Effects of Divalent Cations and ATP on the Kinetic Properties of the Sulfhydryl Groups of Sarcoplasmic Reticulum Membranes and Purified Ca-ATPase

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Abstract. The modification of SR membranes and purified Ca-ATPase with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) reveals about 11 mol of SH-groups per mol enzyme. The sulfhydril groups of both preparations are divided into two classes (kinetic sets). These classes of SH-groups of SR membranes display pseudo-first-order rate constants (**k**) of 0.16 and 0.015 min^{-1} (25 °C, pH 7.0, 0.75 mmol/l NBD-Cl). The corresponding values of **k** for modification of these sulfhydryl groups of purified Ca-ATPase are 0.11 and 0.027 min^{-1}, respectively. In the presence of Mg^{2+} (E^{Mg}-state of enzyme) all fast sulfhydryl groups of SR membranes are modified with **k** 0,09 min^{-1}. Under these conditions, however, there are but little changes in the reactivity of fast SH-groups of the purified enzyme. Binding of Ca^{2+} to SR membranes (E^{Ca}-state) results in an enhancement of the fast SH-groups number by 1, and all fast SH-groups are modified with **k** 0.34 min^{-1}. In the presence of this ion the number of fast SH-groups of purified Ca-ATPase is decreased by 2, and their modification constant is increased to 0.17 min^{-1}. The addition of ATP to both preparations results in similar effects. At low ATP concentration (30 μmol/l, E^{ATP}-state) the number of fast SH-groups is decreased by 1 in both preparations, modification constants of these groups being of 0.12 and 0,09 min^{-1} for SR membranes and purified Ca-ATPase, respectively. In the presence of high ATP concentration (3 mmol/l, E^{ATP}_{ATP}-state) the number of the fast SH-groups is decreased more : by 2 for SR membranes and by 1 for purified Ca-ATPase. Ca-ATPase activity of SR membranes and the purified enzyme is lost as a result of NBD-Cl modification. The pseudo-first-order constants of modification exceed by the factor of 4 the values of **k** for modification of fast SH-groups. In the presence of high ATP concentrations, the value of **k** for Ca-ATPase inactivation in SR membranes is decreased to 0.07 min^{-1}, that is equal to the modification constant of the fast SH-groups. The rate of the Ca-ATPase inactivation in the purified enzyme does not change virtually in the presence of ATP.

Key words: Sarcoplasmic reticulum — Ca-ATPase — Conformational changes — SH-groups

Introduction

Ca-pumping ATPase, as an intrinsic component of the sarcoplasmic reticulum membrane, plays a key role in the regulation of intracellular Ca^{2+} level. This enzyme is responsible for the active uptake of Ca^{2+} from the sarcoplasm of skeletal muscle cells (Tada et al. 1978; Hasselbach 1979). The calcium-dependent ATP hydrolysis requires the presence both of Ca and Mg ions. By changing the concentrations of Mg^{2+} and Ca^{2+}, Ca-ATPase is converted into various enzymic states (E^{Mg}, E^{Ca}) (Murphy 1978). The complex-dependence of Ca-ATPase on ATP concentration was noted early by several investigators (Yamamoto and Tonomura 1967; Lopina and Boldyrev 1977, Taylor and Hattan 1979). Ca-ATPase activity is observed at micromolar ATP concentrations. Hower, the rate of Ca-ATPase is not saturable at micromolar level, but continues to increase with the increase in ATP concentrations up to millimolar range. A possible reason for this complex kinetics is the allosteric coupling between two active sites oR active site and effector site which also binds ATP. Therefore, with increasing of ATP concentrations Ca-ATP-ase is converted from one conformational state (E^{ATP}) to another (E^{ATP}_{ATP}) (Lopina and Boldyrev 1977; Taylor and Hattan 1979). A possible approach for obtaining information on the mechanisms of enzyme operation is the chemical modification of amino acid residues in the enzyme molecule by means of groups-specific reagents. With the aid of N-ethylmaleimide and other thiol reagents evidence has been obtained for the importance of SH-groups for Ca-ATPase operation (Hasselbach and Seraydarian 1966; Murphy 1976b). Recently, chemical modification has also been used to investigate conformational changes of enzyme (Yoshida and Tonomura 1976; Murphy 1978).

The reagent 7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl) has been used to modify sulfhydryl groups of Ca-ATPase (Eckert et al. 1977). This paper deals with a detailed investigation of the modification of SR membranes and purified Ca-ATPase with NBD-Cl. Modification of the SH-groups was used as a tool for investigation of conformational changes of Ca-ATPase in the presence of different concentration ATP and co-substrates (Ca^{2+} and Mg^{2+}). The effect of NBD-Cl modification of ATPase activity gives additional informations about structure and function of this enzyme.

Materials and Methods

Reagents: EGTA, imidazole, sodium dodecylsulphate (SDS) and Coomassie brilliant blue R-250 were obtained from „Sigma"; ATP, histidine, dithiothreitol and kit for polyacrylamide gel electrophoresis were purchased from „Reanal" (Hungary). All the other reagents were from „Sojuzreaktiv" (USSR) of „chemical pure" grade.

Preparation of SR membranes. The SR membranes were isolated from rabbit white skeletal muscles by differential centrifugation. The muscles (50 g) were dissected and homogenized in 200 ml solution

containing 100 mmol/l KCl, 5 mmol/l histidine (pH 7.0) for 2 min in Type 302 homogenizer („Mechanika Precysyjna", Poland) at 8,000 rpm. Homogenate was centrifuged at 30,000 × g in "Beckman J-21B" centrifuge for 1 h. The pellet was rehomogenized as described above. The homogenate was centrifuged at 2,000 × g for 20 min and the pellet was discarded. Supernatant was passed through 6 layers of gauze and KCl was added to filtrate up to final concentration 0.5 mol/l. This solution was kept at 4°C for 40—60 min under stirring and afterwards centrifuged for 20 min at 8,000 × g. The obtained supernatant was again centrifuged for 60 min at 45,000 × g. The resulting pellet was taken up in 6 ml of solution containing 0.25 mol/l sucrose, 5 mg/ml bovine serum albumine, 5 mmol/l histidine (pH 7.0) and was suspended by hand in a glass teflon homogenizer. SR preparations were frozen in aliquots at −20°C.

Purification of Ca-ATPase. Purified Ca-ATPase was obtained by the method described by Ikemoto et al. (Ikemoto et al. 1971) with some modification (Lopina et al. 1976). The SR membranes (10—20 mg) were suspended in a solution containing glycerol 1.09 mol/l, dithiothreitol 2 mmol/l, Tris-HCl (pH 8.2) 20 mmol/l and Triton X-100 (the final protein to Triton ratio was 2:3). This solution kept at 4°C for 10—20 min and subsenquently centrifuged at 100,000 × g for 40 min in "Beckman J-75" ultracentrifuge. Thereafter, $CaCl_2$ was added to the supernatant up to final concentration 4 mmol/l and the suspension was kept at 4°C overnight. Ca-binding proteins were sedimented by centrifugation at 100,000 × g for 60 min. The detergent was removed by passing the supernatant through a Sepharose 4B column (3 × 40 cm) equilibrated with a solution containing glycerol 1.09 mol/l, dithiothreitol 2 mmol/l, $CaCl_2$ 4 mmol/l and Tris-HCl (pH 8.2) 10 mmol/l. This procedure resulted in formation of vesicles containing only Ca-ATPase protein and lipids. The membrane-bound enzyme appeared immediately following the void volume and was clearly separated from a second protein peak. Ca-ATPase was sedimented by centrifugation at 80,000 × g for 50 min. The pellet was resuspended in solution containing 0.25 mol/l sucrose and 30 mmol/l imidazole (pH 7.0) to a protein concentration of 2—5 mg/ml. The preparations of purified enzyme were kept frozen at −20°C. Protein was assayed according to Lowry et al. (1951).

The SDS-polyacrylamide gel electrophoresis. Samples of membrane fractions equivalent to 100 μg of protein were solubilized in a solution containing 20 per cent sucrose, 0.06 mol/l β-mercaptoethanol and 0.07 mol/l SDS. E.ectrophoresis was carried out according to Laemmly (1970) with Bromphenole blue dye as a tracking agent at a current 3 mA per tube for 1 h and then 6 mA per tube for 3 h. The gels were stained with Coomassie brilliant blue R-250 for 3 h and destained in 1.15 mol/l acetic acid. The peaks were integrated for area assuming that it is proportional to the amount of protein. Phosphorylase b (m. w. 100,000), bovine serum albumine (m. w. 64,000), egg albumine (m. w. 45,000), troponin (three subunits with m. w. of 37,000, 26,000 and 18,000) and cytochrome c (m. w. 12,900) were used as standards for relative mobility estimation. Densitometric scanning was done by a „Karl Zeiss Jena" JRI-65m densitometer (GDR) at 550 nm.

Determination of ATPase activity. The total ATPase activity of SR membranes was measured in medium containing 100 mmol/l KCl, 5 mmol/l oxalate, 20 μmol/l $CaCl_2$, 3 mol/l $MgCl_2$, 3 mmol/l ATP, 30 mmol/l imidazole (pH 7.0 at 25°C) and 20—30 μg/ml SR protein. Mg-ATPase activity was assayed under similar conditions, but in absence of $CaCl_2$ and presence of 0.5 mmol/l EGTA in the medium. Ca-dependent ATPase activity was estimated as a difference between the total and Mg-ATPase activities.

Ca-ATPase activity of the purified enzyme was measured in a medium containing: 100 mmol/l KCl, 0.5 mmol/l EGTA, 0.5 mmol/l $CaCl_2$, 3 mmol/l $MgCl_2$, 3 mmol/l ATP, 30 mmol/l imidazole (pH 7.0 at 25°C) in the presence of 5—10 μg enzyme-protein per ml. Mg-dependent ATP hydrolysis was not observed in these preparations. In both cases the reaction was started by addition of protein and was

stopped after 1 min by addition of an equal volume of acetic buffer (pH 4.2). The amount of P_i liberated was measured according to Rathbun and Betlach (1969).

Reaction with NBD-Cl. The modification of the SH-groups of SR membranes and purified Ca-ATPase by NBD-Cl was followed by measuring the absorbance in a "Pye Unicam" SP-1700 spectrophotometer at 420 nm, i. e. at the absorbtion maximum characteristic for the S-NBD-cysteinyl-ATPase (Eckert et al. 1977). The incubation mixture contained 100 mmol/l KCl, 0.5 mmol/l EGTA, 30 mmol/l imidazole (pH 7.0) and other components as indicated in the legends to tables and figures. The experiments on Ca-ATPase inactivation by NBD-Cl were carried out under similar conditions with protein concentration of 0.2—0.3 mg/ml and NBD-Cl concentration of 0.75 mmol/l (a 50—100 fold the actual concentration of excess over sulfhydryl groups to provide conditions for pseudo-first-order reaction). Absorbance was recorded 10 s after mixing of components and then within 2—3 h with 3-min intervals. The number of sulfhydryls was calculated using the molar extinction coefficient $\varepsilon = 13 \times 10^3\ \mathrm{mol}^{-1}\ \mathrm{cm}^{-1}$ (Eckert et al. 1979). The experimental results were presented in semilogarithmic plots and analyzed to distinguish different classes of sulfhydryl groups. For this aim latter linear portion of the curve was extrapolated to zero time. The curve thus obtained was subtracted from the original curve and this process was repeated until only a straight line remained. Each straight line represent the reaction rate of one class of SH-groups, and the value of absorbance extrapolated to zero time is proportional to the number of SH-groups in each class. The above procedure represents a graphical solution of the equation

$$A_f - A_t = \Sigma(A_{n_0} - A_{n_t}) = \Sigma(A_{n_0} e^{-k_n t}),$$

where A_f and A_t are the absorbances observed at the end of the reaction and time t, respectively; A_{n_0} and A_{n_t} are the corresponding values for individual classes of SH-groups, $\mathbf{k}_n$ is the pseudo-first-order rate constant for this class of SH-groups (Murphy 1976 b).

Results

Characterization of SR membranes and purified Ca-ATPase. Electrophoresis on polyacrylamide gels containing SDS revealed in SR membranes several protein components (Fig. 1), which were already identified by other authors (Mac Lennan

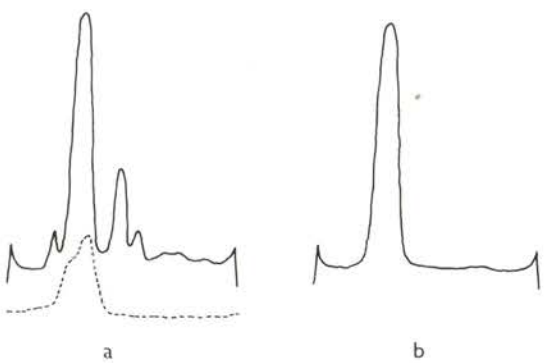

Fig. 1. Gel electrophoresis of proteins of SR membranes (a) and purified Ca-ATPase (b). (—) scan of the gel at 550 nm, (---) scan of the gel at 420 nm. The gels were loaded with 100 μg of protein.

et al. 1974; Antunes-Madeira and Madeira 1978). The major component represents the Ca-ATPase (molecular weight of approximately 100,000), which accounts for 80 per cent of the total protein of SR membranes. The other bands were identified as Ca-binding proteins — Calsequestrin and M_{55} (the mobilities of Ca-binding proteins in this gel system correspond to the protein with m. w. about 70,000 and 55,000) and so-called acidic proteins with m. w. between 10,000—30,000 (Antunes-Madeira and Madeira 1978). The purified Ca-ATPase contains only one protein with m. w. of 100,000 (Fig. 1.).

In Table 1 some enzyme properties of SR membranes and purified Ca-ATPase are presented. The SR preparations possess Ca-uptake ability. Since their Ca-ATP-

Table 1. Activity of Ca-dependent and Mg-dependent ATPases of SR membranes and purified ATPase preparations

	μmol P_i/mg of protein × min (37°)	
	Ca-dependent ATPase	Mg-dependent ATPase
SR membranes	3.14 ± 0.4	0.44 ± 0.05
Purified Ca-ATPase	10.7 ± 1.6	absent

ase activity was shown to be considerably reduced after filling the vesicles with Ca^{2+}, the activity of the latter enzyme was measured in the presence of oxalate which increases the Ca-accumulation capacity of the vesicles. Besides, Ca-ATPase activity the SR membranes possess also Ca-independent Mg-ATPase activity, which represents at about 10 per cent of the total ATPase activity (Table 1). Purification of the Ca-ATPase was followed by an approximately 3-fold increase in activity of their enzyme. On the other hand, Mg-dependent activity was completely absent in the purified preparations of Ca-ATPase. It should be noted, than in contrast to SR membranes the purified Ca-ATPase preparations have no Ca-accumulation capability, presumably owing to the leakiness of the membranes for Ca^{2+} (Ikemoto 1975).

Recently it was shown that NBD-Cl modifies only the SH-groups of SR membranes and the purified Ca-ATPase (Eckert et al. 1977; Lopina et al. 1979). The reaction of NBD-Cl with SR membranes and purified Ca-ATPase reveals, that in these fractions there are 9.1 and 11.7 mol of SH-groups per 10^5 g of the protein, respectively. These SH-groups might belong to cysteine since it is known, that some minor components of SR membranes contain this amino acid (Mac Lennan et al. 1973). In our experiments the distribution of NBD-S-derivates between the components of SR membranes was investigated. After modification of membranes by NBD-Ch and resolution of labelled proteins by gel electrophoresis, this label was found to be included only in the 100,000 dalton protein. The absorbance scan at 420 nm of the gels with labelled membranes is shown in the Fig. 1. Approximately

80 per cent of the total stain and 100 per cent of the total label was found in the 100,000 dalton protein. This distribution of the label allowed the calculation of the total number of SH-groups per mol of Ca-ATPase not only for purified preprations, but also for SR membranes. Accordingly, the number of SH-groups per mol was found to be Ca-ATPase 11.4 and 11.7 for SR membranes and purified Ca-ATPase, respectively. These data indicate, that the purification of the enzyme does not change the total number of NBD-accessible SH-groups.

A semilogarithmic plot for the reaction of NBD-Cl with SR-membranes (the changes of absorbance as a function of time) is demonstrated in Fig. 2 and becomes linear about 30 min after mixing the reactants. Analysis of these data by extrapolation and substraction of the linear portion of the curve results in resolution of a second faster component of the reaction. These two distinguishable components (kinetic sets) in SR membranes represent 6.5 and 4.9 mol of sulfhydryl groups per mol of Ca-ATPase with pseudo-first-order rate constants of 0.16 and 0.015 min^{-1}, respectively (Table 2). The reaction pattern obtained for the purified Ca-ATPase is quite similar to those obtained for SR membranes. The number of fast and slow SH-groups in preparations of the purified Ca-ATPase is 5.4 and 6.3 mol/mol

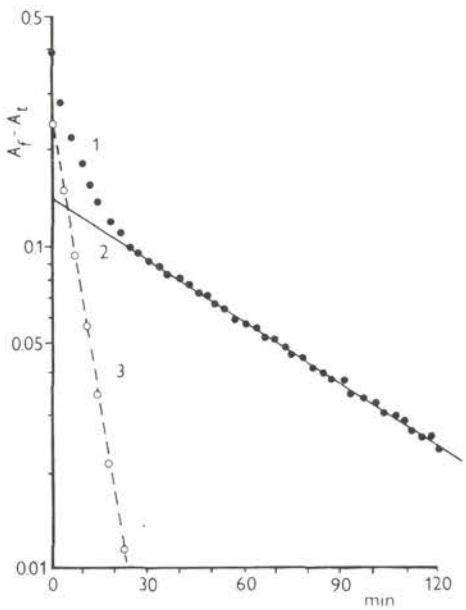

Fig. 2. Semilogarithmic plots of the difference in the final absorbance (A_f) and absorbance at time t (A_t) during the reaction of SR with NBD-Cl. Curve 1 (●), observed absorbance difference; Curve 2 (—), extrapolated portion of the final slope of curve 1; Curve 3(-○-), difference between curves 1 and 2. For reaction conditions, see "Materials and Methods".

Table 2. The kinetic parameters of modification of SH-groups and inactivation of Ca-ATPase by NBD-Cl in SR membranes and purified Ca-ATPase. The average data on 5 preparations of protein are given. The incubation media contain: 100 mmol/l KCl, 0.5 mmol/l EGTA, 30 mmol/l imidazole (pH 7.0), 0.75 mmol/l NBD-Cl 0.3 mg/ml protein.

	Additions to reactior mixture	Fast SH-groups		Slow SH-groups		
		mol per mol of ATPase	modification constants min^{-1}	mol per mol of ATPase	modification constants min^{-1}	Inactivation constants min^{-1}
SR	None	6.5	0.16	4.9	0.015	0.68
	+1 mmol/l $MgCl_2$	6.4	0.09	4.8	0.019	—
	+0.5 mmol/l $CaCl_2$	7.6	0.34	3.4	0.016	—
	+30 μmol/l ATP	5.1	0.12	6.0	0.016	0.13
	+3 mmol/l ATP	3.0	0.07	7.8	0.016	0.07
Purified Ca-ATPase	None	5.4	0.11	6.3	0.027	0.44
	+1 mmol/l $MgCl_2$	5.6	0.10	6.0	0.028	—
	+0.5 mmol/l $CaCl_2$	3.8	0.17	7.4	0.058	—
	+30 μmol/l ATP	4.5	0.09	6.6	0.029	—
	+3 mmol/l ATP	3.6	0.07	7.8	0.029	0.36

ATPase and the corresponding rate constants are 0.11 and 0.027 min^{-1}, respectively.

Influence of Ca^{2+}, Mg^{2+} and ATP on the kinetic properties of the SH-groups. Although the rate constants and number of SH-groups in each class investigated without ligands differs from those obtained in the presence of ATP or divalent cations, the general pattern and the total amount of SH-groups are the same in the presence or absence of each ligand in the reaction mixture. In the presence of Mg^{2+} (E^{Mg}-state) the number of fast and slow sulfhydryl groups does not change in any of the preparations. However, in the case of SR membranes these fast and slow SH-groups become respectively less or more reactive (Table 2). The addition of Mg^{2+} induced but little effect on the reactivity of SH-groups in preparations of purified Ca-ATPase.

In the presence of Ca^{2+} (E^{Ca}-state) in SR membranes there was observed a conversion of one SH-group from the slow to the fast set (Table 2). Moreover, the whole class of fast sulfhydryl groups reacted with NBD-Cl more rapidly. The latter was manifested by an increase in the respective rate constant from 0.16 to 0.34 min^{-1}. On the other hand, binding of Ca^{2+} to purified enzyme enables near 2 additional SH-groups of slow type to be revealed, while the number of fast thiol groups is decreased by 2. Both the fast and the slow classes of SH-groups in this preparation react with NBD-Cl in the presence of Ca^{2+} more repidly (Table 2).

ATP exerted a similar, concentration-dependent effect on the reactivity of thiol groups in both preparations. At low ATP level (E^{ATP}-state) in both preparations an amount of about 1 mol SH-groups per mol ATPase was converted from fast to the slow set (Table 2), and decrease in reactivity of all fast thiol groups was observed. ATP at high concentrations (E^{ATP}_{ATP}-state) induces the appearance of additional slow SH-groups as compared to that at low ATP level. An increase of ATP concentration from 30 μmol/l to 3 mmol/l resulted in decrease of the number of fast SH-groups from 5.1 to 3.0 mol per mol ATPase in SR membranes and/or from 4.5 to 3.6 mol per mol ATPase in purified enzyme preparations. This would represent an interconversion of 2 fast thiol groups to slow ones in SR membranes, but, however, only one in the purified enzyme. Under these conditions the remaining fast SH-groups in both preparations become equally reactive with a rate constant of 0.07 min^{-1}.

The inhibition of Ca-ATPase by NBD-Cl. The modification of SH-groups in both preparations was accompanied by an inhibition of enzyme activity. The diminution of Ca-ATPase activity under these conditions was in both cases characterized by pseudo-first-order reaction kinetics with rate constants of inactivation being 0.68 and 0.44 min^{-1} for the SR membranes and the purified Ca-ATPase, respectively (Table 2).

Investigation the effect of ATP in different concentrations on the inactivation of Ca-ATPase revealed that in SR membranes ATP decreases the rate of enzyme inactivation. The value of rate constant for decrease of Ca-ATPase activity in these preparations was diminished from 0.68 to 0.13 min^{-1} at ATP concentration of 30 µmol/l (E^{ATP}-state) as well as to 0.07 min^{-1} at 3 mmol/l ATP (E^{ATP}_{ATP}-state). At high ATP level the rate constant for the loss of Ca-ATPase activity was equal to the rate constant of the fast SH-groups modification. This may be interpreted in such a way that in SR membranes, in the presence of ATP, only one fast SH-group is responsible for the enzyme activity. In both, the SR membranes and in purified Ca-ATPase, in absence of ATP the rate constant of modification of fast SH-groups was proved to be about 4-times lower than the rate constant of Ca-ATPase inactivation. It should be noted than ATP does not provided any protection of the Ca-ATPase activity in the purified enzyme preparations (Table 2).

Discussion

The reagent NBD-Cl has been used recently to modify a number of enzymes (Fergusson et al. 1975a; Fergusson et al. 1975b; Eckert et al. 1977). It is known, that NBD-Cl can interact not only with cysteine, but with lysine and tyrosine residues, too. The nature of the groups modified may be identified by the absorbance spectrum of the sample. The absorbance maximum, characteristic for protein-S-NBD-derivative, is highly distinctive from those for -O-NBD- or -N-NBD-derivatives (Fergusson et al. 1975a; Fergusson et al. 1975b). It was demonstrated in our previous paper, that under applied conditions only the SH-groups of SR membranes are modified by the above label (Lopina et al. 1979). Similar result excluding any intramolecular transfer of NBD from sulphur to nitrogen has been reported also by Eckert et al. for Ca-ATPase purified according to Mac Lennan (Eckert et al. 1977). Comparing the NBD-labelled preparations of SR membranes and the purified enzyme, it was found that in both cases NBD is incoporated into only one 100,000 dalton protein fraction. Thus it may be concluded that all changes in kinetic properties of NBD-accessible SH-groups observed in our investigation are associated excluseively with changes in conformation of Ca-ATPase protein.

The data presented further show, that a removal of about 20 per cent of protein during purification procedure results in a 3-fold increase in the Ca-ATPase activity. There are at least 3 possible explanations for this phenomenon: i) the elimination of an inhibitory protein; ii) changes in the lipid environment i. e., partial removal of lipids surrounding the Ca-ATPase molecule or alteration in their arrangement after detergent treatment; iii) the removal of some protein that might control the orientation of Ca-ATPase in the membrane (like Racker's proteolipid).

In spite of the fact that our data are lacking any direct evidence, the two latter explanation assuming conformation changes of the Ca-ATPase molecule seem to be more plausible. Both, the purified Ca-ATPase and the SR membranes exhibit about 11 NBD-axcessible SH-groups, and only one fast SH-group has been converted to slow one during the purification procedure, but the reactivity of SH-groups of both classes has been considerably altered (Table 2).

Using NBD-Cl-modification the existence of 4 different conformational states of Ca-ATPase in dependence on binding of Mg^{2+}, Ca^{2+} and ATP has been demonstrated. As it is seen from Table 2, Mg^{2+} alone (E^{Mg}-state) induces only a little alteration in the reactivity of SH-groups. This is not very surprising, since Mg^{2+} is required for the formation of true substrate complex Mg-ATP (Vianna 1975) and also for dephosphorylation of the phosphoenzyme (Kanazawa et al. 1971), but in both cases Mg^{2+} affects the Ca-ATPase only in the presence of other ligands.

By contrast, Ca^{2+} by itself (E^{Ca}-state) produces a significant alteration in SH-groups reactivity. In the SR membranes one slow SH-group is converted into the fast one, and the reactivity of all fast groups is markedly increased (Table 2). In earlier paper we could demonstrate that the above effect of Ca^{2+} is induced by its binding in a high affinity site with K_d of about 5×10^{-7} mol/l (Lopina et al. 1979). It was also shown by other investigators that SR vesicles are capable to bind Ca^{2+} with high affinity even in the absence of ATP (Chevallier and Butow 1971; Meissner 1973; Ikemoto 1975). Accordingly, the demonstrated alteration in SH-groups reactivity which follows the binding of Ca^{2+} with these sites seems to promote conformational changes of Ca-ATPase even when ATP is absent.

On the other hand, effect of Ca^{2+} on the purified Ca-ATPase leads to conversion of 2 SH-groups from the fast to the slow set, nevertheless, all remaining SH-groups react with NBD-Cl more rapidly (Table 2). The different effect of Ca^{2+} on the purified Ca-ATPase appears to be stipulated with the different permeability of membranes of these preparations for Ca^{2+}. In the case of SR membranes Ca ions bind only on the outside of membrane, while in the purified enzyme all of the Ca-binding sites are accessible to Ca ions (Ikemoto 1975). Thus the influence of Ca^{2+} on the purified Ca-ATPase apparently reflects the sum of its effects on several Ca-binding sites.

The activation of Ca-ATPase at high levels of ATP was already investigated and interpreted from several points of view (Neet and Green 1977; Taylor and Hattan 1979). The occurrence of a low affinity ATP-binding site, that would be either effector site or hydrolytic site of the neighbouring molecule of the Ca-ATPase was postulated. However, several reports have presented evidence, that Ca-ATPase preparations have 2 ATP-binding sites with different K_d for this nucleotide (Dupont 1977; Taylor and Hattan 1979). Spin label studies indicate, that the conformational change of Ca-ATPase does occur in the presence of high

ATP concentration, presumably after phosphorylation of the enzyme (Landgraf and Inesi 1969; Pang et al. 1974). Our results obtained with NBD-Cl, however, point to two distinct conformational states of the Ca-ATPase induced by low and high ATP concentrations in the absence of other ligands (E^{ATP}, E^{ATP}_{ATP}-states). This would mean, that namely ATP binding without phosphorylation of the enzyme induces these conformational changes. ATP at low concentrations exerts similar effects on the reactivity of SH-groups in both preparations (Table 2). These effects are apparently induced by ATP binding in a hydrolytic (high affinity) site, which is not affected during purification.

The effect of high ATP concentrations on SR membranes and purified Ca-ATPase is different: an increase in ATP concentration from 30 μmol/l to 3 mmol/l transforms only 1 SH-group from fast to the slow one in the case of purified enzyme, while in the same conditions 2 fast SH-groups are converted to slow one in SR membranes (Table 2). In preparations of purified enzyme no protective effects of ATP on Ca-ATPase inactivation have been observed. The effect of ATP in high concentration seems to be caused by occupation of a low affinity binding site, which affects the properties of an other high affinity binding site.

Their interaction, resulting in conformational change of the enzyme, is probably prevented during purification procedure-induced alterations.

The question, whether the low affinity site for ATP binding is an effector site located on the same molecule of a hydrolytic site on the neighbouring molecule of the enzyme is according to the available data impossible to answer in satisfactory way.

There is much evidence that the Ca-ATPase is an oligomeric (tetrameric) system (Murphy 1976a; Vanderkooi et al. 1977; Pick and Racker 1979). As it might be seen from Table 2, the rate of Ca-ATPase inactivation in the absence of ATP exceeds 4 times the rate of modification of the fast SH-groups. This would be suggestive to the fact that, Ca-ATPase is a tetramer and the modification of 1 fast SH-group located on one of the subunits leads to inactivation not only of that particular subunit, but the whole oligomeric complex. In the presence of ATP in high concentrations the rates of modification of the fast SH-groups and of inactivation of Ca-ATPase in SR membranes are equal. This might be explained by independent inactivation of each subunit of the oligomeric complex under these conditions. The purification-induced changes of Ca-ATPase conformation i. e. the changes of interaction between the different subunits in the oligomeric complex apparently exclude the protective effect of high ATP concentrations on inactivation of the enzyme. Thus it may be assumed that the purification procedure changes the quaternary structure of Ca-ATPase.

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