The fluorescent reagent, S-mercuric N-dansyl-cysteine, reacts specifically with thiols of the purified Ca\(^{2+}\)-ATPase of the sarcoplasmic reticulum, producing an increase of fluorescence intensity at 500 nm (Amax = 335 nm). The reaction is stoichiometric, and the increase of the fluorescence intensity is proportional to the number of blocked thiols. Twelve reactive thiols per 10^5 daltons of ATPase peptide fall into roughly three classes. Blocking of the most reactive thiol entails little inhibition of enzyme activity. Blocking of the five thiols reacting next (intermediate class) results in almost complete inhibition of both phosphorylated intermediate formation and ATP hydrolysis. The second order rate constants of the reaction of thiols have been determined by stopped flow studies. The most reactive thiol and the six least reactive thiols can each be treated as a single class with respect to the rate constant; five thiols of intermediate reactivity appear to have different rate constants (k2, k3, ..., k6). Of these constants, k1, corresponding to the most reactive thiol, does not change with [Ca\(^{2+}\)]. Upon increasing [Ca\(^{2+}\)] from 10^{-6} to 10^{-5} M, k2 increases and k11 decreases; the changes roughly parallel the activation of ATPase activity and the Ca\(^{2+}\) binding to the high affinity \(\alpha\) sites (Ikemoto, N. (1975) J. Biol. Chem. 250, 7219-7224). Upon further increase of [Ca\(^{2+}\)] k1 decreases and k11 increases, in parallel with the inhibition of ATPase activity and with the Ca\(^{2+}\) binding to the low affinity \(\gamma\) sites.

Important functions of the sarcoplasmic reticulum, e.g. the formation of the phosphorylated enzyme intermediate (1-5), hydrolysis of ATP (6, 7), and the rate of Ca\(^{2+}\) uptake (6, 8, 9), are all activated in the same range of the Ca\(^{2+}\) concentration (half-activation 1 to 5 x 10^{-7} M). In contrast, higher concentrations of Ca\(^{2+}\) (~mm), which presumably prevail inside the sarcoplasmic reticulum in the steady state of Ca\(^{2+}\) transport (10, 11), inhibit both Ca\(^{2+}\) uptake and ATP hydrolysis (12), but not formation of phosphoenzyme (4, 13).

Our recent studies of the SR\(^{1}\) (14, 15) have permitted assignment of various roles in the regulation of enzyme activity to several types of Ca\(^{2+}\) binding sites located on the purified ATPase enzyme. Binding of Ca\(^{2+}\) to the high affinity \(\alpha\) sites (K\(_{a}\) = 3 x 10^6 M\(^{-1}\)) activates the enzyme reaction, while the binding to the low affinity \(\gamma\) sites (K\(_{\gamma}\) = 1 x 10^2 M\(^{-1}\)) inhibits it. Recent reports have suggested that Ca\(^{2+}\) binding to the high affinity sites would induce some changes of conformation of the SR ATPase. According to the work of Murphy (16, 17) both the thiol reactivity with Nbs and the intrinsic tryptophan fluorescence (cf. Dupont, Ref. 18) are increased by Ca\(^{2+}\) (half-activation ~10^{-6} M). Champeil et al. (19, 20) have shown that the mobility of a spin-labeled derivative of iodoacetamide attached to the SR membrane is increased in the range of Ca\(^{2+}\) concentration in which the activation of phosphorylation takes place, and that in the same [Ca\(^{2+}\)] range thiol reactivity with reagent decreases.

This study was initiated in attempts to reveal details of the mechanism by which the binding of Ca\(^{2+}\) to the high affinity \(\alpha\) sites and to the low affinity \(\gamma\) sites produce activation and inhibition of the enzyme, respectively. The use of fluorescent thiol reagent, S-mercuric N-dansyl-cysteine (Dns-Cys-Hg) (21), in the stopped flow studies on the purified ATPase of SR, has permitted us to distinguish several classes of thiols whose kinetic constants vary with [Ca\(^{2+}\)]. The results show here suggest that the [Ca\(^{2+}\)]-dependent changes of the thiol reactivity reflect changes in the enzyme conformation controlled by the Ca\(^{2+}\) binding, which may have an important bearing on the mechanism by which the enzyme activity is regulated by [Ca\(^{2+}\)].

**EXPERIMENTAL PROCEDURES**

**Preparation of the Purified Enzyme**—Fragmented SR was prepared from rabbit skeletal fast-twitch (white) muscles (22). The Ca\(^{2+}\)-ATPase was purified from the fragmented SR solubilized with Triton X-100 using the method described previously (15) except that 7.2 mM \(\beta\)-mercaptoethanol was used rather than thiourea during solubilization of the membrane. The column-purified ATPase was sedimented at 78,500 x g for 60 min, and homogenized in 0.3 M sucrose, 20 mM Tris/maleate (pH 7.0), final protein concentration 10 mg/ml. The homogenate was lyophilized to remove the last trace of \(\beta\)-mercaptoethanol, and stored in a freezer. Before use, the lyophilized preparation was homogenized in deionized water of the same volume as the original solution in a tight fitting glass hand homogenizer at 0°C. During storage for 2 months at least 90% of the ATPase activity remained.

**Preparation of Dns-Cys-Hg**—Dns-Cys-Hg was prepared according to the method of Leavis and Lehrer (21) with the modification that HgCl and HCl rather than HgNO\(_3\) and HNO\(_3\) were used in the reaction with N-dansyl-cysteine in the presence of 10 mM HCl. Dns-Cys-Hg solutions, 1.0 to 2.5 mM in 10 mM HCl, were stored at 4°C. We noticed that these solutions became turbid but cleared up upon dropwise addition of 1M Tris base. Complete dissolution of Dns-Cys-Hg aggregates was essential for the thiol-specific reactivity of the reagent, without this procedure Dns-Cys-Hg aggregates seemed to be bound nonspecifically in hydrophobic domains of the enzyme preparation.
Titration of Free Thiols—To determine the number of free thiols of the purified Ca"+ -ATPase, a 3-ml solution containing 5 μM purified ATPase, 0.1 M KCl, 1 mM EDTA, 1 mM Nbs (100 μM Tris-Cl, pH 8.0) was incubated at 22°C for 30 min. The enzyme, being present in the form of vesicles, was sedimented at 105,000 × g for 45 min. A125 of the supernatant fraction was determined, and the concentration of free thiol was calculated using ϵ = 1.36 × 10^4 M^{-1} cm^{-1} (23). The number of thiols is given in this paper in terms of 10^7 daltons of protein (24-26). For the determination of free thiols after Dns-Cys-Hg incorporation, a 1-ml portion of the reaction mixture (see legend to Fig. 1) was mixed with a 1-ml solution containing 2 mM EDTA, 0.32% SDS, 0.4 mM Nbs, 200 mM Tris-Cl, pH 8.0. After incubation for 60 min at 22°C, A128 was determined.

Stopped Flow Studies—The reaction of Dns-Cys-Hg with the purified ATPase was studied in a Durrum stopped flow apparatus equipped with a Jarrell Ash 0.25-m excitation monochromator under the conditions described in the figure legends. Increase of the dansyl fluorescence (λex = 500 nm, λem = 335 nm) resulting from binding of Dns-Cys-Hg to thiols was monitored with a photomultiplier through a cutoff filter which excluded light below 430 nm. The oscilloscopic trace was triggered at the time of start of the flow actuator, which occurred normally 10 to 12 ms before the time of stopping the flow. During the initial 10 ms the fluorescence decreased, presumably due to dilution of the Dns-Cys-Hg solution upon mixing; at about the time when the flow stopped the fluorescence intensity showed the lowest level (cf. Fig. 4A), which was taken as the fluorescence at the zero reaction time.

The stoichiometric titration shown in Fig. 1 was carried out by obtaining uncorrected fluorescence emission spectra with a Perkin-Elmer fluorescence spectrophotometer MPF-4 with the use of a 1-cm cell at 22°C. The protein concentration was determined according to the method of Lowry et al. (27) with bovine serum albumin as a standard.

Method of Estimating Number and Kinetic Constants of Thiols—The differential equation of second order reaction of a single class of equivalent sites (s) reacting with a reagent (p), rate constant k, producing a product x(t), can be written as dx/dt = k(s - x) - p - x), or in its integrated form,

\[(s - p) \ln \left(\frac{(1 - x/s)}{(1 - x/p)}\right) = kt = f(t)\]

(1)

If there are n classes of sites, each of which has s members, characterized by rate constants \(k_i\) (i = 1, 2, ..., j, ..., n), substituting \(x = \Sigma_j s_j\) and \(s = \Sigma_j s_j\) into Equation 1 above and differentiating, one sees that the slope of the \(f(t) - df(t)/dt\) plot, provided \(p \geq \Sigma_j s_j\) and if \(p \leq \Sigma_j s_j\) the final slope \(df(t)/dt\), \(t \geq k_s\), with time will vary with \(p\). On substitution \(dx/dt = k_s (s - x) - p - x\), one obtains

\[df(t)/dt = \Sigma_j k_j (s_j - x_j) \frac{s_j}{s} (s - x)\]

(2)

If the rate constants differ considerably, e.g. \(k_1 > k_2 > ... > k_n\), the reagent will react with the classes in a sequential fashion starting from class 1. In this case,

\[dx/dt)_{\text{init}} = k_s p\]

(3)

\(k_s\) can be determined from the initial slope of the \(x\) versus \(t\) plot knowing the values of \(s\) and \(p\). When all classes but class \(i\) have reacted with reagent, namely \((s - x) = 0\), i.e. \(i = 1, 2, ..., j, ..., n\), the final slope \(df(t)/dt\), \(t \geq k_i\), can be obtained from the final slope of the \(f(t) - df(t)/dt\) plot, provided \(p \geq \Sigma_j s_j\) and if \(p \leq \Sigma_j s_j\), the final slope \(df(t)/dt\), \(t \geq k_i\), the rate constant of the \(j\)th class can be determined in the following way. At the time when the reagent has reacted with the 1st through the \((j - 1)\)th classes, namely \((s - x) = 0\), i.e. \(j = 1, 2, ..., x_0, ..., x_{j-1}\), are nearly zero. The \(df(t)/dt\) value at this time, defined as \([df(t)/dt]_{j-1}\), can be written from Equation 2, as

\[df(t)/dt = \Sigma_j k_j s_j k_i / s_i\]

Therefore, \(k_i\) can be determined from the \(df(t)/dt\) values when \((j - 1)\)th and \(j\)th classes have reacted knowing the \(s\) value of each class.

RESULTS

Reaction of Dns-Cys-Hg with Thiols—Upon addition of Dns-Cys-Hg that shows the emission maximum at 555 nm (λex = 335 nm) to the purified Ca"+ -ATPase of SR there is an increase in fluorescence with a shift of the emission maximum to 500 nm. Fig. 1 depicts the results of adding increasing amounts of the reagent at [Ca"+] = 1.5 × 10^{-3} M; the emission spectra were recorded after the emission intensity became maximal (30 s to 3 min). Upon addition of dithiothreitol a spectrum characterized by an emission maximum at 555 nm and having a much lower fluorescence intensity, which is basically identical with that of free Dns-Cys-Hg and having a much lower fluorescence intensity, which is basically identical with that of free Dns-Cys-Hg and having a much lower fluorescence intensity, which is basically identical with that of free Dns-Cys-Hg at the same concentration with neither protein nor dithiothreitol, is recorded. This suggests that dithiothreitol causes detachment of the probe from the enzyme.

The fluorescence intensity of the probe reacted with the enzyme increases in proportion to the amount of added probe up to 12 mol/10^7. Upon further additions the increase of the fluorescence intensity becomes small and roughly parallels the curve for the free probe. The number of free thiols that had not reacted with Dns-Cys-Hg was determined by Nbs in the presence of SDS (Fig. 2). With no Dns-Cys-Hg but with SDS present 16 thiols are titrated per 10^7 daltons protein. The number of free thiols decreases linearly with the amount of probe added and levels off at 4 mol of thiols/10^7 g enzyme. In the native conformation of the enzyme, namely in the absence of SDS, approximately 12 thiols are titrated with Nbs. These results indicate that the 12 thiols are reactive with Dns-Cys-Hg and react stoichiometrically with the reagent.

The same type of experiment shown in Figs. 1 and 2 was...
carried out also at a lower and a higher concentration of Ca^{2+}, namely 10^{-9} and 10^{-2} M. Regardless of [Ca^{2+}] the number of reactive thiols was 12 and the reaction was stoichiometric. Furthermore, the high fluorescence intensity at 500 nm characteristic of the protein-attached Dns-Cys-Hg is proportional

**Fig. 2.** The relation between the increase of fluorescence intensity and the number of thiols blocked with Dns-Cys-Hg. The emission intensity at 500 nm in Fig. 1 was plotted against the amount of Dns-Cys-Hg added. For the determination of the extent of thiol blockage by Dns-Cys-Hg, a 1-ml sample treated with various amounts of Dns-Cys-Hg as described in the legend to Fig. 1 was mixed with a 1-ml solution containing 2 mM EDTA, 0.32% SDS, 0.4 mM Nbsl, 200 mM Tris-HCl, pH 8.0. After incubation for 60 min at 22°C, AdP was determined (23). ○, fluorescence intensity (λ_em = 500 nm, λ_ex = 335 nm) of the probe attached to protein; ●, that (λ_em = 500 nm, λ_ex = 335 nm) of the probe dissociated from protein by addition of 20 mM dithiothreitol; □, number of thiols available to Nbsl in the presence of SDS. For details see text.

**Fig. 3.** Inhibition of ATPase activity and phosphoenzyme formation by Dns-Cys-Hg. Various amounts of Dns-Cys-Hg, in the ratios indicated on the abscissa, reacted with 10 μM purified SR Ca^{2+}-ATPase in a 1-ml solution of 0.3 M sucrose, 0.1 M KCl, 5 mM MgCl_{2}, 1 mM EGTA, 0.9 mM CaCl_{2} (free [Ca^{2+}] = 1.5 × 10^{-6} M), 0.1 M Tris/maleate, pH 7.0, for 10 min at 22°C. For the assay of ATPase activity, a 0.1-ml fraction of the Dns-Cys-Hg/enzyme mixture was diluted with a 1.0-ml solution containing 0.1 M KCl, 5 mM MgCl_{2}, 5 mM ATP, 1 mM EGTA, 0.9 mM CaCl_{2}, 0.1 M Tris/maleate, pH 7.0 at 22°C. For the assay of the phosphoenzyme formation, the reaction was started by diluting a 0.1-ml fraction of Dns-Cys-Hg/enzyme mixture with 1 ml of 0.1 M KCl, 0.2 mM MgCl_{2}, 10 mM CaCl_{2}, 10 μM [γ-^{32}P]ATP, 0.1 M Tris/maleate, pH 7.0 at 0°C. After incubation for 2 min, the reaction was stopped by adding 6.7% trichloroacetic acid, and the filters retaining ^{32}P-labeled enzyme were dried and counted as described previously (15).

**Fig. 4.** Time course of the rapid reaction of thiols of the purified ATPase with Dns-Cys-Hg. A, reaction was started by mixing 2.32 μM purified ATPase with 5.2 μM Dns-Cys-Hg in 0.1 M KCl, 5 mM MgCl_{2}, 1 mM EGTA, 0.9 mM CaCl_{2} (free [Ca^{2+}] = 1.5 × 10^{-6} M), 20 mM Tris/maleate, pH 7.0 at 22°C with the use of stopped flow apparatus. Increase in the fluorescence intensity was monitored as described under "Experimental Procedures." Two sets of time courses were recorded at different time scales: bottom trace, 50 ms/unit of time scale; top trace, 2 s/unit. B, stopped flow reaction of Dns-Cys-Hg with the enzyme was carried out in the same way as above except that various amounts of Dns-Cys-Hg were mixed with the enzyme: numbers on each curve indicate molar concentration of Dns-Cys-Hg added per mol of enzyme. The number of blocked thiols was calculated from the F/F_{o} values determined from the oscilloscopic trace (see text).
to the number of blocked thiols. This excludes the possibility that the presence of EGTA which presumably has a high affinity for mercury might result in various degrees of dissociation between the mercury and cysteine moieties of Dns-Cys-Hg depending upon the proportion of EGTA and calcium.

Effect of Thiol Blocking on the Enzymic Activity—We have studied the effect of blocking of thiols on the phosphoenzyme formation and the rate of ATP hydrolysis at various Dns-Cys-Hg/enzyme ratios at 1.5 x 10^{-6} M Ca^{2+} (Fig. 3). It appears that blocking of the most reactive thiol, approximately one per 10^5 daltons of ATPase protein, has little inhibitory effect on the enzyme activity. On blocking the next five thiols, almost complete inhibition of both the phospho-enzyme formation and ATP hydrolysis takes place. The type of experiment shown in Fig. 3 has also been carried out at 1 x 10^{-9} M Ca^{2+} and 1 x 10^{-7} M Ca^{2+}. The results were basically the same as shown in Fig. 3.

Kinetically Distinguishable Classes of Thiols—The time course of the fluorescence increase during the Dns-Cys-Hg incorporation has been investigated by the stopped flow method. Fig. 4A shows an example of stopped flow trace of the experiment. As concluded from the data in Fig. 2, the reaction of Dns-Cys-Hg with the protein thiols is stoichiometric and the fluorescence intensity is proportional to the number of blocked thiols. Thus, applying the method of calculation outlined, if $F_p$ is the maximum fluorescence for $\mu$mol of Dns-Cys-Hg per 10^5 g, $x = F/F_m$ when $F$ is the fluorescence intensity at the time $t$.

Fig. 4B shows the time course of blocking of thiols with various amounts of added Dns-Cys-Hg. In the initial phase 0.5 to 1.0 thiol is rapidly blocked. In the subsequent phase, blocking proceeds rather slowly, and levels off when all added Dns-Cys-Hg is incorporated. This suggests that approximately one thiol per 10^5 daltons of ATPase protein has considerably higher reactivity with Dns-Cys-Hg than the other 11 thiols. Presumably this is the thiol whose blocking has little effect on phospho-enzyme formation and ATP hydrolysis (cf. Fig. 3).

Fig. 5 shows plots of $(s - p)^{-1} \ln \left(1 - \frac{x}{s}\right)/(1 - \frac{x}{p})$ versus $t$ (cf. Equation 1) of the data shown in Fig. 4. As expected, the slope of the plots varies as a function of time. Following the initial steep phase, the slope (namely $k$) decreases rapidly and approaches the final slope. The final slope becomes smaller at larger values of the probe/enzyme ratio. However, at the probe/enzyme ratio ≥ 6, there is little or no further decrease in the final slope.

As discussed in “Method of Estimating Number and Kinetic Constants of Thiols,” when the reagent has reacted with all thiols but the least reactive class, the final slope, namely $(df/dt)/dt$, is identical with the rate constant of the least reactive class. By this criterion, after blocking six thiols the remaining thiols, namely from the 7th to the 12th thiols, can be treated as a single kinetic class, and the calculated rate constant defined as $k_{4-12}$ is 1.2 x 10^3 M^{-1} s^{-1}.

The relation between the $(df/dt)/dt$ value and the number of blocked thiols is shown in Table I. The $(df/dt)/dt$ value when a certain number of thiols is blocked is basically the same regardless of the amount of probe mixed at the beginning of the reaction. However, as more thiols are blocked, $(df/dt)/dt$ decreases sharply, and upon blocking approximately two thiols it reaches about the value of the final slope with $p ≥ 6$. This would suggest that the rate constants of five thiols, $n = 2, \ldots, 6$, vary in the range from a higher value to about $k_{12}$. The rate constant (M^{-1} s^{-1}) of these thiols has been calculated according to Equation 4 using the data shown in Table I: $k_2 = 1.21 x 10^5$; $k_3, 1.43 x 10^4$. Values of $k_4$ to $k_9$ are rather close to $k_{12}$, and the method of calculation, which deals with kinetic classes having significantly different rate constants, would not permit accurate estimation of $k_4$ to $k_9$ nor of $k_5$, particularly in the case when $k_2$ decreases and $k_{12}$ increases as shown later. For this reason, only the $k_2$ value is used as the constant of an intermediate class in the following analysis.

The results described above suggest that there are several kinetically distinguishable classes of thiols characterized by $k_1$, $k_2$, $k_3$ to $k_6$, and $k_{12}$. However one cannot exclude the possibility that blocking any one or two of the 12 reactive thiols might reduce the rate constant of the others (negative cooperativity).

[Ca^{2+}] Dependence of the Rates of Probe Incorporation—The rate of incorporation into the most reactive thiol ($k_1$) is virtually independent of [Ca^{2+}] (Fig. 6A), whereas $k_2$ (Fig. 6B) and $k_{7-12}$ (Fig. 6C) show appreciably large changes depending upon [Ca^{2+}]. The value of $k_2$ increases in the range of 10^{-10} to 10^{-8} M Ca^{2+} in which the activation of enzyme activity (cf. Refs. 6, 7, 14) and Ca^{2+} binding to the high affinity $\alpha$ sites (14, 15) take place; $k_3$ decreases in the range of [Ca^{2+}] ≥ 10^{-4} M, again parallel with the inhibition of ATPase activity (6, 7, 12, 14) and with Ca^{2+} binding to the low affinity $\gamma$ sites (14, 15). The [Ca^{2+}]-dependent changes of $k_{7-12}$ show a reciprocal relation to those of $k_3$, namely $k_{7-12}$ decreases in the

![Fig. 5. Second order expression of the process of thiol blockage with Dns-Cys-Hg. The data of the same experiment shown in Fig. 4 were calculated as described in the text. Values calculated by using the left side of Equation 1 were plotted as a function of reaction time.](http://www.jbc.org/)

### Table I

<table>
<thead>
<tr>
<th>Dns-Cys-Hg/enzyme</th>
<th>Number of blocked thiols/10^5 daltons</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.17</td>
</tr>
<tr>
<td>2</td>
<td>2.24</td>
</tr>
<tr>
<td>3</td>
<td>3.36</td>
</tr>
<tr>
<td>4</td>
<td>4.48</td>
</tr>
<tr>
<td>5</td>
<td>5.60</td>
</tr>
<tr>
<td>6</td>
<td>6.72</td>
</tr>
</tbody>
</table>

The values of the slope, namely $(df/dt)/dt$, were determined at the times when various numbers of thiols indicated had been blocked. Data were taken from the experiment shown in Figs. 4 and 5. The average value of the data shown in each column is indicated at the bottom line with the standard deviation.
Fluorescent probe, Dns-Cys-Hg (21), has several new advantages over the same emission characteristics regardless of the thiol class of ATPase, however, the protein-associated Dns-Cys-Hg shows that Dns-Cys-Hg-labeled (Na⁺ + K⁺)-ATPase (44).

Dns-Cys-Hg is in a nonpolar domain regardless of the classes of thiol described here. A similar finding has been reported in experiments on SR Ca²⁺-ATPase is stoichiometric, and unlike the reaction with other reagents, in which the number of blocked thiols has to be determined at various times of reaction, the number of thiols to be blocked can be controlled by the amount of reagent mixed with the enzyme. Because of the rapidity of the reaction, the enzyme would be less susceptible to the secondary effects, such as oxidation, compared with the conventional pseudo-first order analysis of thiol reaction with covalently reacting reagents, which normally requires an excess of reagent over the enzyme and rather lengthy times of reaction. Because of the relatively large increase in fluorescence intensity upon incorporation of the probe into the protein, the time course of thiol blocking can be easily monitored. Furthermore, the fact that various thiols of the purified ATPase react at distinctly different rates with the probe, has permitted the blocking of different classes of thiol in a sequential fashion.

According to the work of Leavis and Lehrer (21), the spectral property of the protein-attached Dns-Cys-Hg varies depending upon the environment of the attached probe. Thus, Dns-Cys-Hg may serve as a probe for the studies of microenvironment of protein thiols (21). In the case of SR Ca²⁺-ATPase, however, the protein-associated Dns-Cys-Hg shows the same emission characteristics regardless of the thiol class (cf. Fig. 1). This would indicate that the thiol-attached Dns-Cys-Hg is in a nonpolar domain regardless of the classes of thiol described here. A similar finding has been reported in the Dns-Cys-Hg-labeled (Na⁺ + K⁺)-ATPase (44).

Attempts to classify the thiols in terms of reactivity with various thiol reagents and of their involvement in the ATPase activity have already been made by other workers in studies of the purified ATPase and the sarcoplasmic reticulum membrane (Table II). Assignment of several kinetic classes shown in this study is in reasonable agreement with the previous reports. It appears that blocking the most reactive thiol, characterized by $k_7$, probably identical with the class that reacts rapidly with N-ethylmaleimide and NbE (Table II), has little or no effect on ATPase activity. Upon further blocking of five thiols, both the phosphoenzyme formation and ATP hydrolysis rate are almost completely inhibited; these thiols may be equivalent with the reported intermediate class (Table II). The 7th through 12th thiols characterized by $k_{7-12}$ would correspond with the reported slow class.

We have found that the rates of incorporation of Dns-Cys-Hg into each of these classes are changed as a function of [Ca²⁺] during the incorporation. The changes of $k_2$ and $k_{7-12}$ in the [Ca²⁺] range of 10⁻⁸ to 10⁻⁴ M and at [Ca²⁺] higher than 10⁻⁴ M parallel the Ca²⁺ binding to the α sites ($K_a = 3 \times 10^5 M^{-1}$) and that to the γ sites ($K_{α} = 6 \times 10^5 M^{-1}$), respectively. Thus, it appears that the Ca²⁺-dependent changes of $k_2$ and $k_{7-12}$ reflect the changes in conformation of the enzyme molecule controlled by binding of Ca²⁺ to the two types of sites. The conformational states could be expressed as $α(−) γ(−), α(+) γ(−)$, and $α(+) γ(+)$, which predominate at pH 9.0, 5.7, and 2.7, respectively. The symbols + and − indicate occupied and empty sites, respectively. To facilitate further discussion, values of $k_1$, $k_2$, and $k_{7-12}$ in the three conformational states are tabulated in Table III. The $α(−) γ(−)$ conformation is clearly distinguishable from the $α(+) γ(−)$ and $α(+) γ(+)$, by its higher value of $k_2$ and the lower value of $k_{7-12}$. The $α(−) γ(−)$ and $α(+) γ(−)$ conformations are somewhat similar to each other but there is an appreciable difference, in that the former has a considerably higher value of $k_2$.

The important point in this study is that the three conformational states described above have an intimate relation to the activation and inhibition of the enzyme activity. Upon the addition of ATP, the enzyme in the $α(+) γ(−)$ state carries out a whole cycle of the ATPase reaction, as seen from the activation of both the phosphoenzyme formation and ATP hydrolysis; whereas in the $α(+) γ(+) state the enzyme forms the phosphorylated intermediate but the reaction steps in which phosphoenzyme is decomposed are inhibited, and in...
TABLE II
Thiol classes of purified Ca2+-ATPase and of sarcoplasmic reticulum

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Ref.</th>
</tr>
</thead>
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<tr>
<td>N-Ethylmaleimide</td>
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</tr>
<tr>
<td>Nbs2</td>
<td>31</td>
</tr>
<tr>
<td>Dns-Cys-Hg</td>
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TABLE III
The rates of Dns-Cys-Hg incorporation into thiol classes at three representative states of enzyme

<table>
<thead>
<tr>
<th>Thiol class</th>
<th>pCa</th>
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<td>1</td>
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<tr>
<td>2</td>
<td>5.7</td>
</tr>
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<td>3</td>
<td>2.7</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Fast (k1)</th>
<th>(2.1 ± 0.4) x 10^6 M^-1 s^-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermediate (k2)</td>
<td>(3.4 ± 0.4) x 10^4 M^-1 s^-1</td>
</tr>
<tr>
<td>Slow (k12)</td>
<td>(1.4 ± 0.1) x 10^3 M^-1 s^-1</td>
</tr>
</tbody>
</table>

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N Ikemoto, J F Morgan and S Yamada


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