Specific Cross-Links between Fragments of Proteolyzed Na,K-ATPase Induced by *o*-Phthalaldehyde†

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ABSTRACT: We have used *o*-phthalaldehyde (OPA) to cross-link adjacent fragments of "19 kDa membranes", a tryptic preparation of Na,K-ATPase lacking the ATP site but retaining cation occlusion sites. Treatment with OPA of "19 kDa membranes" or detergent-solubilized membranes containing occluded Rb ions [Or, E., Goldshleger, R., Tal, D. M., and Karlish, S. J. D. (1996) *Biochemistry 35*, 6853−6864] yielded cross-linked products of 25 and 31 kDa. Both species contained the 19 kDa fragment of the α subunit (transmembrane segments M7−M10). In addition, the 25 kDa product contained the fragment including M5−M6, while the 31 kDa product contained a 16 kDa fragment of the β subunit. Cross-linking was unaffected by the absence or presence of ligands (Na, Rb, or Mg and ouabain). Cross-linking was largely abolished in thermally inactivated "19 kDa membranes". When proteolytic digestion of the 25 and 31 kDa products was combined with antibody binding, PKA-dependent phosphorylation, and sequencing of fragments, approximate positions of the cross-links were established. In the 25 kDa product, the cross-link was located within the short cytoplasmic segment Asn831−Arg841 of the 19 kDa fragment preceding M7 and within Ala749−Ala770 preceding M5. Thus, M7 and M5 are likely to be in close proximity. In the 31 kDa product, the cross-link was located in the extracellular loop of the α subunit between M7 and M8, close to residues which are known to interact with the β subunit. Functional implications of the interactions between the fragments of the α (M5−M6 and M7−M10) and β subunits are discussed.

Na,K-ATPase is a P-type pump located in the plasma membrane, which utilizes ATP to extrude three Na ions from the cytoplasmic side and take up two K ions from the extracellular medium. Renal Na,K-ATPase is a heterotrimer comprising a catalytic α subunit (112 kDa), including 10 transmembrane segments, a glycosylated β subunit (33 kDa) with a single transmembrane segment, and a γ subunit with a single transmembrane segment (≈6.5 kDa) (*2*). The α subunit contains the ATP hydrolytic site and the Na and K transport sites, and the β subunit is also an essential constituent of the active pump unit (*3*). The γ subunit may be a tissue specific regulator (*4*).

An understanding of the coupling mechanism requires knowledge of the structure of the ATP and the cation sites. A simplified and well-defined tryptic preparation of Na,K-ATPase which retains cation occlusion has provided one tool for studying the cation binding domain (*5*). This preparation, known as "19 kDa membranes", is obtained by extensive tryptic digestion of Na,K-ATPase in the presence of Rb and absence of Ca ions. In essence, the treatment removes the two larger cytoplasmic loops, leaving in the membrane distinct fragments consisting mainly of the transmembrane segments with the connecting loops and tails (*6*). The preparation contains a prominent 19 kDa fragment (Asn831−Tyr1016, M7−M10) and several smaller peptides of 8.0−11.7 kDa derived from the α subunit containing pairs of membrane-spanning segments (M1−M2, N-terminal Asp68; M3−M4, N-terminal Ile263; and M5−M6, N-terminal Gln737). About half of the β chain is cut into a 16 kDa fragment (N-terminal Ala5) and a glycosylated ≈50 kDa fragment (N-terminal Gly143). "19 kDa membranes" have no ATP-dependent function but retain full Rb and Na occlusion capacity (*5*) and also the high-affinity site for cardiac glycosides (*7*).

At 37 °C, "19 kDa membranes" rapidly loose their capacity to occlude cations, but they are fully protected against thermal inactivation in the presence of Rb, Na or congeners, or ouabain (*8*, *9*). Binding of cardiac glycosides is also thermally inactivated at the same rate (*7*). By preventing thermal inactivation of "19 kDa membranes", occluded cations also protect all the component fragments against further digestion of their extramembrane loops and tails by trypsin (*10*). The latter result implies that the tryptic fragments in "19 kDa membranes" interact as a cation-stabilized complex. Recently, we have demonstrated the existence of such a complex of tryptic fragments containing occluded Rb cations by solubilizing "19 kDa membranes", prestabilized with Rb and ouabain, with the nonionic

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[1] Abbreviations: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine; OPA, *o*-phthalaldehyde; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, poly(vinylidine difluoride); $C_{12}E_{10}$, polyoxyethylene 10-lauryl ether; FMI, fluorescein 5-maleimide; PMSF, phenylmethanesulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; PKA, protein kinase A.

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detergent $C_{12}E_{10}$ (*1*). The solubilized complex contains all tryptic fragments of the α, β, and γ chains present in "19 kDa membranes" but no other contaminant proteins. In the absence of Rb ions and ouabain, the solubilized complex dissociates into separate fragments and the pair β−19 kDa.

The structure of the cation occlusion sites is determined by the spatial organization of transmembrane segments which donate ligating groups to the occluded cations. Whereas the consensus number of transmembrane segments in the α subunit is 10 (*11*), their spatial organization is unknown. One experimental approach for determining spatial organization could be the use of short cross-linking reagents and a search for proximities between fragments of "19 kDa membranes". By screening a number of cross-linking agents, one might be able to obtain sufficient evidence to enable construction of a low-resolution spatial model of the transmembrane segments. Potentially, cross-linking in "19 kDa membranes" might be compromised by the high protein concentration, which favors intermolecular cross-linking reactions. However, the availability of the detergent-solubilized preparation alleviates this difficulty because the protein can be diluted at will in order to minimize artifactual intermolecular cross-linking, and cross-linking can then be compared in intact and solubilized "19 kDa membranes". "19 kDa membranes" have been used recently in studies utilizing Cu-phenanthroline to induce disulfide bridges between fragments (*12*, *13*). The β subunit (Cys44) was found to be cross-linked to the 19 kDa fragment (M7−M10) which was also cross-linked to the fragment containing M1−M2. However, the cross-linked cysteine residues were not identified.

OPA is used widely in chromatography for fluorogenic detection of picomole quantities of amino acids (*14*). OPA reacts with thiols and primary amines, yielding highly fluorescent 1-alkylthio-2-alkylisoindoles (*15*, *16*). In addition to its major use, OPA has also been employed as a short length (∼3 Å) cross-linker (*17*−*19*). In this study, we have treated "19 kDa membranes", either solubilized or intact, with OPA, observed two cross-linked products of 25 and 31 kDa, identified their component peptides, and located the cross-linked regions.

A preliminary report of this work has appeared (*20*).

MATERIALS AND METHODS

Na,K-ATPase was prepared from pig kidney red outer medulla by the rapid procedure of Jørgensen (*21*) and stored at −70 °C. Protein concentration and ATPase activity were determined as described by Jørgensen (*21*). Specific activities were 13−18 units/(mg of protein). Before being used, enzyme was thawed and dialyzed overnight at 4 °C against 1000 volumes of 25 mM histidine and 1 mM EDTA (Tris) at pH 7.0. "19 kDa membranes" were obtained by digestion of Na,K-ATPase with trypsin as described by Capasso et al. (*6*) and finally resuspended at 3 mg/mL in 2 mM RbCl, 25 mM imidazole, and 1 mM EDTA (pH 7.5). The specific Rb occlusion capacity of "19 kDa membranes" was 6−6.5 nmol/(mg of protein). Solubilized "19 kDa membranes" were prepared using $C_{12}E_{10}$ at a ratio of 2.2 w/w as described by Or et al. (*1*).

Cross-Linking with OPA. "19 kDa membranes" (0.5 mg/mL) in 5 mM RbCl, 4.1 mM imidazole, and 170 μM EDTA (pH 7.5) or solubilized "19 kDa membranes" (0.4 mg/mL) were mixed with a 1% volume of 6 mM OPA dissolved in water and incubated at 20−22 °C for 40 min. The reaction was stopped with $^1/_9$ volume of 20% SDS, and the mixture was precipitated with 4 volumes of methanol at −20 °C.

Gel Electrophoresis. $C_{12}E_{10}$-solubilized protein samples were precipitated overnight at −20 °C with 4 volumes of methanol/ether (2:1). "19 kDa membrane" suspensions were solubilized with 2% SDS prior to precipitation with 4 volumes of methanol. Precipitated protein was collected by centrifugation at 9700*g* for 1 h at 4 °C, left to dry, and dissolved in loading buffer. Samples were resolved by Tricine-SDS−PAGE (*22*) as described by Capasso et al. (*6*). Unless indicated otherwise, 1 mm thick, 12 cm long 10 or 16.5% T/3% C gels were used.

Purification of OPA Cross-Linked Products. Membranes (15 mg) were cross-linked with OPA and methanol-precipitated as described above. The OPA cross-link is unstable under acidic conditions (data not shown), and therefore, the following procedure was used to cut out the cross-linked products from unfixed and unstained gels. Pellets were dissolved in nonreducing loading buffer and FMI (final concentration of 100 μM) was added to 10% of the amount. After 30 min at room temperature, in the dark, β-mercaptoethanol and glutathione were added, and the two portions were recombined and resolved on two 1.5 mm thick, 23 cm long 10% Tricine gels. One lane at the edge from each gel was cut and stained with Coomassie blue. Each gel was then appropriately aligned with its stained stripe and illuminated under long-wavelength UV light. The position of the Coomassie blue-stained bands relative to the FMI-labeled bands was used as a guide for cutting out the regions containing the fragments of interest. Proteins were eluted from acrylamide into 50 mM NH_4HCO_3 and 0.1% SDS using a Bio-Rad model 422 Electro-Eluter at 8 mA/tube overnight. Eluted proteins were precipitated with methanol after adjusting the pH to 7.0 with acetic acid. Pellets were resuspended in 50 mM Tris-HCl (pH 8.0) and 0.1% SDS, and aliquots were analyzed on a minigel to check the amounts and purities of the eluted proteins.

Proteolytic Digestion of Peptides. Eluted protein (0.1 mg/mL) in 48 mM Tris-HCl (pH 8.0) and 0.07% SDS was digested at 37 °C with trypsin (10% w/w) in the presence of 2 M urea and 10 mM $CaCl_2$. Reactions were stopped with 1 mM PMSF plus trypsin inhibitor (31 μg/mL) in 49 mM Tris-HCl and 1 mM EDTA (pH 8.0). Chymotryptic digestions of eluted protein (5−10% w/w) were carried out at 37 °C in 25 mM Tris-HCl, 1 mM EDTA (pH 8.0), and 0.05% SDS. Digestions were stopped with 5 mM PMSF.

Phosphorylation by PKA was carried out for 30 min at 30 °C in a volume of 40 μL containing 4 μg of eluted protein in 20 mM Tris-HCl (pH 7.5), 0.02% SDS, 10 mM $MgCl_2$, 0.1% octyl glucoside, 50 μM [γ-^{32}P]ATP (11.5 μCi), and 50 ng of the catalytic subunit of PKA. Reaction was stopped with 4 volumes methanol, and protein was precipitated at −20 °C. After transfer of proteins from the gel to PVDF, autoradiography was carried out using a Fuji BAS 1000 phosphorimager.

Sequencing and Immunoblotting. Bands of interest were cut out of a 16.5% T/6% C gel, equilibrated in 0.7 M Tris-HCl (pH 8.9) for 1 h, and separated on a second 1.5 mm thick 10% Tricine gel. Electroblotting from unfixed and unstained gels onto PVDF followed the procedure of

Matsudaira (*23*) as described by Capasso et al. (*6*). Sequencing of peptides blotted onto strips of PVDF was carried out with an Applied Biosystems model 475A protein sequencer with an on-line model 120A phenylthiohydantoin analyzer. For immunoblotting, protein samples were separated on 10% or 16.5/3% Tricine gels and blotted onto PVDF sheets, which were then reacted with the indicated antisera as described (*6*). Immunoblots were stained with diaminobenzidine with metal ion enhancement (*24*).

Materials. TPCK-trypsin (bovine pancreas) was obtained from Worthington, and α-chymotrypsin and OPA were purchased from Merck. Trypsin inhibitor (type 1-S from soybean), PMSF, ouabain, thioglycolate, Tricine, $C_{12}E_{10}$, and molecular mass markers (2.5−16.9 kDa) were bought from Sigma. FMI was purchased from Molecular Probes and octyl glucoside from Calbiochem. Electrophoresis grade reagents for SDS−PAGE were purchased from Bio-Rad. PVDF paper was from Millipore. Antiserum raised against the 16 kDa fragment of the β chain (Ala5−Arg142, referred to as anti-16 kDa) was prepared here using methods described previously (*1*). Antisera recognizing Leu815−Gln828 (anti-M5−M6) and Asn889−Gln903 (anti-L7/8), the synthetic peptides Lys1012−Tyr1016Y (anti-KETYY), and also the catalytic subunit of PKA were gifts from colleagues (see the Acknowledgment). [γ-^{32}P]ATP was obtained from DuPont-NEN.

RESULTS

Cross-Linking of Peptide Fragments of "19 kDa Membranes" with OPA. "19 kDa membranes" were solubilized with $C_{12}E_{10}$ (2.2 w/w) in the presence of 5 mM Rb ions and 10 mM ouabain to preserve the functionally intact complex (*1*), and the soluble protein was then treated with OPA (Figure 1). Two major cross-linked products with M_r's of 25 and 31 kDa were observed. Yields of the products increased gradually to a maximum after 30 min, and their formation was paralleled by a decrease in the amount of the 19 kDa fragment (Figure 1, 5−40). Judged by the intensity of the Coomassie stain, the yield of each of the two products is 10−15% of the total protein or 20−30% overall. When $C_{12}E_{10}$-solubilized "19 kDa membranes" were denatured with SDS prior to cross-linking with OPA, the cross-linked products were not observed (Figure 1, SDS), demonstrating that cross-linking requires an intact complex. OPA treatment of intact "19 kDa membranes" yielded the same cross-linked products as the detergent-solubilized preparation and with a similar yield (Figure 1, Sol vs Mem). Thus, the 25 and 31 kDa products are the result of genuine intramolecular cross-linking. For convenience, further work (Figures 2−7) utilized the intact "19 kDa membranes". We have detected fluorescence (λ_{ex} = 340 nm, λ_{em} = 398 nm) after treatment of $C_{12}E_{10}$-solubilized "19 kDa membrane" with OPA (*17*, *18*). However, denaturation with SDS prior to treatment with OPA did not abolish fluorescence, whereas SDS abolished the formation of the 25 and 31 kDa products (Figure 1). Therefore, we have not continued attempts to follow cross-linking by fluorescence. By comparison with the specific cross-links induced by OPA, incubation of solubilized or intact "19 kDa membranes" with terephthalaldehyde did not produce any specific cross-links (not shown).

For identification of the peptide components of the 25 and the 31 kDa cross-linked products, OPA-treated "19 kDa membranes" were resolved on a gel and immunoblotted with antibodies directed against specific peptide sequences or fragments (Figure 2, arrows point to pairs of bands with the same electrophoretic mobility). Both cross-linked peptides contain the 19 kDa C-terminal peptide of the α chain (Asn831−Tyr1016) since they were both recognized by the antibody raised against Lys1012−Tyr1016Y (anti-KETYY), as was the unreacted 19 kDa fragment. The 31 kDa product was recognized by antiserum raised against the 16 kDa fragment of the β chain (anti-16 kDa), as was the unreacted 16 kDa fragment and the intact β subunit. The 25 kDa product also reacted with anti-Leu815−Gln828, recognizing the fragment containing the M5−M6 pair (anti-M5−M6), as did the unreacted 8 kDa fragment containing M5−M6. Neither the 25 nor the 31 kDa products were recognized by antibodies directed against the γ chain or against the fragment containing the M1−M2 pair (not shown). The immunoblot also reveals some minor cross-linked products such as the ≈24 kDa band which runs below the 25 kDa peptide and recognizes the anti-16 kDa antibody (seen also in Figure 4). This ≈24 kDa band should contain a second component (e.g. M1−M2, M3−M4, or the γ subunit), although we did not identify any other fragments, due probably to the small amount present. Our subsequent efforts to locate the positions of the cross-links have focused only on the two major 25 and 31 kDa products. Attempts to identify

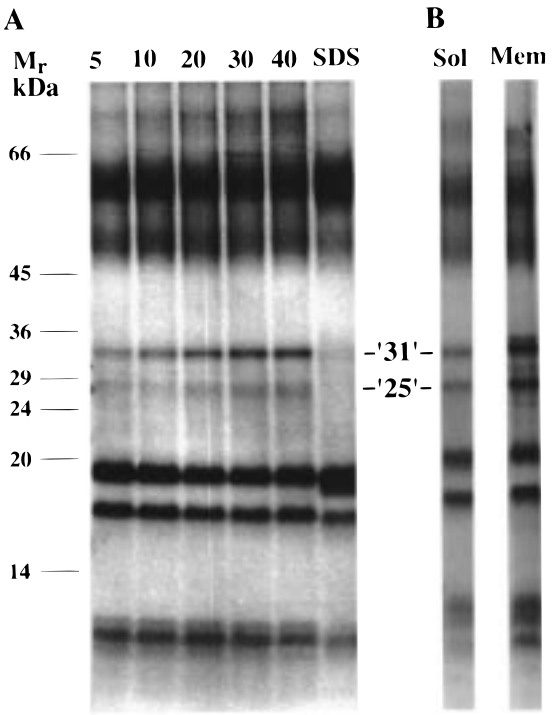

FIGURE 1: Treatment of "19 kDa membranes" with OPA produces 25 and 31 kDa cross-linked products. Solubilized "19 kDa membranes" (0.4 mg/mL) were treated with 60 μM OPA at room temperature. Aliquots (250 μL) were removed after 5, 10, 20, 30, and 40 min, mixed with $^1/_9$ volume of 20% SDS, and precipitated with 4 volumes of methanol/ether (2:1). SDS-solubilized "19 kDa membranes" were denatured with 2% SDS and then treated with 60 μM OPA for 40 min. M_r values are molecular mass standards. In the Sol lane, solubilized "19 kDa membranes" were treated with OPA as above for 30 min. In the Mem lane, "19 kDa membranes" (0.4 mg/mL) in 25 mM imidazole, 1 mM EDTA, and 5 mM RbCl (pH 7.5) were treated with OPA as above for 40 min. Samples were resolved on long 10% gels and stained with Coomassie blue. The numbers 25 and 31 denote the two cross-linked bands.

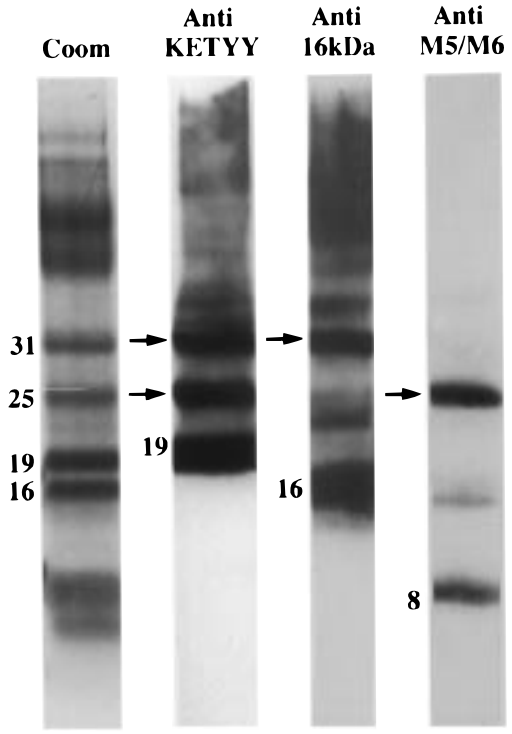

FIGURE 2: Peptide components of the 25 and 31 kDa cross-linked products. Samples (17 μg) of "19 kDa membranes" which had been cross-linked with OPA were resolved on a short 10% gel, transferred to PVDF, and immunostained with anti-KETYY, anti-16 kDa fragment (β subunit), and anti-M5−M6. The Coom lane depicts the Coomassie stain of the OPA-treated preparation (50 μg). The numbers 25 and 31 denote the two cross-linked bands. The numbers 19, 16, and 8 denote the remanining unreacted components.

components of the 25 and 31 kDa products by sequencing were unsuccessful, presumably because treatment of polypeptides with OPA blocks the N termini.[2]

The effect of the conformational state on the yield of the 25 and 31 kDa cross-linked products was assessed by treatment of "19 kDa membranes" with OPA in the presence of Rb, Na, or Mg and ouabain or without any ligand (Figure 3). The polypeptides were probed with anti-KETYY since the 19 kDa fragment is a component of both cross-linked products. Apart from a slight decrease in yield in the presence of Na ions, addition of ligands had no effect on the amount or nature of the cross-linked products.

Further evidence for the specificity of the cross-links was obtained by looking at the result of OPA treatment of "19 kDa membranes" which had been preincubated at 37 °C under various conditions (Figure 4). The capacity to occlude Rb is rapidly and irreversibly lost upon incubation at 37 °C and is associated with disorganization of the complex of fragments (*7*, *9*). Rb ions fully protect against thermal inactivation (*8*, *9*). Thermal inactivation also leads to selective dissociation of the M5−M6 fragment from the membrane, and the presence of Rb ions prevents dissociation of the fragment (*26*). We have reported recently that Ca ions prevent the release of the M5−M6 fragment, although they do not protect against the thermal inactivation (*9*, *10*). Thus, "19 kDa membranes" were incubated at 37 °C for 60 min in the absence or presence of Rb ions or the presence of Ca ions, and they were then treated with OPA under

[2] OPA reacts with primary amines to form phthalimidines (*25*).

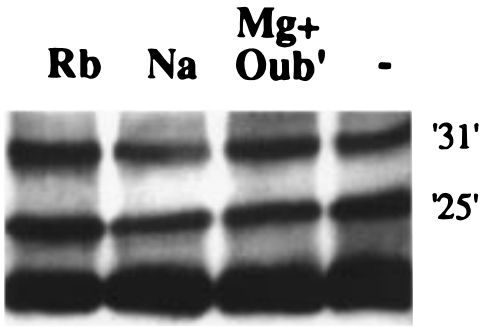

FIGURE 3: Lack of effect of occluded cations or ouabain on cross-linking by OPA. "19 kDa membranes" [0.5 mg/mL in 20 mM imidazole and 0.8 mM EDTA (pH 7.5)] were incubated for 40 min in the presence of either 5 mM RbCl, 100 mM NaCl, 0.2 mM ouabain, and 1 mM $MgCl_2$ or without any ligand and were then treated with 60 μM OPA for 40 min. Samples (25 μg) were dissolved with 2% SDS; protein was precipitated with 4 volumes of methanol, dissolved with sample buffer, and resolved on a 10% Tricine gel, which was then blotted onto PVDF and stained with anti-KETYY. The numbers 25 and 31 denote the two OPA cross-linked products.

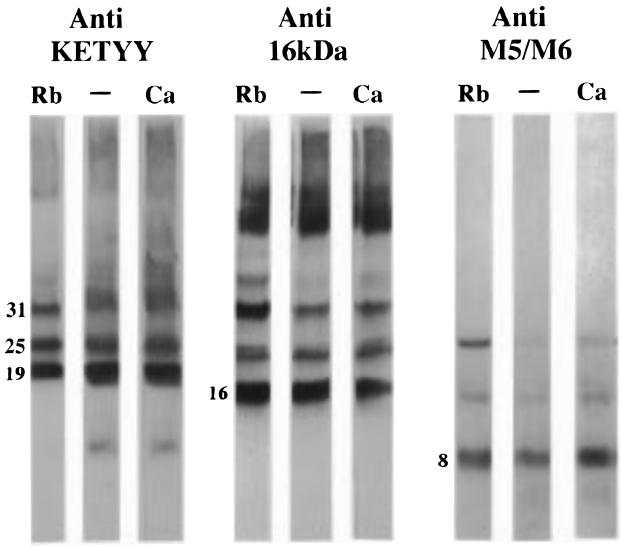

FIGURE 4: Thermal inactivation of "19 kDa membranes" interferes with OPA-mediated cross-linking. Two hundred microliters of "19 kDa membranes" [0.5 mg/mL in 21 mM imidazole and 0.84 mM EDTA (pH 7.5)] was incubated for 1 h at 37 °C either in the presence of 5 mM RbCl (Rb) or 2 mM $CaCl_2$ (Ca) or with no ligand (−). Membranes were then precipitated at 50 000 rpm for 45 min and were resuspended in 200 μL of 25 mM imidazole, 1.0 mM EDTA, and 5 mM RbCl (pH 7.5). One hundred fifty microliters of resuspended membranes (Rb, −, and Ca) was treated with 60 μM OPA for 40 min, dissolved with 2% SDS, and precipitated with 4 volumes of methanol. Each pellet was dissolved in loading buffer, split into three 25 μg samples, resolved on the same 10% gel, and immunoblotted with anti-KETYY, anti-16 kDa, or anti-M5−M6, respectively.

identical conditions. The samples were then resolved on a gel and immunoblotted with anti-KETYY, anti-16 kDa, and anti-M5−M6 (Figure 4). The result is that thermal inactivation reduced the yields of the specific 25 and 31 kDa cross-linked products (compare Rb with − and Ca). The reduction in the yield of cross-links was most striking when fragments were probed with the anti-16 kDa or anti-M5−M6 but was less pronounced when they were probed with anti-KETYY. In the latter case, the two bands appear more diffuse, suggesting that the 19 kDa fragment may be unselectively cross-linked by OPA to other fragments following thermal

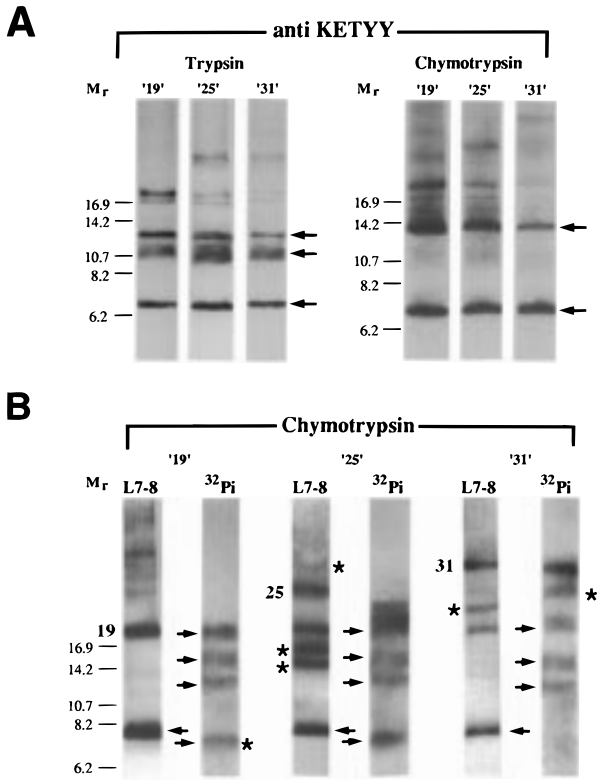

FIGURE 5: Identification of the cross-linked region in the 19 kDa peptide. (A) The 19 kDa fragment and the 25 and 31 kDa cross-linked products were eluted from gels and were digested with trypsin at 37 °C for 6 min or with chymotrypsin at 37 °C for 5 min (see Materials and Methods). Samples (1.5−2 μg per lane) were resolved on a 16.5/3% Tricine gel, blotted onto PVDF, and immunostained with anti-KETYY. Positions of marker peptides (6.2−16.9 kDa) are indicated. (B) The 19 kDa fragment and the 25 and 31 kDa cross-linked products were phosphorylated with the catalytic subunit of PKA at 30 °C for 30 min as described in Materials and Methods, and protein was precipitated with methanol. Pellets were dissolved in 50 mM Tris-HCl (pH 8.0) and 0.1% SDS and digested with chymotrypsin for 30 min at 37 °C. Samples (~4 μg) were then resolved on a 16.5/3% Tricine gel, blotted onto PVDF, and washed twice for 30 min with 100 mL of 0.4 M K_2HPO_4 (pH 7.5). The PVDF sheet was exposed to FUJI X-ray RX film at −70 °C with intensifying screens over the weekend ($^{32}P_i$). The PVDF sheet was then immunostained with an antibody recognizing residues Asn889−Gln903 (anti-L7/8). Arrows (→ or ←) or asterisks (*) point to bands with the same or different electrophoretic mobilities, respectively.

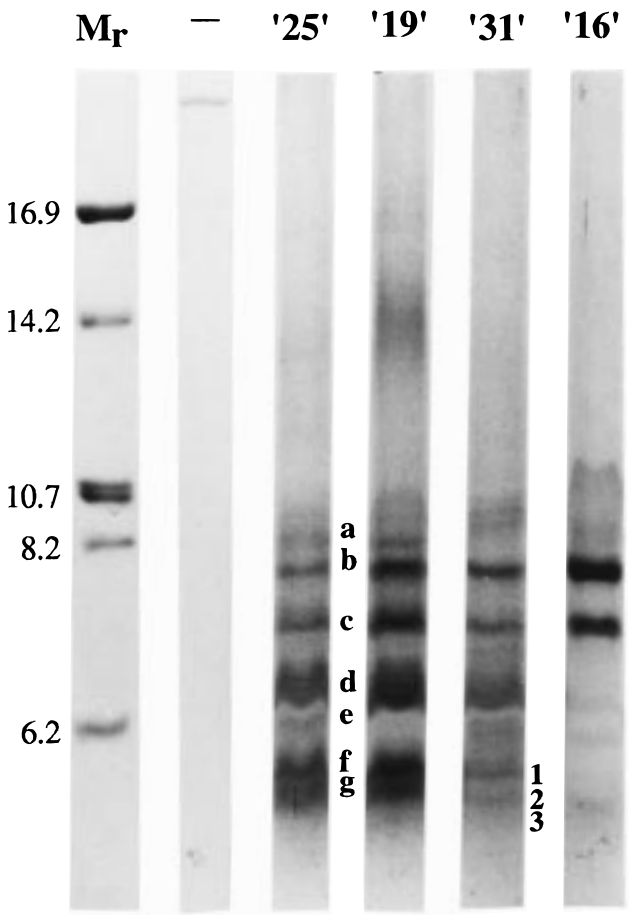

FIGURE 6: Tryptic peptides derived from the 25 and 31 kDa cross-linked products. The 25 kDa cross-linked species (60 μg), 19 kDa fragment (25 μg), 31 kDa cross-linked product (90 μg), and 16 kDa fragment (20 μg) were each digested with trypsin at 37 °C for 12 h (see Materials and Methods). Mixtures were then separated on a long 16.5/6% Tricine gel at 33 mA for 21 h, and the gel was stained with Coomassie blue. M_r values are molecular mass standards. The − lane is control digestion with trypsin only. Fragments labeled a−g in lane 25 and 1−3 in lane 31 were sequenced (see Table 1).

denaturation. Although one might have attributed the lower yield of the 25 kDa product to dissociation from the membrane of the M5−M6 fragment (Figure 4, Rb vs −), the amount of both cross-links was greatly reduced upon thermal inactivation even in the presence of Ca ions, when no such loss of the M5−M6 fragment occurs (Figure 4, Rb vs Ca). Thus, the lower yield of the 25 kDa cross-linked product is due to thermal disruption of the interaction between the M5−M6 and M7−M10 fragments. Similarly, the lower yield of the 31 kDa product is due to disruption of the interaction between the M7−M10 fragment and the β subunit.

Location of the Cross-Linked Region(s) of the M7−M10 Fragment. The rationale behind the experiments in Figures 5−7 is that, after proteolytic digestion of the 25 and 31 kDa products, fragments containing the cross-linked segments of the 19 kDa peptide should be larger than those obtained by digestion of the 19 kDa peptide itself. Conversely, fragments containing segments which are not involved in cross-linking should be the same size as those derived from the 19 kDa peptide. Mild conditions of proteolysis were chosen so that intermediates of the digestion would be detected and to avoid cleavage of antibody recognition sites used for identification of fragments. In Figures 5−7, arrows (→ or ←) denote bands with the same electrophoretic mobility in all lanes being compared while asterisks (*) denote new bands which are not present in all lanes being compared. The reader should find it easier to follow the planning and interpretation of the experiments by referring to Figure 8.

In the experiment of Figure 5A, the 19 kDa fragment and the 25 and 31 kDa cross-linked products were extracted from gels and partially digested with trypsin or chymotrypsin, and digests were then separated on a gel, blotted onto PVDF, and probed with anti-KETYY. Digestion of 19, 25, and 31 kDa peptides produced similar patterns of tryptic fragments recognizing anti-KETYY, including three major bands of 6.8, 10.6, and 12.6 kDa (←), and also a similar pattern of chymotryptic fragments with major bands at 7.0 and 13.6 kDa (←). By comparison with known proteolytic fragments which bind anti-KETYY (see ref *11*), it can be inferred that the 12.6 and 13.6 kDa fragments contain M8−M10, the 10.6

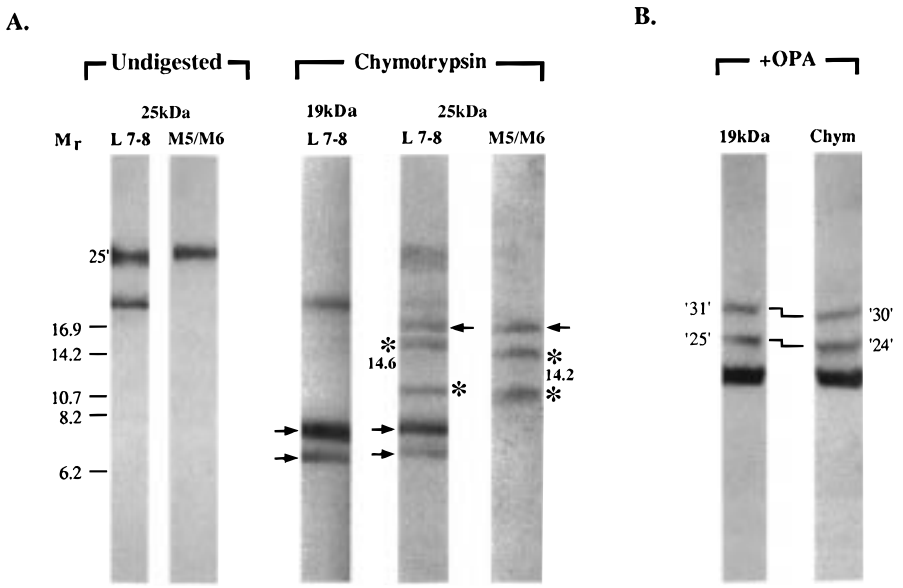

FIGURE 7: Identification of the cross-linked regions of the M5−M6 fragment. (A) The 25 kDa cross-linked product either was left undigested or was digested with chymotrypsin at 37 °C for 5 min (see Materials and Methods). Samples (~4 μg per lane) were resolved on a 16.5/3% Tricine gel, blotted onto PVDF, and immunostained with anti-M5−M6 or anti-L7/8. In the lane marked 19 kDa, the 19 kDa fragment was digested with chymotrypsin and immunostained with the anti-L7/8. (B) "19 kDa membranes" at 1.5 mg/mL in 12.5 mM imidazole, 1.4 mM EDTA (pH 7.5), and 20 mM Tris-HCl (pH 8.0) were treated with chymotrypsin (1:5 w/w) at 20 °C for 90 min. Digestion was stopped with 15 volumes of 1 mM PMSF and 0.2 mM TPCK in 150 mM KCl and 25 mM Tris-HCl (pH 7.5). After 10 min, membranes were precipitated at 250000*g* for 30 min at 4 °C. Pellets were resuspended again in 1 mL of the same buffer, incubated for 10 min at room temperature, and precipitated again. "19 kDa membranes" (19 kDa) or membranes treated with chymotrypsin (Chym) were resuspended at 0.5 mg/mL in 5 mM RbCl, 25 mM imidazole, and 1 mM EDTA (pH 7.5) and treated with 60 μM OPA for 40 min (+OPA). Treated samples (30 μg) were resolved on a short 16.5/3% gel and immunoblotted with anti-KETYY. Arrows (→ or ←) or asterisks (*) point to bands with the same or different electrophoretic mobilities, respectively.

kDa fragment contains M9−M10, and the 6.8 and 7.0 kDa fragments contain M10 (see Discussion and Figure 8). Thus, the result in Figure 5A excludes location of either cross-link in the 19 kDa peptide from a region between the N terminus of the 13.6 kDa fragment and the C terminus of the α subunit.[3]

Additional evidence for the locations of cross-linked regions of the 19 kDa peptide has been obtained by utilizing the PKA phosphorylation at Ser936 on the short uncleaved loop between M8 and M9 (*27*, *28*) and an anti-peptide antibody raised against the sequence Asn889−Gln903 in the loop between M7 and M8 (referred to as anti-L7/8, Figure 5B; *29*). The 19 kDa fragment and the 25 and 31 kDa cross-linked products were first phosphorylated with PKA and [γ-^{32}P]ATP, and they were then digested with chymotrypsin for 30 min.[4] Digests were resolved on a gel and blotted onto PVDF, which was first autoradiographed and then immunostained with anti-L7/8. Adjacent lanes depict the $^{32}P_i$ label and anti-L7/8 recognition of the chymotryptic fragments of the 19, 25, and 31 kDa peptides. Comparison of the cleavage patterns of the 25 kDa product with that of the 19 kDa peptide revealed two unique bands of 14.6 and 16.7 kDa (*, 25, L7/8) which bind anti-L7/8 and did not appear in the digest of the 19 kDa fragment, and also two peptides (apparent M_r's of 7.5 and 19 kDa) with the same mobility in both lanes. Thus, these 14.6 and 16.7 kDa bands must contain cross-linked fragments of the M7−M10 and M5−M6 peptides. Comparison of the ^{32}P-labeled bands (apparent M_r's of 19, 14.9, 13.6, and 7 kDa) did not reveal differences between the digests of the 19 and 25 kDa peptides, and the ^{32}P-labeled fragments do not overlap with the 14.6 and 16.7 kDa fragments which bind anti-L7/8 (*). Thus, the cross-linked chymotryptic fragments do not contain the phosphorylation site, Ser936, and ^{32}P-labeled fragments do not contain cross-links (see Discussion). Comparison of the digests of the 31 and 19 kDa peptides was most informative. First, it can be seen that the 7 kDa ^{32}P-labeled fragment observed in digests of the 19 kDa peptide was not seen in digests of the 31 kDa peptide, and instead, a new 24 kDa ^{32}P-labeled fragment appeared (fragments marked *, 19, and 31, $^{32}P_i$). Evidently, the 24 kDa ^{32}P-labeled band contains the ^{32}P-labeled 7 kDa fragment cross-linked to the 16 kDa fragment of the β subunit. Second, anti-L7/8 recognized a unique 23 kDa band, which did not appear in the other digests and does not overlap with the ^{32}P-labeled 24 kDa fragment (*****, 19, and 31, L7/8). This 23 kDa band must also contain cross-linked fragments of the M7−M10 peptide and the β subunit. Thus, the experiment revealed two chymotryptic fragments, 23 and 24 kDa, containing the cross-link to the β subunit and either the anti-L7/8 epitope or the PKA phosphorylation site, respectively. Third, the 7.5 kDa fragment which recognizes anti-L7/8 does not appear to contain the cross-link in the 31 kDa product. Taken together, the evidence in panels A and B of Figure 5 leads to the conclusion that the cross-link is located outside the entrance to transmembrane segment M8 between two closely positioned chymotryptic cleavage sites (see Discussion and Figure 8A).

[3] The minor fragments above the position of the 13.6 kDa fragment seen in chymotryptic digests of 19 and 25 kDa peptide, but not in that of the 31 kDa product, hint that the cross-link in the 25 kDa product is located near the N terminus of the 19 kDa fragment.

[4] Chymotrypsin was used rather than trypsin to avoid cleavage at the arginine residues immediately preceding Ser936.

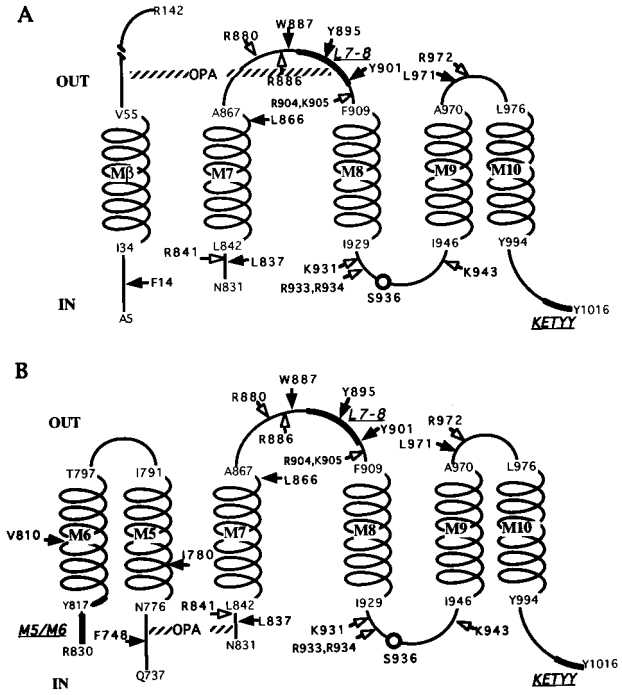

FIGURE 8: Topological models of cross-linked fragments. (A) The 31 kDa product: the M7−M10 fragment and the 16 kDa fragment of the β subunit. (B) The 25 kDa product: the M7−M10 fragment and the M5−M6 fragment. Chymotryptic and tryptic cleavage sites are represented by solid or empty arrows, respectively. Peptide segments used to produce antibodies are depicted by lines of double thickness and are indicated by M5−M6, L7/8, or KETYY, respectively. Ser936 is the residue phosphorylated by protein kinase A. The approximate positions of cross-links are depicted by striped bars.

Table 1: N-Terminal Sequences of Tryptic Peptides of the 25 and 31 Cross-Linked Products

peptide	apparent M_r (kDa)	N-terminal sequence	transmembrane region
a	8.8	no sequence obtained	
b	8.3	β, V^{71}APPGLTQ	extracellular
c	7.4	α, L^{842}ISMAYGQIG	M7
d	6.9−6.5	α, M^{973}YPLKPT	M10
e	6.2	no sequence obtained	
f	5.6	α, L^{842}ISMAYGQIG	M7
		α, R^{934}NSVFQQGMK	M9
		α, M^{973}YPLKP	M10
g	5.3	α, N^{935}SVFQQ	M9
1	5.5	α, L^{842}ISMA	M7
		α, R^{934}NSVFQ	M9
2	5.2	α, L^{842}ISMAYGQIG	M7
		α, N^{935}SVFQQGMK	M9
3	5.0	α, N^{944}KILIF	M9

Amino acid sequencing of peptides obained by extensive tryptic digestion of the 25 and 31 kDa cross-linked products confirms the conclusions based on Figure 5 and extends them in the case of the 25 kDa product (Figure 6 and Table 1). The pattern of tryptic fragments (Figure 6) of either cross-linked product appears similar to that of the 19 kDa fragment. One fragment, e (6.3 kDa), derived from the 25 kDa product seems to contain the cross-link because it was not observed in the digest of the 19 kDa peptide itself. However, no sequences were obtained from fragment e which has blocked N termini. Fragments a−g and 1−3 of trypsinised 25 and 31 kDa species, respectively, were sequenced, and the results are presented in Table 1. Peptides d, f, g, and 1−3 contain segments containing M9 or M10, thus confirming that the cross-links are not located in these segments. Peptide d appears to consist of a nest of fragments, but a unique N terminus was found, Met973; i.e., the C terminus is probably ragged. Fragment b is a fragment of the β subunit and is presumably derived from a contaminant in the 25 kDa cross-linked product, such as that seen in Figures 2 and 4. The important additional information in Table 1 comes from the finding that peptides c and f derived from the 25 kDa product have Leu842 as the N terminus. Both of these fragments were also found in digests of the 19 kDa peptide, and therefore, they do not contain the cross-link. Note in particular peptide c (M_r of 7.4 kDa) which extends from Leu842 to about Arg904.

Taken together, the results of Figures 5 and 6 and Table 1 allow one to conclude that the cross-linked region in the 25 kDa product must be located in the short cytoplasmic segment from Asn831 to Arg841 preceding M7 (see Discussion and Figure 8B).

Location of the Cross-Linked Regions of the M5−M6 Fragment. The conclusions about the position of the cross-link in the M7−M10 component of the 25 kDa product indicate that a cytoplasmic segment of the M5−M6 fragment must be the partner. Both N and C termini of the M5−M6 fragment face the cytoplasmic side (*30*). To determine which end of the M5−M6 fragment is cross-linked to the M7−M10 fragment, the 25 kDa product was digested with chymotrypsin for 5 min, resolved on a gel, and immunoblotted with anti-L7/8 and anti-M5−M6 raised against the sequence Leu815−Gln828 at the C terminus of this fragment (*28*) (Figure 7A). The undigested 25 kDa cross-linked product is effectively stained by both antibodies (Figure 7A, undigested 25). After digestion of the 25 kDa product, five bands were produced which are recognized by anti-L7/8, but two of the bands (→) were also found in digests of the 19 kDa fragment. The three bands of 16.7, 14.6, and 10.8 kDa were not found in digests of the 19 kDa fragment and thus contain the cross-linked fragments (Figure 7A, chymotrypsin, 25 kDa, L7/8).[5] After digestion, three bands (16.7, 14.2, and 10.6 kDa) were recognized by anti-M5−M6 and must contain the cross-link because they are larger than the uncross-linked M5−M6 fragment (8 kDa). Of these three fragments, only the 16.7 kDa fragment has the same mobility as the 16.7 kDa band recognized by anti-L7/8 (←). By contrast, the 14.6 kDa cross-linked fragment (*) which was recognized by anti-L7/8 is not recognized by the anti-M5−M6, and it is clearly resolved from the 14.2 kDa fragment (*) which is recognized by anti-M5−M6 but not by anti-L7/8. Thus, Leu815−Gln828 must have been removed from the M5−M6 component present in the 14.6 kDa cross-linked fragment (and the 14.2 kDa fragment contains a component of the 19 kDa peptide which lacks Asn889−Gln903). The result of Figure 7A demonstrates that the cross-linked region is located between the N terminus of the M5−M6 fragment (Gln737) and the entrance to M5, and not after M6 (see Figure 8B)

Finally, we have been able to place a further limit on the cytoplasmic segment of the M5−M6 fragment involved in the cross-link (Figure 7B). The experiment utilizes "19 kDa

[5] The 16.7 and 14.6 kDa bands are the same as those seen in Figure 5B.

membranes" digested with chymotrypsin in a controlled fashion as we described recently (*31*). Digestion at room temperature truncates 12 amino acids from the N terminus of M5−M6 (new N-terminal Ala749) and also 10 amino acids from the N terminus of the 16 kDa fragment of the β subunit (new N-terminal end Ile15), without affecting Rb occlusion. This preparation was treated with OPA. We observed the two cross-linked products with unchanged yields compared to those in "19 kDa membranes" (Figure 7B, compare 19 kDa and Chym), but the sizes of the two cross-linked species, 24 and 30, were slightly reduced, consistent with the truncations of the M5−M6 and 16 kDa fragments. Thus, the experiment localizes the region of cross-linking in the 25 kDa product between Ala749 and the entrance to M5 (Ala770).

DISCUSSION

Specific Cross-Linking of Fragments of "19 kDa Membranes". OPA reacts with thiols and primary amines to form 1-alkylthio-2-alkylisoindoles which are highly fluorescent (*15*, *16*). OPA was found to inactivate the catalytic subunit of cAMP-dependent protein kinase and also cGMP-dependent protein kinase in a substrate protectable manner (*17*, *18*). Spectroscopic data suggested that the inactivation by OPA resulted from concomitant modification of two closely spaced (∼3 Å) lysine and cysteine residues within the active site, leading to the formation of an isoindole derivative. However, the chemical specificity of OPA is not absolute. Reaction of OPA with primary amines can be followed by addition of cyanide instead of a thiol, producing 1-cyano-2-alkylisoindoles (*32*). Kuralay et al. (*33*) provide evidence that amino acids with nucleophilic centers other than thiols can be reaction partners for fluorescent derivatization of proteins by OPA. In our case, the protein segments that were cross-linked by OPA (cf. Figure 8) contain no cysteine residues, and OPA may have mediated cross-linking in "19 kDa membranes" by reaction with primary amines and other nucleophiles.

It is striking that, although "19 kDa membranes" contain numerous lysines, cysteines, and other residues with nucleophilic side chains (e.g., serine and threonine), treatment with OPA yielded only two major cross-linked products of 25 and 31 kDa (Figure 1). Both species contained the 19 kDa fragment which is cross-linked either to the peptide containing M5−M6 or to the 16 kDa fragment of the β chain, respectively (Figure 2). This selectivity, as well as the finding that the same products are formed upon treating intact and solubilized "19 kDa membranes" (Figure 1), provides strong indications that the cross-linked fragments and residues are indeed close (≈3 Å) to each other in "19 kDa membranes". Furthermore, when "19 kDa membranes" were first denatured with SDS (Figure 1) or were thermally inactivated (Figure 4) and were then treated with OPA, the two cross-linked products largely disappeared.

Identification of Cross-Linked Regions. Figure 8 depicts the topological organization of the M7−M10 fragment and the 16 kDa fragment of the β subunit (Figure 8A) or the M5−M6 fragment (Figure 8B) and the inferred location of the cross-linked regions, as well as antibody binding sequences, the PKA phosphorylation site, and presumed proteolytic cleavage sites. The inferences that particular regions of the α subunit are involved in the cross-links rest on a combination of positive evidence and exclusion of the other regions.

The experiment in Figure 5A excludes location of the cross-links in both the 31 and 25 kDa products from the region between the N terminus of the 13.6 kDa chymotrytpic fragment and the C terminus of the α subunit. Previously, we described chymotrytic fragments of the α subunit which extend to the C terminus and run with apparent M_r values of 15.7, 13.1, 9.6, and 7.8 kDa on 10% Tricine gels (or as 16.2, 14.9, 9.3−9.8, and 6.8−7.1 kDa on 16.5%/6% gels) and similar tryptic fragments with M_r values of 14.4, 12.1, 9.9, and 7.4 kDa (*11*). The N termini of the chymotryptic fragments were determined to be Arg880, Glu902, Gln939, and Arg972, respectively. On the basis of this scale, the chymotryptic cleavage sites of the 13.6 and 7 kDa chymotryptic fragments in Figure 5A can be assigned as Tyr901 preceding M8 and Leu971 between M9 and M10 and those of the 12.6, 10.6, and 7 kDa tryptic fragments as Arg904 and Lys905 preceding M8, Lys931, Arg933, and Arg934 between M8 and M9, and Arg972 between M9 and M10 (see Figure 8). Thus, both cross-links in the 19 kDa peptide are located between its N terminus, Asn831, and the N terminus of the 13.6 kDa fragment, Tyr901, which lies outside the entrance to M8 (Phe909).[6]

The inference that the cross-link in the 19 kDa component of the 31 kDa product lies between the two closely located chymotryptic cleavage sites before M8 (Figure 8A) is inescapable if one is to reconcile the evidence in Figure 5A with that in Figure 5B. The salient findings in Figure 5B are the observation of the two chymotryptic fragments containing the cross-link, that either recognize anti-L7/8 but do not contain Ser936 (23 kDa) or contain Ser936 but do not recognize anti-L7/8 (24 kDa), respectively, and also a 7.5 kDa fragment that recognizes anti-L7/8 which does not contain the cross-link. One chymotryptic site has been assigned as Tyr901, and the most likely position of the other is Tyr895. On the basis of this assumption, the 23 kDa band would contain a fragment extending from the N terminus of the 19 kDa peptide, Asn831 (or a nearby chymotryptic site such as Leu837), to Tyr901 cross-linked to the 16 kDa fragment of the β subunit, while the 24 kDa band would contain a Gly896−Leu971 fragment cross-linked to the 16 kDa fragment of the β subunit. The 7.5 kDa fragment would extend from Asn831 (or Leu837) to Tyr895. An implication of the hypothesis is that the segment ^{889}NDVEDSY^{895} constitutes the major determinant of the anti-L7/8 antibody. This is not unreasonable because three charged residues are located in this segment. Sequences of tryptic fragments of the 31 kDa product (Table 1, fragments 1−3), found also in digests of the 19 kDa peptide itself, provide further support that the cross-link is not located in segments containing M9 or in the 5.2 and 5.5 kDa fragments with N termini Leu842 at the entrance of M7 and extending to Arg880 (fragment 2, 5.5 kDa) or Arg886 (fragment 1, 5.5 kDa) in the loop between M7 and M8 (Figure 8A). The evidence that the

[6] Note that if one allows for the possibility of nonclassical tryptic cleavages (i.e., not at Lys or Arg) then the chymotryptic and tryptic cleavage sites could be a few residues closer to the entrance of M8 than depicted in Figure 8. However, this would not affect the basic interpretation of Figure 5A or the logic of the arguments presented below.

latter two fragments do not contain the cross-link does not contradict the inferred location of the cross-link in Figure 8A, which is C-terminal to Arg886 and before M8.

The inference that the cross-link in the 19 kDa component of the 25 kDa product lies within the short cytoplasmic segment Asn831−Leu842 before M7 (Figure 8B) rests on the evidence in Figure 5A discussed above, together with that in Figure 5B, and particularly on the sequencing data in Table 1. The sequencing of tryptic fragments of the 25 kDa product (Table 1), which were also observed in digests of the 19 kDa peptide alone, supports the conclusion from Figure 5A that the cross-link is not located in segments containing M9 or M10, but it adds the important piece of information that the cross-link can also be excluded from the 7.4 kDa fragment (c) extending from Leu842 to Arg904 or Lys905 just before the entrance of M8. The 7.5 kDa chymotryptic fragment seen in Figure 5B which is not cross-linked but does recognize anti-L7/8 provides a similar but less direct argument. Any slight doubt on the overlap of the exclusion regions defined by the fragments in panels A and B of Figure 5 and Table 1, and the possibility that the cross-link might be located near M8, is removed by the observation in Figure 5B that the PKA phosphorylation site, Ser936, is not found in any fragments of the 25 kDa product containing the cross-link. On the contrary, the two cross-linked chymotryptic fragments (14.6 and 16.7 kDa) recognize anti-L7/8 but do not contain Ser936. According to Figure 8B, these two fragments would both include segments Asn831−Tyr895/Tyr901 and either the full-length M5−M6 fragment (16.7 kDa) or a truncated form (14.6 kDa). Since the evidence in Figure 5A excludes the cross-link from the segment Tyr901−Tyr1016 and that in Table 1 and Figure 5B excludes it from the segment Leu842−Arg904/Lys905, the only remaining possibility is that the cross-link lies within Asn831−Arg841, leading into M7 (*28*). The finding that the one tryptic peptide in Figure 6 which contains the cross-link, e, could not be sequenced, and our observation that OPA blocks the N termini of cross-linked products, strengthens the conclusion that the cross-link is located near the N terminus of the 19 kDa peptide.

The experiment in Figure 7 demonstrates that, in the 25 kDa cross-linked product, the cytoplasmic segment Ala749−Ala770 preceding M5 provides the partner for the cross-linked residue in the cytoplasmic segment preceding M7. The 16.7 kDa chymotryptic fragment seen in Figure 7A which contains the cross-link and recognizes both anti-L7/8 and anti-M5−M6 could consist of a fragment of the 19 kDa peptide (Asn831−Tyr901) and the M5−M6 fragment (Ala749−Arg830) (see Figure 8B). Observation of the 14.6 kDa fragment which contains the cross-link and recognizes anti-L7/8 but not anti-M5−M6 indicates that chymotrypsin removed 15−20 residues from the C terminus of the M5−M6 fragment, including the epitope Leu815−Gln828 and any residues which could be involved in cross-linking (e.g., Lys826 and Ser822). This is the result which makes it unlikely that the partner is located in the cytoplasmic segment which follows M6 and contains the epitope. The 14.6 kDa fragment could include the segment Asn831−Tyr901 cross-linked to the segment extending from Ala749 to a chymotrytpic site such as Val810. The 14.2 kDa fragment containing the cross-link which recognizes anti-M5−M6 but not anti-L7/8 could consist of the M5−M6 fragment Ala749−Arg830 and a fragment extending from Asn831 to a chymotrytpic site such as Trp887 (*11*). In Figure 7A, the 10.6 and 10.7 kDa bands which recognize anti-M5−M6 and anti-L7/8, respectively, have similar electrophoretic mobilities, but these cannot represent the same fragment containing the cross-link, due to size considerations (see Figure 8B). The 10.6 kDa band could consist of the M5−M6 fragment Ala749−Arg830 cross-linked to a fragment of the 19 kDa peptide extending from Asn831 to a chymotryptic site such as Leu866. The 10.7 kDa band could consist of Asn831−Tyr901 cross-linked to a fragment extending from Ala749 to a chymotryptic site such as Ile780.

Functional and Structural Implications of Interactions between the Fragments. (a) Interactions between α *and* β *Subunits*. The β subunit of Na,K-ATPase is required for posttranslational processing and maturation of the α subunit, including its correct folding, stabilization, and expression of the functional holoenzyme at the plasma membrane (see ref *3* for a review). Major structural determinants for assembly with the extracellular domain of the β subunit are contained within a stretch of 26 residues between M7 and M8, within the α subunit (*34*, *35*). Using the yeast two-hybrid system, a conserved four-amino acid sequence, ^{894}SYGQ^{897}, within the loop between M7 and M8 of the α subunit has recently been shown to be crucial for the α−β intersubunit interaction (*36*). In addition to the strong extracellular α−β subunit interactions, there is also evidence for interactions within the membrane-spanning region or between cytoplasmic segments (*31*, *37*−*39*).

The specificity of cross-linking in forming the 31 kDa product and the location of the cross-link in a short segment of the α subunit near M8 (between Tyr895 and Tyr901) fit very well with the finding that the sequence ^{894}SYGQ^{897} represents the major site of α−β subunit interactions at the extracellular surface. Residues in the β subunit which interact with the α subunit have not been mapped in detail, although work with truncated β subunits and mutations of the cysteines suggested that a region up to and including the first S−S bridge could be involved (*40*, *41*). The two-hybrid work restricts the location further to residues between Ser61 after the transmembrane segment and Cys125 before the first S−S bridge (*36*). As mentioned in the introductory section, disulfide bridge mediated cross-linking of the 19 kDa peptide and the 16 kDa fragment of the β subunit has been described (*12*, *13*). We have now established that Cys44 in the transmembrane segment of the β subunit is cross-linked to one of the cysteines in M8 (Cys911 or Cys930) (E. Or, R. Goldshleger, and S. J. D. Karlish, manuscript in preparation). This observation is an indication of the existence of the proximity of transmembrane segments M8 and Mβ in addition to the intimate connection between the residues just outside M8 and the β subunit.

In addition to the structural effects, the extracellular interaction of the α and β subunit is known to affect activation of the enzyme by K or Na ions and also Rb(K) occlusion (*37*, *38*, *42*−*44*). Reduction of S−S bonds in the extracellular domain of the β subunit inactivates Na,K-ATPase activity or Rb occlusion (*42*, *43*). Disruption of α−β interactions and inactivation of the enzyme via indirect effects on the α subunit is a likely mechanism (*42*), but it has also been proposed that the β subunit is directly involved in gating cation occlusion (*43*). The loss of the 31 kDa cross-

linked product upon thermal inactivation of cation occlusion (Figure 4B) is consistent with the notion that the extracellular $\alpha-\beta$ interaction stabilizes the cation occlusion domain. The lack of an effect of occluded cations (or ouabain) on the efficiency of cross-linking (Figure 3) shows that the ligands do not change the relative positions of the interacting residues of α and β subunits and makes it unlikely that the β subunit is directly involved in cation occlusion or "gating". Presumably, it is the loop between M7 and M8 of the α subunit which interacts with M4−M6 containing the cation sites so that disruption of the $\alpha-\beta$ interaction alters the disposition of this loop and inactivates cation occlusion.

(b) Interactions between M5−M6 and M7−M10 Segments of the α *Subunit.* The experiments demonstrate the existence of interacting cytoplasmic segments adjacent to transmembrane segments M5 and M7. Thus, it is likely that the proximity exists also at the membrane surface and extends to the interior of the membrane.

Site-directed mutagenesis studies have established the central role of M4−M6 in coordination of occluded cations (*45*−*47*) and of different transmembrane and extracellular segments of the α subunit, including M5 and M6, in binding of ouabain (*48*, *49*). Biochemical studies with "19 kDa membranes" have shown that both occluded cations and ouabain protect against thermal inactivation of Rb occlusion (*8*, *9*) and against further proteolysis of the 19 kDa fragment (*5*, *6*, *8*, *10*, *50*), and they also prevent dissociation of the M5−M6 hairpin from the membrane (*26*). Therefore, we have proposed (*9*) that Rb ions or ouabain strengthens preexisting interactions between the M5−M6 and the M7−M10 regions and so protect against structural perturbations, and conversely, disruption of these interactions is responsible for thermal inactivation of Rb occlusion. The recent work (*9*) demonstrates that dissociation of the M5−M6 hairpin is not the direct cause of the loss of Rb occlusion (as suggested in refs *51* and *52*) but rather is a result of thermal inactivation leading to disruption of its interactions with other fragments, presumably M7−M10. The present experiments strengthen the latter conclusion by providing direct evidence for an interaction between the M5−M6 hairpin and the M7−M10 domain and disruption of the interaction upon thermal inactivation (Figure 4B). Of course, the cytoplasmic interaction detected by OPA may be only one of several interactions between these fragments.

Dissociation of the M5−M6 fragment from "19 kDa membranes" has been used to postulate a "piston-like" movement of the M5−M6 pair in the normal active cation transport cycle (*52*). We have pointed out that, because the fragment dissociates from an inactive, partly disordered, protein complex, the phenomenon cannot be taken as evidence for such a piston-like movement in normal function (*9*). The finding that the yield of the 25 kDa cross-link product is largely unaffected by Rb and Na ions or ouabain (Figure 3) provides a much stronger argument. If occlusion or deocclusion of Rb or Na ions were associated with a transmembrane sliding movement of the M5−M6 fragment relative to M7−M10, significant in magnitude compared to the cross-linking distance of OPA ($\approx$3 Å), one could have expected the efficiency of cross-linking to be very different in conformations with or without cations. The result does not exclude the possibility that occlusion and deocclusion of cations are associated with twisting, bending, or stretching movements provided that these do not significantly affect the M5−M6−M7−M10 interaction detected by OPA.

Whereas the role of M4−M6 as major donors of side chains that coordinate occluded cations is well established (*45*−*47*), the role of the M7−M10 transmembrane segments is an open question. The M7−M10 region may support the proper folding and stability of the M5−M6 hairpin (*52*), but there is also evidence that suggests the C-terminal region plays a more direct and dynamic role in cation occlusion. A direct interaction with the occluded Rb ions is supported by strong protection afforded by Rb ions against proteolysis of the M7−M10 fragment (*5*, *6*), and the preference for K(Rb) over Na ions in shark rectal gland Na,K-ATPase (*53*) for protecting this fragment against proteolysis. In addition, exchange of the 102 C-terminal residues of Na,K-ATPase (M8−M10) into the corresponding region of SERCA-ATPase confers a high affinity for K-dependent stimulation of Ca-ATPase (*54*), implying recognition of K ions by the C-terminal domain of Na,K-ATPase. It is possible that the M4−M6 segments provide ligating residues common to both K and Na ions, whereas the M7−M10 domain donates extra ligating residues which confer selectivity for K ions (*20*). The mobility of the M7−M10 domain is indicated by covalent labeling of carboxyl (*55*) or cysteine residues (*51*, *56*, *57*) in M9 and M8 which is affected by the conformational state of the protein. These mobile segments may move in concert with the M5−M6 region. However, the way in which movements of the transmembrane segments bring about transport of the cations is unknown.

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