Molecular and antigenic properties of cytochrome b_5 from slow-muscle sarcoplasmic reticulum

Giovanni SALVIATI, Sergio SALVATORI, Romeo BETTO and Alfredo MARGRETH*
*Consiglio Nazionale delle Ricerche Centro di Studio per la Biologia e la Fisiopatologia Muscolare,
Istituto di Patologia Generale, Università di Padova, Via Loredan 16, 35100 Padova, Italy*

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NADH–cytochrome b_5 reductase and cytochrome b_5 associated with slow-muscle sarcoplasmic reticulum and liver microsomal fraction were identified with discrete protein bands of molecular weights 33 000 and 16 700 by polyacrylamide-gel electrophoresis. Purified detergent-extracted cytochrome b_5 from muscle sarcoplasmic reticulum is indistinguishable from liver microsomal cytochrome b_5 with respect to spectral properties, pI values and immunological reactivity with antibody to the liver cytochrome b_5 . Reaction of the antibody with membrane-bound cytochrome b_5 inhibits the sarcoplasmic-reticulum NADH–cytochrome c reductase activity.

The occurrence of a cytochrome b_5 -linked stearyl-CoA desaturase system has been demonstrated in the isolated sarcoplasmic reticulum of rabbit slow-twitch muscle, following earlier observations (Margreth *et al.*, 1971). By these enzymic properties, as well as the electrophoretic pattern of protein composition (Margreth *et al.*, 1977), slow-muscle sarcoplasmic reticulum appears to be much more diversified than that of fast-twitch muscle and to contain intrinsic membrane proteins shared by the smooth endoplasmic reticulum of other cells.

Our previous results (Salviati *et al.*, 1979) showed that the NADH–cytochrome b_5 reductase and cytochrome b_5 of slow-muscle sarcoplasmic reticulum and of liver microsomal fraction are similar in several properties. This interpretation is further supported by the results now reported, which demonstrate, in particular, the presence of cytochrome b_5 with identical molecular and antigenic properties in both membranes.

Materials and methods

Sarcoplasmic-reticulum fragments were isolated from 10% (w/v) homogenates of rabbit slow-twitch muscle (soleus and semitendinosus) by methods identical with those reported by Salviati *et al.* (1979) and were washed once with 0.6 M-KCl solution. The final suspension in 0.3 M-sucrose/1.0 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH buffer, pH 7.5 (about 5.0 mg of protein/ml), was stored frozen at -20°C before use. Rabbit

liver microsomal fraction was isolated by the procedure of Jones & Wakil (1967). Detergent-extracted cytochrome b_5 (D-cytochrome b_5) and NADH–cytochrome b_5 reductase were purified from the membrane preparations by the methods of Strittmatter *et al.* (1978) and Mihara & Sato (1978) respectively. The supernatant was passed through a column of DEAE-cellulose (Whatman DE-52) to separate the two proteins. D-cytochrome b_5 was eluted from the column with 90 mM-Tris/acetate buffer, pH 8.1, containing 0.9 mM-EDTA, 2% (w/v) Triton X-100 and 70 mM-NaSCN, and purified by chromatography on a DEAE-cellulose and Sephadex G-75 and G-25 columns (Strittmatter *et al.*, 1978). The yields of D-cytochrome b_5 , measured by differential spectrometry with dithionite (Salviati *et al.*, 1979), were about 8 μg /mg of protein and 2 μg /mg of protein for liver microsomal fraction and slow-muscle sarcoplasmic reticulum respectively. Liver and muscle D-cytochrome b_5 had identical spectral properties, with a 409 nm minimum, a 424 nm maximum and two smaller absorption peaks at 526 nm and 556 nm. Liver NADH–cytochrome b_5 reductase was further purified by chromatography on a DEAE-Sephadex A-50 column and then on a hydroxyapatite column (Mihara & Sato, 1978). NADH–cytochrome c and NADH–potassium ferricyanide reductase activities were measured as described by Salviati *et al.* (1979).

Antibody against liver D-cytochrome b_5 was raised in adult hens. Each animal received four intramuscular injections, at weekly intervals, of 0.5 mg of liver D-cytochrome b_5 incorporated into

* To whom correspondence should be addressed.

complete Freund's adjuvant (Difco, Detroit, MI, U.S.A.). The animals were bled 7 days after the last injection. Specific antibodies were isolated from immune sera by affinity chromatography, with a column of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) coupled with D-cytochrome b_5 (5 mg of protein/g of gel) (Biral *et al.*, 1979). Antibody was tested with purified D-cytochrome b_5 from liver and muscle by an enzyme-linked immunosorbent assay method under the general conditions described by Biral *et al.* (1979). Microtitre wells were coated with antigen (0.7 µg/ml). Inhibition of NADH-cytochrome b_5 activity by antibody was determined as described by Remacle (1978).

Polyacrylamide-gel electrophoresis of liver and muscle membrane preparations, and of purified D-cytochrome b_5 and NADH-cytochrome b_5 reductase, was conducted by the procedure of Laemmli (1970) with an exponential polyacrylamide gel gradient prepared by mixing two solutions of 5% and 22.5% acrylamide in a chamber (Buchler universal density-gradient mixer) of constant volume (5.5 ml).

Isoelectrofocusing of D-cytochrome b_5 was performed by the procedure of O'Farrell (1975) with 1% Ampholines (0.8% of pH range 5–7 and 0.2% of pH range 3.5–10) (LKB Produkter, Stockholm, Sweden).

Results and discussion

The biochemical properties of the isolated sarcoplasmic reticulum from rabbit slow-twitch muscle have been described previously (Salvati *et al.*, 1979). Slab-gel electrophoresis with exponential polyacrylamide gradient, however, reveals a more complex peptide composition of the sarcoplasmic-reticulum membranes than disc-gel electrophoresis by the procedure of Weber & Osborn (1969), owing to a better resolution of peptides of molecular weight below 50 000. On comparison of slow-muscle sarcoplasmic reticulum with liver microsomal fraction (Plate 1), major peptides can be identified characteristic of each type of membrane, e.g. peptides of apparent molecular weights about 100 000, 65 000 and 55 000, corresponding to the Ca^{2+} -dependent ATPase, calsequestrin and the high-affinity Ca^{2+} -binding protein (Michalak *et al.*, 1980), which are ubiquitous components of the sarcoplasmic-reticulum membranes but are not seen in membranes from non-muscle tissues.

Among the peptides shared by slow-muscle sarcoplasmic reticulum and liver microsomal fraction, two minor bands are shown to co-electrophorese with liver D-cytochrome b_5 and NADH-cytochrome b_5 reductase (Plate 1), of molecular weights 16 700 (Spatz & Strittmatter, 1971) and

33 000 (Mihara & Sato, 1978) respectively. The cytochrome b_5 band appears to be fainter for slow-muscle sarcoplasmic reticulum than for liver microsomal fraction, as expected from the respective contents of cytochrome b_5 (Salvati *et al.*, 1979) and the actual yield of the purified cytochrome from these membranes (see the Materials and methods section). On the basis of these results, the native content of cytochrome b_5 in slow-muscle sarcoplasmic reticulum is about one-quarter that of liver microsomal fraction. Interestingly, the ability for maximum incorporation of the haemoprotein *in vitro* appears much more similar. In a typical experiment, 6.6 mg of sarcoplasmic-reticulum membrane protein, with an original content of 0.29 nmol of cytochrome b_5 /mg of protein, was incubated in the presence of 0.12 mg of D-cytochrome b_5 in 90 mM-Tris/acetate buffer, pH 8.1, as described by Strittmatter *et al.* (1972). The final cytochrome b_5 bound to the sarcoplasmic-reticulum membrane was 8.8 nmol/mg of protein, as compared with a reported value of 10.1 nmol of cytochrome b_5 /mg of protein for liver microsomal fraction (Remacle, 1978). We have already shown in sarcoplasmic-reticulum vesicles enriched with D-cytochrome b_5 by another method (Salvati *et al.*, 1979) that the exogenous cytochrome is readily reduced by the endogenous NADH-cytochrome b_5 reductase in the presence of NADH.

Identification of the flavoprotein NADH-cytochrome b_5 reductase, with protein material in the 30 000-mol.wt. region of the electrophoretic gels of slow-muscle sarcoplasmic reticulum, is supported by further experiments. In these experiments the overall 30 000-mol.wt. material was cut out and pooled from several gels, after electrophoresis by the procedure of Weber & Osborn (1969), and was extracted with sodium dodecyl sulphate. The extracted protein was isolated as described by Weber & Osborn (1975), and then assayed for NADH-potassium ferricyanide reductase activity. In a typical experiment, the recovered reductase activity accounts for 60% of the activity originally present in the sarcoplasmic-reticulum membrane protein loaded on the gel. This result allows us to interpret our earlier correlative data on the relative amount of 30 000-mol.wt. protein and of NADH-cytochrome c and NADH-ferricyanide reductase activities of the isolated sarcoplasmic reticulum from different types of muscles (Margreth *et al.*, 1971, 1974a,b, 1975, 1977; Margreth & Salvati, 1974; Salvati *et al.*, 1979). From our own present electrophoretic data (Plate 1) and other published results (see, e.g., Campbell *et al.*, 1980), it appears, however, that the 30 000-mol.wt. material comprises more than one protein species.

With regard to the structural interrelatedness between liver microsomal and muscle sarcoplasmic-

EXPLANATION OF PLATE 1

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of liver and muscle membrane protein and of purified cytochrome b_5 and NADH-cytochrome b_5 reductase

One-dimensional electrophoresis was performed as described in the Materials and methods section. Lane (i), liver microsomal fraction; lanes (ii) and (v), liver D-cytochrome b_5 ; lanes (iii) and (vi), liver NADH-cytochrome b_5 reductase; lane (iv), isolated sarcoplasmic reticulum from slow muscle. About $25\mu\text{g}$ of membrane protein or $2\mu\text{g}$ of protein (cytochrome b_5 and NADH-cytochrome b_5 reductase) was applied per well. Key: Ca^{2+} -ATPase, Ca^{2+} -dependent ATPase; CS, calsequestrin; M_{55} , M_{55} protein; Fp, NADH-cytochrome b_5 reductase; Cyt. b_5 , cytochrome b_5 .

EXPLANATION OF PLATE 2

Isoelectrofocusing of liver and slow-muscle D-cytochrome b_5 , and two-dimensional electrophoresis of the mixture
 Panel (i) shows isoelectrofocusing over the pH range indicated of 10 μ g of liver (a) and muscle (b) D-cytochrome b_5 . Panel (ii) shows two-dimensional electrophoresis, according to isoelectric point in the first dimension, and according to molecular weight in the second dimension, of a 1:1 mixture of liver and muscle cytochrome b_5 (5 μ g of protein each). For details see the Materials and methods section.

Fig. 1. Enzyme-linked immunosorbent assay of liver and muscle D-cytochrome b_5 .

The test was performed as reported in the Materials and methods section, with anti-(liver D-cytochrome b_5) antibody at the concentration indicated on the abscissa, in microtitre wells coated with antigen solution at a concentration of $0.7\mu\text{g/ml}$. Values reported on the ordinate represent the phosphatase activity bound to the wells measured from the formation of *p*-nitrophenol from *p*-nitrophenyl phosphate at 400nm, after the reaction had been stopped with alkali. O, D-cytochrome b_5 from slow-muscle sarcoplasmic reticulum; ●, D-cytochrome b_5 from liver microsomal fraction.

reticulum cytochrome b_5 , the results in Plate 2 show that preparations of D-cytochrome b_5 from the two membranes have identical isoelectrofocusing properties when analysed by the O'Farrell (1975) method, and both focus as a single protein species at pH5.35. Likewise, in agreement with earlier evidence that microsomal cytochrome b_5 of kidney and liver cells (Raffel & Orrenius, 1971) are antigenically homogeneous, an identity reaction of rabbit liver and slow-muscle cytochrome b_5 can be demonstrated by an enzyme-linked immunosorbent assay (Fig. 1). Furthermore, anti-(liver D-cytochrome b_5) antibody appears to inhibit the NADH-cytochrome *c* reductase activity of the sarcoplasmic-reticulum vesicles (Fig. 2), in a way similar to that reported by Remacle (1978) for liver microsomal fraction, except that maximum inhibition attained with the sarcoplasmic-reticulum vesicles is about 70% instead of 90%.

Since the native content of cytochrome b_5 , as well as of the flavoprotein NADH-cytochrome b_5 reductase, are characteristically much higher in the

Fig. 2. Inhibition of rotenone-insensitive NADH-cytochrome *c* reductase activity of slow-muscle sarcoplasmic reticulum by anti-(liver D-cytochrome b_5) antibody.

Sarcoplasmic-reticulum membrane protein ($13\mu\text{g}$) was incubated for 1 h at 0°C in 0.5 ml of phosphate-buffered saline medium containing the amounts of antibody indicated on the abscissa. NADH-cytochrome *c* reductase activity was determined on 0.1 ml samples of the incubated sarcoplasmic-reticulum vesicles, as reported in the Materials and methods section. Control values refer to the activity found for sarcoplasmic-reticulum vesicles preincubated in phosphate-buffered saline medium in the absence of antibody ($1.27\mu\text{mol}$ of cytochrome *c* reduced/min per mg of protein).

sarcoplasmic-reticulum membranes of slow muscle, as compared with those of fast muscle, it is an interesting question to ask which may be the factors responsible for this specificity, as discussed by Remacle (1978) in a more general context.

We had shown earlier that the sarcoplasmic reticulum isolated from immature muscle has a high NADH-linked electron-transport activity (Margreth *et al.*, 1971). We have found now that the isolated sarcoplasmic reticulum from immature fast muscle of 4-day-old rabbits has a cytochrome b_5 content of 0.16nmol/mg of protein, i.e. in the range of values found for adult slow-muscle sarcoplasmic reticulum, when allowance is made for the different degree of purity of these preparations. It has been speculated that the decrease of cytochrome b_5 in the sarcoplasmic reticulum of fast muscle during postnatal development may be the expression of a specific repressor influence of phasic motor innervation to the muscle (Margreth *et al.*, 1971, 1974b). The

neural influence might in turn be mediated by developmental changes in the membrane phospholipids (Martonosi, 1975; Sarzala *et al.*, 1975). Thus the fatty acid composition of sarcoplasmic-reticulum phospholipids seems to influence other aspects of membrane specificity, related to muscle type, e.g. the differential binding of α -tocopherol to the sarcoplasmic-reticulum membranes of fast muscle and slow muscle (Salviati *et al.*, 1980).

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