Carboxylic ester hydrolases in mitochondria from rat skeletal muscle

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Summary
A mitochondrial pellet, prepared from rat skeletal muscle, contained a number of carboxylic ester hydrolase isoenzymes. The esterases which split α-naphthyl acetate were organophosphate sensitive, whereas two out of three indoxyl acetate hydrolysing enzymes were resistant to both organophosphate and organomercury. The activity of the indoxyl acetate esterases was enhanced by the non-ionic detergents Tween-40 and Lubrol. After freezing, thawing and high speed centrifugation most of the α-naphthyl acetate splitting enzymes were found in the supernatant, indicating that the enzymes are loosely bound to mitochondrial membranes.

Introduction
Naphthyl acetate splitting hydrolases in muscle fibres change their activity both quantitatively and qualitatively during the progression of a number of neuromuscular diseases (Kar & Pearson, 1978; Kar & Pearson, 1979; Bianchi et al., 1982; Kirkeby & Moe, 1987; Kirkeby et al., 1989). However, little is known about the distribution and function of these enzymes in mammalian skeletal muscle. In other tissues the esterases have been reported to be localized in rough endoplasmic reticulum, lysosomes and mitochondria (Sugihara & Deimling, 1973; Böcking & Riede, 1979; Sannes & Randell, 1985). Since non-lysosomal proteases seem to be important for intracellular protein catabolism in muscle (Stauber et al., 1987) we have investigated a possible mitochondrial origin of some of the non-specific esterases present in skeletal muscle fibres.

Materials and methods

Experimental procedures
For the biochemical studies the muscles of the hindlimbs from male Wistar (3 months old) rats were dissected free of fat and connective tissue and minced finely with scissors. For the histochemical procedures the gracilis muscle was removed and specimens from this muscle were frozen in isopentane cooled to −150°C with liquid nitrogen. Sections (6 μm) were cut on a cryostat microtome at −30°C.

Preparation of mitochondrial fraction
All steps were carried out at 4°C. (1) Muscle pieces (36 g) were homogenized for 3×20 s in 360 ml sucrose-histidine containing 18,000 units heparin (Stagni & De Bernard, 1968). (2) The homogenate was centrifuged at 600 g for 10 min and the residue discarded. (3) The supernatant was centrifuged at 8000 g for 30 min and the supernatant discarded. (4) The residue was resuspended in 50 ml sucrose-histidine containing 2500 units heparin and centrifuged at 600 g for 10 min. The residue was discarded. (5) The supernatant was centrifuged at 8000 g for 30 min and the resulting supernatant discarded. (6) The residue (mitochondrial pellet) was gently homogenized by hand with a teflon pestle in 4.5 ml sucrose-histidine.

Electrophoresis
Polyacrylamide gel electrophoresis was performed with the different muscle extracts mentioned above on either native or SDS gels. The native gels were used for demonstration of esterase isoenzymes and the SDS gels for protein detection. The acrylamide gels for the zymograms were 10% slab gels pH 7.6 run in the tris/glycine buffer of Maurer (1971). The concentration of the spacer gel was 4%. The electrophoresis was performed at 20°C for 3 h with constant current of 74 mA. Each lane was loaded with 20 μl muscle extract. The SDS electrophoresis was carried out with a Pharmacia PhastSystem on a 8–25% gradient PhastGel with SDS buffer strips. The Coomassie staining of the muscle proteins was done according to Huekshoven and Dernick (1988).

Staining for esterase activity
The substrates used for non-specific esterases were α-naphthyl acetate (αNA) and 5-bromo- 4-chloro indoxyl acetate (IAC). The gel staining media were as follows. (1) An

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azo-coupling method with 25 ml phosphate buffer (pH 6.3, 0.1 M), 38 mg Fast Blue B dissolved in 4 ml phosphate buffer with 1% Brij, 14 mg αNA in 0.2 ml acetone. (2) The incubation medium for IAc activity consisted of 25 ml tris maleate buffer (pH 7.2, 0.1 M), 8 mg IAc in 3 ml dimethylformamide, 12 mg nitro blue tetrazolium (NBT). For demonstration of esterase activity in the muscle sections we used a polyvinyl alcohol αNA medium (Kirkeby et al., 1988). 100 ml phosphate buffer (0.1 M, pH 6.3) with 5 g polyvinyl alcohol (G18/140 Wacker), 6 ml hexazoniated p-rosaniline and 20 mg αNA dissolved in acetone.

Electron microscopy embedding procedures
About 50 μl of the mitochondrial pellet was resuspended in 1 ml fixative (2.5% glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer at pH 7.2) and fixed for 1 h at 4°C. The suspension was centrifuged at 1500 g for 10 min and the pellet was resuspended in a drop of warm 6% agar, solidified and cut into blocks. The blocks were postfixed in 1% osmium tetroxide for 30 min at 4°C, dehydrated and embedded in polyvinyl alcohol αNA medium (Kirkeby et al., 1988), 100 ml of the mitochondrial fraction. A triplet of bands with weakly expressed activity (lane d) while TOCP and mersalyl inhibited almost completely inhibited the αNA esterases (lane b), while EDTA had no effect on the IAc medium (lanes a and c). EDTA or mersalyl was added to the incubation medium for IAc activity (lane d) while TOCP and mersalyl inhibited the slow migrating band completely (lanes e and f).

The mitochondrial fraction was subjected to freez-