Separation of the tryptic fragments of sarcoplasmic reticulum ATPase with high performance liquid chromatography

Identification of the calcium binding site

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The four tryptic fragments of the Ca^{2+}- and Mg^{2+}-dependent ATPase from sarcoplasmic reticulum were separated on a TSK-G 4000 SW preparative high performance liquid chromatography column in the presence of sodium dodecyl sulfate. Using atomic absorption spectroscopy, the analysis of the protein peaks demonstrated that the calcium binding sites with high affinities are located in the smallest fragment (A_2; 25 kDa).

Sarcoplasmic reticulum *Ca^{2+}-ATPase* *Tryptic digestion* *Ca^{2+} binding site* *HPLC*

1. INTRODUCTION

It has been shown by several groups [1–4] that the sarcoplasmic reticulum ATPase from fast skeletal muscle is split into 4 fragments by the limited action of trypsin. Molecular masses of the fragments are 55, 45, 32 and 25 kDa and they were named, A,B,A_1 and A_2 respectively [5–7]. It has also been demonstrated that the two smaller fragments (A_1 and A_2) are the digestion product of A. The separation of the fragments was only possible in the presence of SDS. Nevertheless, the elution profiles obtained from different columns were not at all satisfactory. Neither Triton X-100 nor deoxycholate was able to dissociate the fragments [2,3]. Therefore, little is known about the functional properties of the individual fragments, with the exception of the A_2 fragment which is located at the NH_2 terminus of the polypeptide chain [8].

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Abbreviations: SDS, sodium dodecyl sulfate; Mops, 4-morpholinepropane sulfonic acid; HPLC, high performance liquid chromatography

This fragment exhibits calcium ion-specific conductivity when incorporated into black lipid membranes [4]. But the presence of SDS during the incorporation and the necessity of millimolar concentrations of calcium raise some doubts concerning the real identity of the calcium ionophore present in the ATPase molecule. We have now separated the tryptic fragments using the HPLC-technique in the presence of SDS and were able to demonstrate that the A_2 fragment contains calcium binding sites with high affinities.

2. MATERIALS AND METHODS

Trypsin and trypsin inhibitor from soybean were purchased from Serva (Heidelberg). Mops was from Sigma Chemie GmbH (München) and ^{45}Ca was from Amersham Buchler (Braunschweig). All other chemicals were analytical grade products.

Sarcoplasmic reticulum vesicles from fast skeletal muscle were prepared as in [8], as modified in [10]. Protein concentration was determined by the Biuret method calibrated with Kjeldahl standards or spectrophotometrically at 280 nm

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($E^{1\%}_{1cm}$ = 10) [11,12]. Sarcoplasmic reticulum ATPase was digested at 20°C in a buffer containing 20 mM Mops (pH 7.0), 50 mM NaCl, 5 mM $MgCl_2$, 0.45 mM EGTA, 0.5 mM $CaCl_2$ and 2.5 mg · ml^{-1} protein. Trypsin at a ratio of 1:10 (w/w) was added for 5 min. Thereafter, trypsin inhibitor at a ratio of 2:1 (w/w; inhibitor:trypsin) and 0.1 mM phenylmethyl sulfonylfluoride were added and the solution was stirred for 20–30 min. Extrinsic proteins were subsequently removed as in [13] using small amounts of deoxycholate (0.3 mg/mg protein). The final pellet was resuspended in the same buffer solution as used for the digestion to about 20–30 mg protein · ml^{-1} and 1 mg SDS/mg protein was added.

Five mg of digested ATPase were injected on a TSK-G 4000 SW column (21.5 × 600 mm) equipped with a TSK GSWGP precolumn (21.5 × 75 mm) and equilibrated with 20 mM Mops (pH 7.0), 50 mM NaCl and 1 mg · ml^{-1} SDS. The flow rate was 3 ml · min^{-1} (pressure = 30 bar). The fractions were subjected to SDS gel electrophoresis and atomic absorption spectroscopy for calcium determination (Perkin Elmer Atomic Absorption Spectrophotometer 4000 connected to a HGA 400 Programmer). SDS gel electrophoresis was performed as in [14], but using 0.1 M Tris-Bicine buffer (pH 8.2) containing 0.1% SDS (R. Lebermann, personal communication).

Flow dialysis was performed as follows: sarcoplasmic reticulum vesicles (5 mg protein · ml^{-1}) were incubated in a buffer solution containing 20 mM Mops (pH 7.0), 50 mM NaCl and 5 mM $MgCl_2$. A small amount of ^{45}Ca was added and the total calcium concentration was determined with atomic absorption spectroscopy. The solution was placed over a dialysis membrane with an area of 2 cm^2 and the flow buffer was pumped below the membrane at 30 ml · h^{-1}. The composition of the flow buffer was 20 mM Mops (pH 7.0), 50 mM NaCl and 5 mM $MgCl_2$. The amount of free calcium in the vesicular suspension and the corresponding amount of radioactivity present in the flow buffer was determined in a separate experiment, either with the specific radioactivity or using atomic absorption spectroscopy. A protein-free solution containing 10^{-4} M calcium was used. Tryptic digestion during flow dialysis was done exactly as described for the separation of the fragments with the HPLC technique.

3. RESULTS

To establish which is the calcium binding fragment of digested sarcoplasmic reticulum ATPase it is necessary to demonstrate that neither trypsin nor SDS displaces the calcium ion from its high affinity binding site. This was shown using flow dialysis and the results are presented in fig. 1. Whereas denaturation of control vesicles for 10 min at 100°C releases the calcium bound to the protein, a 5-min treatment with trypsin which is able to split the protein into the 4 fragments (see below) released only part of the calcium (~30%). Further addition of trypsin-inhibitor and phenylmethyl sulfonylfluoride did not change the elution profile of calcium. In contrast, the addition of 2 mg SDS per mg protein drastically decreased the amount of

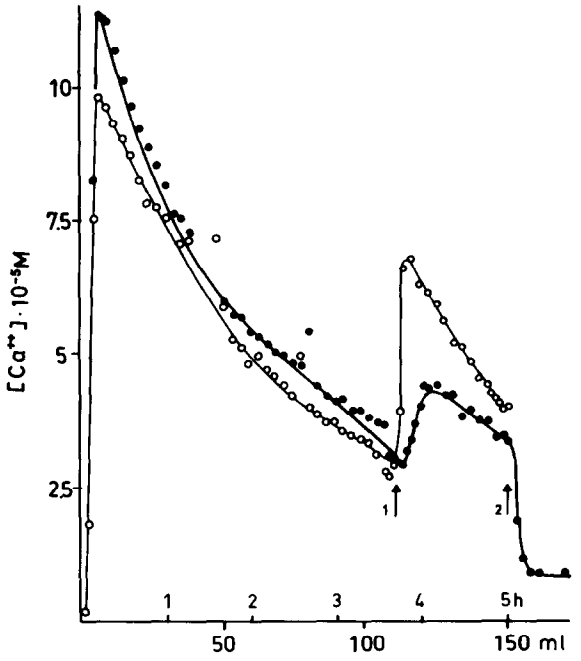

Fig. 1. Flow dialysis experiment with control and digested vesicles. Flow dialysis was carried out exactly as described in section 2. (○—○) Elution of ^{45}Ca from a vesicular suspension (5 mg protein · ml^{-1}). At arrow 1 the vesicles were boiled for 10 min. (●—●) same as (○—○), but at arrow 1 trypsin at a ratio of 1:10 (w/w) was added for 5 min, followed by trypsin inhibitor and phenylmethyl sulfonylfluoride. At arrow 2 the vesicles were solubilized with 2 mg SDS/mg protein. The free calcium concentration present in the vesicular suspension is given in the ordinate.

free calcium in the vesicular suspension. As verified using protein-free solution this was due to a complexation of the calcium with SDS (not shown). Denaturation of the SDS-treated protein for at least 30 min at 100°C did not cause precipitation and consequently no calcium release was observed.

The separation of the tryptic fragments on a TSK-G 4000 SW column using a buffer solution containing 1 $mg \cdot ml^{-1}$ SDS is illustrated in fig. 2. The fragments A_1 and A_2 are clearly separated from the fragments A and B as shown in the inset of fig. 2 by the gel electrophoresis pattern of the protein peaks. Fig. 2 also shows that deoxycholate treatment of digested sarcoplasmic reticulum vesicles removes extrinsic proteins such as calsequestrin. When the calcium content of the fractions eluting from the column was determined by atomic absorption spectroscopy, most of the calcium eluted together with the A_2 fragment and a smaller amount with the fragments A or B. In the peak fraction of fragment A_2 3.3 mol calcium was bound/mol protein if the following values were taken into account: (i) concentration of bound calcium: 2×10^{-5} M (calculated from the total amount and the amount present in the buffer solution); (ii) protein concentration: 0.15 $mg \cdot ml^{-1}$; (iii) molecular mass: 25 kDa.

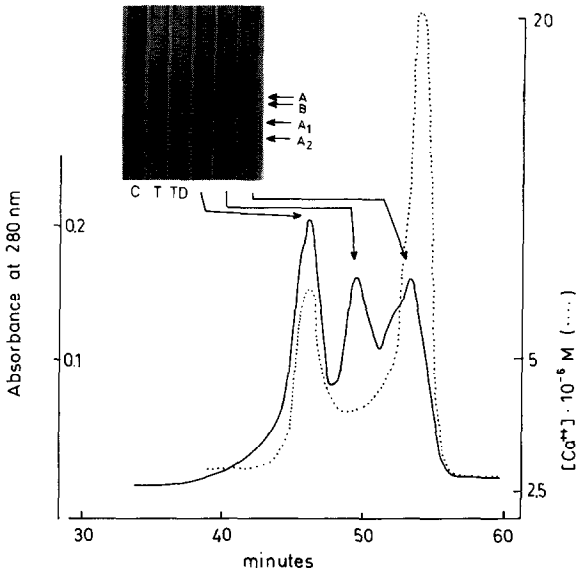

Fig. 2. Separation of the tryptic fragments and determination of the calcium concentration. The HPLC-column was run as described in section 2. (——) Elution profile measured at 280 nm. (– – –) Calcium content of the fractions determined with atomic absorption spectroscopy. Inset: SDS gel electrophoresis of the peak fraction. C, sarcoplasmic reticulum vesicles, T, digested sarcoplasmic reticulum vesicles without deoxycholate extraction (see section 2; TD, digested ATPase protein applied to the column.

4. DISCUSSION

The localization of the calcium binding site in the tryptic fragment of the ATPase of 25 kDa is in agreement with the conductivity measurements done in black lipid membranes [4] and with the localization of the dicyclohexylcarbodiimide binding site in this fragment [15]. The main advantages of the experimental approach presented in this study are: (i) a rapid and good separation of the fragments using the HPLC technique; (ii) no calcium was added to the buffer solution used for the column run. Therefore, the calcium binding was determined at a calcium concentration of about 2×10^{-6} M (see fig.2). Furthermore, the amount of 3.3 mol calcium/mol protein is close to the expected value, taking into account that 2 calcium ions are transported per ATP split, and considering the relatively large uncertainties in protein concentration and M_r determinations. It is also shown in control experiments using flow dialysis that neither trypsin nor SDS is able to displace the calcium ion from its high affinity binding site (fig.1). One could argue that the complexation of calcium with SDS induces the binding of calcium to the fragment A_2. This seems very unlikely, since such a general phenomenon would not only induce calcium binding to the fragments A and A_2, but also to the fragments B and A_1.

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