DISRUPTION OF THE QUATERNARY STRUCTURE OF $(Na^+ + K^+)$-DEPENDENT ADENOSINE TRIPHOSPHATASE BY TRITON X-100[1]

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Received June 9,1976

Summary: $(Na^+ + K^+)$-dependent adenosine triphosphatase (NaK ATPase) consists of two polypeptide chains, a large polypeptide with a molecular weight of about 100,000, and a sialoglycoprotein with a molecular weight of about 40,000. In the presence of Triton X-100 both polypeptides react to form high molecular weight aggregates with apparent molecular weights of 168,000, 200,000 and 260,000. These aggregates arise as a result of disulfide bond formation which results from the autooxidation of sulfhydryl groups on the two polypeptides of NaK ATPase. These data are discussed in light of studies aimed at determining the size and subunit structure of membrane proteins with Triton X-100.

Introduction: Triton X-100 has recently been used to study the size and subunit composition of membrane proteins (1-3). The detergent is generally considered to be nondenaturing, and is thus thought to maintain membrane proteins in their native state. Recently, however, while performing experiments aimed at determining the quaternary structure of the membrane bound enzyme, NaK ATPase, I observed that Triton X-100 causes the formation of large molecular weight aggregates of the enzyme. In the present communication I show that Triton X-100 disrupts the membrane configuration of NaK ATPase which results in the formation of disulfide bonds by autooxidation of sulfhydryl (SH) groups on the two polypeptides of the enzyme. The formation of the disulfide bonds are responsible for the presence of the high

[1]This work was supported by grants from the Massachusetts Heart Association, grant HL-08893 from the National Institutes of Health, and grant GB-36827 from the National Science Foundation.

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molecular weight aggregates. These data are discussed in light of studies aimed at determining the size and subunit structure of membrane proteins utilizing Triton X-100.

Materials and Methods: N-ethylmalemide was purchased from Schwarz/Mann and dissolved in 25 mM Tris-Cl buffer, pH 7.4,immediately prior to use. β-mercaptoethanol was purchased from Sigma Chemical Company. Triton X-100 was from New England Nuclear.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in 5% acrylamide gels essentially according to the method of Davies and Stark (4). It was necessary, however, to add 4% SDS to the electrophoresis sample buffer to prevent artifacts in the electrophoresis profiles of Triton X-100 solubilized NaK ATPase. Bromphenol blue was used as a tracking dye. Molecular weight standards were myosin (MW = 220,000) bovine serum albumin (MW = 68,000), and carbonic anhydrase (MW = 30,000).

NaK ATPase was purified by the angle rotor technique of Jørgensen (5). A modification of the procedure was to centrifuge the sucrose gradients in a Beckman 30 rotor for 3.5 hr at 50,000 x g or in an IEC-A192 rotor for 90 min at 120,000 x g. The specific activity was 900 μmoles of P_i/mg/hr. No ouabain insensitive activity was detected in the presence of 0.1 mM ouabain. NaK ATPase activity in the absence of Triton X-100 was analyzed as described previously (6). When NaK ATPase activity was assayed in the presence of Triton X-100, the method of Post was used (7).

Results: Figure 1B shows a sodium dodecyl sulfate (SDS) polyacrylamide gel which contains NaK ATPase that was incubated in 1% Triton X-100 for 5 min prior to electrophoresis. The sample was prepared in SDS electrophoresis sample buffer not containing β-mercaptoethanol. In addition to the large and small polypeptides of NaK ATPase, there are three other prominent bands with apparent molecular weights of 168,000, 200,000 and 260,000. Simultaneously with the appearance of these bands there was a loss in Coommassie blue staining material running in the regions of both the large and small polypeptides.

A time course experiment revealed the rapid disappearance of the large polypeptide into the high molecular weight aggregates (Fig. 2). At the end of 1 min and 2 min 35% and 50% of the large polypeptide had reacted, respectively. At the end of 4 min the reaction had stopped with 58% of the large polypeptide reacted. If NaK ATPase was incubated with 0.1% Triton X-100, the large polypeptide reacted to form the high molecular weight aggregates but at a much slower rate (Fig. 2).

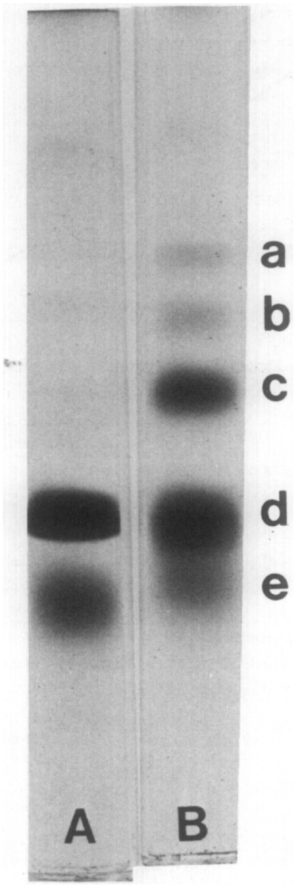

Figure 1. Sodium dodecyl sulfate-polyacrylamide gels of NaK ATPase incubated in the presence (B), or absence (A) of 1% Triton X-100 for 5 min. a,b,c, refer to proteins with apparent molecular weights of 260,000, 200,000 and 168,000. d and e refer to the large and small polypeptides of NaK ATPase, respectively.

At the end of 5 min incubation with 1% Triton X-100, NaK ATPase was assayed for ATPase activity. No activity, however, was detected. Including 50 mM β-mercaptoethanol in the reaction mixture with 1% Triton X-100 had no effect. These findings agree with previous work which showed NaK ATPase to be inactive in the presence of 1% Triton X-100 (8).

It is important to note that the high molecular weight aggregates are observed on SDS-polyacrylamide gels only if β-mercaptoethanol is left out of the electrophoresis sample buffer. If, however, prior to electrophoresis,

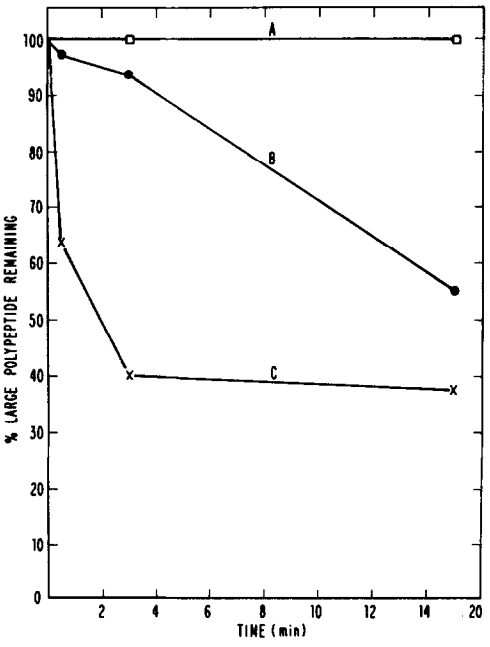

Figure 2. Plot of the percent large polypeptide remaining with time in the absence of Triton X-100 (curve A), or in the presence of 0.1% (curve B), and 1% (curve C) Triton X-100.

Triton X-100 treated NaK ATPase is allowed to sit in electrophoresis sample buffer containing 1% β-mercaptoethanol for 30 min at room temperature, the SDS gel profile reveals only the large and small polypeptide of the enzyme. No high molecular weight aggregates were detected. This finding suggests that the high molecular weight aggregates formed in the presence of Triton X-100 arise as a result of disulfide bond formation involving the two polypeptides of NaK ATPase.

To test the hypothesis that the high molecular weight aggregates arise as a result of disulfide bond formation, NaK ATPase was reacted with 5 mM n-ethylmalemide (NEM) for 30 min at room temperature. NEM is known to react covalently and with a high degree of specificity to SH groups (9). Thus, if Triton X-100 is causing NaK ATPase to aggregate by disulfide bond formation, then prior treatment with NEM before incubation with the detergent should make the SH groups unavailable for reaction. After incubation with NEM,

NaK ATPase was washed two times to remove unreacted NEM, and then incubated with 1% Triton X-100 for 5 min. The sample was subjected to electrophoresis in the absence of β-mercaptoethanol. Surprisingly, the SDS gel profile revealed high molecular weight aggregates similar to those seen in Fig. 1B.

A possible explanation for this finding is that the SH groups involved in the formation of the high molecular weight aggregates may, in the native enzyme, be inaccessible and therefore unable to react with NEM. Thus, Triton X-100 may alter the disposition of NaK ATPase so as to bring into close contact SH groups on the two polypeptides. If once properly positioned the SH groups autooxidize, the net effect of Triton X-100 would be to form disulfide bonds. If this hypothesis is correct, then it is likely that in the presence of Triton X-100 the SH groups involved in forming the disulfide bonds may become accessible to reaction with NEM. In order to test this hypothesis, an experiment was performed similar to the preceding one, except that at the end of 30 min unreacted NEM was not washed away from NaK ATPase. Thus, in this experiment Triton X-100 was added to NaK ATPase in the presence of 5 mM NEM. An SDS-polyacrylamide gel of this sample revealed no high molecular weight aggregates. This finding is consistent with my hypothesis that the SH groups involved in forming the high molecular weight aggregates are in the native enzyme inaccessible and therefore do not react with NEM. Thus, Triton X-100, either by directly perturbing NaK ATPase, or its surrounding lipid environment, drastically alters the accessibility of these SH groups.

The complete absence of the high molecular weight aggregates is surprising since Triton X-100, even in the presence of NEM, might still be expected to form a significant amount of aggregated enzyme. That this was not observed, however, suggests that the reaction of NEM with the SH groups is more rapid than the formation of the disulfide bonds.

Trace metals are known to greatly increase the rate of SH group auto-

oxidation (10). If the high molecular weight aggregates are a result of the autooxidation of SH groups, it may be possible to prevent their formation by including a metal chelator in the reaction mixture. To test this idea, NaK ATPase was incubated with 10 mM EDTA followed by the addition of enough Triton X-100 to give a 1% solution. After 5 min incubation, the sample was subjected to electrophoresis on an SDS-polyacrylamide gel. The results showed the complete absence of the high molecular weight aggregates.

The question arises as to whether trace metals in the absence of Triton X-100 can cause the formation of disulfide bonds, and thus the high molecular weight aggregates. Indeed, previous work from this laboratory has shown that $CuSO_4$ in combination with o-phenanthroline can give rise by disulfide bond formation to oligomeric forms of the large polypeptide when reacted with purified NaK ATPase (11). In no instance, however, in those experiments were aggregates with molecular weights of 168,000 and 260,000 observed. Thus, while trace metals may account for the production of the 200,000 molecular weight aggregate in my experiments, it is unlikely they also produce the aggregates with molecular weights of 168,000 and 260,000.

Discussion: Triton X-100 is currently being used to study the size and subunit structure of membrane proteins (1-3). The underlying assumption on which these studies are based is that the detergent does not disrupt the native structure of membrane proteins. The work I have presented, however, clearly shows that this assumption is not valid for NaK ATPase. The formation of the high molecular weight aggregates seen in my experiments arises as a result of disulfide bond formation between the two polypeptides of NaK ATPase, and is due to a distribution in the native structure of the enzyme in the membrane by Triton X-100. While trace metals are involved in the formation of the disulfide bonds, they alone cannot account for the production of all the high molecular weight aggregates.

Further evidence which suggests that Triton X-100 disrupts the native structure of NaK ATPase comes from cross-linking experiments. In the work

reported here, no attempt was made to determine whether Triton X-100 selectively solubilized the large or small polypeptides of NaK ATPase. However, recent studies from this laboratory have shown that if NaK ATPase is solubilized with Triton X-100 in either the presence or absence of a reducing agent[3], one large and one small polypeptide are solubilized (3). Sucrose gradient sedimentation studies showed the large and small polypeptides to be intimately associated (3). Recently, I have shown by cross-linking experiments that the quaternary structure of native NaK ATPase consists of at least a dimer of the large polypeptide (11). Thus, since Triton X-100 does not solubilize significant amounts of a dimer of the large polypeptide, or other higher possible quaternary forms of the enzyme, it must clearly disrupt the quaternary structure of NaK ATPase.

Taken together all these results suggest that caution should be used in studies aimed at determining the size and subunit structure of membrane proteins with Triton X-100.

Acknowledgments: I wish to thank Dr. Guido Guidotti in whose laboratory this work was done. Also, I wish to thank Bob Jackson and some of the members of Dr. Guidotti's group for their advice and friendship.

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[3]We have recently repeated these experiments in the presence of β-mercaptoethanol and observed similar results (G.J. Giotta and S. Clarke, unpublished observation).