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# Polymorphism of sarcoplasmic-reticulum adenosine triphosphatase of rabbit skeletal muscle

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Antibody was raised in chickens against purified sarcoplasmic-reticulum $Ca^{2+}$-activated ATPase ($Ca^{2+}$-ATPase). The immunological relationship between the $Ca^{2+}$-ATPase of fast-muscle and slow-muscle sarcoplasmic reticulum was investigated by a one-step and a two-step competitive enzyme-linked immunosorbent assay (ELISA). The results show marked antigenic differences between the membrane-bound $Ca^{2+}$-ATPase of the sarcoplasmic-reticulum vesicles from fast muscle and slow muscle, beside differences in the membrane content of ATPase protein.

A still controversial problem is whether the differences in relaxation rates between fast-twitch and slow-twitch skeletal muscles may be accounted for by differences in the total number and density of the $Ca^{2+}$-pump ($Ca^{2+}$-activated ATPase) units in the SR membranes, by qualitative structural differences in the ATPase protein, or both (Beringer, 1975; Heilmann *et al.*, 1977; Wang *et al.*, 1979). The possible existence of this protein in different polymorphic forms according to the type of muscle (Margreth *et al.*, 1975) is supported by study of partial reactions of the $Ca^{2+}$-ATPase in the isolated SR from fast and slow skeletal muscle and from cardiac muscle (Wang *et al.*, 1979; Sumida *et al.*, 1978). De Foor *et al.* (1980) reported a low degree of immunological cross-reactivity between the SR of predominantly fast skeletal muscles and of cardiac muscle from different species. We have studied the antigenic properties of purified SR membranes from pure fast-twitch and slow-twitch muscle of the rabbit with antibody to fast-muscle SR $Ca^{2+}$-ATPase, by an enzyme-linked immunosorbent assay, and show marked qualitative differences in the pattern of reactivity by a competitive inhibition test.

## Materials and methods

SR vesicles were isolated from pure fast-twitch and slow-twitch muscle of the rabbit by methods described previously (Salviati *et al.*, 1979; Margreth *et al.*, 1980). The pelleted material (80000*g* for 60min) was suspended and incubated at 0–4°C overnight with 0.3M-sucrose/1mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.5, and after dilution with an equal volume of 1.2M-KCl/0.3M-sucrose/1mM-Hepes, pH 7.5, was centrifuged at 100000*g* for 60min, and further washed once with 0.25M-sucrose. These purification steps resulted in complete removal of phosphorylase, as judged by polyacrylamide-gel electrophoresis by the method of Laemmli (1970; see below). For further purification, the SR vesicles were centrifuged at 140000*g* for 90min in a linear (0.3–2.0M) sucrose gradient and fractions banding in the concentration range 0.6–0.9M-sucrose were pooled and stored at −20°C, unless immediately used.

These fractions were virtually devoid of mitochondrial inner-membrane fragments, based on measurements of succinate:cytochrome *c* reductase activity (Salviati *et al.*, 1979). Likewise, measurements of plasma-membrane markers, e.g. ability to bind [$^3$H]ouabain, assayed as described by Lau *et al.* (1979) in the presence of 0.05% deoxycholate, and $K^+$-stimulated ouabain-sensitive *p*-nitrophenyl phosphatase activity, assayed as described by Skou (1974), gave values of about 2pmol of [$^3$H]ouabain bound/mg of protein, under steady-state conditions, and of 0.7μmol of *p*-nitrophenol formed/h per mg of protein, i.e. about 60 and 20 times lower, respectively, than the corresponding values for purified sarcolemmal membranes (Adams *et al.*, 1979; Weglicki *et al.*, 1980).

Cholate-treated SR vesicles (Meissner *et al.*, 1973; Salviati *et al.*, 1979) from fast muscle, containing the $Ca^{2+}$-ATPase in virtually pure form

Abbreviations used: SR, sarcoplasmic reticulum; ELISA, enzyme-linked immunosorbent assay.

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(see Fig. 1*a*), were used as the immunogen for the production of antisera in adult hens, essentially as described by Stewart *et al.* (1976). Each animal received three intramuscular injections, given at weekly intervals, and a fourth injection, after 10 days, of 0.5 mg of protein incorporated into Freund's complete adjuvant (Difco, Detroit, MI, U.S.A.). A booster injection was given at 60 days from the beginning of the immunization. The antiserum used in the present study was from a single animal that was bled at 70 days. The $\gamma$-globulin fraction was isolated by a $Na_2SO_4$ precipitation method (Orlans *et al.*, 1961). The specificity of antibody was tested by the gel-electrophoresis-derived-enzyme-linked-immunosorbent-assay (GEDELISA) method described by Lutz *et al.* (1978), after electrophoresis of the SR membranes by the method of Weber & Osborn (1969). It was verified that under these conditions the antibody was reactive with the 100000-mol.wt. $Ca^{2+}$-ATPase protein band, and with no other band.

Polyacrylamide-gel electrophoresis of SR-membrane protein was performed as described by Weber & Osborn (1969) in 10% acrylamide gels, or by a modification of the Laemmli (1970) method using a polyacrylamide exponential-gradient gel (5–18.5%).

Antibody to rabbit fast-muscle $Ca^{2+}$-ATPase was tested by the ELISA assay, under the general conditions described previously for other antigens (Biral *et al.*, 1979; Volpe *et al.*, 1981), in microtitre wells coated with the SR vesicles. For the two-step competitive assay (Engvall, 1980), the conditions were derived from Rennard *et al.* (1980): 0.6 ml of antibody solution (20 $\mu$g/ml) was incubated with an equal volume of suspension of SR vesicles, at various protein concentrations (see legend to Fig. 3), for 16–18 h, at 0–4°C; 0.2 ml of each incubated sample was transferred to microtitre wells coated with fast-muscle SR vesicles (5 $\mu$g/ml) and the ELISA test was carried out as in the one-step procedure (Biral *et al.*, 1979; Volpe *et al.*, 1981).

## Results

SR vesicles from fast and slow muscle were characterized by their protein and enzymic composition. Polyacrylamide-gel electrophoresis (Fig. 1) showed that the SR vesicles were highly enriched with respect to the $Ca^{2+}$-ATPase (mol.wt. 100000), though the peptide pattern was more complex for slow-muscle SR (see Heilmann *et al.*, 1977; Salviati *et al.*, 1979), which contained additional components beside calsequestrin and high-affinity $Ca^{2+}$-binding protein (Meissner *et al.*, 1973; Michalak *et al.*, 1980).

The rate of $Ca^{2+}$ uptake, measured by a murexide method (Scarpa, 1979), in the presence of oxalate,

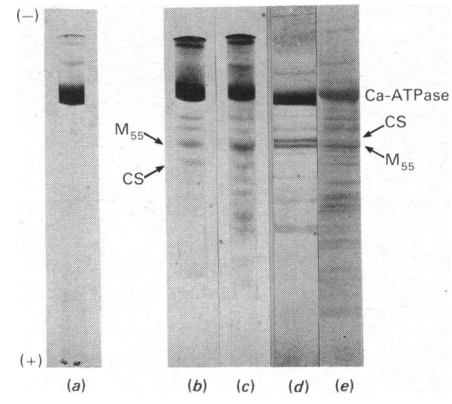


Fig. 1. *SDS/polyacrylamide-gel electrophoresis of SR vesicles from fast and slow muscle*

(*a*) Cholate-treated SR vesicles from fast muscle; (*b*) and (*d*) untreated SR vesicles from fast muscle; (*c*) and (*e*) untreated SR vesicles from slow muscle. Abbreviations: CS, calsequestrin; $M_{55}$, $M_{55}$ protein. Electrophoresis was carried out as described by Weber & Osborn (1969) in (*a*)–(*c*) and by Laemmli (1970) in (*d*) and (*e*). Under the two conditions used, CS had an apparent mol.wt. of 44000 or of 63000, respectively (see Michalak *et al.*, 1980). About 30 $\mu$g (*a*, *d*, *e*) or 40 $\mu$g (*b*, *c*) of protein was applied per gel.

was 1.89 and 0.45 $\mu$mol/min per mg of protein for the SR vesicles from fast and from slow muscle respectively. The corresponding values for the initial velocity of $Ca^{2+}$-ATPase, measured spectrophotometrically (Warren *et al.*, 1974) with a Ca–EGTA buffer at a free $Ca^{2+}$ concentration of about 10 $\mu$M, were 5.4 and 0.7 $\mu$mol of $P_i$/min per mg of protein, on average. Steady-state values of the phosphorylated enzyme E ~ P, measured as described by Meissner *et al.* (1973), after 6 s incubation at 0°C, were 4.4 and 0.6 nmol/mg of protein for fast and slow muscle respectively. In agreement with earlier data in the literature (Heilmann *et al.*, 1977; Wang *et al.*, 1979), slow-muscle SR also exhibited a higher basal (EGTA-insensitive), azide- and oligomycin-insensitive, $Mg^{2+}$-ATPase activity than did fast-muscle SR (1.8 and 0.9 $\mu$mol/min per mg of protein respectively). On the basis of densitometric measurements on the electrophoretic gels in Fig. 1, the ATPase protein accounted for 70–80% of the total membrane protein of fast-muscle SR and for not less than 40–50% of the total protein of slow-muscle SR.

The antigenic relationship between the $Ca^{2+}$-ATPase of the SR vesicles from fast and slow muscle was first investigated by a one-step immunoassay (Biral *et al.*, 1979), with anti-(rabbit fast-muscle $Ca^{2+}$-ATPase), in microtitre wells coated with SR vesicles (see the Materials and methods

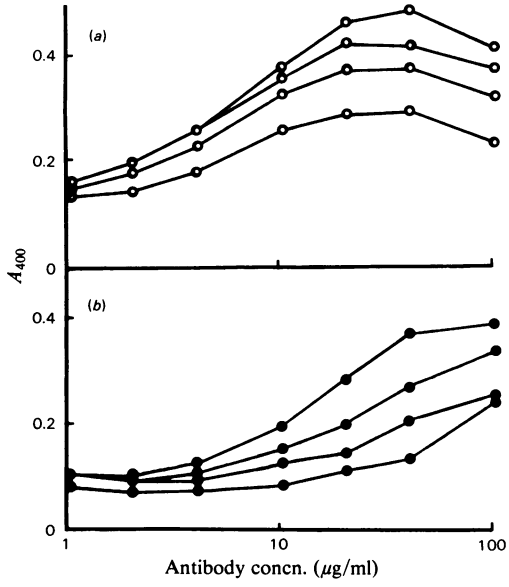


Fig. 2. *Titration of chicken anti-(rabbit fast-muscle $Ca^{2+}$-ATPase) in microtitre wells coated with SR membranes isolated from rabbit fast and slow muscle*

Alkaline phosphatase activity, measured from the formation of yellow *p*-nitrophenol at 400nm (see Biral *et al.*, 1979), is expressed as a function of antibody concentration in microtitre wells coated with SR membranes at protein concentrations of 5, 4, 3 or 2μg/ml for fast muscle (O), and of 5, 4, 2 or 1μg/ml for slow muscle (●) (from top to bottom curve, in the stated order).

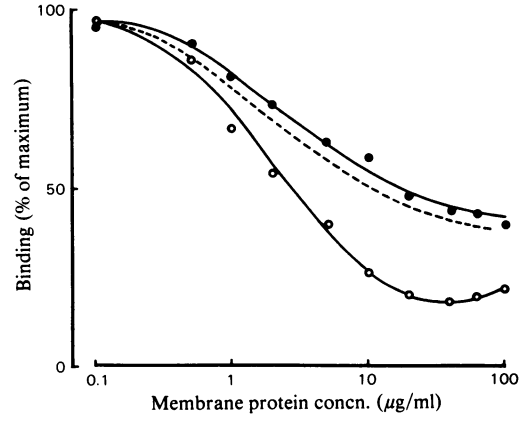


Fig. 3. *Inhibition of binding of anti-(fast-muscle $Ca^{2+}$-ATPase) to microtitre wells coated with SR membranes from fast muscle by prior incubation of antibody with SR membranes from fast and from slow muscle*

ELISA assay was carried out as in Fig. 2. O, Inhibition curve with SR membranes from fast muscle; ●, inhibition curve with SR membranes from slow muscle. The broken line is that for slow muscle normalized by multiplication by 0.6. Percentage values of antibody bound to antigen-coated plate are given as means of three (O) or two (●) immunoassays.

section). Circumstantial evidence obtained by electron microscopy (I. Mussini & D. Biral, unpublished work) of negatively stained SR vesicles had shown that the vesicles under the conditions of the ELISA test underwent some degree of fragmentation, but retained the basic membrane ultrastructure, with the characteristic 3–4nm (30–40Å) outer projections corresponding to the ATPase protein (Stewart *et al.*, 1976).

Titration of anti-(fast-muscle $Ca^{2+}$-ATPase) at several concentrations of SR membrane protein (Fig. 2) gave a typical dose–response curve, as described for ELISA with other antigens (Engvall, 1980). However, both the response pattern and the concentration of antibody for half-maximal saturation of antigen differed markedly between the SR vesicles from fast and slow muscle.

The extent of cross-reactivity between the ATPase protein of fast and slow SR was quantified by a two-step competitive-inhibition test (Fig. 3), i.e. from the ability of either type of SR membrane to inhibit the binding of anti-(fast-muscle $Ca^{2+}$-ATPase) to microtitre wells coated with the immunogen. Fig. 3 shows that 50% inhibition was obtained with the SR from slow muscle at a much higher concentration of total membrane protein. By normalizing the curve for slow-muscle SR to the fast-muscle SR ATPase content (on average 48% and 80% of the total membrane protein respectively), an index of dissimilarity (De Foor *et al.*, 1980) was obtained of about 4.

## Discussion

The present results lend support to the suggestion (De Foor *et al.*, 1980) that a limited number of antigenic determinants are shared by the SR $Ca^{2+}$-ATPase of different types of muscles and that the ATPase of cardiac (De Foor *et al.*, 1980) and of slow-twitch muscle (the present work) possess some unique structural features. Since the activity of the SR $Ca^{2+}$-ATPase does not seem to be modified after binding to specific antibody (Tada *et al.*, 1979), one may have to postulate that these structural features of the ATPase molecule are unrelated to the active site. On the other hand, additional differences between the $Ca^{2+}$-ATPase of cardiac and slow-muscle SR, and of fast-muscle SR, appear to relate to modulation of the ATPase activity by phosphorylation of the regulatory protein phospho-

lamban catalysed by cyclic AMP- and calmodulin-dependent protein kinase (Le Peuch *et al.*, 1979; Margreth *et al.*, 1975; Tada *et al.*, 1980).

At the present time, it cannot be said with any certainty whether the antigenic determinants responsible for the immunological heterogeneity between the $Ca^{2+}$-ATPase of fast-twitch and slow-twitch muscle may be sequential or configurational determinants. In the latter case, particular three-dimensional configurations could be influenced by phospholipid–protein interactions in the micro-environment of the ATPase in membrane-bound form. In spite of these reservations concerning interpretation of the structural basis for the immunological diversity between the SR $Ca^{2+}$-ATPase of fast-twitch and slow-twitch muscle, it is biologically appealing to consider this as an additional example of muscle protein polymorphism under the epigenetic influence of the nerve, i.e. reminiscent of the structural and immunological heterogeneity between fast- and slow-muscle myosins (Gauthier & Lowey, 1979).

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