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INTERACTION OF A NEW FLUORESCENT REAGENT WITH SULFHYDRYL GROUPS OF THE ($Na^+ + K^+$)-STIMULATED ATPase

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SUMMARY

The ($Na^+ + K^+$)-ATPase enzyme of rat brain microsomes can be reversibly inhibited by a new fluorescent sulfhydryl (SH) probe, dimethylaminonaphthalene-cysteine-Hg^+ (Dn-cys-Hg^+). This reagent is more reactive than *N*-ethylmaleimide (MalNEt) toward membrane sulfhydryl groups. A procedure using the two SH reagents sequentially seems to permit a more selective labelling of the SH groups involved in the ($Na^+ + K^+$)-ATPase than is possible by using MalNEt alone. Brain microsomes treated by this method incorporated the fluorescent label within or near the active site of the enzyme. When the probe was bound a blue shift of its fluorescence emission maximum (from 540 to 495 nm) and a 20-fold increase in relative fluorescence occurred. This indicates that the Dn moiety is within a very non-polar region of the membrane.

INTRODUCTION

The ($Na^+ + K^+$)-ATPase enzyme can be inhibited by reaction with a number of sulfhydryl reagents [1]. The reaction of *N*-ethylmaleimide (MalNEt), a sulfhydryl reagent, with the membrane ($Na^+ + K^+$)-ATPase from several sources has been extensively studied [1–5]. MalNEt inhibits the dephosphorylation of the enzyme [2] and under certain conditions can cause an increase in the Na^+-stimulated ADP–ATP exchange reaction [5, 6]. The presence of Na^+ and ATP in the MalNEt reaction medium protects the ($Na^+ + K^+$)-ATPase from inhibition [3–5, 7] and has been used for specific labelling of SH groups related to the enzyme [7].

Only a very small portion of reactive sulfhydryl groups within brain microsomal membranes are probably involved in the functioning of the ($Na^+ + K^+$)-ATPase [7, 8]. Our approach, like that of Hart and Titus [7], has been to react the

Abbreviations: Dn-cys-Hg^+, dimethylaminonaphthalene-cysteine-Hg^+; MalNEt, *N*-ethylmaleimide; GSH, glutathione; SH, sulfhydryl.

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membrane SH groups with MalNEt in the presence of Na^+ and ATP. Under these conditions those membrane SH groups not involved with the enzyme would react with MalNEt. The protecting ligands could then be removed and those SH groups remaining would be reacted with a new fluorescent sulfhydryl reagent, dimethylaminonaphthalene-cysteine-Hg^+ (Dn-cys-Hg^+) [9]. This reagent forms a mercaptide linkage (S-Hg-S) with reactive SH groups.

It has been demonstrated that the fluorescence of Dn-cys-Hg^+ is affected by the type of compound with which it reacts. After reaction with the muscle proteins, tropomyosin or G or F actin, the Dn moiety shows a shift in its emission maximum to shorter wavelengths and there is an overall increase in relative fluorescence which is not seen after its reaction with small molecules such as cysteine and glutathione [9].

There are several classes of sulfhydryls within membranes [10]. There appears to be a class of very reactive SH groups that react with nearly all sulfhydryl reagents, a less reactive class that combines only with strong sulfhydryl reagents, and a class of sulfhydryls that reacts only after the membrane has been denatured or solubilized. Changes in fluorescent characteristics of Dn-cys-Hg^+ after reaction with membrane SH groups should yield information both on the reactivity of the SH groups and on the nature of the environment of SH groups related to the $(Na^+ + K^+)$-ATPase system.

METHODS

Rat brain microsomes were prepared by differential centrifugation in 0.32 M sucrose, pH 7.4 [11] and stored at −80 °C in sucrose. 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 was determined by the Lowry method [12]. Sulfhydryl groups were analyzed by the procedure described by Boyne and Ellman [13]. Dn-cys-$^{203}Hg^+$ was prepared as described by Leavis and Lehrer [9] with the inclusion of ^{203}Hg (New England Nuclear, Spec. Act. 5.8 Ci/g). ^{203}Hg was measured using an auto gamma counter. Fluorescence measurements were made using a Perkin-

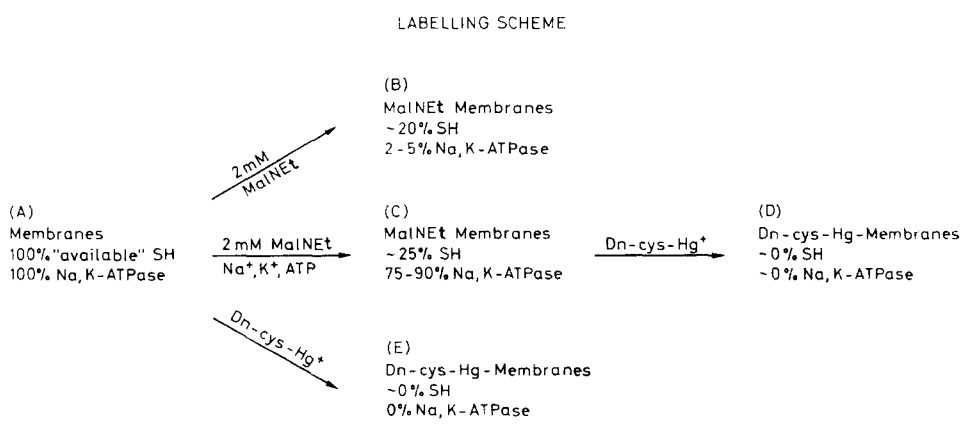

Fig. 1. Scheme for labelling sulfhydryl groups of rat brain microsomes. See text for details.

Elmer MPF-2A Spectrofluorometer and spectra were not corrected for instrumental response.

The reactions of either sulfhydryl reagent with the microsomal preparation (1–3 mg protein/ml) outlined in Fig. 1 were carried out in 30 mM Tris–HCl, 5 mM EDTA (pH 7.4) in a final volume of 2 ml. Incubations were for 45 min at 37 °C. Product B was obtained in the presence of 2 mM MalNEt, whereas product C formed when 2 mM MalNEt plus 120 mM NaCl, 30 mM KCl and 3 mM ATP–Tris were present. Addition of 120–125 μM (3-fold excess of reagent to available SH groups) Dn-cys-$^{203}Hg^{+}$ to products A or C yielded products E and D, respectively. All reactions were stopped by addition of 2 vols. of 5 mM cysteine, 10 mM Tris–HCl (pH 7.4). The preparation was centrifuged at 15 000 rev./min (SS-34 rotor) in a Sorvall refrigerated centrifuge for 20 min. The microsomes were washed until there was either no detectable cysteine in the supernatant or until the ^{203}Hg radioactivity in the supernatant reached background level. Usually 3–4 washes were sufficient.

RESULTS

Comparison of reactivity of MalNEt and Dn-cys-Hg^{+} toward the ($Na^{+}+K^{+}$)-ATPase

The difference in reactivity between Dn-cys-Hg^{+} and MalNEt toward the ($Na^{+}+K^{+}$)-ATPase is illustrated in Fig. 2. The reaction of Dn-cys-Hg^{+} with the membrane SH groups (no ligand protection) was more than 50 % complete in less than 1 min; whereas, 8–10 min were required for 50 % inhibition using MalNEt. A 2 mM MalNEt concentration was needed to produce the same degree of enzyme inhibition produced by 35 μM Dn-cys-Hg^{+}.

Table I shows a second major difference in the reactivity of these reagents toward the ($Na^{+}+K^{+}$)-ATPase. Na^{+} and ATP protect the enzyme against inhibition by MalNEt, while these same ligands promote inhibition of the enzyme by Dn-cys-

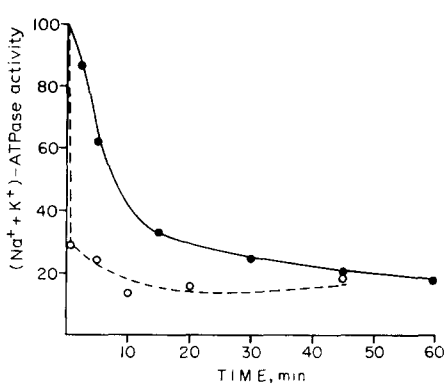

Fig. 2. Inactivation of ($Na^{+}+K^{+}$)-ATPase by Dn-cys-Hg^{+} or MalNEt. Rat Brain microsomes, 1.5 mg protein/ml, in 60 mM Tris · HCl/10 mM EDTA (pH 7.4) were pre-incubated for 5 min at 37 °C. Then either 2 mM MalNEt(●) or 36 μM Dn-cys-Hg^{+} (○) were added and aliquots withdrawn at the indicated times. These were made 5 mM in cysteine and were assayed immediately for ($Na^{+}+K^{+}$)-ATPase activity at 37 °C.

TABLE I

EFFECT OF SULFHYDRYL REAGENTS ON $(Na^+ + K^+)$-ATPase

Rat brain microsomes, 3.5 mg protein/ml, were reacted with 2 mM MalNEt in either (B) 30 mM Tris · HCl/5 mM EDTA buffer (pH 7.4) or (A) buffer with 100 mM NaCl, 30 mM KCl and 3 mM ATP · Tris (pH 7.4) at 37 °C for 45 min. The reaction was stopped by the addition of 2 vols. of 5 mM cysteine/10 mM Tris · HCl (pH 7.4). The cysteine was removed by repetitive washing (see Methods). The sulfhydryl content (Ellman procedure) of each preparation was determined. An aliquot of microsomes from sample (A) was resuspended at 3.5 mg protein/ml in either Tris-EDTA buffer (to yield Sample D) or buffer containing 120 mM NaCl, 30 mM KCl and 3 mM ATP · Tris (sample C). Dn-cys $^{203}Hg^+$ was added in a 1 : 1 molar ratio to reactive sulfhydryl groups present. These were incubated for 20 min at 37 °C and were stopped with cysteine and washed as above. $(Na^+ + K^+)$-ATPase was assayed in all samples and ^{203}Hg was measured by auto gamma counting as described under Methods.

	Sample	Additions	SH reagent	Molar ratio reagent : SH	$(Na^+ + K^+)$-ATPase (μmol Pi formed/mg protein per h)	nmol Dn-cys-^{203}Hg bound/mg protein
A	Microsomes	NaCl KCl, ATP	MalNEt	23 : 1	9.56	–
B	Microsomes	none	MalNEt	23 : 1	0.87	–
C	Sample A above	NaCl, KCl, ATP	Dn-cys-$^{203}Hg^+$	1 : 1	0	1.4
D	Sample A above	none	Dn-cys-$^{203}Hg^+$	1 : 1	4.62	0.708

TABLE II

REVERSIBILITY OF Dn-cys-Hg^+ INHIBITION BY DITHIOTHREITOL

Rat brain microsomes were suspended in 30 mM Tris · HCl/5 mM EDTA buffer (pH 7.4). Two thirds of the suspension were treated with a 3 : 1 molar excess of Dn-cys-$^{203}Hg^+$ available SH groups for 20 min at 37 °C. The remaining third of the original suspension represented the control preparation (A) and the Dn-cys-$^{203}Hg^+$-treated suspension was divided to yield preparations B and C. After the incubation, these preparations were each washed with 5 mM cysteine/10 mM Tris · HCl (pH 7.4) at 5 °C and then with 10 mM Tris · HCl (pH 7.4) until all cysteine was removed. Pellets were resuspended to 1.2 mg protein/ ml in Tris-EDTA buffer and were incubated for 1 h at 25 °C with or without 5 mM dithiothreitol. They were then dialyzed against 1000 vols. of 5 mM Tris · HCl (pH 7.4) at 5 °C for 16 h with one buffer change, and enzyme activity was measured. Values in parenthesis represent percent of recovered enzyme activity relative to the control microsomes (sample A). The ^{203}Hg retained by samples B and C was measured after dialysis.

Sample		Additives	$(Na^+ + K^+)$-ATPase (μmol Pi formed/mg protein per h)	% recovery of ^{203}Hg/mg protein
A	Control microsomes	5 mM dithiothreitol	14.76 (100 %)	–
B	Microsomes reacted with Dn-cys-$^{203}Hg^+$	–	2.65 (18 %)	88
C	Microsomes reacted with Dn-cys-$^{203}Hg^+$	5 mM dithiothreitol	13.60 (92 %)	11.6

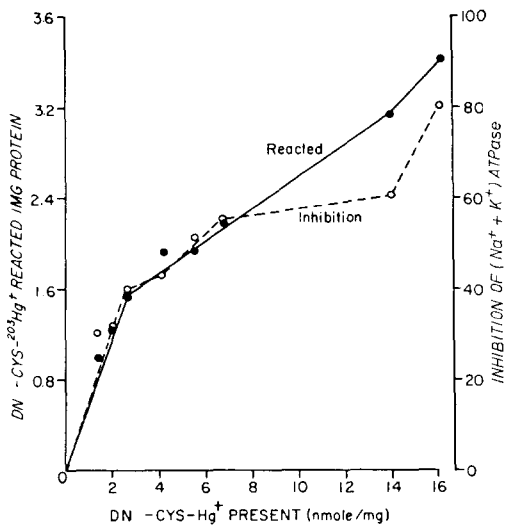

Fig. 3. Reaction of Dn-cys-^{203} Hg^+ with ligand-protected brain microsomes. Ligand-protected rat brain microsomes (Fig. 1, product C) were suspended to 0.7 mg protein/ml in 30 mM Tris · HCl/ 5 mM EDTA (pH 7.4) and contained 9 nmol available SH/mg protein. They were treated with the various concentrations of Dn-cys-^{203} Hg^+ indicated at 37 °C for 20 min and the reaction was stopped with cysteine as in Table I. The microsomes were washed by centrifugation four times with Tris-EDTA buffer and resuspended to the initial volume for ($Na^+ + K^+$)-ATPase assays and measurement of $^{203}Hg^+$ content.

Hg^+. At a 1 : 1 molar ratio of Dn-cys-Hg^+ to "reactive SH groups"* in Tris-EDTA buffer, half of the enzyme activity was lost. However, under the same conditions with Na^+ and ATP in the medium all the enzyme activity was lost and twice the number of sulfhydryl groups were reacted with Dn-cys-Hg^+.

The mercaptide bond formed between the membrane sulfhydryl and Dn-cys-Hg^+ can be broken by the addition of excess dithiothreitol and the enzyme activity can be recovered. Table II shows that 92 % of the ($Na^+ + K^+$)-ATPase activity lost owing to Dn-cys-Hg^+ treatment was recovered after incubation with 5 mM dithiothreitol. Dithiothreitol had no demonstrable effect on a MalNEt-treated enzyme preparation under similar conditions (data not shown).

The stoichiometry of the reaction of Dn-cys-$^{203}Hg^+$ with protected SH groups and the inhibition of the ($Na^+ + K^+$)-ATPase are correlated in Fig. 3. At approximately a 1 : 1 molar ratio of Dn-cys-Hg^+ to available SH groups (Ellman method), there was 50 % inhibition of the enzyme in Tris-EDTA buffer. The degree of inhibition correlated with the amount of Dn-cys-$^{203}Hg^+$ retained by the microsomal pellet after repeated washings, as determined by measurement of ^{203}Hg. The decrease in SH groups in the microsomes, determined by the Ellman procedure, corresponded to the increase of membrane bound ^{203}Hg, indicating that the Dn-cys-^{203}Hg was reacting only with the sulfhydryl groups.

* "Reactive SH groups" refers to the 24–35 nmol SH groups/mg protein found in rat brain microsomes by the procedure of Boyne and Ellman [13] in Tris · HCl buffer (pH 7.4). Additional SH groups became reactive toward Ellman's Reagent after treatment of microsomes with 1 % sodium dodecyl sulfate but are not considered in the present context as "reactive".

The properties of each of these sulfhydryl reagents toward the $(Na^+ + K^+)$-ATPase system may be summarized as follows. (1) MalNEt is required in great excess to inhibit the enzyme, while Dn-cys-Hg^+ reacts rapidly and stoichiometrically with SH groups to produce inhibition of the enzyme. (2) Inactivation by MalNEt is irreversible under the conditions used here while it is possible to remove Dn-cys-Hg^+ by dithiothreitol treatment and to recover most of the enzyme activity. (3) Na^+ and ATP protect the $(Na^+ + K^+)$-ATPase from reaction with MalNEt but in the presence of Dn-cys-Hg^+ these ligands cause increased reactivity toward SH groups, which results in increased inhibition of the enzyme.

Specific labelling scheme for $(Na^+ + K^+)$-ATPase

The procedure for the specific labelling of SH groups in the $(Na^+ + K^+)$-ATPase with Dn-cys-Hg^+ is shown in Fig. 1. Rat brain microsomes were treated with MalNEt in the presence of NaCl, KCl and ATP to protect sulfhydryl groups related to the $(Na^+ + K^+)$-ATPase to yield product C (Fig. 1) while other SH groups not involved with the enzyme reacted with this agent. Under these conditions 75 % of the available SH groups of microsomes were reacted and 75–90 % of the $(Na^+ + K^+)$-ATPase activity was retained. The reaction was stopped by adding 2 vols. of 5 mM cysteine and the protecting ions and cysteine were subsequently removed by repetitive centrifugation. The protected microsomes were then reacted with Dn-cys-$^{203}Hg^+$ to yield product D with all "available" SH groups reacted and totally inhibited enzyme. Reaction of microsomes with MalNEt without protective ligands (product B) inhibited greater than 95 % of the $(Na^+ + K^+)$-ATPase activity and reacted with about 80 % of the available SH groups. On the other hand reaction of microsomes with Dn-cys-Hg^+ inhibited all $(Na^+ + K^+)$-ATPase activity and reacted with all available SH groups. It should be stressed that only a small number of SH groups, about 1–2 nmol/mg protein, were protected by Na^+, K^+ and ATP. The total number of available reactive SH groups in the preparation A (Fig. 1) was between 24 and 35 nmol/mg protein.

Fluorescence of Dn-cys-Hg^+-labelled microsomes

The fluorescence excitation and emission spectra of the protected, labelled microsomes (Fig. 1D) and the corresponding spectra of Dn-cys-Hg^+ reacted with the tripeptide glutathione (GSH) are presented in Fig. 4. The concentration of the fluorescent moiety, Dn, in the membrane preparation was 0.57 μM and of the Dn-cys-Hg-GSH 3.8 μM. The large shoulder on the excitation spectra at 290 nm was due to energy transfer from the membrane proteins to the attached Dn groups. The very large blue shift in the emission spectra from 540 to 495 nm when the Dn was covalently bound to the membrane was not seen when the same fluorescent groups were attached to the glutathione and exposed to aqueous environments.

The relative fluorescence and the shift in the fluorescence emission maximum of several compounds labelled with Dn-cys-Hg^+ are presented in Table III. The relative fluorescence of Dn-cys-Hg-GSH was used as a standard for comparison. Dn-cys-Hg^+ was more fluorescent after it had reacted with GSH than alone in solution. When all the available sulfhydryl groups in the brain microsomes, including those that could be protected by Na^+, K^+ and ATP, were reacted with Dn-cys-Hg^+ (product E, Fig. 1) there was a 13-fold increase in relative fluorescence compared to

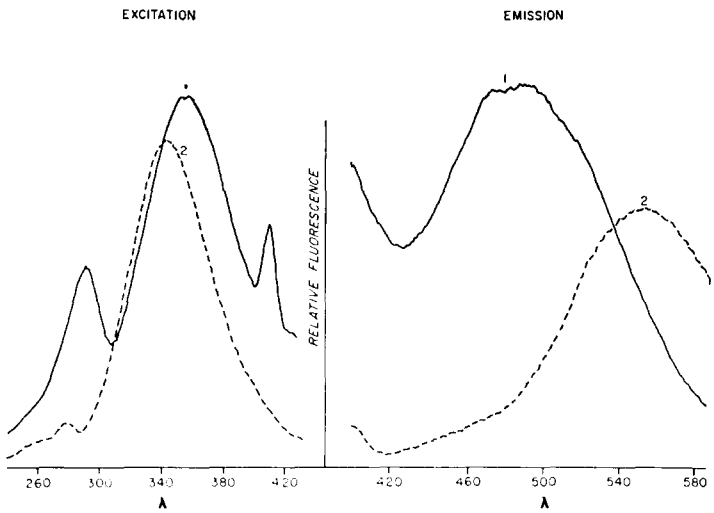

Fig. 4. Excitation and emission spectra of Dn-cys-^{203}Hg-GSH and Dn-cys-^{203}Hg-microsomes. The preparation of Dn-cys-^{203}Hg-GSH (curve 2) was prepared as described in Table III and the final solution was 3.8 μM in Dn. The Dn-cys-^{203}Hg-microsomes (product D, Fig. 1) were 0.57 μM with respect to Dn (curve 1). The excitation and emission spectra of the samples in 400 μl of 30 mM Tris · HCl/5 mM EDTA (pH 7.4) were run in microfluorometer cuvettes. The excitation and emission band pass were set at 7 nm. The excitation spectra were measured at the emission maxima: 540 nm for Dn-cys-^{203}Hg-GSH and 495 nm for Dn-cys-^{203}Hg$^+$-treated microsomes. The emission spectra were also run at the excitation maxima of 340 and 350 nm, respectively.

TABLE III

FLUORESCENCE OF Dn-cys-^{203}Hg$^+$ AND REACTION PRODUCTS

GSH was dissolved in 30 mM Tris · HCl/5 mM EDTA (pH 7.4) and the SH content determined [13]. Then an equimolar amount of Dn-cys-^{203}Hg$^+$ was added and the solution allowed to equilibrate at 25 °C. Further additions of Dn-cys-^{203}Hg$^+$ did not increase the relative fluorescence of the product. The fluorescence of the other products was measured in the same buffer at 25 °C and the fluorescence of Dn-cys-^{203}Hg$^+$-GSH was arbitrarily set at unity. The reported fluorescence has been corrected for the amount of Dn-cys-^{203}Hg$^+$ present in the product. The data on tropomyosin and actin have been taken from the work of Leavis and Lehrer [9].

	Relative fluorescence	Shift of emission maximum
Dn-cys-^{203}Hg-GSH	1.0	0
Dn-cys-^{203}Hg$^+$	0.48	0
Microsomes+Dn-cys-^{203}Hg$^+$ (all available SH reacted, Fig. 1, product E)	13.2	540 → 495
Microsomes reacted with MalNEt with protecting ligands present, then Dn-cys ^{203}Hg$^+$ (Fig. 1, product D)	21.8	540 → 495
Calculated fluorescence due to non-protected SH in microsomes	8.8	–
Dn-cys-Hg-tropomyosin	~ 1.4	535 → 522
Dn-cys-Hg-G or F actin	10	535 → 515

Dn-cys-Hg-GSH. Reacting the microsomal SH groups that had been protected from MalNEt with excess Dn-cys-Hg^+, i.e. the $(Na^+ + K^+)$-ATPase SH groups, produced greater than a 20-fold increase in relative fluorescence. Since the number of SH groups in each preparation was known, it was possible to calculate the increase in fluorescence that was due to the "non-$(Na^+ + K^+)$-ATPase" SH groups. This was estimated to be a 9-fold increase as compared to a 20-fold increase for the $(Na^+ + K^+)$-ATPase SH groups. The increase in fluorescence and emission shift as reported by Leavis and Lehrer [9] for the muscle proteins, tropomyosin and G and F actin are included for comparison. There was no discernable difference in the shift in the emission maximum when all the membrane SH groups reacted (Fig. 1, product E) or when only the protected SH groups (Fig. 1, product D) were reacted with Dn-cys-Hg^+.

Figure 5 shows that the relative fluorescence of labelled protected microsomes undergoes changes when specific ligands that promote conformational changes in the $(Na^+ + K^+)$-ATPase are present. In a solution containing Mg^{2+} and ATP the enzyme can change conformation from an E_1 state to an E_2 or E_2-P state [7]. It is thought that Mg^{2+} is needed for the enzyme to become phosphorylated, but even in the presence of Na^+ and ATP an increase in fluorescence is seen. There was a reproducible increase in fluorescence of the protected Dn-cys-Hg^+-labelled microsomes in each of these conditions. Since the enzyme had been inactivated by its reaction with Dn-cys-Hg^+ it is difficult to prove that conformational changes have actually occurred. However, ionic conditions that are thought to change the conformation of the $(Na^+ + K^+)$-ATPase do change the relative fluorescence of the fluorescent probe. The relative increase in fluorescence and the large shift in the emission maximum

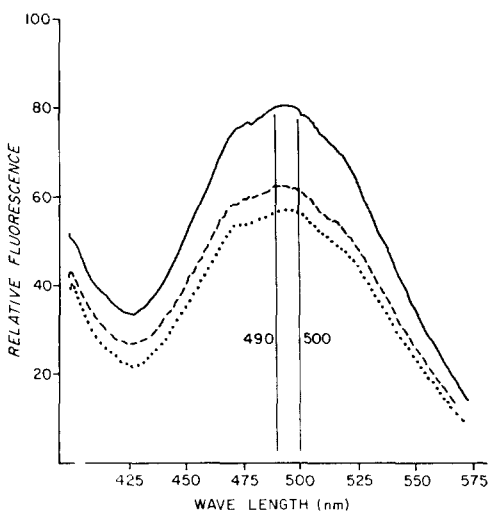

Fig. 5. Emission spectra. Dn-cys-$^{203}Hg^+$-labelled microsomes (Fig. 1, product D) at 0.17 mg protein/ml and containing 12 nmol Dn-cys-$^{203}Hg^+$/mg protein were suspended in 30 mM Tris · HCl (pH 7.4). The initial fluorescence of the suspension in a volume of 400 μl was established and additions of 15 mM $MgCl_2$ and 3 mM ATP · Tris (pH 7.4) (—) or 100 mM NaCl and 3 mM ATP · Tris (pH 7.4) (- - -) were made. An equivalent volume of buffer was added to the control cuvette (· · ·). Excitation was a 350 nm with 8 nm band pass. The emission band pass was 10 nm.

indicate that the Dn portion of the label is within a very non-polar environment within the membrane.

DISCUSSION

Dn-cys-Hg^+ offers several advantages over other commonly used SH reagents. Its reactivity with SH groups is rapid and stoichiometric and permits the titration of these groups to be monitored fluorimetrically. Nanomolar quantities of SH groups can be fluorometrically detected compared with micromolar quantities using dithio-bis-dinitrobenzene sulfonate (Ellman's reagent). In the present experiments we have used a radioactive label as a means of quantitating the reactive SH groups within the membranes. The results obtained by this procedure and the Ellman method were equivalent. The mercaptide linkage may be broken with dithiothreitol, and Dn-cys-Hg^+ could potentially be used as an SH protective reagent in a preparative scheme for enzyme isolation with later removal of the mercaptide.

The present study, as well as that of Hart and Titus [7], has shown that the ligands Na^+, K^+ and ATP protect reactive sulfhydryl groups of the $(Na^+ + K^+)$-ATPase, probably by inducing conformational changes within the enzyme. It is therefore possible to react SH groups unrelated to the active site of the $(Na^+ + K^+)$-ATPase with MalNEt with little loss of enzymatic activity. After removal of the protective ligands the reactive sulfhydryls can then be reacted with another sulfhydryl reagent such as Dn-cys-Hg^+ with complete loss of enzyme activity. This finding, in conjunction with the work of Hart and Titus [7], implies that SH groups of the $(Na^+ - K^+)$-ATPase are being selectively labelled.

Hart and Titus [7] have used a double labelling technique with [^{3}H]MalNEt and [^{14}C]MalNEt in conjunction with preparative polyacrylamide electrophoresis to label the $[Na^+ + K^+)$-ATPase SH groups. They observed specific labelling of a 98 000 dalton protein, which is thought to represent the catalytic subunit of the enzyme [7, 16]. In these studies the conformation of the enzyme was critical to the number of reactive sulfhydryls that were labelled in this peptide. The number varied from 2 to 6 mol SH per active site. In the present study when 1–2 nmol SH/mg protein were reacted with Dn-cys-Hg^+, complete inhibition of the enzyme resulted. This figure is higher than the number of active sites per mg protein, determined by specific binding of [^{3}H]ouabain [14], which was 0.2 nmol/mg or the 0.3 nmol ^{32}P bound as calculated by Alexander and Rodnight [15]. However, Kyte [16] found 19 cysteic acid residues per unit 98 000 dalton peptide. Also, if as suggested by Hart and Titus [7] there are 2–6 nmol of SH groups per active site, the degree of labelling with Dn-cys-Hg^+ is reasonable and suggests specific labelling of a component of the $(Na^+ + K^+)$-ATPase, probably the 98 000 dalton peptide, although the evidence for this is clearly indirect.

Better evidence on the membrane components labelled may be obtained from polyacrylamide gel electrophoresis. However, we have been unable to obtain satisfactory resolution of protein components on sodium dodecyl sulfate-polyacrylamide gels without a dithiothreitol treatment of the microsomal membranes. Dithiothreitol, of course, breaks the mercaptide linkage and precludes localization of the fluorescent label. Further work is being carried out on a more purified preparation of $(Na^+ + K^+)$ ATPase which should decrease the fluorescence background interference due to reaction of Dn-cys-Hg^+ with unrelated SH groups.

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