Reconstitution of the lactate carrier from rat skeletal-muscle sarcolemma

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The lactate carrier was solubilized from purified rat skeletal-muscle sarcolemma with the detergent decanoyl-N-methylglucamid and the solubilized carrier was reconstituted into phospholipid vesicles. Reconstituted proteoliposomes showed a faster rate course of L-lactate uptake than did protein-free liposomes. The rate of L-lactate uptake into the proteoliposomes was inhibited by the lactate-transport inhibitors p-chloromercuribenzenesulphonate, diethyl pyrocarbonate, \(\alpha\)-cyano-4-hydroxycinnamate and quercetin. In contrast, the anion-exchange inhibitor DIDS \((4,4'\text{-di-isothiocyanostilbene-2,2'-disulphonate})\) had almost no effect on the uptake. The extent of L-lactate uptake at equilibrium was not affected by the presence of the transport inhibitors, but was sensitive to osmotic strength. L-Lactate and pyruvate, but not D-lactate, inhibited L-lactate uptake when present at 10-fold excess. The properties of L-lactate transport in reconstituted proteoliposomes were similar to those observed in native sarcolemmal vesicles, i.e. the lactate carrier seems to retain its transport characteristics during the solubilization and reconstitution steps.

INTRODUCTION

During intense glycolytic activity, skeletal muscles produce lactic acid, which is metabolized, accumulated in the muscle fibres or released to the blood. The capacity to release lactic acid could, by decreasing cellular acidosis, be important for fatigue resistance. Released lactic acid can also enter the fibres and thereby serve as substrate for oxidation. Thus the transport of lactic acid across the sarcolemma is important in periods with both lactic acid release and uptake.

It is well established that lactate transport is mediated by a specific H\(^+\)/lactate co-transporter (carrier) (Watt et al., 1988; Juel, 1988, 1991; Juel and Wirbrand, 1989; Roth and Brooks, 1990). However, a full understanding of the mechanisms involved in the transport process awaits the identification and isolation of the carrier protein(s). A main problem associated with this approach stems from the fact that isolation of the sarcolemma membrane in sufficient yield and purity has proved difficult to achieve. Moreover, to purify the protein(s) responsible for lactate transport it is essential to develop reliable procedures for solubilization and subsequent reconstitution into liposomes as a functional assay.

Juel (1991) has purified sarcolemmal vesicles obtained by collagenase treatment (Burton et al., 1988). A unique feature of this membrane preparation, when compared with commonly used procedures (Grindith et al., 1985; Klip et al., 1987), is the large size (median diameter 6 \(\mu\)m) of the vesicles, which makes them very suitable for transport studies (Juel, 1991; Ploug et al., 1993). The aim of the present study was to use the membrane preparation to extract and reconstitute the sarcolemmal lactate carrier in a functional active form. We here describe a reconstitution protocol, based on the method of Poole and Halsestrap (1988, 1992), who partially purified a related lactate carrier from erythrocytes. To our knowledge, functional reconstitution of a carrier protein from skeletal-muscle sarcolemma has not been reported previously.

EXPERIMENTAL

Materials

Phospholipids (phosphatidylcholine; Sigma type X-E) were stored as small portions at \(-20\) °C in chloroform containing 0.1 % (w/v) butylated hydroxytoluene. Stock solutions of diethyl pyrocarbonate (DEPC) (Sigma) in ethanol were prepared daily, and intermediate dilutions of this reagent into buffer were made immediately before use. L-\([^{14}\text{C}]\)Lactate, L-\([^{3}\text{H}]\)lactate and \([^{14}\text{C}]\)sucrose were purchased from Amersham International. \([^{3}\text{H}]\)Nitrendipine was from New England Nuclear. \(\alpha\)-Lactate (lithium salt) was from Boehringer Mannheim. L-Lactate (lithium salt), pyruvate (sodium salt), decanoyl-N-methylglucamid (MEGA-10), aprotinin (A 1153), collagenase (type VII), cholesteral (grade I), nifedipine, quercetin, 4,4'-di-isothiocyanostilbene-2,2'-disulphonate (DIDS), p-chloromercuribenzenesulphonate (PCMS), \(\alpha\)-cyano-4-hydroxycinnamate (CIN), phenylmehanesulphonyl fluoride (PMSF) and CHAPS were from Sigma. Nycodenz was obtained from Nycomed, Oslo, Norway. Percoll and Sephadex G-50 were from Pharmacia, Uppsala, Sweden. All other chemicals were of analytical grade. Minitas filters were purchased from Sartorius. Membra-Fil filters were from Costar. Glass-fibre filters (type GF/C) were from Whatman. Polyacrylamide pre-cast gels and molecular-mass standards were obtained from NOVEX. Scintillation liquids were from Packard.

Animals

Male Wistar rats were kept in a room (24–26 °C) with a 12 h-dark/12 h-light cycle and allowed to eat and drink ad libitum. Rats weighing 200–250 g were killed by a blow to the head and cervical dislocation.

Preparation of sarcolemmal giant vesicles

The protocol for the isolation of sarcolemmal giant vesicles was

Abbreviations used: CIN, \(\alpha\)-cyano-4-hydroxycinnamate; DEPC, diethyl pyrocarbonate; DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonate; MEGA-10, decanoyl-N-methylglucamid; PCMBs, p-chloromercuribenzenesulphonate; PMSF, phenylmehanesulphonyl fluoride.

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described previously (Juel, 1991). In brief, the hindlimb muscles were excised and trimmed of blood vessels, nerves and fat. The muscles were split lengthwise and incubated for 1 h at 34 °C in 140 mM KCl/5 mM Mops (pH 7.4), containing collagenase (150 units/ml) and aprotinin (0.01 mg/ml). The muscles were then washed with KCl/Mops and 10 mM EDTA. Percoll (final concn. 16 %) and aprotinin were added to the suspension of vesicles and cell fragments. A three-layer step-density gradient was used to isolate the vesicles. The upper layer consisted of 1 ml of KCl/Mops. The middle layer consisted of 3 ml of 4 % Nycodenz in KCl/Mops. The vesicle suspension (6 ml) was placed at the bottom of the 10 ml centrifugation tube. After centrifugation (50 g for 45 min) at room temperature (20–22 °C), the vesicles were harvested from the interface of the two upper layers. The vesicles were then diluted with KCl/Mops and spun down (830 g for 30 min). The vesicle diameters were measured by phase-contrast microscopy.

Measurments of lactate transport in sarclemmal giant vesicles

Transport experiments were performed essentially as described by Juel (1991). In brief, the vesicles were loaded with L-[3H]lactate (5 μCi/ml), unlabelled L-lactate (1 mM) and [14C]Sucrose. The latter was used as an extravascular marker. After 30 min incubation the vesicles were spun down (830 g) for 30 min, and the sedimented vesicles were ready for the efflux studies. The efflux of labelled lactate started when 10–25 μl of sedimented vesicles were transferred to 20 ml of efflux medium (140 mM KCl, 5 mM Mops, pH 7.4). During the efflux period, ten vesicle-free samples were obtained by using a syringe mounted with a 0.45 μm-pore-size Minisart cellulose acetate filter. The 3H and 14C radioactivities were determined by scintillation counting after addition of 2.5 ml of Ultima Gold scintillation liquid. The 14C radioactivity was used to compensate for the extravascular lactate.

For inhibition experiments, PCMBS, cit, DEPC, DIDS or quercetin was included in the preincubation medium as well as in the efflux medium. DIDS preincubations were carried out in the dark. When substrate inhibition was studied, 10 mM L-lactate, D-lactate or pyruvate was present in the incubation medium only. All incubations and transport experiments were performed at room temperature (20–22 °C).

Because the efflux is dependent on the surface-to-volume ratio, the efflux from a mixture of vesicles with different diameters is not monoeXponential. The efflux curve is the sum of monoeXponentials, \[ y = \sum\{1 - \exp[-tk(S_0/V_a)]]F_\ell V_a \] where \( y \) is the external accumulated lactate, \( t \) is the time, \( V_a \) is the vesicle volume, \( S_0 \) is surface area, \( F_\ell \) is frequency of vesicles with diameter \( d \), and \( k \) is the rate constant. The sum of exponentials was fitted to the experimental data by the non-linear least-squares regression method. The initial efflux rate was calculated from the curve fit; the flux per cm² of membrane was calculated from the distribution of vesicular diameters observed in each of the experimental groups and from the vesicular lactate space as determined in the individual experiments. The vesicular lactate space was calculated as the difference between the total (internal and external) lactate space and the sucrose space (external space).

Washing of sarclemmal giant vesicles

To remove soluble and loosely membrane-bound proteins, vesicles were frozen and thawed, washed once in hypertonic buffer (300 mM KCl, 25 mM sodium pyrophosphate, 10 mM Mops, 1 mM EGTA, 0.1 mM dithiothreitol, 0.1 mM PMSF, pH 7.4) followed by a wash in hypotonic buffer (20 mM Mops, 1 mM EGTA, 0.1 mM dithiothreitol, 0.1 mM PMSF, pH 7.4). Membranes were pelleted by centrifugation at 40000 g for 20 min and finally suspended in sucrose buffer (250 mM sucrose/10 mM Mops, pH 7.4) and stored at −20 °C. No considerable improvement in yield of protein was seen when the membranes were pelleted by centrifugation in a Beckman TL-100 ultracentrifuge at 200000 g for 60 min.

Membrane characterization

To prepare a crude homogenate, pieces of hindlimb muscles were homogenized (Ultra-Turrax, highest setting) in sucrose buffer, diluted to the appropriate protein concentration and stored in batches at −20 °C.

Contamination with transverse tubules was assessed by measuring the number of [3H]nitrendipine-binding sites, by the method of Fosset et al. (1983). Samples of washed vesicles and homogenate were incubated at 20 °C in 1 ml of medium containing 50 mM Tris/HC1 and 5 mM [3H]nitrendipine (0.4 μCi), pH 7.5. Non-specific binding was measured in the presence of 1 μM nifedipine. After incubation for 45 min, 400 μl samples of the incubation mixture were rapidly filtered through glass-fibre filters under reduced pressure. The filters were immediately washed with 2 x 5 ml of ice-cold solution containing 20 mM Tris/HC1 and 200 mM choline chloride, pH 7.4. The experiments were carried out in duplicate and under dim light because of the light-sensitivity of dihydropyridine derivatives.

SDS/PAGE was performed by the method of Laemmli (1970) in 4–20 % acrylamide/Tris/glycine gradient gels.

Reconstitution procedure

A chloroform suspension of phospholipid/cholesterol (2:1, mol/mol) was dried to a thin film under stream of N2. The lipid was resuspended in buffer A (140 mM KCl, 10 mM Mops, 1 mM EGTA, 0.1 mM dithiothreitol, pH 7.4), and sonicated in a bath sonicator until translucent. The dispersion was centrifuged at 20000 g for 10 min at 4 °C in a Beckman TL-100 ultracentrifuge and the pellet discarded. The supernatant was taken and assayed for lipid phosphate. Washed sarclemmal membranes were solubilized by adding the appropriate volume of a MEGA-10 solution to give a final concentration of 0.5 % (w/v). PMSF (0.1 mM) was present during the solubilization. Non-solubilized protein was removed by centrifugation at 200000 g for 10 min at 4 °C, and the protein content of the supernatant was measured. The solubilized protein and lipid were mixed at a ratio of 200 μg of protein/μmol of lipid phosphate, and the detergent concentration was decreased by passage through a column (30 cm x 1 cm) of Sephadex G-50 (coarse grade) equilibrated in buffer A containing 0.1 mM PMSF. The turbid fractions eluted in the void volume were collected, and diluted 3-fold in buffer A before freezing in liquid N2. This mixture was thawed at room temperature, and the proteoliposomes were collected by centrifugation at 40000 g for 20 min at 4 °C. The pellet was resuspended in buffer A to a protein concentration of approx. 0.5 mg/ml for the transport assay. To serve as controls, protein-free liposomes were prepared by the above procedure after mixing sonicated lipid with a 0.5 % MEGA-10 solution.

Assay of reconstituted transport activity

Lactate transport in reconstituted vesicles was measured as uptake of [14C]-labelled L-lactate essentially as described by Poole and Halestrap (1988). Specifically, a 15 μl portion of proteoliposomes (or protein-free liposomes) was mixed rapidly...
with 45 µl of buffer A containing 0.1 µCi L-[14C]lactate and 1.33 mM L-lactate. After the appropriate time, the uptake was stopped by addition of 800 µl of ice-cold buffer A containing 0.5 mM PCMBs. The suspension was immediately filtered through a 0.45 µm-pore-size Membra-Fil cellulose filter and the filter was washed with 5 ml of ice-cold buffer A. Radioactivity remaining on the filter was determined by scintillation counting after addition of 3.5 ml of Filter Count scintillation liquid. The results were corrected for filter blank. In all experiments, each data point was the average value of triplicate samples. To measure the osmotic sensitivity of the proteoliposomes, increasing amounts of sucrose were added to the tracer solution. The initial rate of transport was determined by measuring the amount of uptake at 15 s after correction for tracer uptake at zero time.

For inhibition experiments, reconstituted vesicles were preincubated with PCMBs, DEPC, DIDS or quercetin for 60 min before samples were used for uptake measurements as described above. DIDS preincubations were carried out in the dark. In the experiments with quercetin this inhibitor also was included in the tracer solution. CIN, L-lactate, D-lactate and pyruvate were present in the tracer solution only. All incubations and transport measurements were performed at room temperature (20–22°C).

Other methods
Protein was measured by the method of Bradford (1976) for samples dissolved in 2% CHAPS to eliminate turbidity. BSA was used as a standard. For reconstituted vesicles, the phospholipids interfered with the assay. This interference was corrected for as described previously (Doyle and McGivan, 1992).

Lipid phosphate was assayed as described by Chen et al. (1956), after digestion with H2SO4/HClO4 (Lynch and McGivan, 1987).

RESULTS AND DISCUSSION
Membrane preparation and characterization
Incubation of muscle bundles in 140 mM KCl solution containing collagenase results in the formation of giant membrane vesicles (Burton et al., 1988). However, the mechanism behind the generation of the membrane spheres is at present unknown. Giant vesicles have for some years been used for patch-clamp studies of ion channels (Burton et al., 1988; Burton and Hutter, 1990; Wareham et al., 1990). More recently, application of a purification procedure, as described in the present paper, has permitted studies of transport of lactate (Juel, 1991) and glucose (Ploug et al., 1993) in these vesicles. Available data suggest that the membrane spheres are primarily, if not solely, oriented right-side-out (Burton et al., 1988; Pilegaard et al., 1993).

Recently we have shown that the giant membrane vesicles are predominantly of sarcolemmal origin (Pilegaard et al., 1993). However, a possible contamination with T-tubule membranes was not ruled out. In the present study we have compared the number of [3H]nitrendipine-binding sites in the washed vesicles with that in a crude homogenate. The former contained 0.53 ± 0.05 (S.E.M.) pmol of binding sites/mg of protein (6 preparations), whereas the latter had 1.61 ± 0.20 pmol/mg of protein. A comparison between this purification index (PI = 0.3) and that of the sarcoclemmal marker enzyme K+-stimulated p-nitrophenylphosphatase (PI = 15.5; Pilegaard et al., 1993) indicates that T-tubule membranes are not a major contaminant. Similar results have recently been published by Ploug et al. (1993).

Figure 1 shows a comparison between the SDS/PAGE patterns of the different fractions obtained during the membrane preparation. The protein pattern of the intact vesicles (lane 2) was much simpler than that of a crude homogenate (lane 1). Myosin was absent from the vesicles, and many other protein bands were greatly diminished. Washing of the lysed vesicles only slightly changed the protein pattern (lane 4). Moreover, the supernatant from the hypertonic wash (lane 3), which contains approx. 85% of the total vesicular protein (Ploug et al., 1993), had essentially

![Figure 1](image_url)  
**Figure 1** SDS/PAGE protein analysis of fractions obtained during the reconstitution procedure

Isolated fractions (12 µg of each) were subjected to SDS/PAGE in a 4–20% acrylamide gradient gel. The proteins were stained with Coomassie Brilliant Blue. Lanes: 1, crude homogenate; 2, sarcolemmal giant vesicles; 3, supernatant from the hypertonic wash; 4, washed sarcolemmal vesicles; 5, solubilized protein; 6, reconstituted proteoliposomes. The kDa values represent the migration of molecular-mass standards.

![Figure 2](image_url)  
**Figure 2** Time course of L-lactate uptake in proteoliposomes (a) and in protein-free liposomes (b)

Reconstitution and assay of transport activity were performed as described in the Experimental section. Assays were performed after preincubating the reconstituted vesicles in the absence (○) or in the presence of 0.5 mM PCMBs (●) or 0.1 mM DEPC (■). Values are means ± S.E.M. of 3 separate reconstitution experiments.
the same pattern as the washed vesicles. These results indicate that the sarcolemmal vesicle membranes, even after the washing procedure, consist predominantly of non-intrinsic proteins. However, two minor bands of approx. 100 and 50 kDa, which were increased in the washed vesicles, are probably identical with the sarcolemma marker proteins Na+/K⁺-ATPase (Walaas et al., 1988) and SL50 (Jorgensen et al., 1990) respectively.

### Reconstitution of L-lactate transport

To reconstitute the lactate carrier from rat skeletal-muscle sarcolemma, we modified the procedure of Poole and Halestrap (1988, 1992), which was based on a method used to reconstitute the Na⁺-dependent neutral amino acid transport from bovine renal brush-border membranes (Lynch and McGivan, 1987). The criteria that we have used to assess the preservation of transport properties in this system are inhibitor-sensitivity and substrate specificity. Moreover, we have compared the results obtained in reconstituted vesicles with those obtained in native sarcolemmal vesicles.

Proteins from purified skeletal-muscle plasma membranes were solubilized with MEGA-10 before reconstitution into liposomes. When 0.5% MEGA-10 was used, approx. 70% of the total protein was extracted. Solubilization of the proteins apparently did not change the protein pattern, when compared with the washed vesicle membranes (Figure 1, lanes 5 and 4). Mixing solubilized protein with liposomes at a ratio of 200 μg of protein/μmol of lipid phosphate yielded proteoliposomes with a ratio of approx. 60 μg of protein/μmol of lipid phosphate. The proteoliposomes showed a slightly different protein pattern when compared with the solubilized fraction (Figure 1, lanes 6 and 5).

Figure 2 compares the time courses of L-lactate uptake in proteoliposomes and in protein-free liposomes. In proteoliposomes the rapid uptake of L-lactate is greatly inhibited by the well-known lactate-transport inhibitor PCMBS (Figure 2a). Likewise, DEPC, which is a potent inhibitor of lactate transport in cardiac sarcolemma (Troper and Philipson, 1989), inhibits the L-lactate uptake in proteoliposomes. In the experiment depicted in Figure 2a, 0.5 mM PCMBS and 0.1 mM DEPC inhibited the initial rate of uptake by 39 ± 5% and 46 ± 3% (means ± S.E.M.) respectively. The inhibitor-insensitive component of uptake is believed to be due to diffusion of the undissociated lactic acid. The data of Figure 2(a) also show that the inhibitors have no effect on the L-lactate uptake into the vesicles at equilibrium (30 min). In protein-free liposomes the L-lactate uptake is slow (Figure 2b); the time course is comparable with that of the inhibitor-insensitive component of uptake in proteoliposomes. Moreover, the inhibitors do not affect the L-lactate uptake into protein-free liposomes.

To confirm that the observed L-lactate uptake is due to membrane transport rather than binding to the surface of the proteoliposomes, equilibrium uptake of L-lactate was measured as a function of extrasynaptic osmolarity. As shown in Figure 3, when the L-lactate uptake is plotted against the inside/outside osmolarity ratio, a straight line is obtained. The intersection with the ordinate represents L-lactate binding to the proteoliposomes at infinite osmolarity. In our preparation, this binding makes up about 5% of the total uptake, whereas the remaining 95% represents transport into an osmotic space.

An estimate of reconstituted transport activity can be made by using DEPC-sensitive lactate uptake at 15 s; this was 12 ± 2 nmol/min per mg of protein in the experiment shown in Figure 2a.

### Characteristics of transport activity in proteoliposomes and in native sarcolemmal vesicles

#### Inhibitor sensitivity

We compared the effect of different inhibitors on the initial rate of lactate transport in proteoliposomes and in native sarcolemmal vesicles. Figure 4 shows dose-response curves for some inhibitors of lactate transport. In proteoliposomes maximal inhibition was obtained in the presence of 0.1 mM DEPC (Figure 4a). Higher
Table 1  Comparison of the effects of substrates on carrier-mediated L-lactate transport in proteoliposomes and in native sarcolemmal vesicles

<table>
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<tr>
<th>Substrate</th>
<th>Proteoliposomes</th>
<th>Native sarcolemmal vesicles</th>
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<tbody>
<tr>
<td>L-Lactate</td>
<td>65 ± 6 (5)</td>
<td>47 ± 7 (10)</td>
</tr>
<tr>
<td>D-Lactate</td>
<td>8 ± 6 (5)</td>
<td>30 ± 9 (11)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>62 ± 4 (3)</td>
<td>34 ± 5 (7)</td>
</tr>
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concentrations of this inhibitor were not used, because of its non-specific effects on L-lactate uptake in protein-free liposomes (results not shown). In native sarcolemmal vesicles 100% inhibition of the carrier-mediated transport was defined as the inhibition exerted by 0.5 mM PCMBs + 10 mM CN- (Figure 4b). Higher concentrations of PCMBs (1 mM) were not used, because it made the native vesicles leaky. The data of Figure 4 show that DEPC is a potent inhibitor of lactate transport in proteoliposomes as well as in native vesicles. In the former, half-maximal inhibition (IC50) was seen at approx. 3 μM DEPC, whereas in the latter this value was 30 μM. PCMBs inhibited lactate transport in proteoliposomes and in native vesicles, with IC50 values of approx. 0.2 mM and 0.3 mM respectively. The non-transportable lactate analogue CN- also inhibited lactate transport in both systems. Quercetin, which is an inhibitor of lactate transport in different tissues (Belt et al., 1979; Jennings and Adams-Lackey, 1982; Edlund and Halestrap, 1988; Rosenberg et al., 1993), was found to be a very potent inhibitor in native sarcolemmal vesicles, with an apparent IC50 of approx. 3 μM. However, in proteoliposomes the effect of quercetin was much less pronounced. DIDS (0.1 mM), an inhibitor of the inorganic-anion transporter, was found to have only a very little effect on lactate transport in proteoliposomes as well as in native vesicles. This finding confirms earlier results (Roth and Brooks, 1990) suggesting that the sarcolemmal lactate carrier is different from the lactate carrier in erythrocytes.

Substrate specificity

The carrier specificity of the substrate was determined by measuring the inhibitory effect of 10 mM L-lactate, D-lactate or pyruvate on the transport of 1 mM L-lactate. In proteoliposomes L-lactate and pyruvate were equally potent inhibitors of the transport, whereas D-lactate had almost no effect (Table 1). These data are in full agreement with the substrate specificity found for lactate uptake in small sarcolemmal vesicles (Roth and Brooks, 1990). However, in giant sarcolemmal vesicles L-lactate was only a slightly better inhibitor than D-lactate, with pyruvate being as effective as D-lactate (Table 1). The reason for the discrepancy in substrate specificity in the giant vesicles and in the proteoliposomes is at present unknown.

Conclusions

The present study represents the first demonstration of a successful solubilization and reconstitution of the lactate carrier from rat skeletal-muscle sarcolemma in a functionally active form. The carrier seems to retain its characteristics during the solubilization and reconstitution process. Our procedure is recommended for the identification and isolation of the sarcolemmal lactate carrier, a step which is essential for the understanding of the mechanisms involved in lactate transport.

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