Ca\textsuperscript{2+}-dependent Effect of ATP on Spin-labeled Sarcoplasmic Reticulum*

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Vesicular fragments of sarcoplasmic reticulum (SR) were labeled with the \(-\)SH-directed spin label 2,2,6,6-tetramethyl,1-amino(N-iodoaceticamide). Colorimetric titrations of the remaining \(-\)SH residues and determinations of unbound spin label indicated that primarily 3 residues/enzyme molecule were labeled under saturating conditions. This labeling was accompanied by minimal losses in activity, providing precautions were taken to prevent sulfhydryl oxidation during the labeling process.

Additions of ATP produced a new “highly constrained” component in the ESR spectrum of the labeled SR, an effect not noted in previous studies. It is demonstrated that the changes produced by ATP are reversible, and require both substrate binding and Ca\textsuperscript{2+} binding. However, hydrolysis of the substrate is not required. It is further demonstrated that the labeled residue(s) responsible for the spectral change is not in the immediate vicinity of the ATP binding site.

It is apparent that the observed spectral change is related to a conformational effect of ATP and Ca\textsuperscript{2+} on the ATPase protein, which is associated with a large free energy change occurring on binding. It is also suggested that the conformational effect extends to a significant distance from the nucleotide binding site and may be a precursory step to Ca\textsuperscript{2+} translocation.

Furthermore, postulation as to the steps in the complex mechanism responsible for the observed change has been inconclusive. It should be pointed out that different labeling conditions were used by the investigators, although all labels were \(-\)SH-directed. Furthermore, in only the more recent work (1, 6) has any attempt been made to determine which or how many residues, or both, can be labeled, or to compare the observed spectral effects directly to specific activity measurements, done under conditions used in obtaining the spectra (1).

With the present work we have demonstrated the occurrence of specific effects related to substrate and Ca\textsuperscript{2+} binding at the active site of the ATPase enzyme which was labeled with an iodoaceticamide spin label. Moreover, we have attempted to quantitatively monitor the labeling process by: (a) following the decrease in signal from the free label as the reaction proceeds, and (b) making use of recently developed procedures for titrating free sulfhydryl residues on SR with DTNB (7). In doing so, we have found that several sulfhydryl residues are quite susceptible to oxidation and, unless proper precautions are taken, inaccurate information is obtained from labeling studies.

EXPERIMENTAL PROCEDURES

Materials - SR vesicles were obtained from the white skeletal muscle of rabbit hind legs, using methods previously described (8). The vesicles, suspended in a buffered sucrose solution (30% sucrose, 10 mM Mops at pH 6.761), were stored at 4° until used. Under these conditions both the ATPase activity and Ca\textsuperscript{2+} pumping activity remain stable for about 1 week. 2,2,6,6-Tetramethyl,1-amino(N-iodoaceticamide) was obtained from Byk-Gulden, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and adenyl-5-yl imidodiphosphate (AMP-PNP) were purchased from Sigma. Both reagents were used without further purification.

Methods - Labeled protein was obtained in the following manner: 1 to 2 mg of iodoaceticamide spin label was dissolved in 2.5 ml of Mops buffer (20 mM Mops, pH 6.8) which had been purged with nitrogen. Similarly, an aliquot of SR suspension containing 15 mg of protein was brought to a volume of 2.5 ml with the buffer, and the two solutions were mixed and allowed to incubate under nitrogen at 24°. After 1 to 2 h, the suspension was spun down at 40,000 x g and resuspended in a small amount of the cold Mops buffer. This process was repeated until no trace of spin label was found in the supernatant (two washings were usually sufficient), at which time both the ATPase activity and the number of remaining free sulfhydryl residues were determined by the methods described below.

The ATPase activity of labeled SR was compared to unlabeled SR directly from the fresh preparations, by following the liberation of orthophosphate after the addition of 5 mM ATP and 0.2 mM CaCl\textsubscript{2} to a dilute vesicle suspension (0.2 mg/ml of SR in 20 mM Mops (pH 6.8),
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0.1% Triton, 80 mm KCl, 5 mm MgCl₂, 0.1 mm EDTA). Serial samples were taken from the reaction mixture during a 15-min incubation (25°), the activity of each sample being terminated by the addition of equal volumes of MOPS, MgCl₂, and ATP, added in accordance with the concentration of SR. Generally, 40 mg of SR were suspended in 400 μl of reaction mixture, with 20-μl aliquots being taken for serial analysis. Unless specified, no Triton was added to the buffer and the concentration of CaCl₂, MgCl₂, and ATP were added in accordance with the concentrations used in obtaining a given spectrum.

During some of these activity studies, 1-μl serial samples were taken from the reaction mixture and spotted on polyethyleneimine-cellulose thin layer chromatography sheets. Chromatographic separations discussed above and shown in Fig. 4, could be maintained for at least two full spectral scans. As long as the Ca²⁺ and Mg²⁺ concentrations were kept above the critical level (Fig. 4), these cations had no effect on the spectra.

RESULTS AND DISCUSSION

Labeling of Sulphydryl Residues — Under maximal labeling conditions, we found an average of 3 mol of bound acetamide spin label/mole of ATPase (assuming a molecular weight of 100,000 for ATPase). The number of labeled sulphydryl residues was determined by two separate methods as discussed above. In initial experiments, a large excess of label (0.2 mg/1.5 mg of SR) was used to ensure complete labeling. Titrations with DTNB indicated that only 3 residues could be labeled. In additional experiments aliquots of label varying from 3 to 10 mol/mole of SR-ATPase were used and, as shown in Table 1, difference measurements of the ESR signal from free label as well as titrations with DTNB again showed that only 3 residues could be labeled.

An excess of iodoacetamide was required to fully label the 3 available sulphydryl residues. The enzyme appeared to be primarily labeled after an incubation of 1 h (24°), and only a small degree of additional labeling was observed after 4 h at 24° overnight under refrigeration.

The rate of ATP hydrolysis of labeled enzyme was always found to be identical to that of unlabeled enzyme treated in the same manner, and this activity was very close to that obtained with the fresh preparation of SR, providing precautions were taken to prevent —SH oxidation. Similarly, the labeled SR exhibited the same Ca²⁺ uptake as the unlabeled.

ESR Spectrum of SR — Selected spectra of SR are shown in Figs. 1 and 2, and are typical of the broad asymmetric lines associated with the anisotropic motion of a spin label covalently bound to a protein residue (13-17). As has been previously observed, the spectra of SR suspended in buffer (Fig. 1a and 2a) exhibit the features of a two-component spectrum, consisting of a “more constrained” and a “more mobile” component (1-5, 18). In this case, however, the major component, represented by the broad outer lines labeled R in the figure, is a good deal less constrained than those observed for SR labeled with the maleimide spin label (4-6), or in the spectrum of SR-ATPase.

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<table>
<thead>
<tr>
<th>Number of residues labeled as function of spin label concentration</th>
<th>Mes AcI&quot;/ SH residues labeled/mol of ATPase</th>
<th>+ATP</th>
<th>+AMP-P′N Hex</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>1.4 ± 0.4</td>
<td>2.3 ± 0.3</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>4.5</td>
<td>2.0 ± 0.3</td>
<td>2.3 ± 0.3</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>6.0</td>
<td>9.5 ± 0.3</td>
<td>7.5 ± 0.2</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>9.0</td>
<td>3.1 ± 0.25</td>
<td>3.2 ± 0.3</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>12.0</td>
<td>3.4 ± 0.3</td>
<td>3.0 ± 0.3</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>15.0</td>
<td>2.8 ± 0.4</td>
<td>3.0 ± 0.3</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>18.0</td>
<td>3.0 ± 0.3</td>
<td>3.0 ± 0.3</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>30.0</td>
<td>3.0 ± 0.3</td>
<td>3.0 ± 0.3</td>
<td>3.0 ± 0.3</td>
</tr>
</tbody>
</table>

* Measured by titrations with DTNB only.

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but does not display a secondary activating effect as described are related to binding of these substrates to the catalytic, for ATP (3, 21), it is apparent that the observed spectral effects for the hydrolytic reaction coupled to Ca\(^{2+}\) transport (19, 20), after an extended period of time, as illustrated in Fig. 1 here. Since acetylphosphate functions as a substrate is reversible, the original spectra (no ATP) always returning to the enzyme is responsible for the extended time required for return of the original spectrum. In fact, identical spectral changes were obtained when ADP was added instead of ATP, even in the presence of hexokinase (255 units/0.1 ml of ADP) and glucose (100 mM), which eliminated ATP produced by the action of myokinase contaminants on ADP. On the other hand, addition of AMP produced no spectral change.

ADP is known to bind to SR-ATPase with a comparatively high binding constant and, since ADP is in excess after hydrolysis of ATP (Fig. 3b), it seems likely that the binding of ADP to the enzyme is responsible for the extended time required for return of the original spectrum. In fact, identical spectral changes were obtained when ADP was added instead of ATP, even in the presence of hexokinase (255 units/0.1 ml of ADP) and glucose (100 mM), which eliminated ATP produced by the action of myokinase contaminants on ADP. On the other hand, addition of AMP produced no spectral change.

As a further demonstration that the observed spectral changes are related to general nucleotide binding, as the ADP binding studies suggest, the effects of AMP-P(NH)P were studied. We found that this ATP'\(^ \gamma \) analogue, which is not hydrolyzed by the SR-ATPase, also produces spectral changes. Binding of Nucleotides — On addition of ATP in the presence of Ca\(^{2+}\) and Mg\(^{2+}\), the ratio between A and B peak heights is clearly changed (Figs. 1b and 2b). This change corresponds to a decrease of component B, due to a shift of part of the signal to a broader component indicated by C. This new component is most clearly seen in spectra with minimal contribution from the mobile component A (Fig. 1b). C is not as well resolved when peak A is more prominent (Fig. 2b) and may be missed altogether in such spectra (2, 3). Independently of the resolution of components, however, we found the ratio of line heights under C and B to be nearly the same in all preparations. Variations in the contribution of component A to the total ESR signal are related to SR preparations and to the labeling procedure, but do not occur on addition of nucleotides.

In all cases, additional evidence for the signal broadening induced by ATP may be obtained in the high field portion of the spectrum indicated by D in the figure. Here the outer lines are clearly broadened and the characteristic dip at the outer spectral extremum is shifted upward. This effect is also independent of the prominence of component A.

It has been established in previous studies (3) that the effect of ATP is produced within 1 s after mixing, and that the effect is reversible, the original spectra (no ATP) always returning after an extended period of time, as illustrated in Fig. 1 here. Subsequently, it was found that an identical effect is obtained when acetylphosphate, rather than ATP, is added to the SR suspension (1). Since acetylphosphate functions as a substrate for the hydrolytic reaction coupled to Ca\(^{2+}\) transport (19, 20), but does not display a secondary activating effect as described for ATP (3, 21), it is apparent that the observed spectral effects are related to binding of these substrates to the catalytic, rather than to a secondary site. This is further demonstrated by the Ca\(^{2+}\) requirement for these effects (see below).

The reversal of the ATP spectral effect was monitored by measuring the change of line height under component B relative to the line height under component A. The choice of these two components is due to the comparatively clear resolution of their peaks. Monitoring was done using both full spectral scans and scans covering only the first portion of the spectrum, to obtain data at 1-min intervals.

The ratio between line heights A and B as a function of time is shown in Fig. 3a. It is apparent that the ratios obtained on addition of ATP revert in 2 to 3 h to ratios observed in the absence of ATP. On comparing these changes in line heights (Fig. 3a) with enzyme activity measurements (Fig. 3b) obtained in reaction mixtures identical to those used for ESR spectrometry, it is clear that the time required for return to the original spectrum is longer than that required for hydrolysis of the ATP \(\gamma\)-phosphate. Rather, the spectrum returns to normal only when the nucleotide is degraded to AMP by virtue of myokinase contaminants present in the SR preparations (note in Fig. 3b the results obtained by chromatographic separation of nucleotides).

Ca\(^{2+}\) Dependency — In the above experiments Ca\(^{2+}\) and Mg\(^{2+}\)
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**FIG. 3.**

**a,** change in the ratio of line heights in ESR spectrum on addition of ATP: \( \Delta R = (A/B)_{\text{spectr}} - (A/B)_{\text{max}} \), where \( A \) and \( B \) are defined in text; \( \circ \), measured from full spectral scans; \( \square \), measured from scan of first lines only; \( \ominus \), SR labeled in the presence of ATP; \( \Delta \), 5% Triton X-100 added to buffer. \( b \), ATP degradation as a function of time; conditions the same as for spectra shown in Fig. 1. Symbols refer to concentrations of: inorganic phosphate produced, \( \bullet \); ATP, \( \Box \); ADP, \( \Delta \); and AMP, \( \ominus \).

were always present in excess amounts. Recently, it has been shown that a similar spectral change occurs on binding of acetylphosphate to SR-ATPase, but only in the presence of Ca\(^{2+} \) (1). In order to test the requirement for Ca\(^{2+} \) and Mg\(^{2+} \) with ATP, ADP, and AMP-P\( \text{N} \text{H} \text{P} \), EDTA (10 mM) was added to SR suspensions to chelate endogenous cations, before the addition of nucleotide. Only a small change in the spectrum was observed in this case with any of the three nucleotides. In the concentrated SR suspensions required here, it is possible that all cations are not chelated, even at high EDTA concentrations, and this could account for the small change observed in these conditions (Fig. 4).

In additional experiments, MgCl\(_2\) was added in increasing amounts to a suspension containing ATP (10 mM EDTA). As shown in Fig. 4, at concentrations of Mg\(^{2+} \geq\) EDTA, some additional spectral broadening occurs, but to a much smaller extent than in the presence of Ca\(^{2+} \).

The Ca\(^{2+} \) sensitivity was also investigated by the addition of CaCl\(_2\) to SR suspensions containing ATP, with EGTA and MgCl\(_2\). The observed spectral change was complete at 10 mM CaCl\(_2\) (see inset in Fig. 4) and since this is approximately equal to the EGTA concentration, on initial analysis it seems likely that binding at the high affinity sites, which have been directly linked to ATP hydrolysis (22, 23), is responsible for the observed spectral change. To further distinguish between potential binding sites, a binding constant was calculated from the Ca\(^{2+} \) concentration required for one-half of the enzyme to undergo spectral conversion. This constant was found to be 4.8 \( \mu \text{M} \) \( (K_b) \), for EGTA was obtained from Ref. 24, and endogenous Ca\(^{2+} \) was estimated to be 30 nmol/mg of SR), which agrees well with the Ca\(^{2+} \) binding constants for the high affinity site obtained by other investigators (23, 26), and differs from that observed for lower affinity sites by an order of magnitude (26).

**Labeling in Presence of ATP**—It has previously been reported that ATP protects at least one --SH residue specifically associated with the enzymatic site of SR-ATPase, and conversely that when this residue is labeled with a sulphydryl reagent, enzymatic inhibition occurs (27, 28). Since labeling with iodoacetamide produces no inhibition, it would follow that this certain residue is not affected. To further investigate this possibility, we labeled several SR suspensions in the presence of 10 mM ATP or AMP-P\( \text{N} \text{H} \text{P} \) (10 mM EGTA, no added Ca\(^{2+} \) or Mg\(^{2+} \)). As shown in Table I, neither nucleotide affected the number of residues labeled, nor did they affect the rate of reaction (as determined by the rate of decrease of free ESR signal). Furthermore, spectra of samples in the presence and in the absence of ATP are virtually identical and undergo identical changes upon the addition of nucleotides and Ca\(^{2+} \) (See Fig. 3a).

**Triton X-100**—In some cases varying amounts of Triton X-100 were added to the reaction mixture. At concentrations up to 5%, Triton had no effect on the ESR spectrum, the spectral conversion following the normal pattern (See Fig. 3a). Small amounts of Triton have the general effect of enhancing ATP hydrolysis and it is thought that the detergent solvates lipids in the membrane and to some degree solvates the ATPase molecule. In any case, the disruption of the ATPase membrane system does not effect the spectral change observed here, indicating that the spectra reflect protein conformation rather than membrane structure.

At concentrations greater than 5% Triton the ESR spectrum began to show signs of protein denaturation (31, accompanied by a loss of activity, as determined by concurrent ATP hydrolysis studies.

**CONCLUSION**

The addition of ATP to suspensions of spin-labeled SR has been shown to cause changes in the ESR spectrum, provided Ca\(^{2+} \) is present in the medium. Two other nucleotides, ADP and AMP-P\( \text{N} \text{H} \text{P} \), which are bound but are not hydrolyzed by
the ATPase, produce almost identical spectral effects, which are also Ca\(^{2+}\)-dependent. Care was taken in the latter cases to ensure that hydrolysis of trinucleotides by SR did not occur under conditions used in obtaining the spectra. ADP was combined with a hexokinase-glucose system designed to eliminate any ATP formed from ADP by the action of myokinase contaminants. AMP-P(NH)P-SR suspensions were carefully checked for evidence of hydrolysis. In addition, no spectral conversion, indicating utilization of the nucleotide, occurred after extended periods of time with AMP-P(NH)P suspensions. These experiments definitely indicate that a conformational change occurs in the membrane-bound ATPase, which requires both binding at the ATP site and Ca\(^{2+}\) sites. They also resolve apparent discrepancies between the work of previous investigators, i.e. the observed conformational change requires both nucleotide (3) and the presence of Ca\(^{2+}\) (4).

It is difficult to determine the exact nature of the observed spectral change. An apparent broadening of the constrained component seems due to the appearance of a more constrained component on the shoulder of component B; however, the peak maximum of the new component cannot be resolved. The location of B itself does not appear to change. Unfortunately, unless the spectral components can be resolved, it is difficult to determine the number of labeled residues contributing to each component. Measurements of the degree of labeling only indicate the total number of labeled residues per mol of protein. Since the labeling was done under saturating conditions, however, it is reasonable to assume that the label is primarily confined to 3 accessible residues. These residues would most likely be represented by component B of the spectrum, the much smaller A being attributed to secondary labeling of less reactive sites. It would appear, then, that on the binding of ATP in the presence of Ca\(^{2+}\), the conformation of the enzyme changes in such a manner as to restrict the mobility of 1 or 2 labeled residues (appearance of C) while the rest remain unchanged (retention of B).

The studies with enzyme labeled in the presence of ATP binding site. Therefore, the observed spectral effects reflect conformational changes extending to a significant distance from the effector (nucleotide) site. In this respect it is interesting to note that low temperature studies have recently shown a connection between Ca\(^{2+}\) binding in the absence of ATP and the constrained component of the ESR spectrum (18). It is possible, therefore, that the residues of interest here reflect changes in the vicinity of the Ca\(^{2+}\) binding site concomitant with substrate binding. In fact some evidence of an interaction between Ca\(^{2+}\) and nucleotide binding already exists, in that enzyme activation displays different Ca\(^{2+}\) requirements depending on whether ATP or other substrates (e.g. acetylphosphate) are used (19, 20).

Alignment of the observed conformational changes with the enzyme mechanism is more straightforward. Several studies on steady and transient state behavior (21, 29-31) have shown that the mechanism of Ca\(^{2+}\)-dependent ATPase can be separated at least into the following basic steps:

\[
\begin{align*}
2 \text{Ca}^{2+} + S + E & \rightarrow \text{Ca}_2E + S \quad (1) \\
\text{Ca}_2E + S & \rightarrow \text{Ca}_4E - P + \text{ADP} \quad (2) \\
\text{Ca}_4E - P & \rightarrow \text{Ca}_2 + E + \text{P} \quad (3) \\
\text{E} + \text{P} & \rightarrow \text{E} \cdot \text{P} \quad (4)
\end{align*}
\]

The first step of the reaction mechanism involves binding of the substrate (ATP-Mg) and activator (Ca\(^{2+}\)) to the enzyme on the membrane outer surface. Step 2 corresponds to the transfer of the ATP terminal phosphate to an aspartyl residue (32, 33) of the ATPase protein. This transfer is Ca\(^{2+}\)-dependent (34) and the resulting phosphorylated protein (E-P) is a "high energy" intermediate.

Release of Ca\(^{2+}\) from the transport site of the ATPase protein (Step 3) involves a reduction in affinity of this site for Ca\(^{2+}\), which has been shown to follow enzyme phosphorylation (35, 36). It is apparent that Step 3 includes translocation and release of Ca\(^{2+}\) on the inner side of the membrane to account for the vectorial properties of transport. In addition, since the Ca\(^{2+}\) is released against a concentration gradient, this step must involve free energy expenditure and decay of \(E \cdot P\) to a lower energy level (E-P). Subsequently, hydrolytic cleavage of orthophosphate occurs (Step 4) and the enzyme is allowed to regain its high Ca\(^{2+}\) affinity state (E).

Our experiments indicate that the enzyme protein undergoes a conformational change upon binding both substrate and activator (Step 1). It is important to note that hydrolysis of the substrate is not necessary for occurrence of the conformational change. Hence, binding of the AMP-P(NH)P and ADP can also produce this change. Furthermore, the change is not related to specific steric effect of the adenosine moiety, since acetylphosphate, a substrate for the Ca\(^{2+}\)ATPase, is also effective (31) for the sequential steps of the ATPase.
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reaction, indicate a high free energy change on substrate binding to the enzyme (in fact much larger than that for $E \rightarrow P$ formation). Therefore, it is apparent that the conformational effect produced not only by ATP, but also by nucleotides which are not hydrolyzed (e.g. AMP-P(NH)P), is associated with the large free energy change which occurs on binding. It is tempting to speculate that the conformation of the complex permits Ca$^{2+}$ translocation which is coupled to subsequent transfer and hydrolysis of the ATP terminal phosphate.

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