Biochimica et Biophysica Acta 821 (1985) 137–141
Elsevier

BBA 72787

Preparation of a highly concentrated, completely monomeric, active sarcoplasmic reticulum Ca^{2+}-ATPase

Hans Lüdi * and Wilhelm Hasselbach

Max-Planck-Institut für medizinische Forschung, Jahnstrasse 29, D-6900 Heidelberg (F.R.G.)

(Received May 29th, 1985)

Key words: Sarcoplasmic reticulum; Ca^{2+}-ATPase; Protein-protein interaction; Analytical ultracentrifugation; Detergent

Sarcoplasmic reticulum vesicles from fast skeletal muscle were partially delipidated with sodium cholate at high ionic strength and sedimented in a discontinuous sucrose gradient. Phospholipid content was reduced from 0.777 μmol/mg protein to 0.242 μmol/mg protein. As judged from gel electrophoresis and high pressure liquid gel chromatography, accessory proteins were removed during centrifugation and the Ca^{2+}-ATPase was obtained in an almost pure form. Addition of myristoylglycerophosphocholine (1 mg/mg protein) reactivates ATPase and dinitrophenylphosphatase activity to the same degree obtained with native vesicles. Using the analytical ultracentrifuge it could be demonstrated that the reactivated Ca^{2+}-ATPase was present exclusively in a monomeric state. These results were obtained at high and low ionic strength and up to a protein concentration of 10 mg/ml. Therefore this preparation should be very useful to investigate differences between oligomeric and monomeric Ca^{2+}-ATPase.

Introduction

It has been shown, that sarcoplasmic reticulum Ca^{2+}- and Mg^{2+}-ATPase from fast skeletal muscle exists as an oligomeric protein within the membrane of native vesicles [1]. To test whether protein-protein interactions are essential for ATPase activity and Ca^{2+}-transport, or regulate these processes, one should be able to compare the results obtained using native vesicles with those obtained with a highly active, monomeric Ca^{2+}-ATPase (see, for example, Refs. 2–4). Non-ionic detergents such as dodecyl octaoxyethylene glycol monoether ($C_{12}E_8$) indeed are able to solubilize sarcoplasmic reticulum Ca^{2+}-ATPase in an active form [5]. But under most circumstances, apart from the monomeric Ca^{2+}-ATPase substantial amounts of dimers and oligomers are present after solubilization. This is due to high protein concentrations (> 0.05 mg/ml) [6,7] and to phospholipids still bound to the protein [5]. Therefore it is difficult to establish, if in a given experiment the Ca^{2+}-ATPase was present as a monomeric protein or as a mixture of monomers and oligomers. We now describe the preparation of a concentrated, highly active, pure and homogeneous monomeric Ca^{2+}-ATPase in a myristoylglycerophosphocholine (LL_{14}) containing solution. Native vesicles were delipidated in a discontinuous sucrose density gradient and reactivated with LL_{14}, which has previously shown to be able to monomerize the Ca^{2+}-ATPase protein [1,7] and to support enzyme activity for at least one day [8].

* To whom correspondence should be sent at (present address): Vet.-Pharmakol. Institut, Universität Bern, Länggass-Strasse 124, CH-3012 Bern, Switzerland.

Abbreviations: HPLC, high pressure liquid chromatography; DNPP, 2,4-dinitrophenyl hydrogen phosphate, monolutidinium salt; LL_{14}, myristoylglycerophosphocholine; Mops, 4-morpholinepropanesulfonic acid.

Materials and Methods

Materials. Myristoylglycerophosphocholine (LL_{14}) was from Calbiochem, Lahn-Giessen (F.R.G.), ATP was from Pharma Waldhof GmbH. Düsseldorf (F.R.G.) and sodium cholate was from E. Merck, Darmstadt (F.R.G.). 2,4-Dinitrophenyl hydrogen phosphate (DNPP) was synthesized according to Ramirez and Marecek [9] and was used as its lutidinium salt. All other chemicals were analytical grade products.

Preparations. Sarcoplasmic reticulum vesicles were prepared according to Hasselbach and Makinose [10] as modified by De Meis and Hasselbach [11] and delipidated in a discontinuous density gradient as follows (cf. Ref. 12). Top layer: 10 mg/ml vesicular protein, 5 mg/ml sodium cholate, 5 mM $CaCl_2$, 0.5 M NaCl, 10^{-4} M dithioerythritol. The solution was made up to 5 ml by the addition of a 0.5 M imidazole solution (pH 7.0). Intermediate layer: 5.5 ml of 100 mM imidazole (pH 7.0), 0.3 M NaCl, 0.3 M sucrose, 5 mM $CaCl_2$, 5 mg/ml sodium cholate, 10^{-4} M dithioerythritol. Bottom layer: 2 ml of 100 mM imidazole (pH 7.0), 0.5 M NaCl, 0.5 M sucrose 5 mM $CaCl_2$, 10^{-4} M dithioerythritol. The tubes were centrifuged in a Beckman L8-M centrifuge (SW 40 rotor; Beckman Instruments, Munich (F.R.G.)) at 15°C. A dual program mode was used and set as follows: (i) 20000 rpm for 40 min, acceleration 6, and (ii): 40000 rpm for 90 min. The delipidated Ca^{2+}-ATPase was collected as a pellet and homogenized with a glass-teflon homogenizer in a buffer containing 50 mM imidazole (pH 7.0), 50 or 500 mM NaCl and 10^{-4} M dithioerythritol to give a protein concentration of 20–25 mg/ml. The total recovery of protein was about 50%. Depending on the experiments to carry out 1 mg LL_{14} or 2 mg sodium dodecylsulfate per mg of protein were added and the solution was stored at −20°C.

Sedimentation equilibrium analysis. Delipidated Ca^{2+}-ATPase in LL_{14} or in sodium dodecylsulfate was subjected to sedimentation equilibrium analysis using a Beckman model E ultracentrifuge combined with a scanner to monitor the extinction of the sample. A cell which allows the simultaneous run of three different samples and three different reference solutions was used. The protein concentration of the sample for centrifugation was 10 mg/ml, the temperature was set at 20°C and pictures were taken after 3–5 h of centrifugation at 8000 rpm using a wavelength of 307 nm. Analysis of the data was carried out as described elsewhere [13–15]. The following equations were used:

$$M_r = \frac{2RT}{(1-\bar{V}\rho)\omega^2} \cdot \frac{c_b - c_m}{c_o(r_b^2 - r_m^2)} \quad (1)$$

where

$$1-\bar{V}\rho = (1-\bar{V}_P\rho) + \delta_D(1-\bar{V}_D\rho) + \delta_L(1-\bar{V}_L\rho) \quad (2)$$

$\bar{V}$, effective partial volume; ω, radial velocity of rotation; c_b, concentration of protein at the bottom of the cell; c_m, concentration of the protein at the meniscus; c_o, concentration of the protein at the beginning of the run; r_b and r_m, distance (in cm) from the centre of rotation of cell bottom and cell meniscus, respectively. $\bar{V}_P$, partial specific volume of the protein; $\bar{V}_D$, partial specific volume of the detergent bound to the protein; $\bar{V}_L$, partial specific volume of the lipids bound to the protein. δ_D and δ_L, grams of detergent and lipid bound per gram of protein, respectively. If LL_{14} was used to reactivate Ca^{2+}-ATPase $\bar{V}_P$ was taken as 0.74 [14]. The other terms of Eqn. 2 could be neglected, since they have only minute influence on the calculation of the molecular weight [14]. In the experiments with sodium dodecylsulfate $\bar{V}_D$ has to be taken as 0.87 [13], and δ_D as 1.2 [15].

HPLC gel filtration. These experiments were carried out essentially as described in Ref. 7. Reactivated Ca^{2+}-ATPase (20–25 mg/ml) (i.e. in the presence of LL_{14}) was applied to a LKB TSK-G 4000 SW column (LKB Instruments GmbH, Gräfeling, (F.R.G.)) equilibrated with 20 mM Mops (pH 7.0), 50 mM NaCl and 1 mg/ml LL_{14} and run at a flow rate of 0.7 ml/min resulting in a back pressure of 30 bar.

Assays. Dinitrophenylphosphatase activity was determined as described in Ref. 16. Reactivated Ca^{2+}-ATPase (0.1 mg/ml) was added to a buffer solution containing 50 mM imidazole (pH 7.0), 20 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM dithioerythritol and 0.4 mM DNPP. The reaction was followed spectrophotometrically at 412 nm for a few minutes. ATPase activity was determined in

some experiments in parallel as described in Ref. 17. The ATPase activity exceeded the dinitrophenylphosphatase activity by about 20–30%. Protein concentration was determined by the biuret method using Kjeldahl calibrated standards or spectrophotometrically at 280 nm ($E^{1\%}_{1cm} = 10$) [18,19]. Phospholipid content was determined after digestion of the sample with $HClO_4$. The total amount of inorganic phosphate was measured according to Dittmer and Wells [20] (Bartlett phosphate determination).

Results

Native sarcoplasmic reticulum vesicles were delipidated in a discontinuous sucrose density gradient using sodium cholate and high ionic strength to reduce the critical micellar concentration of the detergent. This procedure decreased the phospholipid content of the vesicles, present as a pellet, from 0.777 μmol/mg protein to 0.242 μmol/mg protein (Table I). The amount of protein recovered in the pellet was about 50% of the protein applied to the gradient. As can be seen from Fig. 1A accessory proteins were removed during centrifugation. The relatively large amount of protein on the top of the gel is due to an infavorable (but inevitable) sample preparation for gel electrophoresis after the readdition of LL_{14}. As shown in Table I LL_{14} at a concentration of 1 mg/mg protein restored the dinitrophenylphosphatase activity to a level obtained with native vesicles. The same results were obtained if instead of dinitrophenylphosphatase activity ATPase activity was measured (not shown). It should be noted, that maximal activity of delipidated Ca^{2+}-ATPase was obtained at 0.5 mg LL_{14}/mg protein, but this amount of LL_{14} was not able to monomerize the Ca^{2+}-ATPase protein (see below). The stability of the reactivated Ca^{2+}-ATPase in 1 mg LL_{14}/mg protein is shown in Fig. 1B. When kept at −20°C the preparation was still 60% active after one week. Fig. 2 shows the analysis of the reactivated Ca^{2+}-ATPase using sedimentation equilibrium centrifugation in an analytical ultracentrifuge. From the slope of the straight line in Fig. 2B the molecular weight (M_r) of the protein can be calculated as outlined in Materials and Methods. A M_r of 110100 ± 18400 (mean of 20 experiments ± S.D.) was obtained, clearly indicating the presence of monomeric Ca^{2+}-ATPase, even at concentrations of 10 mg/ml. For comparison the sedimentation equilibrium analysis was repeated, but 2 mg sodium dodecylsulfate per mg of protein

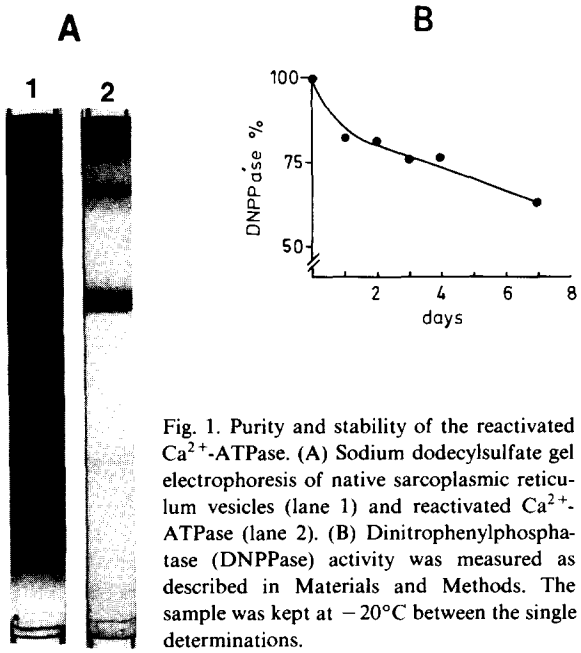

Fig. 1. Purity and stability of the reactivated Ca^{2+}-ATPase. (A) Sodium dodecylsulfate gel electrophoresis of native sarcoplasmic reticulum vesicles (lane 1) and reactivated Ca^{2+}-ATPase (lane 2). (B) Dinitrophenylphosphatase (DNPPase) activity was measured as described in Materials and Methods. The sample was kept at −20°C between the single determinations.

TABLE I

DINITROPHENYLPHOSPHATASE AND PHOSPHOLIPID CONTENT OF NATIVE VESICLES AND DELIPIDATED Ca^{2+}-ATPase

In some experiments ATPase activity was measured in parallel and was found to be 20–30% larger than the dinitrophenylphosphatase (DNPPase) activity. Note: No Ca^{2+}-independent ATPase activity could be detected. Figures are presented as means ± S.D.

Vesicles	DNPPase activity (μmol·mg^{-1}·min^{-1})	Phospholipid content (μmol/mg protein)
Native (control) [a]	0.131 ± 0.032(5)	0.777 ± 0.078(7)
Native + 1 mg/mg LL_{14}	0.469 ± 0.037(3)	–
Delipidated	0.033 ± 0.020(6)	0.242 ± 0.034(8)
Delipidated + 1 mg/mg LL_{14}	0.464 ± 0.038(4)	–

[a] Measured in the absence of a Ca^{2+} ionophore! The number in parenthesis corresponds to the number of experiments.

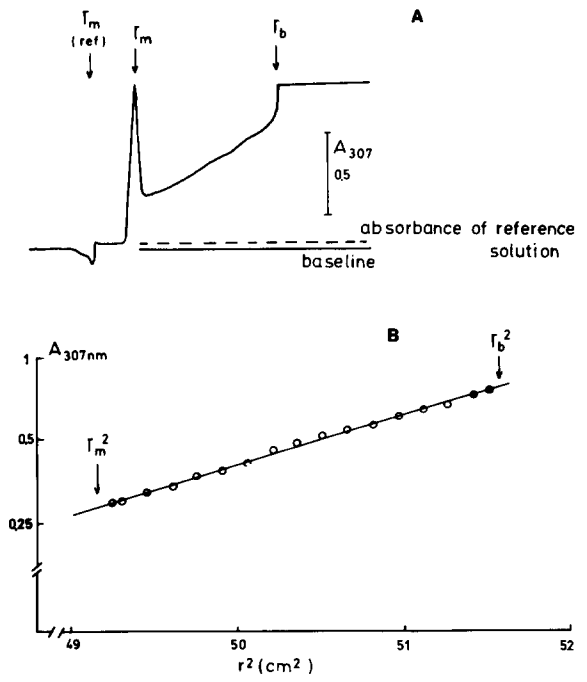

Fig. 2. Sedimentation equilibirum analysis of reactivated Ca^{2+}-ATPase. (A) Original trace of a sedimentation equilibrium run of reactivated Ca^{2+}-ATPase at 8000 rpm after 3 h using a protein concentration of 10 mg/ml. Absorbance was measured at 307 nm due to the high protein concentration used. (B) Analysis of the trace shown in (A). Absorbance at 307 nm (on a logarithmic scale) is plotted against the square of the distance from the centre of rotation. r_m and r_b, distances of the sample meniscus and the cell bottom, respectively. r_m(ref), distance of the meniscus of reference solution.

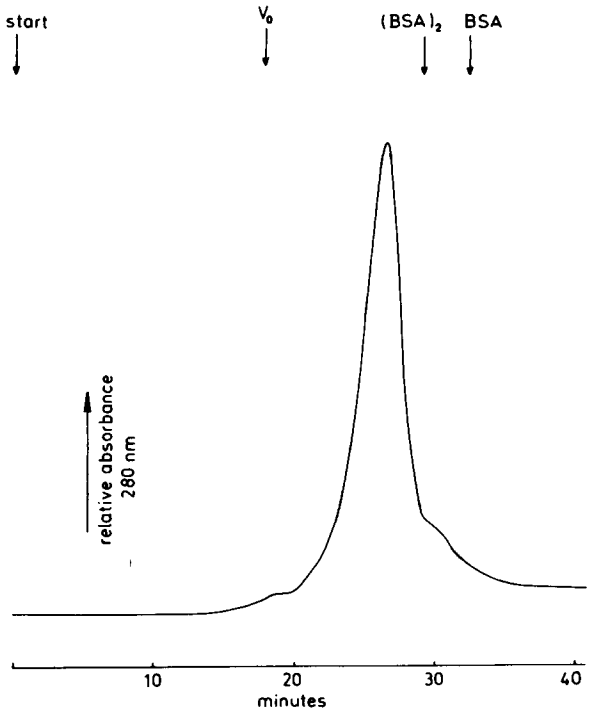

Fig. 3. HPLC gel filtration of reactivated Ca^{2+}-ATPase. Experimental conditions are given in Materials and Methods. Flow rate, 0.7 ml/min; pressure, 30 bar. V_0, void volume of the column. BSA and $(BSA)_2$: peak of bovine serum albumin and bovine serum albumin dimer (see also Ref. 7).

were added to the delipidated Ca^{2+}-ATPase. A molecular weight of 107000 ± 7200 (six experiments $\pm$ S.D.) was obtained. These results were independent of the ionic strength used for the centrifugation, since the same molecular weight was obtained in the presence of 0.5 M or 0.05 M NaCl, respectively. Furthermore no deviation from linearity was observed near the cell bottom, indicating the absence of dimers or higher aggregates in the preparation of the Ca^{2+}-ATPase. These results were obtained even after one week of storage at $-20°C$. To detect a small amount of aggregates, possibly sedimented at the bottom of the cell and not seen in sedimentation equilibrium analysis due to the damping of the scanner, reactivated Ca^{2+}-ATPase was submitted to HPLC gel filtration. The results are shown in Fig. 3. It is obvious that no protein appeared in the void volume, excluding the presence of higher aggregates. From the position of the peak in absorbance the relative molecular weight of the protein-detergent micelle can be estimated to be 200000 (see also Ref. 7). It is concluded from Figs. 2 and 3 that the reactivated Ca^{2+}-ATPase preparation contains more than 95% of the protein in its monomeric state (our estimated limit of detection).

Discussion

The characterization of the monomeric form of the sarcoplasmic reticulum Ca^{2+}-ATPase is often hampered by the fact, that in a given experiment it is not possible to account for the contribution of oligomers, especially if dodecyl octaoxyethylene glycol monoether ($C_{12}E_8$) is included to solubilize the protein. Nevertheless in a numerous amount of studies differences in the kinetic behaviour of the

Ca^{2+}-ATPase in either native vesicles or in a detergent containing solution are reported (see, for example, Refs. 2–4, 6, 14 and references therein). There now seems to be general agreement, that the monomeric Ca^{2+}-ATPase is able to perform ATP splitting and ATP synthesis, but that the kinetic parameters are quite different than those obtained with native vesicles. Therefore a regulatory role of protein-protein interactions was suggested.

We have previously shown that LL_{14} and Triton X-100 are the most suited detergents to solubilize the Ca^{2+}-ATPase in its monomeric form [7]. In contrast to Triton X-100, LL_{14} is able to maintain the activity and the state of aggregation of the reactivated Ca^{2+}-ATPase for several days (Fig. 1). These results could even be improved using the 2-deoxy derivative of LL_{14}, suggesting that the stability of the lysophosphatidylcholine determine the stability of the preparation. This modification of the lysophosphatidylcholine had no influence on the results shown in Figs. 2 and 3 (not shown). Sedimentation equilibrium analysis and HPLC gel filtration are the methods of choice to determine the molecular weight of very concentrated protein samples. Using HPLC gel filtration, a sample containing more than 20 mg protein per ml can be injected. This concentration is only slightly reduced in the peak fraction during the column run. On the other hand very small amounts of aggregates (less than 5% of the total protein) can be detected. The difference obtained in the molecular weight obtained for the monomeric Ca^{2+}-ATPase in sedimentation equilibrium analysis and HPLC gel filtration is due to (i) and inappropriate calibration of the HPLC column (see Ref. 7) and to (ii) the fact that in HPLC gel filtration experiments the molecular weight of the protein-detergent micelle is determined. In conclusion the preparation of monomeric Ca^{2+}-ATPase described above should be very useful to study the properties of a monomeric Ca^{2+}-ATPase with respect to native vesicles.

References

1 Lüdi, H. and Hasselbach, W. (1983) Eur. J. Biochem. 130, 5–8
2 Møller, J.V., Andersen, J.P. and Le Maire, M. (1982) Mol. Cell. Biochem. 42, 83–107
3 Lüdi, H., Rauch, B. and Hasselbach, W. (1982) Z. Naturforsch. 37c, 299–307
4 Martin, D.W., Tanford, C. and Reynolds, J.A. (1984) Proc. Natl. Acad. Sci. USA 81, 6623–6626
5 Dean, W.L. and Tanford, C. (1978) Biochemistry 17, 1683–1690
6 Silva, J.L. and Verjowski-Almeida, S. (1983) Biochemistry 22, 707–716
7 Lüdi, H. and Hasselbach, W. (1984) J. Chromatogr. 297, 111–117
8 Nestruck-Goyke, A.C. and Hasselbach, W. (1981) Eur. J. Biochem. 114, 339–347
9 Ramirez, F. and Marecek, J.F. (1978) Synthesis, pp. 601–603, Georg Thieme, Stuttgart
10 Hasselbach, W. and Makinose, M. (1963) Biochem. Z. 339, 94–111
11 De Meis, L. and Hasselbach, W. (1971) J. Biol. Chem. 246, 4759–4763
12 Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974) Proc. Natl. Acad. Sci. USA 71, 622–626
13 Tanford, C., Nozaki, Y., Reynolds, J.A. and Makino, S. (1974) Biochemistry 13, 2369–2376
14 Martin, D.W. (1983) Biochemistry 22, 2276–2282
15 Hasselbach, W. (1972) in Molecular Bioenergetics and Macromolecular Biochemistry (Weber, H.H., ed.), pp. 148–171, Springer Verlag, Berlin, Heidelberg and New York
16 Hasselbach, W., Lüdi, H. and Migala, A. (1983) Eur. J. Biochem. 132, 9–13
17 Ronzani, N., Migala, A. and Hasselbach, W. (1981) Eur. J. Biochem. 101, 593–606
18 Hardwicke, P.M.D. and Green, N.M. (1974) Eur. J. Biochem. 42, 183–193
19 Swoboda, G. and Hasselbach, W. (1982) Z. Naturforsch. 37c, 289–298
20 Dittmer, J.C. and Wells, M.A. (1969) Methods Enzymol. 14, 482–487