

# Role of the self-association of $\beta$ subunits in the oligomeric structure of $\text{Na}^+/\text{K}^+$ -ATPase

Alexander V. IVANOV<sup>1</sup>, Nikolai N. MODYANOV and Amir ASKARI

Department of Pharmacology, Medical College of Ohio, 3035 Arlington Avenue, Toledo, OH 43614-5804, U.S.A.

The two subunits of  $\text{Na}^+/\text{K}^+$ -ATPase that are essential for function are  $\alpha$  and  $\beta$ . Previous cross-linking studies on the oligomeric structure of the membrane-bound enzyme identified  $\alpha,\beta$  and  $\alpha,\alpha$  associations, but only the former and not the latter could be detected after solubilization. To study the possibility of direct  $\beta,\beta$  association, the purified membrane enzyme and a trypsin-digested enzyme that occludes cations and contains an essentially intact  $\beta$  and fragments of  $\alpha$  were subjected to oxidative cross-linking in the presence of  $\text{Cu}^{2+}$ -phenanthroline. Resolution of products on polyacrylamide gels, N-terminal analysis and reactivity with anti- $\beta$  antibody showed that, in addition to previously identified products (e.g.  $\alpha,\alpha$  and  $\alpha,\beta$  dimers), a  $\beta,\beta$  dimer, most likely linked through intramembrane Cys<sup>44</sup> residues of two chains, is also formed. This dimer was also noted when digitonin-solubilized intact enzyme, and the trypsin-digested enzyme solubilized with digitonin or polyoxyethylene 10-lauryl-

ether were subjected to cross-linking, indicating that the detected  $\beta,\beta$  association was not due to random collisions. In the digested enzyme,  $\text{K}^+$  but not  $\text{Na}^+$  enhanced  $\beta,\beta$  dimer formation. The alternative cross-linking of  $\beta$ -Cys<sup>44</sup> to a Cys residue of a transmembrane  $\alpha$ -helix was antagonized specifically by  $\text{K}^+$  or  $\text{Na}^+$ . The findings (i) indicate the role of  $\beta,\beta$  association in maintaining the minimum oligomeric structure of  $(\alpha,\beta)_2$ , (ii) provide further support for conformation-dependent flexibilities of the spatial relations of the transmembrane helices of  $\alpha$  and  $\beta$  and (iii) suggest the possibility of significant differences between the quaternary structures of the P-type ATPases that do and do not contain a  $\beta$  subunit.

Key words:  $\text{C}_{12}\text{E}_{10}$  solubilization, cross-linking, *Cu-o*-phenanthroline, digitonin, subunit interaction.

## INTRODUCTION

$\text{Na}^+/\text{K}^+$ -ATPase catalyses the coupled active transport of  $\text{Na}^+$  and  $\text{K}^+$  across the plasma membrane of most eukaryotic cells [1,2]. Two subunits of the enzyme,  $\alpha$  and  $\beta$ , are essential for function. Some preparations also contain other subunits that may regulate function [3,4]. The 112 kDa  $\alpha$  subunit, often called the catalytic subunit, is homologous to the  $\text{Ca}^{2+}$ -ATPase of the sarcoplasmic reticulum whose 2.6 Å resolution structure was reported recently [5]. The  $\beta$  subunit, a 34 kDa protein that is highly glycosylated, has no counterpart in the  $\text{Ca}^{2+}$ -ATPase, but does so in some other members of the P-type ATPase family [2].

The quaternary structure-function relationship of  $\text{Na}^+/\text{K}^+$ -ATPase has been a subject of long-standing debates [1,6,7]. That the non-covalent  $\alpha,\beta$  heterodimer ( $\alpha,\beta$  dimer) is the minimum structural unit of the enzyme is generally accepted. It has not been possible to obtain a catalytically competent preparation of the  $\alpha$  subunit in the absence of the  $\beta$  subunit, and early studies [8,9] showed the presence of an  $\alpha,\beta$  complex with 1:1 stoichiometry in membrane-bound and detergent-solubilized preparations, establishing that the detected complex is not due to random collisions within the membrane. There is no unanimity, however, on the status of the higher association states of the  $\alpha,\beta$  dimer. Although some propose that the weight of evidence clearly indicates that the native enzyme of the membrane functions as an oligomer of the  $\alpha,\beta$  dimers [10–15], others disagree and consider the  $\alpha,\beta$  dimer as the structural and functional unit of the enzyme [9,16,17]. In previous studies on the interactions and the proximities of the  $\alpha,\beta$  dimers attention has been focused on contact between multiple  $\alpha$  subunits, perhaps

with the tacit assumption that if the dimers interact they must do so through the larger  $\alpha$  subunits. Here we present data indicating the presence of stable non-covalent  $\beta,\beta$  association in crude, purified and proteolytically cleaved enzyme preparations. The results show that this self-association, like the  $\alpha,\beta$  association, persists in detergent-solubilized preparations that retain partial catalytic activity or ion-occlusion capacity. The findings provide further support for the proposal that  $\text{Na}^+/\text{K}^+$ -ATPase functions as a co-operative oligomer of  $\alpha,\beta$  dimers.

## EXPERIMENTAL

Previously described procedures were used for the preparation of microsomes and the purification of  $\text{Na}^+/\text{K}^+$ -ATPase from the outer medulla of canine [18] or pig [19] kidney. The specific activity [18] of either preparation was at least 1100  $\mu\text{mol}$  of ATP hydrolysed/mg of protein per h. Both enzyme preparations were used in these studies, with nearly identical results. The preparation used in each specific experiment is identified in the relevant figure. The extensively trypsin-digested enzyme (often called 19 kDa membranes) was prepared by a slight modification [20] of the procedure of Karlsh et al. [21]. Native or digested enzyme preparations were solubilized in 0.3% digitonin as described before [20]. Solubilization of the digested enzyme with polyoxyethylene 10-lauryl ether ( $\text{C}_{12}\text{E}_{10}$ ) in the presence of  $\text{Rb}^+$  and ouabain was done according to the procedure of Or et al. [22].

For cross-linking experiments, any of the membrane-bound or solubilized preparations (0.75 mg of protein/ml) were incubated with 1.25 mM *o*-phenanthroline, 0.25 mM  $\text{CuSO}_4$  and 15 mM Tris/HCl (pH 7.4) for 15 min at 24 °C. It was established that

Abbreviations used:  $\text{C}_{12}\text{E}_{10}$ , polyoxyethylene 10-lauryl ether; CuP,  $\text{Cu}^{2+}$ -phenanthroline; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

<sup>1</sup> To whom correspondence should be addressed (e-mail aivanov@mco.edu).

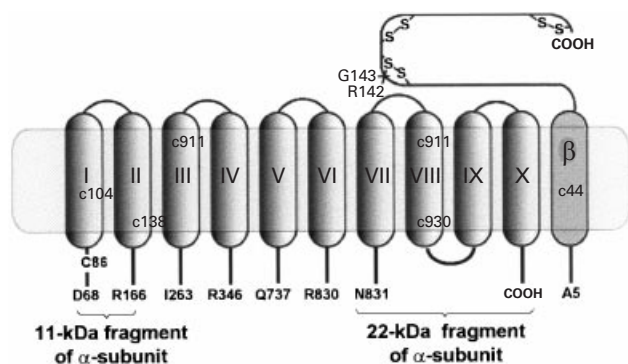
under these conditions the maximal level of cross-linking was obtained for the indicated experiments. Reactions were terminated by the addition of SDS to obtain a final concentration of 5%. Samples were then resolved on gels either immediately or after overnight freezing at  $-20^{\circ}\text{C}$ . Electrophoresis was done on Tricine  $\{N$ -[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}/SDS/polyacrylamide (10%) gels [20]. Unless indicated otherwise, reducing agents were omitted from the gel buffers to preserve the cross-linked products. Gels were stained with Coomassie Brilliant Blue. The presented Western blots were obtained [20] using an anti- $\beta_1$  subunit monoclonal antibody (provided by Dr M. Caplan, Yale University, New Haven, CT, U.S.A.), whose epitope has been determined to be within the C-terminal extracellular domain of the  $\beta$  subunit. The results of critical experiments on the native undigested enzyme using the above antibody were verified (results not shown) with the use of a polyclonal antibody against the N-terminal sequence of the  $\beta_1$  subunit, provided by Dr W. J. Ball (University of Cincinnati, Cincinnati, OH, U.S.A.). Appropriate alkaline phosphatase-conjugated secondary antibodies were used. Electroelution of the peptide bands and N-terminal analyses were done as described before [20]. Each presented stained gel or immunoblot is representative of at least three experiments. In the indicated experiments stained bands were quantitated by densitometry, and the presented curves were fitted with GraphPad software (Prism 3.0).

Digitonin,  $\text{C}_{12}\text{E}_{10}$ , trypsin (type XIII from bovine pancreas), trypsin inhibitor and other chemicals were obtained from Sigma. Canine kidney was obtained from Pel-Freeze Biologicals until the supply was recently discontinued.

## RESULTS

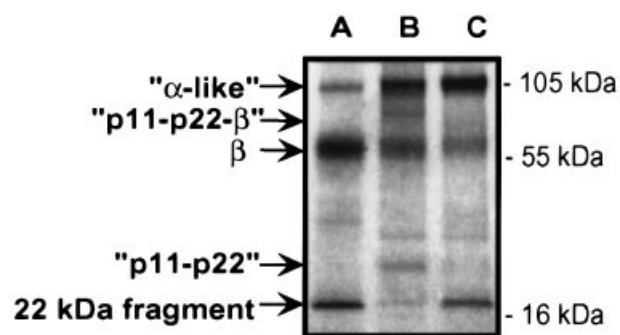
### $\beta_1\beta_1$ Interaction in the extensively trypsin-digested enzyme that retains cation-occlusion capacity

The possibility of detectable self-association of the  $\beta$  subunits was suggested in the course of experiments on this preparation, which contains an essentially intact  $\beta$  subunit, but in which large portions of the cytoplasmic domains of the  $\alpha$  subunit have been removed by proteolysis. Figure 1 is a schematic presentation of



**Figure 1** Schematic representation of an extensively trypsin-digested  $\text{Na}^+/\text{K}^+$ -ATPase that retains ion-occlusion capacity

The preparation contains an essentially intact  $\beta$  subunit (cleaved at  $\text{Lys}^4$ - $\text{Ala}^5$  and partly cleaved at  $\text{Arg}^{142}$ - $\text{Gly}^{143}$  [32–34]) with its one transmembrane helix, and the four indicated fragments of the  $\alpha$  subunit that retain the ten transmembrane helices of the subunit. The indicated N-termini have been determined experimentally, but most of the C-termini of the  $\alpha$  fragments have been estimated based on apparent molecular masses. The indicated cysteines have been implicated in cross-linking reactions of the intramembrane helices. See the Results section for other details.

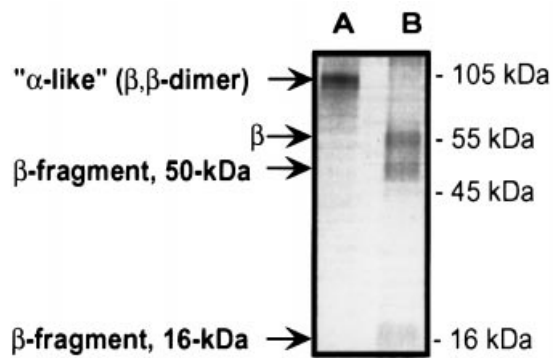


**Figure 2** Cross-linking reactions of the membrane-bound preparation of the extensively trypsin-digested  $\text{Na}^+/\text{K}^+$ -ATPase

The canine kidney preparation was cross-linked in the presence of  $\text{CuP}$ , resolved on 10% gels and stained as described in the Experimental section. Lane A, uncross-linked control (the pattern of this control was the same before and after 15 min of incubation at  $24^{\circ}\text{C}$ ). Lane B, after cross-linking in the absence of  $\text{K}^+$ . Lane C, a sample that was preincubated for 5 min at  $24^{\circ}\text{C}$  with 40 mM  $\text{KCl}$  before being cross-linked as in lane B. Locations of the molecular-mass standards are shown on the right. See the Results section for the identities of the bands designated on the left.

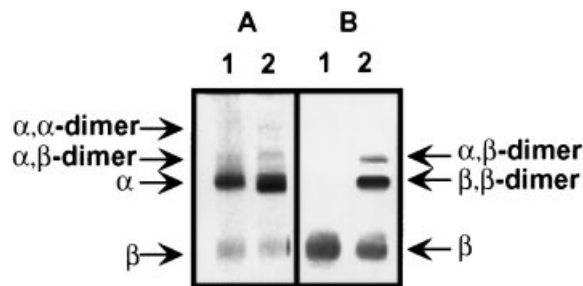
the peptide composition of this preparation, and Figure 2 (lane A) shows the composition of a representative preparation that was resolved on a non-reducing 10% Tricine/SDS gel and stained. As has been established before [20,23–25], the major components of this preparation are the slightly modified  $\beta$  subunit (cleaved at  $\text{Lys}^4$ - $\text{Ala}^5$  and partly cleaved at  $\text{Arg}^{142}$ - $\text{Gly}^{143}$ ) and four fragments of the  $\alpha$  subunit: an 11 kDa peptide containing the transmembrane helices  $\text{H}_1$  and  $\text{H}_2$ , a 9 kDa peptide containing  $\text{H}_3$  and  $\text{H}_4$ , a 10 kDa peptide containing  $\text{H}_5$  and  $\text{H}_6$  and a 22 kDa peptide containing  $\text{H}_7$ - $\text{H}_{10}$  (also referred as the 19 kDa peptide because of its mobility on SDS/PAGE; Figure 1). The preparation also contains a 6.5 kDa  $\gamma$  subunit, which is not shown in Figure 1. Note that in Figure 2 (lane A) the three smallest fragments of the  $\alpha$  subunit and the  $\gamma$  subunit are not shown. These were not well resolved on the bottom of this 10% gel, which was removed from Figure 2, but have been resolved on other gels [23–25]. Also note in Figure 2 (lane A) that above the prominent  $\beta$ -subunit band there is a less-intense band designated  $\alpha$ -like. Because this band, whose mobility is close to that of the  $\alpha$  subunit, was not observed in some preparations, and its amount varied in others, we assumed initially that it was a small fraction of the intact  $\alpha$  subunit that remained due to incomplete proteolysis.

In previous studies [20,25,26] we used the above trypsin-digested enzyme to explore the interactions of the transmembrane helices of the two subunits by chemical cross-linking experiments, and identified some of the cross-linked products. When the preparation shown in Figure 2 (lane A) was subjected to oxidative cross-linking in the presence of  $\text{Cu}^{2+}$ -phenanthroline ( $\text{CuP}$ ; Figure 2, lane B), in addition to the two products identified before (designated p11-p22 and p11-p22- $\beta$ ), we also noted a significant increase in the intensity of the  $\alpha$ -like band. Since this increase clearly could not be due to the intact  $\alpha$  subunit, we attempted to identify its source. Bands from several gels similar to those of Figure 2 (lane B) were electroeluted, combined and subjected to N-terminal sequence analysis. The only two identified sequences (AKEEGSXXKFI and GERKVVXRFKLE) were those of the peptides beginning at  $\text{Ala}^5$  and  $\text{Gly}^{143}$  of the  $\beta$  subunit, respectively. It has been established [23–25] that in these trypsin-digested preparations all  $\beta$  subunits are cleaved at



**Figure 3** Reduction of the cross-linked  $\beta,\beta$  dimer to the  $\beta$  monomer and its fragments

Bands similar to those designated  $\alpha$ -like in lane B of Figure 2 were removed, electroeluted and subjected to gel electrophoresis as indicated in the Experimental section either before (lane A) or after (lane B) reduction in 5% mercaptoethanol and brief immersion in a boiling-water bath. See the Results section regarding the identities of the bands.



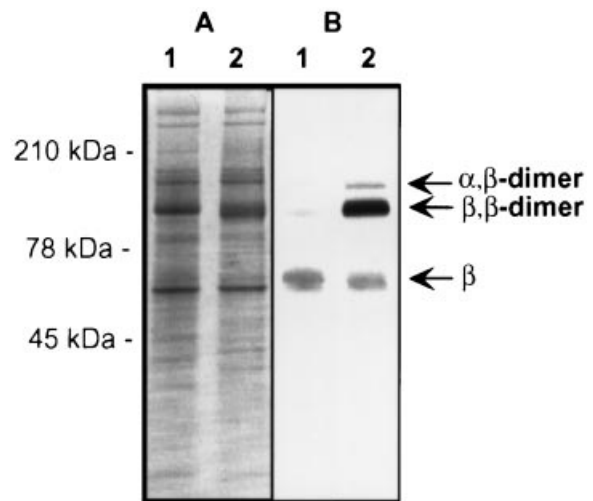
**Figure 4** Cross-linked products of the membrane-bound native  $\text{Na}^+/\text{K}^+$ -ATPase

The purified canine kidney enzyme was cross-linked, resolved on gels and either stained (A) or immunoblotted with an anti- $\beta$  antibody (B) as indicated in the Experimental section. Lanes 1, un-cross-linked control. Lanes 2, after cross-linking.

Lys<sup>4</sup>-Ala<sup>5</sup>. In addition, some are cleaved at Arg<sup>142</sup>-Gly<sup>143</sup>, which is between Cys<sup>125</sup> and Cys<sup>148</sup> that are connected by a disulphide bridge (Figure 1). Therefore, these data indicate that the  $\alpha$ -like band of Figure 2 (lane B) is a cross-linked  $\beta,\beta$  dimer. This was confirmed when the cross-linked product was subjected to electrophoresis under reducing conditions (Figure 3). As expected, the dimer was resolved to  $\beta$  monomers and two bands whose mobilities were consistent with those of the fragments resulting from Arg<sup>142</sup>-Gly<sup>143</sup> cleavage (Figure 3B). The above data also suggest that the small and variable amount of the  $\alpha$ -like band detected in some preparations before cross-linking (Figure 2, lane A) is most likely due to spontaneous oxidative dimerization of the  $\beta$  subunits, and not due to incomplete trypsinolysis of the  $\alpha$  subunit.

### $\beta,\beta$ Interaction in the native enzyme

To determine whether the  $\beta,\beta$  cross-linking noted above was a peculiarity of the trypsin-digested preparation, the purified undigested enzyme was exposed to CuP, subjected to gel electrophoresis, and either stained or immunoblotted with an antibody against the  $\beta$  subunit. The stained gels (Figure 4A) showed the formations of two cross-linked products with mobilities lower



**Figure 5** Formation of cross-linked  $\beta,\beta$  dimer in crude microsomes

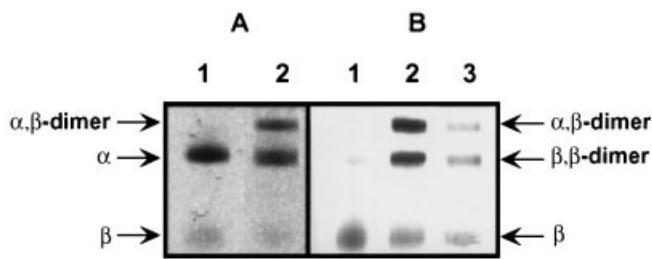
Pig kidney microsomes were cross-linked, resolved on gels and either immunoblotted with an anti- $\beta$  antibody (A) or stained (B) as in Figure 4. Lanes 1, un-cross-linked control. Lanes 2, cross-linked.

than that of the  $\alpha$  subunit. These products have been noted repeatedly in similar cross-linking experiments, and have been identified as the  $\alpha,\beta$  and  $\alpha,\alpha$  dimers [8,9,27,28]. The Western blots (Figure 4B) showed clearly the formation of the  $\beta,\beta$  dimer, and confirmed the formation of the  $\alpha,\beta$  dimer. Evidently, in the early cross-linking studies the formation of the  $\beta,\beta$  dimer was missed due to its co-migration with the intact  $\alpha$  subunit in the gel systems used, and because of the unavailability of the antibody against the  $\beta$  subunit.

In the experiments shown in Figure 5 crude kidney microsomes that were the starting material for the preparation of purified  $\text{Na}^+/\text{K}^+$ -ATPase were exposed to CuP, resolved on gels and stained (Figure 5A) or immunoblotted with the antibody against the  $\beta$  subunit (Figure 5B). The blots show the formation of two cross-linked products with mobilities corresponding to those of the  $\beta,\beta$  dimer and the  $\alpha,\beta$  dimer (Figure 5B, lane 2). That these are the only prominent cross-linked products of the  $\beta$  subunit in crude microsomes containing a multitude of proteins points to the specificity of the noted interactions, and argues against the possibility of the cross-linking reactions being the result of random collisions. Since the crude microsomes were prepared without the use of detergents, these findings also argue against the possibility that cross-linking reactions noted in the purified enzyme are due to the use of low SDS concentrations in the purification procedure, and the SDS-induced partial denaturation of the enzyme.

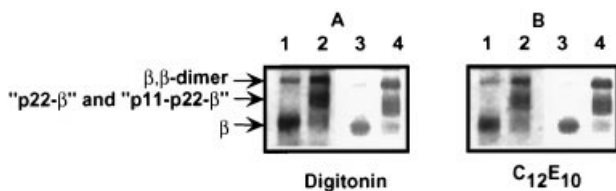
### $\beta,\beta$ Interactions in detergent-solubilized preparations

Because of the well-established difficulty of distinction between stable and collision complexes within a membrane [9,28–30], it was necessary to determine if  $\beta,\beta$  interaction could also be detected in detergent-solubilized preparations. Previous studies showed that solubilization of the native membrane-bound enzyme in digitonin prevents oxidative formation of the cross-linked  $\alpha,\alpha$  dimer but not that of the  $\alpha,\beta$  dimer [8]. In experiments of Figure 6, the purified membrane-bound enzyme was solubilized in digitonin, exposed to CuP, resolved on gels and either stained or immunoblotted. The stained gels (Figure 6A) showed the



**Figure 6** Cross-linked products of the digitonin-solubilized  $\text{Na}^+/\text{K}^+$ -ATPase

Purified pig kidney enzyme was cross-linked, resolved on gels and either stained (A) or immunoblotted with an anti- $\beta$  antibody (B) as in Figure 4. Lanes 1, uncross-linked control. Lanes 2, cross-linked. Lane 3, 10-times-diluted sample that was then cross-linked.



**Figure 7** Cross-linked products of the detergent-solubilized preparations of the extensively trypsin-digested  $\text{Na}^+/\text{K}^+$ -ATPase

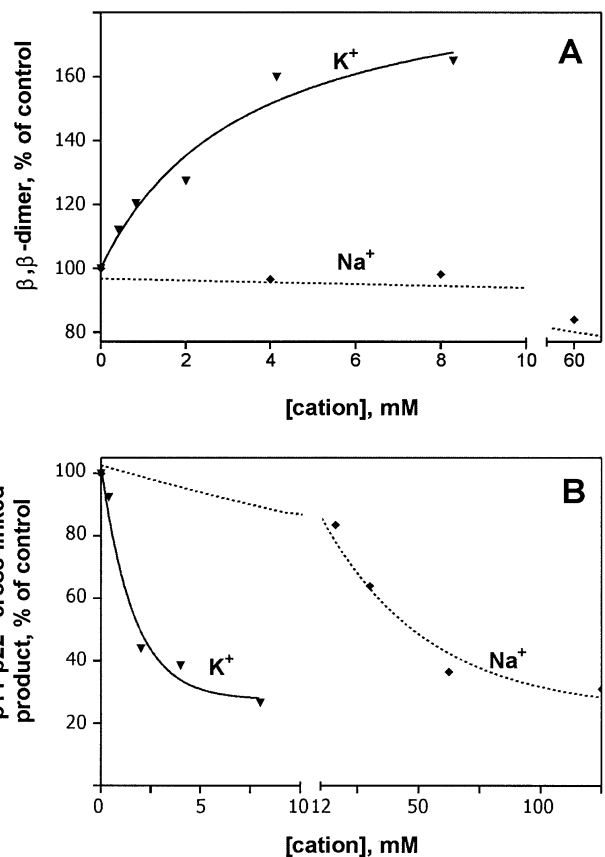
Digested preparations of the pig kidney enzyme were solubilized with either digitonin (A) or  $\text{C}_{12}\text{E}_{10}$  (B), cross-linked, resolved on gels and either stained (lanes 1 and 2) or immunoblotted with an anti- $\beta$  antibody (lanes 3 and 4) as described in the Experimental section. Lanes 1 and 3, uncross-linked controls. Lanes 2 and 4, cross-linked.

expected formation of the  $\alpha,\beta$  dimer, and the blots (Figure 6B) showed that in addition to the  $\alpha,\beta$  dimer a cross-linked  $\beta,\beta$  dimer with  $\alpha$ -like mobility was also formed. When the solubilized enzyme was diluted 5-fold or 10-fold in 0.3% digitonin and then exposed to CuP, the cross-linking pattern was the same as shown in Figure 6(B), lane 3. Clearly, the  $\beta,\beta$  association that is noted in the membrane-bound enzyme (Figure 4B) persists in the solubilized enzyme (Figure 6B) and does not dissociate upon further dilution.

To determine if the  $\beta,\beta$  association detected in the trypsin-digested enzyme (Figure 2) also persists in the detergent-solubilized state, digested preparations were solubilized in either digitonin or  $\text{C}_{12}\text{E}_{10}$ , and subjected to CuP cross-linking. The  $\text{C}_{12}\text{E}_{10}$ -solubilized preparation was used because it has been shown to retain ion-occlusion capacity [22]. Formation of the cross-linked  $\beta,\beta$  dimer in each solubilized preparation was evident (Figure 7), both in stained gels and immunoblots. As expected from previous studies [20,25,26,31], cross-linked products of the  $\beta$  subunit and fragments of the  $\alpha$  subunit were also noted (Figure 7).

#### Ligand sensitivity and yield of the $\beta$ subunit cross-linking

In previous studies on the extensively trypsin-digested preparation, we showed that several specific ligands of the enzyme regulated the CuP-induced cross-linking reactions involving the  $\alpha$ -subunit 22 kDa peptide fragment [25]. Similar experiments were done to examine the ligand sensitivity of the  $\beta,\beta$  interaction. A representative experiment is shown in Figure 2. Comparison of the cross-linking patterns obtained in the absence (Figure 2, lane B) and presence (Figure 2, lane C) of  $\text{K}^+$  show that (i) in



**Figure 8** Effects of varying concentrations of  $\text{Na}^+$  and  $\text{K}^+$  on the formation of the cross-linked  $\beta,\beta$  dimer (A) and p11-p22 dimer (B) in the extensively trypsin-digested  $\text{Na}^+/\text{K}^+$ -ATPase

The canine kidney preparation was cross-linked as in Figure 2, and the effects of varying cation concentrations were quantitated as indicated in the Experimental section.

agreement with previous observations [25] cross-linking of the 22 kDa  $\alpha$  fragment with other  $\alpha$  fragments and with the  $\beta$  subunit are antagonized by  $\text{K}^+$  and (ii) conversion of the  $\beta$  subunit to cross-linked  $\beta,\beta$  dimer is enhanced by  $\text{K}^+$ . When experiments similar to those in Figure 2 were done and the effects of varying  $\text{K}^+$  concentration on the formation of the cross-linked  $\beta,\beta$  dimer and the cross-linked p11-p22 were quantitated,  $\text{K}^+$  stimulated the former with a  $K_{0.5}$  value of approx. 3–5 mM (Figure 8A) and inhibited the latter with a  $K_{0.5}$  value of approx. 1–2 mM (Figure 8B). Significantly, when the effects of varying  $\text{Na}^+$  concentration were examined in similar experiments,  $\text{Na}^+$  had no stimulatory effect on  $\beta,\beta$  dimer formation (Figure 8A), but it inhibited the formation of p11-p22 with a  $K_{0.5}$  value of approx. 30 mM (Figure 8B). The similar qualitative effects of  $\text{Na}^+$  and  $\text{K}^+$ , but with different  $K_{0.5}$  values, on the formation of p11-p22 (Figure 8) are in agreement with the effects of the two ions in preventing the participation of the 22 kDa peptide in cross-linking reactions [25]. The data in Figure 8 demonstrate clearly the specificities of  $\text{Na}^+$  and  $\text{K}^+$  effects on  $\beta,\beta$  association and on interactions among the transmembrane helices of the  $\alpha$  subunit. A full account of the complex effects of the various ligands of the enzyme on  $\beta$  interactions (studies that are in progress) will be reported subsequently.

The incomplete cross-linking of the  $\alpha$  subunits (to each other or to the  $\beta$  subunit) to the maximal extent of about 50% has

been noted previously using different cross-linking reagents and conditions [32,33]. We cross-linked different preparations of either the purified native enzyme, as in Figure 4(A), or the extensively trypsin-digested enzyme, as in Figure 2, and quantitated the amount of the remaining  $\beta$  subunit after product formation had plateaued. This was about 40–60% of the total content of the  $\beta$  subunit prior to cross-linking.

## DISCUSSION

The findings presented here are pertinent to the issue of the oligomeric structure of the membrane-bound Na<sup>+</sup>/K<sup>+</sup>-ATPase; i.e. whether or not there are specific non-covalent associated states of the  $\alpha,\beta$  dimer that are not due to the random collisions of these structural units within the membrane. It has been implied that the resolution of this question may be unnecessary because studies on the enzyme that is solubilized in some non-ionic detergents have shown that the unassociated  $\alpha,\beta$  dimer has full catalytic and transport functions [9,16,34]. There is, however, ample evidence (reviewed in [14]) to suggest that in active solubilized preparations rapid association/dissociation of the  $\alpha,\beta$  dimers may be occurring during a cycle of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity; hence, the uncertainty about the ability of a free-standing  $\alpha,\beta$  dimer to have full activity remains. In view of this we suggest that it is necessary to clarify the nature of the associated states of the  $\alpha,\beta$  dimers, and how these associations are regulated by ligands, in membrane-bound preparations where nearly all studies on the reaction mechanism of the enzyme have been conducted. In turn, such information on the quaternary structure of the enzyme is necessary to clarify the unresolved complexities of the reaction mechanism [13,14]. It is in this context that the present demonstration of the  $\beta,\beta$  interaction, which supports the existence of higher associated states of the  $\alpha,\beta$  dimer, is relevant to the further understanding of this enzyme's function.

### The association state of the native enzyme

The early chemical cross-linking studies on Na<sup>+</sup>/K<sup>+</sup>-ATPase established that a non-covalent  $\alpha,\beta$  dimer is the minimum structural unit of the enzyme [8,9]. At the time, in a number of similar studies on the purified membrane-bound enzyme cross-linked  $\alpha,\alpha$  dimers were also obtained [8,9,27,28]. However, because the formation of these homodimers, but not that of the heterodimers, was prevented upon enzyme solubilization in mild detergents, reasonable suggestions were made that perhaps the  $\alpha,\alpha$  associations detected by cross-linking were due to random collisional events within the membrane [6,9,27,28]. Although this suggestion has not been supported by subsequent extensive cross-linking studies on ligand-regulated  $\alpha,\alpha$  interactions of the membrane-bound enzyme [7,14,28,32,33], there have been continued assertions that the higher homocomplexes of the  $\alpha$  subunit detected by cross-linking in the membrane-bound enzyme are non-specific and due to random collisions within the membrane phase. That such collisions may account for the formation of some cross-links in membrane preparations was realized long ago, but it was also recognized that the probability of these accidental events being responsible for a certain cross-linked product would be greatly diminished if the same product obtained in the membrane preparation could also be obtained in a detergent-solubilized preparation, where a large dilution is achieved by solubilization, and where a further test of dilution may be applied [29,30]. The present findings on the intact enzyme (Figures 4–6), showing that the self-association of the  $\beta$  subunits, like their hetero-association with the  $\alpha$  subunit, persists in the digitonin-solubilized enzyme and withstands the test of further

dilution (see the Results section), indicate that the minimum structural unit in both the membrane-bound and the solubilized states must be the dimer of the  $\alpha,\beta$  dimer, i.e.  $(\alpha,\beta)_2$ . If this is the case, why is it that the cross-linked  $\alpha,\alpha$  dimer is not obtained after solubilization? Since it is well recognized that the absence of cross-linking does not rule out the association of subunits in an oligomer [29,30], in light of the present findings we must conclude that while solubilization does not dissociate the minimum unit of  $(\alpha,\beta)_2$ , it alters the normal alignment of the residues at the  $\alpha,\alpha$  interface. This is in keeping with the known properties of the digitonin-solubilized enzyme. This preparation retains all the partial catalytic activities, albeit with different kinetic properties, but not the Na<sup>+</sup>/K<sup>+</sup>-dependent ATPase activity [8]. The loss of this activity, therefore, must be due to the changes at the  $\alpha,\alpha$  interface rather than the loss of  $(\alpha,\beta)_2$  quaternary structure. With remarkable insight, Liang and Winter [8] proposed this as a possibility without the benefit of having direct evidence for  $\beta,\beta$  association.

Previous studies on the phosphorylation-dependent formation of the cross-linked  $\alpha,\alpha$  dimer or the cross-linked  $\alpha,\beta$  dimer in the membrane-bound enzyme showed that only half of each subunit content participated in these reactions, suggesting the asymmetry of the  $\alpha,\beta$  oligomers [32,33]. The present findings suggest that with regard to  $\beta,\beta$  association, there is also asymmetry within the oligomer. When these findings from the cross-linking studies are considered along with a large body of other data that indicates negative interactions among the enzyme's  $\alpha,\beta$  dimers [13,14,35–37], it is evident that the minimum association state within the membrane must indeed be  $(\alpha,\beta)_4$ , as suggested before [7,32,33,38,39]. Further strong support for this is provided by the recent report of the presence of specific and stable  $\alpha$ -oligomers in the course of the unfolding of the enzyme [40].

### The association state of the extensively trypsin-digested enzyme

This preparation, which was developed and characterized in Karlsh's laboratory [21], has been invaluable in subsequent studies of their laboratory and others on the properties of the ion-occlusion pockets, and on the packing of the transmembrane helices of the enzyme [20,22–26]. As in the case of the undigested native enzyme, it has been suggested that cross-linking products of the membrane-bound state of this preparation, as opposed to those of the detergent-solubilized state, may be due to non-specific interactions [31]. The present demonstration of the cross-linked  $\beta,\beta$  dimer in both states of the preparation (Figures 2, 3 and 7) should remove these doubts. More importantly, the presence of  $\beta,\beta$  association in this preparation clearly questions the validity of the proposal [22] that this digested complex consists of a 'monomer' containing one copy of each fragment of the  $\alpha$  subunit, as well as the  $\beta$  and  $\gamma$  subunits. Because the  $\beta,\beta$  association that exists in the native oligomer persists after trypsinolysis, and since interaction of the  $\beta$  subunit with the  $\alpha$  fragments of this preparation has been noted [20,25,26,31], the following important question arises. What  $\alpha,\alpha$ -contact domains of the native  $(\alpha,\beta)_2$  are retained within the trypsin-digested preparation? Previous work [41] indicates that some  $\alpha,\alpha$  contact must occur within the large central loop that connects H<sub>4</sub> to H<sub>5</sub>. Although most of this loop is missing from the trypsin-digested preparation, the possibility of additional  $\alpha,\alpha$  contact through the transmembrane helices of two  $\alpha$  subunits cannot be ruled out at this time. Thus it remains to be determined if the ligand-regulated associations of the  $\alpha$  fragments, e.g. that between the 11 and 22 kDa fragments ([25] and Figures 1, 2 and 8), represent intra-subunit or inter-subunit interfaces.

### Characteristics of the $\beta,\beta$ -interface

Formation of the cross-linked  $\beta,\beta$  dimer is most likely due to a disulphide bridge between two transmembrane Cys<sup>44</sup> residues. This residue carries the only free thiol group of the subunit, and is also involved in the formation of the disulphide cross-link between the  $\beta$  subunit and one of the cysteine residues of the H<sub>8</sub> transmembrane domain of the  $\alpha$  subunit [26,31]. Such alternative helix interactions clearly indicate the flexibility of the spatial relations of the transmembrane domains, involving helix tilting, rotations or piston movements, as suggested for a variety of other helical membrane proteins [42].

The flexibilities of the  $\beta,\beta$  interface, and those of the other interfaces of the oligomer, are also emphasized by the ligand sensitivities and selectivities of the cross-linking reactions. Our previous studies on the membrane-bound preparations of the purified enzyme and several of its partially cleaved preparations demonstrated the ligand sensitivities of the cross-linking reactions between two intact  $\alpha$  subunits [28,32,33],  $\alpha$  and  $\beta$  subunits [33], fragments of the  $\alpha$  subunits [25,43] and fragments of the  $\alpha$  subunit and the  $\beta$  subunit [25]. The experiments in Figure 8 now add  $\beta,\beta$  interactions to the above list of the ligand-regulated associations, establishing the existence of conformation-dependent movements at intra-subunit and inter-subunit interfaces of the transmembrane helices of the oligomer.

The  $\beta$  subunits of the kidney enzymes used here contain two consecutive GXXXG motifs within the transmembrane sequence (Gly<sup>43</sup>–Gly<sup>47</sup>–Gly<sup>51</sup>). The GXXXG motif was first identified as an essential part of the interacting surfaces of the transmembrane helices of the glycophorin dimer, and was later shown to be present in the interacting transmembrane helices of a number of integral membrane proteins [42]. The presence of two such motifs in the kidney  $\beta$  subunits and all other  $\beta_1$  isoforms of Na<sup>+</sup>/K<sup>+</sup>-ATPase was noted recently, and suggested as a possibility for the  $\beta,\beta$  interface [44]. To see if this was indeed feasible, we modelled the dimer of the two transmembrane domains of the  $\beta$  subunit with the Swiss-PDB viewer program in the framework of the known three-dimensional structure of the dimeric transmembrane domain of glycophorin [45]. The models revealed that the two Cys<sup>44</sup> residues are closely positioned for disulphide-bond formation if the two Gly<sup>47</sup>–Gly<sup>51</sup> motifs are appropriately oriented for dimerization, but not if the Gly<sup>43</sup>–Gly<sup>47</sup> motifs are so oriented. The possible role of these motifs in the flexible interactions of the  $\beta$  subunits remains to be determined.

### Implications for the quaternary structures of the related P-type ATPases

Although it is reasonable to assume the existence of  $\beta,\beta$  association in all Na<sup>+</sup>/K<sup>+</sup>-ATPase complexes that contain the  $\beta_1$  isoform, because of the present uncertainty about the structural features of the  $\beta,\beta$  interface it is not possible to predict whether the complexes that contain other  $\beta$  isoforms are likely to exhibit  $\beta,\beta$  association. There is, however, ample evidence, including that from chemical cross-linking experiments, to indicate that  $(\alpha,\beta)_2$  is also the minimum structural unit of the gastric H<sup>+</sup>/K<sup>+</sup>-ATPase [46]. Finally, since there is a vast and controversial literature on the quaternary structure of the Ca<sup>2+</sup>-ATPase of sarcoplasmic reticulum [47,48], and because of the extensive structural similarities between Ca<sup>2+</sup>-ATPase and Na<sup>+</sup>/K<sup>+</sup>-ATPase [5,49,50], one is tempted to draw comparisons between the associated states of the two enzymes. We suggest that in any quaternary structural comparisons between the two enzymes, it is now appropriate to consider the inevitable differences that must be due to the presence of  $\beta,\beta$  interactions in one but not the

other. Whether these differences prove to be subtle or profound remains to be seen.

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