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Biochimica et Biophysica Acta 1667 (2004) 122–131

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Reconstitution into liposomes of the glutamine/amino acid transporter from renal cell plasma membrane: functional characterization, kinetics and activation by nucleotides

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Received 29 July 2004; received in revised form 9 September 2004; accepted 16 September 2004
Available online 29 September 2004

Abstract

The glutamine/amino acid transporter was solubilized from rat renal apical plasma membrane (brush-border membrane) with $C_{12}E_8$ and reconstituted into liposomes by removing the detergent from mixed micelles by hydrophobic chromatography on Amberlite XAD-4. The reconstitution was optimised with respect to the protein concentration, the detergent/phospholipid ratio and the number of passages through a single Amberlite column. The reconstituted glutamine/amino acid transporter catalysed a first-order antiport reaction stimulated by external, not internal, Na^+. Optimal activity was found at pH 7.0. The sulfhydryl reagents $HgCl_2$, mersalyl and *p*-hydroxymercuribenzoate and the amino acids alanine, serine, threonine, cysteine, asparagine, methionine and valine strongly inhibited the transport, whereas the amino acid analogue methylaminoisobutyrate had no effect. Glutamine, alanine, serine, asparagine, threonine were efficiently translocated from outside to inside and from inside to outside the proteoliposomes as well. Cysteine and valine were translocated preferentially from outside to inside. The K_m for glutamine on the external and internal side of the transporter was 0.47 and 11 mM, respectively; the values were not influenced by the type of the counter substrate. The transporter is functionally asymmetrical and it is unidirectionally inserted into the proteoliposomal membrane with an orientation corresponding to that of the native membrane. By a bisubstrate kinetic analysis of the glutamine antiport, a random simultaneous mechanism was found. The glutamine antiport was strongly stimulated by internal nucleoside triphosphates and, to a lower extent, by pyrophoshate. The reconstituted glutamine/amino acid transporter functionally corresponds to the ASCT2 protein.

Keywords: Plasma membrane; Transport; Liposome; Reconstitution; Glutamine; Amino acid; ASCT2

1. Introduction

Among the numerous amino acid transport systems of the plasma membrane, there is a restricted group of transporters that share the specificity for glutamine (see Refs. [1–5] for reviews). In mammals, these transporters play the important function of mediating glutamine trafficking among different tissues and intestinal and renal (re)absorption. In the last decade the genes coding for the glutamine-specific transport systems have been identified and the encoded proteins have been characterized in expressing cell systems. A general feature of the proteins that mediate glutamine transport is the more or less broad specificity towards different amino acids: thus, the glutamine transporters are also designated as amino acid transporters with specificity towards glutamine. The various transporters can be assigned to different protein families on

Abbreviations: *p*-OHMB, *p*-hydroxymercuribenzoate; MTSET, [2-(trimethylammonium)ethyl] methanethiosulfonate; C_xE_y, alkyl(*x*)-poly(*y*)oxyethylene ether; MeAIB, α-(methylamino)isobutyric acid; ANTP, adenosine 5′ -(β,γ-imido)triphosphate; PLP, pyridoxal-5-phosphate

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doi:10.1016/j.bbamem.2004.09.007

the basis of their structural features. The glutamine-specific transporters can be functionally divided in two groups: sodium-dependent (systems ASCT2/ATB°, N, B°′ $^{+}$, A and y^{+}L) and sodium-independent (Systems L and b°′ $^{+}$) transporters. Among the sodium-dependent transporters, a further distinctive feature is the high (N and y^{+}L) or low (ASCT2) tolerance towards the substitution of Na^{+} by Li^{+}. In general, the transporters are characterized by the different specificity for amino acids and sensitivity to inhibitors; for example, the amino acid analogue MeAIB is used to assess the function of System A [1–5]. In spite of the very large amount of experimental data deriving from the studies on the glutamine transporters, some aspects of the transporter function and regulation are still unclear or unraveled. The procedure of reconstitution into liposomes has often been helpful in clarifying functional properties of membrane transport systems since it allows some types of experiments that cannot be accurately performed in cell systems. To gain further insight on the plasma membrane glutamine transport, we have reconstituted the glutamine/amino acid transporter from renal apical plasma membrane by using the cyclic detergent removal procedure, previously pointed out and extensively used for the reconstitution of mitochondrial carriers [6–8] and recently optimised for a plasma membrane transporter [9]. The reconstituted glutamine/amino acid transporter has been identified as ASCT2, on the basis of the sodium-dependence and the substrate/inhibitor specificity, as compared with previous data in cell systems [10–16]. In the reconstituted liposomes, we have revealed novel functional, kinetic and regulatory properties of this transporter.

2. Materials and methods

2.1. Materials

Amberlite XAD-4, egg yolk phospholipids (3-*sn*-phosphatidylcoline from egg yolk) C_8E_4 and $C_{12}E_8$ were purchased from Fluka; L-[^{3}H]glutamine from Amersham; Sephadex G-75, Triton X-100, Triton X-114, nucleotides and ANTP from Sigma; MTSET from Toronto Research Chemicals (North York, Ontario, Canada). All other reagents were of analytical grade.

2.2. Solubilization of the glutamine/amino acid transporter

Brush-border membranes were prepared from rat kidney and stored as previously described [9,17]. The glutamine/amino acid transporter was solubilized by treating the membrane preparation (50 μl, about 0.15 mg protein) with 1.3% $C_{12}E_8$ in a final volume of 150 μl and centrifuged at 13 000×*g* for 4 min at 4 °C. The supernatant (extract) was used for the reconstitution.

2.3. Reconstitution of the glutamine/amino acid transporter into liposomes

The glutamine/amino acid transporter was reconstituted by removing the detergent with a hydrophobic chromatography column [6,7]. In this procedure, the mixed micelles containing detergent, protein and phospholipids were repeatedly passed through the same Amberlite XAD-4 column. The composition of the initial mixture used for reconstitution was: 25 μl of the solubilized protein (25-35 μg protein in 1.3% $C_{12}E_8$), 75 μl of 10% $C_{12}E_8$, 100 μl of 10% egg yolk phospholipids in the form of sonicated liposomes prepared as described in Ref. [6], 30 mM L-glutamine (except when differently specified in the legends to figures or tables), 20 mM HEPES/Tris pH 7.0 in a final volume of 700 μl. After vortexing, this mixture was passed 16 times through the same Amberlite column (0.5×2.5 cm) preequilibrated with a buffer containing 20 mM HEPES/Tris pH 7.0 and the same type and concentration of the substrate present in the initial mixture. All the operations were performed at 4 °C, except the passages through Amberlite, which were carried out at room temperature.

2.4. Transport measurements

To remove the external substrate, 550 μl of proteoliposomes was passed through a Sephadex G-75 column (0.7×15 cm) preequilibrated with 20 mM HEPES/Tris pH 7.0 and sucrose at an appropriate concentration to balance the internal osmolarity ("unlabeled" proteoliposomes). For efflux measurements, the "unlabeled" proteoliposomes (600 μl), containing 30 mM glutamine, were "prelabeled" by transporter-mediated exchange equilibration [7] by incubation with 10 μl of 0.6 mM [^{3}H]glutamine at high specific radioactivity (2 μCi/nmol) for 60 min at 25 °C; then, the external radioactivity was removed by passing again the proteoliposomes through Sephadex G-75 as described above. Transport was started, in the case of uptake, by adding [^{3}H]glutamine to the "unlabeled" proteoliposomes or, in the case of efflux, by adding non-radioactive substrates to the "prelabeled" proteoliposomes. In both cases, transport was stopped by adding 20 μM mersalyl at the desired time interval. In control samples the inhibitor was added at time zero according to the inhibitor stop method [18]. The assay temperature was 25 °C. Finally, each sample of proteoliposomes (100 μl) was passed through a Sephadex G-75 column (0.6×8 cm) in order to separate the external from the internal radioactivity. Liposomes were eluted with 1-ml 50 mM NaCl and collected in 4 ml of scintillation mixture, vortexed and counted. For the determination of the [^{3}H]glutamine uptake, the experimental values were corrected by subtracting the respective controls (samples inhibited at time zero). For kinetic determinations, the initial rate of transport was measured by stopping the reaction after 10 min, i.e., within the initial linear range of [^{3}H]glutamine uptake into the

proteoliposomes. [^{3}H]glutamine efflux was determined as $cpm_0 - cpm_{30}$, where cpm_0 was the intraliposomal radioactivity at time zero and cpm_{30} was the residual intraliposomal radioactivity 30 min after the start of the transport.

2.5. Other methods

The protein concentration was determined by the modified Lowry procedure [19]. The internal volume of the proteoliposomes was measured as previously described [6,20].

3. Results

3.1. Optimal conditions for reconstitution

To solubilize the glutamine/amino acid transporter in active form, some nonionic detergents were tested: Triton X-100, Triton X-114, $C_{12}E_8$, C_8E_4. The most suitable for the solubilization of the protein in active form was $C_{12}E_8$ at a concentration of 1.3%. Triton X-100 and Triton X-114 led to the extraction of active protein with about 40–60% activity with respect to $C_{12}E_8$. C_8E_4 was ineffective in the solubilization of active transporter (experiments not shown).

The reconstitution procedure has been optimised for the glutamine/amino acid transporter by adjusting the parameters that influence the efficiency of transport protein incorporation into the liposomes i.e., the protein concentration, the detergent/lipid ratio, the number of passages through the same Amberlite XAD-4 column [6,7]. In these experiments, the [^{3}H]glutamine/glutamine (0.1 mM/30 mM) antiport in 20 min has been measured as function of each parameter. In all the experiments, the intraliposomal volume, which gives information on the liposome formation, was also determined. The transport increased with the protein concentration up to 40 μg/ml (Fig. 1); above this value, a reduction of transport was observed. The intraliposomal volume decreased by increasing the protein concentration, indicating that higher concentrations of protein cause the formation of less or smaller liposomes. The transport activity was not critically influenced by the detergent/lipid ratio in the range from 0.4 to 0.9 (w/w); out of this range a decrease of transport was observed. The transport activity increased with the number of columns passages up to 12; in the range from 12 to 18 the activity remained nearly constant. The intraliposomal volume was not influenced by both the detergent/lipid ratio and the number of column passages within the ranges mentioned (experiments not shown). According to the results described, a protein concentration of about 30 μg/ml, a detergent/lipid ratio of 0.78 and 16 column passages were used in the experiments.

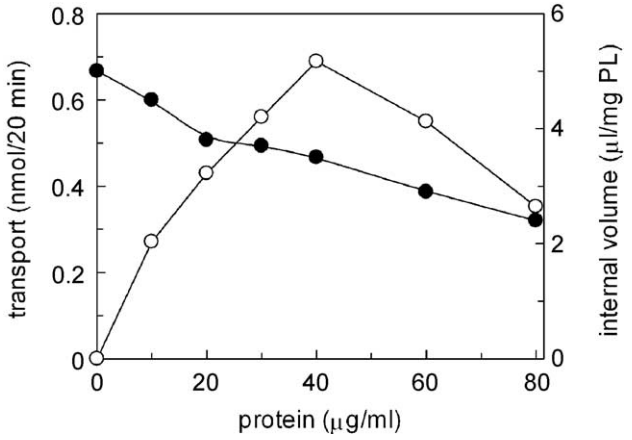

Fig. 1. Dependence of the efficiency of reconstitution of the glutamine/amino acid transporter on protein concentration. The reconstitution was performed as described in Materials and methods except that increasing concentrations of protein were used. Transport was measured as 0.1 mM [^{3}H]glutamine uptake into proteoliposomes containing 30 mM glutamine, in the presence of 50 mM external NaCl, in 20 min (○); (●) internal volume of the proteoliposomes. Similar results were obtained in three different experiments.

3.2. Functional characterization

The dependence of the transport activity of the glutamine/amino acid transporter has been studied as function of the time of incubation of the proteoliposomes with 0.1 mM external [^{3}H]glutamine under different experimental conditions: in the presence or in the absence of intraliposomal glutamine and in the presence or in the absence of external NaCl. The results are summarized in Fig. 2. The accumulation of [^{3}H]glutamine into the proteoliposomes containing internal glutamine increased as function of the time; on the contrary, the glutamine uptake into the vesicles without internal substrate was nearly negligible, indicating that the transport process occurs by an antiport mode. The antiport was strongly stimulated by the presence of external NaCl; in this case the radioisotopic equilibrium was reached at about 100 min; the experimental data describing the transport of glutamine in the absence and in the presence of external NaCl, both fitted a first order rate equation; the first order rate constant, k, resulted to be 0.0029 or 0.024 min^{-1}; the transport rate (calculated as the product of k and the transport at equilibrium) was 0.14 or 1.1 nmol min^{-1} mg $protein^{-1}$ in the absence or in the presence of NaCl, respectively.

The dependence of the transport on the concentration of external or internal cations, as Cl^- salts, was studied. The data of Fig. 3A show that the transport rate of glutamine antiport, starting from about 0.15 nmol min^{-1} mg $protein^{-1}$ at 0 mM salt, was strongly stimulated by external Na^+, reaching a value of about 1.2 nmol min^{-1} mg $protein^{-1}$ at 70 mM NaCl. The experimental data were interpolated by the Hill allosteric kinetic equation; half maximal effect was given by 14.8 mM NaCl (12.6±3.6 from four experiments) and the cooperativity index was 1.18 (1.16±0.04 from four

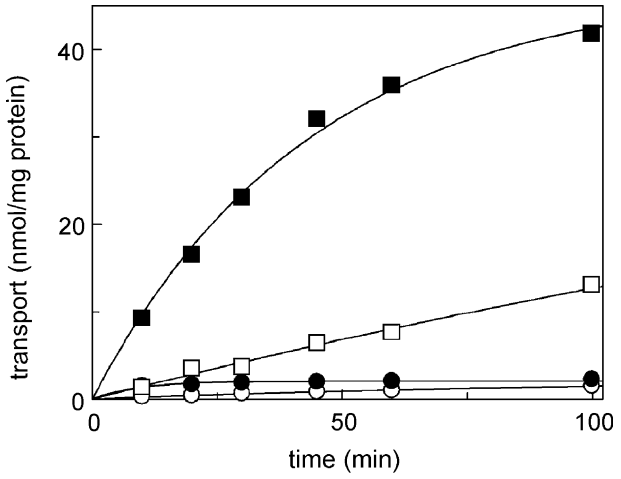

Fig. 2. Time course of [^{3}H]glutamine uptake by reconstituted proteoliposomes. [^{3}H]Glutamine (0.1 mM) was added at time zero to proteoliposomes containing 30 mM internal glutamine (□, ■) or without internal substrate (○, ●), in the presence (●, ■) or in the absence (○, □) of 50 mM external NaCl; the transport reaction was stopped at the indicated times, as described in Materials and methods. Similar results were obtained in three different experiments.

experiments). Li^+ exerted a stimulatory effect much lower than that of Na^+; K^+ had nearly no effect. In a different experiment we studied the dependence of the Na^+-stimulated glutamine antiport on internal NaCl and KCl concentration. As shown in Fig. 3B, neither internal Na^+ nor K^+ stimulated the transport; a slight inhibition effect was observed at higher internal salt concentrations. No stimulation by internal cations was observed even in the absence of external Na^+; it was verified that Cl^- did not exert any effect on the transport (not shown).

The dependence of the glutamine antiport on the pH has been studied (Fig. 4). Maximal transport activity was observed at pH 7. At acidic pH the activity drastically decreased whereas at alkaline pH a slighter reduction of activity was observed.

The inhibition of the glutamine antiport by externally added amino acids and other compounds was studied. As shown in Table 1, alanine, serine, threonine, cysteine, asparagine, methionine and valine strongly inhibited the glutamine transport; the inhibition by these amino acids ranged from 54% to 70% at 1 mM and was virtually complete at a concentration of 10 mM. Glycine, proline, arginine, histidine, glutamate and creatine had a low inhibitory effect. The amino acid analogue MeAIB did not inhibit at all the transporter. The glutamine antiport was also strongly inhibited by some protein modifying reagents. The thiol specific reagents mersalyl, $HgCl_2$ and *p*-OHMB strongly inhibited the glutamine/amino acid transporter, whereas *N*-ethylmaleimide, *N*-phenylmaleimide and MTSET were poor inhibitors. The lysine reagent PLP completely inhibited the transporter at 10 mM concentration. By studying the glutamine transport kinetics in the absence and presence of the inhibiting amino acids reported in Table 1, purely competitive inhibition was found (not shown), indicating that the amino acids interact with the glutamine binding site.

To establish which of the amino acids could be transported by the antiport reaction catalysed by the reconstituted glutamine/amino acid transporter, the experiments of Table 2 have been performed. [^{3}H]Glutamine uptake was measured in proteoliposomes containing different amino acids or, alternatively, [^{3}H]glutamine efflux from prelabeled proteoliposomes was measured in the presence of externally added amino acids. As clearly demonstrated by the data in Table 2, alanine, serine, asparagine and threonine efficiently stimulated both the [^{3}H]glutamine uptake and efflux; cysteine and valine stimulated the efflux much more than the uptake of [^{3}H]glutamine, i.e., they are preferentially translocated from outside to inside; methionine, glycine, histidine, glutamate, arginine, creatine and MeAIB were poorly or not transported.

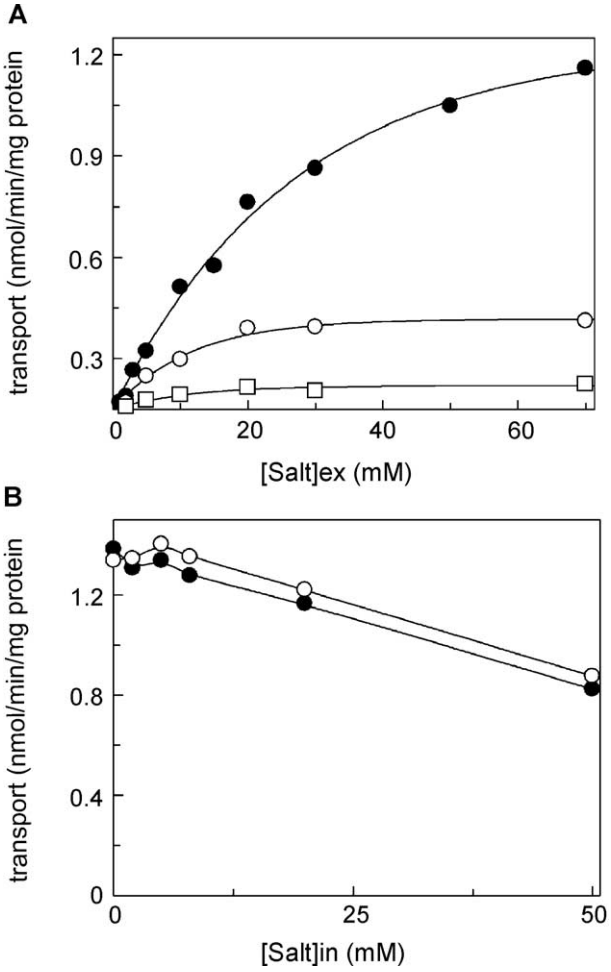

Fig. 3. Dependence of glutamine transport on the concentration of cations in the extraliposomal or intraliposomal compartments. Transport rate was measured as 0.1 mM [^{3}H]glutamine uptake into proteoliposomes containing 30 mM glutamine, as described in Materials and methods. (A) NaCl (●), LiCl (○) or KCl (□) was added at the indicated concentrations outside the proteoliposomes. (B) NaCl (●) or KCl (○) were included at the indicated concentrations inside the proteoliposomes in the reconstitution mixture and the transport rate was measured in the presence of external 50 mM NaCl. Similar results were obtained in three different experiments.

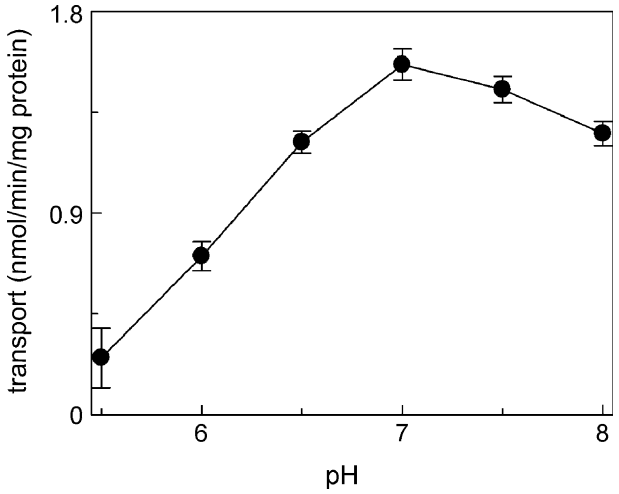

Fig. 4. Effect of pH on the reconstituted glutamine/amino acid transporter. All the experimental procedures from the reconstitution to the transport measurement (see Materials and methods) were performed in 20 mM HEPES/Tris buffer at the indicated pH. Transport rate was measured as 0.1 mM [^{3}H]glutamine uptake in 10 min into proteoliposomes containing 30 mM glutamine, in the presence of 50 mM external NaCl. The results are means±S.D. of triplicate samples of one experiment. Similar results were obtained in three different experiments.

3.3. Kinetics

The half-saturation constants (K_m) of the reconstituted glutamine/amino acid transporter for glutamine and other amino acids are reported in Table 3. The K_i values reported for external serine or alanine are coincident with the K_m since the two amino acids are competitive inhibitors of the glutamine transport. The external affinity of the transporter for glutamine, serine and alanine is much higher (lower K_m) than the internal one. In addition, the K_m values are not influenced by the type of counter substrate. ATP, present inside the proteoliposomes (see also below), led to a slight, but reproducible, reduction of the external K_m for glutamine. When the initial rate of antiport was plotted according to Lineweaver–Burk or to Eadie–Hofstee (not shown, but see Fig. 5) both as function of internal or external substrate concentration, single slopes of the interpolated straight lines were obtained (single external or internal K_m values) indicating that the transporter is fully oriented in the liposomal membrane. The mean value of V_{max} from 10 different experiments was 9.7±2.0 nmol min^{-1} mg protein^{-1}.

To obtain information on the kinetic mechanism of the glutamine antiport, a bisubstrate kinetic analysis was performed. The dependence of the antiport rate on both external and internal glutamine concentration was studied, in a single experiment, by changing the glutamine concentration in both the external and the internal proteoliposomal compartments. The experimental data were plotted according to Lineweaver–Burk as function of the reciprocal external (Fig. 5A) or the reciprocal internal (Fig. 5B) glutamine concentration. In both cases, an intersecting pattern of straight lines was obtained with a common intersection point close to the abscissa. By replot of the apparent V_{max} values extrapolated from the graphs of Fig. 5A and B at infinite internal or external glutamine concentration, respectively, K_m values independent from the counter substrate concentration were derived; their values were 0.46 mM for the external and 12 mM for the internal glutamine, in agreement with the K_m determined at finite counter substrate concentration (Table 3).

Table 1
Effect of inhibitors on the reconstituted glutamine/amino acid transporter

Inhibitor	Concentration (mM)	Residual activity (%)
Alanine	1	45±6.1
	10	<5
Serine	1	42±8.2
	10	<5
Threonine	1	42±6.6
	10	<5
Cysteine	1	39±4.2
	10	<5
Asparagine	1	46±5.7
	10	<5
Methionine	1	30±3.9
	10	<5
Valine	1	35±7.6
	10	<5
Glycine	1	70±7.9
	10	37±6.0
Proline	1	74±9.8
	10	42±6.3
Arginine	1	102±15.6
	10	86±12.5
Histidine	1	69±9.2
	10	27±5.3
Glutamate	1	81±5.5
	10	43±8.1
Creatine	1	99±6.8
	10	84±12
MeAIB	1	95±15
	10	101±16
Mersalyl	0.02	<5
$HgCl_2$	0.02	<5
p-OHMB	0.02	<5
N-Ethylmaleimide	0.1	98±11
	1	81±10
N-Phenylmaleimide	0.1	71±14
	1	21±14
MTSET	0.1	72±14
	1	39±8.2
PLP	1	53±7.2
	10	<5

Transport was measured as 0.1 mM [^{3}H]glutamine uptake into proteoliposomes, reconstituted as described in Materials and methods, containing 30 mM internal glutamine, in the presence of 50 mM external NaCl, in 30 min. The inhibitors were added together with the labelled substrate at the concentrations indicated. Percent of glutamine transport was calculated for each experiment with respect to the control sample (referred as 100%), i.e., in the absence of inhibitor. The results are means±S.D. of the percentages for three experiments. The average transport activity of the control samples of the three experiments analysed was 21±9.0 nmol 30 min^{-1} mg protein^{-1}.

Table 2
Dependence on counter substrate of glutamine transport in reconstituted liposomes

Counter substrate	Glutamine uptake (% of control)	Glutamine efflux (% of control)
Glutamine	100	100
–	<10	<10
Alanine	97±6.1	98±8.0
Serine	83±3.5	101±9.7
Asparagine	93±0.7	99±7.3
Threonine	95±6.0	96±7.3
Cysteine	24±7.3	98±11
Valine	25±4.6	94±8.5
Methionine	<10	52±5.8
Glycine	38±7.9	30±7.1
Histidine	36±9.2	26±6.4
Glutamate	37±6.5	<10
Arginine	<10	19±8.5
Creatine	<10	<10
MeAIB	<10	<10

Transport was measured in the presence of 50 mM external NaCl as 0.1 mM [^{3}H]glutamine uptake in 30 min into proteoliposomes containing 30 mM internal counter substrates or as [^{3}H]glutamine efflux from prelabeled proteoliposomes in the presence of 5 mM of the external counter substrates. Percent of glutamine transport was calculated for each experiment with respect to its control sample (referred as 100%), i.e., in the presence of intraliposomal 30 mM glutamine (uptake) or extraliposomal 5 mM glutamine (efflux). The results are means±S.D. of the percentages for three experiments. The average glutamine uptake in the control samples of the three experiments analysed was 25±6.8 nmol 30 min^{-1} mg protein^{-1}. The average glutamine efflux was 2460±1120 cpm.

3.4. Activation of the glutamine antiport by nucleotides and pyrophosphate

The effect of nucleotides on the glutamine antiporter was studied by analysing the variation of the transport activity induced by the presence of nucleotides in the external or in the internal proteoliposomal compartment. Any of the adenine nucleotides (AMP, ADP, ATP) added outside did not cause significant variations of the transport activity (not shown). The experiments reported in Fig. 6A show the dependence of the transport activity on the concentration of the different adenine nucleotides present inside the proteoliposomes. The transport, reported as percent of the control, was strongly stimulated by internal ATP. The effect was concentration dependent with a maximal activation at 4 mM; at higher ATP concentrations, the activation slightly diminished. ADP exerted a very low effect, whereas AMP had no effect. MgATP also activated the transport but with a lower efficiency compared to the free ATP. Fig. 6B shows the effect of other nucleoside triphosphates, phosphate and phyrophosphate. As clearly evidenced by the experimental data, UTP, GTP and, at a lower extent, CTP (not shown) stimulated the glutamine antiport as ATP. The non-

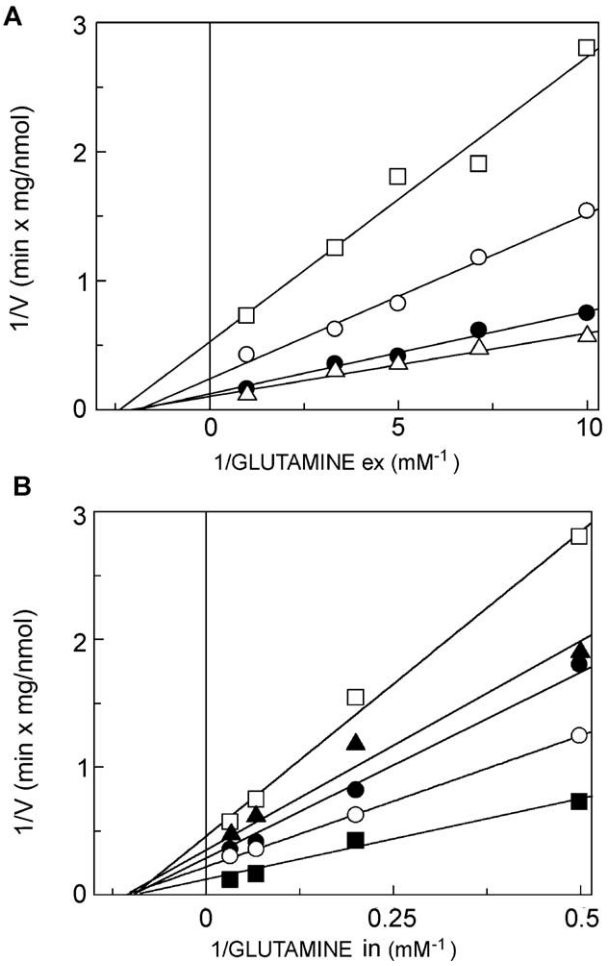

Fig. 5. Bisubstrate analysis of the glutamine/glutamine antiport reaction mediated by the reconstituted glutamine/amino acid transporter. Lineweaver–Burk plots showing the dependence of antiport rate on external (A) and internal (B) glutamine concentration. The concentrations of the counter substrates were as follows: (A) 2 (□), 6 (○), 15 (●) and 30 (△) mM internal glutamine; (B) 0.1 (□), 0.14 (▲), 0.2 (●), 0.3 (○) and 1 (■) mM external glutamine.

Table 3
Half-saturation constants of the reconstituted glutamine/amino acid transporter

Substrate	Counter substrate	K_m or K_i (mM)	Exp. (n)
glutamine$_{ex}$	glutamine$_{in}$	0.47±0.07	10
glutamine$_{ex}$	serine$_{in}$	0.45±0.07	4
glutamine$_{ex}$	alanine$_{in}$	0.50±0.08	3
serine$_{ex}$	glutamine$_{in}$	0.67±0.11[a]	3
alanine$_{ex}$	glutamine$_{in}$	0.67±0.18[a]	3
glutamine$_{ex}$	glutamine$_{in}$ (ATP$_{in}$)	0.34±0.04	3
glutamine$_{in}$	glutamine$_{ex}$	11±1.9	4
glutamine$_{in}$ (ATP$_{in}$)	glutamine$_{ex}$	12±2.1	2
serine$_{in}$	glutamine$_{ex}$	10±2.3	3
alanine$_{in}$	glutamine$_{ex}$	10±1.8	3

K_m values were calculated from double reciprocal plots of the experimental data obtained by measuring the antiport rate as [^{3}H]glutamine uptake in the presence of external 50 mM NaCl. For the determination of external K_m the external glutamine concentration was varied from 0.05 to 5 mM at fixed internal (30 mM) substrate concentration; for the determination of internal K_m the internal substrate concentration was varied from 1 to 30 mM at fixed external (1 mM) substrate concentration. K_i were derived by experiments of external glutamine K_m in the presence of 1 and 2 mM serine or alanine. 4 mM ATP was included inside the proteoliposomes where indicated. The values given are means±S.D. from (n) experiments.

[a] K_i values.

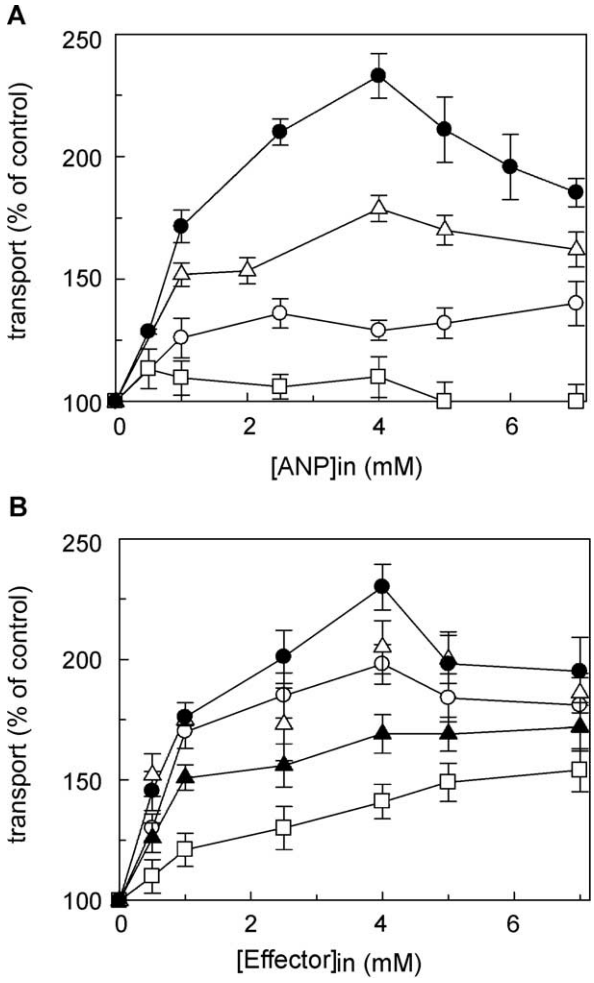

Fig. 6. Effect of nucleotides and pyrophosphate on the reconstituted glutamine/amino acid transporter. Transport was measured as 0.1 mM [^{3}H]glutamine uptake in 20 min into proteoliposomes containing 30 mM glutamine, in the presence of 50 mM external NaCl. The results, expressed as percent of the control (samples without effectors), are means±S.D. of three different experiments. The average glutamine uptake in the control samples of the experiments analysed was 18±6.2 nmol 20 min^{-1} mg protein^{-1}. (A) ATP (●), ADP (○), AMP (□), ATP plus $MgCl_2$ (△) or (B) GTP (●), UTP (△), ANTP (○), pyrophosphate (▲) or phosphate (□), at the indicated concentrations, were added to the reconstitution mixture (internal proteoliposomal compartment). In other experiments it was tested that $MgCl_2$ had nearly no effect on the transporter up to 7 mM.

hydrolysable ATP analogue ANTP also activated the transport with an efficiency comparable to that of the other nucleoside triphosphates. Pyrophosphate activated the glutamine/amino acid transporter, even though less efficiently than nucleotides. Phosphate exerted a slight activation effect that reached a maximum of about 60% activation at a concentration of (not shown) 20 mM. Nearly no effect was exerted by internal ATP in the absence of internal glutamine (not shown), indicating that the activation is specifically exerted on the glutamine antiport and that ATP is not transported in exchange for external glutamine.

In another experiment the protein extract was incubated with 1, 2 or 5 mM ATP before the reconstitution, and then incorporated into the liposomes in the absence of internal ATP. In this case no effect was observed, indicating that the activation was not due to hypothetical covalent ATP-induced protein modification.

The mean value of V_{max} in the presence of internal 4 mM ATP was 21.5±7.8 nmol min^{-1} mg protein^{-1} (from three different experiments). The relatively high standard deviation often associated with the V_{max} values is due to variations in the amount of active transport protein molecules present in the different preparations.

4. Discussion

This paper deals with the functional study of the glutamine/amino acid transporter by reconstitution into liposomes. The reconstituted system presents some advantages with respect to the cell systems [7] and (see Ref. [9] for reference) like the possibility to control the experimental conditions in the internal compartment, which allows the determination of the internal substrate affinity or the study of internal effectors; the reduction of interferences was due to the virtual absence of external and internal enzymes. In addition, the reconstitution procedure used here leads to the formation of proteoliposomes larger than those obtained by other methods like the freeze-thaw sonication [6,7]; this feature allows to increase the accumulation of radioactivity inside the vesicles. Recently, this procedure has been successfully used to define novel functional properties of a plasma membrane transporter [9]. Thus, we have applied this reconstitution procedure, with appropriate modifications, to the glutamine/amino acid transporter that was extracted from renal apical plasma membranes (brush-border) where the glutamine transport is active and plays an important physiological role [21–24].

On the one hand, the basic functional properties of the reconstituted glutamine/amino acid transporter correspond to those of the ASCT2 (ATB$^\circ$) transporter, expressed and characterized in cell systems [10–16]: the Na^+ dependence, the intolerance for Li^+, the antiport mode of transport, the specificity for glutamine, alanine, serine, threonine and some other amino acids (see Tables 1 and 2), the lack of inhibition by MeAIB. On the other hand, some functional differences allow to exclude that the reconstituted glutamine/amino acid transporter corresponds to one of the other known glutamine-specific transporters: systems L and b$^{\circ\prime\,+}$ that are Na^+-independent; systems N and B$^{\circ\prime\,+}$ that exhibit a cotransport mode of substrate translocation; system A that shows trans-inhibition and sensitivity to MeAIB like; systems y^+L that show Li^+ tolerance and specificity for cationic amino acids [1–5]. In addition, the source of the reconstituted transporter corresponds with the cellular localization of ASCT2 in the apical plasma membrane of kidney, among other tissues [5,10,25]. Thus, the reconstituted transporter functionally corresponds to the ASCT2 protein. Interestingly, in the reconstituted system, we have found that some of the amino acids can be translocated

bidirectionally, in agreement with the role of mediating also glutamine efflux, as proposed for the astroglial ASCT2 [12,26,27] and as a general mechanism of small amino acid reabsorption [3].

Novel functional properties of the transporter have been found by means of the reconstituted system. The glutamine/amino acid transporter is inhibited by some thiol and amino specific reagents (Table 1); thus, some Cys and Lys residues must be important for the transport function. The transporter is selective towards the mercurial reagents that, differently from maleimides and MTSET, do not require alkylation reaction. Thus, we hypothesize that the SH residue(s) involved in the inhibition may be located in a small pocket of the protein, accessible to the -Hg^+ residue of the inhibitors, but not to the whole molecules of *N*-ethylmaleimide or MTSET that, consequently, cannot came in close contact with the SH residue for the alkylation reaction. Whether or not this SH residue is located in the active site is a matter of further investigation.

The internal K_m for the substrates, that so far was unknown, is about 20 times higher than the external one; this difference reflects the difference between the extra- and intracellular concentrations of glutamine and other amino acids [28,29]. The difference in K_m values together with the side-specificity of the Na^+-stimulation and the side-specific regulation (see below) is in favor of a functional asymmetry of the transporter. This functional asymmetry reflects the structural asymmetry of the ASCT2 protein that is evidenced by the analysis of its hydropathy profile [10]. Virtually all the reconstituted glutamine/amino acid transporter molecules are inserted in the lipid bilayer of the liposomes with the same orientation (see Results), as it was previously found for the reconstituted OCTN2 [9]. Thus, it seems that this reconstitution procedure allows the unidirectional insertion of transport proteins in the membrane, probably due to a peculiar feature of this method: the slow process of proteoliposome formation that starts from mixed micelles; the insertion of the protein in the micelles is probably influenced by the asymmetrical structure of the protein itself. The orientation of the glutamine/amino acid transporter in the proteoliposomes is the same as in the native membrane on the basis of two observations: the specificity of the activation by Na^+ on the external face of the reconstituted transporter; the comparison of the external K_m in proteoliposomes with the values reported in cells for ASCT2: some of these values are very similar [16] while others are different [10–13], however, all of the values previously reported are more close to the external than to the internal K_m in proteoliposomes. On the basis of the kinetic mechanism of transport here found (see below), the discrepancies in K_m between different measurements in cells cannot be explained by a difference of intracellular amino acid concentration among the various cell systems. It may be that the external K_m measured in the cell systems is influenced by the presence of different concentrations of nucleotides and of other hypothetical still unknown effectors. Among these, Na^+ and other cations, including H^+, may be considered. In preliminary experiments (not shown), we have found that $^{22}Na^+$ is cotransported with glutamine; thus, the concentration of Na^+ might influence the K_m for the amino acids, as it occurs in the case of an ordered system of cotransport. This aspect and the potential effects of cations and pH on the affinity of the reconstituted transporter for various amino acids will be analysed in future investigations.

We have studied the kinetic mechanism of the antiport catalyzed by the glutamine/amino acid transporter by a bisubstrate kinetic analysis [30]. The experimental data, plotted according to Lineweaver–Burk, led to an intersecting pattern of straight lines indicating a simultaneous transport mechanism. According to this mechanism, a ternary complex, $substrate_{out}$–transporter–$substrate_{in}$, is involved in the translocation process. In addition, the common intercept of the straight lines on the abscissa indicates a random type of mechanism, i.e., no order of binding of the substrates is favored for the formation of the ternary complex. This mechanism implies that the K_m on one face of the transporter is not influenced by the concentration and type (see Fig. 6 and Table 1) of the substrate on the other side of the transporter.

Nucleoside triphosphates activate the glutamine/amino acid transporter by interacting with the internal (cytosolic) face of the protein. The transporter has a broad specificity for the base moiety, but a strict requirement for the triphosphate moiety. On the basis of the finding that also pyrophosphate activates the transporter, we hypothesize that the site for nucleotide binding should be constituted by two close sub-sites: one for the binding of the base (nucleoside) moiety, the other for the binding of the pyrophosphate moiety. The sub-sites must be occupied simultaneously for maximal activation, and the distance between the two moieties is critical. The occupancy of only one sub-site leads to a limited (pyrophosphate sub-site) or no (base sub-site) effect. ADP does not fit the appropriate size to interact with both the sub-sites. Regulation by nucleotides (activa-

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                    **** * *
PFPB    381 --------DPHGNVQVA  389
ASCT2     1 MAVDPPKADPKGVVAVD   17
UCP2    264 ---EGPRAFYKG-----  272
UCP1    261 ---EGPAAFFKG-----  269
              * ***  **
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Fig. 7. Local sequence alignment of UCP nucleotide binding motif and pyrophosphate specificity motif with the ASCT2 protein sequence. The local alignment has been performed with the "LALIGN" software (http://www.ch.embnet.org/software/LALIGN_form.html) by aligning the nucleotide binding motif of UCP2 and UCP1 (aa residues 264–272 and 261–269, respectively; see Ref. [37]) and the sequence of a loop critical for pyrophosphate specificity of the pyrophosphate-dependent phosphofructokinase β-subunit PFPB (aa residues 381–389; see Ref. [40]) with the rat ASCT2 protein sequence.

tion or inhibition) was previously observed for other transporters like the mitochondrial UCP [31], the glucose transporter GLUT1 [32], the Na^+-dependent sugar transporter [33], the thiamine transporter in neuroblastoma cell [34], and the vacuolar amino acid transporter [35]. The broad specificity for nucleotide interaction and the stronger effect of free ATP compared to MgATP found for the glutamine/amino acid transporter are, under some aspects, similar to those described for the inhibition of UCP2 [36,37]. Thus, we have performed a local alignment of the nine-amino-acid nucleotide binding motif of UCP1 and UCP2 [38,39] with the ASCT2 amino acid sequence. We have found an overlap of six amino acids (four identical plus the conserved D/E and R/K substitutions) with the amino-terminal end of ASCT2 (Fig. 7). In addition, we have aligned the nine-amino-acid consensus sequence critical for pyrophosphate binding to pyrophosphate-dependent 6-phosphofructokinase [40] with the ASCT2. Also in this case, we have found an overlap of six (five identical plus the conserved substitution H/K) amino acids with the N-terminal sequence of ASCT2 adjacent to those of the nucleotide binding (Fig. 7). Thus, in agreement with the identification of the reconstituted transporter as ASCT2, it may be hypothesized that the amino-terminal end of the protein may constitute at least a part of the sub-sites for the interaction with nucleotides. The N-terminus is located, on the basis of the hydropathy profile [10], on the internal (cytosolic) side according to the internal side-specific effect of nucleotides. In vivo, nucleoside triphosphates may represent sensor molecules of the metabolic state of the cell. It has to be stressed that the activation of the glutamine/amino acid transporter occurs at concentrations within the physiological concentration range of ATP [41,42]; the effect of ATP may be further modulated by Mg^{2+}. A possible physiological role may also be played by pyrophosphate itself, which is known to have some modulatory effects on protein synthesis [43].

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