miscellaneous Findings—Incubations of the cross-linked enzyme samples with 300 mM mercaptoethanol for 1 h at 55 °C resulted in the partial disappearance (about 90%) of the cross-linked products. No attempt was made to see if complete reduction could be achieved. When the digitonin-solubilized enzyme samples were diluted 10-fold with a solution of 3 mg of digitonin/ml, and then cross-linked, the products (identified by their mobilities on gels) were the same as those indicated above. This test of dilution argues against the products being the result of random collisions of the peptides. In limited experiments on enzyme samples solubilized with Triton X-100 or CHAPS, we noted similar cross-linking patterns as those described for the digitonin-solubilized enzyme.

DISCUSSION

Chemical cross-linking techniques that have been used extensively in studies on the quaternary structure of Na^+/K^+-ATPase have suggested the existence of α, β-, and α,α-interactions in the membrane-bound enzyme (23). The α,α-interactions are not noted in cross-linking experiments with most detergent-solubilized preparations of the enzyme (1, 2, 23). Because of this, and the well known difficulty of distinction between stable and collision complexes within the membrane phase (42), there have been disagreements on the meanings of the cross-linked α,α-complexes of the enzyme (1, 2, 23). There are no such ambiguities, however, regarding α,β-associations. In cross-linking experiments with preparations that are solubilized with a variety of detergents, the α,β-dimer is the major or the only product (1, 2, 23, 36–38). It is reasonable, therefore, to assume that this product represents the stable noncovalent α,β-complex, and that localization of the domains involved in oxidative cross-linking of α to β identifies some, if not all, of the α,β-interfaces of the native enzyme.

The original intent of this work was to use preparations with intact β and defined proteolytic fragments of α to identify the regions of α that are in direct contact with β. In the process, it became apparent that the same approach also provided information about the domains of α that are in contact with each other within a single α chain. As in the case of the cross-linked α,β-dimer obtained in detergent-solubilized preparations, it is reasonable to assume that cross-linked products containing α fragments also do not result from the random collisions of the reactants.

Intrasubunit Interfaces of α-Subunit—Our recent studies on the enzyme whose α is cleaved at Arg^{438}-Ala^{439} clearly showed that the two resulting fragments of α remain in contact such that covalent modification of one fragment (phosphorylation of the N-terminal fragment, or reaction of fluorescein isothiocyanate with the C-terminal fragment) regulates the properties of the other (27, 43). The present experiments with this cleaved preparation indicating the cross-linking of the two proteolytic fragments (Fig. 1) confirm the existence of continued interactions across the cleavage site, and show close contact between sulfhydryl-containing regions of the two fragments. However, because there are large numbers of sulfhydryl groups on each fragment (9 on the N-terminal fragment, and 13 on the C-terminal), based on these experiments alone, little can be said about the location of the contact points of the two fragments.

The results of the experiments on the preparation extensively digested by trypsin are more informative. Formation of the cross-linked 11,22-dimer (Fig. 3 and Table I) clearly indicates the proximities of the sulfhydryl groups of these peptides. Although the C-terminal residue of the 11-kDa peptide is not known, its N-terminal residue (Asp^{68}), and its apparent molecular mass indicate that this peptide contains the first two transmembrane helices of α (H_1-H_2), and three cysteines (residues 86, 104, and 138) either within or close to the transmembrane segments. The 22-kDa peptide is the intact C-terminal segment of α containing four cysteines (residues 911, 930, 964, and 983). There are uncertainties regarding the structural organization and the number of transmembrane segments in this part of α (3, 13–15, 30). However, if we assume the most likely 10-span model of α (3), the four cysteines of the 22-kDa peptide would all be within or adjacent to the three transmembrane segments H_8, H_9, and H_{10}. It is reasonable to conclude, therefore, that the formation of cross-linked 11,22-dimer is indicative of close contact between at least one N-terminal helix and one C-terminal helix of α. Based on the estimated relative positions of cysteine residues within these helices (3, 22), two pairs of interacting helices involved in cross-linking are suggested: H_1-H_{10} (Cys^{104} and Cys^{983}) and H_2-H_8 (Cys^{138} and Cys^{930}). It is likely that the disulfide cross-linking of the same helices is also involved in the formation of 48,64-dimer (Fig. 1), and in the change of mobility of the intact α monomer after cross-linking (Fig. 1). To our knowledge, these are the first experimental data to provide concrete information on three-dimensional helix-helix interactions of the enzyme. Efremov *et al.* (20–22) have addressed the important issue of

the spatial organizations of these helices using theoretical and molecular modeling methods. Studies toward the more precise identifications of the cross-linked residues and helices are in progress.

The α,β-Interfaces—Formations of the cross-linked 48,64,β-trimer (Fig. 1) and 11,22,β-trimer (Fig. 2 and Table I) indicate the proximities of β to both N-terminal and C-terminal regions of α. The question of whether β is in direct contact with one or both regions cannot be answered with certainty; however, the experiments with the enzyme cleaved at Leu^{266}-Ala^{267} provide some clarification. The findings with this chymotrypsin-cleaved enzyme are both problematic and revealing. It is puzzling that neither the expected 30-kDa N-terminal product of this cleavage, nor any well defined smaller fragments of this peptide, were identified on the gels we have used here, or in any of the previous studies with the chymotrypsin-cleaved enzyme (28, 39, 41). Perhaps this is indicative of ligand-dependent lability of the topological organization of this segment of α, similar to the recently reported labilities of other putative transmembrane domains of α (14, 45, 46). Regardless of how this matter is resolved by future studies, however, the fact that 83,β-dimer seems to be the only cross-linked product obtained from the chymotrypsin-cleaved α (Fig. 4 and Table I), suggests that the interaction of the N-terminal segment of α with its C-terminal segment is not a requirement for β contact with the C-terminal of α. This, together with the demonstration of 11,22-dimer and 11,22,β-trimer formations (Figs. 2 and 3, and Table I), suggest that the C-terminal end of α is the intermediate in the pathway of interaction between β and the N-terminal end of α.

Which β sulfhydryl(s) cross-links to a sulfhydryl(s) of the 22-kDa segment of α? The β-subunit contains a cysteine (Cys^{45}) within its only transmembrane segment, and three cystines within its extracellular domains (3, 44). If the cysteines of the 22-kDa fragment of α are within the H_8-H_{10} intramembrane domains (as indicated by the favored 10-span model of α), close contact must be between one of these segments and the one intramembrane segment of β. Because of uncertainties on the topology of the C-terminal region of α, however, cross-linkings of extracellular domains of β to those of the 22-kDa segment of α cannot be ruled out.

An important issue is the relation of the present findings to the conclusions of previous studies on the structural requirements for the assembly of Na^+/K^+-ATPase. Several elegant studies on the expressions of α, β, and their mutants in mammalian cells and *Xenopus* oocytes have provided considerable information on the structural elements of the two subunits that are required for the assembly of the α,β-complex (11, 15–19); and have shown the importance of both the transmembrane and the extracellular domains of β for efficient assembly (19). It is appropriate to note two points about these studies: in principle, subunit interactions that are essential to the processes involved in the assembly need not be the same as those of the assembled enzyme of the plasma membrane. Also, while studies on assembly identify domains and residues that are important to α,β-contacts necessary for assembly, they do not distinguish those structural elements that regulate the interface from those that are at the interface. On the other hand, while the present cross-linking experiments reveal some domains and residues that are at interfaces, they do not identify all relevant interfaces. Clearly, these and other experimental approaches have the potential of complementing each other.

Acknowledgments—We thank Dr. W. J. Ball, University of Cincinnati, for the generous gift of the antibodies; and Dr. J. Pohl, Emory University Microsequencing Facility, for amino acid analysis.

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