Oligomerization of sarcoplasmic reticulum Ca^{2+}-ATPase from rabbit skeletal muscle

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Received 18 August 1996; revised version received 17 September 1996

Abstract Although the primary structure and catalytic cycle of the sarcoplasmic reticulum Ca^{2+}-ATPase has been revealed, it is not well understood whether functional Ca^{2+} pump proteins exist in a monomeric or an oligomeric state in native skeletal muscle membranes. Here, we show that the Ca^{2+}-ATPase tends to form high molecular weight complexes, estimated to be dimers and tetramers using immunoblotting of two-dimensionally separated microsomal membranes following crosslinking. This agrees with both electron microscopical and biochemical findings which demonstrate that Ca^{2+}-ATPase clusters are the predominant molecular species in native membranes and that oligomerization may play a role in cooperative kinetics and enzyme stabilization.

Key words: Ca^{2+}-ATPase; Sarcoplasmic or endoplasmic reticulum calcium ATPase; Sarcoplasmic reticulum; Skeletal muscle

1. Introduction

Following contraction of skeletal muscle fibers, the rapid sequestration of calcium ions by the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase plays a central role in muscle relaxation [1]. In conjunction with fiber-type specific variations in the abundance of SR membranes, a differential Ca^{2+}-ATPase isoform expression pattern [2] appears to be the major feature underlying a higher Ca^{2+}-ATPase activity and a higher rate of Ca^{2+}-uptake in fast-twitch muscle as compared to slow-twitch muscle [1]. Three separate genes code for the mammalian sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase (SERCA). While isoform SERCA2b is expressed in slow-twitch skeletal muscle and cardiac muscle, SERCA1 is found in adult fast-twitch skeletal muscle fibers [3,4]. Based on biochemical and structural information, the tertiary model of the 110 kDa Ca^{2+}-ATPase predicts the protein to consist of a cytoplasmic head piece, a stalk domain and a transmembrane domain [3,5].

Although an enormous amount of progress has been made in the detailed elucidation of the molecular characteristics and reaction mechanisms of calcium uptake into the lumen of the SR [6–8], it is still under debate whether functional Ca^{2+}-ATPase molecules exist as monomeric or as oligomeric structures. Analysis of the secondary structure and topology of a single Ca^{2+}-ATPase copy predicts a sufficient number of transmembrane helices to constitute an ion channel [3,5]. Solubilized, monomeric Ca^{2+}-ATPase molecules are capable of performing all steps of the complex reaction cycle [9,10] and Ca^{2+}-induced crystallization results in the formation of monomeric units [11]. Reconstitution studies of Ca^{2+} pumps by Heegaard et al. [12] also indicate a monomeric unit model for the SR Ca^{2+}-ATPase.

In contrast, comparative freeze-fracture electron microscopy showed that 8.5 nm intramembranous particles of the SR membrane represent oligomeric clusters of Ca^{2+}-ATPase molecules as reviewed by Martonosi [13]. In addition, exclusion chromatography of detergent solubilized Ca^{2+}-ATPase preparations [14], fluorescence energy transfer measurements of native and reconstituted membranes [15], kinetic measurements on calcium translocation [16] and radiation inactivation studies of the Ca^{2+}-ATPase [17] all argue in favor of oligomeric SERCA structures. In addition, previous crosslinking studies of SR membranes indicate that a 110 kDa protein exhibits a tendency to form oligomeric forms as reviewed by Andersen [18]. However, these earlier reports are based on the interpretation of protein gels and did not employ immunoblot analysis to unequivocally identify the crosslinked complexes [19–22].

Thus, to address the question of whether the SR Ca^{2+}-ATPase exists in its native membrane environment as oligomeric units, we performed immunoblot analysis of two-dimensionally separated microsomal proteins following crosslinking. An important prerequisite for our analysis was the existence of highly specific antibodies which can accurately identify the Ca^{2+}-ATPase and do not immunologically crossreact with other components of the microsomal muscle membranes. We previously showed, using immunofluorescence microscopy of muscle cryosections, that monoclonal antibody IIH11 exclusively recognizes the fast-twitch isoform SERCA1 [23]. Hence, these antibodies were suitable probes for a reliable analysis of SR Ca^{2+}-ATPase oligomerization as presented in this study.

2. Materials and methods

2.1. Materials

Crosslinkers dithiobis(succinimidyl propionate) (DSP) and dithiobis(sulfosuccinimidyl propionate) (DTSSP) were obtained from Pierce (Rockford, IL). Protease inhibitors, acrylamide/bisacrylamide stock solutions and secondary, peroxidase-conjugated antibodies were purchased from Boehringer-Mannheim UK (Bell Lane, East Sussex). Prestained molecular weight markers were from Gibco BRL (Bethesda, MD) and Immobilon NC-pure nitrocellulose was from Millipore Corporation (Bedford, MA). All other chemicals used were of analytical grade and purchased from Sigma Chemical Company (Dorset, UK).

2.2. Isolation of muscle membranes

All preparatory steps were carried out at 4°C and all isolation buffers contained 1 μM of the following protease inhibitors: pefabloc,

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Abbreviations: DSP, dithiobis(succinimidyl propionate); DTSSP, dithiobis(sulfosuccinimidyl propionate); PBS, phosphate-buffered saline; SERCA, sarcoplasmic or endoplasmic reticulum calcium ATPase; SR, sarcoplasmic reticulum

PII S0014-5793(96)01106-4

antipain, pepstatin, E64, chymostatin, leupeptin, benzamidine and trypsin inhibitor. Freshly dissected skeletal muscles from New Zealand white rabbits were homogenized for 3×20 s in 30 mM Tris-HCl, pH 7.4, 10% (w/v) sucrose, 0.5 mM EDTA using a Kenwood blender. Following centrifugation for 15 min at 1100×*g*, the microsomal fraction was obtained by centrifugation of the supernatant fraction for 35 min at 140000×*g*. Microsomes were resuspended at a final protein concentration of 10 mg/ml and immediately used for crosslinking experiments. Ca^{2+}-ATPase activity was measured as described previously [24] and protein concentration was determined according to Bradford [25] using bovine serum albumin as a standard.

2.3. Chemical crosslinking

Crosslinking of muscle membranes was performed with homobifunctional *N*-hydroxysuccimide crosslinkers according to Lomant and Fairbanks [26]. Microsomes were treated at room temperature in phosphate-buffered saline (50 mM phosphate, 0.9% (w/v) NaCl; PBS) for 30 min at pH 8 using 0, 10, 50, 100, and 200 μg of DSP or DTSSP per mg protein. Crosslinking was terminated by the addition of 50 μl of 1 M ammonium acetate per ml reaction mixture. Samples were then immediately solubilized with 4% (w/v) sodium dodecyl sulfate and protein complexes electrophoretically separated for further analysis.

2.4. Gel electrophoresis

Electrophoretic analyses were performed using a Hoefer electrophoresis unit SE600 (Hoefer Scientific Instruments, San Francisco, CA) with 50 μg protein per lane. For one-dimensional sodium dodecyl sulfate gel electrophoresis, proteins were treated with non-reducing sample buffer as described by Laemmli [27] and separated on 3–12% gradient gels for 2000 Vh. Silver staining of electrophoretically separated proteins was performed as previously described [28]. For two-dimensional gel electrophoresis, 7% rod gels were run under non-reducing conditions for 2000 Vh, subsequently reduced for 10 min using sample buffer complemented with 2% (v/v) β-mercaptoethanol [27], transferred onto the top of 3–12% slab gels and then run for a further 2000 Vh.

2.5. Immunoblot analysis

Electrophoretically separated proteins were transferred to nitrocellulose for 90 min at 100 V by the method of Towbin et al. [29] using a Hoefer Transfor unit TE50X (Hoefer Scientific Instruments, San Francisco, CA). Efficiency of transfer was evaluated by Ponceau-S-Red staining of membranes followed by destaining in PBS and blocking for 2 h in 5% (w/v) fat-free milk powder dissolved in PBS, pH 7.4 (blotto). Membranes were then incubated overnight with monoclonal antibody IIH11 to the fast-twitch Ca^{2+}-ATPase SERCA1 [23], diluted 1:1000 in blotto. Following washing twice for 10 min in blotto, blots were incubated with 1:1000 diluted peroxidase-conjugated secondary antibodies for 1 h, washed as described above and then developed using 4-chloronaphthol as a substrate [30]. Densitometric scanning of immunoblots was carried out on a Molecular Dynamics 300S computing densitometer using Image Quant V3.0 software.

3. Results and discussion

A component of central importance for skeletal muscle relaxation is the intensively studied SR Ca^{2+}-ATPase. To investigate the oligomeric status of the fast-twitch isoform SERCA1, we performed immunoblot analysis of two-dimensionally separated microsomal muscle proteins following crosslinking. We analyzed crude, non-salt treated microsomal preparations so as to avoid the introduction of potential artefacts. Thus, the detection of the potential formation of oligomeric complexes was only limited by the efficiency of crosslinking, the resolution of the two-dimensional gel electrophoresis technique and the sensitivity of the antibodies used in the immunoblotting technique. Pilot crosslinking experiments with DSP led to the use of 10–200 μg crosslinker per mg microsomal protein. Crosslinking conditions were optimized with respect to temperature, pH, buffer and incubation time according to the recommendations of Wong [31]. Monoclonal antibody IIH11 used for our immunoblot analysis was previously shown to be highly specific for SERCA1 [23], which makes it an ideal probe for investigating monomeric and oligomeric structures of the fast-twitch isoform of the SR Ca^{2+}-ATPase.

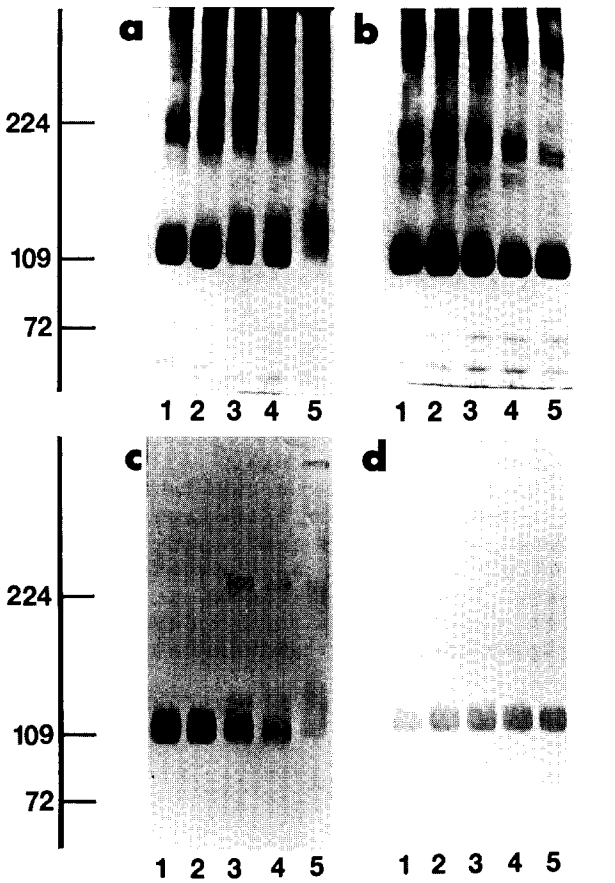

Fig. 1. Crosslinking of SERCA1 Ca^{2+}-ATPase. Shown are silver-stained SDS polyacrylamide gels under non-reducing conditions (a, b) and identical immunoblots stained with monoclonal antibody IIH11 to the fast-twitch isoform SERCA1 of the sarcoplasmic reticulum Ca^{2+}-ATPase from skeletal muscle (c, d). Lanes 1–5 represent the microsomal fraction following crosslinking with 0, 10, 50, 100 and 200 μg crosslinker DSP (a, c) or DTSSP (b, d) per mg protein. Molecular weight standards (MW×10^{-3}) are indicated on the left.

Using the hydrophobic crosslinker DSP, the SERCA1 Ca^{2+}-ATPase isoform was found to form high molecular weight oligomers as illustrated by one-dimensional SDS polyacrylamide gel electrophoresis and immunoblot analysis (Fig. 1). Silver staining revealed that increasing concentrations of DSP in the incubation mixture resulted in a decrease of an approximately 110 kDa protein band and the concurrent appearance of higher molecular weight protein bands (Fig. 1a). Immunoblotting of identical nitrocellulose transfers confirmed that the 110 kDa species of microsomes is partially representative of SERCA1. The amount of monomeric Ca^{2+}-ATPase units decreased with higher concentrations of crosslinker, while two major higher molecular weight oligomeric structures were clearly detectable (Fig. 1c). Comparison with contractile proteins of very high molecular weight such as myosin, nebulin and titin (not shown) suggests that the major crosslinked species of SERCA1 are dimers and tetramers. At the

highest crosslinker concentration shown, the predominant Ca^{2+}-ATPase species was estimated to be a tetramer. Interestingly, the water-soluble analogue of DSP, DTSSP, did not result in significant crosslinking of SERCA1 under identical incubation conditions (Fig. 1d). Hence, crosslinking of Ca^{2+}-ATPase monomers probably occurs in or close to hydrophobic domains of this integral membrane protein. In addition, the immunoblot analysis of untreated microsomal membranes (Fig. 1c,d) suggests that protein bands of apparent 200 kDa and 400 kDa (Fig. 1a,b) do not contain SERCA1 oligomers and that these protein bands must thus represent other abundant high molecular weight muscle proteins. Alternatively,

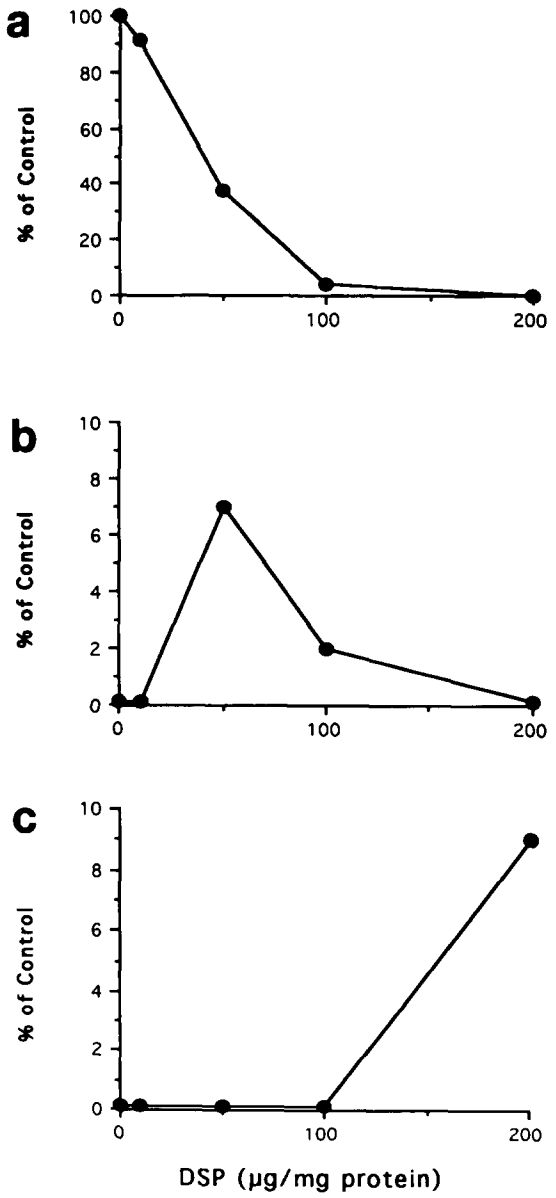

Fig. 2. Densitometric analysis of oligomerization of SERCA1 Ca^{2+}-ATPase. As determined by volume integration of immunoblots (see Fig. 1c), increasing amounts of crosslinker DSP result in the decrease of the 110 kDa SERCA1 monomer (a) and the concurrent increase in oligomerization of SERCA1. At the highest concentration of DSP shown the predominant complex of SERCA1 appears to be a tetramer (c) while dimers (b) are only observed at intermediate crosslinker concentrations.

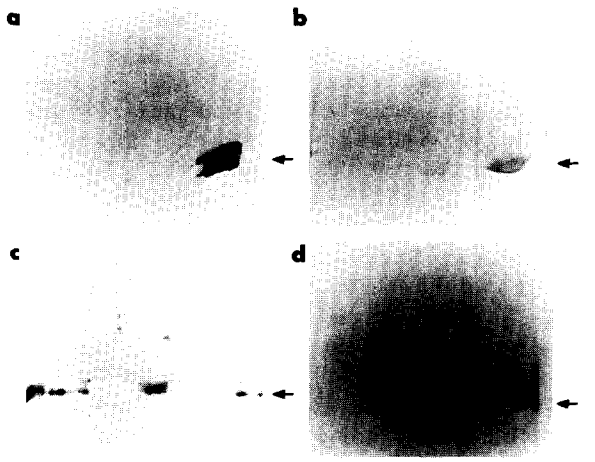

Fig. 3. Immunoblot analysis of SERCA1 Ca^{2+}-ATPase oligomerization. Shown are immunoblots of two-dimensional gels run in the first dimension as 7% rod gels under non-reducing conditions and electrophoresed in the second dimension as 3–12% slab gels under reducing conditions in order to detect crosslinked complexes off the diagonal. Immunoblots were stained with monoclonal antibody IIH11 to SERCA1 and represent the microsomal fraction following crosslinking with 0 (a, b) and 100 (c, d) μg crosslinker DSP (a, c) or DTSSP (b, d) per mg protein. The positions of the 110 kDa SERCA1 species following reduction are indicated by arrows.

monoclonal antibody IIH11 may not recognize native aggregates of the Ca^{2+}-ATPase, although this is unlikely since this antibody detects crosslinked complexes of the enzyme. The densitometric analysis shown in Fig. 2 summarizes the results of our crosslinking analysis of SERCA1 from skeletal muscle SR. Ca^{2+}-ATPase dimers were transiently observed at intermediate concentrations of DSP, while 200 μg DSP per mg protein resulted predominantly in the formation of apparent tetramers.

To confirm the crosslinking of SERCA1 by DSP, two-dimensional electrophoresis with a non-reducing first dimension and a reducing second dimension was performed in order to detect high molecular weight crosslinked complexes of the 110 kDa SERCA1 species off the gel diagonal. As can be seen in the immunoblot analysis of Fig. 3, incubation of microsomal membranes with DSP results in a shift from monomeric 110 kDa SERCA1 to higher molecular weight species. Fig. 3c shows a representative blot of microsomes treated with a crosslinker concentration of 100 μg/mg protein. At this intermediate concentration of DSP, dimers and higher molecular weight complexes are present. Tetramers of apparent 440 kDa appear to represent the predominant complex of the SR Ca^{2+}-ATPase in native skeletal muscle membranes. As already illustrated in Fig. 1d, DTSSP did not significantly crosslink monomeric SERCA1 as evidenced by the lack of immunoreactive protein bands of higher molecular weight off the diagonal in two-dimensional gel electrophoresis (Fig. 3d). Measurement of Ca^{2+}-ATPase activity showed no significant difference between untreated control microsomes and membranes crosslinked with DSP or DTSSP (not shown). This agrees with findings from Kurobe et al. [22] who demonstrated that DSP crosslinking does not inactivate the enzyme. Furthermore, extensive crosslinking of the Ca^{2+}-ATPase seems not to alter the number and appearance of 8.5 nm intramembranous particles in the sarcoplasmic reticulum

membrane as judged by freeze-etching electron microscopy [20].

In analogy to various biochemical findings [14–17] and electron microscopical data which suggest an oligomeric structure for the SR Ca^{2+}-ATPase [13], we provide in this report biochemical evidence that this abundant muscle calcium ion pump exists in its native membrane environment as a quaternary structure in a multi-copy complex of 110 kDa units. Previous crosslinking studies of the sarcoplasmic reticulum used protein gels for the analysis of the microsomal 110 kDa protein [18–22]. Thus, these studies did not unequivocally identify the observed oligomeric complexes as multiple copies of specific isoforms of the Ca^{2+}-ATPase since the 110 kDa band in microsomes or SR is a heterogeneous band consisting of several muscle membrane proteins of similar electrophoretic mobility [32]. In contrast, using immunoblotting, this report eliminated potential erroneous interpretations of oligomeric complexes formed by 110 kDa units which do not represent the SR Ca^{2+}-ATPase.

Although analysis of the primary structure of SERCA1 suggests that sufficient transmembrane domains exist in a monomer to provide an ion channel [3,5], our results demonstrate the existence of Ca^{2+}-ATPase tetramers as being the predominant unit of Ca^{2+} pumping in native SR membranes. It remains to be determined how exactly intermolecular associations between SERCA1 units have an effect on the regulation of calcium handling and relaxation in muscle fibers. Possibly, interactions between 110 kDa SERCA1 units are important for cooperative kinetics and stabilization of the ion transfer process under physiological conditions [13].

Acknowledgements: We wish to thank Cliona Feighery for technical assistance and Dr. Kevin P. Campbell for a generous gift of monoclonal antibody IIH11. This work was supported by grants from the Wellcome Trust, London and the Health Research Board, Dublin.

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