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Reconstitution of the L-leucine-H^+ cotransporter of the plasma membrane from Chang liver cells into proteoliposomes

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L-Leucine is cotransported with H^+ in the plasma membrane of Chang liver cells (Mitsumoto, Y. et al. (1986) J. Biol. Chem. 261, 4549). The leucine transport system was solubilized from the plasma membrane of the cells with octyl glucoside and reconstituted in proteoliposomes prepared by a rapid dilution of a mixture of the solubilized proteins, octyl glucoside and liposomes. The proteoliposomes exhibited H^+-gradient and electrical potential-stimulated leucine uptake. The H^+-gradient-stimulated leucine uptake could be completely inhibited by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH). The stimulatory effect of H^+ gradient on leucine uptake was shown to be mainly due to decrease of the K_m, but not to change of the V_{max}, of the transport kinetics. These results suggest that the leucine-H^+ cotransporter is solubilized and reconstituted into proteoliposomes.

Introduction

The uptake of neutral amino acids in animal cells is carried out by several distinct transport systems, which have overlapping reactivities [1–3]. Na^+-independent amino acid transport system (system L) is most reactive with hydrophobic, branched-chain and aromatic amino acids, such as leucine, isoleucine, valine, phenylalanine and the nonmetabolizable analogue, BCH.

We previously reported in chang liver cells that L-leucine transport mediated by system L is stimulated by electrochemical gradient of H^+ across the cell membrane [4,5], and identified a leucine-H^+ cotransport system which is coupled to an amiloride-sensitive Na^+/H^+ exchanger in intact cells [5].

One approach to purification and further characterization of this transport system is solubilization of relevant membrane proteins from the plasma membrane and reconstitution of the transport function in liposomes. There have been numerous reports of reconstituting functional transport systems of mammalian origins, such as glucose transporter [6,7], cation pump [8,9] and Na^+-dependent amino acid transporter [10–13]. We have established in the present work the procedures for solubilization and reconstitution of the leucine-H^+ cotransporter from the plasma membrane of Chang liver cells and characterized the system.

Materials and Methods

Materials. L-[U-^{14}C]Leucine (348 mCi/mmol) was purchased from (Amersham International, U.K.). Octyl β-D-glucopyranoside (octyl glucoside) was purchased from Dojin Laboratories (Kumamoto, Japan). Asolectin was purchased from Sigma (St. Louis, MO). BCH was purchased from Calbiochem-Behring (San Diego, CA).

Cell culture. Chang liver cells were grown as large-scale culture in suspension culture vessels (4–8 l) with Eagle's minimum essential medium supplemented with 10% (v/v) bovine serum at 37°C for 4–5 days.

Preparation of the plasma membrane fraction. Mixed membrane fraction was separated on a discontinuous sucrose gradient that contained the following layers: mixed membrane vesicles in 1 mM Tris-HCl (pH 7.8) and 2 mM $MgCl_2$ and 30, 40 and 47% (w/v) sucrose in 1 mM Tris-HCl (pH 7.8) and 1 mM $MgCl_2$ (9 ml each). After centrifugation at 100 000 × *g* for 13 h, the membrane fraction was concentrated at the interfaces between 30/40 and 40/47% (w/v) sucrose. The mem-

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; BCH, 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid.

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brane fraction was collected, diluted with 1 mM Tris-HCl (pH 7.8) and 2 mM $MgCl_2$, and pelleted at 100000 $\times g$ for 1 h. The pellet was suspended in 10 mM Tris-HCl (pH 7.4), 0.15 M KCl and 1 mM dithiothreitol (DTT) by passage through 25-gauge syringe. Vesicles were stored in 1-ml aliquots in liquid nitrogen. Compared with the homogenate, the membrane preparations from Chang liver cells were enriched approx. 12-fold with ouabain-sensitive Na^+/K^+-ATPase.

Solubilization and reconstitution. Membrane proteins were reconstituted by the octyl glucoside dilution procedure as previously described for various materials [14–16]. The plasma membrane fraction was suspended (1–2 mg protein) in 1.5% octyl glucoside, 2–4 mg acetone/ether washed asolectin, 0.15 M KCl, 1 mM DTT and 10 mM Tris-HCl (pH 7.4), and incubated for 4°C for 30 min. The suspension was blended on a Vortex mixer, and then centrifuged at 140000 $\times g$ for 1 h. The supernatant (about 0.5–1 mg solubilized membrane protein) was carefully removed with a Pasteur pipet and mixed with bath sonicated liposomes (10–20 mg lipid) containing 1.5% octyl glucoside. The mixture was blended on a Vortex mixer and then incubated at 4°C for 10 min. The suspension was then pipetted directly into 30-fold 0.15 M KCl, 1 mM DTT, 1 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.4) at room temperature. The resultant proteoliposomes were sedimented by centrifugation at 100000 $\times g$ for 1 h. The supernatant was decanted and the pellet was resuspended in experimental medium by passage through a 25-gauge syringe.

Transport assay. Proteoliposomes suspended in 0.15 M KCl, 1 mM DTT, 1 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.4) were incubated for 10 min at 37°C. Amino acid transport activity was measured with 0.3 mM L-[U-^{14}C]leucine (2.5 μCi/ml) by rapid filtration technique using Millipore filter (pore size 0.22 μm) as previously described [5].

Protein determination. Protein concentration was determined by the method of Schaffner and Weissmann [17].

Results and Discussion

When preparations of the plasma membrane from Chang liver cells were treated with 1.5% octyl glucoside in the presence of exogenous acetone/ether washed asolectin as described under Materials and Methods, 60% of the membrane proteins were solubilized. The solubilized fraction was then added to liposomes in medium and diluted 30-fold into octyl glucoside-free buffer. In the present experiments, about 10% of the solubilized proteins were recovered in the reconstituted proteoliposomes after centrifugation following the dilution step.

Fig. 1 shows that the leucine uptake by the proteoliposomes is stimulated by decrease of external pH.

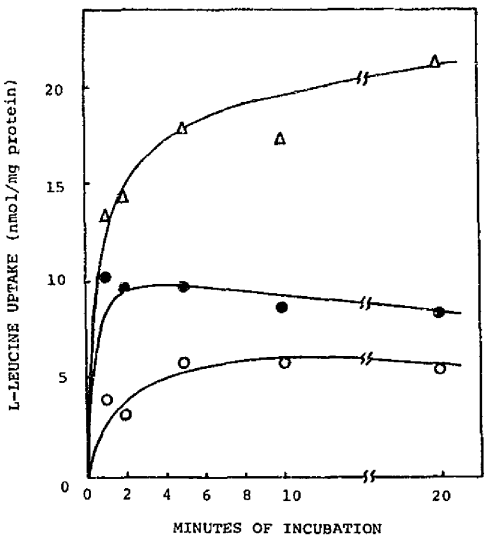

Fig. 1. External pH dependence of leucine uptake by reconstituted proteoliposomes. The leucine uptakes were determined in 10 mM Tris-HCl (pH 7.4) (○), 10 mM Hepes-Tris (pH 6.4) (●) or 10 mM Mes-Tris (pH 5.4) (△) buffers containing 0.15 M KCl, 1 mM $MgCl_2$ and 1 mM DTT. The reconstituted proteoliposomes were incubated for 10 min at 37°C and then the uptake was initiated by addition of 0.3 mM leucine. Each point is expressed as the mean of duplicate samples.

Stimulation of leucine uptake (pH 5.4/pH 7.4) at 1 min of incubation was about 1.5-fold that by native membrane vesicles [4].

As shown in Table I, when the H^+ gradient was dissipated by addition of FCCP, the initial rate of uptake of leucine was almost the same level as in no H^+-gradient. FCCP had no effect on the leucine uptake in the absence of H^+-gradient. These results indicate that the leucine uptake by proteoliposomes was stimulated by an H^+-gradient across the membranes.

Fig. 2 shows concentration dependence of the inhibition of H^+-gradient-stimulated leucine uptake by BCH, a model substrate specific for system L of amino acid transport in mammalian cells [1]. 1-min uptake of leucine

TABLE I

Inhibition by FCCP of H^+ gradient-stimulated leucine uptake by reconstituted proteoliposomes

Each value represents the mean ± S.E. ($n = 4$). n.s., not significant. FCCP, 5 μM.

Experimental condition		(L-Leucine uptake nmol/mg protein per min)	P
external pH	FCCP		
7.4	–	6.45 ± 1.60	–
7.4	+	6.19 ± 0.85	n.s.
5.4	–	13.95 ± 3.03	< 0.05
5.4	+	6.71 ± 0.76	n.s.

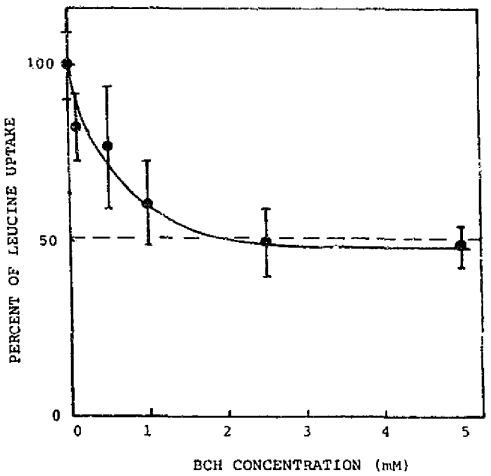

Fig. 2. Inhibition by BCH of H^+-gradient-stimulated leucine uptake by the reconstituted proteoliposomes. The initial rate of leucine uptake (1 min) was determined in 10 mM Mes-Tris (pH 5.4) using the indicated concentrations of BCH. The dotted line indicates leucine uptake without H^+-gradient. Each value represents the mean ± S.E. ($n = 4$).

(1 mM) by intact Chang liver cells was inhibited by 5 mM BCH by about 75%, consistent with our previous data [18]. Leucine uptake without H^+-gradient was not affected by 5 mM BCH (12.67 ± 3.25 and 11.23 ± 2.13 nmol/mg protein per min in the absence and presence of BCH, respectively ($n = 4$). We assume that H^+-gradient-independent and BCH-insensitive leucine uptake is an unmediated process across the membranes. With 2.5 mM BCH, a maximal inhibition was observed, completely abolishing stimulation of leucine uptake due to H^+-gradient.

As shown in Fig. 3, a potassium diffusion potential produced by addition of valinomycin to proteoliposomes possessing a K^+ concentration gradient ($[K^+]_{in} > [K^+]_{out}$) could stimulate leucine uptake without inward H^+-gradient. Therefore, leucine uptake can be driven by electrical potential (inside negative) in reconstituted membranes as in native membrane vesicles [4].

Fig. 4 shows stimulation by an inward H^+-gradient of the initial rate of leucine uptake by proteoliposomes with various concentrations of leucine. The rate is saturable and double-reciprocal plots of its concentration dependence (Fig. 4, inset) indicate a decrease of the K_m value for the uptake kinetics by the H^+-gradient (0.49 mM from 0.90 mM). The V_{max} value for the uptake rate is almost unaltered by the H^+-gradient, 55.81 and 44.13 nmol/mg protein per min in the presence and absence of H^+-gradient, respectively. Therefore, the H^+-gradient imposed across the membrane is considered to increase the affinity of the carrier protein for leucine.

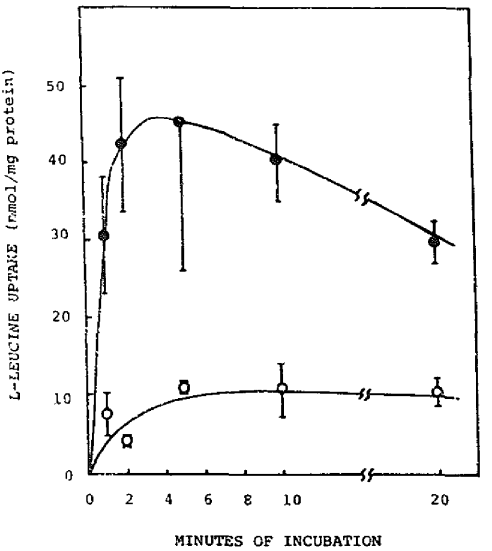

Fig. 3. Stimulation by K^+-valinomycin diffusion potential of leucine uptake by reconstituted proteoliposomes. The leucine uptakes were determined in NaCl instead of KCl with (●) of without (○) valinomycin (3 μM). Each point represents the mean ± S.E. ($n = 3$).

McCormick et al. reported that Na^+-independent leucine exchange systems in Ehrlich ascites cells were reconstituted into proteoliposomes formed by Sephadex G-50 chromatography combined with a freeze-thaw step

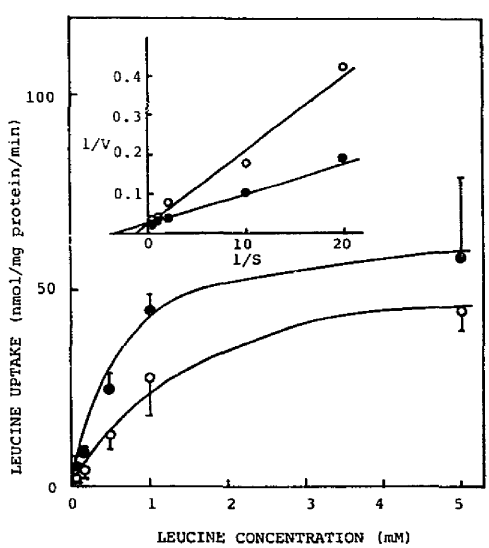

Fig. 4. The effect of H^+-gradient on the kinetics of the leucine uptake by reconstituted proteoliposomes. The initial rates of leucine uptake (1 min) were determined in Tris-HCl (pH 7.4) (○) or Mes-Tris (pH 5.4) (●) buffers. Each point represents the mean ± S.E. ($n = 4$). The inset shows Lineweaver-Burk plots of the data.

[10]. However, Na^+-dependent amino acid transporters were also included in their liposome system. In our proteoliposome system Na^+-dependent glycine and leucine transports were found not to be included (data not shown). Therefore, the rapid dilution technique adopted in the present work and in isolation of many H^+ transporters in bacteria [19], plants [14] and animals [16,20] has been proved to be effective in selective reconstitution of H^+-coupled leucine transport system of Chang liver cells.

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