Quaternary Structure of \((\text{Na}^+ + \text{K}^+)-\text{dependent Adenosine Triphosphatase}^*\)

(Received for publication, September 4, 1975)

GREGORY J. GIOTTA

From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

\((\text{Na}^+ + \text{K}^+)-\text{dependent adenosine triphosphatase} (\text{NaK-ATPase})\) 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. Cross-linking of purified \text{NaK-ATPase} with the \((\text{o-phenanthroline})_2\)-cupric ion complex (CP) results in the reversible formation of dimers, trimers, tetramers, and pentamers of the large polypeptide and loss of NaK ATPase activity. ATPase activity is partially recovered if NaK ATPase is incubated with \(\beta\)-mercaptoethanol after treatment with CP. In contrast to these results, if NaK-ATPase is cross-linked in crude canine kidney microsomes, only a dimer of the large polypeptide is formed. No cross-linking of the sialoglycoprotein to the large polypeptide is detected when NaK-ATPase is cross-linked in purified form. However, when NaK-ATPase is reacted with CP in either purified or microsomal form, the sialoglycoprotein cross-links to itself yielding a high molecular weight aggregate.

The results show that the functional subunit structure of NaK-ATPase consists of at least two large polypeptides.

Sodium-potassium ion-activated adenosine triphosphatase is the enzyme responsible for regulating the cytoplasmic concentrations of sodium and potassium in eukaryotic cells (1–4). The enzyme has been highly purified and shown to consist of two polypeptides (5–8). The larger of the two has a molecular weight of about 100,000 (6–9) and is specifically phosphorylated by ATP during turnover of the enzyme (6, 7, 10). The second polypeptide is a sialoglycoprotein with a molecular weight of about 40,000 (11). Its role in active transport remains unknown. Recent experiments, however, have shown that antibodies directed against the sialoglycoprotein inhibit ATPase activity (12, 13).

Considerable evidence suggests that the molecular weight of native NaK-ATPase is about 250,000. Radiation inactivation experiments have yielded values of 190,000 to 300,000 for the molecular weight of the functional enzyme (14). Gel filtration experiments with NaK-ATPase sonicated in the presence of detergents give values of 300,000 for the molecular weight of the enzyme complex (15, 16). Jørgensen has shown the maximum molecular weight per site of phosphorylation, ATP binding, and ouabain binding to be 137,000, 250,000, and 278,000, respectively (17). Despite these data, however, the subunit structure of NaK-ATPase remains unknown.

One approach to establishing the stoichiometry of the two polypeptides in the enzyme complex is that of chemical cross-linking. In the present communication I describe the covalent cross-linking of NaK-ATPase by the \((\text{o-phenanthroline})_2\)-cupric ion-catalyzed air oxidation of sulphydryl groups (18). The results suggest that the subunit structure of NaK-ATPase is \(\alpha_2\beta_2\).

MATERIALS AND METHODS

Preparation of canine kidney microsomes, as well as the purification of NaK-ATPase by the angle rotor technique, were done as described by Jørgensen (8). A modification of the purification procedure, however, was to centrifuge the sucrose gradients in a Beckman 30 rotor for 31/2 hours at 50,000 \(\times g\), or in an IEC A160 rotor for 90 min at 120,000 \(\times g\). The purified enzyme was stored at \(-70^\circ\) in a solution containing 10% sucrose (w/v), 1 mM EDTA, 1 mM \(\beta\)-mercaptoethanol, and 25 mM imidazole, pH 7.5. The enzyme had an ouabain-sensitive specific activity of 900 \(\mu\)mol of P\(_2\)/mg/hour. Little or no ouabain-insensitive activity was detected if NaK-ATPase activity was measured in the presence of 0.1 mM ouabain.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the method of Davies and Stark (19). Bromphenol blue was used as a tracking dye. Molecular weight standards were myosin \((M_s = 220,000)\), bovine serum albumin \((M_r = 68,000)\), and carboxy anhydrase \((M_r = 30,000)\).

Tritiated sodium borohydride (specific activity 138 mCi/mmol) was obtained from New England Nuclear. \(N\)-Ethylmaleimide was from Schwarz/Mann. 1.10-Phenanthroline (monohydrate) was from Fischer Scientific Co. Sodium periodate and cupric sulfate were purchased from Mallinckrodt Chemical Works. Scintillation counting was done in 10 ml of a solution made up of 50 ml of Eastman scintillation grade tissue solubilizer, 40 ml of Eastman concentrate I for liquid scintillation counting, and 910 ml of toluene.

NaK-ATPase activity was measured as described previously (5). Protein was determined by the method of Lowry (20). Phosphate was assayed as described by Leloir and Cardini (21). Experiments aimed at determining the effect of \(\beta\)-mercaptoethanol on the ATPase activity of \((\text{o-phenanthroline})_2\)-cupric sulfate-treated NaK-ATPase were per-
formed by adding β-mercaptoethanol to a final concentration of 50 mM. The samples were kept for 15 min at room temperature before being assayed. Control samples had an equal amount of β-mercaptoethanol added immediately after the addition of the sulfite acid-molybdate quenching solution.

Reaction of Purified NaK-ATPase and Microsomal Membranes with (o-Phenanthroline)₃-Cupric Sulfate—(o-Phenanthroline)₃-cupric ion oxidation of purified NaK-ATPase was performed in 50 mM triethanolamine-chloride buffer, pH 7.6. The solution contained 200 mM o-phenanthroline, 100 μM cupric sulfate, and 750 pg of NaK-ATPase. After the reaction was allowed to proceed for various periods of time, 50 μl was removed and added to an equal volume of twice concentrated electrophoresis sample buffer containing 1 mM EDTA and 2 mg/ml of N-ethylmaleimide. Samples were then subjected to electrophoresis immediately.

(o-Phenanthroline)₃-cupric ion oxidation of NaK-ATPase in canine kidney microsomes was performed in 4 ml of 50 mM triethanolamine chloride buffer, pH 7.6. The solution contained 60 mg of microsomal protein, and 200 μM in o-phenanthroline, and 100 μM in cupric sulfate. At the appropriate time, 1 ml was removed and added to a beaker, followed by the immediate addition of 20 μl of 100 mM EDTA. NaK-ATPase was then purified from the microsomes by the procedure of Jergensen (8).

Tritiation of Sialoglycoprotein—Tritiation of the sialoglycoprotein of NaK-ATPase was performed essentially according to Van Lenten and Ashwell (22). An aliquot of NaK-ATPase which had been stored at 70 °C was thawed and resuspended in 7 ml of triethanolamine-chloride buffer, pH 7.6. After centrifugation at 100,000 × g for 30 min the pellet was resuspended with gentle homogenization in 1 ml of triethanolamine-chloride buffer. This solution (200 μl) which contained 300 μg of NaK-ATPase was reacted either with or without CP for 60 min. At the end of this time EDTA was added to a final concentration of 1 mM, followed by the addition of MalNEt to a final concentration of 2 mM. The sample was kept for 5 min on ice, followed by the addition of 5 ml of a solution in which was 0.1 M in sodium acetate, 0.15 M in sodium chloride, and 2 mM in MalNEt; pH 5.6. The sample was centrifuged at 100,000 × g for 30 min, and the pellet was resuspended in 200 μl of ice-cold sodium acetate buffer. Two hundred microliters of 0.2 mg/ml of sodium periodate was added to the sample and allowed to react for 8 min on ice in the dark. At the end of this time, 5 ml of 0.05 M sodium phosphate buffer, pH 8.5, containing 2 mM MalNEt, was added, and the sample was centrifuged at 100,000 × g for 30 min. The pellet was resuspended in 200 μl of sodium phosphate buffer followed by the addition of 20 μl of a solution containing 1 mg/ml of tritiated sodium borohydride made up just prior to use in 10 mM NaOH. The sample was allowed to react for 10 min at room temperature, followed by two washes with 0.05 M sodium phosphate buffer, pH 8.5, containing 2 mM MalNEt. Finally, Na dodecyl-SO₄ electrophoresis sample buffer was added and the sample subjected to electrophoresis. After staining and destaining, 2 mm slices of the gel were placed in glass scintillation vials which 10 ml of counting fluid were added. The vials were kept overnight at 45 °C and counted the next morning.

It is important to note that the addition of MalNEt as indicated in the procedure is necessary to prevent the formation of high molecular weight aggregates of NaK-ATPase.

RESULTS

Effect of (o-Phenanthroline)₃-Cupric Sulfate on Large Polypeptide of Purified NaK-ATPase—Fig. 1B shows the sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of purified NaK-ATPase which was incubated in the presence of CP for 2 min at room temperature. In addition to the large and small polypeptides of NaK-ATPase, there is a band with an apparent molecular weight of 200,000 (Fig. 2). Longer incubation times result in the appearance of bands with apparent molecular weights of 300,000, 400,000, and 500,000 (Fig. 1C, Fig. 2). Optical density determinations of NaK-ATPase treated with CP for 10 min, subjected to electrophoresis on Na dodecyl-SO₄ gels and stained for protein with Coomassie blue, revealed a reciprocal relationship between the total amount of stain in the CP-produced bands and the M₄ = 100,000 region. Thus, increases in the amount of stain in the CP-produced bands were accompanied by corresponding decreases in the amount of stain in the M₄ = 100,000 region. A plot of the percentage of loss of the large polypeptide with time (Fig. 3) showed that approximately 22% and 42% of the large polypeptide was lost at the end of 10 and 60 min, respectively. Neither the rate nor the extent of reaction of the large polypeptide was altered if the reaction was performed under molecular oxygen.

CP is believed to catalyze the air oxidation of protein sulfhydryl groups resulting in the formation of disulfide bonds.
Cross-linking of (Na\(^{+} + K^{+}\)) ATPase by the (\(\alpha\)-Phenanthroline)\(_2\)-Cupric Ion Complex

Fig. 3. The effect of CP on the large polypeptide of purified NaK-ATPase. NaK-ATPase was reacted with CP for different periods of time and then subjected to electrophoresis in 3.3% Na dodecyl-SO\(_4\)-polyacrylamide gels. The gels were stained with Coomassie blue, destained, and scanned at 550 nm.

That this reaction is responsible for the formation of the \(M_r = 300,000\) to 500,000 proteins was shown by incubating NaK-ATPase with \(N\)-ethylmaleimide at a concentration of 2 mg/ml for 60 min prior to treating with CP. MalNEt has been shown to react covalently with a high degree of specificity with protein --SH groups (29). Thus if oxidation of --SH groups by CP is responsible for the formation of the high molecular weight proteins, pretreatment with MalNEt should render the --SH groups unavailable for disulfide formation. Indeed, when NaK-ATPase was incubated with MalNEt, treated with CP, and subjected to electrophoresis in Na dodecyl-SO\(_4\) gels, the \(M_r = 200,000\) to 500,000 proteins were not present.

These data suggest that CP produces proteins with molecular weights that are multiples of the large polypeptide by disulfide formation. That this is indeed the case was shown by running gels of NaK-ATPase which had been reacted with CP for 10 min and then incubated in electrophoresis sample buffer with 2% \(\beta\)-mercaptoethanol for 30 min prior to electrophoresis. Optical density determinations of these gels stained with Coomassie blue revealed a complete loss of the \(M_r = 200,000\) to 500,000 proteins and a simultaneous increase in the amount of stain in the \(M_r = 100,000\) region. An alternate approach was electrophoresis of NaK-ATPase which had been reacted with CP for 60 min and then immediate removal of 2-mm slices which contained the \(M_r = 200,000\), 300,000, and 400,000 proteins. The slices were allowed to incubate for 30 min in 100 \(\mu\)l of electrophoresis sample buffer containing 2% \(\beta\)-mercaptoethanol, and then were subjected to re-electrophoresis on a new gel. Fig. 4B shows that about 50% of the \(M_r = 200,000\) protein was reconverted into the \(M_r = 100,000\) large polypeptide. If the gel slice was kept for 90 min in electrophoresis sample buffer containing \(\beta\)-mercaptoethanol prior to electrophoresis, there was complete conversion of the \(M_r = 200,000\) protein to the \(M_r = 100,000\) polypeptide. Thus, the \(M_r = 200,000\) protein is a covalent dimer of two large polypeptides. Similar experiments showed that the \(M_r = 300,000\) and 400,000 proteins could also be converted to the \(M_r = 100,000\) polypeptide. The presence of the sialoglycoprotein was not detected in any of the cross-linked products. Thus, CP results in the production of dimers, trimers, tetramers, and pentamers of the large polypeptide.

Preincubation of NaK-ATPase with MalNEt at a concentration of 2 mg/ml for 60 min prior to reacting with CP completely prevented the loss of the small polypeptide and the formation of material at the gel top. This result suggests that the small polypeptide is capable of being cross-linked by CP into very large molecular weight aggregates, although at a slower rate than that of the large polypeptide. The aggregates completely disappeared from the gel top if prior to electrophoresis the sample was allowed to react for 30 min in electrophoresis sample buffer containing 2% \(\beta\)-mercaptoethanol.

The finding that the small polypeptide was capable of being cross-linked by CP made it necessary to reconsider the possibility that a significant amount of the sialoglycoprotein may be cross-linked to the large polypeptide. Although the previous data strongly suggest that the proteins which under-
went electrophoresis with molecular weights of 200,000 to 500,000 consist of dimers, trimers, tetramers, and pentamers of the large polypeptide, it is likely that the small polypeptide could be involved in forming these oligomeric structures. Similar to other glycoproteins, the small polypeptide stains very lightly with Coomassie blue and therefore by gel scanning techniques could easily escape detection. Indeed, as much as 1 to 2 μg of small polypeptide is not reliably detected by optical density measurements of 3.3% polyacrylamide gels stained with Coomassie blue. Thus, in order to ensure that CP is not cross-linking the small and large polypeptides together, a method was sought to specifically label the small polypeptide. Van Lenten and Ashwell (22) have shown that periodic acid oxidation of sialic acid followed by reduction with tritiated sodium borohydride results in the chemical conversion of glycosidically linked sialic acid to the tritiated 5-acetamido-3,5-dideoxy-L-arabino-2-heptulosonic acid derivative. Previous investigators have used this reaction to label sialoglycoproteins, such as ceruloplasmin (22) and those of the red cell (24). Since the small polypeptide of NaK-ATPase contains a significant amount of sialic acid (11), it was thought that it could be similarly modified. The procedure was to oxidize NaK-ATPase with periodic acid followed by reduction with tritiated sodium borohydride. The samples were then subjected to electrophoresis in sodium dodecyl sulfate 3.3% polyacrylamide gels. The gels were stained with Coomassie blue, destained, sliced into 2-mm sections, and counted. Fig. 5 shows that the procedure selectively modifies the small polypeptide. Virtually all of the radioactivity incorporated migrates with the small polypeptide; little or no radioactivity migrates with the large polypeptide. In order to test whether the sialic acid residues of the small polypeptide are the ones modified by this procedure, NaK-ATPase was treated with 0.5 mg/ml of neuraminidase for 15 min at room temperature. The specific activity of the tritiated small polypeptide was 1000 cpm/μg. Thus, as little as 0.1 to 0.2 μg of the small polypeptide can be detected by slicing and counting the gel. In order to examine the possibility that CP is cross-linking small and large polypeptides together, NaK-ATPase was treated with CP for 45 min, followed by oxidation with periodic acid and reduction with tritiated sodium borohydride. The sample underwent electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel and was stained, destained, sliced, and counted. Fig. 5 shows the results. There are no detectable counts above background running in the molecular weight regions of 200,000, 300,000, 400,000, and 500,000. Also, the number of counts running with the small polypeptide is nearly identical with those of a sample of NaK-ATPase not pretreated with CP (Fig. 5). These results suggest that it is extremely unlikely that CP cross-links the small and large polypeptides together. It is important to note that there is a significant number of counts at the top of both gels which contained NaK-ATPase treated or not treated with CP, followed by oxidation with periodic acid and reduction with tritiated sodium borohydride. In both cases including 2% β-mercaptoethanol in the electrophoresis sample buffer for 30 min prior to electrophoresis caused the disappearance of the radioactivity from the gel tops. Gels which contained NaK-ATPase prereacted with CP always had about twice as many counts at the top of the gel than a sample of NaK-ATPase which was not prereacted with CP. If the gel top was removed and allowed to soak in electrophoresis sample buffer with 2% β-mercaptoethanol for 90 min and then subjected to re-electrophoresis on a new sodium dodecyl sulfate-polyacrylamide gel, all the counts penetrated the gel and migrated in the position of the small polypeptide. Thus, the sialic acid of the small polypeptide carries virtually all of the radioactivity.

Fig. 5. The radioactivity profile of purified NaK-ATPase cross-linked with CP, then oxidized with sodium periodate, and reduced with sodium borohydride. The procedure is described under “Materials and Methods.” Approximately 30 μg of NaK-ATPase treated (○—○), or not treated (x—x) with CP were subjected to electrophoresis in 3.3% Na dodecyl-SCN polyacrylamide gels. The gels were stained, destained, sliced into 2-mm sections, and counted. The bottom trace (●—●) shows the radioactivity profile of a gel which contained 30 μg of NaK-ATPase cross-linked with CP, oxidized with sodium periodate acid, reduced with tritiated sodium borohydride and then treated with 0.5 mg/ml of neuraminidase (Clostridium perfringens) for 15 min at room temperature.
amount of the large polypeptide, respectively. Thus the rate of loss of ATPase activity is more rapid than the rate of loss of the large polypeptide. This suggests that the CP-catalyzed air oxidation of NaK-ATPase probably results in the formation of intrachain as well as interchain disulfide bonds. Both types of disulfide bonds may be responsible for the loss of ATPase activity.

Experiments aimed at determining if the loss of ATPase activity could be reversed were performed by adding β-mercaptoethanol to a final concentration of 50 mM to samples of NaK-ATPase which had been previously incubated with CP for 30 min. The samples were kept at room temperature for 15 min before being assayed for ATPase activity. The results showed that approximately 20% of the ATPase activity could be recovered when compared to samples of NaK-ATPase not incubated with β-mercaptoethanol.

(o-Phenanthroline)₃-Cupric Sulfate Cross-linking of Canine Kidney Microsomes—It is possible that purification of NaK-ATPase may result in significant clustering of the enzyme in the lipid bilayer. Indeed, Jørgensen (2) has recently presented electron micrographs of purified NaK-ATPase which shows regions in the membrane which probably contains clusters of the enzyme. Thus, cross-linking experiments on highly purified NaK-ATPase may not be representative of the true subunit structure of the enzyme. Therefore, experiments were performed to determine if cross-linking NaK-ATPase in kidney microsomes would yield results similar to those found for purified NaK-ATPase. The procedure was to cross-link crude microsomes with CP, followed by purification of NaK-ATPase from the microsomes. Sodium dodecyl sulfate-polyacrylamide gels were then run on the purified material.

Optical density measurements of gels which contained NaK-ATPase incubated with CP for different periods of time revealed the loss of the large polypeptide. Thirty-three per cent and 45% of the large polypeptide had disappeared by the end of 10 and 30 min, respectively (Fig. 7). Simultaneously with the disappearance of the large polypeptide, there appeared a band on the gel with a molecular weight of 200,000. A reciprocal relationship existed between the amounts of stain present in the M₄ = 200,000 and 100,000 regions. Similar to the experiments done on purified NaK-ATPase, including 2% β-mercaptoethanol in the electrophoresis sample buffer for 30 min prior to electrophoresis caused the M₄ = 200,000 protein to revert to the M₄ = 100,000 large polypeptide. Also, pretreatment of the microsomes with MalNEnt for 60 min prior to incubation with CP prevented the formation of the M₄ = 200,000 protein. Thus, these results suggest that the M₄ = 200,000 protein is a covalent dimer of two large polypeptide molecules.

A time course experiment revealed that the large polypeptide was converted into the M₄ = 200,000 dimer, followed by aggregation into higher molecular weight material which stayed at the top of the gel. Thus it is important to note that (a) at any one time only a small fraction of the large polypeptide exists in dimer form, and (b) no bands corresponding to molecular weights of 300,000, 400,000, or 500,000 were detected.

Cross-linking of the small polypeptide by CP in crude kidney microsomes yields results similar to those found when NaK-ATPase is cross-linked after purification. At the end of 10 min there was no detectable loss in the small polypeptide as revealed by optical density measurements. By the end of 30 and 45 min the amount of small polypeptide lost was 10% and 15%, respectively.

**DISCUSSION**

Considerable evidence suggests that the molecular weight of native NaK-ATPase is about 200,000 to 300,000. The present results show that two large polypeptides of NaK-ATPase can be specifically cross-linked to yield an M₄ = 200,000 dimer. Thus the functional subunit structure of the enzyme consists of at least two large polypeptides. Previous cross-linking experiments with dimethylsulphurimide resulted in the cross-linking of one large to one small polypeptide (11). Unfortunately, these experiments were performed on highly purified enzyme. The extent to which cross-linking results obtained on purified enzyme can be taken to represent the in vitro situation is not clear. However, if the αβ structure observed for purified NaK-ATPase is representative of the true subunit structure, then clearly the quaternary structure of NaK-ATPase must be α₂β₂. This finding would agree with Jørgensen, who has shown that purified preparations of NaK-ATPase have a molar ratio of 1 large to 1 small polypeptide (12).

The present communication shows the presence of high molecular weight oligomeric structure of the large polypeptide of NaK-ATPase when the enzyme is cross-linked in purified form. In marked contrast, however, only an M₄ = 200,000 dimer of the large chain is formed when NaK-ATPase is treated with CP in crude microsomes. The difference may be accounted for in one of two ways. First, the procedure used to purify NaK-ATPase may result in stable aggregates of the enzyme in the membrane. Indeed, Jørgensen recently presented electron micrographs suggesting that the purified enzyme may be highly clustered (2). Clustering of the enzyme could conceivably bring in close contact molecules of NaK-ATPase and thus facilitate the formation of oligomers of the large polypeptide. Second, purified NaK-ATPase may be able
to undergo rapid diffusion in the plane of the membrane. If this is true, in the time it takes to do the (o-phenanthroline)cupric sulfate reaction, a single molecule of NaK-ATPase would have sufficient time to undergo a large number of collisions with other enzyme molecules. Thus it is conceivable that (o-phenanthroline)cupric sulfate could give rise to trimers, tetratomers, pentamers, and higher oligomeric forms of the large polypeptide. Indeed, if NaK-ATPase is also capable of rapid diffusion in the microsomal membranes, the absence of oligomeric forms of the large polypeptide greater than dimer in microsomal cross-linked material might be accounted for by the rapid reaction of the dimer with other membrane proteins immediately after its formation. This mechanism would account for the absence of higher molecular weight forms of the large polypeptide greater than dimer, as well as the disappearance of the dimer and the subsequent formation of material at the gel top.

I have presented data which show NaK-ATPase to be composed of subunits. Thus, the question arises as to whether subunits are a general structural requirement for transport by membrane proteins. Experiments on two other transport proteins suggest that this may be the case. Cross-linking experiments on the putative anion carrier of the human red cell membrane (Band 3) have shown this protein to exist as a dimer in the membrane (28). Also, preliminary cross-linking experiments in our laboratory on the calcium ATPase of sarcoplasmic reticulum have shown it to be composed of dimers. Thus, taken together, all these data strongly suggest that the unit of function for membrane transport proteins is that of an oligomeric structure.

Acknowledgments—I wish to express my sincere gratitude to Dr. Guido Guidotti in whose laboratory this work was carried out. Also, I wish to thank Robert Jackson and Siu Tong for critically reading the manuscript, Kurt Drickamer for bringing to my attention the procedure for modification of sialic acid, and the Cardiovascular Research Unit of Massachusetts General Hospital for the generous gift of canine kidneys.

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Quaternary structure of (Na+ + K+)-dependent adenosine triphosphatase.
G J Giotta

J. Biol. Chem. 1976, 251:1247-1252.

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