Cardiac and skeletal muscle mitochondria have a monocarboxylate transporter MCT1

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Conventional wisdom is that lactate is produced in working muscle as a result of oxygen insufficiency, but numerous findings on different species are discordant with the prevailing view (15). For instance, lactate is always present in blood, muscle, and other tissues, and lactate production occurs in contracting skeletal muscle under fully aerobic conditions (10). Arterial lactate concentration ([lactate]) is low in humans (8, 9) and rats (12, 24) and gluconeogenesis (~20%) (9, 18). Studies using mass balance measurements (8), tracers (9, 12, 24), or enzyme activities (10) showed that lactate is produced in contracting skeletal muscle as a result of oxygen insufficiency. The ability of lactate to become the major fuel for the heart during physical exercise when arterial [lactate] rises has been attributed to high mitochondrial density and prevalence of heart-type lactate dehydrogenase (LDH-H4, LDH-1). However, the equilibrium constant of LDH is very high (3.6 × 10^4 M^{-1}), and in cytosol the concentrations of pyruvate and H^+ are not limiting. Consequently, it is difficult to understand how net lactate-to-pyruvate conversion is accomplished in cytosol, even in well-perfused tissues with high mitochondrial densities.

Recently, the ability of tissues to oxidize lactate led to the hypothesis of an "intracellular lactate shuttle" (6) in which cytosolic lactate could be taken up and oxidized within mitochondria of the same cell. Isolated heart, skeletal muscle, and liver mitochondria were shown to oxidize lactate directly (7), and evidence was provided to show roles of mitochondrial LDH and a lactate/pyruvate transporter MCT to permit lactate oxidation. Although results obtained by using electrophoresis and electron microscopy clearly showed several LDH isoforms, particularly LDH-1, abundant in rat cardiac and soleus muscle mitochondria (7), the matter of which, if any, of the known MCT isoforms (13, 27) were present in striated muscle mitochondria was not addressed.

To determine whether MCT1 (13) could be the mitochondrial lactate/pyruvate transporter, we purified rat cardiac and skeletal muscle mitochondria and other cellular fractions with an antibody to MCT1. MCT1 was selected because its abundance in striated muscle is associated with mitochondrial density (13, 25, 27). Also, as with LDH (7), we attempted to visualize MCT1 in muscle mitochondria in situ by immunolocalization and electron microscopy.

**METHODS**

**Mitochondrial Isolation**

Subsarcolemmal mitochondria and cell membranes. Subsarcolemmal (SM) and interfibrillar mitochondrial (IM) fractions were obtained from Sprague-Dawley rat hearts and hindlimb skeletal muscles as described previously (3, 7), but proteolytic digestion with Nagarse or trypsin was not used. Simply, to isolate the SM fraction, the muscle homogenate was subjected to a low-speed (800 g) centrifugation for 10 min, and the supernatant was filtered through cheesecloth to eliminate erythrocytes, nuclei, hair, and other debris and the matrix containing the IM population. The supernatant was then centrifuged at high speed (8,000 g) to precipitate SM. The latter procedure was repeated twice to wash the fraction. In addition, the supernatant from the final high-speed spin was saved as it contains sarcolemmal (SL), microsomal, and other nonmitochondrial cell membranes.

IM. The IM population was isolated by resuspension of the pellet from the first low-speed spin. The resulting pellet was resuspended, and mitochondria were freed from myofibrils by Teflon-glass homogenization. A low-speed centrifugation (800 g) was used to precipitate erythrocytes and other nonmitochondrial debris. The supernatant containing IM was centrifuged at high speed (9,000 g) and washed by...
were minced into small (glutaraldehyde in 0.1 M sodium cacodylate buffer where they and immediately fixed in 2% paraformaldehyde and 0.1% sodium-anesthetized rats, prepared for electron microscopy, longus (EDL) muscles were harvested from pentobarbital

**Western Blotting**

Sarcolemmal and mitochondrial fractions were probed with a rabbit polyclonal antibody to the COOH-terminal of rat MCT1 (N'-CPLQNSSGDPAEESPVC') (13, 19) and Western blotting. Erythrocyte ghosts were used as positive controls. To evaluate the extent of contamination from sarcolemmal MCT1, mitochondrial and cell membrane fractions were probed with an antibody to GLUT-1 (gift from M. Kern and G. L. Dohm). Purity of mitochondrial fractions as well as the extent of cross-contamination of other cell membranes by mitochondrial debris was evaluated by Western blotting with a mouse monoclonal antibody (20E8-C12) against the subunit IV of the cytochrome oxidase (COx) (Molecular Probes, Eugene, OR). Gels were scanned using a Bio-Rad GS-700 laser densitometer, and bands were quantified by software analysis using Molecular Analyst (Bio-Rad Laboratories).

**Electron Microscopy**

Tissue fixation. Heart, soleus, and extensor digitorum longus (EDL) muscles were harvested from pentobarbital sodium-anesthetized rats, prepared for electron microscopy, and immediately fixed in 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer where they were minced into small (=1-mm²) pieces as previously described (7). The samples were then dehydrated in a graded acetone series and embedded into LR white resin by using microwave processing. After polymerization, thin (60-nm) sections were cut using an Research and Manufacturing Company MTX ultramicrotome with a diamond knife. Sections were immunolabeled and stained.

Immunolabeling and transmission electron microscopy. Sections were placed on formvar, carbon-coated nickel grids, and labeled by using a 1:100 dilution of MCT1 antibodies. For secondary antibody labeling, a 1:20 dilution of goat anti-rabbit antibody conjugated to 15-nm gold particles was applied. Negative controls received only the secondary antibody. This form of control was used to demonstrate that the secondary antibody did not bind nonspecific to the tissue. Immunolabeled sections were then stained by using 2% aqueous uranyl acetate and Reynolds's lead citrate solutions. Grids were viewed by using a J EOL 100CX transmission electron microscope operating at 80 kV. Density of MCT1 labeling in muscle mitochondria and in surrounding cell compartments was determined by scanning micrographs into Adobe Photoshop 4.0 and quantitated by NIH Image 1.61 (7); n = 2 rats.

**RESULTS**

**Heart**

Autoradiographs of Western blots performed after probing of heart mitochondrial preparations with an antibody to MCT1 (44 kDa) reveal presence of MCT1 in both SM and IM mitochondria as well as SL membranes (Fig. 1). The antibody also responded strongly to erythrocyte membranes that were used as a positive control (not shown). These positive responses were blocked by the polypeptide used as the antigen in antibody production.

 Autoradiographs of Western blots performed after probing of SM and IM preparations showed strong reactions to the COx antibody (17.2 kDa) (Fig. 1). In contrast, the antibody to COx did not react to erythrocytes or to SL fractions.

 **Skeletal Muscle Mitochondria**

Results obtained on rat skeletal muscle mitochondria (Fig. 2) were similar to those obtained on heart mitochondria (Fig. 1). The antibody to MCT1 reacted strongly to SM, IM, and SL fractions. Antibodies to GLUT-1 reacted positively to the SL membrane fraction but weakly to mitochondrial fractions. In contrast, the antibody to COx reacted strongly to mitochondrial, but not sarcolemmal, fractions. Similar results have been obtained on human vastus lateralis muscle biopsies (H. Dubouchaud, G. E. Butterfield, E. E. Wolfel, B. C. Bergman, and G. A. Brooks, unpublished observations).

**Electron Microscopy**

Micrographs of rat heart (Fig. 3), soleus, and EDL (not shown) yielded immunolabeling of inner mitochondrial membranes by MCT1. In heart and soleus muscle, the labeling density was not significantly different in mitochondria and in surrounding areas. In EDL, labeling in mitochondria was less than in surrounding areas (P < 0.05). Negative control experiments produced no labeling.
DISCUSSION

Isolated rat cardiac and skeletal muscle mitochondria readily oxidize lactate and pyruvate in vitro, and the rate of lactate oxidation is ~10% greater than that of pyruvate (7). Oxidation of both lactate and pyruvate by muscle mitochondria is blocked by the MCT inhibitor cinnamate, whereas the LDH inhibitor oxamate blocks lactate, but not pyruvate, oxidation (7). Therefore, the ability of muscle mitochondria to directly oxidize lactate requires both mitochondrial LDH and an MCT. Previously we showed (7) LDH-1 (heart type) and other LDH isoforms to be abundant in soleus and cardiac muscle mitochondria. Now, using both Western blotting and immunolocalization followed by electron microscopy, we show that MCT1 is abundant in subpopulations of rat cardiac and skeletal muscle mitochondria.

Our polyclonal antibody to MCT1 was developed in rabbit against a 16-amino acid polypeptide from the COOH-terminal of MCT1 (13, 19). Analyses of human and mouse Genbank data indicate that the amino-acid sequence used has no significant homology with any protein other than MCT1. Furthermore, the polypeptide sequence used has low homology with other known MCT sequences (27). Because our antibody to MCT1 reacts readily with mitochondria, and the reaction was blocked by the polypeptide used in antibody production, we conclude that mitochondria in rat cardiac and skeletal muscle contain MCT1.

Results of our efforts to visualize MCT1 in cardiac and skeletal muscle mitochondria by immunolocalization and electron microscopy (Fig. 3) corroborate and confirm those obtained with Western blotting (Figs. 1 and 2). Negative control experiments, involving application of only the secondary antibody, did not show labeling of the muscles studied and preclude the possibility of accidental labeling. On a relative basis, results obtained by using our anti-MCT1 antibody and Western blotting (Figs. 1 and 2) yielded stronger results than those obtained by using the same antibody and immunolabeling and electron microscopy (Fig. 3). This apparent difference is likely due to the conformation of MCT1 in mitochondrial membranes, which shields the antibody from the COOH-terminal.
Biochemical evidence from diverse sources provides additional support for a functional role of MCT1 in striated muscle mitochondria. Cinnamate is well established as an inhibitor of pyruvate transport in several systems that include liver mitochondria and erythrocytes (16). Furthermore, we have shown that cinnamate blocks lactate and pyruvate transport by sarcolemmal vesicles (28). Most recently, we have shown that cinnamate blocks lactate and pyruvate oxidation in ADP-stimulated mitochondria (7). Evidence that MCT1 is in mitochondria, that MCT1 is cinnamate sensitive, and that cinnamate blocks mitochondrial lactate and pyruvate oxidation makes MCT1 a candidate for the mitochondrial lactate/pyruvate transporter. Most importantly, inhibition of mitochondrial lactate and pyruvate oxidation by cinnamate means that the mitochondrial MCT is functionally oriented, not present as the result of isolation artifact.

We probed muscle mitochondria for MCT1 because it is known to be abundant in muscle tissues high in oxidative capacity (13). However, the family of 7 or more related MCTs that are putative cell membrane MCTs possess 12 membrane spanning regions (13, 27). In contrast, previously identified mitochondrial MCTs possess six transmembrane segments (22). Therefore, it is likely that mitochondria possess MCTs coded for by two gene families.

Rat and human skeletal muscle is known to contain two lactate transporters, MCT1 and MCT4 (27). In contrast to our results indicating presence of MCT1 in muscle mitochondria, autographs of Western blots, performed after probing of muscle mitochondria preparations with an antibody to MCT4, did not indicate presence of that isoform in mitochondria. Similar results obtained on human vastus lateralis biopsies (H. Dubouchaud, G. E. Butterfield, E. E. Wolfel, B. C. Bergman, and G. A. Brooks, unpublished observations) lead us to conclude that in muscle MCT1 occupies sarcolemmal and mitochondrial domains, whereas MCT4 is the constitutive sarcolemmal lactate transporter.

The cell-to-cell lactate shuttle hypothesis (5) is supported by demonstration of lactate exchange between producing and consuming cells and tissues (1, 29). The intracellular lactate shuttle hypothesis (6) is supported by observations that isolated mitochondria readily oxidize exogenous lactate (7) and that working muscle (1, 29) and heart (14) consume and oxidize lactate. Additionally, there is growing support from 1H- and 13C-nuclear magnetic resonance spectroscopy for direct mitochondrial oxidation of lactate in vivo. The importance of lactate oxidation in glioma cells has been recognized (4) and an astrocyte-neuron lactate shuttle proposed (26). Similarly, magnetic resonance spectroscopy data showing preferential lactate uptake and oxidation by mammalian hearts (23, 30) and skeletal muscles have been obtained (2).

Although apparently very different in their interpretation regarding a mitochondrial domain for MCT1 or the function of an intracellular lactate shuttle in striated muscle, our data are consistent with those of McCullagh et al. (25). On muscle homogenates, they observed high correlations among concentrations of MCT1, LDH-1, and other mitochondrial marker enzymes. For striated muscle mitochondria, our results to date (7), and Figs. 1–3, show essentially the same results. However, we interpret those results to indicate mitochondrial domains for LDH and MCT1 that facilitate function of an intracellular (cytoplasmic-to-mitochondrial) lactate shuttle in myocytes.

The present results as well as those of Johannsson et al. (20), who were able to visualize MCT1 in rat heart, are consistent with the possibility that more than one lactate transporter is expressed in striated skeletal muscle cells. In the aggregate, our results and those of others are consistent with diverse cellular locations of MCTs in striated muscle cells, with MCT1 abundant in mitochondria.

The process of muscle tissue fractionation for mitochondrial isolation involves risk of cross-contamination of cell components during homogenization and other procedures. MCT1 is known to be abundant in both erythrocytes and sarcolemmal membranes. For that reason, we took precautions to remove erythrocytes and erythrocyte membrane fragments. Thus, whereas probing of muscle homogenates with an antibody to MCT1 is likely to suffer from contamination by erythrocytes, it is unlikely that our mitochondrial preparations endured contamination by erythrocyte remnants.

More problematic in terms of contamination of isolated mitochondria with sarcolemmal remnants in our experiments is the potential for coalescence of cellular membranes from adjacent organelles during cell fractionation. Our previous work (21) indicates that, in muscle, the mitochondrial apparatus exists as an extended reticulum. Thus the process of cell fractionation and separation of SM and IM provides ample opportunity for membrane fragments from adjacent organelles to fuse into discrete mitochondria as they reform after cell fragmentation. Therefore, to assess the extent of cross-contamination during mitochondrial isolation, we probed sarcolemmal and mitochondrial fractions with an antibody to GLUT-1.

Western blot analyses (Figs. 1 and 2) showed slight, if any, contamination of isolated cardiac or skeletal muscle mitochondrial fractions with the sarcolemmal marker GLUT-1. For heart, SM densitometric analyses of gels loaded with equivalent amounts of protein gave a ratio of MCT1 in the SM fraction to MCT1 in the SL fraction of 0.74. In contrast, the ratio of GLUT-1 band density in the SM fraction compared with GLUT-1 in the SL fraction was 0.12. For IM, densitometric analyses of gel bands gave a ratio of MCT1 in the IM fraction to MCT1 in the SL fraction of 0.23. Correspondingly, the ratio of GLUT-1 band density in the IM fraction compared with GLUT-1 in the SL fraction was 0.02. Additionally, similar ratios were obtained after band-density analysis of muscle mitochondria. Consequently, MCT1 appearing in SM and IM fractions cannot be from SL contamination; otherwise, the ratios would be the same, not different by almost an order of magnitude. Moreover, mitochondrial fractions reacted.
strongly to our probe for COX, whereas cell membrane fractions reacted poorly or not at all to this antibody. Thus it is unlikely that results obtained with Western blots are attributable solely to contamination of mitochondria by cell membranes during isolation.

In conclusion, our present and previous results (7) show roles for mitochondrial MCT and LDH in facilitating mitochondrial lactate oxidation. These results support the presence of an intracellular lactate shuttle in skeletal muscle. In cytosol of working muscle, the large negative change in standard free energy for LDH along with abundance of pyruvate, H⁺, and NADH favor lactate formation. Lactate flux from cytosol to mitochondria is favored by the concentration gradient between compartments and because H⁺ concentration is low and NAD⁺ concentration is high within the mitochondrial matrix when the electron transport chain is activated to maintain chemiosmotic gradient. Operation of a cytoplasmic-to-mitochondrial lactate shuttle in working muscles and beating hearts permits glycolysis to progress to lactate without the penalties of acidosis and other cells that influence tissue respiratory capacity (11, 17) profoundly affect the ability to clear lactate and other metabolites.

We thank the staff of the University of California, Berkeley, Electron Microscope Laboratory for their generous support, and S. L. Lehman and P. Licht for critical commentary.

This work was supported by National Institutes of Health Grants DK-19577 and AR-42906.

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Received 11 june 1999; accepted in final form 16 August 1999.

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