Reconstitution studies of amino acid transport system L in rat erythrocytes

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In many cell types, including human erythrocytes, membrane transport of hydrophobic amino acids such as leucine and phenylalanine is mediated primarily by Na^+-independent system L. In this paper we demonstrate that erythrocytes from the rat have a 400-fold higher system L transport capacity than human erythrocytes. We have exploited this high transport activity to achieve the first successful reconstitution of an erythrocyte amino acid transporter into phospholipid vesicles. Rat erythrocyte membranes were depleted of extrinsic membrane proteins, solubilized in 50 mM n-octyl glucoside and reconstituted into egg-yolk phospholipid vesicles by a gel filtration freeze–thaw protocol. Optimal reconstitution of transport activity occurred at lipid/protein ratios of 25–35:1. At a lipid/protein ratio of 25:1, one-half of the total uptake of L-[^{14}C]leucine (0.2 mM, 25 °C) was inhibited by 2 mM phloretin and thus judged to be carrier-mediated. This component of L-leucine uptake was inhibited by non-radioactive L-phenylalanine and L-leucine, and only to a very much weaker extent by glycine and L-alanine. Two other inhibitors of system L in intact cells, MK196 and PCMBS (*p*-chloromercuriphenylsulphonate), were also effective inhibitors of phloretin-sensitive L-leucine transport in reconstituted proteoliposomes. Phloretin-insensitive uptake of L-leucine in proteoliposomes occurred by simple diffusion across the lipid bilayer.

INTRODUCTION

Unlike most other classes of plasma membrane transport systems, no specific high-affinity inhibitors of mammalian amino acid transporters are known, and systems have been identified and characterized largely on the basis of substrate specificity and cation/anion requirements [1,2]. This lack of suitable covalent or reversibly binding probes, together with a generally low membrane abundance of these systems, has severely hampered attempts to purify amino acid transport proteins. At the present time, the only viable strategy for identification of relevant proteins in solubilized membrane extracts has been reconstitution of the transport function into phospholipid vesicles. By this approach, enriched preparations of several Na^+-dependent amino acid transport proteins have been obtained [3–6], but no equivalent progress has been reported with respect to mammalian Na^+-independent amino acid transporters. cDNAs have been isolated recently from rat and rabbit kidney which, when expressed in *Xenopus* oocytes, elicit increases in Na^+-independent neutral and cationic amino acid transport activity [7–9]. In contrast to a previously cloned murine fibroblast cationic amino acid transporter which has 14 putative transmembrane domains (622 amino acid residues) [10,11], these cDNAs (79% identity in nucleotide sequence) encode 683- and 677-amino-acid type II membrane glycoproteins with single transmembrane regions. The encoded proteins are thought to be homologous to α-amylases and α-glycosidases, but have apparently no such enzymic activity.

Erythrocytes from different mammalian species express a diverse array of different Na^+-dependent and Na^+-independent amino acid transport systems, and vary widely in their amino acid transport capacity [12,13]. With respect to molecular studies of amino acid permeation, these cells have the additional advantage that they lack intracellular organelles and have a well defined membrane protein composition. Amino acid transport by erythrocytes has been most thoroughly and widely investigated in humans [12,13]. With respect to Na^+-independent amino acid transport, human erythrocytes have a high activity of system L, a transporter with a wide tissue distribution which is selective for hydrophobic amino acids such as leucine and phenylalanine, and lower activities of two other Na^+-independent systems (T and y^+) [14–20]. System T is selective for aromatic amino acids and system y^+ transports dibasic amino acids.

In the present study we have quantified the system L transport capacity of erythrocytes from another mammalian species, the rat. We report here that the estimated membrane abundance of system L transporters in these cells is more than two orders of magnitude higher than that in human erythrocytes and approaches, or exceeds, the abundance of the human erythrocyte glucose transporter (2×10^5 copies/cell). This paper also describes conditions for the reconstitution of the system L transport protein.

MATERIALS AND METHODS

Materials

L-[U-^{14}C]Leucine and other radioactive amino acids were obtained from Amersham International (Amersham, Bucks., U.K.), or from Amersham Canada Ltd. (Oakville, Ontario, Canada). Cellulose nitrate filters (pore size 0.45 μm; HAWP 025 00) were obtained from Millipore (Canada) Ltd. (Mississauga, Ontario, Canada). Crude egg-yolk phospholipid was prepared by the method of Dawson [21], assayed as described by Chen et al. [22] and stored at −70 °C under N_2 in chloroform containing 0.1% (w/v) butylated hydroxytoluene. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or BDH Inc. (Vancouver, British Columbia, Canada) or Merck (Darmstadt, Germany). Protein molecular mass standards for SDS/PAGE were from Pharmacia LKB Biotechnology (Baie d'Urfe, Quebec, Canada).

Uptake experiments (intact cells)

Rat and human blood was collected into heparin and washed three times in incubation medium (150 mM NaCl, 15 mM Mops and 5 mM glucose, pH 7.4). The buffy coat was discarded. To

Abbreviation used: PCMBS, *p*-chloromercuriphenylsulphonate.
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deplete erythrocytes of intracellular amino acids, the cells were adjusted to a haematocrit of 10% in incubation medium and incubated for 2 h at 37 °C, after which they were washed a further three times in incubation medium and stored on ice at a haematocrit of 20% until required. Uptake experiments were performed by an n-dibutyl phthalate separation method used routinely in this laboratory [23]. The stopping solution contained 2 mM phloretin [18]. Blank values were obtained by mixing ice-cold phloretin-treated cells with ice-cold radiolabelled permeant, followed by immediate centrifugation through oil. Iso-osmolarity at different extracellular amino acid concentrations was maintained by adjusting the NaCl concentration of the incubation medium. The water content of rat erythrocytes, determined using ^3H_2O and the impermeant amino acid L-[^{14}C]glutamate as an extracellular space marker, was 70.4% (v/v). Kinetic parameters were determined by Enzfitter, a non-linear regression data analysis program (Elsevier Biosoft).

Preparation of membranes

Rat erythrocyte ghosts were prepared as described previously [24] and depleted of extrinsic membrane proteins by treatment with 0.1 mM EDTA (pH 11.2) for 15 min on ice [24]. Protein-depleted membranes were washed twice with ice-cold buffer containing 20 mM Na-Mops (pH 7.4) and stored at −70 °C until required.

Reconstitution

Reconstitution was by the procedure of McGivan and co-workers [25,26] and Poole and Halestrap [27], modified by the substitution of n-octyl glucoside for Mega-10 as solubilizing detergent. Protein-depleted membranes (5 mg of protein/ml) were solubilized for 5 min at 4 °C in an equal volume of reconstitution buffer [20 mM Na-Mops (pH 7.4), 1 mM EGTA, 0.1 mM dithiothreitol and 0.1 mM phenylmethanesulphonyl fluoride] containing 50 mM n-octyl glucoside. Solubilized membranes were centrifuged at 150000 *g* for 20 min at 4 °C, and the supernatant was assayed for protein content by the method of Peterson [28], after precipitation with trichloroacetic acid, using BSA as standard. Liposomes were prepared from crude egg-yolk phospholipid. Lipid was dried under a stream of nitrogen, redissolved in 1 ml of diethyl ether and subsequently dried under N_2 to remove residual traces of chloroform. The dried lipid was suspended in 1 ml of reconstitution buffer and sonicated at room temperature under N_2 until translucent.

Solubilized membrane proteins were mixed with sonicated liposomes at 4 °C at a ratio of 1 mg of protein/25 μmol of lipid phosphate. Detergent was removed by passing the mixture (2 ml) through a Sephadex G-50 (coarse grade) column (1 cm × 30 cm) equilibrated with reconstitution buffer at 4 °C. Turbid fractions collected in the void volume were pooled together and diluted 4-fold with reconstitution buffer. The mixture was frozen in liquid N_2 and thawed in a water bath at 25 °C Proteoliposomes were then collected by centrifugation at 50000 *g* for 30 min at 4 °C. The white pellet of proteoliposomes was finally resuspended in a small amount of reconstitution buffer for the transport measurements. The size of proteoliposomes was determined using a BI-90 Particle Sizer (Brookhaven Instruments).

Transport experiments (proteoliposomes)

Uptake of L-[^{14}C]leucine by proteoliposomes was measured at 25 °C. Briefly, 30 μl of pre-warmed proteoliposome suspension (1 mg of protein/ml) was incubated with an equal volume of pre-warmed reconstitution buffer containing L-[^{14}C]leucine (0.2 mM final concentration 5 μCi/ml). After a pre-determined time interval, uptake was terminated by adding 0.5 ml of ice-cold reconstitution buffer containing 2 mM phloretin, followed by immediate filtration through a Millipore nitrocellulose filter (0.45 μm) on a manifold connected with a vacuum assembly. The filter was washed rapidly with 3 × 1 ml ice-cold aliquots of phloretin-containing reconstitution buffer and then dissolved in 5 ml of Ready Safe liquid scintillation cocktail (Beckman Instruments) for 2 h before measurement of radioactivity. Blanks were processed identically to samples, except that 1 ml of ice-cold phloretin-containing reconstitution buffer was mixed with proteoliposomes before addition of the radioactive solution and filtered immediately.

SDS/polyacrylamide-gel electrophoresis

SDS/PAGE in 1.5 mm-thick slab gels was performed by the method of Thompson and Maddy [29] using the Laemmli buffer system [30]. Gels were stained with Coomassie Blue and scanned at 633 nm by laser densitometry (Gelscan XL; Pharmacia) for comparison with molecular mass standards.

RESULTS

Uptake of leucine, phenylalanine and valine by human and rat erythrocytes

Figure 1 shows the ability of human erythrocytes to transport L-leucine, L-phenylalanine and L-valine at 37 °C when the three amino acids were present at an initial extracellular concentration of 0.2 mM. In agreement with previous studies from this and other laboratories [14,16,18,20], amino acid uptake by human erythrocytes proceeded rapidly, reaching the equilibration value of 200 μmol/l of cell water in the first 5–10 min of incubation for

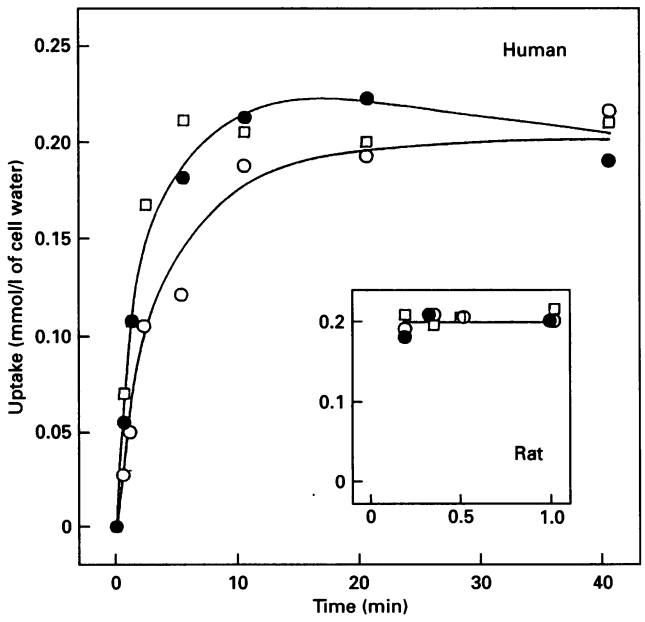

Figure 1 Time courses of L-leucine (●), L-phenylalanine (□) and L-valine (○) uptake by human and by rat erythrocytes

Uptake was measured at 37 °C and 0.2 mM initial extracellular amino acid concentration. Values are means of triplicate determinations.

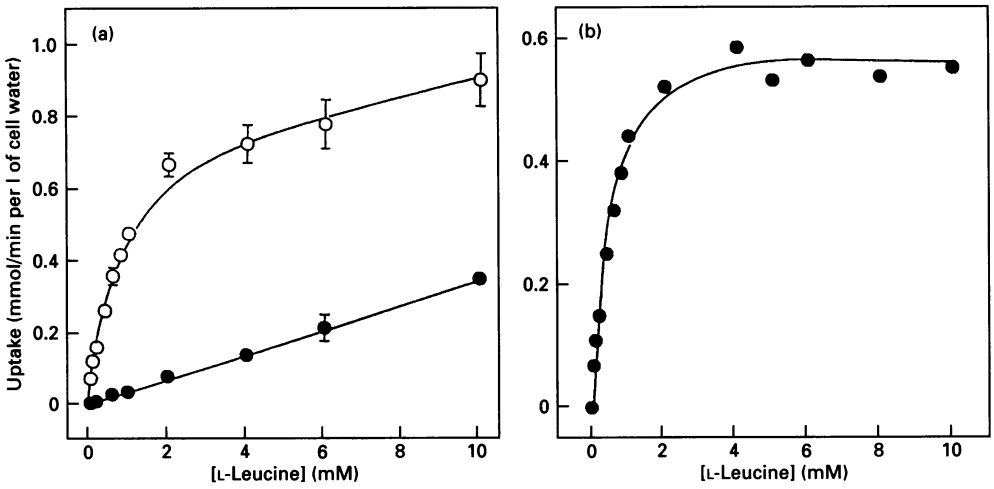

Figure 2 Concentration-dependence of L-leucine uptake by rat erythrocytes

Initial rates of L-[^{14}C]leucine uptake by rat erythrocytes at 1 °C (10 s incubation) were measured in the absence (○) and in the presence (●) of 50 mM non-radioactive L-phenylalanine (**a**). Each data point is the mean ± S.E.M. of triplicate determinations. (**b**) Phenylalanine-sensitive component of leucine uptake. Kinetic parameters for system L-mediated leucine uptake are given in the text.

leucine and phenylalanine, and between 20 and 40 min of incubation for valine. Previous experiments have shown that leucine and phenylalanine uptake by these cells is mediated largely (> 95%) by system L [14,16,18,20]. Valine is also a system L permeant, but has a lower affinity for the transporter than the other two amino acids [14]. The inset to Figure 1 shows that uptake of leucine, phenylalanine and valine by rat erythrocytes was considerably more rapid than in human cells, all three amino acids fully equilibrating with intracellular water within the first 10 s of incubation at 37 °C.

Rapid equilibration with intracellular water (< 30 s) was also observed for leucine uptake by rat erythrocytes when we reduced the incubation temperature, first to 25 °C and then to 10 °C. To obtain measurable initial rates of leucine uptake in these cells for subsequent kinetic and inhibitor studies, it was necessary to decrease the incubation temperature to 1 °C and to use short (10 s) incubation periods. At this temperature, the initial rate of uptake of 0.2 mM leucine by rat erythrocytes was typically 180 μmol/min per l of cell water, i.e. 30 μmol/l cell water in 10 s.

Kinetic and inhibition characteristics of leucine transport by rat erythrocytes

Various non-radioactive amino acids (5 mM extracellular concentration) were tested as inhibitors of [^{14}C]leucine uptake (0.2 mM) by rat erythrocytes at 1 °C. As expected, transport of leucine was strongly inhibited by L-phenylalanine, non-radioactive L-leucine and L-valine (91%, 86% and 80% inhibition respectively (means of triplicate determinations; S.E.M. < 2%)). In contrast, L-tryptophan, which is transported largely by system T in human erythrocytes [19], caused only modest (25%) inhibition of leucine uptake. In human erythrocytes, system L shows only partial stereoselectivity for uptake of L versus D-leucine [20]. In agreement with this, 5 mM D-leucine caused substantial (73%) inhibition of L-leucine uptake by rat erythrocytes. In contrast, small neutral (glycine, L-alanine and L-proline), acidic (L-glutamate) and basic (L-lysine) amino acids caused only slight inhibition of leucine influx (4–9%). Transport was inhibited by phloretin, with an IC_{50} value of 0.25 mM (results not shown).

Figure 2(a) shows the concentration-dependence of leucine influx into rat erythrocytes at 1 °C over the range 0.05–10 mM initial extracellular concentration. Uptake was determined both in the absence and in the presence of excess non-radioactive phenylalanine (50 mM) to distinguish system L-mediated uptake from that occurring by other routes (low-affinity uptake by other transporters and simple diffusion through the lipid bilayer). In the presence of phenylalanine, leucine uptake was linear with respect to concentration, and at the highest concentration tested (10 mM) corresponded to 39% of the total uptake. In contrast, phenylalanine-inhibitable leucine uptake was saturable and conformed to simple Michaelis–Menten kinetics, giving apparent K_m and $V_{max.}$ values of 0.49 ± 0.05 mM and 600 ± 15 mmol/h per l of cell water (Figure 2b). For comparison, kinetic parameters for leucine uptake via system L in human erythrocytes at this temperature from the work of Hoare are K_m 0.28 mM and $V_{max.}$ 2.0 μmol/min per l of cell water (Figure 2 of [17]). There is a relatively small 2.7-fold difference in K_m between the two species, but the expected dramatic difference in $V_{max.}$ (355-fold). Based upon these kinetic parameters, the calculated initial rates of influx of 0.2 mM leucine by system L in the two species at 1 °C are 148 and 0.83 μmol/min per l of cell water in rat and human cells respectively, a ratio of 178-fold. To confirm this very large difference in transport capacity between the two species at 1 °C, we measured the concentration-dependence of system L-mediated leucine uptake by human erythrocytes under conditions identical to those used for rat erythrocytes in Figure 2, except that it was necessary to use a 1 h incubation period in order to achieve a measurable uptake of amino acid into the cells. Kinetic parameters for phenylalanine-sensitive leucine uptake were in good agreement with those of Hoare [17], except that our apparent K_m value (0.43 ± 0.03 mM) was almost identical to that obtained by us for rat erythrocytes at this temperature, and the $V_{max.}$ (1.33 ± 0.2 μmol/min per l of cell water) was slightly lower than that reported by Hoare [17]. Using our values, the $V_{max.}$ difference between the two species is 451-fold (352-fold difference in initial rate at 0.2 mM extracellular amino acid concentration).

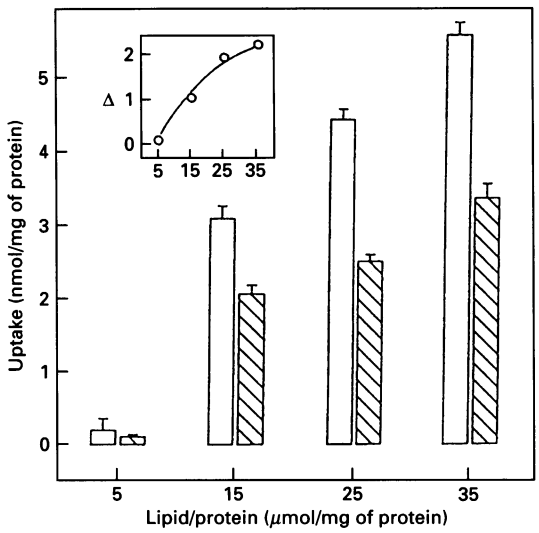

Figure 3 Reconstitution of rat erythrocyte system L into proteoliposomes

Uptake of L-[^{14}C]leucine (0.2 mM; 25 °C; 30 s incubation) into proteoliposomes reconstituted at different lipid/protein ratios was measured in the absence (□) and in the presence ▨ of 2 mM phloretin (15 min preincubation at 25 °C) as described in the text. Values are means ± S.E.M. of triplicate determinations. The inset shows the effect of the lipid/protein ratio on the phloretin-sensitive component of uptake. See the text for other experimental details.

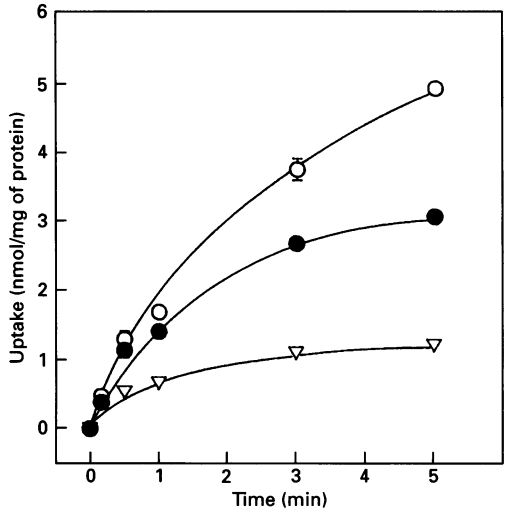

Figure 4 Effect of sucrose on L-leucine uptake by reconstituted proteoliposomes

Time courses of L-[^{14}C]leucine uptake (0.2 mM; 25 °C) by reconstituted proteoliposomes was measured in normal reconstitution buffer (○) and in buffer containing 50 mM (●) and 250 mM (▽) sucrose. Values are means ± S.E.M. of triplicate determinations. See the text for other experimental details.

Reconstitution of rat erythrocyte system L

The presence of high system L activity in rat erythrocytes makes this cell type a potential source of transporter for isolation and purification studies. We therefore investigated conditions for the solubilization of system L from rat erythrocyte membranes and its reconstitution in a functional state into phospholipid vesicles. Our approach towards reconstitution of system L was to use modifications of a gel-filtration freeze–thaw protocol devised originally for the erythrocyte monocarboxylate transporter [27] and for amino acid transport systems from rat liver and bovine kidney plasma membranes [25,26]. Transport (0.2 mM leucine; 25 °C; 30 s incubation) was assayed by rapid filtration of reconstituted egg-yolk phospholipid vesicles through nitrocellulose filters, and is expressed as nmol/mg of reconstituted membrane protein.

Before solubilization, membranes were first treated with 0.1 mM EDTA (pH 11.2) to remove the cytoskeleton and other peripheral proteins. SDS/PAGE determined that this protein depletion procedure resulted in loss from the membranes of bands 1, 2, 4.2, 5 and 6 (nomenclature of Steck [31]). Subsequent extraction of these membranes with 50 mM n-octyl glucoside solubilized 64% of the integral membrane protein, and the SDS/PAGE profile of the solubilized preparation was indistinguishable from that of the starting protein-depleted membranes. Under standard reconstitution conditions (see below), ⩾ 90% of the solubilized protein was incorporated into proteoliposomes which had a mean diameter of 560 nm.

Figure 3 shows that optimal reconstitution of system L transport activity was achieved at lipid/protein ratios of 25–35:1. A lipid/protein ratio of 25:1 was used in all subsequent experiments. Under these conditions, approximately one-half of the total uptake of leucine was inhibited by 2 mM phloretin. Leucine is a lipophilic amino acid. As a control to distinguish between transport of leucine into liposomes and binding of amino acid to the surface of vesicles, we confirmed that the presence of external sucrose (50 and 250 mM) in the extravesicular medium decreased leucine uptake by reconstituted proteoliposomes, indicating that uptake was into an osmotically active space (Figure 4). To demonstrate that phloretin-sensitive L-leucine uptake was mediated by system L, we tested the effects of different non-radioactive amino acids (5 mM) as inhibitors of this component of leucine uptake by reconstituted proteoliposomes. In agreement with the results for intact rat erythrocytes, uptake was markedly inhibited by L-phenylalanine and non-radioactive L-leucine (97 and 77% respectively), and only to a very much smaller extent by glycine and L-alanine (20 and 10% respectively) (Table 1). In contrast, amino acids had no effect on the phloretin-insensitive component of leucine uptake (results not shown). Two other inhibitors of system L in intact cells [MK196 and *p*-chloromercuriphenylsulphonate (PCMBS)] [32] were also effective inhibitors of

Table 1 Inhibition of L-leucine uptake by reconstituted proteoliposomes from rat erythrocyte membranes

Various non-radioactive amino acids and other compounds were tested as inhibitors of phloretin-sensitive L-[^{14}C]leucine transport (0.2 mM) in reconstituted proteoliposomes at 25 °C (30 s incubation). Non-radioactive amino acids and [^{14}C]leucine were added to proteoliposomes simultaneously. MK196 and PCMBS were preincubated with proteoliposomes for 15 min at 20 °C before addition of permeant. Values are means ± S.E.M. of triplicate determinations. Numbers in parentheses are % of control uptake

Inhibitor	L-Leucine uptake (nmol/mg of protein)
Control	2.23 ± 0.10 (100)
L-Leucine (5 mM)	0.52 ± 0.07 (23)
L-Phenylalanine (5 mM)	0.06 ± 0.06 (3)
L-Alanine (5 mM)	2.01 ± 0.39 (90)
Glycine (5 mM)	1.78 ± 0.17 (80)
MK196 (2 mM)	0.37 ± 0.05 (17)
PCMBS (2 mM)	0.45 ± 0.05 (20)

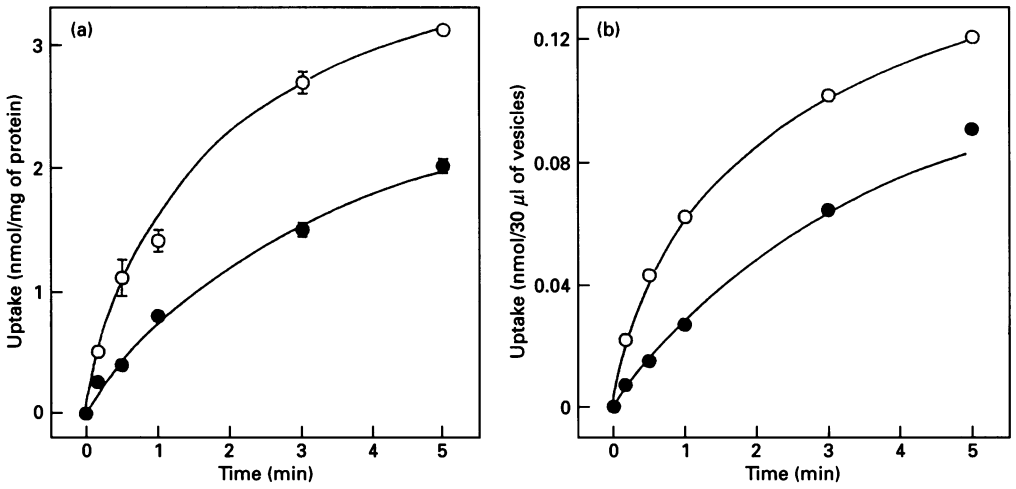

Figure 5 L-Leucine uptake by reconstituted proteoliposomes, liposomes reconstituted with heat-inactivated membrane extract and protein-free liposomes

(**a**) Time courses of L-[^{14}C]leucine uptake (0.2 mM; 25 °C) by normal proteoliposomes (○) and liposomes reconstituted with heat-treated (100 °C for 4 min) membrane extract (●). (**b**) Time course of L-leucine uptake measured in normal proteoliposomes (○) and protein-free liposomes (●). Values are means ± S.E.M. of triplicate determinations. See the text for other experimental details.

phloretin-sensitive leucine transport in reconstituted proteoliposomes (see also Table 1). Since leucine is a relatively hydrophobic amino acid, the residual phloretin-insensitive component of leucine uptake is most likely to represent simple diffusion of the amino acid across the lipid bilayer. This was confirmed in the experiments shown in Figures 5(a) and 5(b), which demonstrate that this component of leucine uptake corresponds to that seen with heat-treated membrane proteins and also with liposomes prepared in the absence of membrane extract.

DISCUSSION

In terms of transport activity, the dominant amino acid transport system present in human erythrocytes is Na^+-independent system L [20]. As illustrated in Figure 1, transport of leucine by this route in human erythrocytes is rapid, with an initial rate of ~ 120 μmol/min per l of cell water (0.2 mM extracellular leucine; 37 °C). Kinetically, the transporter conforms to the simple carrier model of Lieb and Stein [33–35]. The system is also present in the erythrocytes of some other mammalian species (e.g. rabbit), but not ruminants, and shares functional similarities with equivalent transporters in a wide range of other cell types and tissues [1,2]. With respect to transport capacity ($V_{max.}$), system L ranks fourth behind three other human erythrocyte equilibrative transport systems the Cl^-/HCO_3^- exchange transporter (10^6 copies per cell), the glucose transporter (2×10^5 copies/cell) and the nucleoside transporter (10^4 copies per cell) [24,36,37].

If system L has a turnover number equivalent to those determined for the human erythrocyte glucose and nucleoside transport systems [35], then the estimated membrane abundance of system L transport proteins in rat erythrocytes would be ~ 2×10^4 copies/cell based upon $V_{max.}$ values for zero-*trans* influx, or ~ 1×10^4 copies/cell based upon $V_{max.}$ values for equilibrium-exchange influx. In a recent study we used equilibration values for leucine and lysine uptake by right-side-out vesicles to provide a more direct estimate of the membrane abundance of system L and system y^+ amino acid transporters in human erythrocytes [38]. It was found that all of the intravesicular space was accessible to leucine, as judged by comparisons with uridine uptake via the nucleoside transporter, while only 28 % of the total intravesicular space was accessible to lysine uptake by system y^+. Since human erythrocyte membranes generate an average of ~ 10^4 vesicles/cell, these data provide independent evidence that system L transporters are present in human erythrocytes at ⩾ 10^4 copies/cell, while system y^+ is present in smaller amounts (~ 300 copies/cell).

In the present series of experiments, we have demonstrated that rat erythrocytes have a dramatically higher system L transport activity than human erythrocytes. This difference is a $V_{max.}$ effect, with essentially no difference in apparent K_m. While some of the measured (350–450-fold) difference in transport capacity between the two cell types at 1 °C might be a consequence of differences in the temperature sensitivity or turnover number of the transporters in the two species [39], it seem likely that much of the difference can be attributed to differences in the number of copies of the system L transporter present in the two cell types. There are well established precedents for such species differences. For example, large species variations in erythrocyte nucleoside transport activity have been shown to correlate direct with the membrane abundance of nucleoside transporter ligand binding sites [40]. On the basis of these arguments, we can estimate that the number of copies of the system L transporter in rat erythrocyte membranes approaches, or exceeds, that of the human erythrocyte glucose transport system (2×10^5 copies/cell), which accounts for ~ 5 % of the total integral membrane protein [36]. However, compared with human erythrocytes, those from the rat exhibit low glucose and nucleoside transport activities [41,42]. For these different reasons, rat erythrocytes represent a unique potential source of system L transporter for isolation and purification studies.

In common with the situation for other mammalian amino acid transporters, no specific high-affinity inhibitors or other ligands of system L are available. Reconstitution of transporter into liposomes followed by assays of transport activity is the only means presently available to detect system L after its extraction from the plasma membrane. There have been two previous reports of reconstitution of system L activity, both in hepatic cells [26,43]. The present study using rat erythrocyte membranes

is the first to report reconstitution of an erythrocyte amino acid transporter.

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