Characterization of Disulfide Cross-links between Fragments of Proteolyzed Na,K-ATPase

IMPLICATIONS FOR SPATIAL ORGANIZATION OF TRANS-MEMBRANE HELICES*

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This study characterizes disulfide cross-links between fragments of a well defined tryptic preparation of Na,K-ATPase, 19-kDa membranes solubilized with C₁₂E₁₀ in conditions preserving an intact complex of fragments and Rb occlusion (Or, E., Goldshleger, R., Tal, D. M., and Karlish, S. J. D. (1996) Biochemistry 35, 6853–6864). Upon solubilization, cross-links form spontaneously between the α subunit, 19- and 11.7-kDa fragments of the α subunit, containing trans-membrane segments M7-M10 and M1/M2, respectively. Treatment with Cu²⁺-phenanthroline (CuP) improves efficiency of cross-linking. Sequencing and immunoblot analysis have shown that the cross-linked products consist of a mixture of β-19 kDa dimers (~65%) and β-19 kDa–11.7 kDa trimers (~35%). The αβ cross-link has been located within the 19-kDa fragment to a 6.5-kDa chymotryptic fragment containing M8, indicating that βCys⁴⁴ is cross-linked to either Cys⁹¹¹ or Cys⁹³⁰. In addition, an internal cross-link between M9 and M10, Cys⁹⁶⁴-Cys⁹⁸³, has been found by sequencing tryptic fragments of the cross-linked product. The M1/M2-M7/M10 cross-link has not been identified directly. However, we propose that Cys⁹⁸³ in M10 is cross-linked either to Cys⁹¹⁴ in M1 or internally to Cys⁹⁶⁴ in M9. Based on this study, cross-linking induced by α-phthalaldehyde (Or, E., Goldshleger, R., and Karlish, S. J. D. (1998) Biochemistry 37, 8197–8207), and from the literature, we propose an approximate spatial organization of trans-membrane segments of the α and β subunits.

Renal Na,K-ATPase consists of a catalytic α subunit (112 kDa), a glycosylated β subunit (33 kDa), and a γ subunit (~6.5 kDa). The α subunit contains the ATP hydrolytic site and the Na⁺ and K⁺ transport sites. The β subunit is required for correct post-translational processing and stabilizes the α subunit (4). The γ subunit may be a tissue-specific regulator (5). Recent studies on P-type pumps have focused on residues involved in cation or ATP binding, or conformational transitions (3, 6). Cation occlusion sites are located within trans-membrane segments (8), and site-directed mutagenesis suggests that side chains within M4, M5, M6, and M8 ligate cations (6, 9–11). In addition, a variety of studies have defined the topological organization of the catalytic subunits, comprising 10 trans-membrane segments (see Refs. 3 and 7 and references therein).

The spatial organization of trans-membrane segments of the catalytic subunits is unknown although such information is essential for defining the structure of the cation transport path. Recently published structures of Ca-ATPase and H-ATPase (12, 13), at 8-Å resolution, show that the 10 trans-membrane segments are α-helices and most helices are tilted so that their spatial organization changes at different levels in the membrane. A tentative model of helix packing of Ca-ATPase has been proposed based on constraints of trans-membrane topology, site-directed mutagenesis, and disulfide cross-linking (12, 14, 15). Modeling of Na,K-ATPase has also been attempted based on hydrophobic labeling and prediction of helix orientation with respect to lipid (16). Despite these attempts, direct determination of helix proximity is largely lacking. Recently, covalent cross-linking experiments have begun to provide specific information (2, 15).

This paper describes experiments utilizing CuP¹-catalyzed S-S bridge formation. Older work (17–20) showed that treatment of detergent-solubilized Na,K-ATPase leads primarily to a 1:1 αβ cross-linked product. Identification of the cross-linked residues constitutes a formidable task for, whereas the β subunit has only one free cysteine, Cys⁴⁴, located within its single trans-membrane helix (21), the α subunit contains 23 cysteines. The problem could be simplified by using 19-kDa membranes which are obtained by extensive tryptic digestion of Na,K-ATPase. This preparation consists of fragments of the α subunit comprising mainly trans-membrane segments (M1/M2, M3/M4, M5/M6, and M7/M10) connected by the external loops, and a partially cleaved β subunit (8, 22). The fragments contain only 10 cysteines located within or next to trans-membrane segments.

A potential problem in cross-linking membrane proteins is the possibility of intermolecular cross-linking via random collisions in the membrane. Detergent solubilization can overcome this problem because the soluble protein can be diluted. Indeed Sarvazyan et al. (23, 24) have reported that treatment of digitonin-solubilized 19-kDa membranes with CuP yielded two cross-linked products, dimers of fragments containing M7/M10 and M1/M2 (22 kDa:11 kDa) and trimers containing these two fragments with the β subunit (~22 kDa:11 kDa) in 1:1 stoichiometries. These studies established that a cysteine residue within Asn⁸⁰¹-Tyr¹⁰¹⁶ of the α subunit (Cys⁹¹¹, Cys⁹³⁰, Cys⁹⁶⁴, or Cys⁹⁸³) is cross-linked to the β subunit (Cys⁴⁴), but did not identify the cysteine.

19-kDa membranes lack ATP-dependent functions, but re-

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1 The abbreviations used are: CuP, Cu²⁺-phenanthroline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; C₁₂E₁₀, polyoxyethylene 10-lauryl ether; PKA, protein kinase A.
tained cation occlusion and ouabain binding (8, 22, 25). Recently, we described a procedure for solubilizing 19-kDa membranes with the non-ionic detergent C12E10, which preserves intact the complex of fragments with occluded Rb ions and bound ouabain (1). The intact soluble complex contains one copy of each fragment. After solubilizing 19-kDa membranes in the absence of Rb ions and ouabain, neither Rb occlusion nor the complex of fragments are intact (1). Because cross-linking in the latter condition, as in Ref. 23, might reveal non-native interactions between fragments, one of our objectives has been to characterize cross-linking of the C12E10-solubilized intact complex of fragments containing occluded Rb ions. A second objective has been to identify cross-linked cysteines.

**EXPERIMENTAL PROCEDURES**

Na,K-ATPase was prepared from pig kidney red outer medulla (26) and stored at −80 °C. Protein and ATPase activity were determined as described (26). Specific activities were 13–18 units/mg of protein. Before use enzyme was thawed and dialyzed overnight at 4 °C against 1000 volumes of 25 mM histidine, 1 mM EDTA (Tris), pH 7.0. 19-kDa membranes were prepared by tryptic digestion of Na,K-ATPase with trypsin (22), and resuspended at 3 mg/ml in 2 mM RbCl, 25 mM imidazole, 1 mM EDTA, pH 7.5. The specific Rb occlusion capacity was 6 to 6.5 nmol/mg of protein, 19-kDa membranes were solubilized with C12E10 at a ratio of 2.2 (w/w) as described by Or et al. (1). Before cross-linking with CuP the pH was adjusted to 8.0 with RbOH (final concentration, 0.8 mM).

**Cross-linking Catalyzed by CuP—** The solubilized preparation (0.4 mg/ml) was treated at 20–22 °C with CuP (final concentration: 0.5/2.5 mM), added in 5 aliquots every 12 min. After 1 h solid urea was added to 2 M and the pH was readjusted to 8.0 with solid Tris base. Free cysteines were blocked by adding iodoacetamide to 40 mM and free Cu2+ was chelated with 10 mM EDTA (Tris). After 30 min at 20–22 °C the mixture was acidified to pH ~ 6.0 with acetic acid and protein was precipitated with 4 volumes of methanol/ether (2:1) and stored at −20 °C overnight. When analytical amounts were cross-linked, urea, Tris base, and acetic acid were omitted.

**Gel Electrophoresis—** Precipitated protein was collected by centrifugation at 9700 × g for 1 h at 4 °C, dried under a stream of nitrogen, and dissolved in loading buffer. Samples were resolved by Tricine-SDS-PAGE as in Refs. 22 and 27. Either 14-cm short or 23-cm long gels were used. In nonreducing conditions glutathione and mercaptoethanol were omitted from sample and running buffers.

**Purification of CuP-catalyzed Cross-linked Products—** Precipitated protein (15 mg) was dissolved in 2 ml of nonreducing sample buffer and resolved on two 1.5-mm thick long 10% Tricine gels, which were electroblotted onto PVDF (Semi-Phor TE70, Hoefer Scientific Instruments) (28) in 3.3 mM imidazole, 130 mM choline-Cl, pH 7.5, plus 5 mM EDTA and 45 mM choline-CI were treated with CuP. SDS, 19-kDa membranes in 3.3 mM imidazole, 130 mM EDTA, pH 7.5, plus 5 mM RbCl, 10 mM ouabain were denatured with 2% SDS prior to addition of C12E10 and CuP treatment.

**RESULTS**

**CuP-catalyzed Cross-linked Peptides—** Fig. 1 presents a representative experiment showing components of C12E10-solubilized 19-kDa membranes in reducing or nonreducing conditions and also cross-linked products after treatment with CuP. In reducing conditions (Red), untreated C12E10-solubilized 19-kDa membranes reveal the standard 19-kDa and smaller (8–12 kDa) fragments of the α subunit (the latter are not well

FIG. 1. Spontaneous and CuP-catalyzed cross-links detected in C12E10-solubilized 19-kDa membranes. Samples (140 μg) were resolved on a long 10% gel. Red and Non red-solubilized 19-kDa membranes were electrophoresed under reducing or nonreducing conditions, respectively, without prior CuP treatment. Sol, solubilized 19-kDa membranes treated with CuP; Mem, unsolubilized 19-kDa membranes (0.4 mg/ml) in 3.3 mM imidazole, 130 μM EDTA, pH 7.5, plus 5 mM RbCl and 45 mM choline-CI were treated with CuP. SDS, 19-kDa membranes in 3.3 mM imidazole, 130 mM EDTA, pH 7.5, plus 5 mM RbCl, 10 mM ouabain were denatured with 2% SDS prior to addition of C12E10 and CuP treatment.
resolved on this 10% gel), an intact β subunit or its ~50-kDa glycosylated and 16-kDa fragments (22). In nonreducing conditions (Non-red) one major new band appeared, 70–80 kDa, and the intensity of the 19-kDa fragment was less than in reducing conditions. The ~50- and 16-kDa fragments were not seen because they are internally cross-linked between Cys125 and Cys135. After treatment of C12E10-solubilized 19-kDa membranes with CuP (Sol), the intensity of the 70–80-kDa band rose and that of the 19-kDa peptide and β subunit decreased further (compare Sol with Non-Red), suggesting that this band contains both the 19-kDa peptide and β subunit. About 63% of the β subunit underwent cross-linking based on the weight ratio of the cross-linked product and remaining β subunit extracted from gels (~3.1, w/w), and on the composition of the cross-linked product (see Table I). Raising CuP concentration or incubation time did not improve the yield (not shown). CuP treatment of unsolubilized 19-kDa membranes, containing occluded Rb ions, did not produce the 70–80-kDa cross-linked band (Mem). After denaturation of 19-kDa membranes with SDS, CuP treatment did not produce the 70–80-kDa band (SDS), excluding the possibility that the cross-link is the product of a nonspecific association of its components after termination of the reaction. A small amount of a higher molecular weight band (Fig. 1, asterisk) was observed after C12E10 solubilization but the amount was not increased by CuP treatment. This band was also observed with intact 19-kDa membranes. It may represent the product of inter-molecular cross-linking between adjacent complexes, and therefore it was not investigated further. In conditions producing the 70–80-kDa band the remaining 19-kDa fragment ran a little faster than in the other conditions, implying that the peptide is more compact (compare Non-red and Sol with Mem, and SDS). This hints at the possibility of an internal cross-link (see also Table II).

![Fig. 2](image)

**Fig. 2.** CuP-catalyzed cross-links detected in intact 19-kDa membranes. 19-kDa membranes (0.5 mg/ml) were suspended in 25 mM imidazole, 1 mM EDTA, pH 7.5, without (−Rb, Th.in.) or with 30 mM RbCl (+Rb). Membranes were either kept on ice (−Rb) or were incubated at 37 °C for 25 min (+Rb, Th.in.). All samples were then treated with CuP as described under "Experimental Procedures," and then dissolved in 2% SDS and treated with 40 mM iodoacetamide and 10 mM EDTA for 30 min at room temperature. Protein was precipitated with 4 volumes of methanol. The samples were resolved on a 10% gel in nonreducing conditions, transferred to PVDF paper, and then probed with the anti-KETYY antibody.

![Table I](image)

**Table I**

| Fragment | Residue | Average yield |
|----------|---------|---------------|
| β Subunit | Ala5 | 32 |
| 50 kDa | Gly144 | 28 |
| 19 kDa | Asn31 | 9 |
| M1–M2 | Asp56 | 15 |
| N-terminal sequence | Average yield |
| Fragment a | Met573 | 12 |
| | Tyr | 11.7 |
| | Pro | 5.7 |
| | Leu | 9.6 |
| | Lys | 6.5 |
| | Pro | 8.5 |
| | Thr | 7.0 |
| Fragment b | Met573 | 10.3 |
| | Tyr | 2.4 |
| | Pro | 6.2 |

**Fig. 3.** Identification of cross-linked peptides following deglycosylation of the β subunit. Purified CuP cross-linked product was treated or not with N-glycosidase F (+ or −PNGase, respectively), resolved on a short 10% gel and electroblotted onto PVDF. Coom’s lanes were stained with Coomassie (A). Samples treated with PNGase were also probed with antibodies recognizing the 19-kDa fragment (anti-KETYY), the 16-kDa N-terminal fragment of the β subunit (anti-16 kDa), or the 11.7-kDa peptide containing M1/M2 (anti-M1/M2) (B).
The combined average yield of fragments containing M9 is 12.6 pmol/cycle while that of the fragment containing M10 is 11.7 pmol/cycle. These yields are measured against a background of about 1 pmol/cycle, i.e., with a possible error of about 10%. Thus, the experiment demonstrates that M9 and M10 are present in equimolar proportions, suggesting that Cys\textsuperscript{964} within M9 and Cys\textsuperscript{983} within M10 are cross-linked to each other. Sequencing of fragment b led to essentially the same result suggesting that it is a truncated version of fragment a (probably cleaved at Arg\textsuperscript{998} or Arg\textsuperscript{1003}).

The evidence for the internal cross-link Cys\textsuperscript{964}Cys\textsuperscript{983} in Table II excludes either residue as a partner of Cys\textsuperscript{44} of the β subunit, unless one makes an unlikely assumption that Cys\textsuperscript{44} can form S-S bridges with different cysteines in the 19-kDa peptide. Thus, one could predict that Cys\textsuperscript{44} is cross-linked to either Cys\textsuperscript{911} or Cys\textsuperscript{930} in M8. However, direct evidence was not obtained because we detected no cross-linked band containing sequences from both α and β subunits. A hypothesis to explain the paradox could be that the broad band (** in Fig. 4) contains cross-linked fragments of both α and β subunits, but the latter are also cross-linked by the internal S-S bridges to other glycosylated fragments of the β subunit. Sequencing of this band (** ) showed indeed that it consists of a heterogenous mixture of peptides which precludes simple interpretation (not shown).

The hypothesis just proposed was tested as follows (Figs. 5, 6, and Table III). The 70–80-kDa cross-linked product, or the 19-kDa fragment, were first incubated with protein kinase A and [γ-\textsuperscript{32P}]ATP, in order to phosphorylate Ser\textsuperscript{936} in the PKA site RRNS (32, 33), and the labeled proteins were then digested with chymotrypsin.\textsuperscript{2} The digestion products were resolved on a 16.5% gel, blotted onto PVDF paper, which was first autoradiographed (Fig. 5, \textsuperscript{32P}) and then also immunostained with an antibody recognizing residues Asn\textsuperscript{889}Gln\textsuperscript{903} in the loop between M7 and M8 (Fig. 5, L7–8). For greater ease of understanding, the scheme in Fig. 5 marks the positions of the Asn\textsuperscript{889}Gln\textsuperscript{903} epitope and the phosphorylation site of PKA in relation to the trans-membrane segments, M7-M10. In nonreducing conditions only broad, poorly defined bands of \textsuperscript{32P} labeled material (*) were observed (Non-red). However, in reducing conditions, a discreet \textsuperscript{32P} labeled band of 6.5 kDa appeared (Red). The same 6.5-kDa band appeared also in a chymotryptic digest of \textsuperscript{32P} labeled 19-kDa fragment resolved under reducing conditions (19 kDa). Thus the 6.5-kDa band is a fragment of the α subunit containing Ser\textsuperscript{906} which is cross-linked to the broad band of material in nonreducing conditions and is released by reduction of an S-S bridge. By contrast to the result with PKA phosphorylation of Ser\textsuperscript{936}, the anti-Asn\textsuperscript{889}Gln\textsuperscript{903} antibody recognized an 8.0-kDa chymotryptic fragment, the size of which was not affected by reduction (Fig. 5, L7–8). Thus, this fragment is not a cross-linked species and does not contain the PKA site at Ser\textsuperscript{936} (compare L7–8 with \textsuperscript{32P}).

In principle, the 6.5-kDa \textsuperscript{32P} labeled cross-linked fragment might extend forward from Ser\textsuperscript{936} and include M9 (containing Cys\textsuperscript{911} and Cys\textsuperscript{930}) or backward from Ser\textsuperscript{936} and include M8 (containing Cys\textsuperscript{911} and Cys\textsuperscript{930}). In order to distinguish between these possibilities we have sequenced the fragment, repeating the experiment of Fig. 5 with 50-fold more protein (Fig. 6). The CuP cross-linked product (0.3 mg) was labeled with \textsuperscript{32P} using PKA, digested with chymotrypsin, and protein was separated on a 16.5%/6% gel. Most of the digest was separated under reducing conditions (Red), but for comparison a portion was separated in nonreducing conditions (Non-red). Because a large amount of protein was loaded onto each lane, the labeled fragment could

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\textsuperscript{2} Chymotrypsin was used rather than trypsin in order to avoid cleavage before the PKA phosphorylation site.
not be expected to run as a sharp band even in the reducing conditions, as in Fig. 5, and indeed the autoradiograph in Fig. 6 (Red) showed a relatively broad band of labeled material in the region of 6–7 kDa. The broad labeled band was transferred to PVDF, and sequenced (Table III). A mixture of two sequences was found, N terminus Glu902 corresponding to a fragment containing M8 and N terminus Arg972 corresponding to a fragment containing M10, respectively. Only the former fragment contains Ser936, and hence the radioactive label. Thus, Figs. 5, 6, and Table III demonstrate that Cys 44 of the β subunit is cross-linked to either Cys 911 or Cys 930 in M8 (see “Discussion”).

**DISCUSSION**

**Cross-linked Fragments of 19-kDa Membranes**

CuP treatment of 19-kDa membranes solubilized with C12E10 in the presence of Rb ions and ouabain led to appearance of one major cross-linked band, which consists of a mixture of β:19-kDa dimers (~65%) and β:19 kDa:11.7-kDa trimers (~35%) (Figs. 1 and 3, Table I). These cross-links reflect proximities of fragments within the intact detergent-solubilized complex. The fact that cross-links were formed in detergent-solubilized but not in native 19-kDa membranes could imply either that the detergent induced a degree of rearrange-ment of trans-membrane segments which permit cross-links between the relevant cysteines or that cysteines embedded in lipid, such as Cys44 of the β subunit, are not reactive in native membranes due to insufficient exposure to oxygen and CuP, and only become exposed to oxygen or CuP after solubilization. However, the crucial point is that Rb occlusion is fully preserved in these C12E10-solubilized 19-kDa membranes (1). Therefore the organization of trans-membrane segments in the soluble complex of fragments, and proximities revealed by cross-linking (M8/Mβ, M9/M10), must be essentially similar to those in native 19-kDa membranes and Na,K-ATPase.

CuP treatment of digitonin-solubilized proteolyzed dog kidney Na,K-ATPase produces cross-linked fragments corresponding to a trimer of β:22 kDa:11 kDa (1:1:1) and, in lower amounts, a dimer of 22 kDa:11 kDa (23). Our study confirms the finding of a β:19 kDa:11.7-kDa trimer, although in lower yield than a β:19-kDa dimer, but a 19 kDa:11.7-kDa dimer was not observed (see Fig. 1). Presumably, the similarities and differences between the two sets of observations reflect the state of the soluble complex. Solubilization of pig kidney 19-kDa membranes with C12E10, in the absence of Rb ions and ouabain, leaves the β-M7/M10 pair tightly associated but the M5/M6 and M3/M4 fragments dissociate, while the M1/M2 fragment shows an intermediate degree of interaction with the β:19-kDa pair (1). Thus, solubilization of 19-kDa membranes by digitonin in the absence of Rb ions and ouabain (23) should not have preserved an intact complex. Tighter association of the 11-kDa fragment with the β:22-kDa pair could explain why

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**TABLE III**

| N-terminal sequence | Average yield pmol/cycle |
|---------------------|--------------------------|
| Arg972 Glu902       | 5.4                      |
| Met Tyr Pro Leu Lys Pro Thr | 4.1                  |

**Fig. 5.** Phosphorylation and chymotryptic digestion of the CuP-catalyzed cross-linked product. The CuP cross-linked product (10 μg, 0.25 mg/ml) was labeled with 15 μCi of [γ-32P]ATP using PKA (see “Experimental Procedures”). The protein was precipitated with methanol and then digested with chymotrypsin at 0.2 mg/ml for 1.5 h. The digest was divided and either reduced (Red) or not (Non-red). The 19-kDa fragment (2 μg, 0.1 mg/ml) was also labeled with [γ-32P]ATP, digested with chymotrypsin, and then reduced. Samples were resolved on a short 16.5% T, 6% C gel, and blotted onto PVDF. The sheet was autoradiographed using a Fuji BAS 1000 PhosphorImager (32Pi) and then immunostained with an antibody recognizing residues Asn889-Gln903 (L7–L8).

**19kDa fragment**
been obtained by utilizing PKA to label Ser 936 with $^{32}$Pi (32, 33). After chymotryptic digestion of the $^{32}$Pi-labeled cross-linked product (300 μg, 0.2 mg/ml) was labeled with 51 μCi of [γ-$^{32}$P]ATP by PKA, precipitated with methanol, and then digested at 1 mg/ml with chymotrypsin for 70 min. Samples (50 μg) were resolved on a long 16.5% T, 6% C gel under reducing (Red) or nonreducing conditions (Non-red). The gel was autoradiographed using a Fuji BAS 1000 PhosphorImager. The 6–7-kDa $^{32}$P-labeled band was transferred to PVDF and sequenced.

**Fig. 6. Preparation of the $^{32}$P-labeled chymotryptic fragment of the cross-linked product used for N-terminal sequencing.** The CuP cross-linked product (300 μg, 0.2 mg/ml) was labeled with $^{32}$Pi and isolated (33). After chymotryptic digestion of the $^{32}$Pi-labeled cross-linked product, reducing conditions released a 6.5-kDa $^{32}$Pi-labeled fragment, with N terminus Glu 902 (Figs. 5 and 6, Red; Table III). This 6.5-kDa fragment includes Cys911 and Cys930 in M8 and ends before Cys964 in M9. In nonreducing conditions the fragment is cross-linked to a mixture of partially digested glycosylated fragments of the β subunit, held together by internal S-S bridges (Figs. 4–6).

Proximity of M8 and Mβ is compatible with prior evidence that α and β subunits interact strongly at the extracellular surface primarily within the short sequence SYGQ outside M8 (34, 35). It is also compatible with our finding that o-phthalaldehyde cross-links α and β subunits near M8 (2). Although we cannot say which cysteine in M8 is cross-linked to the β subunit, Cys911 is a more likely candidate. A helical wheel representation of M8 (Fig. 7) reveals a sector of about 100° with short or non-hydrophobic side chains (Gly43, Cys44, Gly47, Gly51, Gly54, shaded boxes) near the extracellular surface. The nonhydrophobic sector could participate in a protein-protein interface with the remaining hydrophobic surface facing the lipid. Thus Mβ may contact M8, including Cys911, near the extracellular surface.

The cross-link between M7/M10 and M1/M2 fragments has not been identified. Based on topological considerations, Sarvazyan et al. (23) proposed that Cys983-Cys104 (M1-M10) or Cys138-Cys930 (M2-M8) are likely pairs. At first sight a Cys983-Cys104 cross-link appears incompatible with the Cys983-Cys964 internal cross-link (Table II). However, the observation of a mixture of β-M7/M10 dimers and β-M7/M10-M1/M2 trimers (Figs. 1 and 3 and Table I) leads to the following hypothesis. Assume, that M1, M9, and M10 are in proximity, so that S-S bridges can form either between Cys983 in M10 and Cys964 in M9 or between Cys984 in M1 and either Cys983 or Cys964. Then 19-kDa fragments containing the Cys983-Cys964 internal cross-link could not be cross-linked to the M1/M2 fragment via Cys983-Cys104 or Cys964-Cys104. Thus, the mutually exclusive formation of S-S bridges provides an economical explanation of the mixture of cross-linked product. By contrast, the assumption of a Cys138-Cys930 cross-link does not readily explain this observation. Sarvazyan et al. (23, 24) observed the β:22 kDa:11 kDa trimer with 1:1:1 stoichiometry and the 22 kDa:11-kDa dimer, but no β:22 kDa dimer. This could imply that the internal Cys964-Cys983 cross-link was not formed in their conditions.

**Spatial Organization of Membrane Spanning Helices of α and β Subunits**

Fig. 8 presents a tentative proposal for spatial organization of trans-membrane segments of Na,K-ATPase. The arrangement is based on direct and indirect evidence on helix proximity, indications that trans-membrane helices of class II P-type pumps are separated into domains, and the necessity for M1/M2, M3/M4, M5/M6, and M9/M10 to be paired due to their...
M4, M5, and M6—Site-directed mutagenesis indicates that these helices form the main cation occlusion sites in Ca-ATPase and Na,K-ATPase, supplying ligating groups to occluded cations (6, 11, 40, 41). Recent cross-linking studies on Ca-ATPase have shown directly that M4 and M6 interact intimately (15). Thus M4, M5, and M6 are drawn in triangular contact (as in Refs. 14 and 15).

M7, M8, and Mβ—Proximity of M7 to M5 is strongly implied by our recent finding of a cross-link between cytoplasmic segments just preceding M7 and M5 (2). M6 and M7 are connected by a fairly short cytoplasmic loop, and charged residues within the loop may form the entrance to occlusion sites in M4, M5, and M6 (42). Therefore M7 is also placed near M6. As argued above, M7 and M8 are unlikely to be widely separated and proximity of M8 to M4, M5 and M6, is suggested by mutations of Glu908 in Ca-ATPase (9, 43). Thus M7 and M8 are placed together. Mβ interacts with M8 as required by the CuP cross-linking (βCys44-Cys951 or perhaps βCys44-Cys930), and the evidence for interaction between β and α subunits near the entrance to M8 (35, 2). As seen in Fig. 7 a likely surface of interaction of Mβ occupies a sector of only 100° with the remaining hydrophobic sector facing the lipid. This is suggestive of the triangular contact in Fig. 8 with Mβ placed between M7 and M8.

M9 and M10—The M9/M10 pair are internally cross-linked through Cys964 and Cys983 (Table II). Helices M6 and M9 are connected by a fairly short cytoplasmic loop. Thus, M9 is placed near M8. M9 is also placed near cation sites, i.e. M4, M5, and M6, in order to explain cation-protected modification of Glu908 by dicyclohexylcarbodiimide (44), conformation-dependent changes in fluorescence of N-p(2-benzimidazolylphenyl)-maleimide, covalently attached to Cys964 (45), and chemical modification of Cys964 (46). A direct interaction of M10 with M5/M6 is suggested by chemical modification of Cys983 following dissociation of the M5/M6 hairpin from dog kidney 19-kDa membranes (46, 47) and there is evidence implying association of the M5/M6 and M9/M10 hairpins of H,K-ATPase (39).

M1, M2, and M3—The major constraints on the locations of these helices are the indications for the two domains and the necessity for M3 to be close to M4 and M2 to M1. We have proposed above that M1 is cross-linked either to M10 (Cys104, Cys930) or M9 (Cys104, Cys983) by an interaction of the M1-M10 interaction (Cys104-Cys983) depicted in Fig. 8 is a more likely possibility due to other constraints on the interactions of M9 referred to in the previous paragraph.

Comparison of Fig. 8 with the helix packing model of Ca-ATPase (12) shows a general similarity in positions of M1-M7 as well as a major difference in that M8, M9, and M10 are placed on opposite sides of the molecules. Conceivably the position of M7 relative to M8-M10 is affected by the β subunit which interacts strongly with the L7/8. However, it is equally likely that the uncertainty is due to lack of detailed information. Two structural constraints used in Ref. 12, namely proximity of M4-M6 based on S-S bridges (15) and orientation of nonconserved residues of trans-membrane helices toward the lipid, fit either arrangement. A third line of evidence involved placing helices identified as the cytoplasmic stalk, S2-S5, above M2-M5 in the middle of the molecule (12). Without comparable structural data for Na,K-ATPase it is not known whether this constraint applies. Specific predictions in Fig. 8, are that M4 and M6 are largely surrounded by neighboring helices rather than lipid (see Ref. 47), M1 is close to M10, and there is no proximity between M1/M2 and M7/M8. Clearly, it now becomes necessary to devise a means to test specific predictions and assumptions in order to refine the models.
Cross-linking of Proteolyzed Na,K-ATPase

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