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Environment of the Sulfhydryl Groups in Bovine Heart Mitochondrial H^+-ATPase

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Abstract

Electron transport particles and purified H^+-ATPase (F_1–F_o) vesicles from beef heart mitochondria have been treated with two classes of thiol reagent, viz. membrane-impermeable organomercurials and a homologous series of *N*-polymethylene carboxymaleimides (Mal–$(CH_2)_x$–COOH or AMx). The effect of such treatment on ATP-driven reactions (ATP-P_i exchange and proton translocation) has been examined and compared to the effects on rates of ATP hydrolysis. The organomercurials inhibited ATP–P_i exchange and one of them (*p*-chloromercuribenzoate) inhibited ATPase activity. Of the maleimide series (AMx), AM10 and AM11 inhibited both ATP-P_i exchange and ATP-driven membrane potential, but not ATPase activity. The other members of the series were essentially inactive. *N*-Ethylmaleimide was intermediate in its efficacy. Passive H^+ conductance through the membrane sector F_o was 50% blocked by AM10, slightly blocked by AM2 and *N*-ethylmaleimide, and unaffected by the other members of the AMx series. The data imply that one –SH near the membrane surface and one –SH about 12 Å from the surface are functional in proton translocation through the H^+-ATPase.

Key Words: H^+-ATPase; beef heart mitochondria; sulfhydryl groups; maleimides; P_i–ATP exchange; proton translocation

Introduction

The inhibition of P_i–ATP exchange activity in submitochondrial particles by organomercurial compounds was first observed by Cooper and Lehninger (1957). Since *N*-ethylmaleimide also inhibited the reaction (Sanadi *et al.*,

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1968), the role of –SH compounds in the ATP synthase reaction became of considerable interest. The involvement of a vicinal dithiol in the reaction was proposed on the basis of inhibition of P_i–ATP exchange and other energy-linked reactions by Cd^{2+} (Fluharty and Sanadi, 1960; Pringle and Sanadi, 1984) and arsenicals in the presence of excess monothiol compounds (Fluharty and Sanadi, 1960; Joshi and Hughes, 1981), and by carboxypyridine disulfide (Blanchy *et al.*, 1978).

Most of the recent studies on the role of thiol groups in the P_i–ATP exchange reaction have utilized purified preparations of ATP synthase (F_1–F_0, or H^+-ATPase) (Stiggal *et al.*, 1979a; Joshi *et al.*, 1979; Hughes *et al.*, 1982), as well as the resolved individual F_0 (membrane sector) and F_1 (soluble ATPase) segments. There is evidence that *N*-ethylmaleimide (Godinot *et al.*, 1981; Hughes *et al.*, 1983), Cd^{2+}, and arsenicals (Joshi and Hughes, 1981) cause inactivation of P_i–ATP exchange in H^+-ATPase by binding to coupling factor B (F_B),[4] a dithiol protein (Joshi and Hughes, 1981; Stiggal *et al.*, 1979b; Hughes *et al.*, 1979) associated with F_0 (Joshi *et al.*, 1979). When F_B of the H^+-ATPase is inactivated by –SH reagents or is depleted by extraction, the oligomycin-sensitive ATPase activity is not inhibited; in fact it is slightly stimulated (Joshi *et al.*, 1979). These results would confine the inhibitory effects to the proton translocation reaction.

It is interesting that the coupling factor activity of purified F_B as well as the P_i–ATP exchange activity of H^+-ATPase are inhibited by both membrane-impermeable mercurials (Lam and Yang, 1969; Godinot *et al.*, 1981) and hydrophobic membrane-permeable thiol reagents (Godinot *et al.*, 1981, Sanadi, 1982). The data have led to the suggestion that one of the –SH groups of F_B is in a hydrophobic environment (Sanadi, 1982). Whether other mitochondrial –SH groups are functional in proton translocation, and where such –SH group(s) might be located are questions which remain unanswered.

With the use of reagents containing a polar group and a nonpolar –SH-reactive group separated by hydrocarbon spacers of different length, it has recently become possible to probe the depth of functionally active –SH groups within the membrane phase (Abbot and Schacter, 1976; Biber and Hauser, 1979). We have employed the series of *N*-polymethylene carboxymaleimides (Mal–$(CH_2)_x$COOH, or acid maleimides, AMx, where x has values from 1 to 11) synthesized by Griffiths *et al.* (1981) to explore the position of the –SH group involved in ATP-driven reactions of the H^+-ATPase. These reagents were previously used for studies of the –SH group functional in the phosphate transport activity of mitochondria (Griffiths *et al.*, 1981).

[4] F_B, coupling factor B from beef heart mitochondria; oxonal VI, bis-(3-propyl-5-oxoisoxazol-4-yl)pentamethineoxonol; ETP_H, electron transport particles.

Materials and Methods

Electron transport particles (ETP_H) were prepared from frozen beef heart mitochondria (Linnane and Ziegler, 1958) and extracted with lysolecithin to yield a vesicular preparation of H^+-ATPase ((F_1–F_o) according to Hughes *et al.*, 1982. Treatment of the H^+-ATPase with 3.5 M sodium bromide following the procedure of Tzagoloff and Meagher (1971) afforded the membrane sector F_o. Oligomycin-sensitive ATPase activity was assayed by the rate of inorganic phosphate liberated (Tzagoloff *et al.*, 1968), and the ATP–P_i exchange reaction was measured according to Joshi *et al.* (1979). ATP-driven membrane potential was measured indirectly with the potential-sensitive dye oxonol VI (donated by Drs. B. Chance and L. Bashford) as we have previously described (Hughes *et al.*, 1982; Pringle and Sanadi, 1984; see also the legend to Fig. 4). Passive proton conductance through F_o was measured by the quenching of 9-aminoacridine fluorescence after reconstituting the complex into K^+-loaded asolectin liposomes by a freeze/thaw/sonication procedure (Sanadi *et al.*, 1984). Thus, 20 mg of asolectin which had been dried from $CHCl_3$ solution in a thin film under nitrogen was sonicated to clarity in 1 ml of Tricine buffer (10 mM, pH 7.5) containing 200 mM KCl. F_o (1 mg) in 10 ml of Tricine (10 mM, pH 7.5) containing 1 mM dithiothreitol was spun down and the resulting membrane fragments were resuspended with the liposomes. The mixture was frozen in liquid nitrogen, thawed, and briefly sonicated for a few seconds. Aliquots (25–50 μl) of proteoliposomes were diluted into 2 ml of Tricine buffer containing 200 mM NaCl instead of KCl, and 5 μM 9-aminoacridine. Fluorescence was measured on a Perkin-Elmer MPF-44A spectrofluorimeter using excitation and emission wavelengths of 365 and 451 nm, respectively. A potassium diffusion potential was initiated by the addition of 50 ng of valinomycin (10 μg/ml in ethanol). The AMx series of maleimides were dissolved in dimethylsulfoxide and used as 100 mM stock solutions. Organomercurial compounds were dissolved in glycylglycine (pH 11.5) as 200 or 500 mM stock solutions. Radiolabeling F_1–F_o with ^{115}Cd and subsequent SDS PAGE electrophoresis were carried out as in Sanadi *et al.* (1984). Prior to ^{115}Cd treatment, one sample (3 mg) of F_1–F_o was incubated with 10 mM AM10 in 8 ml of buffer for 30 min at room temperature. This and a control sample were spun down, resuspended (10 mg/ml), and then subjected to ^{115}Cd labeling and electrophoresis.

Results

For comparative purposes, experiments have been carried out with both ETP_H and the H^+-ATPase to study the effects of the acid maleimide

compounds (AMx) on P_i–ATP exchange, a reaction which essentially reflects the membrane potential generated by the proton pump. Figure 1A shows that incubation of ETP_H with 2 mM AMx for 15 min produced variable inhibition, which was most pronounced with AM10 and AM11. In the next series of experiments (Fig. 1B), purified H^+-ATPase was used under three different conditions of incubation, i.e., concentrations and incubation times were varied. The activity of control samples was approximately 600 nmol · min^{-1} · mg $protein^{-1}$ in P_i–ATP exchange. It would appear that in a concentration range of 250–500 μM and 15 min incubation, suitable distinction between the different maleimides can be seen. In the third series, titration curves were established for AM10 and AM11 (Fig. 1C). At very low concentrations (10–20 μM), there was a slight stimulation of P_i–ATP exchange activity. Such stimulation has also been observed with low levels of oligomycin (Sanadi, 1982) and has been attributed to the blocking of leaky channels in defective H^+-ATPase complexes in the membrane. In a separate experiment, AM10 was treated with β-mercaptoethanol and then incubated with F_1–F_o at 500 μM. Where the AM10-treated sample was inhibited 92% in ATP–P_i exchange, the sample treated with β-mercaptoethanol/AM10 was only inhibited by 7%. The loss of exchange activity is therefore attributed to an –SH/maleimide interaction and not merely to a nonspecific membrane perturbation. In this context, it is worth noting that Griffiths *et al.* (1981) did not find a correlation between inhibition of phosphate transport and the membrane partition coefficient of the AMx series. It is interesting to note that *N*-ethylmaleimide, a membrane-permeable thiol reagent, produces significantly less inhibition that AM10 or AM11 under the same conditions. If the local concentrations of AM10 or AM11 maleimide groups were higher than that of *N*-ethylmaleimide near the reactive –SH, this difference in activity could be accounted for.

From these observations, it would appear that the AMx-sensitive thiol group is located in the bilayer region some distance from the surface of the membrane. The effect of the AMx series (500 μM, 15 min incubation) on the ATP hydrolysis activity of H^+-ATPase was examined, and little or no inhibition was observed with any of the compounds tested (data not shown).

The effects of various organomercurials were tested on both P_i–ATP exchange and ATPase activities. All of the compounds (*p*-chloromercuribenzoate, *p*-hydroxymercuribenzoate, *p*-chloromercuribenzenesulfonate, and mersalyl) exhibited biphasic inhibition curves with respect to exchange activity, i.e., activity dropped sharply to 40% at ca. 25–50 μM mercurial and then more gradually to about 20% at ca. 800 μM (data not shown). Prior addition of dithiothreitol or 2,3-dimercaptopropanol prevented inhibition, but thiol addition subsequent to the mercurial produced only partial reversal. It

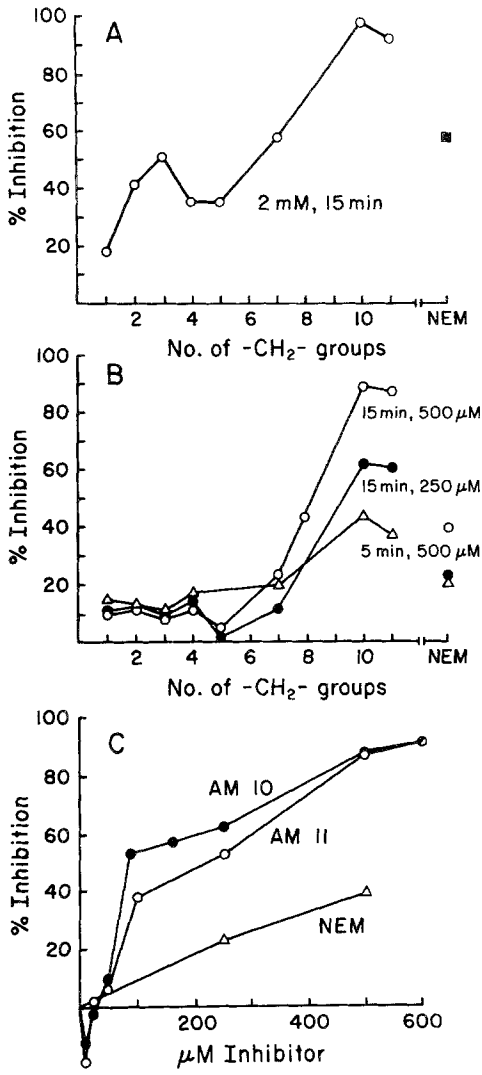

Fig. 1. Inhibition of ATP–P_i exchange activity by AMx thiol reagents. (A) ETP_H (200 μg) in 100 μl Tricine/sucrose buffer (pH 8.0) were incubated with 2 mM of the appropriate maleimide (100 mM in dimethylsulfoxide) for 15 min at 4°C, and assayed for exchange activity as previously described (Joshi *et al.*, 1979). The results are plotted in terms of the degree of inhibition of exchange activity versus the number of methylene groups in the alkyl chain of the maleimide. (B) Purified H^+-ATPase was assayed as in (A) with the exception that the thiol reagent concentrations and incubation times are as shown in the figure, viz. 250 μM, 15 min (●); 500 μM, 5 min (△); 500 μM, 15 min (○). (C) Inhibition of ATP–P_i exchange by AM10 (●), AM11 (○), and, for comparative purposes, NEM (△).

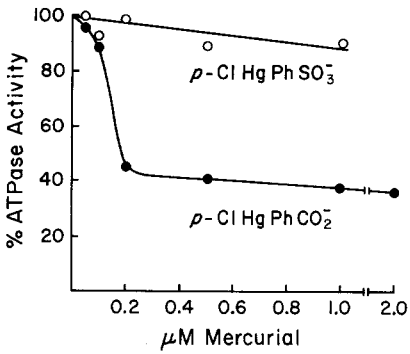

Fig. 2. Effect of organomercurials on ATPase activity of mitochondrial H^+-ATPase. *p*-Chloromercuribenzene sulfonate (O) and *p*-chloromercuribenzoate (●) were dissolved in glycylglycine (250 μM, pH 11.5). Results are expressed as percentages of control ATPase activity which, in this experiment, was 3.3 μmol · min^{-1} · mg $protein^{-1}$ at 37°C.

was also observed that *p*-chloromercuribenzoate inhibited ATPase activity whereas the sulfonate analogue did not (Fig. 2). Godinot *et al.* (1981) have reported strong inhibition of ATPase activity in Complex V by the former compound. On the other hand, none of the mercurials inhibited the ATPase activity of the soluble F_1-ATPase, suggesting that the *p*-chloromercuribenzoate-sensitive thiol may become exposed on the binding of F_1 to F_o.

A number of reagents are known to react with vicinal dithiols in preference to single thiol groups under appropriate conditions (Stocken, 1947; Gaber and Fluharty, 1972), e.g., arsine oxides, arsenite, and Cd^{2+}. These ligands remain bound to the dithiols as cyclic mercaptides. Oxidizing agents such as iodosobenzoate and copper *o*-phenanthroline, although highly specific to –SH groups, do not show such preference since their inhibitory effect is not seen in the presence of monothiol compounds. These oxidants would also promote disulfide formation between –SH groups on different molecules brought together by collision, e.g., glutathione (Kosower *et al.*, 1969). Using carboxypyridine disulfide, Blanchy *et al.* (1978) have provided evidence that the dithiol sensitive to inhibition of P_i–ATP exchange in porcine heart mitochondria is located at the inner face of the inner membrane within an apolar region. We have examined the effect of a similar, though membrane-permeable reagent, 2,2′-dipyridyl disulfide. The inhibition of P_i–ATP exchange increased gradually to 20% at 200 μM reagent and then to 70% at 500 μM (data not shown).

Kantham *et al.* (1983) have recently demonstrated parallel inhibition of the activity of purified coupling factor B (F_B) and the P_i–ATP exchange activity of H^+-ATPase by copper *o*-phenanthroline. Their data pointed to F_B inactivation as the cause of the loss of exchange activity. Figure 3 shows that inactivation of P_i–ATP exchange activity of H^+-ATPase by excess copper chelate follows pseudo-first-order kinetics with $k = 0.12\ min^{-1}$. These data are consistent with a single kinetic class of thiols involved in the inhibition and

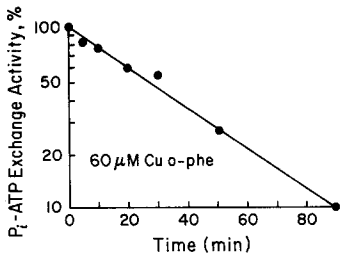

Fig. 3. Kinetics of inhibition of ATP–P_i exchange activity by copper *o*-phenanthroline. Purified H^+-ATPase was incubated with 60 μM copper *o*-phenanthroline, and aliquots were removed for assay of exchange activity as in Fig. 1. Results are expressed as percentages of the control value versus time.

with an intramolecular oxidation. As was reported by Kantham *et al.* (1983), we found no significant effect on ATPase activity even with 2 mM copper *o*-phenanthroline.

Membrane potential in the vesicular H^+-ATPase preparation can be monitored by measuring the absorbance change caused by the redistribution of the potential-sensitive dye oxonol VI. The assay system for this measurement using dual-wavelength spectroscopy has been standardized by Pringle and Sanadi (1984). The absorbance change brought about by the addition of ATP to H^+-ATPase vesicles ($A_{603-630\ nm}$) is inhibited strongly by AM10 and

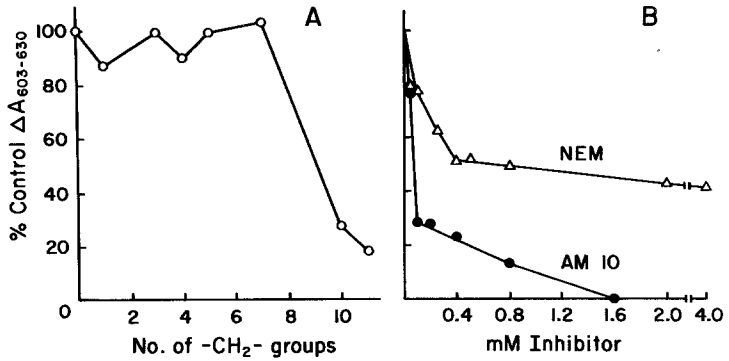

Fig. 4. Effects of AMx reagents on ATP-driven changes in Oxonol VI absorbance. (A) H^+-ATPase (50 μl containing 200 μg protein) was suspended in 1.95 ml of Tris acetate (40 mM, pH 7.5) containing 250 mM sucrose and 2 mM $MgCl_2$. Aliquots of maleimide inhibitor were added to 200 mM and the mixture was incubated for 15 min at room temperature. 2 μl oxonol VI (1.5 mM in ethanol) was added and the mixture allowed to equilibrate for 30 sec after which the reaction was initiated by the addition of ATP (2 μmol) and ADP (0.2 μmol). The change in steady-state absorbance between 603 and 630 nm ($\Delta A_{603-630}$) was monitored as a function of time. The results are plotted as percentages of the control value ($\Delta A = 0.039$) versus chain length of the reagent. (B) The assay was carried out as in (A) in the presence of increasing concentrations of AM10 (●) and, for comparative purposes, NEM (△). Results are expressed as percentages of control ΔA versus inhibitor concentration.

AM11, and much less, if at all, by the other members of the series. This can be seen in Fig 4A, which shows the magnitude of the absorbance change ($\Delta A_{603-630}$) as a function of AMx chain length. The steady-state membrane potential (oxonol VI response) is much more sensitive to inhibition by AM10 than by *N*-ethylmaleimide (Fig 4B), presumably because the –SH-reactive moiety of AM10 is situated at the appropriate depth within the membrane bilayer. These data are consistent with the results on the inhibition of P_i–ATP exchange activity.

Finally, the effect of the AMx series of maleimide alkanoic acids on passive proton conductance through the membrane sector, F_o, of the H^+-ATPase complex was examined. Purified F_o was reconstituted into K^+-loaded asolectin liposomes, as described in Materials and Methods, and a potassium diffusion potential was generated by valinomycin addition. The accompanying influx of protons through the mitochondrial proton channel was monitored by the self-quenching of 9-aminoacridine fluorescence (Sanadi *et al.*, 1984). The effect of AM10 on passive proton conductance through F_o is illustrated in Fig. 5A which shows fluorescence quenching traces for control F_o-proteoliposomes, and those incubated with 500 μM AM10 or 1.2 μM oligomycin. Under the experimental conditions, the thiol reagent exerted a block of ca. 50% in the degree of steady-state fluorescence quenching. As a check on the specificity of the observed quenching in terms of proton conductance through the F_o complex, Fig 5A also shows that oligomycin was able to inhibit the valinomycin response almost completely (i.e., 83% block with 1 μg inhibitor). In Fig. 5B, we compare the efficacies of several members of the AMx series, and again AM10 would seem to be more potent than any of the other members, although AM2 exerted a moderate blocking effect. In terms of absolute potency, however, passive proton conductance through F_o was less sensitive to inhibition by the AMx reagents than P_i–ATP exchange or ATP-driven proton translocation with F_1–F_o. The titration curve in Fig. 5C shows that even at saturating concentrations (>500 μM), AM10 only reduces the steady-state fluorescence quenching by a factor of two. Nevertheless, the previously observed distinction between AM10 and *N*-ethylmaleimide was maintained. Figures 5B and 5C include data for the latter compound which show it to be significantly less potent as a conductance blocker than AM10.

Discussion

Two types of –SH inhibitor have been used in the present study to characterize the environment and location of the –SH groups functional in the proton-translocating activity of the bovine mitochondrial H^+-ATPase. Firstly, we have employed membrane-impermeable organomercurials (Gaudemer

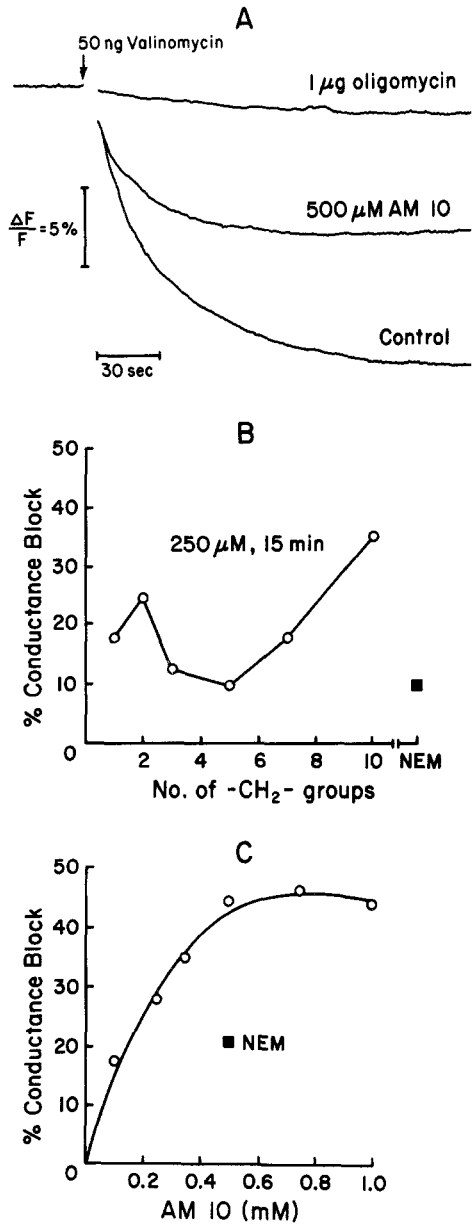

Fig. 5. Effect of AMx reagents on passive H^+ conductance through F_o. (A) 50 μl of K^+-loaded proteoliposomes (Materials and Methods) containing 15.25 μg mitochondrial F_o was added to 2 ml of Tricine (10 mM, pH 7.5) containing 200 mM NaCl and 5 μM 9-aminoacridine. Samples were incubated for 15 min at 20°C in the presence of AM10 (500 μM) or oligomycin (1.2 μM). The record shows the decrease in fluorescence intensity (due to self-quenching) after the addition of 50 ng valinomycin. (B) 25-μl aliquots of F_o proteoliposomes were assayed as in (A) after being incubated with the AMx reagents (250 μM, 15 min). Results are plotted as percentages of control steady-state fluorescence quenching versus inhibitor chain length. The degree of quenching in the absence of inhibitor was 12%. (C) Titration curve for passive conductance block by AM10. Assay conditions were as for (A) and (B). Note the degree of inhibition by *N*-ethylmaleimide in (B) and (C).

and Latruffe, 1975) which react with those –SH groups in the exterior surface of the vesicle. From this group, *p*-chloromercuribenzene sulfonate blocks proton pumping but does not affect oligomycin-sensitive ATPase activity of either ETP_H or the purified H^+-ATPase, suggesting the presence of at least one readily accessible –SH. Secondly, we have used the series of alkanoic acid maleimides ($Mal(CH_2)_xCOOH$), or AMx, where $1 < x < 11$. In this homologous series, the –SH-reactive group of each member is positioned for interaction at different depths within the membrane bilayer, assuming that the terminal carboxyl group is anchored at the membrane surface and interacts with the polar headgroups of the phospholipids. A similar approach has been used for a number of years with spin-labeled fatty acids as probes of "fluidity" at different depths within a lipid bilayer (Hubbell and McConnell, 1971). AM10 and AM11 show the strongest inhibitory activity on proton pumping, P_i–ATP exchange, and passive proton conductance, indicating the location of a second functional –SH approximately 12 Å from the membrane surface. If these two –SH groups are the same ones which chelate with Cd^{2+} or arsenic (Stiggall *et al.*, 1979b; Joshi and Hughes, 1981; Sanadi *et al.*, 1984) this would require flexibility in the region of the protein within the membrane phase in order that the two –SH groups behave as a vicinal dithiol.

We have observed that $^{115}Cd^{2+}$ added to H^+-ATPase or F_o in amounts that inhibit its proton conductance is recovered exclusively at the position of F_B in SDS-PAGE (Sanadi, 1982; Sanadi *et al.*, 1984). When the H^+-ATPase was treated first with copper *o*-phenanthroline, $^{115}Cd^{2+}$ binding was totally eliminated, showing that Cd^{2+} and the oxidant compete for the same dithiol. A similar experiment was conducted on a sample of F_1–F_o treated with AM10 under conditions whereby the ATP-driven change in membrane potential (oxonol VI absorbance change) was totally abolished (Materials and Methods). There was no difference in ^{115}Cd labeling between control and AM10-treated samples (data not shown). Thus, the –SH group sensitive to AM10 is not a part of the Cd^{2+}-sensitive dithiol on coupling factor B (Sanadi *et al.*, 1984).

Although H^+-ATPase has several –SH groups which are labeled by [*N*-^{3}H]ethylmaleimide, inactivation of its P_i–ATP exchange activity correlated best with labeling of F_B (Hughes *et al.*, 1983). Organomercurials also inactivate F_B (Lam and Yang, 1969). The present report shows that at least one additional –SH group within the membrane sector (F_o) of the H^+-ATPase is functional in those energy-linked reactions of the enzyme complex which involve transmembrane proton conductance. The nature of this –SH, located ca. 12 Å within the lipid bilayer, is unknown, although a possible candidate is the DCCD-binding proteolipid which, in bovine mitochondria, contains a single –SH group (Sebald and Hoppe, 1981).

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