HETEROGENEITY OF SH GROUPS IN SARCOPLASMIC RETICULUM

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SUMMARY: The incorporation of $^{14}C$ N-ethylmaleimide reveals fast and slow-reacting sulfhydryl groups in sarcoplasmic reticulum. Two proteins react with the label: a fast-reacting glycoprotein recently isolated (Ikemoto, Cucchiaro and Garcia (1976) J. Cell Biol. 70, 290a), and the Ca$^{2+}$-ATPase. Labeling sarcoplasmic reticulum with a maleimide spin label gives a similar pattern. The spectra of maleimide-spin-labeled sarcoplasmic reticulum have both 'strongly' and 'weakly' immobilized components. Maleimide-spin-labeled purified Ca$^{2+}$-ATPase, or sarcoplasmic reticulum labeled first with N-ethylmaleimide, and then with maleimide spin label, show spectra devoid of the 'weakly' immobilized component; the latter is enhanced in partially purified glycoprotein obtained from spin-labeled sarcoplasmic reticulum. This indicates that spectra from maleimide-spin-labeled sarcoplasmic reticulum do not reflect exclusively the state of the Ca$^{2+}$-ATPase enzyme.

Covalent labeling of the sulfhydryl groups present in fragmented SR with paramagnetic analogs of maleimide and iodoacetamide has been widely used to study structural changes in the Ca$^{2+}$-ATPase enzyme. Spectral changes produced by varying the temperature (1), the concentration of Ca$^{2+}$ (2), and the concentrations of ATP and ADP (3-8) have been reported. The underlying assumption behind this type of experiment is that only the Ca$^{2+}$-ATPase enzyme, which is the predominant protein component of SR, reacts with the spin label. The observed spectral changes are then interpreted as being a reflection of structural changes in the enzyme. We have obtained evidence for the presence of a highly reactive thiol group in a minor glycoprotein component (9) of the SR vesicles, labeling of which presumably contributes significantly to the spectra of SR labeled with a maleimide spin label.

MATERIALS AND METHODS

SR vesicles were obtained from rabbit white muscle (10). The Ca$^{2+}$-ATPase

1Abbreviations used: SR, sarcoplasmic reticulum; Mal NEt, N-ethylmaleimide.
enzyme was purified from a Triton X-100 extract of the SR vesicles as described previously (11). To label SR with \(^{14}C\) Mal NEt, the vesicles (10 mg/ml) were incubated for variable lengths of time with 6.2 mol \(^{14}C\) Mal NEt per 10\(^5\) g of protein, in a solution containing 0.3M sucrose, 20 mM Tris maleate, pH 7.0, at 0\(^\circ\)C. The reaction was terminated by dilution with 30 vol of ice-cold 0.3M sucrose, 20 mM Tris maleate, pH 7.0 and the vesicles were sedimented at 100,000 x g by centrifugation for one hour. The pellets were rinsed with this same solution and resuspended to a protein concentration of 10-20 mg/ml.

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The labeled SR vesicles were solubilized with Triton X-100 as described elsewhere (11), using a weight ratio of 1.5 mg of Triton per mg of protein. The labeled SR vesicles, the supernatant obtained after Triton treatment (containing most of the ATPase enzyme), and the Triton-insoluble fraction (enriched in the glycoprotein) were further fractionated by electrophoresis on SDS-containing 10% polyacrylamide gels (12). To measure the extent of \(^{14}C\) Mal NEt incorporation into the ATPase and the glycoprotein, the gels were sliced in 2 mm fractions. Each gel slice was incubated for 4 hours at 50\(^\circ\)C in tightly sealed scintillation vials containing 0.87 ml of NCS tissue solubilizer (Nuclear Chicago) plus 0.13 ml of water. After cooling, 15 ml of toluene scintillation solution was added. The degree of \(^{14}C\) Mal NEt incorporation into each protein component was expressed as the ratio of the counts associated with the protein peak divided by the peak area, assuming that the intensity of the Coomassie blue stain is proportional to the amount of protein.

EPR spectra of the maleimide-spin-labeled SR, and of the resulting Triton-soluble and Triton-insoluble fractions, were recorded with a Varian E-109E spectrometer. The modulation amplitude was 2 gauss, the microwave power setting was 20 mW, and the temperature was maintained at 4\(^\circ\)C with a V4540 Variable Temperature Controller. The concentration of bound spin label was determined by double integration of spectra, using a Nicolet 1074 computer calibrated with a 0.5 mM solution of spin label.

RESULTS AND DISCUSSION

The time course of incorporation of \(^{14}C\) Mal NEt into SR (Fig. 1) indicates that, after a very rapid incorporation of 1 mol of Mal NEt per 10\(^5\) g of protein, a gradual increase in label incorporation takes place, reaching 5 mol of Mal NEt per 10\(^5\) g of SR protein in 16 hours (Fig. 1, inset).

The analysis of the distribution of radioactivity in SDS-gel electrophoretograms of the \(^{14}C\) Mal NEt-labeled SR reveals significant amounts of radioactivity only in two protein bands, the 100,000-dalton Ca\(^{2+}\)-ATPase band and a 30,000-dalton glycoprotein recently isolated from SR (9). The ratio of the counts incorporated into each protein band as a function of time is given in Table I. Even though the glycoprotein content of SR is considerably lower than that of the Ca\(^{2+}\)-ATPase, at short times the glycoprotein contributes significantly to the total counts incorporated into SR. The relative contribution of
the glycoprotein decreases with time, from one-half of the counts in the ATPase after 10 minutes, to only one-tenth after 16 hours.

The time course of incorporation of $^{14}$C Mal NEt into the Ca$^{2+}$-ATPase and the glycoprotein, analyzed in terms of the SH mol blocked per $10^5$ g of protein, is also shown in Fig. 1. The specific incorporation of label into the glycoprotein is much faster than into the Ca$^{2+}$-ATPase; after 10 min of incubation with the label, 2 SH mol per $10^5$ g of glycoprotein have been labeled, as opposed to only one per $10^5$ g of the Ca$^{2+}$-ATPase. However, after overnight incubation, only 3 SH per $10^5$ g become labeled in the glycoprotein as compared to about 12 in the Ca$^{2+}$-ATPase.

In view of these results, the reaction of SR with SH-directed spin labels
TABLE I
Distribution of \(^{14}\text{C}\) Mal NET in two SR protein components as a function of time

<table>
<thead>
<tr>
<th>Reaction time</th>
<th>(^{14}\text{C}) Mal NET incorporation (cpm in glycoprotein band/ cpn in (\text{Ca}^{2+})-ATPase band)</th>
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<tbody>
<tr>
<td>10 min</td>
<td>0.50</td>
</tr>
<tr>
<td>30 min</td>
<td>0.36</td>
</tr>
<tr>
<td>3 hr</td>
<td>0.26</td>
</tr>
<tr>
<td>16 hr</td>
<td>0.12</td>
</tr>
</tbody>
</table>

SR vesicles were incubated with 6.25 moles of \(^{14}\text{C}\) Mal NET per 10\(^5\) g of protein, as described in Materials and Methods. The labeled SR components were separated by electrophoresis in 10\% polyacrylamide gels containing SDS. The incorporation of \(^{14}\text{C}\) Mal NET into the glycoprotein and the \(\text{Ca}^{2+}\)-ATPase was determined by slicing and counting the 30,000-dalton and the 100,000-dalton bands, respectively.

might result in labeling both the glycoprotein and the \(\text{Ca}^{2+}\)-ATPase. This would result in EPR spectra containing contributions from two different protein species, complicating the interpretation of the spectra. We performed experiments designed to investigate this possibility. The time course of maleimide spin label incorporation into SR is shown in Fig. 2. Under conditions similar to those used to measure the incorporation of \(^{14}\text{C}\) Mal NET, the incorporation of maleimide spin label reached lower values than those obtained for \(^{14}\text{C}\) Mal NET; even after overnight incubation only about 2.5 SH mol per 10\(^5\) g of SR protein are blocked by the maleimide spin label, as opposed to about 5 in the case of \(^{14}\text{C}\) Mal NET. To analyze whether the spin label was incorporated into both the ATPase...
Fig. 2. SR was incubated with 5.0 mol of maleimide spin label per $10^5$ g of protein for different lengths of time, as indicated in the abscissa. The amount of label incorporated into the different fractions was calculated as described in Materials and Methods. Key: $\square$, SR; $\bigcirc$, glycoprotein; $\triangle$, Ca$^{2+}$-ATPase.

and the glycoprotein, the spin-labeled SR was solubilized with Triton X-100 (11) and EPR spectra were obtained from the resulting supernatant (enriched in the Ca$^{2+}$-ATPase and devoid of glycoprotein) and the pellet, containing all the glycoprotein. The concentrations of spin label in both fractions were determined as described above. The results (Fig. 2) indicate that at short times twice as much label per g of protein is found in the Triton-insoluble residue, as compared to the Triton-soluble fraction, but after overnight incubation the label concentration in the Triton-soluble fraction is 1.5 times higher than in the insoluble fraction. These results are, in general, in agreement with those shown in Fig. 1 for the incorporation of $[^{14}C]$ Mal NEt. Although it is not possible to ascertain from these data precisely how much spin label gets incorporated into each protein species, since this would require isolation and purification of each component from the spin-labeled SR, it is conceivable that the fast-reacting SH groups
A: maleimide-spin-labeled SR, containing 1.0 moles of label per 10^5 g of protein. B: Triton-insoluble residue obtained from maleimide-spin-labeled SR (1.32 mol of label bound per 10^5 g). C: SR spin-labeled (1.06 mol of label bound per 10^5 g) after labeling with [14C] Mal NEt (1.0 mol bound per 10^5 g). D: Ca^{2+}-ATPase spin-labeled after purification from SR (0.98 mol bound per 10^5 g).

present in the Triton-insoluble fraction are part of the glycoprotein peptide and are the same groups that show high reactivity towards [14C] Mal NEt.

It is worth noting at this point that the Ca^{2+}-ATPase activity of SR is progressively inhibited as the number of SH groups blocked increases. About 50% inhibition was obtained when one SH per 10^5 g of SR protein was blocked with [14C] Mal NEt.

The EPR spectrum of SR labeled with maleimide spin label (1 SH blocked per 10^5 g) is shown in Fig. 3A. As extensively described in the literature for SR labeled either with maleimide or iodoacetamide spin labels, two spectral components are present: one 'strongly' immobilized (S) and one 'weakly' immobilized (W). A quantitative analysis of the relative contributions of 'strongly' and 'weakly' immobilized components is given in Table II. The spectrum of the Triton-insoluble fraction obtained from spin-labeled SR (Fig. 3B) is different from the SR spectrum in that it shows a much larger contribution of the 'weakly' immobilized component (Table II), suggesting that this component might be partly or totally a signal arising from the glycoprotein, which is enriched in the insoluble fraction. The spectra shown in Figs. 3C and 3D strengthen this
Spectral parameters from different SR fractions labeled with maleimide spin label

<table>
<thead>
<tr>
<th></th>
<th>W/S</th>
<th>$2T_n$</th>
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<tbody>
<tr>
<td>A - SR</td>
<td>0.40 ± 0.01</td>
<td>67.3 ± 0.1</td>
</tr>
<tr>
<td>B - Triton-insoluble fraction</td>
<td>0.70 ± 0.01</td>
<td>66.4 ± 0.1</td>
</tr>
<tr>
<td>C - Mal NEt-SR</td>
<td>0.18 ± 0.01</td>
<td>67.7 ± 0.1</td>
</tr>
<tr>
<td>D - Purified Ca$^{2+}$-ATPase</td>
<td>0.17 ± 0.01</td>
<td>67.7 ± 0.1</td>
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The spectral parameters are taken from the spectra shown in Fig. 3, A through D. For details, see Legend to Fig. 3.

These results indicate, first, that the fast-reacting SH groups of SR are responsible for the 'weakly' immobilized signal; and, second, that the major portion of this signal component probably comes from label bound to the glycoprotein. This last proposition is based on the following evidence: a) Figs. 1 and 2 indicate that the glycoprotein contains fast-reacting SH groups; b) the 'weakly' immobilized signal is enhanced in the spectrum from the Triton-insoluble residue (Fig. 3B), and c) this signal is virtually absent from the spectrum of the purified Ca$^{2+}$-ATPase enzyme. Furthermore, it has been recently determined in
our laboratory that the glycoprotein contains 2.5 cysteic acid residues per mol, assuming a molecular weight of 30,000.

These observations suggest that the two principal parameters (see Fig. 3 and Table II) used in the past to characterize the spectra of spin-labeled SR do not depend exclusively on the state of the \( \text{Ca}^{2+} \)-ATPase enzyme. Therefore, it might not be valid to interpret changes in these parameters as indicators of conformational changes in the enzyme. More reliable results may be obtained by first blocking the fast-reacting SH groups with \( \text{Mn NEt} \) before spin-labeling the SR.

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REFERENCES