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Sulfhydryl Group Modification of Sarcoplasmic Reticulum Membranes†

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ABSTRACT: Modification of calcium-translocating sarcoplasmic reticulum membranes (SR) with 5,5′-dithiobis(2-nitrobenzoate) (Nbs_2) reveals four classes (kinetic sets) of sulfhydryl groups. Of the 25 mol/1.5×10^5 g of SR protein (i.e., containing 1 mol of ATPase protein) estimated in the presence of sodium dodecyl sulfate, 8 mol are unreactive, while 7, 8, and 2 mol display pseudo-first-order rate constants (k_1) of 0.16, 0.68, and 8.3 min^{-1}, respectively (25 °C, pH 7.8, 4 mM Nbs_2). Under these conditions, the Ca-ATPase activity is lost with $k_1 = 0.73$ min^{-1}, whereas the Ca-independent ATPase activity is essentially unchanged. These results are little changed by the presence of Mg^{2+} or Ba^{2+} in the modification mixture, while Ca^{2+} or Sr^{2+} causes all 16–17 reactable sulfhydryls to be modified with $k_1 = 0.50$ and 0.53 min^{-1}, respectively. The corresponding values for the loss of Ca-ATPase activity are 0.53 and 0.67 min^{-1}; this suggests that blocking of only one of the 16–17 SH groups inactivates the enzyme, i.e., that there is a single "essential" SH group. The midpoint of the transition between the Ca^{2+}-free and Ca^{2+}-modification patterns occurs at a free Ca^{2+} concentration of about 0.9 μM, implying that it is Ca^{2+} binding at the active sites ($K_D = 0.1$–1.0 μM), rather than at the low-affinity nonspecific sites, that effects a conformation change in the ATPase protein (which contains >90% of the cysteines). A calcium-induced conformation change is also suggested by increased ultraviolet absorbance spectrum of the purified ATPase protein upon calcium binding. If protein–lipid interaction is disrupted with deoxycholate or Triton X-100 (which does not destroy the Ca-ATPase activity and hence presumably leaves the tertiary structure of the ATPase protein largely intact), 95% of the sulfhydryls react with Nbs_2 considerably faster; thus, at 2 mg/ml of deoxycholate, 14 groups react with $k_1 > 20$, 5 with $k_1 = 2.3$, and 5 with $k_1 = 0.4$ min^{-1}. These results suggest that the inaccessibility of SH groups in the absence of detergents is due to extensive interaction of the bilayer phospholipids with the ATPase protein.

Sarcoplasmic reticulum membranes, isolated in vitro as vesicles, are of interest both because of their biological role in the release and uptake of calcium in muscle, and, more generally, because their relatively few protein components and limited range of activities make them well suited for study as a model for active transport and protein–bilayer interactions (for a recent review, see Mac Lennan and Holland, 1975). As the functional unit which effects calcium translocation across SR[1] membranes, the ATPase protein appears to be intimately associated with the phospholipid bilayer, as evidenced by investigations using electron spin resonance (Inesi et al., 1973), detergent solubilization (Mac Lennan, 1970), electron microscopy (Deamer and Baskin, 1969; Inesi and Scales, 1974), and tryptic digestion (Migala et al., 1973; Thorley-Lawson and Green, 1973; Stewart and Mac Lennan, 1974; Inesi and Scales, 1974; Louis et al., 1974). The latter two techniques further suggest that the ATPase is more strongly associated with outer, cytoplasmic phospholipids of the bilayer and projects from this surface into the aqueous phase. This asymmetric, cytoplasmic localization has also been inferred from x-ray diffraction experiments on centrifugally packed SR membranes (Dupont et al., 1973); similar studies (Worthington and Liu, 1973; Liu and Worthington, 1974), however, have been interpreted to indicate predominant association with the inner half of the bilayer.

The reactivity of protein functional groups, being dependent on their environment, has been widely used to measure their degree of accessibility, and to clarify their roles in maintaining the structural and functional integrity of globular proteins. For membrane-bound proteins there is the additional potential for estimating the nature and extent of interaction with the phospholipids. Furthermore, to the degree that structural changes result from the presence of, e.g., cations, substrates, or detergents, these alterations may be detected from the extent and rate of chemical modification. In this paper is reported a kinetic study of the reaction of SR with the sulfhydryl group specific reagent Nbs_2 (some of the features of these SH groups were reported on earlier by Hasselbach and Seraydarian,

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[1] Abbreviations used are: SR, sarcoplasmic reticulum; Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); Doc, sodium deoxycholate; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; ADP, ATP, adenosine di- and triphosphates; ATPase, adenosine triphosphatase.

1966). Our results suggest that a change in protein conformation occurs upon binding of Ca^{2+} to the active site.

Materials and Methods

A previously described method (McFarland and Inesi, 1971) was used to prepare SR vesicles from rabbit hindleg muscles. Stored at 0 °C in 10 mM Mops, 0.88 M sucrose, pH 7.0, the preparations exhibited on sodium dodecyl sulfate gel electrophoresis one major band (70% of the protein, the ATPase) of about 10^5 daltons and two minor bands (cf. Inesi and Scales, 1974). ATPase activities were determined at 25 °C in 50 mM potassium maleate, 50 mM KCl, 5 mM potassium oxalate, 3 mM $MgCl_2$, 2 mM ATP, 0.02 mg/ml of Triton X-100, pH 6.8, using the method of Fiske and Subbarow (1925) to estimate orthophosphate. From these total activities, calcium-independent ($CaCl_2$ omitted) values were subtracted to obtain the calcium-dependent activities.

Nbs_2 modifications were carried out at 25 °C on SR (0.3–0.5 mg/ml of protein) at pH 7.80 ± 0.02 in 0.1 M Tris-HCl, containing 0.8 mM EDTA and 35 mM sucrose. Other additions, if any, are mentioned in the figure legends. When present, divalent cation concentrations are expressed as that which is in excess over the EDTA concentration. The reference solution was identical except it contained no SR. Upon the addition of Nbs_2 to 4 mM (a minimal 50-fold excess over the sulfhydryl concentration to obtain pseudo-first-order rates) the absorbance vs. time was recorded with a thermostated Aminco DW-2 spectrophotometer at 412 nm, where the nitromercaptobenzoate product absorbs maximally (Ellmann, 1959). Turbidity changes were recorded at 550 nm; only when the divalent cation concentrations were significantly greater than 1 mM were the turbidity changes more than 1% that of the absorbance change at 412 nm. The number of moles of sulfhydryls was calculated using $\epsilon = 13\,600$ (Ellmann, 1959), and is expressed per 1.5×10^5 g of protein, since the ATPase protein (which has ~95% of the cysteines of SR; see below) has a molecular weight of about 105 000 and constitutes 70% of the total protein (Inesi and Scales, 1974).

Semilogarithmic plots of the data were analyzed for distinguishable classes of sulfhydryls by extrapolating later, linear portions to $t = 0$, subtracting this line from the curve, and repeating this process until only a straight line remained. Each straight line represents the reaction rate of a class of SH groups, and the extrapolated absorbance at $t = 0$ is proportional to the number groups in that class. This is a graphical solution to the equation $A_f - A_t = \Sigma(A_{fi} - A_{ti}) = \Sigma(A_{fi}e^{-tk_{1i}})$, where A_f and A_t are the observed absorbances at the end of the reaction and time t, respectively, A_{fi} and A_{ti} are the corresponding values for an individual class of groups, and k_{1i} is the pseudo-first-order rate constant for this class of groups. The number of sulfhydryls per SR ATPase is then $A_{fi}/(\epsilon[SR])$. The kinetic constants are reported as pseudo-first-order values (k_1, see Table I).

The permeability of SR to Nbs_2 under the same conditions used for modification was estimated by filtering aliquots through Millipore Type HA 0.45-μm filters, rinsing with 0.3 M sucrose–10 mM Mops, pH 7.0, at 0 °C, dissolving the SR in 1 mg/ml of sodium dodecyl sulfate–3 mM mercaptoethanol–0.1 M Tris, pH 7.8, and reading the absorbances of the liberated nitromercaptobenzoate at 412 nm. The values obtained were corrected for nitromercaptobenzoate released from the protein. The binding of Mg-[8-^{14}C]ADP to Nbs_2 modified and unmodified SR was determined at 25 °C by scintillation counting aliquots of SR-containing and Millipore-filtered solutions. Free calcium concentrations in the 1–100 μM range were estimated with the aid of a Ca-EGTA buffering system, using a dissociation constant at pH 7.8 and 25 °C of 8×10^{-9} M (calculated from the data in Martell, 1971). Because SR contains calsequestrin which exhibits an absorbance change upon calcium binding (Ostwald et al., 1974), calcium-dependent difference spectra were obtained using the purified ATPase protein (Mac Lennan, 1970). A solution of the protein at pH 7.5 in 0.1 M Mops, 1 mg/ml of Doc, and 0.8 mM EDTA was passed through a 0.45-μm filter; difference spectra at 25 °C were obtained before and after adding $CaCl_2$ to 0.9 mM (and an equal volume of H_2O to the reference cuvette). The protein concentration was 1.8 mg/ml.

Results

The Sulfhydryl Content of SR. In the presence of sodium dodecyl sulfate the reaction of Nbs_2 with SR shows that there are a total of 25 mol of SH groups/1.5×10^{-5} g of protein, i.e., per mole of ATPase protein (Table I). Since there is little likelihood that any SH groups are inaccessible in sodium dodecyl sulfate, it is taken to be the total sulfhydryl content of SR. This value is consistent with reports of the amino acid analyses of the three SR proteins. The two minor proteins, calsequestrin and the calcium-binding protein, yield 0 (Ikemoto et al., 1974) and 4 (Mac Lennan et al., 1972) mol of cysteic acid per mol of protein, respectively, and are present in roughly one-half the molar amount of the ATPase. Mac Lennan et al. (1971) have reported that the ATPase yields 24 mol of cysteic acid per mole; thus, there are some 25–26 cysteines per 1.5×10^5 daltons of SR protein, almost 95% of them contained in the ATPase protein. (Analysis for disulfides by treatment with $NaBH_4$ using the method of Cavallini et al. (1966) indicates their absence in SR membranes, so that cysteic acid found by amino acid analysis represents cysteinyl residues.)

Kinetics of the Nbs_2 Reaction. A semilogarithmic plot of the absorbance change vs. time for the reaction of Nbs_2 with SR is not linear until 400 s have elapsed (Figure 1a). Analysis of the data by successive extrapolations and subtractions yields pseudo-first-order rate constants which indicate there are four distinguishable classes (kinetic sets) of sulfhydryl groups (Table I). Some eight groups are unreactive, seven react slowly with $k_1 = 0.16$ min^{-1}, another eight are moderately reactive ($k_1 = 0.68$), and two groups are relatively quite reactive with $k_1 = 8.3$ min^{-1}. Although the rate constants and number of groups in each class differ somewhat from those obtained in the absence of divalent cations, the general pattern is much the same with Mg^{2+} or Ba^{2+} in the reaction mixture (Table I).

If Ca^{2+} is the divalent cation present during the reaction, the reactivities of the sulfhydryls changed markedly (Figure 1b). Nine groups are unreactive, while the remaining 16–17 groups are apparently equally reactive ($k_1 = 0.50$ min^{-1}). The two fast and seven slow groups become, respectively, less and more reactive. Strontium can be seen to affect the reaction in a manner almost indistinguishable from that of calcium (Figure 1b).[2]

Since SR is known to have both high and low affinity calcium binding sites (Fiehn and Migala, 1971; Chevallier and Butlow, 1971), it is of interest to see which type is implicated in the differential SH reactivity. As shown in Figure 2, the rate constant of the slow SH groups approaches 0.50 min^{-1} (the Ca-SR value) in range of 100 μM calcium. For the apparent equilibrium between Ca-SR and free SR, $K_D = 0.9 \pm 0.1$ μM. This value may be compared with $K_D = 1.3$ μM at pH 6.5

[2] Van der Kloot and Glovksy (1965) and Weber et al. (1966) have reported that strontium is translocated by SR at a rate similar to that for calcium.

TABLE I: Number of Sulfhydryls (and Their Rate Constants) in Each Kinetic Set and the Rate of Loss of Ca-ATPase Activity for the Modification of SR with Nbs_2.[a]

Additions to Reaction Mixture	Very Fast $n(k_1)$	Fast $n(k_1)$	Moderate $n(k_1)$	Slow $n(k_1)$	Unreactive[e] n	Loss of Ca-ATPase Activity (k_1)
No M^{2+}		1.8 (8.3)	8.1 (0.68)	7.3 (0.16)	7.9	(0.73)
Mg^{2+} [b]		2.0 (5.2)	8.5 (0.68)	6.6 (0.16)	8.0	(0.74)
Ca^{2+} [b]			16.4 (0.50)		8.7	(0.53)
Sr^{2+} [b]			16.0 (0.53)		9.1	(0.67)
Ba^{2+} [b]		3.3 (6.0)	7.3 (0.58)	6.6 (0.19)	7.9	
Sodium dodecyl sulfate[c]	20.8 (20)	4.3 (1.9)			0	
Doc[d]	14.2 (20)	4.5 (2.3)	5.1 (0.40)		1.3	
Doc[d] + Ca^{2+} [b]	12.7 (20)	5.1 (2.3)	6.1 (0.34)		1.2	

[a] Number of sulfhydryls, n, is per 1.5×10^5 daltons of SR protein; pseudo-first-order rate constants, k_1, are in units of min^{-1}. For reaction conditions, see Materials and Methods. [b] Divalent cations present at 0.1 mM. [c] Sodium dodecyl sulfate = 1.6 mg/ml. [d] Doc = 2.0 mg/ml. [e] Calculated by substracting the sum of the reactive groups from the total found in the presence of sodium dodecyl sulfate (25.1).

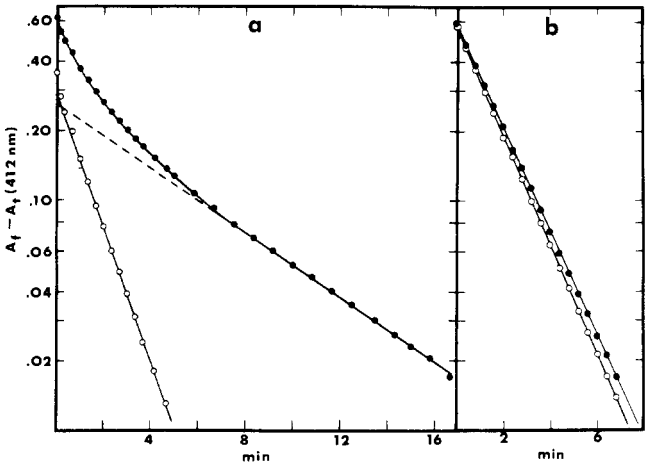

FIGURE 1: Semilogarithmic plots of the difference in final absorbance (A_f) and absorbance at time t (A_t) during the reaction of SR with Nbs_2. (a, left) no divalent cations present; Curve 1 (●), observed absorbance difference; Curve 2 (- - -), extrapolated portion of final slope of curve 1; curve 3 (○), difference between curves 1 and 2. (b, right) With 0.1 mM calcium (●) or strontium (○) present.

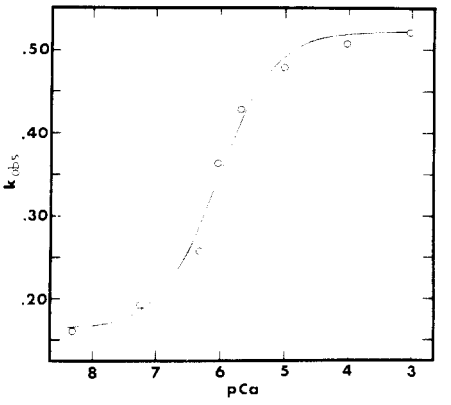

FIGURE 2: Plot of final slope rate constants for Nbs_2-SR modifications as a function of free calcium concentration. The line is calculated using a dissociation constant of 0.9 μM.

(Chevallier and Butlow, 1971). Thus, it is binding to the specific high affinity sites which changes the SR reactivity. Furthermore, since the rate constant is almost unchanged in the range of 0.01–1 mM calcium, binding to low-affinity sites apparently has little effect on the Nbs_2 reactivity.

To test the possibility that Nbs_2 reacts with at least some of the SR sulfhydryls by dissolving in the phospholipids and then reacting with lipid-associated SH groups, the aqueous buffer 1-octanol partition coefficient was determined and found to be about 4000:1. That Nbs_2 does penetrate to the internal space within the vesicles is indicated by influx experiments: under the conditions used for the modifications, Nbs_2 was found to be present inside the vesicles in approximately the same concentration (4 mM) within 30 s after mixing (assuming an internal volume of 4 ml/g of SR protein; Duggan and Martonosi, 1970).

Inactivation of the Ca-ATPase by Nbs_2. The ATPase activities of samples of various stages of modification were determined in order to relate inhibition of the enzyme to the number of sulfhydryls blocked. In Figure 3 are shown the specific activities of SR reacted with Nbs_2 for various times under the same conditions used to obtain the results shown in Figure 1 and Table I. The Ca^{2+}-independent ATPase activity is unaffected by Nbs_2 modification, while the Ca^{2+}-activated hydrolysis is destroyed; the linearity of the semilog plots indicates a first-order loss of Ca-ATPase activity in all cases. The corresponding rate constants indicate that the moderate class of SH groups contains one sulfhydryl that is required for Ca-ATPase activity and that the others of this class are not essential. With Ca^{2+} present the similarity in rate constants for SH modification and loss of enzymatic activity indicates only one of the 16–17 reactable groups is essential. Binding of [^{14}C]ADP to Nbs_2-modified and -unmodified SR was determined; while the latter bound 0.9 mol/1.5×10^5 g of protein with K_D = 20 μM (pH 7.0, 25 °C; cf. Meissner, 1973), the former bound less than 0.1 mol of nucleotide in the range of 1–100 μM ADP, indicating that destruction of Ca-ATPase activity may be simply the result of blockage of substrate binding at the active site.

Effect of Detergents. With sodium dodecyl sulfate present during Nbs_2 modification (Figure 4), over 80% of the SR sulfhydryls react very rapidly ($k_1 > 20\ min^{-1}$). There are four groups, however, which react more slowly ($k_1 = 1.9\ min^{-1}$), suggesting that even at rather high concentrations of sodium dodecyl sulfate (1.2 mg/ml) the proteins are not completely unfolded.

In the presence of Doc, the calcium-activated ATPase activity of SR is maintained (Mac Lennan et al., 1971, and verified during this study up to Doc concentrations of 4 mg/ml),

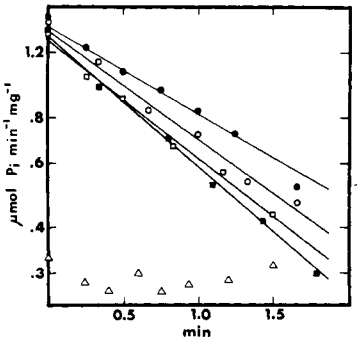

FIGURE 3: Semilogarithmic plots of the ATPase activities of SR reacted for various times with Nbs_2 in the presence or absence of divalent cations. ATPase activities are Ca independent (△) or Ca dependent (all others). Nbs_2 reactions were carried out in the absence of divalent cations (△ and □), calcium (●), strontium (○), or magnesium (■). When present, concentrations of M^{2+} during Nbs_2 reactions were 0.1 mM.

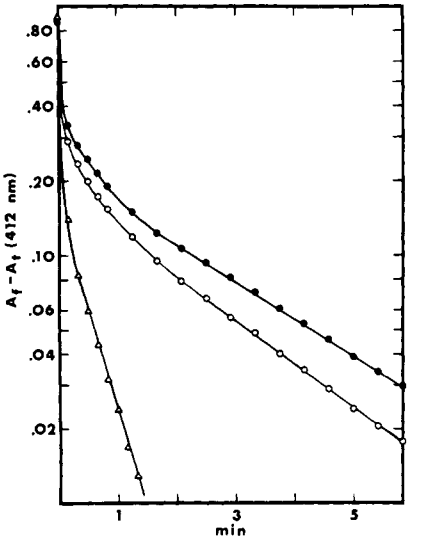

FIGURE 4: Semilogarithmic plots of the difference in final absorbance (A_f) and absorbance at time t (A_t) during the reaction of SR with Nbs_2 in the presence of detergents. Sodium dodecyl sulfate (△), 1.6 mg/ml; Doc (○), 2.0 mg/ml; Doc (●), 2.0 mg/ml plus 0.1 mM $CaCl_2$.

indicating that the essential three-dimensional structure of the enzyme is intact. Semilog plots of the data obtained from the Nbs_2 reaction in the presence of Doc are shown in Figure 4. About 95% (with sodium dodecyl sulfate = 100%) of the sulfhydryls react and do so at considerably greater rates than in the absence of detergents. More than half react at velocities comparable to the very fast groups with sodium dodecyl sulfate present. The number of groups in each kinetic set and their rate constants are listed in Table I. The differential effect of the presence of calcium, though still observable, is lessened, involving 1–2 sulfhydryls rather than the 8–9 seen in the absence of Doc. Similar results were obtained with 1.2 mg/ml of Triton X-100 in place of Doc.

Calcium-Induced Difference Spectrum of the ATPase Protein. The difference spectrum of the purified ATPase protein in the presence and absence of calcium is shown in Figure 5. It features maxima near 279, 286, and 293 nm and is similar to the difference spectrum of the tryptophan chromophore in 20% ethylene glycol (Dyson and Noltmann, 1969). The direction of the change suggests that the environment of this chromophore becomes less polar upon Ca^{2+} binding; the magnitude of $\Delta\epsilon$ is somewhat less than equivalent to that observed for transfer of one tryptophan from water to 20% ethylene glycol (Dyson and Noltmann, 1969).

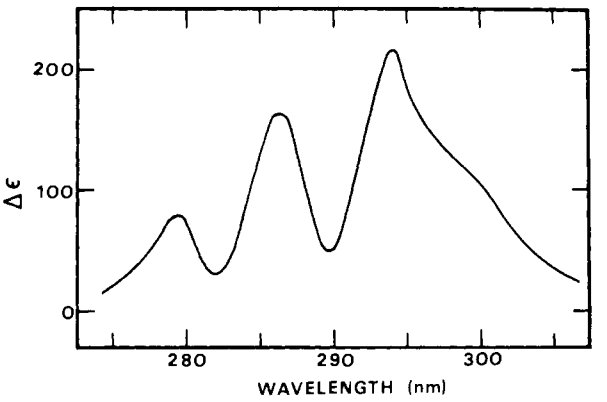

FIGURE 5: Difference spectrum of the purified ATPase protein in the presence and absence of calcium (0.1 mM). $\Delta\epsilon$ is calculated on the basis of mol wt = 105 000.

Discussion

Reaction of SR membranes with Nbs_2 in the presence of sodium dodecyl sulfate indicates that there are 25 mol of sulfhydryls per 1.5×10^5 g of protein. As mentioned under Results, this value agrees well with the results of amino acid analysis. The procedure provides a rapid, simple way to monitor changes in the SH content brought about by other chemical modifications, air oxidation, etc.

In an earlier study of the sulfhydryls of SR, Hasselbach and Seraydarian (1966) found 7 mol/10^5 g of protein reacted with *N*-ethylmaleimide. The corresponding number found here with Nbs_2 is 12 sulfhydryls. Though the difference in the reagent used may account for some of the discrepancy, a more likely reason is the improvement in purification techniques over the last decade. Thus, more recently, Dupont and Hasselbach (1973) have reported finding 10–12 reactable SH per 10^5 daltons (total sulfhydryl content was not determined). The present work also indicates that, contrary to these earlier studies, a turbidity increase (vesicular aggregation) is not a necessary consequence of SH modification, though it occurs if the divalent cation concentration is much higher than 1 mM during modification. An important area of agreement, however, is the finding in the three studies that the rate of inactivation is similar to the rate of reaction of one class of SH groups, implying that blockage of only one of these groups destroys the Ca-ATPase activity. Proximity of this sulfhydryl to the active site is thus suggested and supported by the Nbs_2-induced loss of ADP binding ability and the protective effect of Ca-ATP (Murphy, in preparation).

The observed aqueous 1-octanol partition coefficient of 4×10^3:1 for Nbs_2 suggests that little of the reagent dissolves in nonpolar regions of the phospholipids or proteins, so that access to SH groups likely occurs via polar routes. The apparently rapid influx of Nbs_2 into the intravesicular space implies that the reactivity of sulfhydryls, which might be more accessible by way of this internal compartment, is not lessened by a permeability barrier. That a charged, rather large reagent like Nbs_2 is permeable to SR may seem surprising; Duggan and Martonosi (1970), however, have reported that molecules of comparable size (sucrose) or charge (citrate) penetrate the vesicles. The 8–9 unreactive SH groups, then, are probably unreactive because they are located in nonpolar regions. They become reactive, though, when modification is done in the

presence of Doc or Triton. As reported by McFarland and Inesi (1971) and Mac Lennan et al. (1971), respectively, Triton X-100 and Doc do not destroy the Ca-ATPase activity; in fact, they enhance it. The essential three-dimensional structure of the ATPase is apparently intact in the presence of these detergents; their main effect is the destruction of the bilayer structure and incorporation of the phospholipids into mixed micelles (Dennis, 1974; Walter and Hasselbach, 1973). It would seem to follow that the nonpolar location of the unreactive SH groups is near the protein–phospholipid interface. Since Doc increases the reactivity of almost all of the SH groups, protein–lipid interactions apparently limit accessibility to many other regions of the protein as well.

It also seems reasonable that the fast, moderate, and slow SH groups observed without M^{2+} and with Mg^{2+} and Ba^{2+} differ in their reactivity primarily because they are decreasingly accessible to the aqueous solvent. Conversion to the Ca^{2+} form of the enzyme increases the reactivity of the slow groups, implying a conformational change occurs which makes all of the reactable sulfhydryls about equally accessible. This structural change is also reflected in the apparent shielding of an ATPase protein tryptophan upon calcium binding. Since the SH reactivity changes in the Ca^{2+} concentration range at which it is thought to bind to the ATPase-activating (and possibly translocating) sites (Figure 2), and since the monophasic reaction pattern is specific for the two translocatable ions Ca^{2+} and Sr^{2+} (Figure 1), the conformational change is likely a part of the pumping cycle. The calcium-induced production of ATP from ADP and orthophosphate by the purified SR-ATPase, recently reported by Knowles and Racker (1975), indicates the important role of calcium–protein interactions in the transduction process. Conformational differences between intermediates are also implied by modification patterns in the presence of acetyl phosphate and ATP (Murphy, in preparation).

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