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The glutamine/amino acid transporter (ASCT2) reconstituted in liposomes: Transport mechanism, regulation by ATP and characterization of the glutamine/glutamate antiport

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Abstract

The glutamine/amino acid transporter solubilized from rat renal apical plasma membrane (brush-border membrane) with $C_{12}E_8$ and reconstituted into liposomes has been previously identified as the ASCT2 transporter. The reconstituted transporter catalyses an antiport reaction in which external glutamine and Na^+ are cotransported in exchange with internal glutamine (or other amino acids). The glutamine–Na^+ cotransport occurred with a 1:1 stoichiometry. The concentration of Na^+ did not influence the Km for glutamine and vice versa. Experimental data obtained by a bi-substrate analysis of the glutamine–Na^+ cotransport, together with previous report on the $glutamine_{ex}$/$glutamine_{in}$ pseudo bi-reactant analysis, indicated that the transporter catalyses a three-substrate transport reaction with a random simultaneous mechanism. The presence of ATP in the internal compartment of the proteoliposomes led to an increase of the Vmax of the transport and to a decrease of the Km of the transporter for external Na^+. The reconstituted glutamine/amino acid transporter was inhibited by glutamate; the inhibition was more pronounced at acidic pH. A kinetic analysis revealed that the inhibition was competitive with respect to glutamine. Glutamate was also transported in exchange with glutamine. The external Km of the transporter for glutamate (13.3 mM) was slightly higher than the internal one (8.3 mM). At acidic pH the external but not the internal Km decreased. According with the Km values, glutamate should be transported preferentially from inside to outside in exchange for external glutamine and Na^+.

Keywords: Plasma membrane; Transport; Liposome; Reconstitution; Glutamine; Glutamate; ASCT2

1. Introduction

The plasma membrane transporters for glutamine and neutral amino acids are object of interest for their involvement in the glutamine homeostasis in mammals (see refs. [1–5] for reviews). Even though several genes coding for the glutamine and neutral amino acid transporters have been identified and cloned, the functional and regulatory properties of the transporters are still matter of investigation. Among the experimental approaches for the functional characterization of the plasma membrane glutamine transporters, the most widely used so far is the measurement of substrate uptake into cell systems (over)expressing a specific transporter [6–12]. Recently, the glutamine/amino acid transporter extracted from brush border membranes of rat kidney has been reconstituted into liposomes by the procedure of cyclic detergent removal [13]. The reconstituted transporter has been identified as ASCT2 (or its homologous ATB° [14]), since the basic functional properties found in proteoliposomes were coincident with those described for the ASCT2 transporter in previous studies performed with cell systems [5–12,14] and the source of the reconstituted transporter corresponds with the cellular localization of ASCT2 in the kidney among other tissues [5,6,14]. In particular, the reconstituted glutamine/amino acid transporter, as the ASCT2, shows sodium dependence and low tolerance towards the substitution of Na^+ by Li^+, insensitivity to the amino acid analogue α-(methylamino)isobutyric acid (MeAIB), specificity for neutral amino acids and for glutamate

Abbreviations: $C_{12}E_8$, octaethylene glycol monododecyl ether; DEPC, Diethyl pyrocarbonate

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that are transported by an antiport mode and shows maximal transport at pH 7 [13]. Differently, among the amino acid transporters that accept glutamine: systems L and $b^{o,+}$ are not dependent by sodium, systems y^+L and N accept Li^+ in the place of Na^+, system A is inhibited by MeAIB, system $B^{o,+}$ mediate glutamine uniport, system B°AT1 mediates net uptake of amino acids, does not accept glutamate and shows optimal activity at alkaline pH [1–5,14–16]. Novel functional properties of the glutamine/amino acid transporter have been revealed by means of the reconstituted system, like the functional asymmetry, the sensitivity to SH reagents and the activation by intraliposomal ATP. The transporter was inserted unidirectionally in the proteoliposomal membrane, right-side-out with respect to the cell membrane; thus, the properties of the internal side of the reconstituted transporter correspond to those of the intracellular side and vice versa.

In this paper, kinetic and functional aspects of the glutamine/amino acid transporter have been further investigated. The understanding of the kinetic mechanism of the $glutamine_{ex}$–$Na^+{}_{ex}$ cotransport reaction together with previous data on the analysis of the glutamine/glutamine antiport allowed to define the overall kinetic mechanism of the $glutamine_{ex}$–$Na^+{}_{ex}$/$glutamine_{in}$ reaction catalysed by the transporter. The kinetic parameters that are influenced by the binding of ATP to the internal side of the transporter have been revealed. The involvement of glutamate in the transport function of the glutamine/amino acid transporter has been investigated.

2. Materials and methods

2.1. Materials

Amberlite XAD-4, egg yolk phospholipids (3-*sn*-phosphatidylcholine from egg yolk) and $C_{12}E_8$ were purchased from Fluka; L-[^{3}H]glutamine from Amersham; Sephadex G-75, from Sigma. 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 [13,17]. 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 [18,19]. 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 previously described [18], 30 mM L-glutamine, 20 mM HEPES/Tris pH 7.0 (except when differently specified) 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 of the same composition of the initial mixture with the exception of protein, detergent and phospholipid. 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 were passed through a Sephadex G-75 column (0.7×15 cm) preequilibrated with 20 mM HEPES/Tris pH 7.0 (except when differently specified) 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 [19] 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 [20]. 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 the determination of the [^{3}H]glutamine efflux, the experimental values were subtracted from 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 rate, expressed as cpm/min, was determined by fitting the experimental data in a first order rate equation from which the initial transport rate was calculated as the product of k (first order rate constant) and A (the effluxed radioactivity at equilibrium).

2.5. Other methods

The protein concentration was determined by the modified Lowry procedure [21].

3. Results

To further characterize the functional properties of the reconstituted glutamine/amino acid transporter (ASCT2), the ability to accept intraliposomal leucine and phenylalanine as countersubstrate for external glutamine was tested. The specificity towards the two amino acids is a feature to distinguish B°AT1 from ASCT2 [15]. The uptake of labeled glutamine in proteoliposomes containing internal 30 mM leucine or phenylalanine has been compared to control samples, i.e., proteoliposomes containing 30 mM internal glutamine. The percent radioactivity taken up in the presence of leucine and phenylalanine was 15±2.6% and 20±1.6% of the radioactivity taken up in the presence of glutamine (three experiments). These data further indicated that the reconstituted transporter was different from the B°AT1 transporter, in agreement with previous findings ([13] and see Introduction).

The stoichiometry of the sodium-glutamine co-transport in exchange for glutamine, as counter-substrate, has been determined in double labeling experiments. The time course of the uptake of [^{3}H]glutamine and [$^{22}Na^+$] (as Cl^- salt), at equal concentrations, in proteoliposomes containing 30 mM glutamine was measured. Very similar experimental data were

found for the glutamine and Na^+ uptake (Fig. 1) indicating a 1:1 stoichiometry of transport. This is in agreement with the lack of cooperative effects in the experiment of Fig. 3.

In the Na^+-dependent glutamine antiport, the concentration of Na^+ may influence the Km for glutamine and vice versa. To gain insights into this aspect, the glutamine–Na^+ cotransport has been investigated by a pseudo bi-reactant kinetic analysis [22–24], varying the concentrations of the two external substrates, glutamine and Na^+, keeping the concentration of internal glutamine constant and close to saturation. Transport rates as function of the external glutamine (Fig. 2A) or Na^+ (Fig. 2B) concentration were reported in double reciprocal plots (Lineweaver–Burk plots). The intercepts of the straight lines with the abscissa were very close to each other, indicating that the concentration of Na^+ had nearly no influence on the Km of the transporter for external glutamine and the glutamine concentrations had nearly no influence on the Km for Na^+. The patterns of intersecting straight lines indicated that the glutamine–Na^+ cotransport follows a simultaneous mechanism. In this mechanism the two substrates are translocated simultaneously to the opposite side of the membrane, differently from the ping-pong mechanism in which the translocation of the substrates occur in different consecutive steps. Concentration independent Km for external glutamine and Na^+, and Kis values, (dissociation constant of the pseudo-binary complex transporter-substrate), could be derived from secondary graphs obtained by plotting the values of the ordinate intercepts or the slopes of the straight lines of Fig. 2A and B, as function of the reciprocal substrate concentrations (not shown). The Km values extrapolated from the replots were 0.43 mM for external glutamine and 10 mM for Na^+. The Kis values obtained for glutamine and Na^+ were 0.30 and 7.1 mM, respectively. Thus, the affinity of the transporter for the substrates is similar, whether it is in the free or in the second substrate-bound form. Furthermore, the ratio between Km and Kis for each substrate is constant and close to 1. These features together with the finding of common intersections close to the abscissa (Fig. 2A and B) are typical of the random simultaneous mechanism [22–24] in which, differently from the ordered mechanism, the substrates bind to the transporter without any preferential order.

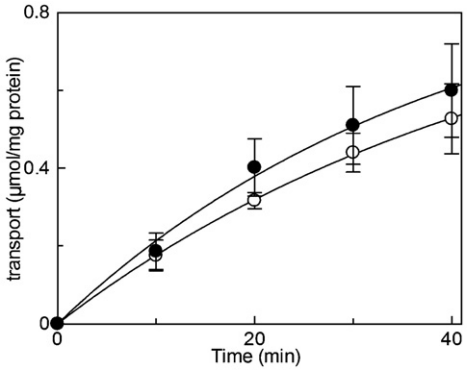

Fig. 1. Time course of [^{3}H] glutamine and [$^{22}Na^+$] uptake by reconstituted proteoliposomes. 5 mM [^{3}H] glutamine (○) and 5 mM [$^{22}Na^+$] (●) were added at time zero to proteoliposomes containing 30 mM internal glutamine; the transport reaction was stopped at the indicated times, as described in Materials and methods. Data are means±S.D. of three different experiments.

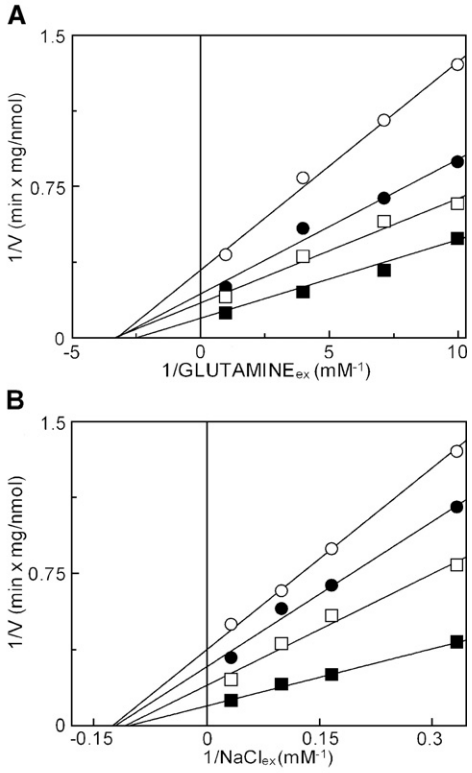

Fig. 2. Bi-substrate analysis of the glutamine–Na^+ cotransport mediated by the reconstituted glutamine/amino acid transporter. Lineweaver–Burk plots showing the dependence of glutamine$_{ex}$–Na^+_{ex}/glutamine$_{in}$ antiport rate on external glutamine (A) or NaCl (B) concentrations. In (A) the concentrations of NaCl were 3 (○), 6 (●), 10 (□) and 30 (■) mM. In (B) the concentrations of glutamine were 0.1 (○), 0.14 (●), 0.25 (□) and 1 (■) mM. The concentration of internal glutamine was constant (50 mM). Similar results were obtained in three different experiments.

It was previously shown that ATP present in the intraliposomal compartment activates the glutamine antiport catalysed by the reconstituted glutamine/amino acid transporter; the presence of the nucleotide did not affect significantly the external or the internal affinity of the transporter for the neutral amino acid substrates [13]. To ascertain whether ATP exerted any effect on the affinity of the transporter for Na^+, the dependence of the transport rate on Na^+ concentration in the presence (or absence) of ATP was determined; the data were plotted according to Michaelis–Menten (Fig. 3). Besides the increase of transport rate in agreement with the previous finding [13], a clear reduction of the Km for Na^+ was detected in the presence of internal ATP. The Km decreased from 16.7 to 6.5 mM (14.4±2.1 to 7.0±0.6 mM from 3 experiments). Neither in the presence nor in the absence of ATP were cooperative effects observed.

It is known from previous studies in cell systems that the ASCT2 transporter shows higher affinity for the amino acid glutamate at acidic pH as compared to neutral or alkaline pH. This is a characteristic feature of this transport system [6,25].

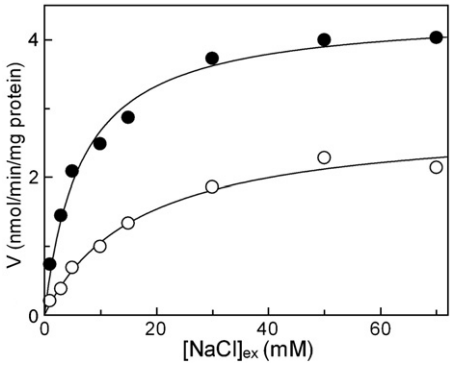

Fig. 3. Dependence of glutamine antiport rate on the concentration of external NaCl: effect of ATP. Antiport rate was measured as 0.1 mM [^{3}H]glutamine uptake into proteoliposomes containing 30 mM glutamine, as described in Materials and methods, in the presence (●) or absence (○) of 4 mM internal ATP. NaCl was added at the indicated concentrations outside the proteoliposomes. Similar results were obtained in three different experiments.

The interaction of glutamate with the transporter has been further investigated in this work. The inhibition by glutamate of the glutamine antiport catalysed by the reconstituted transporter, has been compared to the inhibition by serine and alanine in the range from pH 5.5 to pH 8.5. The results are reported in Fig. 4 as percent inhibition. The inhibition by glutamate strongly increased at acidic pH, whereas the inhibition by serine and alanine was nearly independent of the pH. Other neutral amino acids like threonine and asparagine behaved as alanine (not shown). An inhibition kinetic analysis was performed by studying the effect of glutamate at different glutamine concentration at pH 6.0 and pH 7.0. The data plotted according to Lineweaver–Burk revealed competitive inhibition patterns both at pH 6.0 (Fig. 5A) and at pH 7.0 (Fig. 5B). The inhibition was clearly more pronounced at pH 6.0. The average Ki of the transporter for glutamate were derived from the graphs: their values at pH 6.0 and pH 7.0 were 7.7±2.3 mM and

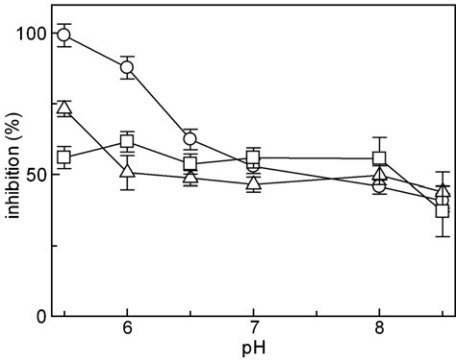

Fig. 4. pH dependence of the inhibition of the glutamine/amino acid transporter by glutamate. All the experimental procedures from the reconstitution to the transport measurement (see Materials and methods) were performed in 20 mM HEPES/Tris buffer at the indicated pH. Transport was measured as 0.1 mM [^{3}H] glutamine uptake in 10 min into proteoliposomes containing 30 mM glutamine, in the presence of 50 mM external NaCl and in presence of 16 mM external glutamate (○), 0.8 mM external serine (Δ), 0.8 mM external alanine (□). The results are expressed as percent inhibition with respect to the control without added inhibitor. Data are means±S.D. of three different experiments.

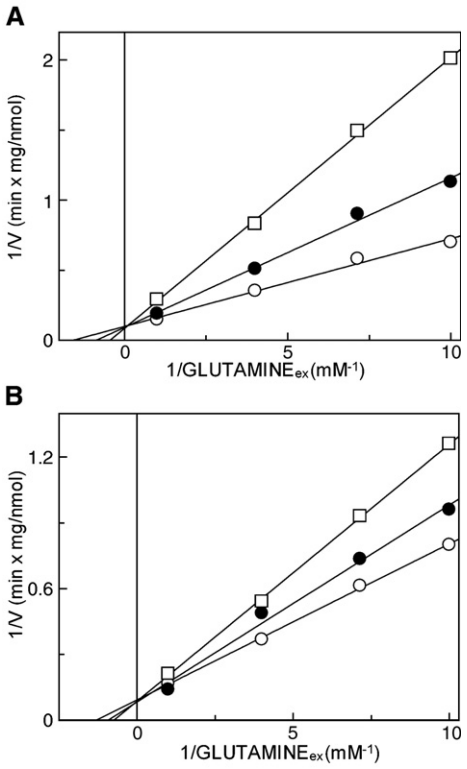

Fig. 5. Kinetic analysis of the inhibition of the reconstituted glutamine/amino acid transporter by glutamate. The uptake rate of [^{3}H] glutamine at the indicated concentrations was measured in proteoliposomes containing 30 mM glutamine in absence (○) or in the presence of 8 (●) mM or 16 (□) mM external glutamate. All the experimental procedures from the reconstitution to the transport measurement (see Materials and methods) were performed in 20 mM HEPES/Tris buffer at pH 6.0 (A), or at pH 7.0 (B). Similar results were obtained in three different experiments.

20.5±0.7 mM, respectively. According to the competitive nature of the inhibition, glutamate should be transported as previously described for the expressed ASCT2 [6] and for the reconstituted transporter [13] even though with a lower efficiency in respect to the other neutral amino acids. To further investigate this important aspect, the dependence of the glutamate$_{ex}$/glutamine$_{in}$ antiport on the pH was studied by measuring the efflux of labeled glutamine from proteoliposomes in the presence of external glutamate (see Materials and methods). As shown in Fig. 6A, a maximum of activity was observed at pH 6.0. The transport decreased at more alkaline pH and was very low at pH 5.5. This dependence was clearly different from the pH dependence of the homologous glutamine/glutamine antiport previously reported [13], in which maximal activity was observed at pH 7.0. In a further experiment the pH dependence of the glutamine$_{ex}$/glutamate$_{in}$ antiport was studied by measuring the uptake of labeled glutamine into proteoliposomes containing internal glutamate (Fig. 6B). In this transport mode, a maximum was observed at pH 7.0 with a slight decrease of activity at more acidic pH and a stronger decrease at pH 5.5 and at alkaline pH; the dependence of this transport mode was more similar to that observed for the glutamine/glutamine antiport [13]. To measure the Km of the transporter for external glutamate, the rate of the glutamate$_{ex}$/

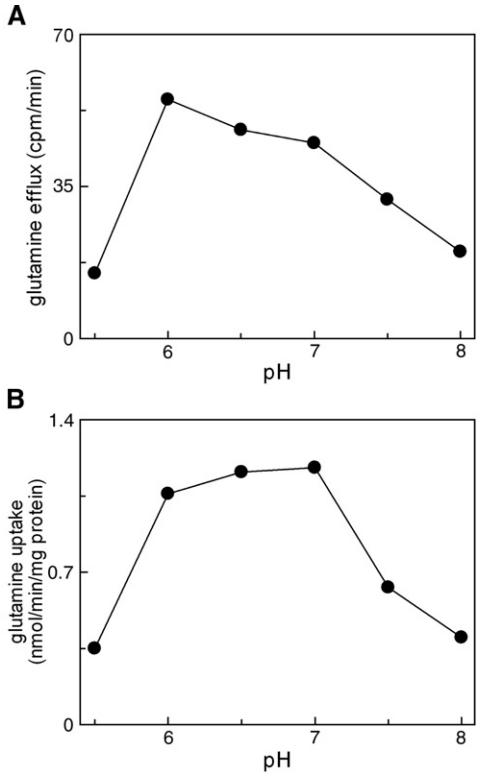

Fig. 6. Effect of pH on the glutamine/glutamate antiport. (A) The glutamate$_{ex}$/glutamine$_{in}$ antiport rate has been measured as 30 mM [^{3}H] glutamine efflux from proteoliposomes (as described in Materials and methods) in the presence of external 16 mM glutamate and 50 mM NaCl. (B) The glutamine$_{ex}$/glutamate$_{in}$ antiport rate has been measured as 0.1 mM [^{3}H]glutamine uptake into proteoliposomes containing 30 mM glutamate in 10 min. 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. Similar results were obtained in three different experiments.

glutamine$_{in}$ antiport was measured at different external glutamate concentrations; to measure the internal Km for glutamate, the rate of the glutamine$_{ex}$/glutamate$_{in}$ antiport was measured at different internal glutamate concentrations. The Km values for the zwitterion glutamic acid were derived from the concentration of the zwitterion at the different pH, calculated on the basis of the pKa 4.25, of the distal carboxyl group. At pH 7.0 the concentrations of the zwitterionic species were ten times lower that those at pH 6.0. The Km for glutamate was also measured in the presence of ATP. The internal and external Km for glutamine have been measured at pH 6.0 and compared to those previously determined at pH 7.0. The data are summarized in Table 1. The Km of the transporter for external glutamate was 13 mM at pH 7.0; the Km decreased to about half at pH 6.0. The Km values for the zwitterions were much lower than those for glutamate. These values clearly followed the concentration of the zwitterions. The presence of internal ATP did not cause appreciable variations of the Km. Neither ATP nor pH influenced the internal Km for glutamate. Differently from glutamate, both the external and the internal Km for glutamine at pH 6.0 did not change as compared to the values measured at pH 7.0. Aspartate has been tested as inhibitor of the glutamine/glutamine antiport both at pH 6.0 and pH 7.0 in experiments performed (not shown) as those described for glutamate in Fig. 4. A very low inhibition was observed at pH 7.0 with a Ki of about 60 mM and nearly no inhibition was detected at pH 6.0. To investigate the possible involvement of His residues in the transport, DEPC, a specific His reagent, has been tested for its ability to inhibit the reconstituted glutamine/amino acid transporter. The reagent added to the reconstituted proteoliposomes inhibited the glutamine/glutamine antiport measured as 0.1 mM [^{3}H] glutamine uptake into proteoliposomes containing 30 mM glutamine: 1, 2 and 3 mM DEPC lead to a transport inhibition of 25±10%, 55±12% and 82±21%, respectively.

4. Discussion

The renal glutamine/amino acid transporter reconstituted into liposomes and identified as ASCT2 catalyses a Na^+ dependent antiport reaction in which glutamine (or other neutral amino acids) and Na^+ are translocated from the external to the internal compartment of the proteoliposomes in exchange for internal glutamine or other neutral amino acids [13]. Even though the reconstituted brush-border extract should contain several membrane proteins, the activity of the glutamine/amino acid transporter was discriminated by using appropriate experimental procedures: labeled glutamine was used to test the transport, thus excluding several transporter that do not accept this amino acid; the antiport of glutamine with itself or other amino acids has been measured under condition of concentration gradient in favour of the unlabeled compounds. This strategy allows a several fold radioactivity accumulation with respect to the net uptake, thus limiting the possible

Table 1
Half-saturation constants of the reconstituted glutamine/amino acid transporter for glutamate and glutamine

Substrate	pH	External Km (mM)	Internal Km (mM)
Glutamate	6.0	7.5±2.2	9.1±2.0
Glutamate	7.0	13.3±2.9	8.3±2.1
Glutamic acid (zwitterion)	6.0	0.13±0.042	0.16±0.032
Glutamic acid (zwitterion)	7.0	0.022±0.0049	0.014±0.0035
Glutamate (ATP$_{in}$)	6.0	7.2±2.1	8.1±1.2
Glutamate (ATP$_{in}$)	7.0	13.1±3.1	9.2±1.0
Glutamine	6.0	0.50±0.03	9.9±2.1
Glutamine	7.0	0.47±0.07 from [13]	11.0±1.9 from [13]

Internal Km values for glutamate 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 into proteoliposomes containing different concentrations of glutamate ranging from 2 to 40 mM. For the determination of external Km the efflux of [^{3}H]glutamine from prelabeled proteoliposomes was determined in the presence of external glutamate concentrations ranging from 1 to 30 mM (see Materials and methods). 4 mM ATP was included inside the proteoliposomes where indicated. Km values for glutamine 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 into proteoliposomes containing glutamine; the external or the internal glutamine concentration was varied from 0.1 to 2 mM or from 2 to 40 mM to measure the external or the internal Km, respectively. All the experimental procedures from the reconstitution to the transport measurement (see Materials and methods) were performed in 20 mM HEPES/Tris buffer at pH 6.0 or at pH 7.0. The values given are means±S.D. from 3 different experiments.

interference of net uptake systems; the discrimination was further improved by the use of the specific inhibitor, mersalyl, that allows to subtract the unspecific transport or transport mediated by mersalyl insensitive transporters. In addition, the reconstituted system was optimised with respect to parameters like the detergent, phospholipid and protein concentrations, that are critical for each single transporter [13,19]. Thus, the possible interference of other transporters, if present, is very low. In this paper it has been found that the glutamine–Na^+ cotransport occurs with 1:1 stoichiometry. The concentration of Na^+ has nearly no influence on the affinity of the transporter for glutamine and vice versa. The reaction catalysed by the glutamine/amino acid transporter (ASCT2) can be assimilated to a ter-reactant reaction in which external glutamine, external Na^+ and internal glutamine are the three substrates. This special case of kinetic mechanism can be analysed by combinations of pseudo-bi-reactant analyses in which the concentrations of two of the substrates are varied keeping the concentration of the third substrate constant and close to saturation [22,23]. This type of kinetic analysis can be applied to transport systems as well [24]. In a previous report the bi-reactant kinetic analysis of the $glutamine_{ex}$/$glutamine_{in}$ antiport, was carried out studying the dependence of the antiport rate on both external and internal glutamine concentration, keeping the Na^+ concentration constant and close to saturation. The experimental data were in favour of a random simultaneous mechanism [13]. The pseudo-bi-reactant analysis described in the previous paper together with the pseudo-bi-reactant analysis of the glutamine–Na^+ cotransport here described, allows a comprehensive interpretation of the kinetic mechanism of the transport catalysed by the glutamine/amino acid transporter: it is a random simultaneous mechanism. This is evidenced by the intersecting patterns of the straight lines with a common interception close to the abscissa, observed in both the pseudo-bi-substrate kinetic analyses and by the constant ratio between Km and Kis of the transporter for the substrates glutamine and Na^+. This mechanism implies the formation of a complex involving one transporter molecule with two glutamine (or other amino acids, on the internal and on the external side of the protein) and one Na^+ (on the external side). The formation of this complex does not require any preferential order for the binding of the substrates and the three molecules are translocated simultaneously [22–24]. Thus, the transporter protein should possess an internal site for the substrate and two external sites, one for the amino acid substrate, the other one for Na^+. Differently, an ordered simultaneous transport mechanism was found for the B°AT1 transporter [26]. At this stage it is not possible to establish whether the transporter has a monomeric or an oligomeric structure and hence whether the transport pathway involves one or more molecules for each transported substrate molecule. The ASCT2 protein shows a significant identity (29%) of the amino acid sequence (not shown) with that of the *Pyrococcus horikoshii* glutamate transporter, the structure of which has recently been resolved [27]. Furthermore the hydrophobicity profiles of the two proteins are very similar (not shown and see ref. [6]). Thus, it is very likely that the ASCT2 has a tertiary structure similar to that of the glutamate transporter. On the light of this similarity, the ASCT2 transporter may have a oligomeric structure as the glutamate transporter. The glutamate transporter catalyses a net co-transport of glutamate and Na^+. It, most probably, has one binding site for glutamate and one for Na^+ on each monomer. The co-transport of the amino acid molecules and the Na^+ ions from the external environment to the cytosol should occur by the opening of a transport pathway from the substrate binding site to the cytoplasm [27]. Differently, the ASCT2 is an antiporter. Thus, the simultaneous transport of glutamine and Na^+ towards the cytoplasm and of another amino acid in the opposite direction should occur with a different molecular mechanism: (i) each transporter subunit should have an additional substrate binding site on the cytosolic side thus allowing the simultaneous translocation of the three molecular species through a single transport pathway or (ii) the oligomeric structure may act in the case of ASCT2 in a concerted molecular mechanism in which two active subunits have the substrate binding sites exposed on opposite sides; the external site binds the amino acid and Na^+, the internal site binds the internal amino acid; the translocation, in this case, should occur through different transport pathways.

In a previous study on the reconstitution of the renal glutamine/amino acid transporter it was found that intraliposomal ATP stimulates the transport [13]. In the present study the mode of action of ATP in the regulation of the transport has been investigated. ATP increases the Vmax of the transporter (see also ref. [13]) and decreases the Km for external Na^+. Thus, the interaction of ATP with the internal side, probably located at the N-terminus of the transporter [13] will determine conformational changes that on the one hand will determine an enhanced mobility of the protein induced for example by a looser conformation, leading to the Vmax increase; on the other hand are transmitted to the external site of Na^+ that, in the modified conformation, exhibits higher affinity (lower Km) for Na^+; this may be caused by little shifting of the amino acid residues that interact with Na^+ . The transmission of conformational changes from one side to the opposite side of a membrane protein would require a particular type of intermembrane structure with a low degree of rigidity. This feature has been observed in the glutamate transporter of the plasma membrane in which the binding of Na^+ on the external side induces conformational changes on the opposite side [28]. The intermembrane structure of the *Pyrococcus horikoshii* member of the glutamate transporters, that should be similar to the ASCT2 transporter (see above), shows the properties of low degree of rigidity. It is characterized by the presence of short transmembrane segments that are connected within the membrane by loops that facilitate conformational changes [27]. From a physiological point of view, the effect of ATP on the affinity of the transporter for Na^+, together with the effect on the Vmax, may play an additional regulatory role in those kidney districts in which the tubular NaCl concentration is lower.

In studies performed with cells, it was proposed that the ASCT2 transporter accepts glutamate, preferentially at acidic pH [6] and that it is more sensitive to glutamate inhibition at pH 6.0 than at pH 7.0 [25]. In the present work the effect of

glutamate has been kinetically analysed both as inhibitor and as substrate. The inhibition by glutamate was clearly more pronounced at acidic pH in agreement with the previous data, differently from the neutral amino acids. The analysis of the inhibition revealed that the glutamate binds to the transporter at the same site of glutamine thus suggesting that both the substrates are transported by the same pathway. The glutamate$_{ex}$/glutamine$_{in}$ antiport mode was more efficient at acidic pH. This is in line with the higher affinity of the transporter for external glutamate at acidic pH as it is evidenced by both the Ki and the Km values (see Fig. 4 and Table 1). An explanation to this behavior may be given by the interaction of glutamate with the transporter as protonated zwitterionic species. However, both the external and internal Km for the zwitterion glutamic acid at pH 7 were about one order of magnitude lower than those at pH 6 indicating that the transported species is not the zwitterion glutamic acid but glutamate. Thus, the different affinity of the transporter for glutamate may be determined by a different state of protonation of amino acid residues with a pKa close to 6, in the external substrate binding site. A residue that fulfils this property is His. At acidic pH, this hypothetical His residue is protonated and has a positive charge that should facilitate the interaction of glutamate with the substrate binding site, but should have little or no influence on the binding of neutral amino acids. The presence of such a residue only in the external binding site may explain the different pH dependence of the opposite glutamine$_{ex}$/glutamate$_{in}$ antiport mode that shows higher transport activity at pH 7.0; this is also in line with the internal Km for glutamate that is not influenced by the pH. In agreement with the presence of a hypothetical His residue that is involved in the transport function, it has been observed that DEPC, an His specific reagent, inhibited the transport activity of the reconstituted glutamine/amino acid transporter. The amino acid sequence of the ASCT2 protein contains six His residues. At least three of these residues are exposed to the cytosolic side of the transporter, as it can be deduced from the hydrophobicity profile (not shown and see ref. [6]). Aspartate, differently from asparagine, does not inhibit the transporter, indicating that the size of the molecule is critical for the interaction with the substrate binding site only in the case of negatively charged substrates. Differently from glutamine, the Km of which is much lower on the external than on the internal site, glutamate showed similar internal and external Km. This feature of the transporter may have physiological implications. The external site of the transporter that exhibits an affinity for glutamine much higher than that for glutamate, will bind glutamine preferentially. The internal site, that has similar affinity for glutamine and glutamate, will bind glutamine and glutamate as well. Since the internal face of the reconstituted transporter corresponds to the intracellular side [13], the physiological mode of transport should be a flux of glutamine from the tubule lumen to the cytosol and a flux of glutamate from the cytosol to the lumen. A glutamate/glutamine transport in the opposite mode (glutamate from the lumen to the cystosol) may become more significant only when the pH of the lumen shifts towards strong acidic values. The glutamine/glutamate antiport mode may have significant role in tissues different from kidney in which ASCT2 is also expressed. In rat lens, a glutamine/glutamate counterflow is required: this function may be performed by the single transporter ASCT2 [29]. ASCT2 may also play an important role in the glutamate/glutamine cycle and astrocyte/neuron communication in the central nervous system. ASCT2 may contribute in the release of glutamine synthesized in the astrocytes by the glutamine/glutamate antiport function [8,30]. This transport function of ASCT2 may explain the previous finding of glutamine efflux, induced by glutamate, observed in astroglia but not in synaptosomes [31]. This paper represents an evidence in favour of the expression of ASCT2 in kidney, that is still not very clear [32]. However, it cannot be excluded that the reconstituted transporter may be a still unknown protein (ASCT-like or even B°-like protein with functional properties different from those described for B°AT1 [15,26]) or an isoform of ASCT2 specifically expressed in kidney, with some functional differences with respect to isoforms expressed in other tissues.

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