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CONFORMATIONAL CHANGES OF PURIFIED (Na^+ + K^+)-ATPase DETECTED BY A SULFHYDRYL FLUORESCENCE PROBE *

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Summary

The fluorescent sulfhydryl reagent *S*-mercuric-*N*-dansyl cysteine (Dn-Cys-Hg^+) has been used to label a purified preparation of the (Na^+ + K^+)-ATPase obtained from the electric organ of *Electrophorous electricus*. The labelled (Na^+ + K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3), although reversibly inhibited, was capable of undergoing conformational changes associated with the active enzyme that could be monitored fluorometrically. The presence of ligands (Na^+ + Mg^{2+} + ATP or Mg^{2+} + P_i) which are known to convert the enzyme from the E-1 state to the E-2-*P* state brought about large (97—100%) increases in fluorescence of the dimethylaminonaphthalene sulfonyl (Dn) label. An E-2 state could be achieved by the addition of Mg^{2+} which caused only a 32.3% increase in fluorescence over the E-1 state. Neither AMP nor TTP with or without Mg^{2+} or Na^+ or P_i added without Mg^{2+} had any effect on the Dn fluorescence. If the enzyme was denatured, no fluorescence changes were observed. Small changes in the polarization of fluorescence of the Dn moiety were observed under all the conditions used. These small polarization changes and the large increases in the fluorescence intensity suggest that the enzyme can change conformational states in the presence of appropriate ligands and these conformational changes may take place in a relatively limited region of the protein's structure.

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Abbreviations: Dn, dimethylaminonaphthalene sulfonyl; Dn-Cys-Hg^+, *S*-mercuric-*N*-dansyl cysteine; AMP, adenosine monophosphate; TTP, thymidine triphosphate; PNPPase, *p*-nitrophenylphosphatase; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

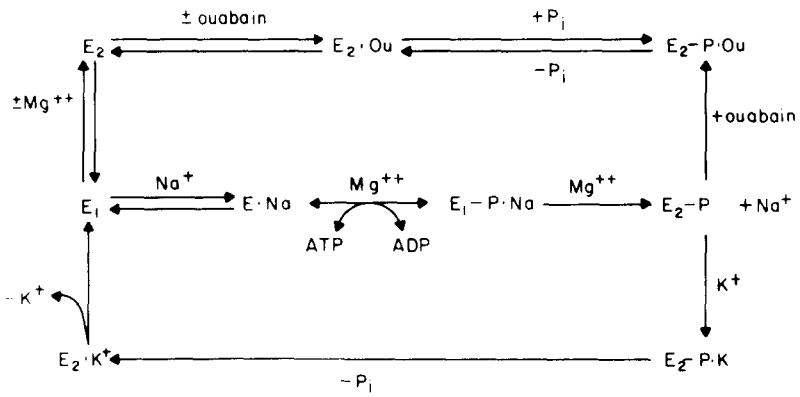

Fig. 1. Cycle for ion transport by the ($Na^+ + K^+$)-ATPase and for binding of ouabain (cf. ref. 6).

Introduction

The reactive chemical species within the active sites of enzymes have been the subject of intense interest and experimental study. The ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) enzyme system couples the transport of Na^+ and K^+ across cell membranes to the hydrolysis of ATP to maintain ionic gradients. This enzyme possesses several sulfhydryl groups, some of which are necessary for the hydrolysis and transport process [1—3]. Work in this laboratory has shown that the ($Na^+ + K^+$)-ATPase within rat brain synaptosomes can be reversibly inhibited by the reaction of its sulfhydryl groups with the fluorescent reagent Dn-Cys-Hg^+ [4]. This compound forms a mercaptide bond, R_1-S-Hg-S-R_2, with the available sulfhydryl groups. However, the ($Na^+ + K^+$)-ATPase represents only a small portion of the total material of synaptosomes and interference due to non-ATPase material was a serious handicap in obtaining optical measurements. To minimize these problems, we have used a purified enzyme preparation obtained from the electric organ of the electric eel following the procedures described by Dixon and Hokin [5].

The following diagramatic scheme (Fig. 1) has been proposed by Sen et al. [6] to explain the coupling of ion transport to the hydrolysis of ATP and the binding of the specific inhibitor ouabain. This scheme predicts an enzyme conformational change by the ($Na^+ + Mg^{2+}$)-stimulated phosphorylation with ATP from a resting E-1 state to an E-1-*P* state. The enzyme is transformed to the E-2-*P* state in the presence of Mg^{2+}. Potassium mediates the dephosphorylation of the enzyme and its return to the resting conformation E-1. Via the upper route (Fig. 1) Mg^{2+} alone could shift the conformation to an E-2 state. Our goal was to label a purified preparation of the ($Na^+ + K^+$)-ATPase with a covalent fluorescent probe near or within the active site and determine if the above proposed conformational changes could be detected fluorometrically.

Methods

Purification ($Na^+ + K^+$)-ATPase. Purified ($Na^+ + K^+$)-ATPase enzyme material was obtained using the methods described by Dixon and Hokin [5] as modified

by Perrone et al. [7]. Electric eels were purchased from World Wide Scientific Animals, Ardsley, N.Y. and were killed the day following arrival. The eels were stunned by cooling their water bath to approx. 4°C with crushed ice. They were decapitated, and the electric organ was dissected away from other tissues. A portion was used fresh, and the remainder was cut into small pieces, frozen, and stored in liquid nitrogen.

The electric organ tissue (100 g) was homogenized in 200-ml volumes of N_2 purged 50 mM Tris · HCl buffer containing 1 mM ATP (pH 7.4) for two 1-min periods in a Virtis Model 45 tissue homogenizer. After straining the homogenate through two layers of gauze to remove connnective tissue, it was centrifuged at 6500 rev./min in a Sorvall refrigerated centrifuge for 30 min. The top floating lipid material and the pellet were combined and resuspended to 100 ml with 1 mM Tris/EDTA, 1 mM Na_2ATP, pH 7.4, and centrifuged in a Spinco 30 rotor at 29 000 rev./min. The sediment was taken up in 25 ml of 3.2% Lubrol WX, 1 mM Tris/ATP, pH 7.0, and homogenized in a Teflon and glass homogenizer. The solution was kept at 4°C for 30—40 min and was then centrifuged for successive 30 and two 60-min periods at 39 000 rev./min in a Spinco 40 rotor. The final extract was a slightly yellowish transparent solution. It was made 20 mM in NaCl by addition of 1 M NaCl to stabilize the enzyme [5].

Discontinuous sucrose gradients consisted of 5 ml of buffer; 4 ml of Lubrol extract; 8 ml each of 5, 10, and 15% sucrose, and a 4 ml cushion of 35% sucrose. All solutions contained 10 mM Tris · HCl, pH 6.5, 20 mM NaCl, 1 mM EDTA, and 1 mM ATP, and were prepared in Spinco SW-27.2 tubes. The samples were centrifuged at 27 000 rev./min for 16 h and during this period the gradients became continuous. The samples were fractionated and protein and enzyme analyses were run immediately to determine which fraction contained maximal (Na^+ + K^+)-ATPase activity. Usually, the highest specific activity was found between 9 and 14% sucrose, a specific gravity of 1.035—1.05. These fractions were pooled and pressure concentrated and washed with ten volumes of buffer in a Diaflo concentrator using an XM-50 membrane. The concentrated protein fraction was made 1 M in $(NH_4)_2SO_4$ plus 1 mM ATP for 68 min at 30°C and then was diluted with buffer to 0.4 M $(NH_4)_2SO_4$. The samples were centrifuged at 39 000 rev./min in Spinco 40 rotor for 90 min over a 0.10 ml cushion of 35% sucrose. The pellet was dialyzed versus 5 mM Tris, pH 7.4, and frozen in aliquots at −80°C. The ammonium sulfate pellet was abbreviated ASP.

Polyacrylamide electrophoresis. This was carried out essentially as described by Fairbanks et al. [8]. An aliquot of each fraction was dialyzed overnight against a solution containing 1% sodium dodecyl sulfate (SDS), 10 mM Tris and 1 mM EDTA, pH 8, at room temperature and was then incubated for 30 min at 40°C in the same buffer containing 40 mM dithiothreitol. The dithiothreitol treatment was omitted with those samples containing Dn-Cys-Hg^+-labeled protein. Electrophoresis was carried out in a Hoefer system (Biorad Model 300) at room temperature (20°C) using 5 × 90 mm gels with Pyronin Y as tracking dye. The gels were run at constant current for 1 h at 1 mA per gel and then for an additional 2—3 h at 4 mA per gel. After completion of a run the length of the gel was measured, and the dye was marked by insertion of a wire. The gels were

stained and destained with Coomassie Blue as described by Fairbanks et al. [8] Relative mobilities for molecular weight determinations were established according to Weber and Osborn [9] as used by Stahl [10]. Densitometric scanning was done at 550 nm with a Gilford Linear Transport Scanner at 0.3 cm/min with the 0.05 × 2.36 slit plate.

Assays. The $(Na^+ + K^+)$-ATPase was assayed using [^{32}P]-ATP as substrate [11]. ^{32}P was counted in Brays solution using a liquid scintillation counter. Protein analysis was determined by the Lowry et al. method [12]. PNPPase was assayed as previously described [13]. Sulfhydryl groups were analyzed by the procedure described by Boyne and Ellman [14]. Dn-Cys-$^{203}Hg^+$ was prepared as described by Leavis and Lehrer [15] with the inclusion of ^{203}Hg (New England Nuclear, Specific Activity 5.8 Ci/g). ^{203}Hg was measured using an auto gamma counter. Lubrol was analyzed by the method outlined by Garewall [16] correcting for phospholipid in sample. The reaction of the Dn-Cys-Hg^+ with enzyme solutions was carried out as follows. Dn-Cys-Hg^+ in 1 mM HNO_3 was neutralized with 500 mM Tris · HCl, 4 mM EDTA, pH 7.4, and the enzyme solution was added immediately in the molar ratios of Dn-Cys-Hg:SH group indicated. The reactants were kept at 4°C for 20—30 min then were initially dialyzed versus 10 mM Tris, 1 mM EDTA, 1 mM cysteine, pH 7.4, and then for 16 h versus the same buffer without cysteine.

The procedure of Post et al. [17] was followed for the formation of the phosphorylated intermediate. The reaction at 0°C was run for 10 s and was then stopped with the addition of two volumes of 5% trichloroacetic acid, 1 mM ATP and 1 mM H_3PO_4. The samples were collected on 0.45 μm Schleicher and Schuell membrane filters. These were dryed and counted. The Dn-Cys-Hg^+ used in the study did not contain $^{203}Hg^{2+}$ as this nuclide emits a β particle of similar energy to ^{32}P.

Results

The stained SDS-polyacrylamide gels were scanned to assess the number of components in the final ammonium sulfate pellet. The scan (Fig. 2) shows two major bands of protein, one at 100 000 daltons and a second at 55 000. The arrows indicate the positions of protein molecular weight standards. These two protein bands represent 68% of the total area of the scans. To obtain the best resolution of the protein bands in these gels, the proteins were treated briefly with 40 mM dithiothreitol [8]. The Dn-Cys-Hg^+ label unfortunately dissociates from the enzyme under these conditions. The Dn-Cys-Hg^+-labeled ammonium sulfate pellet was run without treatment with dithiothreitol which decreased the resolution of the labeled proteins but retained the radioactive label. The absorbance scan at 550 nm of the labeled samples and the distribution of radioactivity within the gels is seen in traces b and c. Some of the labeled protein is unresolved at the top of the gel, but of that resolved, all the ^{203}Hg appears in the 100 000-dalton protein and none in the lower molecular weight species. The 100 000-dalton component of the $(Na^+ + K^+)$-ATPase is known to be phosphorylated by [^{32}P]ATP (cf. ref. 18).

The specific enzymic activity of the ammonium sulfate pellet obtained in our laboratory was lower than originally reported [5]. Our usual $(Na^+ + K^+)$-

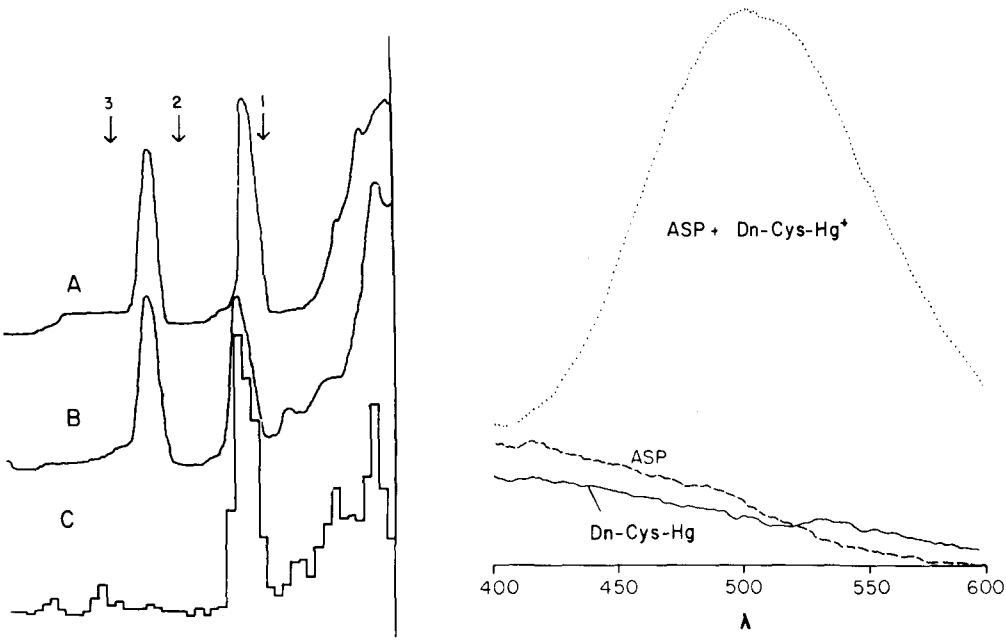

Fig. 2. SDS-polyacrylamide gel electrophoresis of ammonium sulfate pellet (ASP). Absorbance at 550 nm is shown in A and B. A is the sample treated with dithiothreitol and then electrophoresed. The arrows mark the position s of protein molecular weight standards which were run separately; 1, β-galactosidase, M_r 130 000; 2, pyruvate kinase, M_r 57 000; 3, glyceraldehyde phosphate dehydrogenase, M_r 37 000. Scan B is of the ammonium sulfate pellet that had been labeled with Dn-Cys-^{203}Hg and run without dithiothreitol treatment. C shows the distribution of radioactivity (Dn-Cys-^{203}Hg) within the gel of sample B.

Fig. 3. Fluorescence emission spectra before and after formation of the mercaptide bond. Individual reactants (ammonium sulfate pellet (ASP), 67 μg/ml and Dn-Cys-Hg$^+$, 3.6 μM) and the mixture were in 400 μl of 10 mM Tris · HCl, 0.2 M sucrose, pH 7.4. Excitation was at 355 nm with band pass set at 8:8.

stimulated activity was between 250 and 370 μmol P_i/mg protein per h where as the maximum literature values reported were from 1200 to 1900. Our preparation could be stimulated to higher activities by the addition of phosphatidylserine or dithiothreitol, but these were still below literature values. The inclusion of dithiothreitol prevented some of this loss, but this reagent could not be totally separated from the protein, and it interfered with subsequent procedures, so it was omitted.

Fluorescent reaction ammonium sulfate pellet and Dn-Cys-Hg$^+$

Fig. 3 shows the fluorescence spectra of the individual reactants and the product of the reaction of Dn-Cys-Hg$^+$ with the ammonium sulfate pellet. Following the reaction, there is a 17-fold increase in the relative fluorescence of the Dn group. The excitation spectra shows there is energy transfer in the excited electronic state from the protein to the Dn moiety. The concentration of the Dn-Cys-Hg$^+$ was 3.6 μM in both cases. As previously reported [4], there is a shift in the emission maximum of Dn-Cys-Hg$^+$ from 540 to 500 nm after i reacts with the enzyme.

Relationship of (Na^+ + K^+)-ATPase activity and the Dn-Cys-Hg^+ reaction to the number of SH groups

The relationships between the reaction of the enzyme's sulfhydryl groups, its inactivation, and the corresponding fluorescence changes are seen in Fig. 4. The inhibition of the (Na^+ + K^+)-ATPase (curve A) was essentially complete when one half of the sulfhydryl groups had reacted.

The major Dn fluorescence (curve E), exciting at 355 nm with emission measured at 500 nm, increases with the number of protein sulfhydryl groups that were reacted (Curve B), as expected. The relative fluorescence reached a maximum when all the SH groups were reacted and showed a slight decrease after that point. Earlier studies [4] showed a 1:1 stoichiometry between the Dn-^{203}Hg reacted and the loss of SH groups as determined by the Ellman procedure indicating only the SH groups were reacted at this concentration of reagent. One half of the fluorescence maximum was reached when the (Na^+ + K^+)-ATPase was completely inhibited.

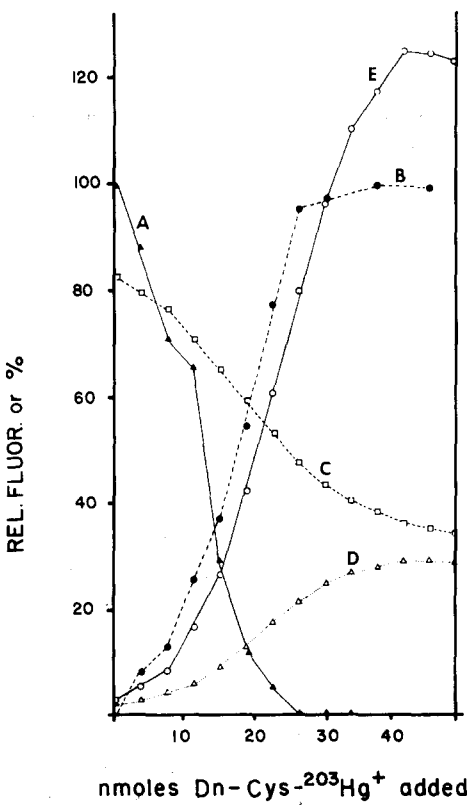

Fig. 4. Titration of the ammonium sulfate pellet with Dn-Cys-^{203}Hg$^+$. The ammonium sulfate pellet (0.31 mg/ml) was suspended in 10 mM Tris · HCl, 0.2 M sucrose, pH 7.4. ▲———▲, inhibition of the (Na^+ + K^+)-ATPase expressed as percent control (curve A); ●———●, percent of maximum Dn-Cys-^{203}Hg reacted per mg protein (curve B); □———□, protein intrinsic fluorescence, excitation at 295 nm and emission at 340 nm (curve C); △· · · · ·△, Dn fluorescence, excitation 290 nm and emission 500 nm (curve D); ○———○, Dn fluorescence, excitation at 355 nm and emission 500 nm (curve E). Excitation band pass was 8 nm and emission bandpass was 10 nm.

Intrinsic protein fluorescence can be quenched by the addition of heavy metals such as Hg^{2+} [19]. The probe used in this study can then have two separate effects on the protein fluorescence: the quenching by Hg^{2+} and also the phenomenon of energy transfer from the protein's aromatic amino acids to the Dn group of the probe. Curve C shows that the protein fluorescence, was quenched by the reaction. At the point where the enzyme was completely inhibited this intrinsic fluorescence was decreased by 65% of its initial value.

Curve D shows the increase in Dn fluorescence observed at 500 nm when the sample was irradiated at the protein's excitation maximum, 290 nm. This curve indicates that energy transfer is occurring between the protein and the Dn group. A plateau was reached as all the SH groups were reacted as seen in curve B. The predominant effect of the probe on the protein fluorescence is that of energy transfer.

When the Dn group was irradiated at its excition maximum, 355 nm, the fluorescence at 500 nm increased as shown in curve E.

Relationship ($Na^+ + K^+$)-ATPase partial reactions and reaction with Dn-Cy-Hg^+

The ($Na^+ + K^+$)-ATPase can be considered as a kinase and a phosphatase. The initial ($Na^+ + Mg^{2+}$)-dependent formation of the phosphorylated enzyme as well as the potassium-dependent dephosphorylation of the phosphatase can be experimentally established. The former is obtained by measurement of the acid-stable ^{32}P enzyme complex from [^{32}P]ATP while the latter may be demonstrated by the use of the synthetic substrate *p*-nitrophenylphosphate. Table I shows the effect of reacting of Dn-Cys-Hg^+ with the ammonium sulfate pellet on the overall hydrolysis of ATP, the K^+-stimulated *p*-nitrophenylphosphatase activity, and on the formation of the phosphorylated intermediate.

The PNPPase was rapidly inhibited by the reaction of the sulfhydryl groups. The phosphorylated intermediate was unaffected at a ratio of Dn-Cys-Hg^+ to SH of 0.66:1 while the PNPPase was inhibited 87% at this concentration. Some formation of the phosphorylated intermediate remains at a ratio of Dn to SH of greater than 1:1, which is past the point where both the ($Na^+ + K^+$)-ATPase and the PNPPase were totally inhibited.

TABLE I

Effects of reaction of the ($Na^+ + K^+$)-ATPase sulfhydryl groups with Dn-Cys-Hg^+ upon enzymic reactions. Procedures for the assays are given in Methods. The data presented are average values of samples run in triplicate from a single enzyme preparation and as such show a single representative experiment.

Ratio Dn-Cys-Hg^+ enzyme-SH	($Na^+ + K^+$)-ATPase		PNPPase		^{32}P intermediate	
	nmol P_i formed mg protein per h	Percent control	μmol PNP formed mg protein per h	Percent control	pmol/mg protein	Percent control
Control (0)	289	100	5.08	100	730	100
0.22	269	92.9	1.46	28.8	766	106
0.33	185	64	0.575	11.3	735	100
0.45	37.9	13.1	0.295	5.8	732	100
0.67	4.7	1.6	0.030	0.5	564	77
1.11	0	0	0	0	125	17

Fluorescence-detected conformational changes

The active (Na^+ + K^+)-ATPase can respond to specific ions and/or with the addition of substrate by undergoing proposed conformational changes, as outlined in Introduction (Fig. 1). A study was made to determine if the enzyme inactivated with Dn-Cys-Hg^+ would still undergo similar changes and whether these changes could be detected with the probe. The enzyme was assumed to be an E-1 conformation when it was suspended in 10 mM Tris buffer, pH 7.4. With the addition of NaCl, the enzyme could change to an E-1-Na^+ state. As seen in Fig. 5, there is an increase of the Dn fluorescence with addition of NaCl (100 mM). Most of this increase in fluorescence is due to the increase in ionic strength and could be duplicated with KCl or choline chloride. The addition of $MgCl_2$ alone (10 mM) or with NaCl caused a 32.2 ± 3.4% increase in the Dn fluorescence. This may reflect the change to a E-2 conformation.

In the presence of NaCl (100 mM) and $MgCl_2$ (10 mM) the addition of ATP (1 mM) caused a dramatic 96.6 ± 5.6% increase in Dn fluorescence. If the enzyme was phosphorylated, this would likely represent the E-2-*P* state. It is theoretically possible to arrive at this same conformation by the reverse path, through the addition of $MgCl_2$ and P_i. Addition of P_i (1 mM) to a Dn-Cys-Hg

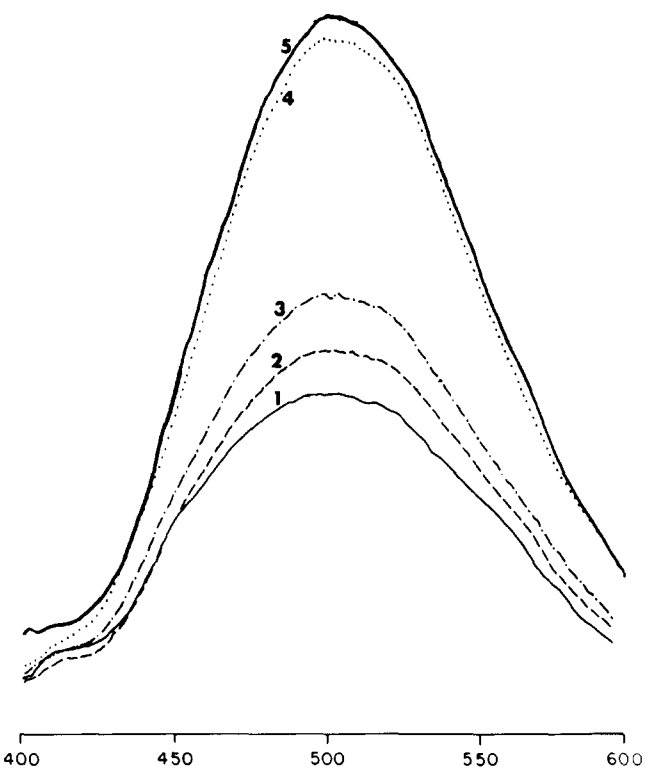

Fig. 5. Fluorescence emission spectra of Dn-Cys-Hg-ammonium sulfate pellet in 10 mM Tris · HCl/0.2 M sucrose, pH 7.4 (curve 1) and with sequential addition to final concentrations: 100 mM NaCl (curve 2); NaCl + 10 mM $MgCl_2$ (curve 3); NaCl + 10 mM $MgCl_2$ + 1.5 mM ATP (curve 4) or 10 mM $MgCl_2$ + 1.5 mM $MgCl_2$ + 1.5 mM H_3PO_4 (curve 5). The protein concentration was 0.067 mg/ml. Excitation was at 355 nm with bandpasses of 8 nm (excitation) and 10 nm (emission).

ammonium sulfate pellet suspension containing Mg^{2+} also shows a 101.4 ± 3.1% of the Dn fluorescence. Insignificant fluorescence changes were observed with the addition of P_i to any solution tested without Mg^{2+}.

Homologs of ATP were introduced to determine the specificity of the fluorescence changes to the (Na^+ + K^+)-ATPase conformational changes. AMP (1 mM), used for the presence of the adenine moiety, had no measurable effect on fluorescence in the presence of Na^+ and Mg^{2+}. Thymidine triphosphate (1 mM) added to similar solutions with Na^+ and Mg^{2+} or to Mg^{2+} alone had insignificant effects on the Dn fluorescence.

On several occasions the increase in Dn fluorescence with either Mg^{2+} and P_i or Na^+, Mg^{2+} and ATP were not elicited from aliquots of the same labeled enzyme preparation. In each of these cases, the labeled sample had been treated in a manner that would decrease or inactivate the native enzyme. These treatments included: freezing and thawing several times, 56 h at 0°C or dialysis at room temperature for prolonged periods. It appeared that the Dn-Cys-Hg^+-labeled enzyme could be denatured if not handled in a manner similar to that for an active enzyme. This was substantiated by treating 'denatured' labeled enzyme and 'inactive' labeled enzyme (which underwent fluorescent changes), with dithiothreitol. This treatment removed the label and, after separation of the Dn-Cys-Hg-DTT from the enzyme, analysis were run. The 'inactive' enzyme recovered 72—90% of control (Na^+ + K^+)-ATPase enzymatic activity while the denatured enzyme remained inactive. The denatured form of the enzyme did not respond to ionic conditions as did the inactivated labeled material.

Polarization of fluorescence

Information concerning the freedom of rotation of the fluorescent moiety can be obtained by examining the polarization of fluorescence. This parameter is independent of the fluorochrome's concentration. Table II shows the polarization of fluorescence of the Dn group covalently attached to the ammonium sulfate pellet material in the solutions previously discussed. The value of 0.354 ± 0.008 in 10 mM Tris buffer (the E-1 state) indicates that the probe is rather rigidly constrained. Values of between 0.1 and 0.2 are typically obtained for fluorescent probes bound to the surface of the membranes [20,21]. The addition of the cations, Mg^{2+}, or Na^+ caused a slight reduction in the

TABLE II

Effects of buffer composition on the polarization of fluorescence of the Dn group in Dn-Cys-Hg-ammonium sulfate pellet. Each value is the average of at least four measurements with a standard deviation of ±0.008 or less. All data were from a single enzyme preparation. The concentration of ligands were as in Fig. 5.

Media	Polarization
Buffer, 10 mM Tris, 0.2 M sucrose, pH 7.4	0.353 ± 0.008
+ $MgCl_2$	0.334
+ Mg^{2+} + P_i	0.332
+ Mg^{2+} + ATP	0.334
+ Na^+	0.326
+ Na^+ + ATP	0.337
+ Na^+ + ATP + Mg^{2+}	0.359

polarization probably due to neutralization of local charge groups relaxing the membrane structure. The important feature of this table is that only small changes, less than 10%, in the polarization of fluorescence occurs in the presence of Mg^{2+} and P_i or Na^+, Mg^{2+}, and ATP.

Discussion

Using the procedure for the purification of the ($Na^+ + K^+$)-ATPase described by Hokin and co-workers, we were able to obtain a relatively pure enzyme preparation. This was determined by SDS gel electrophoresis which also showed that our SH label was only present on one of the major peptides of the enzyme. Although the preparation used here did not have a specific enzyme activity as high as reported by Perrone et al. [7], it was at least 80% pure and we were able to overcome most of the difficulties encountered when brain synaptosomal suspensions were used as a source of the enzyme.

The observation that only the sulfhydryl groups of the 98 000-dalton protein were labeled with the Dn-Cys-Hg^+ agrees with the preparative polyacrylamide work of Hart and Titus [22]. These authors reacted radioactive *N*-ethylmaleimide with rabbit kidney microsomes, and found that the 56 000- and 70 000-dalton proteins did not react with NEM while the 98 000-dalton protein did react with the reagent. The 98 000-dalton peptide is also phosphorylated [23] and is possibly the catalytic subunit of the enzyme. We conclude that the fluorescent changes observed in the present study were associated with the catalytic subunit of the ($Na^+ + K^+$)-ATPase.

The fact that the ($Na^+ + K^+$)-ATPase was totally inactivated when one half of the sulfhydryl groups were reacted further substantiates previous work [24] which indicated that more than one SH group is within the active site region of the enzyme. Changes in net ($Na^+ + K^+$)-ATPase activity would naturally reflect alteration in any of its partial reactions. Examination of the partial reaction of the ($Na^+ + K^+$)-ATPase indicates that inactivation of the ($Na^+ + K^+$)-ATPase by Dn-Cys-Hg^+ occurred at the dephosphorylation step while the phosphorylation was quite insensitive to this SH reagent. Data in Table I show a greater percentage decrease in the PNPPase activity as compared to the percentage decrease in overall enzyme activity. In absolute units of μmol hydrolyzed per unit, the decrease with the synthetic substrate is less than the overall reaction. This reinforces the concept that the dephosphorylation step is the point of blockage by the reagent. Banerjee et al. [24] using *N*-ethylmaleimide as a sulfhydryl reagent concluded that separate sulfhydryl groups are involved in the two partial reactions of the enzyme.

In the present study inactivation of ($Na^+ + K^+$)ATPase by reaction of 50% of the enzyme's sulfhydryl groups with Dn-Cys-Hg^+ was accompanied by dramatic changes in fluorescence of the bound probe. The chief fluorescent changes observed were a 20-fold increase in relative fluorescence and a shift in the emission maximum from 540 to 500 nm. Similar changes were seen when the reagent reacted with the ($Na^+ + K^+$)-ATPase of rat brain synaptosomes [4]. The high value of the polarization of fluorescence indicates that the fluorochrome has restricted freedom of rotation. This would be expected if it were located in a cleft or pore of the structure or if the naphthalene portion of the molecule

were bound to the surface by hydrophobic type interactions at a non-polar region. If the Dn group was protruding from the surface into the aqueous environment the polarization of fluorescence would be much lower. The small changes in the polarization of the Dn group under conditions that caused large relative fluorescence changes may indicate that the conformational changes that occur are within a restricted region of the enzyme matrix. This data would also seem to favor the idea that the probe is within a cleft of the structure rather than on the surface.

The conformational changes of the $(Na^+ + K^+)$-ATPase outlined in Fig. 1 have been shown to cause corresponding changes in the fluorescence of the bound probe. Assuming that the E-1 relaxed state is the lowest energy conformation, the presence of Na^+ caused a slight change in conformation but the specificity is open to question. The presence of Mg^{2+} caused a 30% increase in fluorescence of the probe and can shift the enzyme to an E-2 state (Fig. 1). The greatest fluoresence change was observed in the presence of compounds that would cause an E-2-*P* state, either Na^+, Mg^{2+} and ATP or Mg^{2+} and P_i. The fluorescence of this latter state was twice that of the initial E-1 state. From the fluorescence data the E-2-*P* state reached by the middle pathway of Fig. 1, Na^+, Mg^{2+} and ATP could not be distinguished from the state reached by the reverse of the upper route, (Mg^{2+} and P_i). This should not be taken as proof that these states are identical but only that the site of the fluorescence probe is similar.

The data in Table I indicates the Dn-Cys-Hg^+ inhibits the $(Na^+ + K^+)$-ATPase by blocking the dephosphorylation of the E_2-*P* state in a manner similar to NEM. The fluorescence changes observed in the presence of Mg^{2+} and P_i indicate that the enzyme is also in an E_2-*P* conformation. This would imply that although dephosphorylation is blocked the reverse reaction can occur. As stressed above the fluorescent data only reflect the environment of the probe. A covalent phosphate ester bond may not be necessary for the enzyme to change conformation.

The dramatic increases in the relative fluorescence of the Dn groups in the labeled enzyme under conditions that would lead to conformational changes in the native enzyme indicates that the labeled 'inactive' enzyme is capable of undergoing conformational changes. These inferred conformational changes appear to be specific to the $(Na^+ + K^+)$-ATPase since homologs of ATP will not elicit these changes and if the enzyme has been denatured, no changes were observed. In the present study since all the $(Na^+ + K^+)$-ATPase activity was inhibited at a point where only 25% the kinase activity was lost, it was possible that the Dn-Cys-Hg^+-labeled enzyme was being phosphorylated and the fluorescence changes observed were due to the E-2-*P* state. One interpretation of the fluorescent changes is that the Dn group is being shifted into a more non-polar region of the structure which agrees with the polarization of fluorescence data.

The techniques reported here for the fluorescent labeling of the active site of an enzyme and detection of subsequent conformational changes by fluorescence may be generally useful in studying other SH-requiring enzymes. In the present case the net $(Na^+ + K^+)$-ATPase activity was inhibited by reaction with the Dn-Cys-Hg^+ but the enzyme was still apparently able to undergo conformational changes in the presence of appropriate ligands. This presents good

physical evidence that specific ligands can influence the conformational site of the enzyme.

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