Detection of Cytochrome b_5 in Bovine Skeletal Muscle by Electrophoretic Immunoblotting Technique

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In erythrocytes, methemoglobin is reduced by NADH-cytochrome b_5 reductase using cytochrome b_5 as an electron-transfer mediator¹⁾. On the other hand, the mechanism of metmyoglobin reduction in muscle has not been solved clearly. In the preceding paper²⁾, we purified so-called metmyoglobin reductase³⁾ from bovine skeletal muscle and identified it as NADH-cytochrome b_5 reductase. Metmyoglobin, as well as methemoglobin, is reduced by NADH-cytochrome b_5 reductase in the presence of cytochrome b_5 *in vitro*^{2,4)}. However, cytochrome b_5 has not yet been fully observed in muscle³⁾, although sufficient amounts of cytochrome b_5 in erythrocytes have been shown⁵⁾. In this study, we detected cytochrome b_5 in bovine skeletal muscle via the electrophoretic immunoblotting technique.

Materials and Methods

Materials

Bovine skeletal muscle and liver were obtained immediately after slaughter, and stored at -20°C until use. Bovine erythrocyte cytochrome b_5 was prepared via the procedures described in the preceding paper²⁾. CNBr activated Sephrose 4 B was purchased from Pharmacia Fine Chemicals.

Preparation of anti-cytochrome b_5 antibodies

The antigen, purified cytochrome b_5 (1 mg) derived from bovine erythrocytes, was mixed with Freund's complete adjuvant (1 : 1, v/v) and injected subcutaneously into the back of the rabbit. Boosters were given in the same way at 15 and 36 days after initial vaccination. Blood was collected one week after the last administration.

Purification of anti-cytochrome b_5 antibodies was performed by affinity chromatography. Purified cytochrome b_5 was immobilized to CNBr activated Sepharose 4 B (1 g

牛骨格筋におけるチトクロム b_5 の電気泳動免疫ブロッティング法による検出：有原圭三・伊藤 良
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per 4 mg of cytochrome). The cytochrome-Sepharose conjugate was packed in a column and stored at 4°C in phosphate buffered saline (PBS). To purify anti-cytochrome b_5 antibodies, rabbit antiserum approaching the total capacity of the cytochrome-Sepharose conjugate was loaded into the column. The column was then washed with PBS. Immunoabsorbed antibodies were detached with 3 M NaSCN. The antibody-containing fractions were dialyzed against distilled water and lyophilized.

Electrophoretic immunoblotting technique

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a vertical slab unit according to the method described by LAEMMLI⁶⁾, using 12.5% gel (separating part) in the presence of 0.1% SDS. Samples were dissolved in LAEMMLI's sample buffer containing 2% SDS and 5% mercaptoethanol by heating for 5 min in boiling water bath. Muscle and liver were sliced with scissors and homogenized with LAEMMLI's sample buffer by use of a potter homogenizer before heating.

Immediately after SDS-PAGE, the proteins were transferred from the gel to Clearblot P-Membrane (Atto Co., Tokyo) via a procedure outlined by KYHSEANDERSEN⁷⁾, using a home made horizontal electroblotting apparatus. Blotting was carried out with 25 mM Tris-192 mM glycine-20% (v/v) methanol buffer (pH 8.3) at 100 mA for 60 min.

After electroblotting, blots on the membrane were immunostained using the Bio-Rad Immun-Blot assay kit according to the description of the instruction manual. Rabbit anti-cytochrome b_5 antibody (first antibody), goat anti-rabbit IgG-horseradish peroxidase conjugate (second antibody) and 4-chloro-1-naphthol (horseradish peroxidase development reagent) were used.

Results and Discussion

Bovine skeletal muscle homogenate was subjected to SDS-PAGE, and the separated bands of proteins were transferred onto the membrane and detected by anti-cytochrome b_5 antibodies (Fig. 1, lane B). For comparison, purified bovine erythrocyte cytochrome b_5 and the homogenate of bovine liver which contains the most cytochrome b_5 in the body were also used (Fig. 1, lane A, C). The immunostaining in this study was highly specific. In fact, purified erythrocyte cytochrome was well stained and with liver homogenate, a single band was revealed. The muscle cytochrome b_5 migrated as a single band behind erythrocyte cytochrome b_5 and co-migrated with liver cytochrome b_5 . Based on the position of protein standards, the apparent molecular weight values of erythrocyte and of liver cytochrome b_5 were 15,500 and 18,500, respectively.

Cytochrome b_5 has been reported to be present in erythrocytes, as a water-soluble protein in the cytosol, where it is involved in the reduction of methemoglobin with an NADH-cytochrome b_5 reductase¹⁾. By contrast, in the liver, cytochrome b_5 exists in a microsomal membrane-associated form⁸⁾. Structural studies of purified cytochrome b_5 from bovine erythrocytes demonstrated that the erythrocyte cytochrome corresponds to the hydrophilic domain of liver cytochrome^{9,10)}. The erythrocyte protein corresponds to the liver protein without its hydrophobic tail. Other properties are

Cytochrome b_5 in Bovine Skeletal Muscle

Fig. 1. Detection of cytochrome b_5 by electrophoretic immunoblotting technique using anti-cytochrome b_5 antibodies. Lanes contained: lane A, purified bovine erythrocyte cytochrome b_5 (30 ng); lane B, bovine skeletal muscle homogenate (1.25 mg tissue); lane C, bovine liver homogenate (6.25 μ g tissue). Bands on the left are Bio-Rad's Pre-Stained SDS-PAGE Standards used for a visual check of electrophoretic transfer efficiency. Numbers on the right indicate molecular weight values of protein standards (Bio-Rad SDS-PAGE low molecular weight standards).

indistinguishable from each other. From the comparison of SDS-PAGE mobilities in this study, the muscle cytochrome b_5 seems to be present as a membrane protein.

HAGLER *et al.*³⁾ attempted isolation of cytochrome b_5 from a bovine heart muscle, but found no detectable levels present. Judging from the intensities of immunostained bands of several diluted solutions of the liver homogenate, skeletal muscle contained only about 1/500 times as much as cytochrome b_5 of the liver (data not shown). This is the probable reason for their failure to isolate cytochrome b_5 . An accurate quantitative analysis of cytochrome b_5 in the muscle is now in progress in our laboratory via an immunoblotting assay.

In our preceding paper²⁾, we showed the presence of NADH-cytochrome b_5 reductase in bovine skeletal muscle. And furthermore, in this study, we also demonstrated the presence of cytochrome b_5 in the muscle. Therefore, it is suggested that the enzymatic reduction of metmyoglobin in muscle is preceded by an NADH-cytochrome b_5 reductase system similar to methemoglobin reduction in erythrocytes, although the form of muscle cytochrome b_5 (membrane-bound form) is different from that of erythrocytes protein (water-soluble form).

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