Ca^{++} TRANSPORT BY MEMBRANE-BOUND MONOMERIC Ca-ATPase OF SARCOPLASMIC RETICULUM

V. B. Ritov and N. S. Shcherbakova

UDC 612.744.16:546.41]:612.744.015.1]
.014.2:576.311.336

KEY WORDS: sarcoplasmic reticulum; ATPase; proteoliposomes.

Ca-dependent ATPase, responsible for active Ca^{++} transport, is the chief protein component of membranes of the sarcoplasmic reticulum (SPR) of fast skeletal muscles. The molecular weight of the catalytic polypeptide of Ca-ATPase is 100,000. Methods of obtaining the catalytically active monomeric form of the enzyme in a solution of detergents have now been developed [5, 9, 10]. These preparations can be used to determine the enzymic properties of monomeric ATPase, but they are unsuitable for the study of transport function because the ATPase exists in the form of mixed protein–detergent micelles. The discovery of a transport function in the case of monomeric ATPase is very interesting because it enables a choice to be made between the two models of the Ca^{++}-transporting unit at present available: oligomeric [13] or monomeric [12].

This paper describes a method of obtaining proteoliposomes containing the monomeric form of Ca-ATPase and it is shown that the monomeric form of the enzyme can transport Ca^{++}.

EXPERIMENTAL METHOD

Fragments of SPR from rabbit skeletal muscles and a membrane preparation of Ca-ATPase were obtained by the method in [1]. Proteoliposomes with different protein/lipid ratios were obtained by mixing a solution of ATPase and ovolecithin in medium containing 0.45 M sucrose, 0.5 M KCl, 15 mM Tris-HCl, pH 7.5, at 25°C and choleate in a concentration 10-15 mg/ml. The lecithin/choleate and protein/choleate ratios in the solutions were 1:0.5 and 1:1.5, respectively. The mixture of ATPase and ovolecithin was incubated for 30 min at 4°C and then passed twice through a column with the anion-exchange resin Dowex 2×4 (20-50 mesh) at 25°C to remove choleic acid. The column was equilibrated with ATPase-solubilizing medium not containing choleate. Protein SH-groups were determined by Ellman's method in medium containing 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, at 25°C, and 1 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). The protein concentration was 0.025 mg/ml. The proteoliposomes were treated with the bifunctional reagent 1,3-difluoro-4,6-dinitrobenzene (DFDNB) in medium containing 0.3 M sucrose, 0.5 M NaCl, 10 mM triethanolamine-HCl, pH 7.4, at 37°C and 1 mM DFDNB for 3 min. The protein concentration was 0.5 mg/ml. The reaction was stopped by the addition of glycine in a concentration of 50 mM. Disc electrophoresis in polyacrylamide gel was carried out in the presence of sodium dodecylsulfate [7]. To determine Ca^{++} transport the method of chlortetracycline fluorescence was used [4]. The fluorometric determination was carried out in medium containing 1 mM $MgCl_2$, 100 mM KCl, 1 mM ATP, 2 μM chlortetracycline, and 15 mM imidazole, pH 6.7, at 34°C. The protein concentration was 0.02-0.03 mg/ml. The protein concentration was determined with biuret reagent and with Amido black.

EXPERIMENTAL RESULTS

Electron-microscopic analysis, using a method of negative staining of material passed through the anion-exchange resin, showed that they contain closed vesicles with a mean diameter of about 100 nm. Most vesicles were formed by a single membrane. The method used thus allowed proteoliposomes to be reconstructed from a solution of protein and lipid. The writers showed previously by the freeze-etching method that the number of intramembranous particles on a shear surface of reconstructed proteoliposomes is considerably reduced if the lipid/pro-

Laboratory of Physical Chemistry of Biomembranes, Biological Faculty, M. V. Lomonosov Moscow University. (Presented by Academician of the Academy of Medical Sciences of the USSR S. E. Severin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 93, No. 4, pp. 21-23, April, 1982. Original article submitted November 4, 1981.

0007-4888/82/9304-0405$07.50

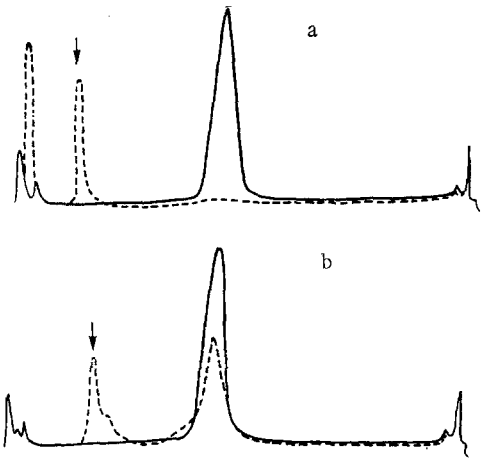

Fig. 1. Densitograms of gels after electrophoretic separation of control specimens of proteoliposomes (continuous line) and after treatment with DFDNB (broken line). a) Proteoliposomes with a protein/lipid ratio of 1:0.6; b) the same, with a ratio of 1:10. Arrow indicates beginning of separating gel.

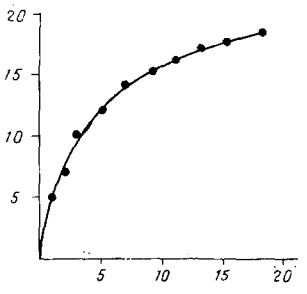

Fig. 2

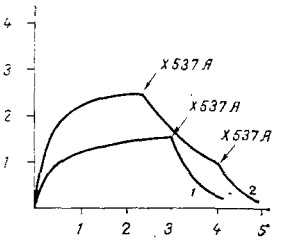

Fig. 3

Fig. 2. Titration of SH-groups of Ca-ATPase in reconstructed proteoliposomes (protein/lipid ratio 1:10 w/w) with DTNB. Abscissa, incubation time (in min); ordinate, modified SH-groups (in moles SH-groups/mole protein).

Fig. 3. Changes in intensity of fluorescence of chlortetracycline during Ca^{++} transport by reconstructed proteoliposomes. 1) Protein/lipid ratio 1:3, 2) 1:10. Abscissa, time (in min); ordinate, intensity of fluorescence (in relative units).

tein ratio rises [2]. This indicates that the ATP concentration in the membrane can be altered by dilution with lipid. To discover whether dissociation of the enzyme into monomers takes place on dilution, a method based on the use of the bifunctional cross-linking reagent DFDNB was adopted. Proteoliposomes with different lipid/protein ratios were incubated with excess of DFDNB and then subjected to electrophoretic separation. To estimate the degree of monomericity of the ATPase in the preparation, the ratio of the area of the peak of the catalytic polypeptide in the specimen treated with DFDNB to the area of the corresponding peak in the control specimen was used. The DFDNB concentration and incubation time were chosen so that practically complete cross-linking of the catalytic polypeptide took place in specimens with a low lipid/protein ratio. Densitograms of gels after electrophoretic separation of control specimens of proteoliposomes and after treatment with DFDNB are shown in Fig. 1. As Fig. 1a shows, when the protein/lipid ratio was 1:0.6, treatment with DFDNB led to the practically complete disappearance of the catalytic polypeptide, and most of the protein remained on the boundary between the separating and concentrating gels. Treatment of proteoliposomes obtained from a solution of ATPase and phospholipid in the ratio of 1:10 under the same conditions led to much less cross-linking of protein (Fig. 2b). The quantity of monomer of the catalytic polypeptide in preparations diluted with lipid was as a minimum 65%, but the possibility cannot be ruled out that the percentage of the monomeric form of ATPase in the reconstructed proteoliposomes was higher, for at the incubation temperature used (37°C) cross-linking of monomers can also take place as a result of diffusion collisions. The decrease in

the degree of cross-linking of the catalytic polypeptide on dilution with phospholipid was probably not connected with screening of groups reactive in relation to DFDNB. As Fig. 2 shows, all 18 SH-groups of the catalytic polypeptide, diluted with phospholipid in the ratio of 1:10 w/w remained accessible for DTNB [14], from which it can be postulated that reactive groups of ATPase preserve their accessibility for DFDNB also, a substance belonging to the class of bifunctional reagents passing through the membrane [15].

The results thus show that the method of separation used in fact allows the ATPase concentration in the membrane to be reduced, in agreement with earlier data obtained by electron microscopy [9]. Under these circumstances the ATPase in the diluted specimens exists chiefly in monomeric form.

To study whether the monomeric form of ATPase is capable of active Ca^{++} transport, the Ca^{++}-transporting function of proteoliposomes with different protein/lipid ratios was compared. The kinetics of Ca^{++} transport, measured as fluorescence of chlortetracycline, by proteoliposomes obtained by diluting the catalytic polypeptide with phospholipids in the ratios of 1:3 and 1:10 w/w is shown in Fig. 3. It will be clear from Fig. 3 that the Ca^{++}-transporting function of proteoliposomes with a large excess of phospholipid was better than that of proteoliposomes containing less phospholipid. This suggests that the monomeric form of ATPase in the membrane can not only hydrolyze ATP, but can also transport Ca^{++} actively. The appearance of a Ca^{++}-gradient in the reconstfucted proteoliposomes is due to the fact that addition of the Ca ionophore X537A leads to rapid outflow of Ca^{++} from the proteoliposomes (Fig. 3).

Two models of organization of the Ca^{++}-transporting pump of the SPR are currently being examined in the literature. According to one, Ca^{++} transport is carried out through a channel in the core of the catalytic polypeptide [12]. The other model postulates Ca^{++} transport through a channel formed in the region of contact between two or more catalytic polypeptides [13]. Many attempts have been made to detect oligomers of SPR Ca-ATPase by means of cross-linking reagents [3, 8, 11]. Most of these investigations have shown that a spectrum of oligomers of different composition — from dimers to aggregates of high molecular weight — is formed by the action of cross-linking reagents. This is probably due to the considerable contribution of diffusion collisions between ATPase molecules as a result of their high concentration in the membrane to the cross-linking process. Position of proteoliposomes with a high lipid/protein ratio enables the ATPase concentration in the membrane and, correspondingly the cross-linking with the bifunctional reagent on account of random collisions to be reduced. Moreover, it was found that ATPase exists in diluted preparations not as oligomers of a definite composition, such as dimers or tetramers, but in monomer form. Hence, if oligomers exist also in SPR membranes, the bond between the subunits in them is rather weak and can easily be broken by the action of detergent and phospholipid. The most important result is that the existence of ATPase in monomeric form does not affect its ability to transport Ca^{++}. Accordingly there is no need to regard the possible oligomeric organization of Ca-ATPase in the SPR membrane as an essential condition for the performance of its transport function.

LITERATURE CITED

1. O. M. Alekseeva and V. B. Ritov, Biokhimiya, 43, 1582 (1979).
2. N. S. Shcherbakova, V. B. Ritov, Yu. S. Tarakhovskii, et al., Biokhimiya, 45, 1503 (1980).
3. G. Bailin, Biochim. Biophys. Acta, 624, 511 (1980).
4. A. H. Caswell and S. Warren, Biochem. Biophys. Res. Commun., 46, 1757 (1972).
5. W. L. Dean and C. Tanford, Biochemistry (Washington), 19, 1683 (1978).
6. M. D. Hardwicke and N. M. Green, Eur. J. Biochem., 183, 183 (1974).
7. V. K. Laemmli, Nature, 227, 680 (1970).
8. C. F. Louis, M. J. Saunders, and J. A. Holroyd, Biochim. Biophys. Acta, 493, 78 (1977).
9. M. Le Maire, E. L. Kirsten, K. E. Jorgensen, et al., J. Biol. Chem., 253, 7051 (1978).
10. J. V. Moller, K. E. Lind, and J. P. Andersen, J. Biol. Chem., 255, 1912 (1980).
11. M. Poo and R. A. Cone, Nature, 247, 438 (1974).
12. A. E. Shamoo, T. L. Scott, and T. Ryan, J. Supramol. Struct., 6, 345 (1977).
13. M. Tada, T. Yamamoto, and Y. Tonomura, Physiol. Rev., 58, 1 (1970).
14. D. A. Thorley-Lawson and N. M. Green, Biochem. J., 167, 739 (1977).
15. M. G. P. Vale, Biochim. Biophys. Acta, 468, 114 (1977).