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PROPERTIES OF DETERGENT-SOLUBILIZED AND MEMBRANOUS (Ca^{2+} + Mg^{2+})-ACTIVATED ATPase FROM SARCOPLASMIC RETICULUM AS STUDIED BY SULFHYDRYL REACTIVITY AND ESR SPECTROSCOPY

EFFECT OF PROTEIN-PROTEIN INTERACTIONS

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Summary

(1) Sulfhydryl reactivity and electron spin resonance spectra of nitroxide maleimide spin labels, covalently attached to sarcoplasmic reticulum ATPase, were examined on both detergent-solubilized and membranous material. Monomeric and oligomeric ATPases were prepared by the use of dodecyloctaethylene glycol monoether as a solubilizing detergent.

(2) Immediately after solubilization, the reaction curve of monomeric ATPase with 5,5′-dithiobis(2-nitrobenzoate) was characterized by positive cooperativity (S-shaped as a function of time). In contrast, the SH reactivity of both oligomeric and membranous ATPases obeyed usual first-order kinetics and could be analyzed in terms of three classes of reactive site. All enzymatically active ATPase preparations responded to addition of ADP with a decrease in SH reactivity. During enzymatic inactivation of monomeric ATPase, the SH-modification rate was dramatically enhanced with loss of cooperative features. Ca^{2+} removal from the high-affinity sites stimulated SH reactivity before inactivation had taken place.

(3) ESR spectroscopy indicated less motional constraints on monomeric than on oligomeric and membranous ATPases. Arrhenius plots of ESR spectral

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoate); Tes, *N*-tris(hydroxymethyl)-2-aminoethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; $C_{12}E_8$, dodecyloctaethylene glycol monoether.

parameters suggest a conformational transition in both membranous and solubilized ATPases at about 22°C. This transition was also present in EGTA-, but not in heat-inactivated ATPase. Although SH reactivity of monomeric ATPase was dramatically enhanced by EGTA inactivation, the results of ESR, circular dichroism and analytical ultracentrifugation experiments indicate limited conformational changes induced by EGTA treatment.

(4) The data indicate marked differences in the properties of monomeric ATPase on the one hand and oligomeric and membranous enzymes on the other hand. They are consistent with previous functional evidence for the presence of ATPase in an associated state in the membrane (Møller, J.V., Lind, K.E. and Andersen, J.P. (1980) J. Biol. Chem. 255, 1912–1920).

Introduction

The properties of detergent-solubilized (Ca^{2+} + Mg^{2+})-activated ATPase of sarcoplasmic reticulum are of interest, as detergents are important tools in the purification and separation of membrane proteins, and as these compounds may be useful as perturbating agents in the study of lipid-protein and protein-protein interactions of membranous enzymes. The non-ionic detergent, dodecyloctaethylene glycol monoether ($C_{12}E_8$), can solubilize the sarcoplasmic reticulum ATPase in a highly enzymatic active from that persists for several hours [1] or days in the presence of glycerol [2]. It is thus possible by chemical and physical methods to compare the conformational properties of the solubilized enzyme in the active state with those of membranous ATPase. We have shown [3] that the rate of sulfhydryl modification by 5,5'-dithiobis(2-nitrobenzoate) (DTNB) is a useful method for characterization of different functional states of membranous ATPase. Similar studies by Murphy [4] and Thorley-Lawson and Green [5] also included some experiments on ATPase solubilized by deoxycholate and Triton X-100. Both papers reported an enhancement of SH reactivity following dispersal with detergent which was considered to be the result of removal of some constraints on the SH groups imposed by the lipid structure. In the present paper, the conformational properties of ATPase, solubilized in $C_{12}E_8$, are studied by DTNB modification and electron spin resonance (ESR) spectroscopy of covalently bound spin labels. We find that oligomeric ATPase resembles membranous ATPase in many respects, while the monomeric form of the enzyme exhibits some unique properties which suggest that during solubilization of the sarcoplasmic reticulum with $C_{12}E_8$ there is disruption of protein-protein interactions.

Methods

Materials

Sarcoplasmic reticulum from rabbit skeletal muscle was prepared as previously described [6] and freed of proteins other than (Ca^{2+} + Mg^{2+})-ATPase by extraction with a low concentration of deoxycholate by method 2 of Meissner et al. [7]. Dodecyloctaethylene glycol monoether was obtained from Nikko Chemicals (Tokyo) and [1-^{14}C]dodecyloctaethylene glycol monoether with a

specific activity of 50 mCi/mmol from C.E.A., Saclay. Spin-labeled chemicals from Syva, Palo Alto, included a long-chain maleimide nitroxide (3-[[2-(2-maleimidoethoxy)ethyl]carbamoyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl, No. 114) and a short-chain maleimide nitroxide (3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl, No. 110). DEAE-cellulose (DE-52) was obtained from Whatman Ltd., Kent (U.K.), and the ATP analog: adenosine 5'-[β,γ-imino]triphosphate (Ado*PP*[NH]*P*) from Boehringer. DTNB, EGTA, nucleotides and acetyl phosphate were from Sigma Chemical Co., MO. Other reagents used were analytical reagent grade.

Preparation of detergent-solubilized ATPase

Monomeric ATPase. Usually, membranous ATPase (suspended in 1 mM Hepes (pH 7.5) and 0.3 M sucrose at a protein concentration of 12—18 mg/ml) was added to $C_{12}E_8$, solubilized in 0.01 M Tes and 0.1 M NaCl, adjusted to pH 7.5, to produce a final ratio of protein to detergent of 1 : 2. In many experiments the medium in addition contained glycerol (20%, v/v) as a precaution against inactivation. Unsolubilized material (comprising approx. 25—35% of the protein) was removed by centrifugation in a Beckman Airfuge for 20 min at 130 000 × *g*. In the SH-modification experiments the concentrations of protein and detergent were 2 and 4 mg/ml, respectively, during solubilization. In the spin-label experiments the ATPase vesicles were labeled with nitroxide before solubilization with $C_{12}E_8$, and considerably higher concentrations of protein and detergent were used (see below).

The solubilized ATPase preparations were capable of hydrolyzing about 5 μmol ATP/mg protein per min at 20°C and pH 7.5. According to previous investigations the major part of the ATPase is present in monomeric form, but with some tendency to aggregate, dependent on protein concentration [1,2]. After dilution with 10 vol. of 0.5 mg $C_{12}E_8$/ml, 0.01 M Tes (pH 7.5) and 0.1 M KCl (i.e., the same medium as used for the SH-modification experiments except for the absence of DTNB, see below), the protein exhibited a single boundary in the analytical ultracentrifuge with a sedimentation coefficient ($s_{20,w}$) of 5.0 S, characteristic of monomeric ATPase [1].

Oligomeric ATPase. 5 mg ATPase were solubilized by 10 mg $C_{12}E_8$ as described above and then applied to a column (0.9 × 3.8 cm) of DE-52, equilibrated with 0.05 mg $C_{12}E_8$/ml, 0.01 M Tes (pH 7.5), 0.1 mM $CaCl_2$ and 0.05 M NaCl. Mixed micelles of lipid and $C_{12}E_8$ were eluted by passing 20 ml of equilibration buffer through the column. Then the protein was eluted with the same buffer to which had been added NaCl to a final concentration of 0.4 M NaCl. Peak fractions which after pooling contained approx. 1 mg protein/ml were used for SH modification shortly after preparation. The ATPase in this preparation was in an aggregated state as evidenced by visible turbidity and sedimentation in the analytical ultracentrifuge which indicated size heterogeneity. The slowest moving component had a sedimentation coefficient ($s_{20,w}$) of about 20 S. The lipid content was about 0.1 mg/ml (corresponding to about 14 mol phospholipid per mol ATPase with a molecular weight of 115 000 [6]). The enzyme activity was about 4—5 μmol/mg per min (pH 7.5, 20°C).

In some experiments oligomeric ATPase was prepared from $C_{12}E_8$-solubilized protein by chromatography on Sepharose CL-6B in the absence of any

detergent in the column, as previously described [8]. The eluted oligomer peak contained about 0.22 mg lipid/mg protein (corresponding to about 30 mol per mol protein), and the enzyme activity was usually 2—3 μmol/mg per min. Spin-labeled ATPase in oligomeric form was prepared by the methods described above. Labeling was performed prior to detergent solubilization, and the protein emerging from the column was concentrated to about 10 mg/ml by centrifugation at 200 000 × *g* for 30 min before recording of ESR spectra.

Delipidated ATPase

In one experiment delipidated ATPase was prepared by the DEAE-cellulose chromatographic procedure described in connection with the preparation of oligomeric ATPase. The spin-labeled protein was washed on the column with 50 ml of 5 mg/ml $C_{12}E_8$ and eluted in the presence of this concentration of $C_{12}E_8$. ESR spectra were recorded after concentration of the protein and adjustment of the detergent-to-protein ratio to 2 : 1. The lipid content of the preparation was less than 0.05 mg phospholipid/mg protein. The enzyme activity was about 3 μmol/mg per min (pH 7.5, 20°C) immediately after elution, but decayed rapidly in the absence of glycerol [2].

Sulfhydryl reactivity

The reaction rate of the various ATPase preparations with DTNB was followed at 25°C by measuring the increase in absorbance at 415 nm, relative to that at 480 nm, on an Aminco DW-2 spectrophotometer operated in the dual-wavelength mode. The initial phase of the reaction was measured in separate experiments by the use of the Aminco-Morrow stopped-flow attachment. In most experiments the final reaction media contained 0.5 mM DTNB, 0.05—0.12 mg/ml protein, 0.01 M Tes (pH 7.5), 0.1 M KCl or NaCl together with detergent, ADP, Mg^{2+}, Ca^{2+} or EGTA as indicated in the legends to the figures. Glycerol, when present, was at a concentration less than 2% (v/v) which did not affect SH reactivity. The reaction was started by the addition of a small volume of protein (about 0.1 ml) to 1.1 ml reaction mixture. When the stopped-flow equipment was used the reaction was always started by mixing 1.5 ml protein solution with 1.5 ml of a solution of the same composition except for the omission of protein and inclusion of 1 mM DTNB. In many experiments the reaction was completed by adding 1% (w/v) sodium dodecyl sulfate (SDS) to obtain the total number of SH groups. The results were plotted as moles of sulfhydryl groups modified per mole ATPase, calculated from a molar absorbance difference of dye at 415 and 480 nm of 10 700 $M^{-1} \cdot cm^{-1}$ and a molecular weight of the protein of 115 000 [6]. The molar absorbance difference was determined by comparison with the increase in light absorbance of a cysteic acid solution at 412 nm after reaction with DTNB, using 13 600 $M^{-1} \cdot cm^{-1}$ [9] as the molar absorbance coefficient at this wavelength. When possible, the results were analysed according to:

$$N_\infty - N_t = \sum_{i=1}^{j} n_i \cdot e^{-k_i t} \qquad (1)$$

where N_t and N_∞ are the numbers of sulfhydryl groups modified at time t and the total number of SH groups, respectively, j is the number of kinetic classes

and n_i and k_i are the number of SH groups in the ith class and an average value for their pseudo first-order rate constant.

Spin labeling and ESR spectroscopy

ATPase was labeled in the membranous form by incubation of ATPase vesicles (12—18 mg/ml) for 4 h at room temperature (pH 7.5) with 1—2 mol of label No. 114 or 110 per mol ATPase. The reaction was followed on a Varian E-3 EPR spectrometer to ensure that more than 90% of the label had reacted at the end of the incubation period. Unreacted label was removed by centrifugation in a Beckman Airfuge for 20 min at 130 000 × g followed by resuspension in 150 μl buffer (0.01 M Tes, 0.1 M NaCl, 0.1 mM $CaCl_2$, pH 7.5). After five washes the pellet was suspended in the same buffer at a protein concentration of 10—20 mg/ml before recording of ESR spectra. In experiments with $C_{12}E_8$-solubilized ATPase 20—40 mg/ml $C_{12}E_8$ was added to the buffer in the last step and insoluble material was removed by an additional centrifugation.

To ensure that no non-protein bound label was present after the washing procedure, the solubilized ATPase in three control experiments was bound to DE-52 columns as described above and washed thoroughly with 1 mg $C_{12}E_8$/ml, dissolved in 0.05 M NaCl, 0.01 M Tes (pH 7.5). No ESR signal could be detected in the fractions collected during washing, and all the label originally present emerged together with the protein when this was eluted by raising the concentration of NaCl to 0.4 M. The extent of labeling was in some cases checked by measuring the number of remaining SH groups with DTNB in the presence of 1% SDS as described above. The ATPase activity was not reduced by the spin labeling.

Spectra were obtained on a Varian E-3 EPR spectrometer equipped with a variable temperature accessory. The actual temperature was determined with a thermocouple to within ±1°C. The spectrometer settings usually were: modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 10 mW; microwave frequency, 9.5 GHz.

Other methods

Protein and phospholipid concentrations were determined according to the method of Lowry et al. [10] and Bartlett [11], as previously described [6]. ATP hydrolysis was measured spectrophotometrically at 20°C by an NADH-coupled assay, using phospho*enol*pyruvate to regenerate ATP [1]. Sedimentation velocity experiments were performed in a Beckman Model E ultracentrifuge, equipped with photoelectric scanner. The samples were examined at 45 000 rev./min on an AN-D rotor. Circular dichroic spectra were recorded at a protein concentration of 0.2 mg/ml on a Jouan dichrograph. Binding of $C_{12}E_8$ was determined with the aid of ^{14}C-labeled detergent from the increase in radioactivity associated with the protein peak by the DEAE-cellulose chromatographic procedure described in connection with the preparation of oligomeric ATPase.

Results

Reaction of ATPase with DTNB

Fig. 1 compares the time course of DTNB modification of fully active pre-

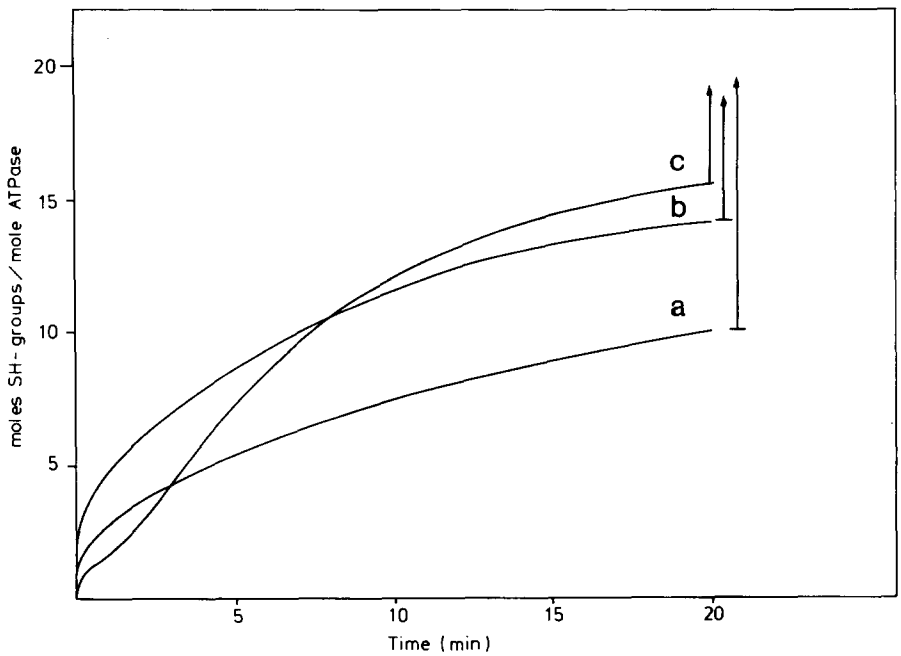

Fig. 1. DTNB modification of solubilized and membranous ATPase. The reaction was measured at 25°C in the presence of 0.5 mM DTNB, 0.01 M Tes (pH 7.5), 0.1 M NaCl, 0.1 mM $CaCl_2$ and $C_{12}E_8$ concentrations as indicated below. Curve a, membranous ATPase (0.07 mg/ml), no detergent present; curve b, oligomeric ATPase (0.08 mg/ml) in the presence of 0.05 mg unbound $C_{12}E_8$/ml; curve c, monomeric ATPase (0.12 mg/ml) in the presence of 0.50 mg $C_{12}E_8$/ml. The curves are the means of three to five experiments. Arrows show the total number of SH groups obtained by addition of 1% SDS.

parations of detergent-solubilized and membranous ATPases. The reaction curves of oligomeric and membranous ATPases are basically similar, both being characterized by a steep initial rise, followed by a gradual decline of the modification rate as a function of time. However, modification of oligomeric ATPase proceeds at a higher rate than for the membranous ATPase. The reactivity pattern of monomeric ATPase is different from both the oligomeric and membranous enzymes. Apart from one to two sulfhydryl groups, which react instantaneously with DTNB, the rate of modification of monomeric ATPase in the initial period is lower than that of both membranous and oligomeric ATPases. But after a few minutes the modification rate increases markedly, resulting in a sigmoidal reaction curve. The number of SH groups, which have reacted after 20 min modification of the monomer, is 15.5 compared to 14.2 and 10.0 in the case of oligomeric and membranous ATPases, respectively. However, as can be seen from the figure, the total number of reactive SH groups, determined by addition of SDS, does not vary significantly between the three preparations. The reaction curves of membranous and oligomeric ATPases can be fitted as a sum of three first-order reactions, while such an analysis is not applicable to the monomeric ATPase. These results agree with those published by Murphy [4]. We previously obtained evidence for only two classes of reactive SH group [3]. This difference is attributable to the higher temperature (25°C) used in the present study together with a better definition of

TABLE I

ANALYSIS ACCORDING TO EQN. 1 OF THE SH MODIFICATION OF MEMBRANOUS AND OLIGOMERIC ATPase (FIG. 1)

The number of SH groups in each class (n_i) and their pseudo first-order rate constants (k_i) are given as means ± S.D. of five experiments.

	Very fast		Fast		Slow		
	n_1	k_1 (min^{-1})	n_2	k_2 (min^{-1})	n_3	k_3 (min^{-1})	Σn_i
Membranous ATPase	1.7 ±0.2	≃10	2.3 ±0.2	0.35 ±0.05	15.3 ±0.3	0.03 ±0.01	19.3 ±1.1
Oligomeric ATPase	3.3 ±0.4	≃13	5.9 ±0.4	0.21 ±0.09	9.4 ±0.5	0.04 ±0.01	18.6 ±0.7

the absorption at the start of the experiment obtained by the stopped-flow attachment. A comparison of the data of Table I indicates that the higher reactivity of oligomeric ATPase relative to the membranous enzyme is due to an increase in the number of SH groups in the fast and very fast reacting classes.

The SH reactivity of oligomeric ATPase prepared on a Sepharose 6B column in the absence of any detergent (results not shown) was very similar to that of the oligomeric preparation shown in Fig. 1, except that the total number of SH groups was less (15.3 ± 1.8 in five experiments), as compared to 18.6 for freshly prepared ATPase in the presence of $C_{12}E_8$ (Table I). The decrease in the number of reactive SH groups is most reasonably attributed to disulfide formation [5] during preparation, which lasted about 30 h. After storage of ATPase for 24 h in the presence of a solubilizing concentration of $C_{12}E_8$, the total number of SH groups usually dropped to about 16.

It has previously been shown that the preservation of ATPase activity of monomeric protein is critically dependent on conditions used for solubilization. Thus, activity is lost quite rapidly at a low protein-to-$C_{12}E_8$-concentration ratio, while it is preserved in the presence of 20% glycerol [1,2]. Fig. 2 shows the SH-modification curves obtained after preincubation for various times of solubilized ATPase in media of different composition. During DTNB modification the level of free Ca^{2+} and $C_{12}E_8$ was the same in all cases (see legend to Fig. 2). Curve a shows the sulfhydryl reactivity of ATPase, which was prepared in the same manner as the monomer in Fig. 1, but stored for 20 h in 20% glycerol before measurement of sulfhydryl reactivity. After this period both the characteristic sigmoidal shape of the sulfhydryl reaction curve and enzyme activity were preserved. The total number of SH groups was reduced, but we have corrected for this effect by showing sulfhydryl modification as a percentage of all reactive SH groups in Fig. 2. Curves b and c show SH modification after preincubation for 30 min and 20 h, respectively, in the absence of glycerol. The shape of curve b is sigmoidal like curve a, but after 20 h (curve c) this feature is lost and replaced by a general increase in SH reactivity. At the same time, enzyme activity is reduced to about 40% of that measured immediately after preparation. The increase in SH reactivity after 20 h is more pronounced at a lower protein-to-detergent ratio (curve d), which leads to

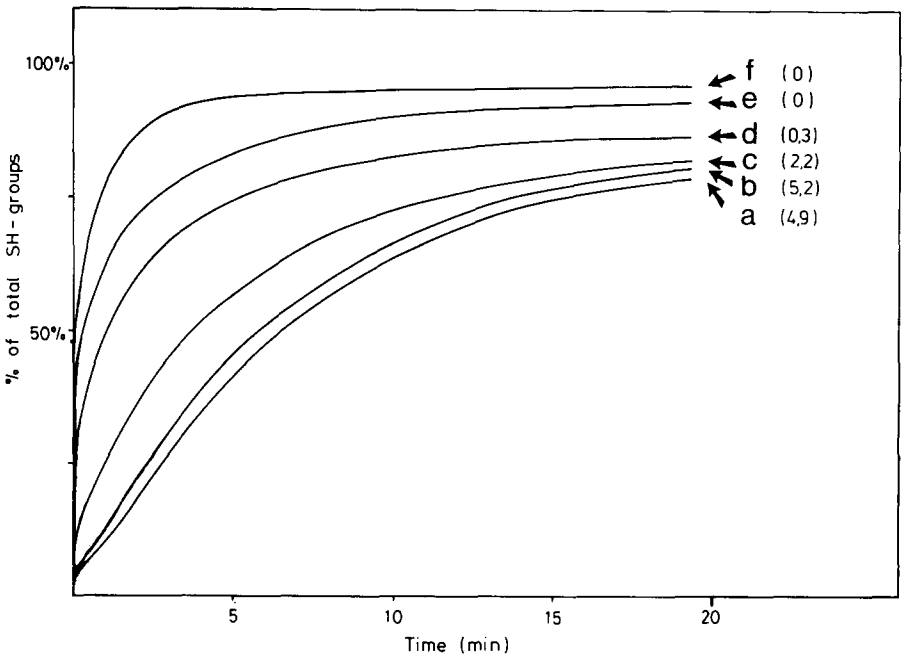

Fig. 2. DTNB modification of solubilized ATPase after preincubation under various conditions. The reaction was measured in the presence of 0.5 mM DTNB, 0.01 M Tes (pH 7.5), 0.1 M NaCl, 0.1 mM $CaCl_2$ and 0.50 mg $C_{12}E_8$/ml at 25°C. Glycerol, when present during DTNB modification, was at a concentration less than 2% (v/v) which did not affect SH reactivity. Conditions used for preincubation were: curve a, 2 mg ATPase/ml solubilized by 4 mg $C_{12}E_8$/ml in the presence of 20% (v/v) glycerol, incubated for 20 h at 22°C; curve b, 2 mg ATPase/ml, solubilized by 4 mg $C_{12}E_8$/ml in the absence of glycerol, 30 min incubation at 22°C; curve c, 2 mg ATPase/ml, 4 mg $C_{12}E_8$/ml, 20 h at 22°C; curve d, 0.1 mg ATPase/ml, 1 mg $C_{12}E_8$/ml, 20 h at 22°C; curve e, 2 mg ATPase/ml, 4 mg $C_{12}E_8$/ml, 1 mM EGTA, 30 min at 22°C; curve f, 2 mg ATPase/ml, 4 mg $C_{12}E_8$/ml, 2 min at 60°C. The curves are means of three to five experiments. ATPase activities, expressed in μmoles/min per mg, are indicated in brackets and were measured after preincubation by adding a small amount of sample to media containing 0.1 mM Ca^{2+} and 5 mM MgATP as previously described [1].

virtually complete inactivation of the enzyme. We have previously found that inactivation of $C_{12}E_8$-solubilized monomer is dramatically enhanced by addition of EGTA to chelate Ca^{2+} [1]. Curve e shows the result obtained on material inactivated by 30 min preincubation in 1 mM EGTA. The stimulation of SH reactivity is even more pronounced than in curve d. However, if 20% glycerol is present together with EGTA several hours of incubation are necessary to observe an effect on the SH-modification rate and enzyme activity (not shown). Curve f shows SH reactivity after heating of the solubilized ATPase at 60°C for 2 min. This treatment results in loss of activity and a drastic stimulation of thiol reactivity. However, inspection of the reaction curve indicates a difference from the other inactivated preparations on the figure. Although the majority of SH groups are modified very quickley, there is also a class which reacts at a distinctly slower rate, resulting in a biphasic reaction curve. This reaction pattern may be the result of aggregation, since visible precipitates were formed at 60°C. In contrast, the major part of the EGTA-inactivated preparation was in monomeric form when examined in the analytical ultracentrifuge (see later results).

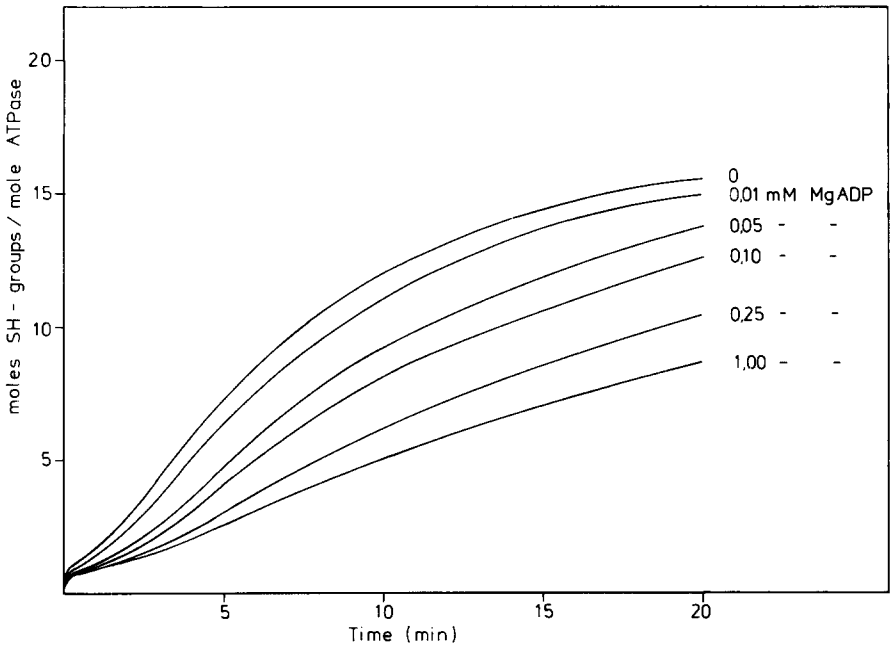

Fig. 3. Representative experiment showing effect of MgADP on DTNB modification of solubilized ATPase. The reaction was measured at 25°C in the presence of 0.5 mM DTNB, 0.01 M Tes (pH 7.5), 0.1 M NaCl, 0.1 mM $CaCl_2$, 0.5 mM free Mg^{2+}, 0.5 mg $C_{12}E_8$/ml, and different concentrations of MgADP, as indicated in the figure.

The SH-modification rate of vesicular ATPase has been shown to be a sensitive indicator of conformational changes occurring during the enzymatic process [3—5]. According to our own findings on membranous ATPase, the most prominent feature is a decrease in modification rates following binding of nucleotide, in the absence of phosphorylation [3]. As shown in Fig. 3, a prominent reduction of the SH-modification rate is also observed for monomeric ATPase in the presence of MgADP. At a nucleotide concentration greater than 0.1 mM, a considerable fraction of the SH groups reacts with DTNB at a reduced rate. The reduction of SH reactivity does not conform to binding of MgADP at the phosphorylation site for which a dissociation constant of about 28 μM has been estimated [1]. One possibility is that it is due to low-affinity binding at a secondary site, the existence of which is suggested by functional studies [1,12,13]. Another possibility is that modification of the instantaneously reacting SH groups changes the affinity of the ATPase for nucleotide so that higher concentrations of MgADP than those expected from the estimated dissociation constant of 28 μM for unmodified ATPase are necessary to obtain the same degree of binding of MgADP. In other experiments we have found that ADP has about the same effect as MgADP on SH reactivity (not shown). Preparations of active oligomer give similar decreases in SH reactivity by addition of ADP as observed previously for membranous ATPase [3], but neither oligomers nor monomeric preparations respond to ADP after inactivation. The non-phosphorylating ATP analog,

Ado*PP*[NH]*P*, at a concentration of 0.5 mM is able to decrease SH-modification rates of active ATPase appreciably, while on the other hand AMP (1 mM), pyrophosphate (5 mM) and acetyl phosphate (1 mM) do not affect sulfhydryl reactivity.

The dramatic effect of 30 min incubation with EGTA on the SH reactivity of monomeric ATPase (Fig. 2) indicates that a distinct conformational change occurs by inactivation of ATPase after lowering of the Ca^{2+} concentration in the medium. To study the inactivation process in more detail we have followed the reaction with DTNB at various EGTA concentrations (Fig. 4). In this series of experiments the solubilized ATPase was not exposed to EGTA before initiation of the reaction with DTNB. In parallel experiments the rate of ATP hydrolysis was determined in media which contained the same concentration of EGTA or Ca^{2+} as the DTNB-modification media. At EGTA concentrations sufficient to prevent ATP hydrolysis (0.1—1 mM), a pronounced stimulation of SH reactivity is observed, while at lower EGTA concentrations intermediate increases in SH reactivity are seen. It is of interest that the change in SH reactivity is correlated with the decrease in the rate of ATP hydrolysis. Thus, the effect of EGTA on SH reactivity is probably related in a direct way to removal

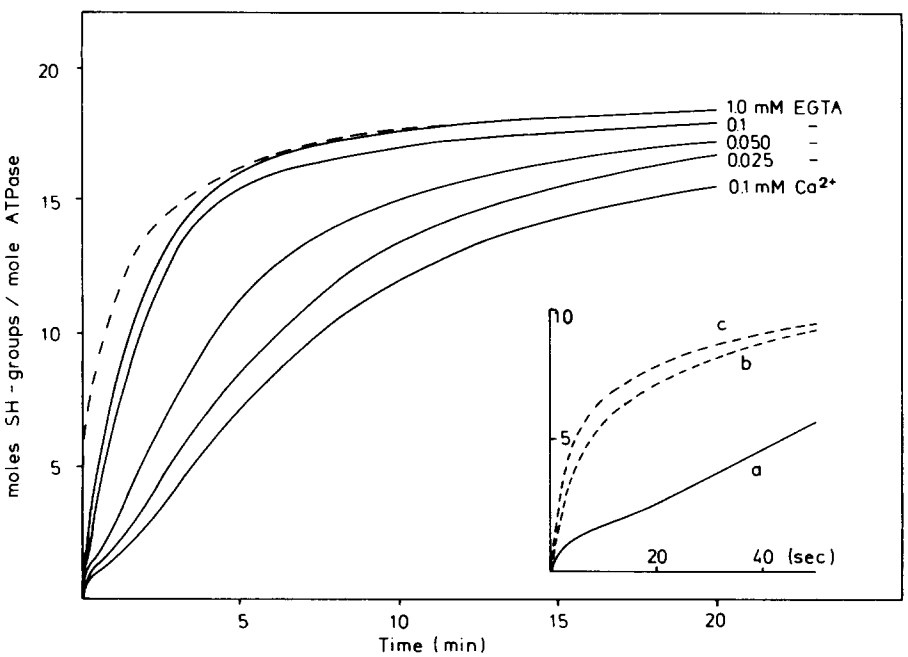

Fig. 4. Representative experiment showing effect of EGTA on DTNB modification of solubilized ATPase. The reaction was measured at 25°C in the presence of 0.5 mM DTNB, 0.01 M Tes (pH 7.5), 0.1 M NaCl, 0.5 mg $C_{12}E_8$/ml and $CaCl_2$ or EGTA as indicated in the figure. Curve e from Fig. 2 is included for comparison (- - - - - -). ATPase activities (μmoles/min per mg) measured in media containing EGTA or $CaCl_2$ concentrations as for DTNB modification were as follows. At 1 mM EGTA and 0.1 mM EGTA, no activity; at 50 μM EGTA, 2.4; at 25 μM EGTA, 3.9; at 0.1 mM Ca^{2+}, 5.1. The inset shows stopped-flow measurements of initial modification rates in the presence of 1 mM EGTA under conditions as in the main figure. Curve a, no preincubation in EGTA; curves b and c (- - - - - -), preincubation in 1 mM EGTA for 8 and 30 min, respectively. Other conditions for preincubation were as in curve e of Fig. 2.

of Ca^{2+} from the high-affinity sites on the protein involved in activation of ATP hydrolysis.

Fig. 4 shows that EGTA affects the initial rate of the reaction with DTNB as measured in the first 30 s after addition of protein to the modification medium. We have previously found that irreversible inactivation of monomeric ATPase in the presence of 1 mM EGTA occurs with a half-life of 6.5 min (Fig. 9 of Ref. 1). In agreement with this, no significant inactivation was observed during the first 30 s after mixing ATPase and EGTA. Therefore, the initial enhancement of SH reactivity by EGTA is anticipated to reflect conformational changes in a protein that is still capable of performing ATP hydrolysis upon readdition of substrates. This view is supported by results obtained with the stopped-flow attachment which are shown in the inset of Fig. 4. Curve a is the initial part of the reaction curve in the presence of 1 mM EGTA (no preincubation) while curves b and c were obtained after 8 and 30 min preincubation, respectively, in 1 mM EGTA (conditions for preincubation as in curve e of Fig. 2). Curve a reveals that the sigmoidal shape characteristic of monomeric ATPase with retention of enzymatic activity persists at the high EGTA concentration, provided that the ATPase has not been exposed to EGTA before initiation of DTNB modification. However, after preincubation of EGTA, no sigmoidality is seen and further enhancement of SH reactivity has occurred. The change appears to be gradual, as in Fig. 2, the stimulation being most pronounced after 30 min incubation with EGTA.

Fig. 5 shows the effect of addition of both 1 mM EGTA and 1 mM MgADP

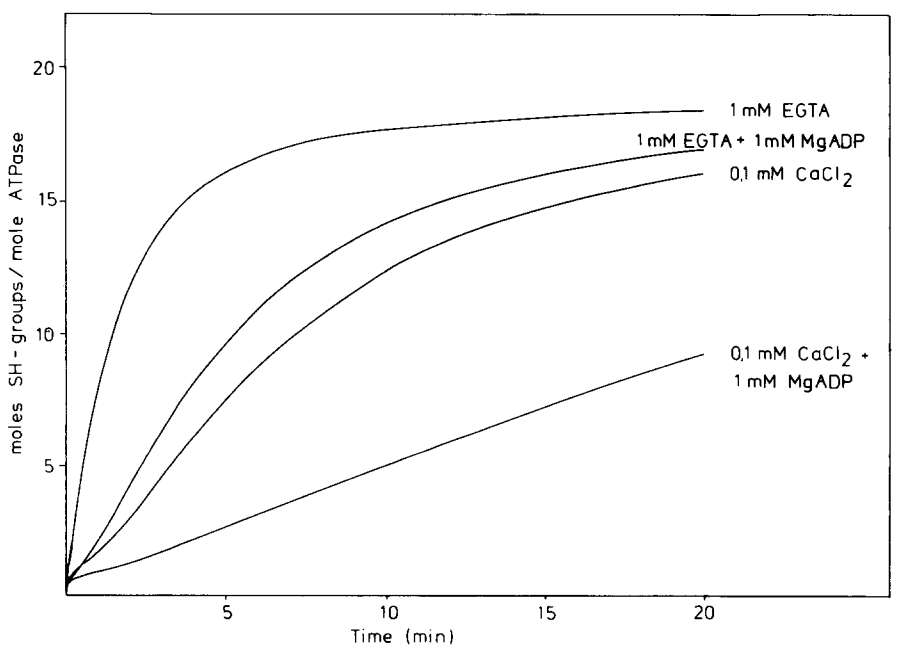

Fig. 5. Representative experiment showing effect of MgADP on DTNB modification in the presence and absence of EGTA. The reaction was measured in 0.5 mM DTNB, 0.01 M Tes (pH 7.5), 0.1 M NaCl, 0.5 mM free Mg^{2+} and 0.5 mg $C_{12}E_8$/ml at 25°C. MgADP and $CaCl_2$ or EGTA were added as indicated in the figure.

on SH reactivity. It is seen that Ca^{2+} chelation gives rise to a similar relative increase in SH reactivity in the presence and absence of MgADP. Since 1 mM MgADP effectively protects the ATPase against the inactivating effect of EGTA [1], the finding adds further support to the conclusion that the initial enhancement of SH reactivity induced by EGTA occurs without inactivation. This does not mean, however, that inactivation and stimulation of SH modification by EGTA are unrelated phenomena. We consider it likely that the graduated stimulation of SH reactivity (inset of Fig. 4) reflects a series of conformational changes among which only the last stages represent inactivated conformations.

ESR spectra of spin-labeled ATPase

Fig. 6 shows the ESR spectra at 20°C of the long-chain nitroxide maleimide (label No. 114) attached to ATPase membranes, $C_{12}E_8$-solubilized ATPase, and oligomeric ATPase prepared by chromatography on a Sepharose 6B column in the absence of detergent. The oligomeric and membranous ATPase give rise to a two-component spectrum containing both an immobilized and a more freely rotating component. The fraction of immobilized label is largest in the oligomeric ATPase prepared by chromatography on a Sepharose 6B column in ATPase is essentially a one-component spectrum, characterized by a faster rotational motion than for the membranous and oligomeric ATPases. The increase in linewidths of the latter preparations may be attributed to steric hindrance of free motion, and to spin-spin interactions [14], resulting from close contact between the ATPase polypeptide chains.

The splitting between the high-field and low-field lines of the mobile component (32.4 G) is the same for all three preparations in Fig. 6 and is consistent

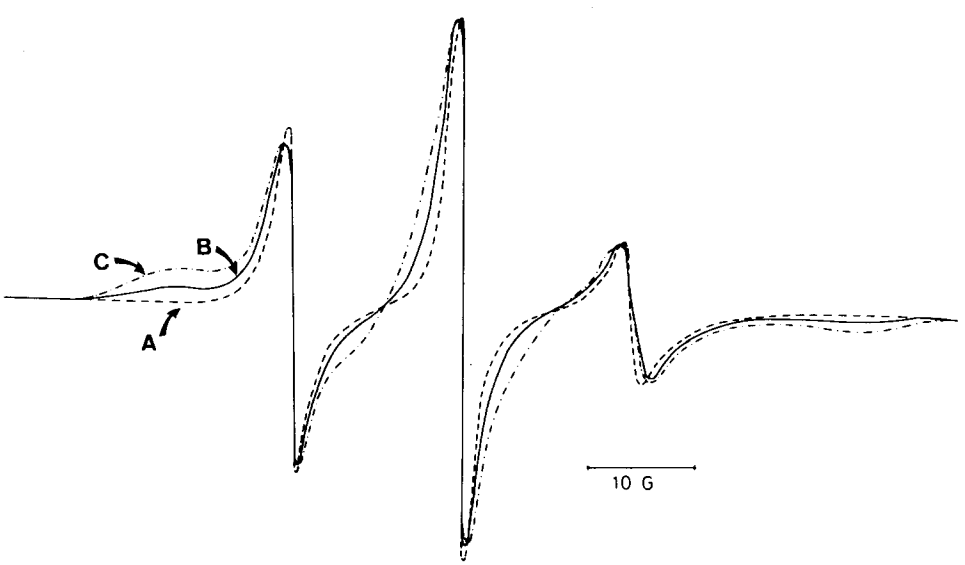

Fig. 6. Representative ESR spectra of spin-label No. 114 attached to membranous, oligomeric and solubilized ATPases prepared as described in Methods. The final samples for ESR spectroscopy contained 10–20 mg ATPase/ml in the presence of 0.1 M NaCl, 0.01 M Tes (pH 7.5) and 0.1 mM $CaCl_2$. Curve a, ATPase solubilized by $C_{12}E_8$ at a weight ratio of 2 mg $C_{12}E_8$/mg ATPase; curve b, membranous ATPase; curve c, oligomeric ATPase prepared in the absence of detergent.

with the label being exposed to a polar milieu [15]. Reduction of the nitroxide with 5 mM ascorbate at 20°C did not reveal any difference in the reduction rate of the mobile and immobile components. This indicates that either there is a rapid equilibrium between the mobile and immobile state, or the immobilized label is in a position permanently accessible to ascorbate. (The latter possibility would suggest exposure to the aqueous phase despite the motional constraints imposed on the label).

Spectra obtained with the oligomer prepared in $C_{12}E_8$ were very similar to those of detergent-free oligomer (Fig. 6C). Therefore, the presence of an immobilized component in the oligomeric preparations may be the result of protein-protein interactions. The aggregational state of the $C_{12}E_8$-solubilized ATPase (Fig. 6A) is somewhat uncertain due to the high protein concentration which may give rise to reversible aggregation [1,2]. However, in some experiments we recorded spectra in which the protein and detergent concentrations were lowered to 2 and 4 mg/ml, respectively. This protein concentration is the lower limit for recording useful ESR spectra. Under these conditions, we found from sedimentation velocity scans in the analytical ultracentrifuge that 80% of the ATPase was present as a monomer with a sedimentation coefficient of 4.8 S. Except for the low amplitude, we did not detect any change in the spectrum from that presented at the protein concentration of 10 mg/ml (Fig. 6A).

Fig. 7 shows results obtained at various temperatures with the short-chain nitroxide maleimide (label No. 110) attached to membranous and $C_{12}E_8$-solubilized ATPase. In this case, the immobile component of the spectrum was more prominent and was also present in the $C_{12}E_8$-solubilized ATPase. The data are presented as Arrhenius plots of the ratio between the amplitude of the strongly immobilized and the weakly immobilized component (h_s/h_w). As in the case of label No. 114, the amount of immobilized component decreases

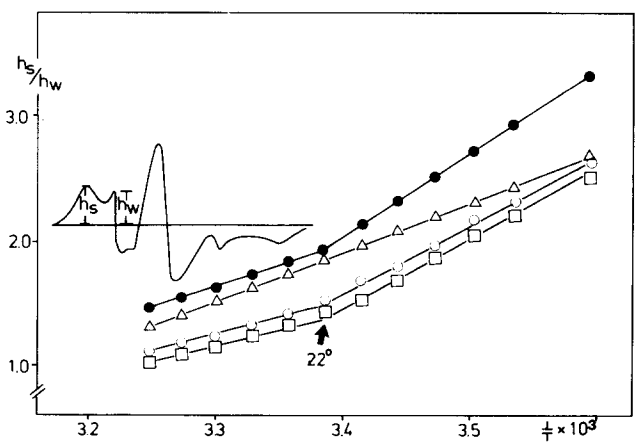

Fig. 7. Arrhenius plot of h_s/h_w for membranous and solubilized ATPases labeled with spin-label No. 110. The definition of h_s and h_w is indicated in the inset. (●———●) Membranous ATPase, 0.1 mM $CaCl_2$; (△———△) heat-denatured solubilized ATPase, 0.1 mM $CaCl_2$, 2 mg $C_{12}E_8$/mg ATPase; (○———○) active solubilized ATPase, 0.1 mM $CaCl_2$, 2 mg $C_{12}E_8$/mg ATPase; (□———□) inactivated solubilized ATPase, 1 mM EGTA, 2 mg $C_{12}E_8$/mg ATPase. All samples were in the usual electrolyte medium containing 0.1 M NaCl, and 0.01 M Tes (pH 7.5).

upon solubilization, giving rise to a downward displacement of the curves of solubilized ATPase relative to that of membranous ATPase. The data indicate the presence of a break at about 22°C, similar to that described previously for ATPase reconstituted in dimyristoyl phosphatidylcholine [16]. Such breaks have been attributed to a transition in the fluidity of lipids in contact with the protein [16—18]. The interaction of ATPase with solubilizing amounts of detergent (at minimum 0.25 g $C_{12}E_8$ bound/g protein, unpublished results) does not change the position of the break point in the h_s/h_w plot. Also, after delipidation to less than 0.05 g phospholipid/g protein (see Methods), the break point is unchanged (not shown). Therefore, we consider it most likely that the break is due to a direct effect of temperature on the protein conformation. A similar conclusion was reached by Dean and Tanford [2] from studies of Arrhenius plots of ATPase activity of membranous and lipid-depleted ATPases. Fig. 7 includes two curves of $C_{12}E_8$-solubilized ATPase inactivated by heating at 60°C for 2 min or by addition of 1 mM EGTA. After heating, the break in the Arrhenius plot disappears and h_s/h_w increases. The effect of EGTA on the ESR spectrum of $C_{12}E_8$-solubilized ATPase is less conspicuous, the only change being a small decrease in h_s/h_w. The preservation of the break in the Arrhenius plot upon addition of EGTA suggests retention of a characteristic conformational feature of the protein, despite the irreversible inactivation of ATPase activity. In contrast to the solubilized ATPase, we never observed any effect of EGTA on the ESR spectrum of membranous ATPase.

Other measurements on $C_{12}E_8$-solubilized ATPase

A limited conformational change of EGTA-inactivated ATPase is supported by examination in the analytical ultracentrifuge which showed that the major part of EGTA-treated ATPase (approx. 70%) had a sedimentation coefficient ($s_{20,w}$) of 4.7 S, which is of the same magnitude as that of the enzymatically active monomer [1,8]. The remaining part of the ATPase gave rise to an

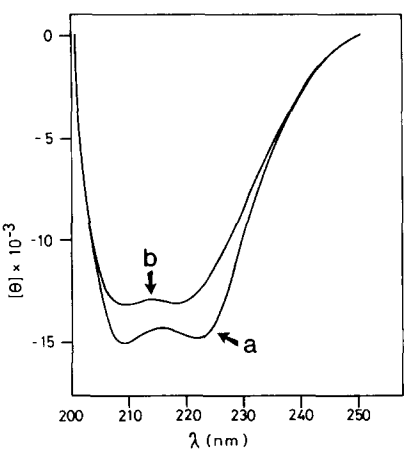

Fig. 8. CD spectra at 20°C of solubilized ATPase in the presence and absence of EGTA. 0.2 mg ATPase/ml was solubilized by 0.4 mg $C_{12}E_8$/ml in 0.1 M NaCl, 0.01 M Tes (pH 7.5) buffer. Unsolubilized material was removed as described in Methods. Curve a, no further additions; curve b, 1 mM EGTA was added just before recording the spectrum.

overlapping component in the sedimentation profile, indicating limited aggregation. Furthermore, detergent binding at a concentration of free $C_{12}E_8$ of 0.2 mg/ml was 0.18 g/g EGTA-treated ATPase, to be compared with binding of 0.25 g/g enzymatically active ATPase. Definite evidence of a conformational change following inactivation with EGTA was obtained from circular dichroic spectra in the far ultraviolet region (Fig. 8) It is seen that there is a modest decrease in the ellipticity after inactivation with EGTA, and that the spectrum is characterized by a less pronounced double-trough feature. These changes indicate a decrease of the α-helix content after inactivation with EGTA, and are similar to those previously observed by inactivation of ATPase in the presence of deoxycholate [6].

Discussion

The present study demonstrates that the sulfhydryl reactivity of detergent-solubilized ATPase is very dependent on the conditions used for solubilization and that treatment with detergent need not result in enhanced modification rates with DTNB as reported in previous studies [4,5]. A low modification rate seems to be associated with solubilization of ATPase by $C_{12}E_8$ in monomeric form when enzyme activity is relatively stable (addition of glycerol to the solubilization medium or a high protein concentration before the start of the experiment). This result suggests that conformational changes of the protein occurring after solubilization are at least as important for an enhanced sulfhydryl reactivity as an increased exposure of buried SH groups resulting directly from disruption of the lipid bilayer structure, as previously suggested [4,5].

As far as we are aware, our results on monomeric ATPase provide the first unambiguous demonstration of cooperative features in the sulfhydryl modification rate of a protein (Fig. 1). It seems reasonable to suppose that the basis for this behaviour is the instability of the system. Initially, all the sulfhydryl groups, apart from one to two very rapidly reacting groups, are in a protected state. But after modification of a few of the more slowly reacting groups conformational changes take place, resulting in an increased modification rate and S-shaped reaction curves. It is of interest to note that the cooperative mode of reaction exclusively seems to be a property of monomeric ATPase. In spite of protein-protein interactions that might shield some of the SH groups, oligomeric preparations invariably were characterized by higher modification rates in the initial reaction period, and the curves could be analyzed in terms of three classes of reactive sulfhydryl group, as in the case of membranous ATPase. Since the level of detergent binding of the oligomeric preparation is quite high (approx. 80% of that of the monomer, unpublished results), the conformation of monomeric ATPase probably differs from that of oligomeric and membranous enzyme, although the monomer has retained full enzymatic activity. This is consistent with structural [19,20] and functional [1] evidence for an oligomeric arrangement of the ATPase in the membrane. The spin-label results (Fig. 6) are also consistent with contact between ATPase polypeptide chains in the membrane, since the distinct feature of highly aggregated ATPase (an immobilized component and linebroadening) were also present in the spin-

labeled ATPase vesicles, but not in $C_{12}E_8$-solubilized ATPase. However, immobilization was less pronounced in vesicular ATPase than in the oligomer, suggesting aggregation to a limited extent in the membranous form.

All types of enzymatically active ATPase preparations in the solubilized state are characterized by a marked reduction of SH-modification rates in the presence of ADP or ATP. This is also a characteristic feature of vesicular ATPase [3,5,21—23] but it was not found after inactivation. Monomeric ATPase differs from vesicular ATPase in being highly susceptible to inactivation in the presence of EGTA. We obtained evidence that a major part of the enhancement of SH reactivity of monomeric ATPase, induced by EGTA, occurred immediately after addition of EGTA at a stage before inactivation had taken place (Fig. 4). Several authors [4,24—26] have reported reversible changes in SH-modification rates of sarcoplasmic reticulum vesicles induced by the presence of EGTA. However, our previous observations on purified vesicles [3] showed only a negligible effect of the concentration of free Ca^{2+} on the SH reactivity. Two recent studies [24,25] using *N*-ethylmaleimide as modification reagent distinguish SH groups with opposite responses to Ca^{2+}. One particular SH group (SH_F) is protected by Ado*PP*[NH]*P* and becomes more reactive at low concentrations of free Ca^{2+}. Although comparison with the present study is difficult due to the difference in reactivity of *N*-ethylmaleimide and DTNB, it is evident that the SH_F group behaves qualitatively in the same way as most of the SH groups on the monomeric ATPase studied here. This, together with the cooperative mode of reaction, suggests that SH_F may be a critical thiol group, which triggers the modification of a number of SH groups because of the instability of the system. The triggering may be counteracted in the case of oligomeric and membranous ATPases due to stabilization by protein-protein contacts and the lipid structure. In this way the difference between monomeric and membranous ATPases in the response to EGTA may be explained solely on the basis of a difference in the secondary changes following the primary event: a change in reactivity of SH_F. However, it is difficult to rule out a more fundamental difference in the conformational response to Ca^{2+} removal, which might have functional significance, if small amounts of monomer are present in the native sarcoplasmic reticulum membrane in equilibrium with the oligomers.

The stimulation of SH reactivity, that we detected after inactivation (Fig. 2), is more clearly a result of secondary changes. Our studies on EGTA-inactivated ATPase argue against the occurrence of major conformational changes as a result of the inactivation. Thus, we did not observe any change in f/f_{min} as evidenced by identical $s_{20,w}$ values for the active and inactive monomer. Furthermore, ESR spectra of the short-chain maleimide nitroxide, attached to ATPase, showed a break in the Arrhenius plots at 22°C both in the active and inactive state. The circular dichroic spectrum indicated some reduction in α-helix content, and there was a moderate decrease in the binding capacity of ATPase for $C_{12}E_8$ after EGTA inactivation. This decrease in $C_{12}E_8$ binding is probably too small to account for the increase in SH reactivity by an uncovering of non-exposed SH groups, since all SH groups of the protein seem to be affected by EGTA (Fig. 2). Most likely, limited conformational changes take place during inactivation. Thus, the dramatic increase in SH reactivity induced by EGTA (Figs. 2 and 4) attests to the extreme sensitivity of DTNB as

a probe of conformational changes. This is in marked contrast to the small change caused by EGTA in the ESR spectral parameter, h_s/h_w, of $C_{12}E_8$-solubilized ATPase as shown in Fig. 7.

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References

1 Møller, J.V., Lind, K.E. and Andersen J.P. (1980) J. Biol. Chem. 255, 1912—1920
2 Dean, W.L. and Tanford, C. (1978) Biochemistry 17, 1683—1690
3 Andersen, J.P. and Møller, J.V. (1977) Biochim. Biophys. Acta 485, 188—202
4 Murphy, A.J. (1976) Biochemistry 15, 4492—4496
5 Thorley-Lawson, D.A. and Green, N.M. (1977) Biochem. J. 167, 739—748
6 Le Maire, M., Jørgensen, K.E., Røigaard-Petersen, H. and Møller, J.V. (1976) Biochemistry 15, 5805—5812
7 Meissner, G., Conner, G.E. and Fleischer, S. (1973) Biochim. Biophys. Acta 298, 246—269
8 Le Maire, M., Lind, K.E. Jørgensen, K.E., Røigaard, H. and Møller, J.V. (1978) J. Biol. Chem. 253, 7051—7060
9 Ellman, G. (1959) Arch. Biochem. Biophys. 82, 70—77
10 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
11 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
12 Dupont, Y. (1977) Eur. J. Biochem. 72, 185—190
13 Vianna, A.L. (1975) Biochim. Biophys. Acta 410, 389—406
14 Rousselet, A. and Devaux, P.F. (1978) FEBS Lett. 93, 161—164
15 Morrisett, J.D. (1976) in Spin Labeling: Theory and Applications (Berliner, L.J., ed.), pp. 306—308, Academic Press, New York
16 Kleeman, W. (1976) in Structural and Kinetic Approach to Plasma Membrane Functions (Nicolau, C. and Paraf, A., eds.), pp. 83—84, Springer-Verlag, Berlin
17 Eletr, S. and Inesi, G. (1972) Biochim. Biophys. Acta 290, 178—185
18 Hesketh, T.R., Smith, G.A., Houslay, M.D., McGill, K.A., Birdsall, N.J.M., Metcalfe, J.C. and Warren, G.B. (1976) Biochemistry 15, 4145—4151
19 Vanderkooi, J.M., Ierokomas, A., Nakamura, H. and Martonosi, A. (1977) Biochemistry 16, 1262—1267
20 Scales, D. and Inesi, G. (1976) Biophys. J. 16, 735—751
21 Yoshida, H. and Tonomura, Y. (1976), J. Biochem. 79, 649—654
22 Martonosi, A. (1976) FEBS Lett. 67, 153—155
23 Murphy, A.J. (1978) J. Biol. Chem. 253, 385—389
24 Yamada, S. and Ikemoto, N. (1978) J. Biol. Chem. 253, 6801—6807
25 Kawakita, M., Yasuoka, K. and Kaziro, Y. (1980) J. Biochem. 87, 609—617
26 Champeil, P., Buschlen-Boucly, S., Bastide, F. and Gary-Bobo, C. (1978) J. Biol. Chem. 253, 1179—1186