Monomer of Sodium and Potassium Ion Activated Adenosinetriphosphatase Displays Complete Enzymatic Function†

William S. Craig*

ABSTRACT: The distribution of sodium and potassium ion activated adenosinetriphosphatase [(Na^+ + K^+)-ATPase] among the various oligomeric forms present in a given solution is assessed unambiguously by cross-linking with glutaraldehyde. Purified enzyme dissolved in a solution of a nonionic detergent, octaethylene glycol dodecyl ether, remains dispersed and unaggregated after removal of the bulk of the detergent. Increases in the aggregation of the enzyme, which have been previously observed upon the addition of substrates to such a solution, are found to be due to changes in ionic strength rather than a consequence of the initiation of turnover. Furthermore, conditions are described that produce solutions containing stable, enzymatically active mixtures of the smaller oligomers of the asymmetric unit, $\alpha\beta$. Cross-linking by glutaraldehyde while the enzyme is turning over demonstrates that at least one of these oligomers is responsible for the observed enzymatic activity. A determination of which oligomers are present in each fraction from a glycerol gradient demonstrates that the profiles of the enzymatic activity and the concentration of monomer coincide. In addition, the monomer can form the sodium-dependent, phosphorylated intermediate of the mechanism for this enzyme. Finally, a preparation of (Na^+ + K^+)-ATPase, dissolved in solutions of the same nonionic detergent, can be prepared in which the predominant species (>85%) is the monomer. The enzyme in this solution exhibits high specific activity, and its apparent Michaelis constants for the cationic substrates are very similar to those of the purified, membrane-bound enzyme. It is concluded from these results that a monomer of the $\alpha\beta$ asymmetric unit is fully capable of catalyzing (Na^+ + K^+)-ATPase activity, and hence active transport, in the native enzyme. A reassessment of proposed molecular mechanisms for active transport is made in light of these discoveries.

The molecular features of the mechanism responsible for the active transport of sodium and potassium ions across the plasma membrane are poorly understood. Theoretical arguments proposing that the channel through which the cations pass lies along the dyad axis formed by the juxtaposition of two identical asymmetric units have been made (Kyte, 1975). Whether or not this is actually the case, however, must be determined experimentally. Crucial evidence addressing this question would be an experimental definition of the smallest unit of the enzyme necessary to catalyze active transport.

The enzyme responsible for this process (Skou, 1960), sodium and potassium ion activated adenosinetriphosphatase [(Na^+ + K^+)-ATPase],[1] is composed of a catalytic polypeptide, designated α, and a sialoglycoprotein, designated β, which are present in an equimolar ratio (Kyte, 1972; Liang & Winter, 1977; Craig & Kyte, 1980). Chemical cross-linking has established that the two polypeptides are both members of the same specific complex (Craig & Kyte, 1980). In addition, an estimate of the molecular weight of the protein portion of the minimum asymmetric unit, $\alpha\beta$, from which this complex is constructed is 177 000 ± 33 000 (Craig & Kyte, 1980), a value consistent with recent evaluations of the stoichiometries of the sites for ligands (Moczydlowski & Fortes, 1981a; Kyte, 1972), although other values have been reported (Freytag & Reynolds, 1981).

It has been claimed that, in solutions of nonionic detergents, (Na^+ + K^+)-ATPase forms monodisperse solutions containing only dimer (Hastings & Reynolds, 1979), dimer (Esmann et al., 1980), or monomer[2] (Brotherus et al., 1981). The shortcomings of the methods used to estimate protein molecular weight in these studies have been discussed (Kyte, 1981). It is also the case that the measurements were never performed on enzyme that was turning over. More to the point, when glutaraldehyde cross-linking was used to assess unambiguously the aggregation state of (Na^+ + K^+)-ATPase, solutions of enzyme in nonionic detergents, prepared as described in these earlier publications, were found to contain a rather complex mixture of different oligomers (Craig, 1982), and no evidence for monodispersity was obtained. Furthermore, in the presence of assay substrates, striking changes in the distribution among the oligomers of the dissolved (Na^+ + K^+)-ATPase occur (Craig, 1982). Taken together, these results bring into serious question earlier claims that this or other active-transport enzymes can function as monomers (Brotherus et al., 1981; Møller et al., 1980).

This report describes a preparation of (Na^+ + K^+)-ATPase that is dissolved in a solution of nonionic detergent, that retains enzymatic activity, and that contains a mixture of the smaller oligomers. When this preparation is applied to a density gradient, the sedimentation velocity profile of the enzymatic activity correlates precisely with the distribution of a monomeric species identified by the glutaraldehyde cross-linking assay. Furthermore, this monomer is capable of forming the

† From the Department of Chemistry, D-006, University of California, San Diego, La Jolla, California 92093. *Received April 26, 1982.* This research was supported by Grants HL-17879, GM-07169, and AM-07233 from the National Institutes of Health, Grant PCM78-24284 from the National Science Foundation, and Grant-in-Aid AHA 81-1003 from the American Heart Association, all of which provide support to the laboratory of Jack Kyte.

* Address correspondence to this author at the Department of Biochemistry, Dartmouth College, Hanover, NH 03756.

[1] Abbreviations: (Na^+ + K^+)-ATPase, sodium and potassium ion activated adenosinetriphosphatase; $C_{12}E_8$, octaethylene glycol dodecyl ether (3,6,9,12,15,18,21,24-octaoxahexatriacontan-1-ol); $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

[2] From hereon, monomer, dimer, trimer, and tetramer will refer to the oligomers of the asymmetric unit, $\alpha\beta$, $(\alpha\beta)_2$, $(\alpha\beta)_3$, and $(\alpha\beta)_4$, respectively.

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covalent, phosphorylated intermediate of the enzymatic reaction. Finally, a preparation of enzyme dissolved in a solution of nonionic detergent has been produced in which the majority (>85%) of the macromolecules present are identified as monomers by cross-linking and yet full enzymatic activity is expressed.

Experimental Procedures

Materials. Octaethylene glycol dodecyl ether ($C_{12}E_8$) was obtained from Nikkol Chemical Co. and was recrystallized from hexane before use. Glutaraldehyde was obtained from Polysciences and was a gift of Dr. K. Tokuyasu, University of California, San Diego. [γ-^{32}P]ATP was purchased from the Amersham Corp. as the triethylammonium salt with a reported specific radioactivity of 30 Ci mmol^{-1}. It was diluted with nonradioactive ATP before use. Standards for sedimentation velocity in gradients of 10–30% glycerol, listed with their sources and sedimentation coefficients, were human transferrin (gift of Dr. Paul Saltman, University of California, San Diego), 6.1 S; lactate dehydrogenase from rabbit muscle (Sigma), 7.2 S; and aspartate transcarbamylase from *Escherichia coli* (gift of Dr. H. K. Schachman, University of California, Berkeley), 11.7 S. Bovine serum albumin, ovalbumin (Sigma Chemical Co.), and phosphorylase *a* (gift of James Stull, University of California, San Diego) were used as polypeptide standards for gel electrophoresis.

Enzyme Preparations. Three distinct preparations of (Na^+ + K^+)-ATPase, purified, membrane-bound (Na^+ + K^+)-ATPase, supernatant–$C_{12}C_8$ enzyme, and soluble (Na^+ + K^+)-ATPase, were used in these experiments. Purified, membrane-bound (Na^+ + K^+)-ATPase was prepared by published procedures from canine renal medulla (Jørgensen, 1974; Munson, 1981). The usual specific activity of this enzyme was 900–1100 μmol of P_i (mg of protein)$^{-1}$ h^{-1} at 37 °C, and $NaDodSO_4$–polyacrylamide gels of the preparation contained only the α and β subunits of (Na^+ + K^+)-ATPase. Supernatant–$C_{12}E_8$ enzyme was prepared as described previously (Craig, 1982) by employing a ratio of 3 mg (5.6 μmol) of detergent (mg of protein)$^{-1}$ in a solution buffered with imidazolium chloride at pH 7.1. The specific activities routinely obtained were 700–1200 μmol of P_i (mg of protein)$^{-1}$ h^{-1} upon dilution into normal assay medium at 37 °C.

Finally, soluble (Na^+ + K^+)-ATPase, the third preparation, is produced by a modification of the second procedure. Supernatant–$C_{12}E_8$ enzyme is prepared at a ratio of 1.2–1.5 mg of detergent (mg of protein)$^{-1}$ in a solution containing 100 mM NaCl and 5% glycerol. Immediately before a centrifugation at 100000*g* for 30 min at room temperature in a Beckman Airfuge, a step sufficient to remove >95% of purified, membrane-bound (Na^+ + K^+)-ATPase from the supernate, 0.5 volume of 20 mM imidazolium chloride, pH 7.1, is added to the sample. After centrifugation, the sample is dialyzed for 60 min at 3 °C against the same buffer, containing 5% glycerol, to further reduce the ionic strength. This dialysis results in the removal, from the sample, of <2% of the detergent. When assayed at 11 °C in 10 mM NaCl and 2 mM KCl, this enzyme routinely displayed a specific activity of 16–25 μmol of P_i mg^{-1} h^{-1}. Under the same conditions of low temperature and less than saturating concentrations of cations, purified, membrane-bound (Na^+ + K^+)-ATPase had a specific activity of 20 μmol of P_i mg^{-1} h^{-1}.

Enzymatic Assays. Supernatant–$C_{12}E_8$ enzyme was assayed at 37 °C by direct addition into detergent-free medium such that the diluted concentration of detergent was below its critical micelle concentration. This assay for strophanthidin-sensitive (Na^+ + K^+)-ATPase activity has been described (Kyte, 1971b). It was modified by the method of Peterson (1978) to eliminate interference from nonionic detergents during the formation of the phosphate–molybdate complex. The concentrations of NaCl and KCl in the assay were 100 and 20 mM, respectively. On the other hand, when either fractions from a gradient of 10–30% glycerol or soluble (Na^+ + K^+)-ATPase were assayed, the reaction, always performed in a low ionic strength medium, was initiated by the addition of assay substrates (0.1 volume) such that the final concentrations of sodium and potassium were 10 and 2 mM, respectively, and the final concentration of MgATP was 3 mM. Often the volume of the sample assayed in this manner was as small as 10 μL, and the volumes of quench solutions were adjusted accordingly. In addition, these assays were often carried out at reduced temperature (21 or 11 °C), and incubation times were varied accordingly. Nevertheless, product formation was consistently linear over the reaction period of the assay. Protein determination was by the Lowry method whose extinction coefficients were determined by quantitative amino acid analysis as previously described (Kyte, 1971b). Fractions from the gradients of 10–30% glycerol were analyzed directly for protein by quantitative amino acid analysis.

Assay for Detergent. The concentration of $C_{12}E_8$ in fractions from the gradient was estimated by measuring the absorbance at 320 nm of the complex formed between the detergent and ammonium cobaltothiocyanate (Garewal, 1973). A standard curve was derived from weighed samples of recrystallized detergent.

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gels were prepared with 3.6% acrylamide and 0.1% methylenebis(acrylamide) according to the method of Shapiro et al. (1967) as modified by Weber & Osborn (1969). Samples were prepared by the addition of an amount of 20% $NaDodSO_4$ sufficient to provide at least a 5-fold weight excess over protein, incubated 12 h at 22 °C, and submitted to electrophoresis. Gels were stained and then scanned, and peak areas were integrated as previously described (Craig & Kyte, 1980). Samples that had been phosphorylated with [γ-^{32}P]-MgATP were submitted to electrophoresis on 3.6% acrylamide gels buffered at pH 2.4 (Avruch & Fairbanks, 1972). The solutions in the reservoirs of the apparatus were buffered at pH 7.4 with Tris–acetate (Fairbanks et al., 1971).

Cross-Linking with Glutaraldehyde. This procedure has been previously described (Craig, 1982). Typically, glutaraldehyde is added to samples to provide a final concentration of 12 mM. Following a 45-min incubation at room temperature, the reaction is quenched by the addition of an 8-fold molar excess of glycine. After several minutes, the polypeptides are denatured with $NaDodSO_4$ in preparation for electrophoresis.

Samples cross-linked during turnover of the enzyme were prepared by adding substrates in 0.1 volume such that the final concentrations of NaCl and KCl were either 100 and 20 mM, respectively, or 10 and 2 mM, respectively. The final concentration of MgATP was always 3 mM, and additional $C_{12}E_8$ (40 μM) was added to some samples. Furthermore, a sample in which 100 mM choline chloride was substituted for the NaCl and KCl was also prepared as a control for the effects of ionic strength. After an incubation for 5 min at either 21 or 11 °C, glutaraldehyde was added to all of the samples (0.1 volume) to produce a final concentration of 12 mM. Following an additional incubation for 60 min at the same temperature, the reaction was quenched, and the polypeptides were prepared for electrophoresis. Duplicate samples, lacking only glutaraldehyde, were assayed for enzymatic activity over at least a

30-min period under conditions identical in temperature, dilution, and substrate concentrations with those utilized for the cross-linking reaction.

Samples that had been phosphorylated at 11 °C with [γ-^{32}P]MgATP were cross-linked rapidly by the addition of 0.1 volume of 0.8 M glutaraldehyde. The high concentration was used to hasten the reaction so that after 3 min at 11 °C it could be quenched and the polypeptides could be denatured by the addition of 0.1 volume of 20% $NaDodSO_4$.

Sedimentation on Glycerol Gradients. Samples (200 μL) of supernatant–$C_{12}E_8$ enzyme (2.5 mg mL^{-1}), containing 100 mM KCl or NaCl and 5% glycerol, were applied to linear gradients of 10–30% glycerol (3.8 mL) containing 20 mM imidazolium chloride, pH 7.1. The gradients (path length 5.5 cm) were subjected to centrifugation at 60000 rpm in a Beckman SW60 rotor at 4 °C for 7 h. After centrifugation, the bottom of the tube was punctured, fractions (90–120 μL) were collected, and each fraction was assayed for $(Na^+ + K^+)$-ATPase activity and $C_{12}E_8$ concentration. The fractions containing the majority of the ATPase activity (Figure 1) were pooled. In separate experiments, individual fractions were assayed and also cross-linked with glutaraldehyde during enzyme turnover. The positions of the sedimentation velocity standards in companion gradients were determined by measuring A_{280} of individual fractions.

Phosphorylation of $(Na^+ + K^+)$-ATPase Oligomers. Samples (45 μL) of soluble $(Na^+ + K^+)$-ATPase (1.9 mg mL^{-1}, <11 mM $C_{12}E_8$),[3] samples (50 μL) of pooled, active fractions from a glycerol gradient, or samples (50 μL) of individual fractions were phosphorylated at 11 °C with 170 μM [γ-^{32}P]MgATP (0.25 Ci $mmol^{-1}$), 13 mM NaCl or 4 mM KCl and 40 μM additional $C_{12}E_8$. The reaction was initiated by the addition of 0.1 volume of 1.7 mM [γ-^{32}P]MgATP. After a 15-s incubation at 11 °C, glutaraldehyde was added such that the final concentration in the reaction mixture was 80 mM. At the end of a 3-min incubation at 11 °C, 0.1 volume of 20% $NaDodSO_4$ was added to denature the polypeptides. Each sample (final volume 75 μL) was immediately transferred to the top of a column (0.9 mL) of Sephadex G-50 (medium). The column was prepared in a disposable syringe (1 mL), equilibrated in 10 mM sodium phosphate, pH 2.4, and 0.1% $NaDodSO_4$, and submitted to centrifugation beforehand at 700 rpm in a clinical centrifuge for 3 min (Penefsky, 1977). After the samples were loaded atop the prespun columns, they were submitted to centrifugation for 3 min, and the effluent was collected. The pH of the original reaction mixture was 7.1 while that of the effluent was 2.5–3.0. In a separate experiment, samples (20 μL) of soluble $(Na^+ + K^+)$-ATPase were diluted 2-, 5-, and 10-fold with 20 mM imidazolium chloride, pH 7.1, containing 40 μM additional $C_{12}E_8$ and phosphorylated under the above conditions. After being cross-linked, the polypeptides were denatured, and the samples (280 μL) were eluted from columns (1.9 mL) of G-50 previously equilibrated at pH 2.4. After pyronin Y (tracking dye) and glycerol were added to final concentrations of 1 μg mL^{-1} and 10%, respectively, each of these effluents was submitted to electrophoresis on 3.6% $NaDodSO_4$–polyacrylamide gels buffered at pH 2.4, and the gels were scanned at 280 nm and sliced into 2.5-mm discs that were added to 0.5 mL of distilled water and assayed for radioactivity (Parker & Elerick, 1966). Identical, nonradioactive samples, run on $NaDodSO_4$–polyacrylamide gels, were stained for protein and then destained and scanned at 550 nm.

[3] Since much of the detergent is lost with the pellet, the notation <11 mM $C_{12}E_8$ has been adopted when referring to the detergent concentration in the supernate.

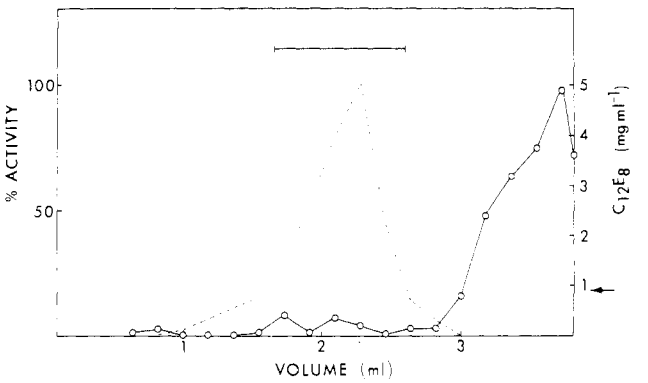

FIGURE 1: Removal of detergent from supernatant–$C_{12}E_8$ enzyme on a linear glycerol gradient. A sample of supernatant–$C_{12}E_8$ enzyme (200 μL of 2.5 mg of protein mL^{-1} and <22 mM $C_{12}E_8$) with a specific activity of 600 μmol of P_i mg^{-1} mL^{-1} at 37 °C was submitted to centrifugation through a linear gradient of 10–30% glycerol buffered with 20 mM imidazolium chloride, pH 7.1. Fractions were assayed for both $(Na^+ + K^+)$-ATPase activity (dashed line) and $C_{12}E_8$ concentration (solid line). The critical micelle concentration of the detergent (90 μM) is indicated on the right-hand axis of the ordinate with an arrow. The activity of the peak fraction from the activity profile was 280 μmol of P_i mL^{-1} h^{-1} at 37 °C. The bar in the upper part of the figure indicates the region of the gradient that was pooled.

Results

Removal of Detergent and Detection of Active Oligomers in Supernatant–$C_{12}E_8$ Enzyme. Supernatant–$C_{12}E_8$ enzyme (Craig, 1982) was subjected to centrifugation through a gradient of 10–30% glycerol containing no detergent. Individual fractions from the gradient were assayed for $(Na^+ + K^+)$-ATPase activity by the direct addition to substrates, and for $C_{12}E_8$ concentration (Figure 1). The bulk of the enzymatic activity present on the gradient has been separated from the region containing $C_{12}E_8$ concentrations greater than the critical micelle concentration. The fractions containing active enzyme were pooled as indicated for further experiments. The yield of activity from gradients of this type was 30–40%, based on the total activity of the supernatant–$C_{12}E_8$ enzyme added.

For determination of the oligomeric composition of this enzyme while it was turning over in the assay, aliquots of the pool from this gradient were incubated in a solution containing 3 mM MgATP, 100 mM NaCl, and 20 mM KCl, in a solution containing 100 mM choline chloride, or in buffer alone. After 5 min at 21 °C, the samples were cross-linked and the products examined by electrophoresis (Figure 2). The numerals 1, 2, 3, and 4 in Figure 2A indicate the relative electrophoretic mobilities of the covalent products derived from the stable, noncovalent oligomers, monomer, dimer, trimer, and tetramer, respectively, present in the solution prior to the addition of the glutaraldehyde (Craig, 1982). Evidence has been presented previously that these cross-linking patterns provide an accurate catalog of the molecular species present in solutions of protein and detergent (Craig, 1982), and it can be concluded that the pool, before addition of assay reagents, contained a mixture of monomer, dimer, trimer, and tetramer, approximately in the proportions indicated in Figure 2A. On the other hand, when a sample was cross-linked under the same conditions with which the enzyme was turning over actively in a companion tube (62 μmol of P_i mg^{-1} h^{-1} at 21 °C), the majority of the protein had aggregated rapidly to oligomers greater in size than a tetramer (Figure 2B). Under the same conditions of low temperature, purified, membrane-bound $(Na^+ + K^+)$-ATPase had a specific activity of 176 μmol of P_i mg^{-1} h^{-1}. Similar aggregation was also observed, however, when high ionic

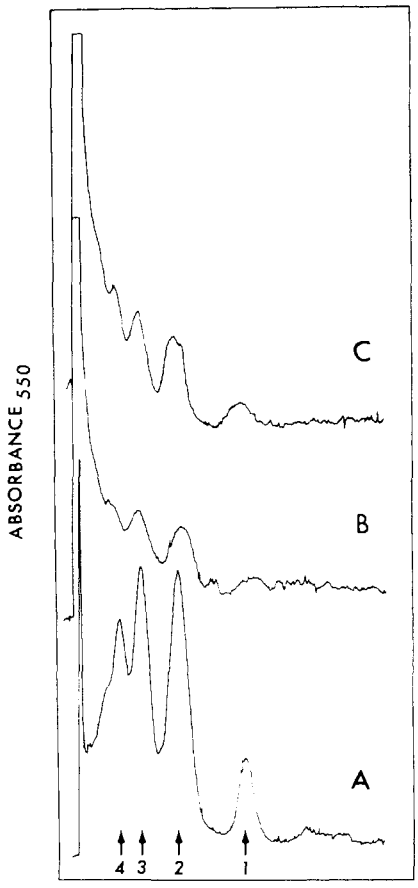

FIGURE 2: Scans of $NaDodSO_4$–polyacrylamide gels of active, supernatant–$C_{12}E_8$ enzyme cross-linked in the presence (B) or absence (A) of substrates or in the presence of choline chloride (C). To aliquots (50 μL of 0.3 mg of protein mL^{-1}) of pooled, active fractions from a gradient (Figure 1) was added 0.1 volume of either buffer alone (20 mM imidazolium chloride, pH 7.1), buffer containing substrates such that the final concentration in the solution would be 3 mM MgATP, 100 mM NaCl, and 20 mM KCl, or buffer containing 3 mM MgATP and choline chloride such that its final concentration would be 100 mM. After an incubation of 5 min at 21 °C, the samples were cross-linked with 12 mM glutaraldehyde for 60 min at 21 °C. After the reaction was quenched, the polypeptides were denatured with $NaDodSO_4$ and submitted to electrophoresis on 3.6% polyacrylamide gels. The gels were stained and scanned at 550 nm. The positions of the products from the cross-linking observed on the gel are indicated by the arrows labeled 1, 2, 3, and 4. The direction of electrophoresis is from left to right.

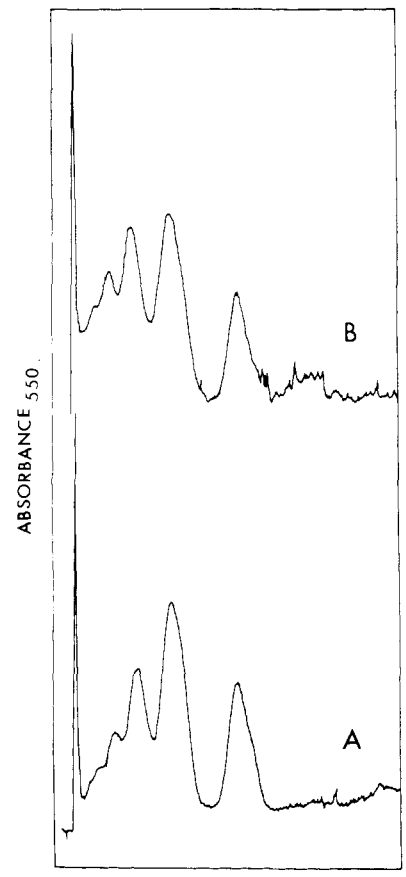

FIGURE 3: Scans of $NaDodSO_4$–polyacrylamide gels of active, supernatant–$C_{12}E_8$ enzyme in the presence (B) or absence (A) of substrates at low ionic strength. To aliquots (50 μL of 0.3 mg of protein mL^{-1}) of pooled, active fractions from a gradient (Figure 1) was added 0.1 volume of either buffer alone (20 mM imidazolium chloride, pH 7.1) or buffer containing substrates such that the final concentrations would be 3 mM MgATP, 10 mM NaCl, 2 mM KCl, and 40 μM additional $C_{12}E_8$. After an incubation of 5 min, the samples were cross-linked and submitted to $NaDodSO_4$–polyacrylamide gel electrophoresis. The direction of electrophoresis is from left to right.

strength was established in another sample with choline chloride (Figure 2C), a cation which cannot react with (Na^+ + K^+)-ATPase. This suggests that the aggregation observed when the sample was added to assay reagents (Figure 2B), which has been previously reported (Craig, 1982), is not a consequence of or a prerequisite for enzyme turnover, but rather a consequence of the high ionic strength imposed on the sample by the salts.

After some experimentation, it was discovered that when an aliquot of the pooled fractions from the gradient was cross-linked in the presence of an enzymatic assay medium of low ionic strength containing 40 μM additional $C_{12}E_8$, 3 mM MgATP, 10 mM NaCl, and 2 mM KCl in 20 mM imidazolium chloride, pH 7.1, aggregation of the protein could be prevented under conditions where the enzyme was turning over (Figure 3). It was learned that a decrease in the ionic strength, the addition of $C_{12}E_8$ to a level just below that which inhibits enzymatic activity, and a reduction of the temperature to either 21 or 11 °C are all necessary to produce conditions where no significant aggregation occurs while enzyme in an identical companion tube is turning over actively (48 μmol of P_i mg^{-1} h^{-1} at 21 °C in the experiment described). Under the same conditions of low temperature and less than saturating concentrations of cations, purified, membrane-bound (Na^+ + K^+)-ATPase had a specific activity of 77 μmol of P_i mg^{-1} h^{-1}. These results show that some, or all, of the oligomers smaller in size than a pentamer are responsible for the observed (Na^+ + K^+)-ATPase activity.

It is appropriate at this point to note that, although the profile of enzymatic activity presented in Figure 1 was obtained with an assay involving dilution and leading to aggregation (Figure 2B), the same profile is also obtained with the assay just described, which does not cause aggregation (Figure 3). This further supports the conclusion that the aggregation is largely irrelevant to turnover of the enzyme.

Distribution of Oligomers after Centrifugation through Detergent-Free Glycerol Gradients. A sample of supernatant–$C_{12}E_8$ enzyme was subjected to centrifugation through a gradient of 10–30% glycerol lacking detergent. The results of both activity assays and cross-linking assays of individual fractions are presented in Figure 4. Very little separation of monomer, dimer, trimer, and tetramer was achieved. This result, however, is entirely consistent with earlier, more extensive ones demonstrating that, as the concentration of detergent present in a gradient is lowered, the oligomers sediment with nearly indistinguishable sedimentation coefficients (Craig,

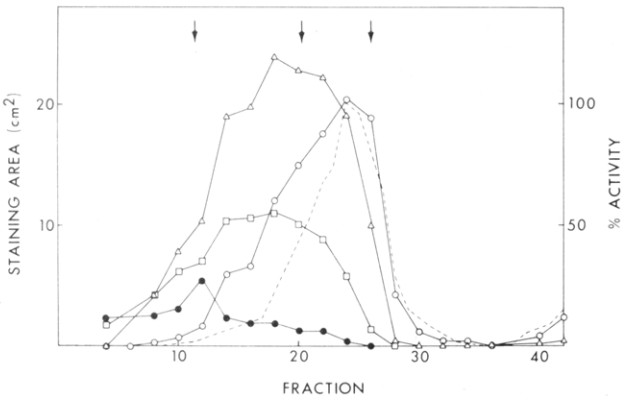

FIGURE 4: Separation of supernatant–$C_{12}E_8$ enzyme on a linear gradient of 10–30% glycerol. A sample (200 μL) of supernatant–$C_{12}E_8$ enzyme [5.6 μmol of $C_{12}E_8$ (mg of protein)$^{-1}$] with a specific activity of 1100 μmol of P_i mg^{-1} h^{-1} at 37 °C was submitted to centrifugation through a linear gradient of 10–30% glycerol buffered with 20 mM imidazolium chloride, pH 7.1. Fractions were assayed for $(Na^+ + K^+)$-ATPase activity (dashed line) and analyzed with the glutaraldehyde cross-linking assay, during turnover, for the presence of the various complexes, monomer, dimer, trimer, and tetramer. The specific enzymatic activity at the peak of the profile was 730 μmol of P_i mg^{-1} h^{-1} at 37 °C. Stained $NaDodSO_4$–polyacrylamide gels of each of the cross-linked fractions were scanned in order to quantify the amount of each complex present in the individual fractions (Craig & Kyte, 1980). Indicated in the graph is the absolute staining area of the covalent complexes α-β (○), $(\alpha$-$\beta)_2$ (△), $(\alpha$-$\beta)_3$ (□), and $(\alpha$-$\beta)_4$ (●) derived from the respective noncovalent oligomers present in each fraction assayed. The positions of sedimentation standards in companion gradients were determined and are indicated on the graph by the upper arrows. The standards were human transferrin (6.1 S), lactate dehydrogenase (7.2 S), and aspartate transcarbamylase (11.7 S) in that order from right to left.

1982). When the same fractions represented in Figure 4 were submitted to cross-linking after storage at 4 °C for 48 h, the distribution of oligomers remained unchanged. This result, as well as ones presented previously (Craig, 1982), rules out any significant interconversions of complexes during the course of these experiments.

In all of the experiments performed with gradients, fractions were present, which, when cross-linked during turnover, were found to contain only $\alpha\beta$ monomers (Figure 4, fractions 28–32). These fractions generally contained less than 50 μg mL^{-1} protein, and their specific activity was difficult to determine. Nevertheless, within the experimental error of quantitative amino acid analysis on these very dilute solutions (±20%), the specific activities were consistently greater than 15 μmol of P_i mg^{-1} h^{-1}, when assayed at 11 °C. Under the same conditions of low temperature and less than saturating concentrations of cations, purified, membrane-bound $(Na^+ + K^+)$-ATPase had a specific activity of 20 μmol of P_i mg^{-1} h^{-1}.

The most provocative feature of Figure 4 is that the profile of enzymatic actively closely parallels the profile of the monomeric species identified by the glutaraldehyde cross-linking assay. Since both the assay of enzymatic activity and the cross-linking were performed under identical conditions, these results suggest that it is a monomer that is responsible for the ATPase activity. There is an even more striking indication that this is the case. In the gel scans of several gradient fractions (Figure 5), two barely resolved covalent components, designated A and B, can be observed. They can be distinguished, however, not only by differences in their electrophilic mobilities but also by the differences in the sedimentation coefficients of their noncovalent precursors because these can be resolved partially on the gradients. This latter observation establishes the unique identities of the two noncovalent complexes from which the two covalent complexes are derived.

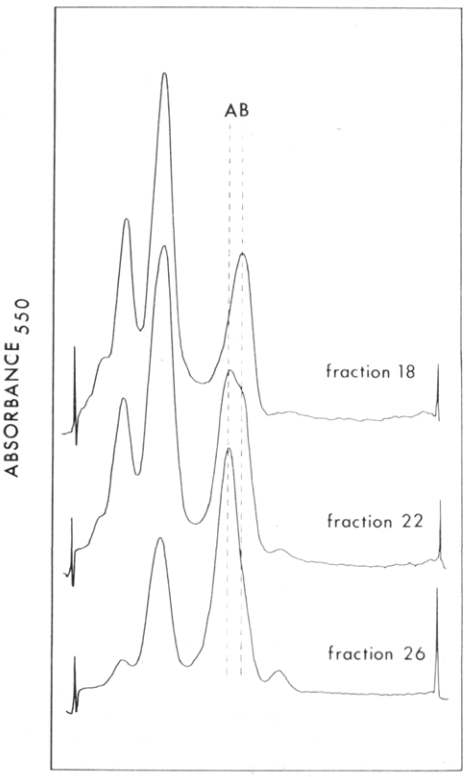

FIGURE 5: Scans of $NaDodSO_4$–polyacrylamide gels of cross-linked fractions from a gradient of 10–30% glycerol. Supernatant–$C_{12}E_8$ enzyme was submitted to centrifugation through a linear gradient of 10–30% glycerol (Figure 4). The fractions were cross-linked during turnover of the enzyme and then submitted to electrophoresis. Scans of the gels from several fractions are presented. Indicated by the dashed lines are the electrophoretic positions of the slower (A) and faster (B) covalent complexes. The direction of electrophoresis is from left to right.

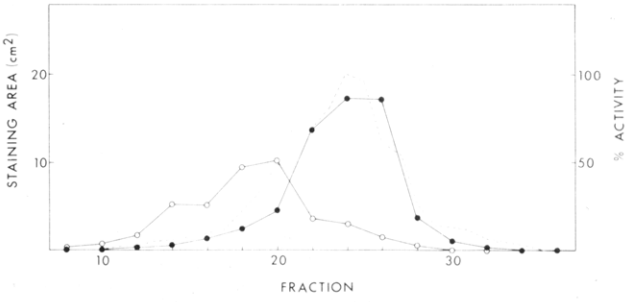

FIGURE 6: Coincidence of the distribution of $(Na^+ + K^+)$-ATPase activity and the monomeric $\alpha\beta$ unit on a gradient of 10–30% glycerol. A sample of supernatant–$C_{12}E_8$ enzyme was submitted to centrifugation through a linear gradient of 10–30% glycerol (Figure 4). Aliquots from the fractions were either assayed for $(Na^+ + K^+)$-ATPase activity or cross-linked during turnover and submitted to electrophoresis. From scans of stained gels, the absolute staining area of each covalent complex was determined. Indicated in the graph is the distribution of the absolute staining area of the covalent complex α-β, that possesses the slower electrophoretic mobility [(A) in Figure 5] (●) and that of the covalent complex, α-β, that possesses the faster electrophoretic mobility [(B) in Figure 5] (○). Enzymatic activity is indicated by the dashed line. The activity of the peak tube in the profile was 440 μmol of P_i h^{-1} mL^{-1}.

Nevertheless, both covalent complexes have electrophoretic mobilities that are so close to that expected for a monomer of the $\alpha\beta$ asymmetric unit (0.51) (Craig, 1982) that the difference in their respective mobilities is smaller than that which would occur as the result of the presence of an additional polypeptide, either α or β, in the larger covalent complex. Therefore, both components A and B are presumed to be covalent complexes of an $\alpha\beta$ unit.

The $(Na^+ + K^+)$-ATPase activity in the gradient can be assigned to only one of these components. In Figure 6, the distribution of enzyme activity over the same gradient as that displayed in Figure 4 has been plotted along with the distribution of only the individual integrated areas of the two monomers, A and B. It can be seen that the profile of enzymatic activity now corresponds precisely to the profile of the monomer possessing the slower electrophoretic mobility, A. This indicates that in this gradient it is only this one particular monomeric species that is active, the remaining oligomers being incapable of $(Na^+ + K^+)$-ATPase activity. If it is assumed that only the one form of the monomer is active, it can be calculated from the profile of activity in Figure 6 that the specific activity of this enzyme assayed at 11 °C is 24 μmol of P_i mg^{-1} h^{-1}. Under the same conditions of low temperature and less than saturating concentrations of cations, purified, membrane-bound $(Na^+ + K^+)$-ATPase had a specific activity of 20 μmol of P_i mg^{-1} h^{-1}.

Phosphorylation of $(Na^+ + K^+)$-ATPase Oligomers with $[\gamma\text{-}^{32}P]ATP$. Since it was not possible to isolate completely and quantitatively the individual oligomers on detergent-free gradients of glycerol, the proteins in individual fractions from a gradient were phosphorylated with $[\gamma\text{-}^{32}P]$ATP (Figure 7) in order to investigate further the identity of the active species. In those fractions for which duplicate samples exhibit $(Na^+ + K^+)$-ATPase activity, the $\alpha\beta$ monomer is competent to form a covalent, phosphorylated intermediate, whose phosphorylation is dependent on sodium (Figure 7D,F). Taken together with the data demonstrating the exact coincidence of enzyme activity with one of the two monomers present on the gradient, it can be concluded that this monomer is the sole complex responsible for the $(Na^+ + K^+)$-ATPase activity in this particular gradient. It should be mentioned, however, that higher oligomers can display enzymatic activity under other circumstances (Figure 2).

The dimer and trimer present in the experiments described in Figure 7, although incapable of a high rate of turnover, are, nonetheless, capable of forming covalent, phosphorylated intermediates. The levels of phosphorylation of these species, however, appear to be less than that of the monomer (Figure 7). A possible complication of this situation would be the existence of a reaction interconverting the several oligomers present, perhaps initiated by the phosphorylation of the enzyme itself. Such an equilibrium should be sensitive to changes in the total concentration of the protein. So that this possibility could be explored, soluble $(Na^+ + K^+)$-ATPase was prepared at a ratio of 1.5 mg of detergent (mg of protein)$^{-1}$. Aliquots of this enzyme were diluted either 2-, 5-, or 10-fold with 20 mM imidazolium chloride, pH 7.1, containing 40 μM additional $C_{12}E_8$. Each sample, as well as an undiluted control, was incubated with 170 μM $[\gamma\text{-}^{32}P]$MgATP and 13 mM sodium and cross-linked, and the phosphorylation pattern was examined (Figure 8). In this experiment, the position of the monomer (R_f = 0.43 at pH 2.4) was determined by scanning the gel at 280 nm prior to slicing. It is evident from the unchanged distributions of radioactivity at the various dilutions that no interconversion between the oligomers occurs within the course of the experiment. Considered together with the results that show no change in the cross-linking pattern taking place upon addition of substrates at low ionic strength (Figure 3), these results further demonstrate that there is no change in quaternary structure coupled to or associated with the turnover of this enzyme.

Monomeric, Soluble $(Na^+ + K^+)$-ATPase. Soluble $(Na^+ + K^+)$-ATPase (1.8 mg mL^{-1} protein, <11 mM $C_{12}E_8$), which exhibited high specific activity (20 μmol of P_i mg^{-1} h^{-1} at 11 °C), was prepared. Under the same conditions of low temperature and less than saturating concentrations of cations, purified, membrane-bound $(Na^+ + K^+)$-ATPase had a specific activity of 19 μmol of P_i mg^{-1} h^{-1}. A sample (20 μL) of this soluble $(Na^+ + K^+)$-ATPase was cross-linked in the medium used for the enzymatic assay of a companion sample. After the cross-linking reaction was quenched and the polypeptides were denatured, the sample was submitted to polyacrylamide

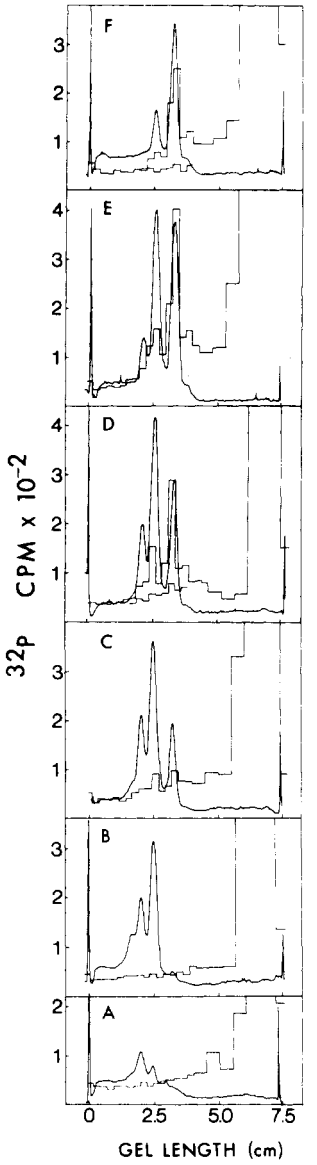

FIGURE 7: Scans of and distribution of radioactivity over NaDodSO_4–polyacrylamide gels of fractions from gradients of 10–30% glycerol phosphorylated with $[\gamma\text{-}^{32}P]$MgATP. Supernatant–$C_{12}E_8$ enzyme was submitted to centrifugation through a linear gradient of 10–30% glycerol. Aliquots (50 μL) of fractions were phosphorylated for 15 s at 11 °C in a medium containing 170 μM $[\gamma\text{-}^{32}P]$MgATP and 13 mM NaCl. After the enzyme was rapidly cross-linked in 80 mM glutaraldehyde, it was denatured with NaDodSO_4 and the unbound radioactivity removed by gel filtration. Following electrophoresis on 3.6% polyacrylamide gels buffered at pH 2.4, the gels were sliced and the slices assayed for ^{32}P. The profiles of radioactivity are superimposed upon scans of stained gels of duplicate, nonradioactive samples. The fraction numbers and their absolute enzyme activities (in micromoles of P_i per hour per milliliter) were fraction 4, 0 (A); fraction 8, 5 (B); fraction 12, 60 (C); fraction 14, 131 (D); fraction 16, 268 (E); and fraction 18, 114 (F). Thirty-one fractions were collected from the gradient (3.8 mL). Fractions 14 and 16 also were phosphorylated in the presence of 4 mM KCl (lower bar graphs). The direction of electrophoresis is from left to right.

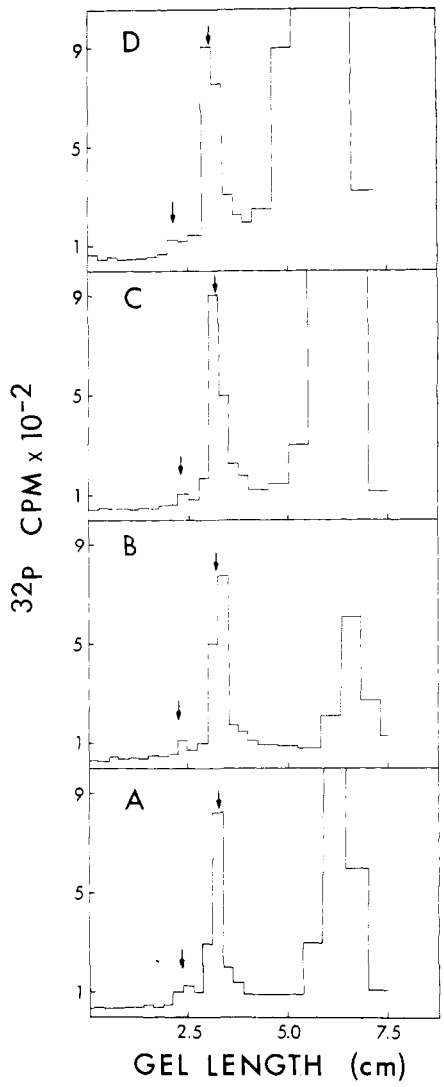

FIGURE 8: Distribution of radioactivity over $NaDodSO_4$–polyacrylamide gels of soluble $(Na^+ + K^+)$-ATPase phosphorylated at decreasing enzyme concentration. A sample of soluble $(Na^+ + K^+)$-ATPase (20 μL of 1.9 mg of protein mL^{-1} and <11 mM $C_{12}E_8$) was added to a volume of solution containing 20 mM imidazolium chloride, pH 7.1, and 40 μM additional $C_{12}E_8$ such that the final protein concentration (in milligrams per milliliter) was 0.95 (B), 0.38 (C), or 0.19 (D). The samples, along with an undiluted control (A), were phosphorylated at 11 °C with 170 μM [γ-^{32}P]MgATP and 13 mM NaCl and cross-linked with 80 mM glutaraldehyde, and the polypeptides were denatured. After removal of unbound radioactivity, they were submitted to electrophoresis. The gels were sliced and the slices assayed for radioactivity. The positions of the covalent monomer and dimer, α-β and $(\alpha$-$\beta)_2$, were determined, before slicing, by scanning the gel at 280 nm, and they are indicated by the arrows (right and left, respectively). The direction of electrophoresis is from left to right.

gel electrophoresis. A scan of the gel at 280 nm, which yields a quantitative measurement of protein distribution, is presented in Figure 9. It can be seen that under the conditions used to produce soluble $(Na^+ + K^+)$-ATPase, nearly all (<85%) of the protein is present as the monomer identified by the glutaraldehyde cross-linking assay. A small amount of dimer (<10%) and un-cross-linked α and β are also present.

When another sample of soluble $(Na^+ + K^+)$-ATPase was incubated with 170 μM [γ-^{32}P]MgATP in the presence of either 13 mM NaCl or 4 mM KCl and then cross-linked, greater than 80% of the radioactivity of the sodium-dependent, phosphorylated intermediate had a relative electrophoretic mobility coincident with that of the covalent monomer (Figure 10). Taken together, these results support the conclusion that

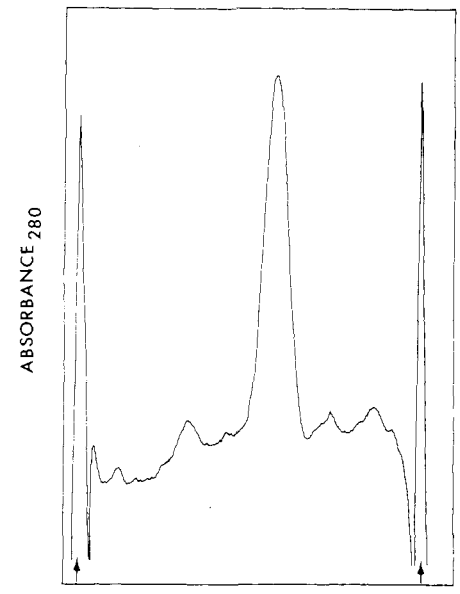

FIGURE 9: Scan of a $NaDodSO_4$–polyacrylamide gel of cross-linked soluble $(Na^+ + K^+)$-ATPase. A sample (25 μL) of soluble $(Na^+ + K^+)$-ATPase (1.9 mg of protein mL^{-1}) was cross-linked during turnover with glutaraldehyde at 11 °C. After denaturation of the enzyme with $NaDodSO_4$, the sample was submitted to electrophoresis on a 3.6% polyacrylamide gel. After electrophoresis, the gel was scanned at 280 nm without staining. The direction of electrophoresis is from left to right.

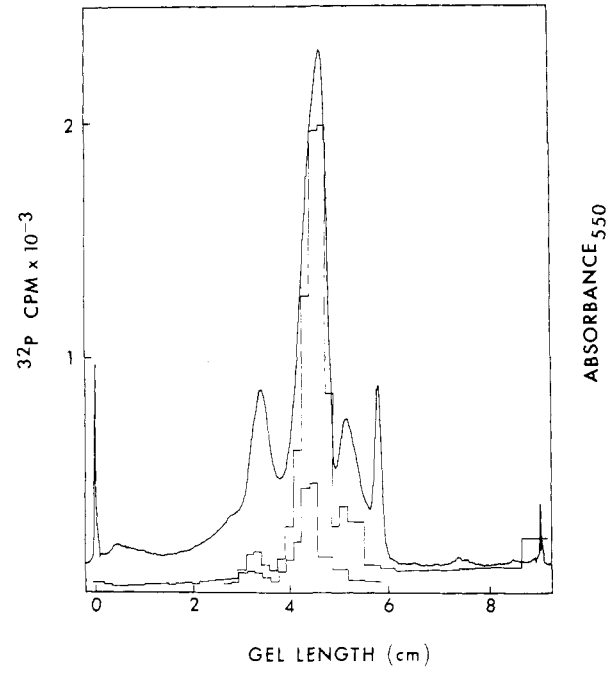

FIGURE 10: Scan of and distribution of radioactivity over a $NaDodSO_4$–polyacrylamide gel of soluble $(Na^+ + K^+)$-ATPase phosphorylated with [γ-^{32}P]MgATP. A sample (45 μL) of soluble enzyme was phosphorylated with 170 μM [^{32}P]MgATP in the presence of either 13 mM NaCl or 4 mM KCl. Slices of the gels were assayed for ^{32}P, and the distribution over the gel of the radioactivity incorporated in the presence of NaCl (upper profile) or KCl (lower profile) is shown superimposed upon a scan of a stained gel of a duplicate, nonradioactive sample. The direction of electrophoresis is from left to right.

soluble $(Na^+ + K^+)$-ATPase is a preparation of the enzyme that contains, almost exclusively, $\alpha\beta$ monomers and that the $\alpha\beta$ monomers present in this solution are fully active.

Apparent Michaelis Constants of Soluble $(Na^+ + K^+)$-ATPase for Na^+ and K^+. So that it could be determined

Table I: Michaelis Constants of Membrane-Bound and Soluble ($Na^+ + K^+$)-ATPase

enzyme preparation	K_m (mM) sodium[a]	potassium[b]
purified, membrane bound[c]	5.5 ± 0.8	0.07 ± 0.03
soluble[d]	7.4 ± 1.4	0.12 ± 0.02

[a] Measured at a constant potassium concentration of 2 mM. [b] Measured at a constant sodium concentration of 10 mM. [c] Protein concentration was 0.25 mg mL^{-1}. [d] Protein concentration was 0.27 mg mL^{-1}.

whether or not the interaction between the enzyme and its cationic substrates had been altered by the detergent required to dissolve it, the apparent Michaelis constants of soluble ($Na^+ + K^+$)-ATPase for Na^+ and K^+ were determined. The enzymatic activity of samples (10 μL) of purified, membrane-bound ($Na^+ + K^+$)-ATPase (0.25 mg mL^{-1}) and soluble ($Na^+ + K^+$)-ATPase (0.27 mg mL^{-1}) was measured by direct addition of substrates so that the concentration of one of the monovalent cations was varied while that of the other was fixed. Sodium concentration was fixed at 10 mM and potassium concentration varied from 0.25 to 2 mM, or potassium concentration was fixed at 2 mM and the sodium concentration varied from 0.5 to 13 mM. In all assays, the concentration of MgATP was held at 3 mM, and the temperature was 11 °C. The Michaelis constants (K_m) for each cation were estimated from reciprocal plots of the data (Table I). It can be seen that the Michaelis constants for sodium are nearly identical in the two different preparations while the Michaelis constants for potassium change by less than a factor of 2. It is clear that the entry and exit of the cation substrates into and from the enzyme are unchanged after it is dissolved in the detergent solution.

Discussion

Previously, chemical cross-linking with glutaraldehyde has been shown to determine accurately the quaternary structure of both a soluble, cytoplasmic enzyme, lactate dehydrogenase (Hermann et al., 1981), and a membrane-bound enzyme, ($Na^+ + K^+$)-ATPase, when it is dissolved in solutions of detergent (Craig, 1982). Furthermore, this method not only unambiguously assesses the molecular complexity of the state of aggregation of ($Na^+ + K^+$)-ATPase, something other techniques have failed to do (Hastings & Reynolds, 1979; Esmann et al., 1980; Brotherus et al., 1981), but also readily detects changes in the distribution among the oligomers brought about by variations in the solution conditions (Craig, 1982). In particular, glutaraldehyde cross-linking can be employed even in the presence of substrates, that is, while the enzyme is turning over actively, and it is this advantage that is exploited in the present attempt to determine the minimum functional unit of ($Na^+ + K^+$)-ATPase.

The results from preliminary experiments, in which ($Na^+ + K^+$)-ATPase dissolved in solutions of detergent was cross-linked in the presence of either substrates or choline chloride (Figure 2), suggested that the severe aggregation of the $\alpha\beta$ oligomers that had been observed previously when dissolved enzyme was assayed (Craig, 1982) had been due to the high ionic strength of the assay solution and was not a necessary consequence of enzyme turnover. The isolation of a mixture of oligomers, all smaller than a pentamer, which are capable of rapid turnover and yet remain unaggregated (Figure 3), supports this explanation.

When the situation is examined in more detail, there are a number of results that support the conclusion that the $\alpha\beta$ monomer identified by the cross-linking assay is the major or exclusive active component in many of the soluble preparations of ($Na^+ + K^+$)-ATPase examined here. Since the cross-linking of the fractions from the gradients was performed under conditions identical with those of the companion assays of activity, the profile of enzymatic activity within the gradient must correspond to the distribution of the component, or components, in the mixture that is responsible for that activity, provided the electrophoresis can resolve differences in the structures of the cross-linked products derived from active and inactive complexes, respectively. In fact, two species of monomer, which differ subtly in hydrodynamic properties, are resolved (Figure 5), and it is clear from the results presented in Figure 6 that only the distribution of one of them correlates with that of the enzymatic activity. This identifies it as the active complex.

In most active fractions, however, other oligomers, as well as monomers, are present, and the possibility that the activity in a given gradient only fortuitously parallels the monomer distribution and that it actually is attributable to an oligomer other than the monomer must be entertained. Yet, the facts that the monomer can be phosphorylated in a sodium-dependent reaction (Figure 7) and shown not to participate in any rapid, concentration-dependent reactions between itself and any of the higher oligomers (Figure 8) further support the conclusion that it alone is responsible for the observed activity.

Finally, soluble ($Na^+ + K^+$)-ATPase can be prepared in which the predominant (>85%) oligomer identified by the glutaraldehyde cross-linking assay is the monomer (Figure 9), and yet the preparation displays high specific activity, 20 μmol of P_i mg^{-1} h^{-1} at 11 °C. Under the same conditions of low temperature and less than saturating concentrations of cations, purified, membrane-bound ($Na^+ + K^+$)-ATPase has a specific activity of 19 μmol of P_i mg^{-1} h^{-1}. Less than 10% of the oligomers present in the sample are higher oligomers (only dimers are seen), and, consequently, more than 90% of the phosphorylated intermediate is monomeric (Figure 10). If the monomer were not active, this would require a minimum enzymatic activity for the dimer of 200 μmol of P_i mg^{-1} h^{-1} at 11 °C in order to account for the observed activity. This number represents a lower limit since some inactive enzyme is also present, yet it is still far in excess of the activity of purified enzyme under these conditions.

Although the results presented earlier (Craig, 1982) provided strong evidence for the conclusion that all of the several oligomers of ($Na^+ + K^+$)-ATPase in a given solution are accurately and quantitatively identified and cataloged by the glutaraldehyde, the possibility that certain specific complexes might fail to be cross-linked and thereby be overlooked should be considered. Particularly relevant to the conclusions drawn above is the question of whether or not an enzymatically active dimer exists whose structure only permits cross-linking to occur quantitatively within each of its asymmetric units and yet not between them. If this possibility is entertained, it would follow that the only product that would result from the reaction of this active dimer with glutaraldehyde would be the covalent heterodimer, α-β. Furthermore, it would be predicted that, as observed in Figure 6, the distribution of the A form of α-β on the gradient of 10–30% glycerol would coincide with the distribution of enzymatic activity but that this active form would be a dimer.

If this suggestion were indeed the explanation for the results presented in Figures 4–6, several improbable circumstances

would have to occur simultaneously. First, as stated, both the α-polypeptides and β-polypeptides within this active dimer would have to fail entirely to cross-link with their symmetrically displayed twins. The static electrophoretic pattern of covalent oligomers observed when concentrations of glutaraldehyde ranging from 8 to 80 mM are employed, the promiscuous nature of this reagent, and the presence of more than 100 lysine residues within each asymmetric unit (Kyte, 1972) all argue against this possibility but cannot disprove it. Second, the sedimentation coefficient of the active dimer would have to be significantly different from that of the inactive dimer. In fact, the active, presumably globular dimer would have to possess a sedimentation coefficient that was smaller than that of the inactive, presumably denatured monomer (Figure 6). Such an observed difference, in the unexpected direction, between the sedimentation coefficients of monomer and dimer would require rather unusual structural irregularities in one or both of the complexes. Since some partial activities remain with the inactive oligomers (Figure 7), any large changes in structure, which would be significant enough to affect the sedimentation coefficients of the complexes, seem to be unlikely. Third, inactive dimers and higher oligomers present within the gradient would have to be altered structurally during inactivation in such a way that cross-linking is then permitted to occur between the twins within a given complex. If this were the case, it would also require that these structural changes be correlated directly to the loss of ($Na^+ + K^+$)-ATPase activity. It appears from the above arguments that the existence of an enzymatically active dimer that can escape detection in the cross-linking assay is unlikely. Taken together, this discussion reinforces the conclusion that monomers of the $\alpha\beta$ asymmetric unit, dissolved in solutions of $C_{12}E_8$, are capable of full ($Na^+ + K^+$)-ATPase activity.

It will never be possible to measure directly the transport of cations when purified ($Na^+ + K^+$)-ATPase is dispersed from the membrane in solutions of detergent. Nevertheless, experimental results obtained with such preparations can be considered to be valid measurements of actual active transport provided certain chemical characteristics of the enzyme remain unchanged. The following discussion describes correlations that support the conclusion that the turnover of ($Na^+ + K^+$)-ATPase and the coupled active transport of sodium and potassium are obligatorily linked events.

The structure–activity relationships of the cardiotonic steroids are the same for active cation transport and ($Na^+ + K^+$)-ATPase activity (Dunham & Glynn, 1961; Abeles, 1969), and potassium ion can partially relieve this inhibition for both processess (Dunham & Glynn, 1961; Glynn, 1957). Sodium and potassium ions must both be present for turnover to occur (Post et al., 1960), and likewise, the normal transport of these ions is obligatorily coupled (Hodgkin & Keynes, 1955; Post & Jolly, 1957; Mullins & Brinley, 1969). The concentrations of these cations that are required for half-maximal activation of ($Na^+ + K^+$)-ATPase (Post et al., 1960) correspond quite closely with those required for active cation transport (Post et al., 1960; Post & Jolly, 1957). Furthermore, potassium, when acting at the inner surface of the plasma membrane (Hoffman, 1962), and sodium, when competing at the outer surface (Post et al., 1960), will inhibit active cation transport, and these ions will also act as competitive inhibitors of ($Na^+ + K^+$)-ATPase activity (Skou, 1960; Post et al., 1960). Finally, sealed vesicles formed with purified phospholipids and ($Na^+ + K^+$)-ATPase will actively accumulate sodium when MgATP is present in the medium, and both these processes are inhibited by the cardiotonic steroids as well (Goldin, 1977). These direct correlations are strong evidence in support of this conclusion that the turnover of the enzyme and the active transport of the cations must occur simultaneously.

Furthermore, it is reasonable to assume that the same obligatory relationship exists in the soluble form of the enzyme since the apparent Michaelis constants of this soluble enzyme for the cationic substrates are very close to those of the purified, membrane-bound enzyme (Table I). If it did not exist and if the translocation of the cations in their respective directions between the cytoplasmic and extracytoplasmic sides of the protein were somehow absent from the mechanism of the soluble form of the enzyme, the kinetic parameters concerned with the cation substrates should have changed to reflect this. In addition, it is unclear how the enzyme could even complete a catalytic cycle, as it has been shown the soluble enzyme is fully competent to do, if an intermediate step in that cycle had been deleted. Specifically, it is known that the rate-limiting step in the overall reaction of ($Na^+ + K^+$)-ATPase is the movement of potassium from the extracytoplasmic to the cytoplasmic surface of the protein (Moczydlowski & Fortes, 1981b). Were this step eliminated, significant changes in V_{max} should be observed, but they are not. It is for all of these reasons that it can be presumed that each time the enzyme turns over, regardless of its physical state, it is also catalyzing active transport. It follows that, since the enzyme functions normally when it is a monomer in the soluble form, it catalyzes active transport as a monomer in the native, membrane-bound form, even if it is present in an oligomer under these circumstances.

A curious aspect of the behavior of the dissolved enzyme on a gradient of glycerol lacking detergent (Figure 4) is that all of the higher oligomers are inactive species. Since there is, a priori, no reason for the aggregate of a functional monomer to be inactive, the results suggest that these oligomers are probably formed from monomers that have lost the capacity for activity. The sequence of events that may be occurring would be a partial denaturation that produces the inactive monomer from the active monomer (Figure 6) followed by the aggregation of this denatured protein. The fact that no changes in the distribution of the protein among the various species are ever observed unless the free concentration of detergent is varied (Craig, 1982) requires that all of these processes occur initially as the detergent is added or while it is being removed on the gradient. As soon as the free detergent concentration ceases to change, no further denaturation and aggregation occur, and a stable distribution with no interconversion of species is established. It is the case, however, that some of these oligomers are capable of forming phosphorylated intermediates (Figure 7), and this suggests that some partial activities, for example, ATP-dependent Na^+–Na^+ exchange, may be retained. Nevertheless, the results confirm that only the monomer is capable of catalyzing the normal level of sodium- and potassium-dependent ATP hydrolysis.

Because a rotational carrier mechanism for the active transport of sodium and potassium has been eliminated (Kyte, 1974), it is now believed that the pathway for the movement of these cations through ($Na^+ + K^+$)-ATPase is provided by a channel (Jardetsky, 1966). The theoretical suggestion (Kyte, 1974) that the channel walls are formed from two $\alpha\beta$ asymmetric units had gained support from several experimental observations. Results from radiation inactivation (Kepner & Macey, 1968; Ellory et al., 1979) and ligand binding stoichiometries (Perrone et al., 1975; Jørgensen, 1977; Petersen et al., 1978) had indicated a protein mass (active site)$^{-1}$, which, when compared with the estimated masses of the constituent

polypeptide chains, was consistent with the existence of a single active site for each dimer, $(\alpha\beta)_2$, or "half-of-sites" behavior (Fersht, 1975). In addition, chemical cross-linking experiments, while unequivocally establishing that α and β are equimolar components of a specific complex (Kyte, 1972; Liang & Winter, 1977; Craig & Kyte, 1980), also provided evidence for the existence of a unique α_2 dimer (Kyte, 1975; Giotta, 1976; Liang & Winter, 1977) in the same complex. Finally, it was claimed that functional, discrete complexes that have apparent protein molecular weights of 380 000 ± 21 000 (Hastings & Reynolds, 1979) and 256 000 ± 23 000 (Esmann et al., 1980) had been produced by detergent treatment of membrane-bound ($Na^+ + K^+$)-ATPase. The inadequacies of the techniques used in the above studies have been discussed previously (Craig & Kyte, 1980; Kyte, 1981), and it is now clear that none of the results can provide any support for the conclusion that the channel is formed at a dimer interface. Furthermore, half-of-sites behavior, the only direct, previously available evidence that suggested the necessity for a dimeric structure, has been eliminated on the basis of improved titrations of the active site (Moczydlowski & Fortes, 1981a). Finally, while it had been claimed that homogeneous solutions of dimers (Hastings & Reynolds, 1979; Esmann et al., 1980) or monomers (Brotherus et al., 1981) could be prepared in nonionic detergents, it was subsequently shown that all of these preparations contain a very similar, complex mixture of oligomers of the $\alpha\beta$ unit (Craig, 1982). Clearly, as has been noted earlier (Craig & Kyte, 1980; Moczydlowski & Fortes, 1981b), the hypothesis invoking a channel formed between two identical subunits is not supported by the available experimental evidence.

It is also very unlikely that the β-polypeptide participates in the formation of the cation channel. Both the site of phosphorylation (Kyte, 1971a) and the binding site for cardiac glycoside (Ruoho & Kyte, 1974) reside upon the α-polypeptide of ($Na^+ + K^+$)-ATPase. In addition, Ca^{2+}–ATPase from skeletal muscle and ($H^+ + K^+$)-ATPase from gastric mucosa, mammalian enzymes that also catalyze active transport and that probably share a recent common ancestor with ($Na^+ + K^+$)-ATPase [for a discussion of this point, see Kyte (1981)], are constructed from only α-polypeptides. It follows that it is only the α-polypeptide of ($Na^+ + K^+$)-ATPase that is responsible for active transport. Taken together with the results presented here, it appears to be the case that only a single polypeptide chain is required to form the channel through which the cations must pass.

The earlier conclusion (Craig & Kyte, 1980) that channels similar to the pores found in gramicidin A (Urry et al., 1971) or amphotericin B–cholesterol complexes (De Kruijff & Demel, 1974) might not be found in proteins may have been overstated. It should not elimate them from consideration as models of the channel involved in active-transport enzymes. They, in fact, gain added appeal in light of the present results. Such a channel formed, as with these ionophores, within the secondary structure may exist along a single stretch of amino acid sequence passing from one side of the protein to the other. On the other hand, the walls of a channel could be formed by the clustering of several individual secondary structures within the α-polypeptide. It has been proposed that such a channel exists in the transmembrane protein bacteriorhodopsin (Engelman et al., 1980), wherein several helical, transmembrane segments (Henderson & Unwin, 1975) would form the walls lined with carboxylic acid side chains strategically positioned to bind and transport protons. Unfortunately, in either case, the observed or hypothetical channels are simple models and could not completely fulfill the requirements of the channel in ($Na^+ + K^+$)-ATPase. They are, however, by exclusion, models that will serve to initiate the experimental approaches needed to solve this problem.

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Chloroacetaldehyde-Treated Ribo- and Deoxyribopolynucleotides. 1. Reaction Products†

J. T. Kuśmierek‡ and B. Singer*

ABSTRACT: The in vitro reaction of the vinyl chloride metabolite chloroacetaldehyde (CAA) with cytosine and adenine residues in ribo- and deoxyribopolynucleotides leads to the formation of the relatively stable hydrated etheno derivatives 3,N^4-(N^4-α-hydroxyethene)cytosine (εC·H_2O) and 1,N^6-(N^6-α-hydroxyethene)adenine (εA·H_2O). Under physiological conditions the hydrates are slowly converted to 3,N^4-ethenocytosine (εC) and 1,N^6-ethenoadenine (εA). The half-life at pH 7.25 of εC·H_2O in poly(rC) is 4.9 h at 50 °C and of εA·H_2O in poly(rA) is 1.4 h at 37 °C. These dehydration rates in polymers are similar to those for hydrates in monomers. The reactivity of A and C residues is greatly suppressed in double-stranded polymers. Adenine residues are about 10 times less reactive in poly(rA)·poly(rU) than A in single-stranded polymers. Under similar reaction conditions no reaction of C residues in poly(rC)·poly(rG) was detected. In vinyl chloride exposed cells, where CAA is formed, the cyclic etheno derivatives of A and C are likely to occur preferentially in single-stranded regions of nucleic acids, with the hydrate forming a major proportion of the modification.

Chloroacetaldehyde (CAA)[1] has long been used to prepare fluorescent etheno derivatives of cytidine and adenosine. Although the reaction mechanism postulates two intermediates, these are unstable, particularly in acid. Most previous workers used pH 3–5 for modification reactions and thus obtained only the etheno compounds (Kochetkov et al., 1971; Barrio et al., 1972; Secrist et al., 1972). Later Wiewiórowski and coworkers (Biernat et al., 1978; Krzyżosiak et al., 1979) did isolate the hydrated intermediates and determined, at pH 3–7, the conditions and rates for dehydration leading to etheno derivatives in monomers. These rates of dehydration were much slower under physiological conditions than those in more acid solution. Although Krzyżosiak et al. (1981) had indications that the hydrates were formed in CAA-treated tRNA, they did not isolate these compounds but studied their stability in the RNA. Nevertheless, investigators have reacted polynucleotides with CAA or cells with vinyl chloride, where CAA is formed, and have assumed that only the etheno derivatives would be present. We now report that after reaction of CAA with poly(rC), poly(dC), poly(rA), and poly(dA) at neutrality, the hydrate is the predominant product and is only converted to the etheno derivative when heated several hours at elevated temperatures. It would thus appear that when CAA-treated polynucleotides are directly used for transcription studies, both hydrate and dehydrated etheno compounds can be present. Therefore, transcription results with CAA-modified polymers are likely to represent data for both types of compounds rather than for only the etheno compounds (Barbin et al., 1981; Hall et al., 1981). In the succeeding paper (Kuśmierek & Singer, 1982), we report the effect on transcription of each derivative separately.

Materials and Methods

Materials. Chloroacetaldehyde (CAA), 45% in water solution, was an ICN Pharmaceuticals product. 3,N^4-Ethenocytidine 5′-diphosphate and 1,N^6-ethenoadenosine 5′-di-

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* Address correspondence to this author at the Laboratory of Chemical Biodynamics, University of California, Berkeley, CA 94720.
‡ Permanent address: Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw 02532, Poland.

[1] Abbreviations: HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; CAA, chloroacetaldehyde; εCyd, 3,N^4-ethenocytidine (5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidine); εCyd·H_2O, hydrated, 3,N^4-ethenocytidine, 3,N^4-(N^4-α-hydroxyethane)cytidine (2-hydroxy-2,3,5,6-tetrahydro-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidine); εAdo, 1,N^6-ethenoadenosine (3-β-D-ribofuranosylimidazo[2,1-*i*]purine); εAdo·H_2O, hydrated 1,N^6-ethenoadenosine, 1,N^6-(N^6-α-hydroxyethene)adenosine (7,8-dihydro-8-hydroxy-3-β-D-ribofuranosylimidazo[2,1-*i*]purine); εC, εC·H_2O, εA, and εA·H_2O stand for modified base residues in polymers. The use of H_2O in the abbreviations represents the hydrated form of εA and εC, rather than water of hydration.

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