Thermal Denaturation of the Na,K-ATPase Provides Evidence for α-α Oligomeric Interaction and γ Subunit Association with the C-terminal Domain

Claudia Donnet, Elena Arystarkhova, and Kathleen J. Sweadner‡
From the Laboratory of Membrane Biology, Neuroscience Center, Massachusetts General Hospital, Charlestown, Massachusetts 02129

Thermal denaturation can help elucidate protein domain substructure. We previously showed that the Na,K-ATPase partially unfolded when heated to 55 °C (Arystarkhova, E., Gibbons, D. L., and Sweadner, K. J. (1995) J. Biol. Chem. 270, 8785–8796). The β subunit unfolded without leaving the membrane, but three transmembrane spans (M8-M10) and the C terminus of the α subunit were extruded, while the rest of α retained its normal topology with respect to the lipid bilayer. Here we investigated thermal denaturation further, with several salient results. First, trypsin sensitivity at both surfaces of α was increased, but not sensitivity to V8 protease, suggesting that the cytoplasmic domains and extruded domain were less tightly packed but still retained secondary structure. Second, thermal denaturation was accompanied by SDS-resistant aggregation of α subunits as dimers, trimers, and tetramers without β or γ subunits. This implies specific α-α contact. Third, the γ subunit, like the C-terminal spans of α, was selectively lost from the membrane. This suggests its association with M8-M10 rather than the more firmly anchored transmembrane spans. The picture that emerges is of a Na,K-ATPase complex of α, β, and γ subunits in which α can associate in assemblies as large as tetramers via its cytoplasmic domain, while β and γ subunits associate with α primarily in its C-terminal portion, which has a unique structure and thermal instability.

The Na,K-ATPase has two obligatory subunits, α and β (reviewed in Ref. 1). The α subunit (112 kDa) is a polytopic membrane protein homologous in structure to the Ca2+-ATPase of sarcoplasmic reticulum, which was recently determined to 2.6 Å (2). The β subunit (34 kDa) is a single-span membrane protein with a large extracellular portion that is glycosylated and stabilized by disulfide bonds (reviewed in Ref. 3). Both α and β are required for the assembly and function of the enzyme, and once assembled, it has not been possible to separate them without irreversible loss of activity. In the renal medulla the Na,K-ATPase is also associated with a 64–70 kDa single-span membrane protein known as the γ subunit (reviewed in Ref. 4). This protein is not present in all Na,K-ATPase preparations, and in fact it is not even present in all segments of the mammalian nephron (5). It has been demonstrated to act as a regulator of Na,K-ATPase properties, however, influencing affinity for both Na+ and K+ (5–7) and affecting conformation (reviewed in Ref. 4). Its association with α and β, when it occurs, is very stable, surviving extensive proteolytic degradation and detergent solubilization along with the ability to occlude K+ (8). Whether the Na,K-ATPase is normally a protomer (one copy of each subunit) or a dimer or tetramer of αβγ units in the membrane remains a controversial matter.

Thermal denaturation of the Na,K-ATPase has produced some surprising results. From the literature, thermal denaturation of membrane proteins differs from that of soluble proteins in one important respect. Those segments of a membrane protein that are embedded in the lipid bilayer, whether α helices or β sheets such as in porins, are normally much more stable to thermal denaturation than the extramembranous portions (reviewed in Ref. 9). This is not because the lipid bilayer provides superior stabilization or a greater heat sink, but because of the absence of water, which provides the competing H-bond donor and acceptor groups that are instrumental for unfolding these internally bonded structures (10). Consequently much higher temperatures are required to denature the membrane domain. In this context, the observation that relatively mild heating (50 °C in β-mercaptoethanol or 55 °C without) caused a radical reorganization of part of the transmembrane domain of the Na,K-ATPase α subunit while unfolding the β subunit ectodomain was surprising (11, 12). Here we have investigated further the consequences of heat denaturation of the Na,K-ATPase. Our observations on the degree of denaturation of other portions of the Na,K-ATPase α subunit are in line with the literature, but some unexpected observations were made that are pertinent to the tertiary and quaternary structure of the enzyme.

EXPERIMENTAL PROCEDURES

Enzyme Preparations

Pig and dog renal medulla Na,K-ATPase were purified by extraction of contaminating proteins with low concentrations of SDS followed by centrifugation on sucrose gradients by the method of Jørgensen (13). The purified enzyme, which remains membrane-embedded throughout the procedure, was stored at −70 °C in 250 mM sucrose, 30 mM histidine, 1 mM EDTA (pH 7.2). Final specific activities were 600–2,000 mol/min/mg of protein. Pig and dog α, differ by only 21 amino acid substitutions; the β subunit antibody used works better on dog, necessitating the use of that species in a few experiments.

Heating

Purified enzyme or crude microsomes were diluted to 0.5 mg of protein/ml in buffer containing 30 mM histidine, 1 mM EDTA, and 250 mM sucrose (pH 7.2 at room temperature) and incubated for 30 min at 55 °C in a thermal block incubator. Subsequently samples were kept on ice.

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ATPase Activity Assay

Na,K-ATPase activity was measured at 37 °C in media containing 3 mM Tris-ATP, 3 mM MgCl₂, 140 mM NaCl, 20 mM KCl, 30 mM histidine (pH 7.2 at room temperature). Na,K-ATPase activity is defined as the ouabain-sensitive difference in Pᵢ released per mg of protein per hour. Pᵢ release was measured colorimetrically (14).

Gel Electrophoresis

Gel electrophoresis was normally carried out with the buffer system of Laemmli on either 5 or 10% polyacrylamide gels (except for gradient 5–15% gels which were used to visualize the γ subunit of the Na,K-ATPase). Samples were reduced with 5% β-mercaptoethanol, and sample preparation was at room temperature. For determination of the molecular weights of the aggregates, a 3.5% polyacrylamide gel with 0.2% SDS was used with the Weber and Osborn buffer system (15). This was necessary because the high molecular weight standards (cross-linked phosphorylase B, Sigma) ran anomalously on Laemmli gels. A Tricine 1 peptide gel system was used in some experiments for resolution of the γ subunit (16).

Electrophoretic transfer to nitrocellulose was performed in a buffer containing 25 mM Tris, 170 mM glycine, 20% methanol, and 5% SDS at 100 mA overnight at 4 °C. Nitrocellulose was quenched with Tris, land). Mouse antibody 9A7, which recognizes the C terminus of the γ subunit, was the gift of Dr. Melitta Schachner, (ETH Zurich, Switzerland). Mouse antibody 9A7, which recognizes α₂, α₃, and α₁ subunits of the intracellular L2–3 loop, was obtained from Dr. Maureen McEnery, Case Western Reserve University (Cleveland, OH). Proteide-directed antibody ETYY against the α₂ subunit C terminus was the gift of Dr. Jack Kyte, University of California, San Diego. Antibody 8A, which is specific for the β₁ subunit, was obtained from Dr. Michael Caplan, Yale University Medical School (New Haven, CT). We thank all our colleagues for generous gifts of antibodies. Antibody RCT-G1, which recognizes the C terminus of the γ subunit, was described earlier (5).

Proteolysis

V8 Protease—Purified pig or dog enzyme (0.5 μg) was incubated with V8 protease (Sigma) (0.1 μg) in a buffer consisting of 30 mM histidine, 1 mM EDTA, and 250 mM sucrose, for 30 min at 37 °C. Digestion was stopped by diluting with 1 volume of 2 × electrophoresis sample buffer acidified with 0.3% trichloroacetic acid, which was effective at preventing digestion during gel electrophoresis. The acidification of the sample lasts only until it penetrates the gel, but it appears to promote irrevers-}

subunit after heating to 55 °C that we previously deduced from observations reported earlier (11). The experiments were performed in sealed rat medullary vesicles so that protein probes (protease, antibody, and kinase) had access only to the extracellular surface. M1-M7 remain anchored to the membrane, but M8-M10 unfolded into the extracellular space, exposing the C-terminal epitope (ETYY), the cytoplasmic protein kinase A site (PKA), and new tryptic cleavage sites (arrows). The protein kinase C site (FKC) and other known tryptic cleavage sites, T1, T2, T3 (17), T4 (64), and T19 (65), remained inaccessible in the vesicle.

Vesicles were incubated with TPCK-trypsin at a ratio of 1:3, trypsin: vesicle protein, for 30 min. The buffer was 250 mM sucrose, 25 mM MOPS (pH 7.4), 1 mM Tris-EDTA. In some experiments, vesicles were pre-equilibrated with 10 mM RbCl to verify the stabilization of structure by this K⁺ analog (data not shown). Proteolysis was at 37 °C, and the reaction was stopped by addition of acidified sample buffer. Samples were then directly loaded on SDS gels. Enzyme in open vesicles is extensively digested and does not contribute to the results (11).

RESULTS

Heat Denaturation of the Na,K-ATPase—Fig. 1A shows the predicted topology of the Na,K-ATPase α, β, and γ subunits based on the literature, and indicates the locations of the epitopes of each of the antibodies used in this study. Fig. 1B diagrams the extent of denaturation of the rat Na,K-ATPase α subunit after heating to 55 °C that we previously deduced from investigating the sensitivity to proteolysis at the extracellular surface in sealed right-side-out rat renal medulla vesicles (11). Normally the Na,K-ATPase is highly resistant to proteolysis at

1 The abbreviations used are: Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MOPS, 4-morpholinepropanesulfonic acid; TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone; SERCA, sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase.

2 C. Donnet and K. J. Sweadner, unpublished observations.
the extracellular surface except for two sites on the β subunit, which nonetheless stays intact because the fragments are connected and stabilized by disulfide bonds. After heating, the last three transmembrane spans of the α subunit, and the associated cytoplasmic segments, became accessible to extravascular trypsin (arrows). The protein kinase C phosphorylation site and several tryptic cleavage sites (T1, T2, T3, T4, and T19) remained inaccessible from the outside of the vesicles, indicating that the integrity of the vesicles was unaffected. Simultaneously, the β subunit extracellular domain became very sensitive to trypsin, but its cytoplasmic N terminus and transmembrane span remained protected by the vesicles. For simplicity, the diagram shows only the truncated portion of β that remained after proteolysis. After heating (and without digestion) the C terminus of the α subunit became accessible to an antibody (ETYY). Heating also caused the protein kinase A phosphorylation site, which is on the cytoplasmic surface of the α subunit between M8 and M9, to become accessible to kinase from the extracellular side. The presence of RhCl or KC1 protected against the partial denaturation of both α and β subunits. Similar observations of K⁺ protection and the extrusion of M8 and M9 were made by others with pig kidney Na,K-ATPase (12).

Some issues remained unaddressed by the experiments summarized in Fig. 1. One was whether the cytoplasmic domains were really as unperturbed as implied in the figure, and another was the fate of the γ subunit. In addition, we fortuitously observed SDS-resistant aggregation or cross-linking of the α subunits as a consequence of the heat denaturation event.

**Proteolytic Sensitivity of Heated Enzyme**—The experiments described first were performed with purified enzyme in open membrane fragments instead of right-side-out medullary vesicles so that we could assess the extent to which protease sensitivity increased in the cytoplasmic domains of the α subunit. An increase in sensitivity to trypsin after heating was readily demonstrated (Fig. 2). Normally only a few tryptic cleavage sites are exposed in the Na,K-ATPase, and the accessibility of the sites is influenced by whether NaCl or KCl is present, causing the enzyme to adopt E1 or E2 conformations. In E2 (KCl), cleavage occurs first at T4, then at T2 and T4, and secondarily at some other sites near those (Fig. 1). A concentration of trypsin and length of time were chosen that permit digestion at T1, T2, and T4, but not at T19. For these experiments the enzyme was heated in the absence of KCl (because KCl prevents the denaturation), but KCl was added prior to addition of the protease. Digestion produced the usual set of fragments from the unheated control, stained with antibodies against the N terminus (6F) and C terminus (ETYY). An antibody that we have mapped to the C-terminal half of the L4–5 loop, 9A7, stained the same set of fragments that were stained by ETYY. After heating, trypsin completely digested both the N-terminal and C-terminal epitopes, leaving blank lanes. Stain with the 9A7 antibody, however, revealed that there was a small amount of almost intact α subunit (which must have been cleaved at both ends) and some residual fragments of the same size as in the control. More than 90% of the stain was gone, however. The conclusion is that multiple additional tryptic cleavage sites were exposed.

**Irreversible Aggregation of α but Not β or γ**—It can be seen in...
aggregation, just as it prevented the physical changes illustrated in Fig. 1B.

It is difficult to accurately estimate the molecular weights of large proteins, and large cross-linked proteins are known to behave anomalously on Laemmli gels, so the Weber-Osborn buffer system was used on slabs of 3.5% polyacrylamide. Fig. 5 shows the migration of α as monomers, dimers, trimers, and tetramers after heating, as identified by mobility calibrated with cross-linked phosphorylase b (Sigma) in the high molecular weight range. In this gel system, larger aggregates were not observed, suggesting that they might have been Laemmli gel artifacts.

The next figure makes two important points. First, heating produced the same aggregates in crude kidney microsome preparations (Fig. 6B) as it did in purified enzyme (Fig. 6A), again detected with two different Na,K-ATPase-specific antibodies. The microsomes were isolated by differential centrifugation alone, and were not treated with SDS like the purified enzyme. This makes it much less likely that the aggregation is a trivial consequence of removing other membrane-associated proteins. Second, the SDS-resistant aggregates contained no detectable β subunit. Since α and β are normally inseparable, this indicates that the aggregation was the result of specific interaction between α subunits, not simply the production of a physical state like cooked egg. Nor did β subunits aggregate with one another in an SDS-resistant form. The same was true for the γ subunit, as illustrated in Fig. 7. In this case the samples were electrophoresed in parallel on 5% polyacrylamide gels to resolve the aggregates of α (Fig. 7A) and on 5–15% gradient gels to resolve the γ doublet, which is only 7.5 kDa (Fig. 7B). It can be seen, however, that no higher molecular weight forms of γ were detected in either type of gel. The same result was obtained with Tricine gels (not shown). In summary, the SDS-resistant aggregation or cross-linking event is clearly very selective, since it creates multimers of the α subunit and excludes the two proteins that have the most intimate association with α.

**Fate of the γ Subunit in Right-side-out Vesicles—**Our previous work on heat denaturation in right-side-out rat renal vesicles indicated that a portion of the α subunit from M8 to the C terminus was extruded from the membrane into the extracellular space (Fig. 1B). The N terminus of the β subunit, which is on the cytoplasmic surface, did not become accessible to protease. The γ subunit (in all the species that have been sequenced) has potential trypsic cleavage sites on both sides of the membrane. The extracellular site, which removes the alternatively spliced N terminus and a few more amino acids, is accessible in right-side-out vesicles (6, 22). We too previously detected cleavage in right-side-out vesicles by a shift in the gel mobility of the γ subunit (5). Our antibody to γ, RCT-G1, binds to an epitope at the C terminus, and there are several potential trypsic cleavage sites that would destroy or remove the epitope if trypsin had access to the C-terminal end. This sets the stage to test the hypothesis that during heating the γ subunit remains anchored in the membrane (like the β subunit) or comes out (like M8, M9, and M10 of the α subunit).

Aggregation of the vesicle α subunit was promoted by heating just as it was in purified enzyme and crude microsomes, and the ETYY epitope on aggregated α was very sensitive to digestion from the outside of the vesicles (Fig. 8A). In the present experiments, some Na,K-ATPase α subunit remained completely undigested (20–30% as determined by densitometry), but we did not detect any ETYY-stained fragments, consistent with extensive digestion of the majority of the C terminus that was extruded to the extracellular surface (Fig. 8A). Goldshleger et al. (12) reported that while M8 and M9 left the

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**Fig. 3. Heating does not increase sensitivity to V8 protease.** Purified dog kidney enzyme was heated as described in the legend to Fig. 2. Control and heated samples were incubated with protease for 30 min where indicated, and then prepared for electrophoresis either in the usual way, or with addition of trichloroacetic acid to acidify the samples just before electrophoresis. Each panel shows stain by a different antibody, ETYY against the C terminus, 2F12 against the L2–3 loop, and 8A against the β subunit. There was no detectable digestion of α or aggregates by V8 protease, although digestion of β was enhanced by heating. The gels were of 10% polyacrylamide.

Figs. 2 and 3 that some high molecular weight aggregates of the α subunit were present in the heated samples. There was also some aggregation seen in the trichloroacetic acid-acidified controls in Fig. 3, and it is notable that the aggregates were stable to such harsh acidic detergent conditions. In the next experiments, we used more porous polyacrylamide gels to investigate the structure and composition of the heat-induced aggregates.

Fig. 4 illustrates the basic observation that Na,K-ATPase heated in the absence of KCl forms a series of SDS-resistant aggregates that can be resolved on 5% polyacrylamide. It was frequently observed that a very small amount of similar bands was found in unheated preparations, which suggests that heating was accelerating a process that occurred to a small but detectable extent during normal sample manipulation. In this experiment 4–5 extra bands were resolved, but the extent of aggregation varied between experiments, as will be seen in other figures. Two different α-subunit-specific antibodies were used to identify the high molecular weight bands, one that binds in the N-terminal half and the other at the C terminus. The presence of 15 mM KCl (or RbCl, not shown) abolished the
membrane, at least some of M10 and the C terminus retained their normal topology because proteolytic fragments stained with ETYY could be detected after digestion from the extracellular surface. Minor differences in protocol probably account for the difference between laboratories, since we performed the digestion at 37 °C instead of room temperature, and this may have resulted in better exposure of tryptic sites near ETYY in a molten globule-like physical state in the extracellular space.

In parallel we examined the trypsin sensitivity of the γ subunit after heating right-side-out pig kidney vesicles (Fig. 8B). First, it can be seen that the γ doublet (γ-a and γ-b) was reduced to a single band in the control (unheated) vesicles, consistent with digestion at a lysine in the extracellular space, (K)GDVD for pig γ (25). In our previous work we used 16% polyacrylamide gels and the faster-migrating digested product was better resolved (5); here on a 12% gel it ran only slightly faster than the γ-b band. In the experiment shown, a 74% loss of the subunits α C-terminal epitope (ETYY) was accompanied by a loss of 68% of the γ subunits C-terminal stain, as determined by densitometry (compare the 2nd and 4th lanes). This indicates that γ, like M8-M10, is extruded from the membrane, allowing the epitope at the C terminus to be exposed to trypsin in the extravesicular space. In experiments performed with rat renal medulla right-side-out vesicles, almost all of the γ subunit epitope was digested (data not shown). We also confirmed with both pig and rat vesicles that the cytoplasmic N terminus of the β subunit, and its transmembrane span, were not digested and remained with the vesicles (data not shown).

**DISCUSSION**

**A Framework for Thinking About the Na,K-ATPase Transmembrane Domain**—Fig. 9A uses the Ca^{2+}-ATPase structure to illustrate the probable organization of the Na,K-ATPase membrane domain. The spans that are extruded during heat denaturation, M8, M9, and M10, are grouped in the Ca^{2+}-ATPase at the edge of the membrane domain, and highlighted in yellow. The view is from the extracellular surface. The Na,K-ATPase has been known to be homologous to the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) ever since the cDNAs were sequenced, but whether they share the same topology in the C-terminal membrane domain has been controversial. Now that the Ca^{2+}-ATPase structure is known (2), it is clear that there are many polar amino acids in the membrane, and discrepancies between hydropathy plots are no longer a

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**Fig. 4. Aggregates of Na,K-ATPase α subunit stable to gel electrophoresis.** Purified pig kidney Na,K-ATPase was heated for 30 min at 55 °C with or without KCl, and prepared for electrophoresis on Laemmli gels of 5% polyacrylamide. Higher molecular weight bands were seen that stained with two different Na,K-ATPase-specific antibodies, and these bands were much more pronounced when heating was performed in the absence of KCl.

**Fig. 5. Formation of dimers, trimers, and tetramers.** Pig kidney Na,K-ATPase was heated without KCl as described in the legend to Fig. 4, but electrophoresed on Weber-Osborn gels with three kinds of molecular weight markers. A shows a blot stained with anti-ETYY antibody against α, and B the plot of electrophoretic mobility versus molecular weight of the standards. The standards were Bio-Rad prestained, high molecular weight range; Amersham Pharmacia Biotech rainbow prestained, high molecular weight range, and Sigma cross-linked phosphorylase b. The latter is unreliable on Laemmli gels. The mobilities of the Na,K-ATPase bands are consistent with being multiples of 110 kDa.
strong argument that the Ca$^{2+}$-ATPase and Na,K-ATPase structures should be different. In a gapped-BLAST alignment of the Na,K-ATPase $\alpha$ subunit with SERCA1a, there is significant homology in all major domains (other than a few deletions and insertions), including in the C-terminal end and the connecting loops and tail. Thus the gapped-BLAST alignment is consistent with the adoption of the same fold. Homologous C-terminal topology for the individual transmembrane hairpins of Na,K-ATPase is now supported by biochemical evidence (12, 24). Cross-linking data suggests proximity of M1-M2 to M8–10 in the Na,K-ATPase (25, 26), and this can be accommodated in the model by the fact that M2 is adjacent to M9, and both have cysteine residues that could be the cause of the observed cross-link. (The appearance in Fig. 9A that M4b separates M2 from M9 is misleading, because that portion of M4 is actually in the cytoplasm, below the plane of the membrane-embedded segments.) In addition, there is a cross-link between M9 and M10 in the Na,K-ATPase that could also be accommodated by this model. The figure shows predicted positions of the $\beta$ and $\gamma$ subunit transmembrane spans, based on data discussed below.

**Insights from Thermal Denaturation**—Heat denaturation of the intact Na,K-ATPase probably occurs in stages. According to the data of Goldshleger et al. (12), heating at 45 °C with 1-butanol and $\beta$-mercaptoethanol exposes the extracellular L7–8 and L9–10 loops to digestion. Heating at 50 °C with dithiothreitol or at 55 °C without it next exposes the intracellular L8–9 loop at the extracellular surface (11, 12). We also observed (here and earlier) that the C terminus was exposed to the extracellular side, and since Goldshleger et al. (12) did not, the extrusion of M10 and the C terminus might be the last step in the denaturation of the C-terminal portion of the protein. In this study we obtained evidence that the cytoplasmic domains of the $\alpha$ subunit are also perturbed to the extent that they become much more sensitive to digestion by trypsin. Denaturation is unlikely to take the form of complete unfolding, however, because of the continued resistance of the cytoplasmic domains to digestion by *Staphylococcus* V8 protease on both sides of the membrane.

Instability of the organization of the C-terminal part of the transmembrane domain of P-type ATPases has now been observed in several contexts. Exposure of the L9–10 loop on the cytoplasmic side of the membrane was observed in Na,K-ATPase (12), H,K-ATPase (27), and Ca$^{2+}$-ATPase (28) after various experimental manipulations, although it is unambiguously luminal in the SERCA1a crystal structure. Exposure of the L7–8 loop on the cytoplasmic side of the membrane has been observed in Ca$^{2+}$-ATPase after low concentrations of a nondenaturating detergent-like C$_{12}$E$_{6}$ and protease K treatment (29), and it too is luminal in the crystal. M7, M8, M9, and M10 apparently leave the membrane to the cytoplasmic side principally after proteolytic cleavage of cytoplasmic loops (12, 27, 28). Association with the Na,K-ATPase $\beta$ subunit may favor loss to the extracellular surface when proteolytic cleavage is not a factor (11, 12).

Calorimetry has been performed on H,K-ATPase and Na,K-ATPase, but the thermal transitions that have been observed...
are not easy to reconcile with the structural changes that were observed here with protease sensitivity and changes in the exposure of defined sites. Gasset et al. (30) observed two peaks of heat capacity at 53.9 and 61.8 °C in gastric H,K-ATPase. We would have predicted that the heat transition at 53.9 °C corresponds to the physical changes we observed at 55 °C, but protection by K⁺ confounds this interpretation. It is well established that K⁺ stabilizes the Na,K-ATPase to heat (31), and it specifically increases the protease resistance of the C-terminal end of H,K-ATPase as well as Na,K-ATPase (32, 33). Of the two peaks of heat capacity detected in intact gastric H,K-ATPase, the lower temperature transition (53.9 °C) occurred in the presence or absence of K⁺, while the higher temperature transition (61.8 °C) disappeared in K⁺. This would suggest that the 61.8 °C transition represents the denaturation event described here at a significantly lower temperature. Gasset et al. (30) also observed no transitions in enzyme digested at the cytoplasmic surface with proteinase K, up to a temperature of 80 °C. The interpretation was that the observed heat capacity signals were derived from denaturation events occurring in the cytoplasmic loops of the α subunit, which is consistent with the concept that secondary and tertiary structures that are embedded in the membrane have very high stability (9). It was not ruled out, however, that portions of the membrane domain became so unstable after protease K that they were already denatured at a lower temperature, or even digested away. It is also puzzling that no transition was seen for the β subunit.

Somewhat different results were reported for thermal transitions in dogfish shark Na,K-ATPase (34). They saw denaturation as measured by a shift in amide I spectrum in Fourier transform infrared spectroscopy commencing at 58 °C in intact enzyme. Enzyme that had been extensively trypsinned in RbCl (19-kDa membranes) had a transition at 57 °C, while enzyme trypsinned in NaCl had a biphasic response at 57 and 84 °C. Again, no unique K⁺-protected transition was seen near 55 °C.

Protection by K⁺ probably has its basis in large conformation changes. K⁺ induces the E2 conformation in the Na,K-ATPase. In the Neurospora proton pump, a highly homologous P-type ATPase, it has been shown that about 175 residues are shielded from solvent proton exchange in the E2 conformation (35). This is physical evidence for a more compact, less exposed structure in E2. Comparison of two SERCA structures and a Neurospora enzyme structure gives shape to this concept, in that the 8-Å Ca²⁺-ATPase structure crystalized in decavanadate has a compact “head” for the cytoplasmic domain, while the 2.6-Å structure, crystalized in Ca²⁺, has three spread-out A-, P-, and N-domains (2, 36). The Neurospora structure ap-
pears to be similar (37). We infer that an “open” conformation is more labile to heat and more susceptible to aggregation.

**Oligomers of Na,K-ATPase**—It has been demonstrated that αβ particles (protomers) are capable of hydrolyzing ATP and of carrying out active ion transport (38–40), but there is much evidence that suggests that the enzyme normally associates as dimers (αβ), or as tetramers (αβ)4. This was initially proposed as a result of cross-linking experiments (41–43), but is supported with many other kinds of evidence as well: by saturation transfer EPR (44) and fluorescence energy transfer between subunits (45, 46), gel filtration (47), co-precipitation (48), kinetic evidence that could be best explained by the interaction of active sites (49), by measurements of the binding of different nucleotide analogs that suggest the existence of as many as four nonidentical sites (50), and by electron microscopy of solubilized particles (50). With electron microscopy of two-dimensional membrane crystals, Na,K-ATPase units (αβ) have been observed in both monomeric and dimeric associations (51, 52).

Prior physical evidence for association of Na,K-ATPase into dimers or tetramers has been received cautiously because of the possibility that the association was secondary to experimental manipulation: partial inactivity, nonspecific aggregation in detergents, or the slow capture of freely diffusing units by chemical cross-linking. The heat denaturation of membrane proteins can result in aggregation into complexes that are stable to SDS, and thus migrate at higher apparent molecular weights in SDS gels (53). With this approach we found evidence that the enzyme normally associates as a tetramer through specific interactions between its α subunits. While β and γ subunits may participate in quaternary interactions and even form SDS-sensitive aggregates, they did not participate in formation of SDS-resistant multimers (Fig. 9B).

Stable aggregation presumably followed more conventional denaturation. Reversible association of Na,K-ATPase α subunits as a consequence of heating has been reported previously, when enzyme was first heat-denatured and then solubilized in the detergent C12E8 and sedimented in an analytical centrifuge (31). Those aggregates dissociated in SDS for gel electrophoresis, however. It is possible that such aggregation occurred here as an intermediate step in the formation of SDS-resistant aggregates, and we observed that enzyme activity was lost significantly faster than SDS-resistant aggregates were formed (data not shown). That the aggregates proved to be more tryptophan-sensitive than unaggregated material in the same samples also implied that denaturation preceded aggregation and that not all of the α was equally denatured at the time point used. Aggregation of detergent-solubilized Na,K-ATPase units has also been reported (31, 54), but is unlikely to be related to the events observed here. The formation of anomalously-migrating α subunit forms after heating Na,K-ATPase in SDS sample buffer (55) is also not related to the observations reported here.

The aggregates were stable to SDS, even at pH 2. Spontaneous disulfide-mediated cross-linking of Na,K-ATPase α subunit and fragments of α has been observed (26), but the aggregates studied here were resistant to reducing agents and unlikely to be due to disulfide bonds. Slower migrating species eventually formed, but it is not clear whether they were higher-order aggregates or internally aggregated forms with reduced electrophoretic mobility. We did not observe aggregates so large that they failed to enter the gel.

**Predicted Transmembrane Organization of Na,K-ATPase Protomers (αβγ)**—In Fig. 9A we show a model of the predicted organization of α, β, and γ transmembrane spans based on the known structure of the SERCA1a Ca2+ -ATPase (2) and on the behavior of the β and γ transmembrane spans during heat denaturation. In the figure, γ’s span is shown associated with M8-M10. The γ subunit is closely enough associated with α to be labeled by cardiac glycoside derivatives with reactive groups (reviewed in Ref. 56). The fact that the γ subunit, like the C-terminal segment of α, was selectively lost from the membrane upon heating could be interpreted two ways. As an optional regulatory subunit, γ might not be tightly bound to the Na,K-ATPase, and it could be hypothesized that its membrane association is spontaneously labile to heat. The highly hydrophobic nature of γ’s membrane span (57), however, makes it unlikely that it would spontaneously leave the membrane in mild heat unless it were bound to something more soluble. The γ subunit has been observed to dissociate from extensively digested Na,K-ATPase destabilized by calcium (8), but the centrally located M5-M6 hairpin of α was also dissociated under those and similar conditions (58, 59), and conditions have been observed in which the M5-M6 hairpin was lost but not the γ subunit (60). Such disruption must reflect major disintegration of the complex of membrane spans. Further evidence that the loss of γ is linked to α structure is that it is prevented by K+, just as α and β denaturation is prevented. The data thus suggest that γ is associated with M5-M10 rather than other more firmly anchored transmembrane spans.

β’s span is shown associated with M7 because M7 is close to the known extracellular α-β association site in the L7–L8 loop (61, 62), and because β and M7 remain anchored during heat denaturation. Since the β subunits extracellular domain denatures but its transmembrane and cytoplasmic domains do not leave the membrane with M8-M10, it must be associated with transmembrane spans that remain in the membrane or with a cytoplasmic domain of α. M7 does not have a cysteine residue available for oxidative cross-linking, but β has been shown to form a disulfide cross-link to M8 (25, 26, 63). As pointed out by the authors, however, this occurred preferentially after treatment by low molecular weight alcohols or extensive digestion, not in native enzyme. In the Ca2+-ATPase structure, M8 is more buried than M7. Consequently it appears likely that β’s proximity is primarily to M7 and secondarily to M8 in the Na,K-ATPase structure. This is a refinement of the position of β proposed by Or et al. (26) and Ivanov et al. (63) based on cross-linking studies alone.

The picture that emerges is of a Na,K-ATPase complex of α, β, and γ subunits in which α can associate in assemblies as large as tetramers (Fig. 9B), probably via a cytoplasmic domain, while β and γ subunits associate with α primarily in its C-terminal portion, which has a unique structure and thermal instability. In the high resolution Ca2+-ATPase structure, membrane spans 1–6 are associated with the large A and P domains, while spans 7–10 are associated only with smaller extramembranous loops which themselves are not part of self-contained, α-helix, or β-strand-containing secondary structures. This C-terminal structure, an assembly of contributing segments, helps to explain the relative ease with which the C-terminal end of the Na,K-ATPase undergoes radical reorganization. We conclude that α subunits associate upon heating, but that the β and γ subunits are either too distant or too easily dissociated to participate in aggregation.

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