Interaction of SDS with Na\(^+\)/K\(^+\)-ATPase

SDS-SOLUBILIZED ENZYME RETAINS PARTIAL STRUCTURE AND FUNCTION*

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Because nearly all structure/function studies on Na\(^+\)/K\(^+\)-ATPase have been done on enzymes prepared in the presence of SDS, we have studied previously unrecognized consequences of SDS interaction with the enzyme. When the purified plasma membrane-bound kidney enzyme was solubilized with SDS or TDS concentrations just sufficient to cause complete solubilization, but not at concentrations severalfold higher, the enzyme retained quaternary structure, exhibiting \(\alpha\alpha\gamma\gamma\), \(\alpha\beta\alpha\beta\), \(\beta\beta\), and \(\gamma\gamma\) associations as detected by chemical cross-linking. The presence of solubilized oligomers was confirmed by sucrose density gradient centrifugation. This solubilized enzyme had no ATPase activity and was not phosphorylated by ATP, but it retained the ability to occlude Rb\(^+\) and Na\(^+\). This, and comparison of cross-linking patterns obtained with different reagents, suggested that the transmembrane domains of the enzyme are more resistant to SDS-induced unfolding than its other domains. These findings (α) indicate that the partially unfolded oligomer(s) retaining partial function is the intermediate in the SDS-induced denaturation of the native membrane enzyme having the minimum oligomeric structure of \(\alpha\beta\gamma\) and (b) suggest potential functions for Na\(^+\)/K\(^+\)-ATPase with intrinsically unfolded domains. Mixtures of solubilized/partially unfolded enzyme and membrane-bound enzyme exhibited cross-linking patterns and Na\(^+\) occlusion capacities different from those of either enzyme species, suggesting that the two interact. Formation of the partially unfolded enzyme during standard purification procedure for the preparation of the membrane-bound enzyme was shown, indicating that it is necessary to ensure the separation of the partially unfolded enzyme from the membrane-bound enzyme to avoid the distortion of the properties of the latter.

Na\(^+\)/K\(^+\)-ATPase is the energy-transducing ion pump that maintains the normal physiological gradients of Na\(^+\) and K\(^+\) across the plasma membrane of most higher eukaryotic cells (1, 2). Two subunits of the enzyme (α and β) are essential for function, and some preparations also contain other subunits (FXYD proteins) that regulate function (3, 4). It is generally accepted that the noncovalent α,β-heterodimer is the minimum structural unit of the enzyme. There are disagreements, however, on whether this dimer is also the functional unit or whether higher association states of α,β-dimer are involved in function (1, 2, 5, 6).

In the course of the continuation of our studies on the oligomeric structure of Na\(^+\)/K\(^+\)-ATPase, we suspected the persistence of specific subunit interactions in the SDS-solubilized enzyme, suggesting that the solubilized enzyme may be partially unfolded rather than completely denatured. In the broad biochemical context, this was not remarkable. Despite the common assumption that SDS is a strong universal protein denaturant, it has been known for a long time that some proteins may retain structure in SDS (see Refs. 7–9, and references therein), and that the hydrophobic domains of some membrane proteins may be more resistant than their hydrophilic domains to the denaturing effects of SDS (10). In the case of Na\(^+\)/K\(^+\)-ATPase, however, exploring the possibility of its partial unfolding by SDS was of special interest for several reasons. First, the existence of partially unfolded oligomeric enzyme as intermediate in its heat denaturation had already been suggested (11, 12). Hence, it seemed that studies on detergent-induced partial unfolding might provide useful complementary information on the folding/unfolding pathways of the enzyme. Second, because of the increasing evidence in support of the physiological role of the intrinsically unfolded protein domains (13–15), it seemed of interest to assess the functional status of any partially unfolded state of Na\(^+\)/K\(^+\)-ATPase. A neglected early work (16) had already suggested that the SDS-solubilized enzyme may retain functional competence. Third, and most importantly, studies on SDS-induced partial unfolding of Na\(^+\)/K\(^+\)-ATPase seemed to be of special relevance to all research on the structure/function relationship of this enzyme because over the past 30 years, ever since the classical work of the Aarhus group on the purification of the enzyme (17–20), the great majority of such research has been done with membrane-bound preparations that are purified by treating enzyme-rich membranes with SDS. Under the standard conditions of these purification schemes (21), SDS is known to remove impurities and permeabilize sealed vesicles, but is assumed to have no deleterious effects on Na\(^+\)/K\(^+\)-ATPase. Obviously, undetected partial unfolding during purification could distort enzyme properties. In fact, from time to time such an effect of SDS has been suggested as an explanation of apparent negative cooperativity and half-site reactivity of the enzyme (22, 23). We deemed it necessary, therefore, to reexamine the consequences of SDS interaction with the enzyme to assess the fidelity of what are considered to be the established properties of the native enzyme. Here, we report the results of our initial studies in these directions, showing that the SDS-solubilized enzyme retains quaternary structure and partial function, that such a partially unfolded enzyme is indeed produced during the commonly used purification procedures, but that this artifact may be removed from the purified membrane-bound enzyme by dilution and washing. Our findings on the properties of the SDS-solubilized enzyme also help in the clarification of a number of issues regarding the quaternary structure/function relationship of the native enzyme.
enzyme, and point to the intriguing possibility of enzyme molecules with natively unfolded domains being involved in the regulation of the functions of this enzyme.

EXPERIMENTAL PROCEDURES

Purified membrane-bound \( \text{Na}^+/\text{K}^+ \)-ATPase was prepared from the microsomes of the pig kidney outer medulla by a modification of the method of Jørgensen (19, 21), using the angle rotor version, as described previously (54). The specific activities of the preparations used were in the range of 900–1300 nmol of ATP hydrolyzed/mg. Enzyme activity, and formation of the acid-stable phosphoenzyme intermediate, were assayed as described previously (24).

Cross-linking was performed through the modification of previously described procedures (25, 26) by exposing membrane-bound or detergent-treated enzyme (0.8 mg/ml) in 15 mM Tris-HCl (pH 7.4) to Cu\(^{2+} \) (0.25 mM CuSO\(_4 \), and 0.625 mM o-phenanthroline) for 15 min at 24 °C. The reaction was terminated by the addition of Tris-EDTA to the final concentration of 30 mM, followed by the addition of the sample buffer to achieve 2% SDS concentration. The amino-reactive cross-linkers, 1 mM BS3 or 1 mM DSS, were similarly reacted with the enzyme, but for 30 min. These reactions were terminated by the addition of a large excess of Tris (200 mM) followed by the sample buffer. Cross-linked samples and appropriate controls were subjected to Tricine/SDS-PAGE (26) on 10% gels, or on 4–12% NuPage precast gels from Invitrogen. The running buffers contained 0.1% SDS. Reducing agents were omitted from the system. The resulting gels were either stained with Coomassie or subjected to Western analysis, using specific antibodies. Blots were visualized by chemiluminescence. For quantitation of the blots, multiple exposures were used to ensure that comparisons were made within the linear range of the assay. When selected cross-linking reactions with the amino-reactive reagents were repeated in 10 mM phosphate buffer, the results were qualitatively the same as those performed in 15 mM Tris buffer.

For sucrose density gradient sedimentation experiments, enzyme sample (0.2 ml) containing the purified enzyme (0.1 mg), dissolved either in 0.1% SDS or 0.15% TDS or 3% SDS, were layered on top of 11 ml of 5–20% linear gradient of sucrose in 0.1% SDS and 15 mM Tris-HCl (pH 7.4). The sample was centrifuged at 40,000 rpm for 22 h at 4 °C in SW-41 rotor (Beckman). Each gradient was divided into 22 equal fractions. A sample of each fraction was dissolved in the sample buffer containing 2% SDS, subjected to SDS-PAGE on 4–12% gradient gel with the running buffer containing 0.1% SDS. Gels from each fraction were assayed for α, β, and γ subunits by Western blots. Samples (0.3 ml) of marker proteins dissolved in 3% SDS were also sedimented as indicated above in sucrose gradients containing 0.1% SDS. Collected fractions were subjected to SDS-PAGE, and the gels were stained with Coomassie. The markers were: bovine serum albumin (66 kDa), β-galactosidase (116 kDa), myosin heavy chain (205 kDa), and phosphorylase b monomer and its cross-linked oligomers (monomer-hexamer; 97–584 kDa).

Rb\(^+ \) occlusion experiments and assays were done as we have described previously (27). Briefly, the purified enzyme (150 μg/ml) was incubated in a solution containing 0.5 mM \(^{86}\text{RbCl}, 0.2 \text{ mM succrose}, 1 \text{ mM EDTA}, and 12 mM histidine (pH 6.8), at 4 °C for 1 h. The indicated concentration of TDS was then added, and either immediately or 1 h later the enzyme was passed through a cation exchange column (transit time, 1 min) at 4 °C. \(^{86}\text{Rb} \) carried with the enzyme in the eluate was counted by conventional procedures. Corrections for nonspecific binding were made with appropriate controls (27). Oligomycin-dependent Na\(^+ \) occlusion was according to procedures similar to those of Esmann (28) and the above procedures used for Rb\(^+ \) occlusion. The enzyme (300–500 μg/ml) was incubated in a solution containing the indicated concentrations of NaCl (0.2–3 mM), 0.2 mM succrose, 1 mM EDTA, 12 mM histidine (pH 6.8), and 50 μg/ml oligomycin, at 4 °C for 1 h. The indicated concentration of TDS was then added, and either immediately or 1 h later the enzyme was passed through the cation exchange column as indicated above, and counted. For each experimental condition, an identical enzyme sample was carried through the entire procedure but without oligomycin. The counts from such samples were considered to represent nonspecific binding and were used for the correction of occlusion values. That oligomycin-dependent Na\(^+ \) occlusion represents occlusion of this ion by the phosphorylated enzyme in the course of the normal reaction cycle has been established (28, 38).

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1 The abbreviations used are: CuP, complex of Cu\(^{2+} \) and o-phenanthroline; BS3, bis(sulfosuccinimidyl)suberate; DSS, disuccinimidyl suberate; TDS, Tris salt of dodecyl sulfate; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

**FIG. 1.** Solubilization of purified membrane-bound Na\(^+\)/K\(^+\)-ATPase by varying concentrations of SDS or TDS. A, the membrane enzyme (1.2 mg/ml) was suspended in 15 mM Tris-HCl (pH 7.4) and the extent of solubilization was determined by centrifugation at 100,000 × g for 30 min, and assay of released protein after the addition of the indicated concentrations of SDS (■) or TDS (□). At one TDS concentration solubilization of the α subunit was also assayed by Western analysis (○). The indicated values are mean ± S.E. of three determinations. B, solubilization was measured by change of light scattering of the membrane enzyme (1.2 mg/ml) suspended in 15 mM Tris-HCl (pH 7.4) upon addition of the indicated concentrations of SDS. Both sets of experiments (A and B) were done at 24 °C.

Monoclonal antibody against α, subunit (αEF) was obtained from the Developmental Studies Hybridoma Bank, University of Iowa, and that against β, subunit (clone 464.8) from Upstate Biotechnology. A polyclonal antibody specific for γ subunit was raised against the 14 C-terminal residues of rat βγ by procedures similar to those described before (29). SDS, TDS, oligomycin, and the marker proteins used in the sedimentation studies were obtained from Sigma. BS3 and DSS were purchased from Pierce. \(^{86}\text{Rb} \) and \(^{22}\text{Na} \) were obtained from PerkinElmer Life Sciences.

Protein was assayed by Peterson’s modification of the Lowry method (30). Data were analyzed and plotted with the help of GraphPad Prism software.

**RESULTS**

Much of the data presented below are the results of experiments on the purified membrane-bound kidney enzyme prepared by the standard treatment of kidney microsomes with SDS (see “Experimental Procedures”). These experiments were initiated with the prevailing assumption of the field that this purified preparation represents the native state, i.e. its structure/function has not been altered irreversibly during purification. We shall examine the validity of this assumption below (see “Discussion”).

**SDS- and TDS-solubilized Enzymes Retain Quaternary Structure**—In experiments of Fig. 1, the purified membrane-bound enzyme was exposed to different SDS or TDS concentrations, and extent of solubilization was determined either by the assay of solubilized protein and immunoreactive α subunit after centrifugation, or by decrease in light scattering. TDS was also used because we wished to have the option of testing the
major cross-linked products formed from the native enzyme are
following. 1) In agreement with previous observations (25), the
results of Fig. 2 and the test of dilution clearly indicate that,
enzyme rather than the random collisions of the subunits. The
result of the associated states of the subunits of the solubilized
shown). This indicates that the cross-linked products are the
products formed from SDS- and TDS-solubilized enzymes are
similar, and include
bic domains are more resistant to TDS-induced unfolding than
contact domains that are within the intramembrane hydropho-
subunit domain structure and subunit association with increasing
detergent binding. Interestingly, traces of $\beta$-$\beta$-dimer were
detectable even in the enzyme that was cross-linked in 3% TDS,
suggesting the different sensitivities of inter-subunit domain
structures to the denaturing effects of the detergent.
The cross-linked products obtained in experiments of Figs. 2
and 3 were sensitive to reduction by mercaptoethanol (data not
shown), confirming the expectation based on our previous stud-
ies (25, 26) that CuP cross-linking detects the juxtaposition of
the amino-reactive reagent BS3. Experiments were
done as described in the legend to Fig. 1 and under “Experimental
Procedures.”

Effects of Na$^+$ or other ligands on the solubilized enzyme.
Under the conditions used in these experiments, complete solu-
ubilization of the enzyme protein was obtained at approxi-
mately 3.3–3.9 mM dodecyl sulfate (0.1–0.11% SDS; 0.13–
0.15% TDS). Because the critical micellar concentration of SDS
under conditions similar to those used here is in the range of
1.2–7.1 mM (31), the data of Fig. 1 are consistent with the
three-stage mechanism of solubilization of membrane proteins
and the involvement of detergent micelles in the complete
solubilization of the enzyme (31).

It has been established that the noncovalent association of $\alpha$
and $\beta$ subunits survives the solubilization of the enzyme in
mild nonionic detergents such as digitonin and C$_{12}$E$_{5}$. Such
association in solubilized preparations was first demonstrated
in early chemical cross-linking studies (25, 32, 33), and subse-
sequently confirmed by other experimental approaches (34, 35).
To assess the possibility of continued subunit interactions in
the dodecyl sulfate-solubilized enzyme, we solubilized the en-
zyme in 0.15% TDS or 0.1% SDS, exposed these and the control
membrane-bound enzyme to the cross-linking reagent CuP,
terminated the reactions by the addition of a large excess of
EDTA followed by 2% SDS, and subjected the mixtures to gel
electrophoresis. The resulting stained gels and the blots probed
with antibodies to $\alpha$ subunit and $\beta$ subunit (Fig. 2) showed the
following. 1) In agreement with previous observations (25), the
major cross-linked products formed from the native enzyme are
$\alpha$, $\alpha$-dimer and higher oligomers of $\alpha$. 2) The major cross-linked
products formed from SDS- and TDS-solubilized enzymes are
similar, and include $\beta$, $\beta$-dimer, $\alpha$, $\alpha$-dimer, $\alpha$, $\alpha$-dimer,
and higher oligomers containing both subunits. When the enzyme
that was solubilized in 0.15% TDS was diluted 5- or 10-fold in
0.15% TDS, and then exposed to CuP as in Fig. 2, the cross-
linked products were the same as those of Fig. 2 (data not
shown). This indicates that the cross-linked products are the
result of the associated states of the subunits of the solubilized
enzyme rather than the random collisions of the subunits. The
results of Fig. 2 and the test of dilution clearly indicate that,
TDS-solubilized enzyme to CuP caused the formation of a γ-containing product with mobility close to that of α subunit (lane 5), suggesting that it must be either an α,γ-dimer or a β,β,γ-trimer. The former is most likely because the only free sulfhydryl group of each β is involved in the formation of the β,β-dimer (26). When the native enzyme was exposed to either BS3 or DSS, a γ-containing product with the expected mobility of an α,γ-dimer was obtained (lanes 3 and 4); however, this product was not obtained when the TDS-solubilized enzyme was exposed to these amino-reactive reagents (lanes 6 and 7). The findings of Fig. 6 indicate the existence of α,γ-association in the native enzyme, and show that this association is also retained but altered in the TDS-solubilized state. The cross-linking evidence in support of α,γ-association confirms previous evidence for such association obtained in thermal denaturation experiments (11) and coimmunoprecipitation studies (36, 37). The cross-linking reactions of the native membrane-bound enzyme have been shown to exhibit cation selectivity (25). Experiments were done to see whether such selectivity also exists in the TDS-solubilized enzyme. A full comparison of Na⁺ and K⁺ effects on the reactions of the solubilized enzyme was not possible because of the limited solubility of the K⁺ salt of dodecyl sulfate. However, formation of the cross-linked products induced by CuP showed different sensitivities to varying concentrations of Na⁺ and Mg²⁺. The results presented in Fig. 7 show that (a) although the presence of either ion increases the yields of the cross-linked products of β, the noted changes are obtained at an order of magnitude lower Mg²⁺ than Na⁺ concentrations, clearly showing that the changes are not ionic strength effects but indicative of ion selectivity; and (b) Na⁺ and Mg²⁺ have clearly different qualitative effects on the formation of the cross-linked γ,α-dimer. That the subunit interactions of TDS-solubilized enzyme respond selectively to two of the ligands of the enzyme is also consistent with the retention of partial structure by the solubilized enzyme. The above cross-linking experiments provide information on the minimum size of the oligomeric species retained in the TDS- or SDS-solubilized state. To obtain a better estimate of the molecular size of the soluble oligomers, the enzyme solubilized in 0.1% SDS or 0.15% TDS (Fig. 1), and some marker proteins, were subjected to SDS-PAGE and Western analysis with antibodies against the α, β, and γ subunits. Marker proteins were sedimented through the same gradient. Positions of their peaks, determined by SDS-PAGE of the fractions, are noted by the arrows. When the enzyme solubilized in 0.15% TDS was sedimented through the same gradient, the results (data not shown) were nearly identical to those shown for the sample solubilized in 0.1% SDS.

**FIG. 6.** Formation of the γ-containing cross-linked products resulting from the reactions of the purified membrane-bound enzyme and the enzyme solubilized by 0.15% TDS with the indicated cross-linking reagents. Experiments were done as described in the legends to Figs. 2, 4, and 5, and the blot was probed with the anti-γ antibody. In addition to detected γ and α-γ, the positions of α and β subunits and other cross-linked products noted on identical blots probed with anti-α and anti-β antibodies (data not shown) are indicated by the arrows.

**FIG. 7.** Effects of Na⁺ and Mg²⁺ on the CuP-induced cross-linked products obtained from the enzyme that is completely solubilized by 0.15% TDS. Experiments were done as described in the legends to Figs. 2, 4, and 5, and the blot was probed with the anti-γ antibody. In addition to detected γ and α-γ, the positions of α and β subunits and other cross-linked products noted on identical blots probed with anti-α and anti-β antibodies (data not shown) are indicated by the arrows.

**FIG. 8.** Sucrose density gradient sedimentation of the SDS-solubilized Na⁺/K⁺-ATPase. Purified enzyme was solubilized either in 0.1% SDS (△) or 3% SDS (○) as indicated in the legend to Fig. 1, and then sedimented through a linear 5–20% sucrose density gradient containing 0.1% SDS as described under "Experimental Procedures." Each fraction was subjected to SDS-PAGE and Western analysis with antibodies against the α, β, and γ subunits. Marker proteins were sedimented through the same gradient. Positions of their peaks, determined by SDS-PAGE of the fractions, are noted by the arrows. When the enzyme solubilized in 0.15% TDS was sedimented through the same gradient, the results (data not shown) were nearly identical to those shown for the sample solubilized in 0.1% SDS.
**Fig. 9.** Retention of occluded Rb⁺ by the TDS-solubilized enzyme. Membrane-bound enzyme was preincubated with 0.5 mM ⁸⁶Rb⁺ at 4 °C as described under “Experimental Procedures.” The indicated concentrations of TDS were then added, and samples were assayed for occluded Rb⁺ immediately after TDS addition or 1 h later as described under “Experimental Procedures.” Samples were also assayed for protein solubilization (inset) as described in the legend to Fig. 1. The level of occluded Rb⁺ in the control membrane-bound enzyme was 2.06 ± 0.07 nmol/mg. The indicated values are mean ± S.E. (n = 4).

**Fig. 10.** Retention of occluded Na⁺ by the TDS-solubilized enzyme, and stimulation of Na⁺ occlusion by partial solubilization of the enzyme. Membrane-bound enzyme was preincubated with 1 mM ⁸²Na⁺ at 4 °C as described under “Experimental Procedures.” The indicated concentrations of TDS were then added, and occluded Na⁺ was assayed immediately after the addition of TDS or 1 h later as described under “Experimental Procedures.” Samples were also assayed for protein solubilization (inset) as described in the legend to Fig. 1. The level of occluded Na⁺ in the control membrane-bound enzyme was 0.74 ± 0.04 nmol/mg. The indicated values are mean ± S.E. (n = 4).

(a) the enzyme solubilized at 3% SDS and sedimented in 0.1% SDS is predominantly a mixture of the monomers of the three subunits as expected; (b) the enzyme solubilized at 0.1% SDS or 0.15% TDS and sedimented in the same detergent concentration is a mixture of monomers and significant quantities of associated states of α, β, and γ; and (c) the size of the largest
The maximal level of occluded Na\(^+\) measured was 3.8 ± 0.5 nmoI. The indicated values are mean ± S.E. (n = 4).

**Fig. 11.** Activating effect of a partially solubilizing concentration of TDS on Na\(^+\) occlusion at different Na\(^+\) concentrations. Membrane-bound enzyme was preincubated with \( ^\text{22} \)Na\(^+\) at 4 °C with the indicated Na\(^+\) concentrations and assayed for occluded Na\(^+\), along with controls, 1 h after the addition of 0.06% TDS as described under “Experimental Procedures.” The maximal level of occluded Na\(^+\) measured was 3.8 ± 0.5 nmoI. The indicated values are mean ± S.E. (n = 4).

**Fig. 12.** Different CuP-induced cross-linking patterns of the completely solubilized and partially solubilized samples of the purified Na\(^+\)/K\(^+\)-ATPase. Experiments were done as described in the legend to Fig. 2 with samples treated with 0.15% TDS (completely solubilized) and 0.06% TDS (partially solubilized).

The maximal level of occluded Na\(^+\) measured was 3.8 ± 0.5 nmoI. The indicated values are mean ± S.E. (n = 4).

**TABLE I**

| Solubilized α subunit, % of total microsomal content |
|--------------------------------------------------|
| First supernatant | 28.17 ± 1.23 |
| Second supernatant | 25.04 ± 1.04 |

In experiments of similar design to those of Fig. 9, the effects of TDS-induced solubilization on occluded Na\(^+\) were studied. Oligomycin-dependent occlusion was obtained by incubation of the enzyme with 1 mM \( ^\text{22} \)Na\(^+\) at 4 °C, varying TDS concentrations were added, and occlusion and degree of solubilization were assayed immediately after the addition of TDS or 1 h later (Fig. 10). The results showed the following. 1) As in the case of occluded Rb\(^+\), at TDS concentrations that cause complete or near complete solubilization, significant amounts of occluded Na\(^+\) are retained. Note that at 0.15–0.2% TDS, occluded Na\(^+\) measured within 1 h after addition of TDS is in the range of 33–63% of the Na\(^+\) occluded by the native enzyme in the absence of TDS. 2) Increasing TDS concentrations have biphasic effects on occlusion, i.e. stimulation followed by inhibition. 3) At the lower TDS concentrations used, stimulation of Na\(^+\) occlusion is greater after 60 min of exposure to TDS. These findings not only show the ability of the completely solubilized enzyme to contain occluded Na\(^+\), but also indicate that incomplete solubilization is accompanied by the activation of Na\(^+\) occlusion. Because this activation was unexpected, further studies were done to assess its mechanism. In experiments the results of which are summarized in Fig. 11, the effects of a partially solubilizing concentration of TDS on oligomycin-dependent occlusion at different Na\(^+\) concentrations were examined. The results clearly showed that TDS-induced activation was caused by an increase in apparent Na\(^+\) affinity and not by an increase in the maximal capacity of Na\(^+\) occlusion. Our data of Fig. 11 in the absence of TDS are consistent with previous estimations of the \( K_{0.5} \) of Na\(^+\) for oligomycin-dependent Na\(^+\) occlusion by the native enzyme (28, 38, 39). The combined data of Figs. 10 and 11 suggest that the TDS-solubilized and partially unfolded enzyme may interact with the membrane-bound enzyme, and that one result of this interaction is an increase in Na\(^+\) affinity. Although the indicated interaction is supported by the additional experiments presented below, an alternative explanation for the increase in Na\(^+\) affinity is the effect of dodecyl sulfate counter anion binding to the protein on Na\(^+\) affinity.
Interaction of the Partially Unfolded Enzyme with the Native Enzyme—To see whether such interaction suggested by the Na\(^+\)/K\(^+\)-ATPase occlusion experiments could be verified by a different approach, cross-linking experiments were conducted with the enzyme that was completely solubilized with 0.15% TDS, and the enzyme that was incompletely solubilized with a lower TDS concentration. As evident from the results (Fig. 12), cross-linked products were most abundant in the incompletely solubilized enzyme, supporting the proposition that the partially unfolded enzyme may interact with the native enzyme.

Formation and Fate of the Partially Unfolded Enzyme in the Course of Enzyme Purification—The starting material for the preparation of the purified native enzyme used in the above experiments is a crude microsomal fraction of the kidney outer medulla that is prepared without the use of detergents (21). This preparation is then treated with a “low” concentration of SDS to solubilize impurities, but to leave the enzyme in unsealed membrane fragments (21). In view of the findings on the purified enzyme, it was necessary to assess the possibility of the formation of the partially unfolded enzyme during the purification procedure.

The first question raised was whether or not the SDS-solubilized and partially unfolded enzyme could be obtained by the direct treatment of the microsomes with SDS. The results of experiments comparable with those of Fig. 1, but using microsomes instead of the purified enzyme, showed that solubilizing effects of varying concentrations of SDS on total microsomal proteins were approximately the same as those shown in Fig. 1 (data not shown), i.e. near complete solubilization was obtained at 0.15% TDS. The results of experiments of Fig. 13 in which control microsomes and those treated with 0.15% TDS were subjected to CuP cross-linking showed that the enzyme solubilized in this manner also retained partial structure, exhibiting subunit interactions similar to those of the purified solubilized enzyme (compare Fig. 13 with Fig. 2).

The experiments of Fig. 13, and the fact that the SDS/protein ratios used in standard purification procedures (Fig. 4 and Ref. 21) are within the range of those shown in Fig. 1, suggested that some partially unfolded enzyme may indeed be produced during these procedures. To address this issue directly, we treated microsomes with SDS under the conditions of the original protocols of Jorgensen (21), and we assayed for the production of solubilized enzyme by Western analysis. The data summarized in Table I clearly showed the solubilization of a significant fraction of the enzyme.

In the standard purification procedures, the next step after treatment of microsomes with SDS is the dilution of the mixture and its fractionation/sedimentation on sucrose or glycerol gradients to separate the membrane-bound enzyme from solubilized impurities (21, 54). To determine the fate of the fraction of the enzyme that is solubilized in the first step, we performed the dilution of the SDS-treated microsomes also according to the original protocols (21) and found that upon centrifugation nearly all of the solubilized enzyme remains in the soluble fraction (Table I). Evidently, dilution does not precipitate the solubilized enzyme, and the partially unfolded enzyme formed at the first step of purification is separated along with impurities from the remaining membrane-bound enzyme in the course of the standard purification procedure.

When the CuP-induced cross-linking patterns of completely solubilized microsomes and incompletely solubilized microsomes were compared (Fig. 13), the cross-linked products were more abundant in the latter, confirming the conclusions of similar experiments with the purified enzyme (Fig. 12) that the soluble and partially unfolded enzyme may interact with the membrane-bound enzyme if the two are not separated.

The findings reported here are pertinent to several issues regarding the structure/function relationship of Na\(^+\)/K\(^+\)-ATPase.

Quaternary Structure of the Native Enzyme—Disagreements on the association state of Na\(^+\)/K\(^+\)-ATPase within the native membrane have continued to persist despite the large number of studies and experimental approaches that have been used to resolve this matter (2, 5, 6). The present findings provide further evidence to indicate that the native membrane-bound enzyme is an oligomer of the interactive \(\alpha,\beta\)-dimers.

Chemical cross-linking experiments have been one of the prominent approaches that have been used by several laboratories to determine the quaternary structure of the enzyme (reviewed in Refs. 5, 40, and 41). A well known problem in the interpretation of all cross-linking studies on membrane proteins is the difficulty of knowing whether the noted cross-links are the result of the specific interactions of the protomers within an oligomer, or of nonspecific and random collisions of the proteins in the crowded membrane. This ambiguity is resolved if the cross-linked oligomer obtained in the membrane-bound state is also obtained in a detergent-solubilized preparation where a large dilution is achieved by solubilization, and where a further test of dilution can be applied. In the case of Na\(^+\)/K\(^+\)-ATPase, previous cross-linking experiments on preparations solubilized in several detergents (Triton X-100, digitonin, and C\(_2\)E\(_8\)), have shown the formation of \(\alpha,\beta\)-dimer and \(\beta,\beta\)-dimer but not that of \(\alpha,\alpha\)-dimer (25, 26, 32, 33, 40). The importance of the present cross-linking experiments on the native and the dodecyl sulfate-solubilized enzyme, using three different cross-linking reagents (Figs. 2–6), is the demonstration of the existence of \(\alpha,\alpha\), \(\alpha,\beta\), \(\beta,\beta\), and \(\alpha,\gamma\)-associations in both solubilized and unsolubilized states, thus establishing that the associations detected in the native membrane-bound enzyme are not caused by random collisions of these subunits, but are indicative of the minimum oligomeric structure of \(\alpha,\beta,\gamma\). Independent confirmation of this conclusion is also provided by the gradient sedimentation experiments (Fig. 8), showing that the enzyme solubilized in 0.1% SDS or 0.15% TDS, but not in higher SDS concentrations, contains complexes that are clearly larger than an \(\alpha,\beta,\gamma\)-trimer, but not large enough to be confused with nonspecific denatured aggregates of these proteins.

Partial Unfolding of the Enzyme—The inability of the TDS-solubilized enzyme to hydrolyze ATP or form the phosphointermediate of the reaction cycle (see “Results”) is consistent with the denaturation/unfolding of the extramembrane domains of the \(\alpha\)-subunit on the cytoplasmic side where the ATP binding and phosphorylation sites are located (2). The ability of the TDS-solubilized enzyme to contain occluded ions (Figs. 9–11) shows retention of structure within the transmembrane domains. That most of the extramembrane domains are not essential to the occlusion capacity has been established by extensive studies on the enzyme that has been stripped of most of the cytoplasmic extramembrane domains by proteolysis (2).

The cross-linking experiments with the amino-reactive reagents (Figs. 4 and 5) further support the proposition that the TDS-solubilized enzyme is partially unfolded as indicated above. That the hydrophilic BS3 produces cross-links in the native but not in the solubilized enzyme is consistent with the TDS-induced denaturation of the extramembrane domains, and loss of contact through such domains. On the other hand, production of cross-links with the hydrophobic DSS in both the native and the TDS-solubilized enzyme supports retention of the structure of the transmembrane domains, and subunit contact through these domains. It is appropriate to note that, in addition to the present and previous chemical cross-linking
evidence (5, 26), there is independent evidence in support of contact between α,β-protoomers through both extramembrane and transmembrane domains. Contact between the cytoplasmic extramembrane domains of two α subunits has been indicated by coinmunoprecipitation of the chimeras of α isoforms (44), by “pull-down” assays of interactions between isolated tagged M4M5 cytoplasmic loops (45), and by the closeness of the cytoplasmic P- and N-domains of two adjacent α subunits in two-dimensional crystals, suggesting the association of the two (46). Contact between the single transmembrane helices of two β subunits has been suggested by the functional consequences of the substitution of residues of the putative dimerizing domains of this helix (47), and by close contact between the protomers noted in two-dimensional crystals, consistent with the positions of transmembrane helices of two β subunits (48).

The same crystallographic study (48) also suggests close contacts between several transmembrane domains of two adjacent α subunits.

TDS concentrations higher than those that are just sufficient for solubilization and partial unfolding cause nearly complete disruption of subunit interactions (Fig. 3) and complete inhibition of ion occlusion (Figs. 9 and 10). Evidently, the partially functional and partially unfolded enzyme is an oligomeric intermediate in the unfolding pathway of the oligomeric native enzyme leading to the inactive and unfolded subunit monomers. There is ample evidence for the existence of partially unfolded intermediates in the folding or unfolding of monomeric proteins (49, 50), and there are oligomeric proteins that unfold through partially unfolded oligomeric intermediates (50, 51). However, we are not aware of an active monomer (protomer) that is converted to an unfolded monomer through a pathway that involves the formation of a partially unfolded oligomer. Put in other words, the fact that the unfolding intermediate is an oligomer argues that the native enzyme must also be an oligomer.

Interaction of the Partially Unfolded Enzyme with the Native Enzyme—Although the cross-linking experiments on the solubilized enzyme show interactions between the partially unfolded protoomers (Figs. 2–6), cross-linking experiments of Figs. 12 and 13 on the mixtures of solubilized and unsolubilized enzyme suggest interactions between the partially unfolded and the native enzyme. That such interaction is accompanied by stimulation of Na⁺ occlusion but not Rb⁺ occlusion (Figs. 9 and 10) is consistent with previous observations indicating that various detergents favor the E₁ rather than the E₂ conformation of enzyme (52, 53). It may also be because of the fact that the Rb⁺ concentration used in experiments of Fig. 9 (0.5 mM) is far in excess of K₀.₅ for Rb⁺ occlusion (27), although our limited preliminary data (data not shown) have not revealed significant TDS-stimulated occlusion at Rb⁺ concentrations lower than that used in Fig. 9.

How Native Are the SDS-treated Preparations of the Enzyme?—There is a large body of data obtained by different laboratories from active site/ binding site titration studies, and pre-steady state kinetic experiments that indicate the existence of negative interactions and partial site reactivity in oligomeric Na⁺/K⁺ -ATPase (reviewed in Refs. 5, 6, 41, and 42). Some have dismissed these data as artifacts based on the proposition that the purified preparations used in these studies may have contained significant amounts of denatured or partially denatured enzyme produced by SDS treatment (22) or other unknown manipulations during purification (23). Our findings now demonstrate the production of a partially unfolded enzyme during the standard purification procedure (Table I and Fig. 13). However, our experiments also show that this solubilized and partially unfolded enzyme, although capable of interacting with the membrane-bound enzyme (Fig. 13), is separated from the sedimented purified enzyme in the course of purification (Table I). We conclude that the properties of the membrane-bound Na⁺/K⁺-ATPase preparations that are routinely purified by the classical SDS treatment (21), or its modifications (54, 55) are not distorted by SDS, provided that the preparations are appropriately diluted and washed to be free of the solubilized and partially unfolded enzyme. In the absence of experimental data demonstrating the production of the unfolded enzyme during purification by causes other than SDS, we also conclude that the findings on the purified enzyme showing its ligand-induced negative cooperativity of binding and partial site reactivity (5, 6, 41, 42) are not preparation-induced artifacts. That these findings are additional support for the existence of interactive α,β-protoomers in the native enzyme has been discussed elsewhere (5, 6, 41, 42).

In the original studies on the use of SDS for Na⁺/K⁺-ATPase purification (17–20), it was shown that SDS treatment of microsomes not only removed impurities, but also served the important function of permeabilizing tightly sealed vesicles and revealing the latent activity of such vesicles. Over the years, in a good number of functional studies where the extent of enzyme purity has not been an issue, SDS has been added to crude membrane preparations to achieve permeabilization/activation, and a variety of enzyme functions have been measured in the presence of SDS (e.g. Refs. 56–59). Because of the wide range of SDS and protein concentrations used in such studies, it is difficult to guess how much, if any, partially unfolded enzyme may have been produced, and whether or not any of the measured functions may have been influenced by this unfolding. Further studies are needed to define the conditions under which the permeabilizing effects of SDS without some partial unfolding/solubilization may be obtained.

Other Implications—We know of no other preparation of Na⁺/K⁺-ATPase that has been shown to retain partial structure and function, and be capable of interacting with the unfolded enzyme to alter its function. It is conceivable that extension of the present work may indicate that the preparation described here is a phenomenon peculiar to the interaction of SDS with the enzyme. On the other hand, because intrinsically unstructured proteins or protein domains seem to be involved in protein-protein interactions related to a variety of physiological or pathological phenomena (13–15), it is appropriate to consider the possibility that partially unfolded molecules of Na⁺/K⁺-ATPase that are formed endogenously may have physiologically relevant functions either by interacting with the folded enzyme or by interacting with other proteins. In the latter regard, we note that the signal transducing function of Na⁺/K⁺-ATPase through induced interactions with a number of neighboring proteins is now well established (60, 61), and it has been noted that a major role of unstructured protein domains seems to be in cellular signaling where the plasticity of the unstructured domain becomes advantageous (13). We suggest that the SDS-solubilized and partially unfolded Na⁺/K⁺-ATPase may be a useful model for studies on the potential physiological roles of the intrinsically unfolded forms of the enzyme.

REFERENCES

1. Skou, J. C., and Esmann, M. (1992) J. Bioenerg. Biomembr. 24, 249–261
2. Kaplan, J. H. (2002) Annu. Rev. Biochem. 71, 511–535
3. Sweadner, K. J., Arystarkhova, E., Donnet, C., and Wetzel, R. K. (2003) Ann. N. Y. Acad. Sci. 986, 382–387
4. Geering, K., Béguin, P., Garty, H., Karlsh, S., Fizazi, M., Harisberger, J.-D., and Cranbert, G. (2003) Ann. N. Y. Acad. Sci. 986, 388–394
5. Askari, A. (2000) in Na⁺/K⁺-ATPase and Related ATPases; Excerpta Medica International Congress Series 1207 (Taniguchi, K., and Kaya, S., eds) pp. 17–26, Elsevier, Amsterdam
6. Taniguchi, K., Kaya, S., Abe, K., and Marsh, S. (2001) J. Biochem. (Tokyo) 129, 335–342
