Transient Kinetics of a Ca$^{2+}$-induced Fluorescence Change from Membrane-associated and Solubilized Sarcoplasmic Reticulum Ca$^{2+}$-ATPase*

(Received for publication, June 2, 1980)

William L. Dean and Robert D. Gray
From the Department of Biochemistry, University of Louisville Medical School, Louisville, Kentucky 40292

The kinetics and extent of the fluorescence change induced by Ca$^{2+}$ interaction with the Ca$^{2+}$-ATPase from sarcoplasmic reticulum have been compared by stopped flow fluorimetry for three preparations: sarcoplasmic reticulum; purified ATPase in membrane vesicles; and solubilized, delipidated ATPase. The kinetics of Ca$^{2+}$ release and binding for both purified preparations could be described by a single exponential as has been observed for sarcoplasmic reticulum. The rate and extent of the fluorescence change for the solubilized and membrane-associated preparations are shown to be quite similar to those of the sarcoplasmic reticulum. From these results, it is concluded that all of the Ca$^{2+}$-induced fluorescence change in sarcoplasmic reticulum originates from the Ca$^{2+}$-ATPase. In addition, since the change in fluorescence is probably a result of a conformational change in the ATPase during the Ca$^{2+}$ pumping cycle, the results provide additional evidence that monomeric Ca$^{2+}$-ATPase may be capable of Ca$^{2+}$ transport since the delipidated preparation is monomeric under the conditions used for these experiments. Finally, it is concluded that phospholipid bilayer is not essential for this conformational change.

The Ca$^{2+}$-ATPase from sarcoplasmic reticulum pumps Ca$^{2+}$ against a concentration gradient (Racker, 1972), but the mechanism for the coupling of ATP hydrolysis to active Ca$^{2+}$ transport has not yet been elucidated (Tada et al., 1978). It has, however, been suggested that a conformational change occurs concomitant with Ca$^{2+}$ binding that can be observed by monitoring the intrinsic fluorescence of the proteins present in the sarcoplasmic reticulum (Dupont, 1976; Guillain et al., 1980). Since more than 70% of the protein present in the sarcoplasmic reticulum is the Ca$^{2+}$-ATPase (Meissner et al., 1973), it was concluded that this fluorescence change is mainly a result of Ca$^{2+}$/Ca$^{2+}$-ATPase interactions, although other Ca$^{2+}$ binding proteins are present in the sarcoplasmic reticulum (MacLennan et al., 1972).

In the present communication, the Ca$^{2+}$-induced change in intrinsic fluorescence is reported for a purified, membrane-associated preparation of the ATPase (Meissner et al., 1973) and a delipidated, monomeric preparation, solubilized in C$_{12}$E$_{6}$ and glycerol (Dean and Tanford, 1978). The kinetics and extent of the Ca$^{2+}$-induced fluorescence change for the two purified preparations were determined by stopped flow fluorimetry and are compared with those of sarcoplasmic reticulum. It is concluded that all of the fluorescence change observed in sarcoplasmic reticulum originates from the Ca$^{2+}$-ATPase. Furthermore, since the delipidated preparation is monomeric under the conditions used for these experiments (Dean and Tanford, 1978), the relationship between the state of aggregation of the ATPase, the Ca$^{2+}$-induced conformational change, and the lipid requirement can be explored.

**MATERIALS AND METHODS**

The source and preparation of all materials used in the present study are the same as those reported earlier (le Maire et al., 1976; Dean and Tanford, 1977).

Sarcoplasmic reticulum was isolated from rabbits by the method of Eletr and Inesi (1972), followed by sucrose density gradient centrifugation in an SW 27 rotor (Beckman) carried out as described for the second gradient in the method of Meissner et al. (1973). Purified ATPase vesicles were prepared according to Method 2 of Meissner et al. (1973). Specific activities for this preparation averaged 30 µmol/min/mg at 37°C, somewhat higher than the preparations described earlier (le Maire et al., 1976) owing to the greater purity of the sarcoplasmic reticulum. Delipidation of the purified ATPase was carried out as previously described (Dean and Tanford, 1978) and the phospholipid content was 4 to 6 mol of phospholipid/mol of enzyme. Specific activities of the delipidated preparations in C$_{12}$E$_{6}$ varied from 20 to 25 µmol/min/mg at 37°C. ATPase activity, protein, and phospholipid were determined as described earlier (Dean and Tanford, 1978).

Stopped flow experiments were performed at 4°C with a Durrum-Gibson stopped flow fluorimeter. Excitation was at 295 nm and fluorescence was observed through a 0.5-4 filter (Corning) which excludes light below 300 nm. Fluorescence changes were analyzed by a computer-controlled data collecting system as previously described (Gray, 1974; DeSa, 1972).

**RESULTS AND DISCUSSION**

Dupont and Leigh (1978) observed a 3% decrease in fluorescence for sarcoplasmic reticulum after removal of Ca$^{2+}$ that could be described by a single exponential decay. The results of a similar experiment with purified ATPase vesicles are shown in Fig. 1A. An exponential decrease in fluorescence of 4% was observed following reduction of the Ca$^{2+}$ concentration from 2.0 x 10$^{-7}$ M to less than 10$^{-10}$ M by mixing with EGTA in the stopped flow apparatus. The slow additional decrease in fluorescence (dashed line in Fig. 1A), which was also observed by Dupont and Leigh (1978), is probably a result of sedimentation of the lipid vesicles since it is eliminated when the ATPase is solubilized in C$_{12}$E$_{6}$, as shown in Fig. 1B. The delipidated, monomeric ATPase in C$_{12}$E$_{6}$ and glycerol yielded a similar decrease in fluorescence following Ca$^{2+}$ removal, which can also be described by a single exponential decay (Fig. 1B). These results contradict those of Nakamura et al. (1979), who could not observe a fluorescence change in a solubilized preparation of the ATPase.

Binding of Ca$^{2+}$ to the purified ATPase preparations resulted in an exponential increase in fluorescence of approxi-
FIG. 1. Kinetics of Ca\(^{2+}\) removal from purified ATPase vesicles and delipidated ATPase. \(\text{A, Syringe 1}}\) contained 0.01 M Tris, pH 7.5, 0.1 M KCl, 2.74 M glycerol, 2 \times 10^{-5} \text{ M CaCl}_2, \text{ and 0.17 mg/ml of purified ATPase vesicles. Syringe 2 contained the same components as Syringe 1 except that the ATPase was omitted and CaCl}_2 was replaced with 4.56 \times 10^{-5} \text{ M EGTA. Fluorescence was corrected for the sedimentation of vesicles as shown by the dashed line.}\) Syringe 1 contained the same components as those described for \(\text{A}}\) except that 0.15 mg/ml of delipidated ATPase was used instead of ATPase vesicles and the solution contained 1.86 \times 10^{-5} \text{ M CaCl}_2. Each point is the average of four separate experiments. The solid line was determined by linear regression of a first-order plot of the data. The final pH after mixing was 7.45, a change of 0.05 units.

Fig. 2. Kinetics of Ca\(^{2+}\) binding to purified ATPase vesicles and delipidated ATPase. \(\text{A, Syringe 1}}\) contained 0.01 M Tris, pH 7.5, 0.1 M KCl, 2.74 M glycerol, 1.0 \times 10^{-5} \text{ M EGTA, 5.0 \times 10^{-5} M MgCl}_2, \text{ and 0.09 mg/ml of purified ATPase vesicles. Syringe 2 contained the same components as those described for Syringe 1, but the ATPase was omitted and EGTA was replaced with 1.2 \times 10^{-5} \text{ M CaCl}_2. Each point is the average of four separate experiments.}\) Syringe 1 contained the same components as those described for \(\text{A}}\) except that 1.86 \times 10^{-5} \text{ M CaCl}_2 was added and the ATPase was delipidated (0.08 mg/ml). Syringe 2 is the same as that described for \(\text{A}}\) except for the addition of 1.86 \times 10^{-5} \text{ M CaCl}_2. The points are from a single experiment. The lines were determined as described in the legend to Fig. 1. The final pH after mixing was 7.35, a change of 0.15 units.

Table 1: Apparent rate constants for Ca\(^{2+}\) binding and release

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Experiment</th>
<th>(k_{obs})</th>
<th>Change in fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcoptermic reticulum</td>
<td>Ca(^{2+}) off - Mg(^{2+})</td>
<td>26 \pm 8(^*)</td>
<td>-1.5 - 3.0</td>
</tr>
<tr>
<td>Sarcoptermic reticulum</td>
<td>Ca(^{2+}) off + Mg(^{2+})</td>
<td>20 \pm 6</td>
<td>-1.5 - 2.0</td>
</tr>
<tr>
<td>Sarcoptermic reticulum</td>
<td>Ca(^{2+}) on</td>
<td>2.6</td>
<td>+2.0</td>
</tr>
<tr>
<td>ATPase vesicles</td>
<td>Ca(^{2+}) off - Mg(^{2+})</td>
<td>42 \pm 6</td>
<td>-2.0 - 4.5</td>
</tr>
<tr>
<td>ATPase vesicles</td>
<td>Ca(^{2+}) off + Mg(^{2+})</td>
<td>37 \pm 4.0</td>
<td></td>
</tr>
<tr>
<td>Delipidated ATPase</td>
<td>Ca(^{2+}) off - Mg(^{2+})</td>
<td>42 \pm 6</td>
<td>-2.0 - 4.0</td>
</tr>
<tr>
<td>Delipidated ATPase</td>
<td>Ca(^{2+}) off + Mg(^{2+})</td>
<td>33</td>
<td>-3.5</td>
</tr>
<tr>
<td>Delipidated ATPase</td>
<td>Ca(^{2+}) on</td>
<td>1.6 \pm 0.8</td>
<td>+3.0 - 4.0</td>
</tr>
</tbody>
</table>

\(^*\) Values with standard deviations were obtained from three different preparations.
could be observed after readdition of Ca²⁺ to the delipidated preparation, in contrast to the membrane-associated ATPase which did not lose the ability to exhibit an increase in fluorescence after 10 min in the absence of Ca²⁺. This loss in the reversibility of the fluorescence change for the delipidated preparation was accompanied by a similar loss in ATP-hydrolysis activity. In the absence of Mg²⁺ and Ca²⁺, the delipidated ATPase loses activity quite rapidly. Thus, in the absence of lipid, Ca²⁺ is necessary for the stabilization of the ATPase, and this requirement is only partially replaced by Mg²⁺.

The results of the studies presented in this communication warrant several important conclusions. First, the fluorescence change observed for sarcoplasmic reticulum is indeed a result of Ca²⁺ interaction with the ATPase and not other Ca²⁺ binding proteins in the sarcoplasmic reticulum. Second, the fluorescence change is not a result of changes in membrane structure, as proposed by Nakamura et al. (1979), since a delipidated, soluble ATPase preparation behaves in exactly the same fashion as the membrane-associated ATPase. Finally, since the conformational change detected by the fluorescence change is probably related to Ca²⁺ transport, as concluded by Dupont and Leigh (1978) and Guillain et al. (1980), the present data lend additional support to the possibility that monomeric ATPase is capable of Ca²⁺ transport. The slow rate of the fluorescence change associated with Ca²⁺ binding to the ATPase strongly suggests that the change is associated with a protein isomerization step, in contrast to troponin C, where the Ca²⁺-induced fluorescence change probably results from a direct effect of Ca²⁺ binding on the fluorescing tyrosine residues (Leavis and Lehrer, 1978). In contrast to the slow change in fluorescence of the Ca²⁺-ATPase upon binding of Ca²⁺, the change observed for troponin C is instantaneous.²

REFERENCES

Tada, M., Yamasaki, T., and Tonomura, Y. (1978) Physiol. Rev. 58, 1-70

² R. D. Gray, unpublished observations.