Colocalization of MCT1, CD147, and LDH in mitochondrial inner membrane of L6 muscle cells: evidence of a mitochondrial lactate oxidation complex

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Once thought to be formed as the result of a lack of oxygen, lactate is now known to be formed continuously in the presence of oxygen (6). On the basis of isotope tracer, arterial-venous difference mass balance, and biopsy studies (2, 8) we now know that working skeletal muscle is not only the major site of lactate production but also the major site of its removal, mainly via oxidation. Lactate shuttling between sites of formation and disposal represents a carbon source for oxidation and gluconeogenesis and a vehicle for cell-cell signaling via redox changes (6). The carboxylic acids lactate and pyruvate are exchanged across lipid bilayer membranes by facilitated proton-linked transport (34, 35, 37) involving a family of monocarboxylate transport (MCT) proteins (15). Of the now recognized superfamily of solute transporters (18), the first four isoforms are lactate-pyruvate transporters. The first isoform discovered (MCT1) (15) is thought to facilitate cellular lactate uptake and oxidation (2, 6, 12) and is abundant in sarcosomal and mitochondrial membranes of striated muscle and other cells, including neurons (7, 10, 28, 29). In contrast, the MCT2 isoform, a high-affinity pyruvate transporter (5, 14, 27), is found predominantly in peroxisomes and is highly expressed in liver (29). In adult mammalian muscle, MCT2 appears in sarcosomal and subsarcolemmal domains of oxidative muscle fibers, but MCT2 is not abundant compared with MCT1 (20). MCT3 appears to be specialized for function in the basolateral membrane of retinal epithelium (40). MCT4, the other isoform expressed in muscle, is associated with sarcosomal membranes of fast-twitch fibers (20). As part of the cell-cell lactate shuttle, sarcosomal MCTs are thought to facilitate tissue and cellular lactate exchange and use.

With the accumulating mass of physiological (2, 8) and other data showing tissue lactate exchange and wide diversity of tissue MCT expression (6, 19), there appears to be growing acceptance of cell-cell lactate shuttles (17). However, there are fewer data and less general agreement on the cellular sites of lactate oxidation (17). Nonetheless, implicit in the overall concept of a lactate shuttle is that, once inside a cell, lactate and pyruvate would shuttle among compartments of metabolism. One proposal is that the presence of peroxisomal membrane MCT1 and -2 and matrix lactate dehydrogenase (LDH) permit lactate-pyruvate exchange and regulation of organelle redox and, hence, affect the control of peroxisomal β-oxidation (29). Another proposal is that mitochondrial MCT1 and LDH facilitate lactate uptake and oxidation in the reticulum (7, 9). By cell fractionation and Western blotting we (7, 9, 10) have shown the presence of both MCT1 and LDH in rat liver and muscle mitochondria and human muscle mitochondria (12). In addition, results of studies using electron microscopy show LDH to be localized on the mitochondrial inner membranes (9). Consequently, in mammalian tissues MCT1 and LDH in mitochondria are hypothesized to participate in the functioning of an intracellular lactate shuttle (ILS) in vivo (9). In concordance, results obtained with magnetic resonance spectroscopy show mitochondrial lactate oxidation in isolated beating hearts and working skeletal muscle (8, 11). As well, using a combination of isotope tracers and arterial-venous difference measurements on intact functioning men, we have shown simultaneous lactate production, uptake, and oxidation in working muscle beds (8). Among the many unanswered questions regarding the mechanism of lactate oxidation in vivo is the identity of the mitochondrial chaperon for MCT1 and other associated lactate oxidation complex constituents. Also, the physical locations of mitochondrial MCT1 and its orientation with LDH are unknown. The single-span transmembrane glycoprotein CD147 (Basigin) is considered to be the chaperone protein for MCT1, localizing it to the cell surface (13, 22, 39). However, results of transfection studies (39) showing tight cell membrane associations between MCT1 and CD147 also dem-

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onstrated that CD147 was expressed throughout Cos and Hela cells, where mitochondrial reticulum is known to be extensive. Indeed, we found a faint signal for CD147 in the mitochondrial fraction of rat skeletal muscle (10). Thus we hypothesized that if CD147 served to anchor MCT1 to the mitochondrial inner membrane, this protein complex would provide a means for lactate oxidation in the mitochondrial reticulum.

In the present investigation, using confocal laser-scanning microscopy (CLSM) we determined the locations of mitochondria, LDH, MCT1, and CD147 in rat-derived L6 skeletal muscle cells, where the expression of MCTs is well maintained (25). Western blotting and immunoprecipitation (IP) studies were performed on fractions of L6 cells to confirm the visual interaction of MCT1 and related proteins obtained by CLSM. Results allow us to propose the existence of a mitochondrial lactate oxidation complex involving MCT1, CD147, LDH, and cytochrome oxidase (COX).

**MATERIALS AND METHODS**

**Materials.** Tissue culture medium, serum, and reagents were obtained from GIBCO (Carlsbad, CA). Aprotinin, DTTP, EDTA, EGTA, HEPES, leupeptin, MOPS, Nonidet P-40 (NP-40), pepstatin A, PMSF, sucrose, Tris, and ε-aminocaproic acid were purchased from Sigma-Aldrich (St. Louis, MO). NaCl and NaN3 were purchased from Fisher (Fairlawn, NJ). Na2P2O7·10H2O was purchased from Matheson Coleman & Bell (Norwood, OH).

**Animals.** The University of California, Berkeley Animal Care and Use Committee approved all protocols. Male Sprague-Dawley rats (200–300 g) were fed and housed under standard conditions. Animals were euthanized using Nembutal injection (120 mg/kg ip). Livers and soleus muscles were taken for preparation of cell subfractions to serve as positive controls for immunoreactivity. As well, because results on cultured myocytes may not fully represent conditions in fully differentiated muscles in vivo, measurements on adult tissues were made to relate results on cultured myocytes to our previous results on adult human (12) and rodent tissues (7, 9, 10, 29).

**Cell culture.** L6 muscle cells were grown in monolayers to the stage of myotubes in a 5% CO2 atmosphere at 37°C. Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 1% penicillin-streptomycin, 0.5% glucose, and 5 mM L-glutamine (medium I) was used for growth of L6 cells. Alternatively, cells were grown in 10-cm-diameter dishes or in 6-cm-diameter dishes with circular cover glasses, where the expression of MCTs is well maintained (25). Western blotting and immunoprecipitation (IP) studies were performed on fractions of L6 cells to confirm the visual interaction of MCT1 and related proteins obtained by CLSM. Results allow us to propose the existence of a mitochondrial lactate oxidation complex involving MCT1, CD147, LDH, and cytochrome oxidase (COX).

**CLSM.** CLSM (Zeiss 510 META) was used for immunofluorescent detection of cellular localizations of the mitochondrial reticulum, LDH, MCT1, MCT2, and CD147. Cells grown on circular cover glasses were fixed by acetone for 5 min on ice. After washing with phosphate-buffered saline (PBS), pH 7.4, cells were incubated with 0.2% Triton X-100 in PBS for 5 min. Thereafter, cells were incubated overnight at 4°C with rabbit anti-MCT1 (Brooks, custom antibody), chicken anti-MCT2 (Chemicon International, Temecula, CA), goat anti-CD147 (Research Diagnosis, Flanders, NJ), mouse anti-COX-IV (Molecular Probes, Eugene, OR), and goat anti-LDH (Abcam, Cambridge, MA). Anti-rabbit and anti-chicken Alexa fluor 488 conjugated secondary antibodies (Molecular Probes) were used for MCT1 and MCT2 detection, respectively. For CD147 and LDH, anti-goat Alexa fluor 546 conjugated secondary antibody (Molecular Probes) was used. For COX, anti-mouse Alexa fluor 647 (Molecular Probes) or Cy3 (Chemicon International) was used. Secondary antibodies were incubated for 1 h at room temperature. Additionally, cells were preincubated for 40 min with 500 nM MitoTracker 633 (Molecular Probes). Antibody detection was performed at an emission of 500–530, 550–600, and 640–720 after excitation at 488, 543, and 630 nm, respectively. An oil immersion objective (Zeiss ×63/1.4 numerical aperture) was used. Images represent optical slices of ~1 μm, and laser power and detection gains were set such that signals from single-stained controls would not appear in adjacent channels.

**Preparation of subcellular fractions.** After harvesting with PBS, L6 cells were collected by centrifuging at 700 g for 10 min at 4°C. Cells were gently homogenized in lysis buffer A (250 mM sucrose, 5 mM Na2HPO4, 2 mM EGTA, 100 μM PMSF, 1 μg pepstatin A, 10 μg leupeptin, 20 mM HEPES-Na, pH 7.4) with the use of a loose-fitting Dunce (Teflon-glass) homogenizer and then centrifuged at 600 g for 10 min at 4°C to remove nuclei and debris. The supernatant was centrifuged at 10,000 g for 30 min at 4°C to precipitate mitochondrial fragments. One fraction of the resulting supernatant was used for immunoblots as a cytosolic fraction. Another fraction was diluted with 0.75 vol buffer B (1.167 M KCl and 58.3 mM Na2P2O7·10H2O, pH 7.4) and centrifuged at 230,000 g for 120 min at 4°C. The pellet from this high-speed spin was washed in buffer C (1 mM EDTA and 10 mM Tris, pH 7.4) and then resuspended with 200 μl of buffer C and 66 μl of 16% SDS and centrifuged at room temperature at 1,100 g for 20 min. This supernatant containing the sarcosomal membrane fraction was used for immunoblots. The mitochondrial pellet was washed twice, first by resuspension in buffer A and repelleted at 10,000 g for 30 min at 4°C. This pellet was washed in buffer C and treated as follows: 1) a fraction was resuspended in 200 μl of buffer C and 1% NP-40 and centrifuged at 1,100 g for 20 min at room temperature, and the supernatant containing this mitochondrial fraction was used for immunoblots; 2) a fraction was resuspended in 500 μl of buffer D (10 mM Tris, 1 mM EDTA, 150 mM NaCl, and 10 μg/ml aprotinin, pH 7.4) with 1% NP-40, and this mitochondrial fraction was used for IP; and 3) a fraction was resuspended in 500 μl of buffer D without 1% NP-40; this mitochondrial fraction was used for immunoprecipitation. In addition, a liver mitochondrial fraction was prepared as a positive control for MCT2 (29). Sarcosomal fraction from soleus muscle was prepared for the positive control of MCT1 and CD147 (10).

**Immunoblots.** The presence of MCTs, CD147, and LDH in cell subfractions was determined by standard Western blotting techniques, as described elsewhere (10, 29). The antibodies to MCT1, MCT2, CD147, COX, and LDH were the same as described in CLSM procedure. In addition, identity of the mitochondrial fraction was confirmed by probing for complex I NADH dehydrogenase (NADH: Molecules Probes) as well as COX. Evaluation of contamination of the mitochondrial fraction by sarcosomal remnants was performed by probing with rabbit anti-β1-Na+K+-ATPase (Upstate Serologicals, Charlottesville, VA). MagicMark XP Western Standard (Invitrogen, Grand Island, NY) was used for molecular weight standards.

**Immunoprecipitation.** Repelleted mitochondrial fractions of L6 cells were resuspended in buffer D with or without detergent, 1% NP-40. Agarose conjugated G protein (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the mitochondrial fractions and incubated at 4°C for 30 min. Samples were centrifuged at 2,300 g at 4°C for 1 min, and aliquots of this supernatant were reacted with primary antibodies to MCT1, CD147, COX, NADPHDH, LDH, and rat normal IgG (Santa Cruz Biotechnology) at 4°C for 2 h. The agarose conjugated G protein was then added and incubated at 4°C for 1 h. The pellet was collected by centrifugation at 2,300 g at 4°C for 1 min. The supernatant (lysate) was used for SDS-PAGE. The pellet was washed five times by PBS buffer (pH 7.4). The final pellet from IP was used for SDS-PAGE.
RESULTS

Mitochondrial MCT1, CD147, and LDH detected by immunocytochemistry. L6 cells were well stained with anti-MCT1 conjugated to Alexa fluor 488 (green) and mitochondrial tracker or COX (red), as shown in Fig. 1A. MCT1 was detected by CLSM at both sarcolemma and intracellular domains, with the nucleus being an exception (Fig. 1, A1 and A4). Probes for mitochondria, MitoTracker (Fig. 1A2), and the mitochondrial inner membrane component COX (Fig. 1A5) showed that the mitochondrial reticulum was highly elaborated in L6 cells, particularly in the perinuclear domain. Superposition of the probe for MCT1 with either probe for mitochondria clearly showed colocalization of MCT1 with the mitochondrial reticulum (Fig. 1, A3 and A6, yellow). In contrast to results with MCT1, we detected only faint signals for MCT2 in L6 muscle cells, with the results being essentially the same as with negative controls (micrographs not shown).

Like MCT1 (Fig. 1B1), CD147 was detected throughout myocytes again except in the nucleus (Fig. 1B2). Merged images to assess colocalization of MCT1 and CD147 indicated the pattern of the localization of CD147 to match that of MCT1 (Fig. 1B3). Furthermore, CD147 (Fig. 1C1) and COX (Fig. 1C2) were colocalized at intracellular domains of L6 cells (Fig. 1C3).

LDH was detected throughout the cell (Fig. 1D1). Superposition of LDH and COX signals clearly showed LDH to be colocalized with the mitochondrial reticulum (Fig. 1D3, yellow). Negative controls showed negligible superposition of LDH and COX (micrographs not shown).

Mitochondrial MCT1, CD147, and LDH detected by immunoblotting. The plasma membrane marker β1-Na+/K+-ATPase was detected by immunoblotting (IB) in the sarcolemmal fractions of both the slow-twitch oxidative soleus muscle (positive control) and L6 cells (Fig. 2A). In contrast, there was no immunoreactivity of β1-Na+/K+-ATPase in the mitochondrial fraction of L6 cells, indicating insignificant sarcolemmal contamination of the mitochondrial fraction from L6 cells (Fig. 2A). Signals for the mitochondrial markers COX and NADHDH were detected in the mitochondrial fractions of both liver (positive control) and L6 cells (Fig. 2, B and F). However, there was no immunoreactivity to COX or NADHDH in the sarcolemmal or cytosolic fractions of L6 cells, indicating insignificant contamination of these fractions by mitochondrial remnants (Fig. 2, B and F). In contrast to freedom of L6 mitochondrial fractions from sarcolemmal cross-contamination, and vice versa, some contamination of sarcolemmal fractions from soleus muscle by mitochondrial remnants was detected, which suggests coalescence of juxtaposed sarcolemmal and subsarcolemmal mitochondrial membranes during disruption of adult muscle (Fig. 2, B and F).

MCT1 was found not only in positive controls (liver mitochondria and soleus sarcolemma), but also in both sarcolemmal and mitochondrial fractions of L6 cells (Fig. 2C). Similarly, CD147 was detected in both sarcolemmal and mitochondrial fractions of L6 cells and the mitochondrial fraction of liver as well as the sarcolemmal fraction of soleus muscle (positive control) (Fig. 2D). MCT2 was found in the mitochondrial fraction of liver (positive control) and the sarcolemmal fraction of soleus muscle, but not in L6 cells (Fig. 2E). LDH was found in all cell fractions of L6 cells, soleus muscle, and liver (Fig. 2G).
Interactions of MCT1, CD147, and COX detected by IB after IP. Figures 3 and 4 represent results of IP experiments with (Fig. 3) and without (Fig. 4) detergent. IP of CD147 from the mitochondrial fraction of L6 cells coprecipitated MCT1, whereas only a faint signal was detected in the lysates (Fig. 3). In contrast, IP of CD147 from the mitochondrial fraction of L6 cells did not coprecipitate MCT2 (data not shown). To confirm specificity of CD147-MCT1 binding, the mitochondrial protein COX was used as a negative control of CD147 pellet. Figure 3 shows that there was no COX in the CD147 pellet, but the corresponding lysate contained a relatively large amount of COX, suggesting that CD147 and MCT1 interact specifically in mitochondria of L6 cells. Conversely, IP of COX from mitochondrial fraction of L6 cells did not coprecipitate either MCT1 or CD147 (Fig. 3). However, when the mitochondrial fraction was suspended in buffer D without detergent NP-40, IP of COX from the L6 mitochondrial fraction coprecipitated both MCT1 and CD147 (Fig. 4).

To compare the coprecipitation of MCT1 and CD147 with another mitochondrial inner membrane component, NADHDH, was used for IP from the mitochondrial fraction of L6 cells suspended without detergent (Fig. 4). IP of NADHDH did not coprecipitate either MCT1 or CD147. Showing integrity of the mitochondria inner membrane, NADHDH was coprecipitated far better with anti-COX than with either anti-MCT1 or -CD147 (Fig. 4).

Interactions of MCT1, COX, and LDH detected by IP. Interactions between LDH and putative mitochondrial inner membrane-bound lactate oxidation proteins (MCT1, CD147, and COX) were examined by IP of LDH from the L6 mitochondrial fraction suspended in medium with (Fig. 3) and without detergent (Fig. 4). Whereas with detergent IP of LDH from the mitochondrial cell fraction coprecipitated neither MCT1 nor COX (Fig. 3), in the absence of detergent LDH coprecipitated both MCT1 and COX (Fig. 4). Similarly, IP of COX from the mitochondrial fraction of L6 cells also coprecipitated LDH. However, IP of NADHDH from the mitochondrial fraction of L6 cells did not coprecipitate LDH. Previously, by using secondary labeling with gold particles of anti-LDH and electron microscopy, we showed LDH located on the inner mitochondrial membrane (9). The present results obtained using a different technique confirm an inner mitochondrial membrane location of LDH and further indicate that the mitochondrial lactate oxidation complex is associated with the COX end (complex IV), as opposed to the NADHDH side (Complex I) of the electron transport chain (ETC).

Normal rat IgG (nlgG) was used as a negative IP control in detergent-solubilized mitochondrial fractions (Fig. 3) and in suspension medium without detergent (Fig. 4). MCT1, CD147, COX, NADHDH, and LDH were not coprecipitated with nlgG (Figs. 3 and 4).

DISCUSSION

Using CLSM, Western blotting of cell fractions, and IB after IP from cell lysates we provide evidence of strong associations among mitochondrial MCT1, CD147, LDH, and COX, but not NADHDH, suggesting the presence of a previously unrecognized mitochondrial lactate oxidation complex associated with
Complex IV, the existence of which might explain how tissues with dense mitochondrial networks are capable of taking up and oxidizing lactate. Additionally, in combination with the well-known effect of endurance training on increasing mass of the muscle mitochondrial reticulum, the presence of the lactate oxidation complex helps explain why training exerts effects on lactate accumulation during submaximal exercise and also results in large increases in lactate clearance via oxidation (2).

Commencing with the data on MCT1, we discuss evidence leading to our conclusions regarding the presence and importance of a mitochondrial lactate oxidation complex.

As detailed in MATERIALS AND METHODS, stringent conditions were set in that images (optical slices of ~1 μm) were at the limit of our system, and laser power and detection gains were set such that signals from single-stained controls would not appear in adjacent channels. Superposition of signals for MCT1 and mitochondrial inner membrane constituents (COX and CD147) produced clear evidence for mitochondrial colocalization, particularly in the perinuclear compartment (Fig. 1A). These findings are consistent with results of our recent study (20), where we showed colocalization of MCT1 and COX in both sarcolemmal and interstitial domains in adult rat plantaris muscle fibers. In agreement, on transformed HT-29 incubated colon cells, Wenzel et al. (38) showed mitochondrial MCT1 using CLSM. In contrast to the abundance of MCT1 in L6 cells, our micrographs obtained with CLSM produced only faint signals for MCT2 protein (data not shown). That finding is again consistent with our recent observation of micrographs in rat plantaris muscle obtained with CLSM (20). And finally, with regard to the presence of MCT1 in muscle mitochondria, it is noteworthy that in the seminal paper on discovery of MCT1, Garcia et al. (15) showed good concordance between cellular locations of MCT1 and succinate dehydrogenase (Fig. 6A and 6B, respectively, in Ref. 15), but they did not draw a linkage between cellular locations of MCT1 and mitochondria.

In terms of the significance of the mitochondrial lactate oxidation complex for explaining the physiology of cell-cell and intracellular lactate shuttles, MCT1 is a proton-coupled lactate-pyruvate carrier that facilitates monocarboxylate and hydrogen ion transport down chemical and proton gradients. Furthermore, the chemical and proton gradients across the inner membrane of respiring mitochondrial networks establish the gradients necessary for mitochondrial lactic and pyruvic acid uptake. And finally, it is suggested that the oxidizing environment of mitochondrial COX permits conversion of lactate to pyruvate for subsequent oxidative catabolism of pyruvate in the tricarboxylic acid (TCA) cycle after transport into the mitochondrial matrix (Fig. 5).

Here, we report that CD147 is colocalized with MCT1 throughout the L6 myocytes, including in the mitochondrial reticulum and sarcolemma (Fig. 1, B and C). Supporting our findings obtained with immunocytochemistry, our Western blots of cell subfractions demonstrated that CD147 was expressed in the mitochondrial fraction obtained from L6 cells as well as liver (Fig. 2). Additionally, IP of CD147 from mitochondrial fraction of L6 cells coprecipitated MCT1 (Fig. 3). Previously, by using CLSM, Kirk et al. (22) showed colocalization of MCT1 and CD147 at the cell surfaces of isolated rat cardiac myocytes. And, although their emphasis was on cell surface protein expression, their micrographs showed colocalization of MCT1 and CD147 throughout transfected Hela cells. Thus our results confirm and extend those of Kirk et al. (22) regarding CD147 as a chaperone for MCT1.

In our experiments, we used mild detergent treatment to assess strength of protein-protein binding. It is notable that IP of COX from the mitochondrial fraction of L6 cells suspended in medium without detergent coprecipitated both MCT1 and CD147, suggesting that both MCT1 and CD147 were associated with the mitochondrial inner membrane (Fig. 4). In contrast, NADHHDH, another mitochondrial inner membrane-bound protein, did not coprecipitate either MCT1 or CD147 from mitochondrial fractions of L6 myocytes in the absence (or presence) of detergent (Fig. 4). Additionally, interactions between COX and MCT1 and between COX and CD147 are not as strong as the association between MCT1 or CD147, because COX immunoprecipitated from detergent-solubilized mitochondrial fractions did not coprecipitate either MCT1 or CD147. In aggregate, these findings indicate that the lactate oxidation complex is associated with the terminal COX end of the ETC.

In the ILS mechanism, mitochondrial lactate oxidation is posited to involve LDH (9). In the present study we observed mitochondrial LDH by CLSM (Fig. 1D), Western blotting (Fig. 2) and IB after IP (Figs. 3 and 4). Furthermore, we
examined interactions between LDH and other putative components of a mitochondrial inner membrane-bound lactate oxidation complex (LDH, MCT1, CD147, and COX). IP of LDH from the mitochondrial fraction coprecipitated both MCT1 and COX (Fig. 4). Conversely, IP of COX from mitochondrial fraction of L6 cells coprecipitated LDH (Fig. 4). However, IP of NADHHD from the mitochondrial fraction of L6 cells did not coprecipitate LDH (Fig. 4). Hence, the present results allow us to elaborate upon and extend our previous model (9) of how lactate is metabolized in the mitochondrial reticulum. We propose that lactate oxidation to pyruvate involves LDH and is coupled to the redox change in COX during mitochondrial electron transport (Fig. 5). Findings of simultaneous muscle tissue glucose uptake and glycogenolysis and lactate production, exchange, and oxidation (6) pose numerous problems for understanding metabolic integration, particularly if a cytosolic site of lactate oxidation to pyruvate is assumed. For physical exercise, it is somewhat helpful that fast-glycolytic muscle fibers express MCT4 at the sarcolemma for lactate import and oxidative glycolytic fibers express MCT1 in the sarcolemma and mitochondrial reticulum for lactate import and oxidation (20). However, for rest, when recruitment of fast-glycolytic fibers cannot be invoked, and even for mild to moderate intensity exercise where oxidative fibers are preferentially used, the cellular site of lactate oxidation to pyruvate has been problematic. However, with recognition of the mitochondrial lactate oxidation complex, simultaneous intracellular lactate production in cytosol and oxidation in mitochondria can be understood.

In this effort we took three approaches to demonstrate colocalization of COX, MCT1, CD147, and LDH. In part, the results are new and novel, but as is always the case, findings follow previous efforts of others. Histochemical localization of LDH in mitochondria of rat heart and skeletal muscle is attributable to efforts of Baba and Sharma (1), who in 1971 used electron microscope histochemistry and showed LDH to be associated with the inner membrane and matrix of rat skeletal and cardiac muscle mitochondria. Baba and Sharma were probably the first to speculate on the presence of a “lactate shuttle,” but in the absence of physiological or biochemical data they were unable to expand on the physiological significance of their discovery. In 1978, Mole et al. (30) determined that rat heart homogenates could oxidize lactate 20% more rapidly than pyruvate, whereas with homogenates of white skeletal muscle lactate and pyruvate, oxidation rates were essentially the same. In the 1980s, Brandt, Kline, and colleagues (4, 24) demonstrated presence of LDH in rat liver, kidney, and heart mitochondria. Furthermore, they showed that isolated liver mitochondria were capable of oxidizing lactate at least as fast as pyruvate (24). They interpreted their results as permitting the lactate shuttle (4). Subsequently, we confirmed the ability of isolated muscle mitochondria to oxidize lactate (9) and the intramitochondrial localization of LDH (9, 12). Additionally, we identified the mitochondrial lactate-pyruvate transporter to be MCT1 (7).

Because the muscle mitochondrial mass is arranged as a reticulum, as opposed to discrete vesicles (23), disruption and loss of function are inevitable in the formation of mitochondrial vesicles. For example, tissue homogenization followed by proteolytic digestion is known to affect the ability of mitochondrial preparations to oxidize fatty acids (31). Not surprisingly, some (33, 36) have been unable to either isolate mitochondria that oxidize lactate or contain LDH. As well, others have been unable to prepare mitochondria with immunoreactivity to MCT1 (41). One possibility (Fig. 5) is that mitochondrial LDH is anchored to the outer side of the inner mitochondrial membrane such that much of the protein is located between the inner and outer mitochondrial membranes, thus making it susceptible to loss during isolation. Such an explanation is consistent with results of Fonsot et al. (32), who compared pyruvate and lactate oxidation rates in saponin-skinned fibers from rat skeletal muscle and heart. In their preparations, lactate oxidation was low, approximately one-half that of pyruvate. But, as mentioned by the authors, saponin treatment results in loss of LDH, so any lactate oxidation was likely attributable to LDH persisting in intermyofibrillar mitochondrial domains deep within fibers. Because lactate uptake and oxidation are known to readily occur in the beating human heart (16) and
working skeletal muscle (2), the data of Ponsot et al. (32) serve to illustrate importance of LDH in the lactate shuttle hypothesis. However, because they did not assess the effect of saponin on cytoplasmic and mitochondrial LDH (Fig. 1D), the results of Ponsot et al. (32) are of little relevance as tests of the site of cellular lactate oxidation in vivo. In retrospect, the results of Ponsot et al. (32) on the suppression of muscle and cardiac lactate oxidation after removal of LDH by treatment with saponin are similar to our results with detergent NP-40 (Fig. 3).

Given the tenuous nature of assays of enzymatic function after tissue fragmentation, observations of lactate metabolism in vivo are relevant for evaluating veracity of the concepts articulated here. Studies of lactate oxidation in cardiac and skeletal muscles of humans and other mammals with the use of isotope tracers, classical arterial-venous mass balance techniques, and their combination show lactate shuttling and muscle lactate oxidation in situ and in vivo (6). However, although results on intact individuals show tissue lactate oxidation and functioning of the cell-cell lactate shuttle in vivo, the results provide no information on the cellular site of lactate oxidation. In contrast, results from magnetic resonance spectroscopy (MRS) are possibly more supportive of a mitochondrial, not cytosolic, site of lactate oxidation. Initially, Laughlin et al. (26) reported that infusion of \([^{13}\text{C}]\text{pyruvate}\) into working canine hearts labeled cytosolic alanine and lactate pools. In contrast, \([^{13}\text{C}]\text{lactate}\) did not label either pyruvate or alanine, which would be expected if lactate was oxidized to pyruvate in the cytosol, but instead glutamate, a marker of TCA cycle labeling would be expected if lactate was oxidized to pyruvate in the mitochondria. Butz CE, McClelland GB, and Brooks GA. Active muscle and whole body lactate kinetics after endurance training in men. J Appl Physiol 87: 1684–1696, 1999.

In conclusion, we provide strong evidence that MCT1 is localized in mitochondrial reticulum of L6 muscle cells. Furthermore, CD147, a chaperone protein for MCT1, and LDH were found to be associated with mitochondrial reticulum of L6 myocytes by both immunocytochemistry and Western blotting after cell fractionation. The interaction of these two proteins, MCT1 and CD147, and the presence of LDH in mitochondria was confirmed by IP of mitochondrial fractions from L6 cells. Our findings, obtained using both CLSM and IP, indicate that the terminal mitochondrial electron transport chain constituent COX is oriented to form a complex with MCT1, CD147, and LDH. However, we did not find an association between NADH and MCT1 and LDH. These findings further the understanding of the mitochondrial role in cellular lactate oxidation and may be interpreted to indicate the presence of a terminal mitochondrial ETC component, the lactate oxidation complex.

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REFERENCES


