Distinction of Thiols Involved in the Specific Reaction Steps of the Ca\(^{2+}\)-ATPase of the Sarcoplasmic Reticulum*

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The reaction of N-ethylmaleimide (MalNEt) with the -SH groups of purified Ca\(^{2+}\)-ATPase has been investigated as a function of calcium concentration. Several classes of thiols can be distinguished on the basis of the Ca\(^{2+}\) dependence of the reaction and the effects of thiol blocking on various steps of the ATPase reaction. Initially, MalNEt is rapidly incorporated with no effect on enzyme activity into the first thiol (-SH\(_1\), ~1 mol/10\(^6\) g) at a rate which is independent of [Ca\(^{2+}\)]. The rate of further MalNEt incorporation which leads to inactivation of enzyme activity varies with [Ca\(^{2+}\)]. At Ca\(^{2+}\) = 10\(^{-8}\) M during MalNEt incorporation, the rate of ATP hydrolysis (v), the rate of phospho-enzyme decomposition (k\(_p\)), and the phosphoenzyme level when the phospho-enzyme decomposition is prevented (EP\(_{ca}\)) are all inhibited at about the same rate. Upon increasing [Ca\(^{2+}\)], a second thiol (-SH\(_2\), ~1 mol/10\(^6\) g) increases its reactivity with MalNEt. This leads to the rapid decrease of v and k\(_p\), but the rate of decrease of EP\(_{ca}\) is about the same as that at low [Ca\(^{2+}\)]. These results indicate that blocking of the -SH\(_2\) results in the preferential inhibition of reaction steps in which phospho-enzyme decomposition occurs, and that besides -SH\(_2\), there is another type of thiol or thiols (S\(_0\)) whose reactivity with MalNEt is independent of [Ca\(^{2+}\)] and whose blocking inhibits the phospho-enzyme formation. The [Ca\(^{2+}\)] at a half-maximum change in the reactivity of -SH\(_2\) as determined from the [Ca\(^{2+}\)] dependence of the inhibition of v is 1.6 \times 10^{-7} M. This is basically the same as that of Ca\(^{2+}\) binding to the high affinity a sites. It is suggested that the change of the MalNEt reactivity with -SH\(_2\) reflects the change in enzyme conformation produced by the Ca\(^{2+}\) binding to the high affinity sites.

The blocking of thiols of the sarcoplasmic reticulum (SR) with various covalently or nonevolutely reacting reagents results in inhibition of ATPase activity and Ca\(^{2+}\) uptake (1-10). Analysis of the pseudo-first order kinetics of the thiol reaction with MalNEt or Nbs\(_2\) has shown that there are several distinguishable classes of thiols (3, 6, 8). Earlier work by Hasselbach and Seraydarian (3) has shown that blocking of four thiols/10\(^5\) g of SR protein with MalNEt results in complete inhibition of ATPase and Ca\(^{2+}\) uptake and that ATP specifically prevents the reaction of one of these thiols with concomitant protection of ATPase activity and Ca\(^{2+}\) uptake. According to Yoshida and Tonomura (11), blocking of at most two thiols with MalNEt results in complete inhibition of ATPase. It was suggested that only one thiol might be essential to the enzyme activity (6) since other nonfunctional thiols having the same reactivity cannot be distinguished.

A number of studies have been carried out on the ATPase of SR with a variety of types of thiol-directed reagents in attempts to monitor the conformation of various intermediate enzyme complexes (8, 10-20). Although nucleotides and metals cause some changes in the ESR and fluorescence spectra of the thiol-attached reagents, a definitive interpretation of these observations has not yet been possible. The crucial requirement for the interpretation of these results is the information concerning the number and the type of thiols to which these reagents are attached. Several attempts have been made to specifically label a single functionally important thiol after blocking the other thiols in the presence of ATP (3, 4). However, according to recent reports, the reaction with Nbs\(_2\) of a large number of thiols is reduced by ATP (6-8). The thiol reactivity appears to be considerably influenced by the Ca\(^{2+}\) concentration (6, 10, 21). Our recent stopped flow studies (22) of the reaction of the thiol-directed fluorescent reagent, S-mercuric N-dansyl cysteine (23), with the purified Ca\(^{2+}\)-ATPase of SR suggest that Ca\(^{2+}\) may exert a more selective effect on thiol reactivity than does ATP. Therefore, it appeared worthwhile to investigate whether selective blocking of certain types of thiols might be achieved by controlling [Ca\(^{2+}\)] during the reaction with MalNEt.

As shown here, upon increasing the [Ca\(^{2+}\)] from 10\(^{-8}\) to 10\(^{-5}\) M, the reactivity of one of the thiols of purified ATPase with MalNEt increases in parallel with the binding of Ca\(^{2+}\) to the high affinity a sites (24-26). Blocking of this thiol inhibits the decomposition of phosphoenzyme with little or no inhibition of its formation. Of the other thiols, there appears to be at least one that reacts with MalNEt at the same rate regardless of [Ca\(^{2+}\)] and whose blocking inhibits EP formation. These results suggest that there are at least two functionally important thiols, the blocking of which results in inhibition of different steps of the ATPase reaction. The results also suggest that the change of thiol reactivity with [Ca\(^{2+}\)] reflects a change in enzyme conformation controlled by Ca\(^{2+}\) binding.

EXPERIMENTAL PROCEDURES

Preparations

Fragments of the sarcoplasmic reticulum (SR) were prepared from skeletal white (fast) muscle of rabbit as described previously (27).
Ca"-ATPase was purified from fragmented SR solubilized with Triton X-100 (26).

**Determination of Thiol Blocked with ["C"]MalNEt**

The reaction with MalNEt was started by adding 1 mM ["C"]MalNEt to a solution containing 1 to 2 mg of ATPase protein/ml, 0.3 M sucrose, 0.1 M Tris/maleate (pH 7.0), 1 mM EGTA, and various concentrations of CaCl2, at 22°C for various lengths of time. For the determination of the bound MalNEt, the reaction was stopped by injecting a 0.1-ml fraction of the reaction mixture into 2 ml of 5 mM ["C"]MalNEt. Subsequently, 1 ml of 20% trichloroacetic acid was added. The denatured protein was placed on a Millipore filter (type HA, 0.45 µm) and washed with 3 x 20 ml of 5% trichloroacetic acid containing 1 mM ["C"]MalNEt. The filters were dried, the radioactivity was determined, and the number of blocked thiols calculated as described previously (11).

**Enzyme Assay**

**Colorimetric Assay of P, Liberation**—Initially, the reaction with MalNEt was stopped by adding dithiothreitol, but it was found that dithiothreitol interfered with the colorimetric assay of the liberated Pi (28). Therefore, the reaction was stopped by diluting 0.1 ml of the reaction mixture described above with 2 ml of ATPase assay solution, simultaneously starting the ATPase assay. The ATPase assay mixture contained (final concentrations) 0.1 M KCl, 5 mM ATP, 5 mM MgCl2, 1.2 mM EGTA, 1.0 mM CaCl2, 14 mM sucrose, 48 µM ["C"]MalNEt, 0.1 M Tris/maleate, pH 7.0; the reaction was stopped by adding 6.5% trichloroacetic acid. Since the liberated Pi was proportional to the reaction time at least for 1 min, the ATP hydrolysis rate was normally determined by measuring the liberated Pi after 1 min.

**Radioactive Assay of the P, Liberation and the Formation of Phosphorylated Enzyme**—The reaction with MalNEt was stopped at various times by mixing a 0.2-ml portion with 0.02 ml of 0.3 M dithiothreitol; the concentration of free Ca" was then adjusted to 1.1 µM by adding 0.05 ml of a solution containing appropriate concentrations of EGTA or CaCl2 (or both). The pH was adjusted to 7.0 by adding Tris/maleate, and the ATPase reaction was started by adding 0.1 ml of the mixture to 1 ml of a solution containing 0.11 M KCl, 11 µM ["P"]ATP, 5.5 mM MgCl2, 1.32 mM EGTA, 1.1 mM CaCl2, and 0.11 M Tris/maleate, pH 7.0, at 0°C. The reaction was stopped at various times by adding 6.5% trichloroacetic acid. The liberated Pi and the phosphorylated intermediate (EPm) were determined as described previously (26, 29). In order to determine maximal formation of phosphorylase (EPm), decomposition was prevented by carrying out the reaction in the presence of 5 mM CaCl2 and 0.2 mM MgCl2 (EPm). If the reaction with MalNEt was carried out at pCa 8.3 (Fig. 3a), the inhibition of the ATP hydrolysis rate (v) roughly parallels that of EPm. At pCa 5.0 (Fig. 3b), however, EPm actually increases in the earlier phase of the reaction comconitant with the reduction of v. This suggests that the blocking of —SH2 reduces in fact the rate of those steps of the ATPase reaction in which the phosphorylase is decomposed with no inhibitory effect on the phosphorylase formation step. The rate of phosphorylase decomposition can be estimated from the ratio v/[EPm], assuming that v = kP/[EPm] (31). The plot of ln ([EPm]/[EPm]) versus the time of the reaction with MalNEt is shown in Fig. 4a. Again, fast and slow phases are evident at pCa 5.0, whereas they are indistinguishable at pCa 8.3. The values of the rate of MalNEt incorporation into —SH2 at pCa 5.0 estimated from the logarithmic plots of v and kP are basically the same (Table 1), indicating that the fast phase of reduction of v at pCa 5.0 is primarily due to the inhibition of phosphorylase decomposition which is accounted for by the blocking of —SH2. In contrast to EPm, the EPm, which represents the phosphorylase level with the prevented phosphorylase decomposition step is reduced at about the same rate regardless of [Ca"]. During the reaction with MalNEt (Fig. 4b), this thiol class is designated as S1. F implies that blocking of this class results in the inhibition of phosphorylase formation.

The semilogarithmic plots shown in Fig. 1, b and d permit further analysis of the results described above. Approximately one thiol (0.5 to 0.8, depending on the preparation) per 105 daltons of ATPase peptide reacts during the initial rapid phase (~5 min) as estimated from the extrapolation of the plots shown in Fig. 1b. From a comparison of Fig. 1, b and d, it is clear that during the first 5 min, no changes are detectable in the slopes of the plots in Fig. 1d at either pCa 5.0 or 8.3, indicating that blocking of this thiol, designated as —SH1, does not inhibit the ATPase activity.

The logarithmic plot of ATPase activity versus reaction time with MalNEt (Figs. 1d and 2) reveals several important points. Upon increasing [Ca"], fast and slow phases become discernible (Fig. 2). The fast phase is completed in 20 to 30 min regardless of [Ca"]. However, the extent of the resultant inhibition of ATPase activity increases with [Ca"], and the maximal extent of inhibition occurs at pCa ~ 6. Thus, after the reaction with MalNEt at pCa 5.0 for 30 min, approximately 80% of the original ATPase activity is inhibited (Fig. 1d), while the fast 2.0 thiols are blocked (Fig. 1b). Since —SH2 accounts for ~1 as described above, the actual number of the thiol which is responsible for the fast phase of Fig. 1d seems to be ~1. These data suggest that Ca" increases the reactivity of a thiol whose blocking reduces the ATPase activity by 60%, as determined from the extrapolation of the final slope to t = 0. In order to facilitate further discussion, this thiol is designated as —SH2. At low [Ca"], e.g., at pCa 8.3, the plot can be fitted by a single line; this indicates that —SH2 reacts with MalNEt at about the same rate as do the other thiol or thiols, whose blocking inhibits ATPase activity.

The effect of the reaction with MalNEt on the phosphorylation of the enzyme was also studied under the two different conditions: 1) the same conditions as the experiment of Fig. 1 (EPm) and 2) the conditions under which the decomposition of phosphoenzyme is prevented in the presence of 5 mM CaCl2 and 0.2 mM MgCl2 (EPm). If the reaction with MalNEt is carried out at pCa 8.3 (Fig. 3a), the inhibition of the ATP hydrolysis rate (v) roughly parallels that of EPm. At pCa 5.0 (Fig. 3b), however, EPm actually increases in the earlier phase of the reaction comconitant with the reduction of v. This suggests that the blocking of —SH2 reduces in fact the rate of those steps of the ATPase reaction in which the phosphoenzyme is decomposed with no inhibitory effect on the phosphoenzyme formation step. The rate of phosphoenzyme decomposition can be estimated from the ratio v/[EPm], assuming that v = kP/[EPm] (31). The plot of ln ([EPm]/[EPm]) versus the time of the reaction with MalNEt is shown in Fig. 4a. Again, fast and slow phases are evident at pCa 5.0, whereas they are indistinguishable at pCa 8.3. The values of the rate of MalNEt incorporation into —SH2 at pCa 5.0 estimated from the logarithmic plots of v and kP are basically the same (Table 1), indicating that the fast phase of reduction of v at pCa 5.0 is primarily due to the inhibition of phosphoenzyme decomposition which is accounted for by the blocking of —SH2. In contrast to EPm, the EPm, which represents the phosphoenzyme level with the prevented phosphoenzyme decomposition step is reduced at about the same rate regardless of [Ca"]. During the reaction with MalNEt (Fig. 4b), this thiol class is designated as S1. F implies that blocking of this class results in the inhibition of phosphoenzyme formation.

Previous studies on the number of thiols involved in the ATPase activity of SR have led to contradictory results (3, 11). The contradiction can now be resolved if one considers the effect of Ca". The results of the experiment shown in Fig.
Thiols Involved in Enzyme Reaction of SR-ATPase

FIG. 1. Time course of the thiol reaction with \[^{14}C\]MalNEt (a, b) and the resultant inhibition of ATPase activity (c, d) of the purified Ca\(^{2+}\)-ATPase at two different Ca\(^{2+}\) concentrations. The purified ATPase (2 mg of protein/ml) was incubated in a solution containing 1 mM \[^{14}C\]MalNEt, 0.3 M sucrose, 0.1 M Tris/maleate (pH 7.0), 1 mM EGTA, and 0.02 mM or 0.97 mM CaCl\(_2\) at 22°C for various times as indicated on the abscissa. Calculated concentrations of free Ca\(^{2+}\) were 5 \times 10^{-9} \text{ M (pCa 8.3)} and 1 \times 10^{-5} \text{ M (pCa 5.0), respectively. The bound \[^{14}C\]MalNEt and the ATPase activity were determined as described under “Experimental Procedures.” The ATPase activity of the control samples (i.e., with no MalNEt treatment) was 4.15 pmol of P\(_i\)/mg of protein/min. \(v\) and \(v_0\) are the rate of P\(_i\) liberation of the samples treated with MalNEt for the time \(t\) and without treatment, respectively. The plots shown in b and d were analyzed according to Equation 1:

\[
\ln A = \ln (A_1 \exp(-k_1t) + A_2 \exp(-k_2t))
\]

1 (bound MalNEt per 10\(^5\) g of ATPase protein) are plotted in the way used in the previous reports (Fig. 5). At pCa 5.0, blocking of approximately 1.7 thiols produces 80% inhibition of ATPase. This is consistent with the report that there are one or two “essential” thiols (11). It appears that to inhibit the remaining 20% activity, further blocking of 3.5 thiols is required. At pCa 8.3, blocking of the first 0.5 thiol does not inhibit ATPase activity, but it is inhibited in proportion to the blocking of additional 4.5 thiols; this is consistent with the reported 4 essential thiols (3). Thus, it appears that the controversy in the reported number of the essential thiols is due to the difference in [Ca\(^{2+}\)] during the incorporation.

The Wide Range [Ca\(^{2+}\)] Dependence of Thiol Reactivity—Fig. 6 shows the experiment in which the ATPase activity was determined after the enzyme was allowed to react with MalNEt at various [Ca\(^{2+}\)] concentrations. The major change in the reactivity of -SH with MalNEt takes place in the range of 8 > pCa > 6, and there is little or no change in the range of pCa \(\leq 6\). The \(K_a\) (association constant) value estimated from the [Ca\(^{2+}\)] at the half-maximum change is 6.33 \times 10^6 \text{ M}^{-1}, which is basically identical with the \(K_a\) value of the high affinity \(a\) sites (3 \times 10^6 \text{ M}^{-1}, Refs. 24 to 26). This suggests that
the increase in the reactivity of $-\text{SH}_2$ reflects the change of enzyme conformation induced by $\text{Ca}^{2+}$ binding to the $\alpha$ sites. Contrary to the increased reactivity of $-\text{SH}_2$ deduced from the inactivation of ATPase, the number of blocked thiol at 20 min decreases as $[\text{Ca}^{2+}]$ increases (Fig. 6a, also see Fig. 1a). This would indicate that upon increasing $[\text{Ca}^{2+}]$, the reactivity with MalNEt of some thiols other than $-\text{SH}_1$, $-\text{SH}_3$, and $S_F$ is reduced.

As a consequence of the fact that blocking of $-\text{SH}_2$ inhibits the phosphoenzyme decomposition step, but not the phosphoenzyme formation step, $E_{\text{P}_{\text{M}}}$ is rather increased during the reduction of $v$ as described above. Therefore, the $[\text{Ca}^{2+}]$ dependence of $E_{\text{P}_{\text{M}}}$ on blocking of $-\text{SH}_2$ is exactly the opposite of that of $v$ (Fig. 6b). On the other hand, reduction of $E_{\text{P}_{\text{C}}}$ which reflects the reactivity of class $S_F$ shows no $\text{Ca}^{2+}$ dependence in the range of $8 > \text{pCa} > 2$ (Fig. 6b).

Factors Affecting $[\text{Ca}^{2+}]$-Dependent Thiol Reactivity—We have studied the effects of various concentrations of $\text{MgCl}_2$ and $\text{KCl}$ on the extent of thiol blockage and the inhibition of ATPase activity (Figs. 7 and 8). Although upon increasing the concentration of $\text{MgCl}_2$ the ATPase inhibition is somewhat increased at both $\text{pCa}$ 5.0 and 8.3 (Fig. 7a), the ratio of the extent of ATPase inhibition to the amount of bound MalNEt, or the specific reactivity of $-\text{SH}_2$ is virtually independent of $\text{MgCl}_2$ concentrations. Fig. 8, $a$ and $b$ shows the results of similar experiments with $\text{KCl}$. Again, the specific reactivity of $-\text{SH}_2$ is basically independent of the $\text{KCl}$ concentrations (Fig. 7b). This indicates that $[\text{Ca}^{2+}]$-dependent reactivity of $-\text{SH}_2$ is not influenced by the other ions.

In many of the previous studies on thiol reaction with MalNEt, blocking of the thiol has been carried out at higher pH (e.g., pH 8.5, Refs. 3 and 4; pH 7.5, Ref. 11). The same type of experiment as shown in Fig. 1 was carried out at pH 8.5 at two different concentrations of $\text{Ca}^{2+}$, $\text{pCa}$ 9.0 and $\text{pCa}$ 5.0. The effect of $[\text{Ca}^{2+}]$ on $-\text{SH}_2$, viz. the increased selectivity of MalNEt incorporation produced by higher $[\text{Ca}^{2+}]$, was qualitatively the same as at pH 7.0.

**DISCUSSION**

An earlier report by Hasselbach and Seraydarian (3) that blocking 4 out of approximately 10 reactive thiols per $10^9$ g of SR protein with MalNEt results in complete inhibition of the $\text{Ca}^{2+}$-activated ATPase and $\text{Ca}^{2+}$ transport has stimulated many workers to further investigate the functionally important thiols. Attempts have been made to label one essential thiol with the $[^{14}\text{C}]$MalNEt after blocking “nonessential”
Thiols Involved in Enzyme Reaction of SR-ATPase

Fig. 4. Semilogarithmic plots of the data of the experiment shown in Fig. 3. a, plots of ln ((v)/[EPM0]) versus t. b, plots of ln([EPM]0/[EPM0]) versus t. [EPM0] and [EPMt] are the steady state levels of phosphoenzyme determined at low [Ca2+] and high [Mg2+] of the samples treated with MalNEt for the time t and without treatment, respectively. [EPM]0 and [EPMt] are the steady state levels of phosphoenzyme determined at high [Ca2+] and low [Mg2+] of the samples treated with MalNEt for the time t and without treatment. respectively. vi and v0 are the rates of Pi liberation following the treatment with MalNEt at pCa 8.3 and [Ca2+]t and [Mg2+]o are the steady state levels of phosphoenzyme determined at high [Ca2+] and low [Mg2+] of the samples treated with MalNEt for the time t and without treatment, respectively. ME, MalNEt treatment at pCa 8.3; O, MalNEt treatment at pCa 5.0.

Fig. 5. Relationship between the inhibition of ATPase activity and the number of blocked thiols. The data of the experiment shown in Fig. 1 were replotted. Key: O, MalNEt treatment at pCa 8.3; O, MalNEt treatment at pCa 5.0.

Table I

| pCa | vi | kP | EPc | v | kP | EPc |
|-----|----|----|-----|----|----|-----|
| 8.3 | 0.034 | 0.016 | 0.001 | 0.034 | 0.125 | 0.011 |
| 5.0 | 0.034 | 0.116 | 0.011 | 0.034 | 0.125 | 0.011 |

The first order rate constants of MalNEt reaction with two thiol classes of purified Ca2+-ATPase of SR as determined from the process of enzyme inactivation.

Data were calculated from the semilogarithmic plots shown in Figs. 1d and 5a and b. Three parameters of enzyme activity were used for the estimation of the rate of reaction with MalNEt: vi, the rate of ATP hydrolysis; kP, the rate of phosphoenzyme decomposition; EPc, phosphoenzyme level with the prevented decomposition. For details, see text.

The analysis of the process of thiol blocking with MalNEt at various [Ca2+] has allowed us in this study to distinguish several types of thiols (viz. −SH1, −SH2, and class Sr) even under conditions when MalNEt blocks less than three thiols per 105 daltons of purified ATPase protein. Blocking of −SH1 (~1/106 daltons) has no effect on ATPase. Blocking of −SH2 and class Sr produces inhibition of different steps of the ATPase reaction, viz. phosphoenzyme decomposition and phosphoenzyme formation, respectively. Upon increasing [Ca2+] from 10^-9 M to 10^-4 M, the extent of rapid inhibition of ATPase activity assessed from the extrapolation of the logarthmic plot of v (A1/A0 of legend to Fig. 1) increases from 0 to the maximum value of 0.6 to 0.7. This can be explained by the assumption that as [Ca2+] increases a larger fraction of −SH2 becomes highly reactive. The reactivity of class Sr with MalNEt is independent of [Ca2+] and is approximately the same as that of −SH2 in a less reactive form. Thus, analysis is made on the plot of ln(kP) versus t at pCa > −SH2 and class Sr are kinetically indistinguishable and the plot can be fitted by a single line. At pCa ≥ 6, −SH2 appears as a fast kinetic phase. Since class Sr is crucial for phosphoenzyme formation but −SH2 is not, the slope of the plot of ln(EPC) versus t is independent of [Ca2+] and is roughly identical with that of ln(kP) versus t plots at low [Ca2+].

Although it seems clear that −SH1 and −SH2 are approximately 1 per 10^5 daltons of ATPase peptide, respectively, the present study does not permit us to decide the number of thiols in class Sr. It appears that the thiol reactivity is considerably different depending upon the type of reagent. In order to favor the selective blockage of class Sr, a spin-labeled derivative of iodoacetamide reacts with the SR-ATPase at a faster rate at lower [Ca2+] (10^-4 M) than at higher [Ca2+] (10^-9 M). Furthermore, incorporation of the iodoacetamide derivative produces no
**Fig. 6.** Ca\(^{2+}\) dependence of the number of blocked thiols (a), inhibition of ATPase activity (b), and inhibition of phosphoenzyme formation (c). a, the reaction of 10 \(\mu\)M purified ATPase with 1 mM \(^{14}C\)MalNEt was carried out for 20 min at various Ca\(^{2+}\) concentrations as described under “Experimental Procedures.” b, thiol modification was done with nonradioactive \(^{14}C\)MalNEt, and the reaction was stopped by dithiothreitol as described in the legend to Fig. 3. The two types of phosphoenzyme measurements, viz., \([EP_c]\) and \([EP_m]\), were done as described under “Experimental Procedures.” For definition of \([EP_c]\) and \([EP_m]\), see text. Key: a, ATPase activity after MalNEt incorporation; b, ATPase activity of the control sample that was incubated in the modification solution devoid of MalNEt; c, blocked thiols; d, \([EP_m]\); e, \([EP_c]\).

**Fig. 7.** Effect of MgCl\(_2\) concentration on the reactivity of \(-\text{SH}_2\). The purified ATPase was allowed to react with MalNEt for 15 min as described in the legend to Fig. 1 except that the concentration of MgCl\(_2\) was as indicated. ATPase activity and blocked thiols were determined as described in the legend to Fig. 1 after adjusting MgCl\(_2\) to 5 mM. Key: solid symbols, MalNEt treatment at pCa 5.0; open symbols, MalNEt treatment at pCa 8.3.

inhibition of enzyme activity (18, 20, 21). This suggests that iodoacetamide derivatives would react preferably with the fourth type of thiol described above.

The Ca\(^{2+}\) affinity of the enzyme estimated from the plot of the extent of ATPase inhibition versus pCa, viz., \(6 \times 10^6\) M\(^{-1}\), is essentially the same as the binding constant of the high affinity \(\alpha\) sites \(K_A \approx 3 \times 10^6\) M\(^{-1}\). This would indicate that the [Ca\(^{2+}\)]-dependent increase in the reactivity of \(-\text{SH}_2\) with MalNEt reflects the conformational change of the enzyme molecule produced by the binding of Ca\(^{2+}\) to the \(\alpha\) sites. This supports the view already alluded to that the Ca\(^{2+}\) binding to the \(\alpha\) sites produces a conformational change in the enzyme molecule, which in turn regulates the ATPase reaction (6, 21, 22, 32, 33). The lack of change in the reactivity of \(-\text{SH}_2\) with MalNEt in the [Ca\(^{2+}\)] range in which Ca\(^{2+}\) binding to the low affinity \(\gamma\) sites takes place contrasts with the behavior of other molecular parameters. In addition to the previously described inhibition of ATPase activity, according to our recent results,\(^2\) the rotational motion of the ATPase protein, as determined by the saturation transfer EPR spectroscopy of a spin-labeled derivative of MalNEt attached to protein, decreases in the low affinity [Ca\(^{2+}\)] range. This suggests that some changes of enzyme molecule are taking place in the low affinity [Ca\(^{2+}\)] range, but they do not reflect on the reactivity of \(-\text{SH}_2\) with MalNEt.

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Distinction of thiols involved in the specific reaction steps of the Ca2+-ATPase of the sarcoplasmic reticulum.
S Yamada and N Ikemoto

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