FLUORESCENCE STUDIES OF THE SARCOPLASMIC RETICULUM CALCIUM PUMP.

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SUMMARY :

Intrinsic Tryptophan fluorescence has been used to reveal conformational changes of the Sarcoplasmic Reticulum Calcium pump. It is shown that upon binding of calcium ions the fluorescence is enhanced. The effect being reversed after removal of dependence calcium ions by EGTA. The calcium concentration dependence of this fluorescence change and the effect of inhibitors is compared with the activation of calcium dependent ATPase. We conclude that calcium ions induces a conformational change of the calcium pump and that this change is responsible for the activation of the ATPase activity.

INTRODUCTION :

In skeletal muscle Sarcoplasmic Reticulum (S.R.) the calcium transport is performed by an ATP driven Calcium pump. The fragmented S.R. retains this activity, it has been demonstrated that calcium activates the ATP splitting and that two calcium ions are transported inside the vesicles for every ATP molecule cleaved (1, 2).

Up to now little is known about the molecular events involved in these mechanisms and we have a poor knowledge of the structure of the ionic pump.

We have tried to gain information about the conformational changes which could occur during these events. We report here observations of the modifications of the intrinsic Tryptophan fluorescence upon interaction with calcium ions. It will be demonstrated that this method is very sensitive and allows small conformational changes of the protein to be observed.

From our observations we will deduce the following conclusions :

- Calcium ions, by themselves, modify the conformation of

the protein in the absence of ATP, effects of competitors like Mg^{++} are easily observed.

- The ATPase activity exhibits the same calcium dependence than the fluorescence change.

- When poisonned with SH reagents the molecule becomes insensitive to calcium ions and is blocked in an altered conformation. Subsequently there is an inhibition of the calcium activated ATPase activity.

MATERIALS AND METHODS :

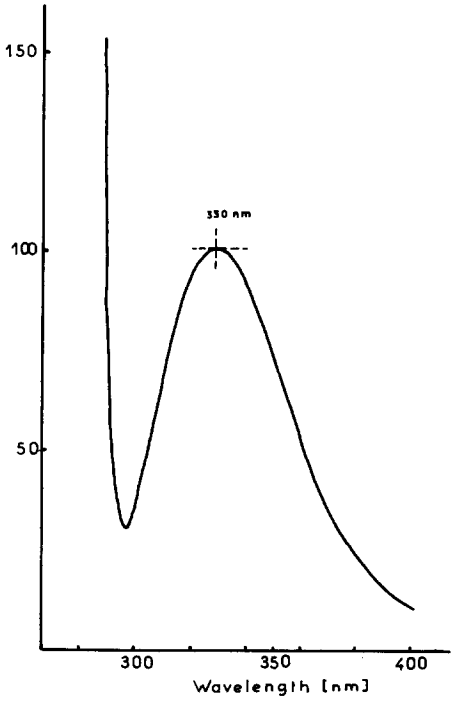
Rabbit S.R. is prepared by the method of Hasselbach and Makinose (3), with a washing step in 0.6M KCl. The vesicle are stored in 0.3M Sucrose in liquid Nitrogen (4). The fluorescence measurements were made on a HITACHI MPF-2A spectrofluorometer in a continuously stired cell at 22°. Unless otherwise described, conditions are the following : 100 to 200 µg of S.R. proteins are diluted in 2 ml of 20 mM Tris-maleate pH 7.15, 100 mM KCl, 5 mM Mg^{++} in a 1 cm cell. The free calcium is adjusted with a calcium-EGTA buffer. The total calcium concentration was kept constant for a given serie of experiment (typically 100 µM) and EGTA was adjusted to give the desired free calcium concentration. The apparent dissociation constant of the calcium-EGTA complex was calculated from (5, 6) and the following values were obtained at pH 7.15 : Kd = 0.10 µM for Mg^{++} = 0. and Kd = 0.13 µM for Mg^{++} = 5. mM. Although these values are taken from accepted stability constants we have found it necessary to mesure the real free concentration of calcium ions in the solution. Complete details of the method used will be published elsewhere. In brief it is based on the fact that when one calcium ion complexes with EGTA, two H^+ are released. The titration of the protons which are produced by an injection of an excess EGTA to the mixture gives directly the concentration of free calcium. Due to the contaminating calcium of water and salts, large discreapancies (up to a factor of 2 to 3) are found between the measured and calculated values of free calcium. This is especially important for low concentration of calcium-EGTA buffer.

ATPase measurements are made with a pH-Stat (Radiometer TTT 1) (4,7). With native vesicles the measurements are quite impossible in a calcium-EGTA buffer : this is due to the fact that the accumulation of Ca^{++} perturb the buffer equilibrium and the OH^- produced by this perturbation interfere with the H^+ liberated by the splitting of ATP. For the measurements at low calcium it is nevertheless necessary to use a calcium-EGTA buffer. We have resolved this problem in using vesicles which have been treated with the calcium inophore X 537-A (8), thus preventing the accumulation of calcium inside the vesicles. After 1 to 2 minutes equilibration a steady state is reached where the calcium-EGTA complex stays stable and the H^+ produced by the ATP splitting is then unperturbed.

The measurements are made under a continuous flow of Argon at pH 7.15 in 1 mM Tris-Maleate, 100 mM KCl, 5 mM Mg^{++}, 100 µM X 537-A, 100 µM Ca^{++} and the desired concentration of EGTA. Titrant is 10 mM KOH and the protein concentration : 30 µg/ml.

RESULTS :

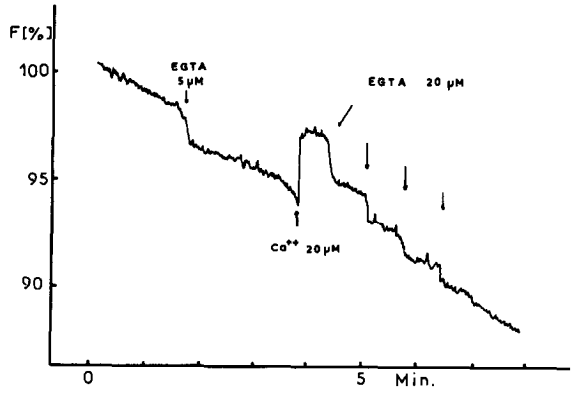
The calcium pump protein posses about 20 Tryptophan residues for a total of about 1000 amino acids (9). The emission spectrum, when excited at 295 nm, has its λ max. at 330 nm (Fig. 1). The intensity of fluorescence is high and concentration as low as few µg/ml can be used.



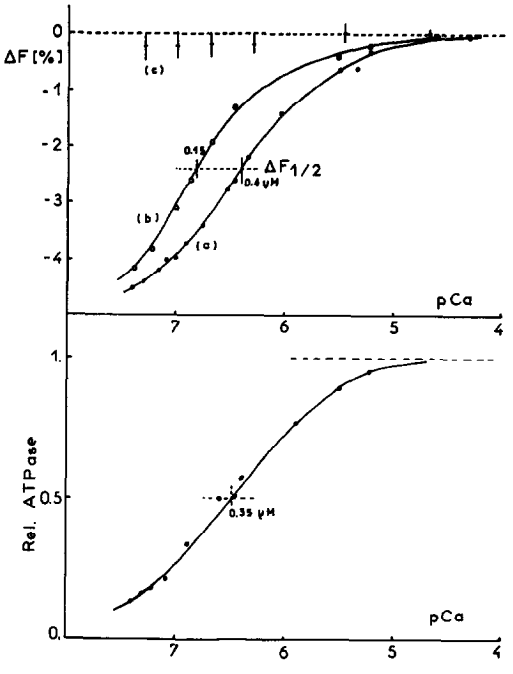
1 - Fluorescence spectrum emitted by the S.R. protein when excited at 295 nm.

Effect of calcium :

The figure 2 shows a record of the fluorescence intensity at 330 nm. An increase of fluorescence is observed upon addition of Ca^{++}, the effect is reversed by successive injection of EGTA. It is important to note that small fluorescence changes have also been observed when varying the pH (ΔF = 0.3% for ΔpH = 0.01). Thus the change of pH resulting from the mixing of calcium to EGTA in the cuvette must be kept as low as possible (or be corrected). When using low calcium-EGTA buffer concentration this effect is negligible



2 - Typical exemple of a record of the fluorescence intensity at 330 nm : effect of Calcium ions and EGTA. A continuous and slow decrease in intensity is observed here just after dilution of the vesicle; it stabilies after 10 to 15 minutes.



3 - Calcium concentration dependence of the fluorescnece change and of the ATPase activity. The standard experimental conditions are described under "Methods". The curve a) has been obtained with 5 mM Mg^{++}, b) without Mg^{++} and points, c) in poisonning the vesicles with 2 - Chloromercury 4 - Nitrophenol.

(about 0.2% for 20 μM Ca^{++}-EGTA). For higher concentrations it is necessary to correct for this effect, another procedure is to use a Ca^{++}-EGTA mixture which has been titrated to the right pH just before injection in the cuvette.

Magnesium ions, per se, do not affect the fluorescence intensity. This is demonstrated in adding Mg^{++} in concentrations as high as 10 mM in the presence of micromolar calcium or in excess EGTA. Effects of Mg^{++} on the fluorescence are only observed at intermediate calcium concentration but they are due to the displacement of calcium from the high affinity calcium sites. The figure 3 shows the fluorescence intensity change for various Ca^{++} concentration in the presence or absence of Mg^{++}. It can be seen that Mg^{++} produces a shift of the half value ΔF 1/2. An evaluation of the inhibitory constant of Mg^{++} gives Ki = 2.5 mM in very close agreement with the value obtained in a calcium binding experiment (Y.D. to be published).

SH Reagents.

We have used 2-Chloromercury 4-Nitrophenol (MNP), (10). With the equivalent of 4 SH groups blocked the calcium induced fluorescence change is inhibited (figure 3), the same amount of poisonning has been previously found to inhibits the calcium activated ATPase (7, 11).

ATPase activity.

The calcium concentration at half maximum activation of the calcium dependent ATPase is found to be 0.35 μM (figure 3). About the same value is obtained for the calcium dependent fluorescence change : (0.4 μM).

Values found in the litterature for the half activation of the Ca^{++}-ATPase range from 10^{-7} to more than 10^{-6}M Ca^{++} (12-14). This discreapancy probably arises from differences in the values adopted for the stability constants of the Ca^{++}-EGTA complex. In measuring the real free calcium concentration we should have eliminated this uncertainty.

DISCUSSION.

The purpose of the measurement of the intrinsic fluorescence modifications was to find a way to follow conformational changes

induced by calcium ions on the calcium pump protein. It has been observed that the concentration dependence of these changes are in very close relation with the activation of the ATPase, furthermore SH poisonning inhibits in the same way the fluorescence change and the calcium dependent ATPase activity. We conclude therefore that the calcium ions produce a modification of the protein's conformation and that this modification is in turn responsible for the activation of the ATPase. ATP binding experiments indicate that this activation is not due to an effect on the affinity for ATP (4).

From calcium binding experiments we have observed that the half saturation of the calcium sites is at a somewhat higher concentration ($Y\frac{1}{2}$ for (Ca^{++}) = 1 μM) than those found for the fluorescence change. In other words this indicates that the calcium induced conformational change might not be directly proportional to the saturation function of the calcium sites. This difference may be explained by assuming that the conformational change observed in fluorescence is followed by a modification of the apparent affinity of the calcium sites. We are now investigating the field open by this interesting observation.

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