Partial purification and reconstitution of the sarcolemmal L-lactate carrier from rat skeletal muscle

Peter J. ALLEN and George A. BROOKS*

Exercise Physiology Laboratory, Department of Human Biodynamics, 103 Harmon, University of California, Berkeley, CA 97420, U.S.A.

Purified sarcolemmal membranes from mixed rat hindlimb muscle were solubilized with octylglucoside and the extract subjected to hydroxylapatite (HA) chromatography. Following protein elution with a sodium phosphate gradient and detergent removal by dialysis, the HA eluate was reconstituted into asolectin liposomes using a freeze–thaw procedure. Specific L-[^{14}C]lactate transport activity eluting from the 0.2 M sodium phosphate fraction was 30-fold higher compared with native sarcolemmal vesicles (31.64 versus 1.06 nmol/min per mg). The reconstituted carrier exhibited Michaelis–Menten saturation kinetics with K_m and $V_{max.}$ values of 46.2 ± 6.6 mM and 498.7 ± 17.2 nmol/15 s per mg respectively. L-Lactate transport activity was inhibited 57% by preincubation of proteoliposomes with 10 mM α-cyano-4-hydroxycinnamate, a known inhibitor of lactate transport. Analysis of the HA eluates by SDS/PAGE showed the presence of a 34 kDa band corresponding to lactate transport activity. Reconstitution of lactate transport activity eluting from the HA column, together with SDS/PAGE analysis suggests the presence of a 34 kDa polypeptide mediating sarcolemmal lactate exchange in rat skeletal muscle.

INTRODUCTION

The existence of a membrane-bound transport mechanism for lactate has been established in skeletal muscle (Juel and Wibrand, 1989; Mason and Thomas, 1985; Roth and Brooks, 1990a,b), hepatocytes (Edlund and Halestrap, 1988; Fafournoux et al., 1985), erythrocytes (Deutike, 1982; Dubinsky and Racker, 1978; Halestrap, 1976; Leeks and Halestrap, 1978), brush-border cells (Friedrich et al., 1991), and cardiac cells (Mann et al., 1985; Trosper and Philipson, 1987). Extensive characterization of these carriers reveals similarities in kinetic properties and inhibitor sensitivities, suggesting the involvement of a family of transport proteins which mediate lactate transport (Poole and Halestrap, 1993). Attempts to detect the lactate carrier protein have resulted in the identification of a range of polypeptides from different tissues: 35–45 kDa in rat erythrocytes, 35–50 kDa in rabbit and guinea-pig erythrocytes, and 40 kDa in rat hepatocytes (Jennings and Adams-Lackey, 1982; Welch et al., 1984; Donovan and Jennings, 1985; Poole and Halestrap, 1992). Presently no attempts to reconstitute and identify the sarcolemmal lactate transporter from rat skeletal muscle have been reported.

In rat skeletal muscle sarcolemmal vesicles the transmembrane movement of lactate exhibits distinct characteristics indicative of carrier-mediated transport, including stereoselectivity for L-lactate, Michaelis–Menten saturation kinetics, and competitive inhibition by monocarboxylate analogues (Roth and Brooks, 1990a,b). Further, lactate exchange across muscle cell membranes is bi-directional, demonstrating increased activity in the presence of proton and lactate gradients, suggesting the presence of an H^+ co-transport (or OH^- counter-transport) mechanism (Roth and Brooks, 1990b; Brown, 1992).

Studies reporting identification of the lactate carrier have relied on two different methodological approaches. The majority of studies utilize specific, potent inhibitors of lactate transport which, following incubation with the membranes, selectively bind to and label the purported polypeptides involved in transport. A second method is a purification scheme involving the reconstitution of transport activity following detergent solubilization of the membrane proteins. This technique allows a more thorough analysis of transport and inhibition properties while providing a more positive identification of the protein component(s) involved in lactate transport.

While several studies have characterized the kinetics of lactate transport in skeletal muscle of rats (Roth and Brooks, 1990a,b), mice (Juel, 1988; Juel and Wibrand, 1989), and humans (Juel, 1991), no study published to date has specifically reported attempts to identify and reconstitute the sarcolemmal lactate transporter into an artificial membrane system. The objectives of the present study were to isolate and partially purify the lactate carrier protein from rat skeletal muscle. To this end, we solubilized and functionally reconstituted the transporter into liposomes following protein fractionation. The study of lactate transport kinetics in a reconstituted system has provided direct evidence for the existence of a membrane-bound carrier, as well as insight into the molecular identity of the protein component(s) responsible for lactate transport in skeletal muscle.

EXPERIMENTAL

Materials

L-[U-^{14}C]Lactate (177.3 mCi/mmol) and D-[U-^{14}C]lactate (89 mCi/mmol) were obtained from ICN Biomedicals (Irvine, CA, U.S.A.). L-Lactic acid and α-cyano-4-hydroxycinnamic acid were from Sigma (St. Louis, MO, U.S.A.). The detergent octyl-β-D-glucopyranoside (ultrol grade) was obtained from Calbiochem (La Jolla, CA, U.S.A.) and stored in a 10% (w/v) stock solution at −30 °C. Crude soybean phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.) prepared in a 50 mg/ml stock solution and stored at −80 °C. Hydroxylapatite

Abbreviations used: HA, hydroxylapatite; KRH, Krebs–Ringers–Hepes medium; DTT, dithiothreitol; FT, flow through fraction; EM, external medium; IM, internal medium.

* To whom correspondence should be addressed.

(HA) resin (Bio-Gel HTP) and pre-cast polyacrylamide minigels were purchased from Bio-Rad (Richmond, CA, U.S.A.).

Membrane preparation and solubilization

Sarcolemmal membrane vesicles from mixed rat hindlimb skeletal muscle were prepared by the method of Grimditch et al. (1985) as modified by Roth and Brooks (1990a). Sarcolemmal vesicles were suspended in a Krebs–Ringers–Hepes (KRH) medium containing 118 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, and 50 mM Hepes, pH 7.4, and stored at −80 °C. Vesicles were solubilized at a protein concentration of 1–1.5 mg/ml with 1.2% (w/v) octyl-β-D-glycopyranoside (octylglucoside), 20% (v/v) glycerol and phospholipid (3:1, lipid to protein ratio). The suspension was vortexed on a high-speed setting for 15 s, incubated on ice for 10 min and then frozen at −30 °C for 20 min. The suspension was then thawed at room temperature and centrifuged in a Beckman 50 Ti rotor at 100000 *g*, 4 °C for 45 min (Wheeler and Hauck, 1985; Brodsky et al., 1993). The supernatant containing the solubilized membrane proteins was collected and kept on ice for the purification step. Aliquots of the supernatant were kept for protein determination and SDS/PAGE analysis.

HA chromatography

Prior to sample application, 600 mg of dry HA resin was packed into a 1 cm × 5 cm column, washed and equilibrated with KRH buffer (pH 7.4) containing 20% (v/v) glycerol, 1 mM dithiothreitol (DTT), and 1.2% (w/v) octylglucoside. A gradient pump connected to a 20 ml syringe was set to a flow rate of 0.6 ml/min for column equilibration and sample application. Chromatography was performed in the cold room at 4 °C. The solubilized membrane proteins (500 μl) were loaded on to the column and, following the removal of non-bound protein, a buffered sodium phosphate step gradient was initiated. Four gradients were utilized each composed of KRH buffer (pH 6.8), 20% (v/v) glycerol, 1.2% (w/v) octylglucoside, and 0.1, 0.2, 0.3, and 0.4 M sodium phosphate respectively. The total volume of each gradient was 1.2 ml. The five eluted fractions including the initial flow through fraction (FT) were collected and immediately placed into Spectrapor1 dialysis tubing (6000 molecular mass cut off) and dialysed for 16–17 h against 1000 vol. of KRH buffer (pH 7.4) containing 20% (v/v) glycerol, 1 mM DTT and 1 μg/ml each of aprotinin and leupeptin. Dialysis buffer was changed once after 4 h. Following dialysis, aliquots of each fraction were taken for protein determination and SDS/PAGE. The remaining samples were used immediately for reconstitution and transport experiments.

Protein determination and reconstitution into liposomes

Protein content of the concentrated sarcolemmal vesicles and column fractions were determined according to Bradford (1976) using BSA as a standard. Liposomes were prepared by dissolving lipids in 20 mM Mops/2 mM 2-mercaptoethanol (pH 7.4) to a final concentration of 50 mg/ml. Prior to reconstitution, the lipid suspension was sonicated at 40 W, until the solution was cleared (approx. 1 min), using a microtip sonicator. Multilamellar lipids were then removed from this suspension by centrifugation in a Beckman 50 Ti rotor at 100000 *g* for 30 min. The supernatant was collected and kept on ice until needed for reconstitution.

Reconstitution was initiated by gently mixing the sonicated and cleared liposomes with the solubilized protein extract to a final lipid to protein ratio of 100:1 (w/w) for all transport experiments. The lipid/protein suspension was placed on ice for 1 min, immersed in liquid nitrogen for 10–20 s, slowly thawed to room temperature (25 °C) and prepared for the transport assay.

Lactate transport assay

All lactate transport experiments were performed at room temperature under zero-*trans* conditions, where substrate and tracer were initially in the external medium (EM) (on the *cis* side of the membrane). The EM consisted of L- or D-tracer lactate in KRH buffer (pH 7.4). The internal medium (IM, on the *trans* side) consisted of the KRH buffer (pH 7.4) which had been equilibrated with the proteoliposomes through dialysis. Therefore, no pH gradient existed between EM and IM.

In preparation for the transport assay, 100 μl of EM was pipetted into the bottom of glass culture tubes, in duplicate for each time point. Reactions were initiated by addition of 100 μl of proteoliposomes to the EM and vortexing. The reaction was stopped by the addition of an isosmotic ice-cold buffered 3 mM $HgCl_2$ stop solution pipetted in at the appropriate time points and the suspension applied to a 0.45 μm pore-size cellulose nitrate membrane filter under reduced pressure. This procedure has previously been shown to be effective in halting both lactate and glucose flux across cell membranes (Grimditch et al., 1985; Roth, 1989; Roth and Brooks, 1990a). The filters were then transferred to glass scintillation vials, dissolved in 600 μl of ethylene glycol monomethyl ether and finally suspended in 15 ml of liquid scintillation counting cocktail. Samples were measured for radioactivity with a Packard Tri-Carb 460 CD Liquid Scintillation System. Transport experiments were performed in duplicate and appropriate standard curves and blank counts were obtained for each experimental series.

All data points were corrected for the amount of tracer bound to the membranes and filters at time zero. This quantity, typically only a few tenths of a nmol per mg of protein, was always subtracted from the net flux values in order to correct for non-specific sequestering of tracer, and was determined by adding 100 μl of the proteoliposome suspension to EM that had been previously diluted with 1 ml of ice-cold 3 mM $HgCl_2$ stop solution. Filtering and washing of the aliquots were then performed as usual. Tests were also performed to determine the quantity of free diffusion of lactate into liposomes without reconstituted carrier activity. Results from these experiments confirmed that free diffusion of lactate into liposomes averaged only two-tenths of 1% over the 60 min time course and as such was deemed insignificant.

SDS/PAGE analysis of proteins

Gel electrophoresis was performed according to the method of Laemmli (1970) using Bio-Rad pre-cast 12% (w/v) polyacrylamide separation minigels. Column fractions were dissolved in buffer containing 0.5 M Tris/HCl (pH 6.8), 10% (w/v) SDS, 10% (v/v) glycerol and 25 mg/ml DTT, boiled for 5 min, allowed to cool and applied to the gel. Electrophoresis was carried out at 150 V (constant voltage) with an E-C 570 power unit. The gel was fixed with 10% (v/v) acetic acid and stained with silver nitrate to visualize polypeptide bands.

RESULTS AND DISCUSSION

Lactate transporter characterization in native vesicles

The uptake of L-lactate into native sarcolemmal vesicles was analysed to establish baseline values of the lactate transporter before protein purification and reconstitution. L-Lactate influx

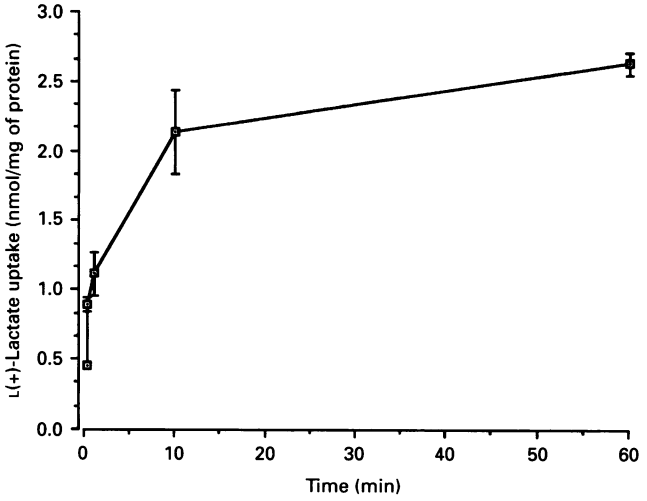

Figure 1 Time-course uptake of 1 mM L-lactate into native sarcolemmal vesicles

External medium contained 1 mM tracer lactate in 280 mM buffered sucrose, pH 7.4. Values are means ± S.E.M. of three different experiments.

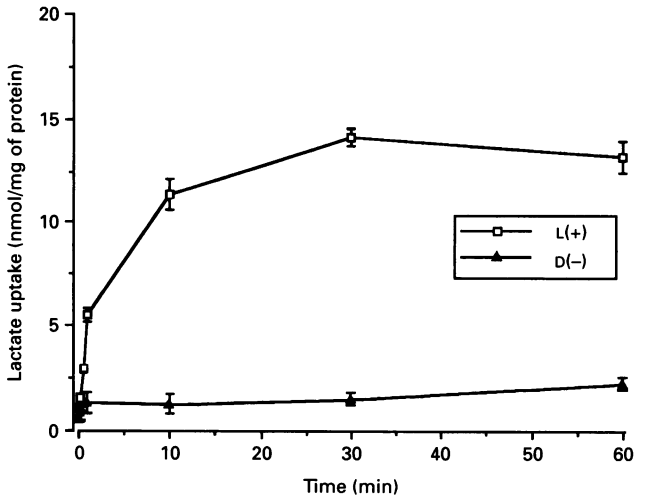

Figure 2 Time course of 1 mM L-(□) and D-(▲) lactate uptake into proteoliposomes

Sarcolemmal vesicles were solubilized with 1.2% (w/v) octylglucoside and the extract dialysed to remove detergent for 17 h against 1000 vol. of KRH buffer (pH 7.4) containing 20% (v/v) glycerol, 1 mM DDT and 1 μg/ml each of aprotinin and leupeptin. Proteins were then reconstituted into liposomes and tested for lactate transport activity. External medium contained 1 mM L- or D-isomer tracer lactate in KRH buffer, pH 7.4. Values are means ± S.E.M. of three different experiments.

activities of native vesicles were examined with both the internal and external media controlled at 25 °C and pH 7.4. The zero-*trans* conditions for these experiments were conducted with lactate present in the EM and followed its flux to the inside of the vesicles.

The uptake of L-lactate into sarcolemmal vesicles as a function of time is shown in Figure 1. This zero-*trans* procedure is the simplest experimental condition and highly reproducible. Membrane vesicles were preloaded with 280 mM sucrose, pH 7.4, and diluted into EM composed of 280 mM sucrose, pH 7.4, containing lactate tracer. Uptake maximum of tracer lactate was reached within a short time period (10 min) and equilibrium was maintained for the extent of the trial (60 min). Although similar results have been shown in prior studies from our laboratory (Roth and Brooks, 1990a) it was necessary for the purposes of the present investigation to establish baseline uptake values with which to compare subsequent purification indices.

Protein solubilization and reconstitution

Preliminary tests were conducted to verify the effectiveness of the reconstitution procedure before any separation techniques were employed. Results from these experiments provided evidence that functional activity of the lactate transport protein was maintained following the solubilization, detergent removal and reconstitution steps. On this basis, protein separation procedures were carried out with subsequent tests intended to characterize the lactate transporter in a reconstituted system.

Figure 2 shows time-course uptake of L- and D-lactate tracer into proteoliposomes following solubilization of vesicles, detergent removal and protein reconstitution into liposomes. L-Lactate uptake maximum was reached rapidly and equilibrium values maintained. Conversely, the uptake of the D(−) isomer

Table 1 Purification of the lactate transport protein from rat sarcolemma

Lactate uptake into native vesicles and proteoliposomes was measured as described in the Experimental section. For the HA chromatography experiments, sarcolemmal vesicles were solubilized with 1.2% (w/v) octyglucoside, applied to the column and eluted with increasing concentrations of buffered sodium phosphate. Each column fraction was then dialysed to remove detergent for 17 h against 1000 vol. of KRH buffer (pH 7.4) containing 20% (v/v) glycerol, 1 mM DTT and 1 μg/ml each of aprotinin and leupeptin. Following detergent removal, each fraction was reconstituted into liposomes and tested for lactate transport activity. External medium contained 1 mM tracer lactate in KRH buffer, pH 7.4. Values are means ± S.E.M. of three (native vesicles) and four (HA chromatography) different experiments.

	Protein		Activity		
Sample	(mg)	(%)	Total (nmol/min)	Specific (nmol/min per mg of protein)	Purification (-fold)
Sarcolemmal vesicles	0.5	100	0.53 ± 0.08	1.06 ± 0.17	
Sarcolemmal vesicles (solubilized)	0.3	60	1.66 ± 0.10	5.54 ± 0.34	5.2
HA eluate:					
0.1 M	0.029	6	0.11 ± 0.04	3.63 ± 1.30	3.4
0.2 M	0.035	7	1.11 ± 0.08	31.64 ± 2.19	29.8
0.3 M	0.025	5	0.24 ± 0.06	9.63 ± 2.24	9.1

into proteoliposomes was slow and constant over time. The shape of the L-lactate uptake curve is similar to that of the native sarcolemmal vesicles as shown in Figure 1, but with higher uptake values reflecting some non-specific protein loss (and purification) during the solubilization step. This initial purification of lactate transport activity was unexpected yet significant as shown from protein visualization by SDS/PAGE. These results are in agreement with previous studies which demonstrate stereoselectivity of the lactate transporter. Taken together these findings demonstrate that functional activity of the lactate transport protein was maintained during solubilization and reconstitution.

HA chromatography

Fractionation of the solubilized sarcolemmal membrane proteins by HA chromatography and subsequent analysis of L-lactate transport activity of each fraction at 1 min is shown in Table 1. The column was equilibrated with KRH buffer (pH 7.4) with 1.2% (w/v) octylglucoside, the sample applied and fractions were eluted with a sodium phosphate gradient between 0.1 and 0.4 M (pH 6.8). Fractions were collected and dialysed against KRH buffer as previously described, reconstituted into liposomes on a 100:1 lipid to protein ratio, and tested for L-lactate transport activity.

The highest activity of L-lactate uptake was observed in the 0.2 M sodium phosphate fraction (31.64±2.19 nmol/min per mg). Lactate transport activity was also examined in the 0.1 and 0.3 M fractions which exhibited uptake of 3.63±1.30 and 9.63±2.24 nmol/min per mg respectively. Conversely, no lactate uptake was observed in the FT or the 0.4 M sodium phosphate fractions following reconstitution. L-Lactate transport activity eluting from the 0.2 M sodium phosphate gradient represents a 30-fold purification over that of non-solubilized, native sarcolemmal vesicles (31.64±2.19 versus 1.06±0.17 nmol/min per mg). These results are shown in Table 1.

The uptake of L-lactate as a function of time into proteoliposomes following reconstitution of the 0.2 M fraction is shown in Figure 3. Lactate uptake reaches equilibrium values over time following rapid uptake. All sample collection, detergent removal and reconstitution procedures described above were employed for this series of experiments.

When the L-lactate uptake rates are plotted as a function of the EM lactate concentration, Michaelis–Menten saturation kinetics are displayed as shown in Figure 4. For these experiments, uptake at 15 s was used as a measure of the initial rate of uptake and is expressed in nmol/15 s per mg of protein. Data were fitted by a non-linear least squares regression analysis to the Michaelis–Menten equation. The apparent K_m and $V_{max.}$ values (expressed as mean±S.E.M. for three different experiments) were 46.2±6.6 mM and 498.7±17.2 nmol/15 s per mg respectively. The K_m value observed in the present study is similar to those reported by Roth and Brooks (1993) in rat skeletal muscle sarcolemmal vesicles from control, sprint-trained and endurance-trained animals (40.1, 37.3 and 46.4 mM respectively).

The effects of L-lactate uptake into proteoliposomes were tested following preincubation with 10 mM α-cyano-4-hydroxycinnamate. The 0.2 M sodium phosphate fraction was collected, dialysed and reconstituted as previously described. Compared with control values, preincubation of proteoliposomes with 10 mM α-cyano-4-hydroxycinnamate prior to the transport assay resulted in a 57% inhibition of L-lactate uptake (13.60±2.18 versus 31.64±2.14 nmol/min per mg respectively; results not shown). These findings are in agreement with transport inhibition studies in purified sarcolemmal vesicles (Roth and Brooks, 1990a).

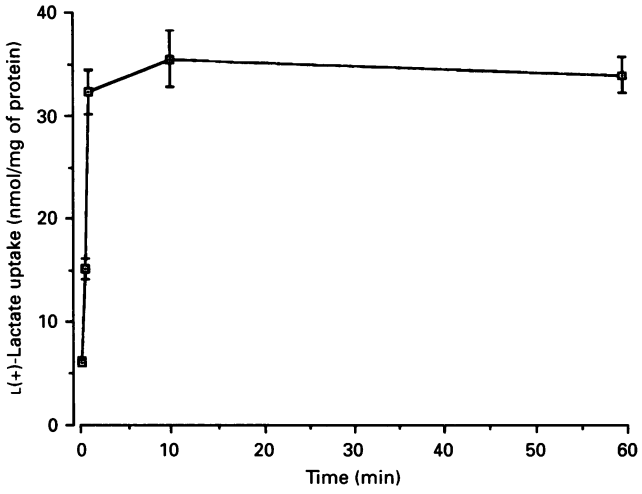

Figure 3 Time course of 1 mM L-lactate uptake into proteoliposomes following HA chromatography

Eluate from the 0.2 M sodium phosphate fraction was collected, dialysed for 17 h against 1000 vol. of KRH buffer (pH 7.4) containing 20% (v/v) glycerol, 1 mM DTT and 1 μg/ml each of aprotinin and leupeptin, reconstituted into liposomes and tested for lactate transport activity. External medium contained 1 mM tracer lactate in KRH buffer, pH 7.4. Values are means±S.E.M. of four different experiments.

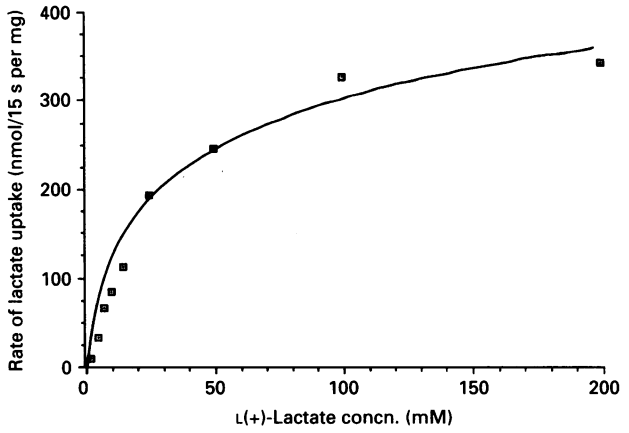

Figure 4 Michaelis–Menten saturation kinetics demonstrating dependence of the initial rate of L-lactate uptake on external medium lactate concentration

Eluate from the 0.2 M sodium phosphate fraction was collected, dialysed for 17 h against 1000 vol. of KRH buffer (pH 7.4) containing 20% (v/v) glycerol, 1 mM DTT and 1 μg/ml each of aprotinin and leupeptin, reconstituted into liposomes and tested for lactate transport activity. Values are means±S.E.M. of three different experiments.

SDS/PAGE

Analysis of the HA eluates by SDS/PAGE is shown in Figure 5. L-Lactate transport activity eluting from the 0.2 M sodium phosphate fraction strongly corresponds to the presence of a band of approx. 34 kDa (lane 6). This band appears in the 0.1 and 0.3 M fractions as well (lanes 5 and 7 respectively). Analysis of SDS/PAGE following HA chromatography, together with

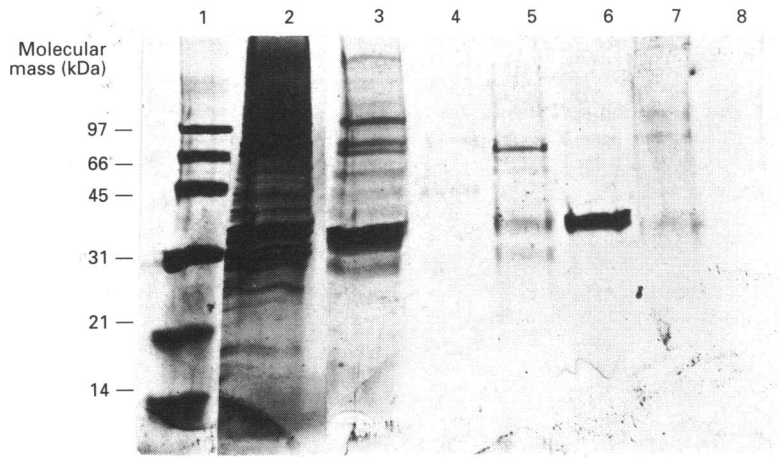

Figure 5 SDS/PAGE analysis showing polypeptide patterns of the native sarcolemmal membrane, octylglucoside extract and HA eluates

Lane 1, molecular-mass markers; lane 2, native sarcolemmal membrane proteins; lane 3, octylglucoside-solubilized extract; lane 4, HA column flow through; lanes 5–8, 0.1–0.4 M sodium phosphate gradient column eluates. Protein contents of lanes 2 and 3 were 3 and 2 μg respectively and lanes 5–7 were 0.5 μg. The gel was stained with silver nitrate.

data from reconstituted transport activity suggests the involvement of a 34 kDa polypeptide mediating lactate transport in rat skeletal muscle. Our present results are in agreement with previous studies which provide evidence suggesting the mediation of lactate transport by polypeptides of 35–45 and 40 kDa in rat erythrocytes and hepatocytes respectively (Jennings and Adams-Lackey, 1982; Welch et al., 1984; Poole and Halestrap, 1992). Further, the purified pyruvate carrier from bovine heart mitochondria is a polypeptide with an apparent molecular mass of 34 kDa (Bolli et al., 1989). Although the affinity for pyruvate in the mitochondrial carrier is greater than that for lactate, the similarities in substrate specificities and inhibitor sensitivities in these different tissues suggests a family of proteins may be responsible for transmembrane monocarboxylate transport.

CONCLUSIONS

Results from this study clearly demonstrate that the lactate transport protein from rat sarcolemmal membranes can be purified and functionally reconstituted into liposomes. Transport activity eluting from the 0.2 M sodium phosphate fraction of the HA column at 1 min represents a purification factor of 30-fold over native sarcolemmal vesicles. The reconstituted lactate carrier protein exhibits Michaelis–Menten saturation kinetics. Further, activity of the reconstituted carrier protein can be inhibited by preincubation with α-cyano-4-hydroxycinnamate, a known inhibitor of lactate transport. Analysis of SDS/PAGE following HA chromatography, together with data from reconstituted transport activity, suggests the involvement of a 34 kDa polypeptide mediating lactate transport in rat skeletal muscle.

The significance of a skeletal muscle carrier-mediated lactate transport process *in vivo* becomes evident in light of studies implicating lactate as an important metabolic intermediate between stored carbohydrate and CO_2 (Donovan and Brooks, 1983; Mazzeo et al., 1986; Brooks, 1991; Stanley, 1991). Integration of data from isotopic tracer and chemical extraction experiments in rats and humans has led to formulation of the 'lactate shuttle' hypothesis (Brooks, 1985, 1986, 1987). According to this hypothesis, the metabolism of lactate at sites anatomically removed from sites of production provides a major source of oxidizable and gluconeogenic substrate. Rather than a metabolic end product resulting from oxygen-limited metabolism, lactate is produced and utilized continuously under fully aerobic conditions in virtually all tissues (Brooks, 1991). Skeletal muscle represents a major site of both lactate production and removal which, due to its relative mass, is by far the most important regulator of whole-body lactate kinetics during rest and exercise.

In a variety of cells including human erythrocytes (DeBruinje et al., 1983; Deutike et al., 1986), rat hepatocytes (Fafournoux et al., 1985; Sestoft and Marshall, 1986), perfused rat skeletal muscle (Watt et al., 1988), mouse soleus muscle (Juel, 1988; Juel and Wibrand, 1989), and rat skeletal muscle sarcolemmal vesicles (Roth and Brooks, 1990b), the lactate transporter exhibits increased activity in the presence of proton and lactate gradients, suggesting the existence of an H^+ co-transport mechanism. In active tissue such as skeletal and cardiac muscle, this lactate–proton coupled transport may be extremely important in maintaining intracellular pH balance while accelerating the efflux of accumulating lactic acid. During exercise, the rapid equilibration of muscle lactic acid into erythrocytes via respective transporters is a purported means by which lactate efflux from muscle is facilitated and elevated plasma lactate concentration is buffered (Fishbein, 1986; Roth, 1991). Consequently, the sarcolemmal lactate transport protein is a key regulator of lactate and proton flux and has considerable metabolic importance during rest, exercise and recovery.

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