J. Membrane Biol. 180, 213–220 (2001)
DOI: 10.1007/s002320010072

The Journal of
Membrane
Biology

Sequential Amino Acid Exchange across $b^{0,+}$-like System in Chicken Brush Border Jejunum

M. Torras-Llort[1], D. Torrents[2], J.F. Soriano-García[1], J.L. Gelpí[2], R. Estévez[2], R. Ferrer[1], M. Palacín[2], M. Moretó[1]

[1]Departament de Fisiologia-Divisió IV, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain
[2]Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain

Received: 23 March 2000/Revised: 29 December 2000

Abstract. In the small intestine, cationic amino acids are transported by y^+-like and $b^{0,+}$-like systems present in the luminal side of the epithelium. Here, we report the characterization of a $b^{0,+}$-like system in the apical membrane of the chicken jejunum, and its properties as an amino acid exchanger. Analysis of the brush border membrane by Western blot points out the presence of rBAT (protein related to $b^{0,+}$ amino acid transport system) in these membranes. A functional mechanism for amino acid exchange across this system was established by kinetic analysis measuring fluxes at varying substrate concentrations both in internal (in) and external (out) vesicle compartments. This intestinal $b^{0,+}$-like system functions for L-arginine as an obligatory exchanger since its transport capacity increases 100–200 fold in exchange conditions, thus suggesting an important role in the intestinal absorption of cationic amino acids. The kinetic analysis of Arg_{in} efflux velocities is compatible with the formation of a ternary complex and excludes a model involving a *ping-pong* mechanism. The binding affinity of Arg_{out} is higher than that of Arg_{in}, suggesting a possible order of binding (Arg_{out} first) for the formation of the ternary complex during the exchange cycle. A model of double translocation pathways with alternating access is discussed.

Key words: L-arginine — Kinetic mechanism — Homoexchange — Heteroexchange — rBAT — Vesicles

Introduction

The transport of cationic amino acids across the plasma membrane is mediated via Na^+-dependent and Na^+-independent transport systems (Palacín et al., 1998). In the brush border membrane of the chicken intestine, we characterized three Na^+-independent amino acid transporters: an L-like system that transports neutral amino acids and is sensitive to BCH (2-aminobicyclo-[2,2,1]-heptane-2-carboxylic acid); a second system, similar to system y^+ transporting cationic and neutral amino acids, which is sensitive to NEM (N-ethylmaleimide) and has been named y^+m for its high capacity to transport L-methionine; and a $b^{0,+}$-like system, which transports cationic and neutral amino acids with an extremely low capacity and shows high affinity for L-lysine ($K_m \approx 2.5$ μM) (Torras-Llort et al., 1996; Soriano-García et al., 1998).

The $b^{0,+}$ transport system is a broad-scope cationic and neutral amino acid transporter and, together with systems y^+L, L and x_c^-, one of the first examples of a heteromeric transporter for organic substrates in vertebrates (Kanai et al., 1998; Mastroberardino et al., 1998; Torrents et al., 1998; International Cystinuria Consortium, 1999; Pineda et al., 1999; Sato et al., 1999; *see* Verrey et al., 1999 and Palacín, Bertran & Zorzano, 2000 for reviews). There is structural and functional evidence that system $b^{0,+}$ is formed by the rBAT protein, a heavy chain of approximately 95 kDa, linked by disulfide bonds to a light chain of ≅40 kDa, referred to as $b^{0,+}$-AT (Chairoungdua et al., 1999; International Cystinuria Consortium, 1999, Verrey et al., 1999). The fact that mutations in $b^{0,+}$-AT and rBAT genes cause nontype I and type I cystinuria, respectively (an inherited aminoaciduria of cystine and dibasic amino acids), supports this hypothesis and is consistent with the role of $b^{0,+}$ system in the renal reabsorption and intestinal absorption of dibasic amino acids and cystine (International Cystinuria Consortium, 1999).

The $b^{0,+}$ system behaves as an obligatory amino acid

Correspondence to: R. Ferrer

exchanger in oocytes expressing rBAT and in the renal OK cell line, which naturally expresses this protein (Chillarón et al., 1996; Mora et al., 1996). The $b^{0,+}$ system has been described as a tertiary transporter energized by the transmembrane gradient of neutral amino acids, mediating the apical absorption of cationic amino acids and cystine (Chillarón et al., 1996). Despite the physiological relevance of this transporter, the mechanism by which the protein binds the substrates at both sides of the membrane remains unknown. We now address this question by examining amino acid exchange across the $b^{0,+}$-like system identified in the apical membrane of the chicken jejunum (Torras-Llort et al., 1996; Soriano-García et al., 1998), using brush border membrane vesicles (BBMV), since in this model external and internal compartments are easily accessible to experimental variations. The results indicate that the chicken intestinal $b^{0,+}$-like system is associated with the presence of rBAT in the apical membrane; they also show that the transport system behaves as an obligatory exchanger with a kinetic mechanism involving the formation of the ternary complex during the exchange cycle.

Materials and Methods

Brush Border Membrane Preparations

Membranes were prepared from the jejunum of 6 wk-old chicken using the Mg^{2+} precipitation method of Kessler et al. (1978) as described elsewhere (Torras-Llort et al., 1996). The composition of the intravesicular medium (in mM) was: 300 mannitol, 20 HEPES/Tris (pH 7.4), 0.1 $MgSO_4 \cdot 7H_2O$ and 0.02% LiN_3. Vesicles were diluted to a final protein concentration of 30–40 mg/ml, frozen and stored in liquid N_2 for no more than 5 months. Each isolation batch corresponds to the jejunum of one chicken and in the *Results* section *n* indicates the number of chickens or membrane preparations.

Western Blotting

Chicken BBMV were isolated in the presence of NEM following the same protocol as above. Brush border membranes from rat kidney, used as positive control, were isolated in the presence of 5 mM NEM by the Ca^{2+} precipitation method described by Malathi et al. (1979). Protein samples were heated to 100°C for 5 min in either the presence or absence of 100 mM DTT (dithiothreitol) and subjected to sodium dodecyl sulfate (SDS)—polyacrylamide gel electrophoresis (7.5% polyacrylamide). The separated proteins were transferred to a nylon membrane and treated with diluted (1:1,000) polyclonal MANRX anti-rBAT antiserum and pre-immune serum PREMAN. Immune serum was generated following Furriols et al. (1993), raised in rabbit against a peptide with sequence highly conserved in the rBAT proteins deduced from the rabbit, rat and human rBAT cDNAs. Peroxidase conjugated anti-rabbit IgG was used as secondary antibody diluted 1:1,000. The signal was detected by an Enhanced chemiluminescence reaction system (ECL, Amersham, USA).

Uptake Measurements

The *trans* effects on the $b^{0,+}$-like exchange activity were studied by measuring amino acid efflux or influx in the presence of various substrates at the *trans* side of the membrane. To isolate this transport activity, vesicles were pre-incubated in the presence of 0.5 mM NEM at 25°C for 15 min as reported elsewhere (Torras-Llort et al., 1996). After pre-incubation, vesicles were diluted and centrifuged (12,000 × *g* for 10 min). The supernatant was discarded and vesicles were resuspended in pre-loading medium containing (in mM): 300 mannitol, 20 HEPES/Tris (pH 7.4), 0.1 $MgSO_4 \cdot 7H_2O$, 0.02% LiN_3 and the appropriate amino acid concentration, for 15 min at 37°C to facilitate amino acid transmembrane equilibrium. Pre-loaded vesicles (final protein concentration of 10–15 mg/ml) were maintained at 4°C before uptake measurements, to reduce substrate efflux. Nontreated vesicles were handled equally to NEM-treated membrane preparations.

Transport experiments were carried out at 37°C using the rapid filtration technique (Torras-Llort et al., 1996). The vesicles were incubated in isotonic conditions (320 mosm/kg) in an incubation medium containing (in mM): 100 KSCN, 100 mannitol, 20 HEPES/Tris (pH 7.4), 0.2 $MgSO_4 \cdot 7H_2O$, 0.02% LiN_3, and the appropriate substrate concentration. Substrate influx or efflux rates were measured for incubation periods in which linearity was ensured (3 sec for L-methionine and 7 sec for L-arginine).

Kinetic Analysis of $b^{0,+}$-like Exchange Mechanism

To study the sequence of substrate binding and release during the exchange cycle, L-arginine homoexchange velocity was measured as a function of substrate concentrations in both external and internal compartments. The resulting kinetic pattern provides the data needed to discriminate between the two basic types of bisubstrate mechanism, namely sequential or *ping-pong* (Cleland, 1970; Dierks, Riemer & Kramer, 1988). The sequential mechanism involves the formation of a ternary complex of the transporter with two substrate molecules; therefore, both substrates must bind to the protein before either of them is released. In contrast, the *ping-pong* (or alternating access) mechanism exposes only one substrate binding site which alternates between opposing membrane surfaces so that one substrate must be released from the protein before the other is bound.

The values of the kinetic parameters of exchange were derived from the experimental data by non-linear regression considering the equations describing the sequential (Eq. 1) and the *ping-pong* (Eq. 2) mechanisms.

$$v = \frac{V_{max}[A][B]}{\alpha K_A K_B + \alpha K_B[A] + \alpha K_A[B] + [A][B]} \quad (1)$$

$$v = \frac{V_{max}[A][B]}{K_B[A] + K_A[B] + [A][B]} \quad (2)$$

where A and B are the exchanged substrates, K_A and K_B are the dissociation constants and V_{max} the maximum capacity of exchange.

The experimental data were also analysed by graphical methods using double-reciprocal plots. This graphical approach allows the analysis of the degree of dependence of kinetic parameters on the internal and external substrate concentrations. The resulting pattern of the reciprocal plots gives further information on the type of exchange mechanism involved; for a *ping-pong* mechanism, the reciprocal plot consists of a set of parallel lines, whereas for a sequential mechanism it consists of intersecting lines (Cleland, 1970).

Simulation of $b^{0,+}$-like Amino Acid Exchanger

L-arginine efflux was simulated by considering system $b^{0,+}$-like an obligatory exchanger with 1:1 stoichiometry. The computer program,

previously designed for oocytes (Chillarón et al., 1996) was adapted to the experimental conditions established here for membrane vesicles. It monitors the amino acid exchange and simulates the experimental influx and efflux rates governed by the exchange mechanisms, i.e. sequential (ordered and random) and *ping-pong.* The computer program is available upon request.

The simulation was prepared as described (Chillarón et al., 1996), considering the following experimental conditions:

(i) The experimental set-up of L-[^{3}H]arginine efflux was reproduced by considering two separate compartments of 0.63 μl/mg protein (intravesicular compartment) and 200 μl (extravesicular compartment, incubation medium) and with protein concentration fixed at 20 mg/ml. Internal and external amino acid concentrations were fixed according to the experimental conditions, considering labeled and unlabeled substrate concentrations individually.
(ii) Transport rates were evaluated taking into account that the limiting step is the translocation of the transporter bound to the substrate (Eleno, Devés & Boyd, 1994); therefore, previous steps would be in equilibrium. In these conditions, the equations describing the kinetic behavior for each exchange mechanism are easier to interpret and allow discrimination between them.
(iii) The negative membrane potential was considered constant during the simulation time (5 sec), and therefore the effects of this parameter were not considered.

Chemicals

L-[^{3}H]-arginine, L-[^{14}C]-methionine and L-[^{14}C]-lysine were from New England Nuclear Research Products (Dreieich, Germany). All unlabeled reagents were from Sigma Chemical (St. Louis, MO).

Data Analysis

The data are reported as means ± SE. All data were compared using analysis of variance and Student's *t*-test. In both cases, $P < 0.05$ denotes significance.

Results

Amino Acid Exchange Activity in Chicken Intestinal BBMV

Exchange activity was studied by measuring L-methionine efflux (50 μM) in the absence or presence of 10 mM L-lysine on the *trans* side of the membrane (Fig. 1). Vesicles were pretreated with 0.5 mM NEM and preloaded with 10 mM BCH to analyze separately the contribution of the L-like, y^+m and $b^{0,+}$-like pathways involved in L-methionine transport (Torras-Llort et al., 1996; Soriano-García et al., 1998). In all conditions tested, the L-methionine efflux increased in the presence of the *trans* amino acid (L-lysine). Assuming that when both BCH and NEM are present, only system $b^{0,+}$-like mediates L-methionine efflux, the 8-fold increase detected should be attributed to this specific transport system. Therefore, although the L-like (BCH-sensitive) and y^+m (NEM-sensitive) systems mediate L-methionine efflux, their activities are not modified by the presence of L-lysine on the *trans* side. In addition, amino acid influx was also enhanced in exchange conditions in the presence of both inhibitors (*data not shown*). These results, together with the low $b^{0,+}$-like transport activity measured in nonexchange conditions, suggest that system $b^{0,+}$-like in the BBM of the chicken jejunum behaves as an amino acid exchanger.

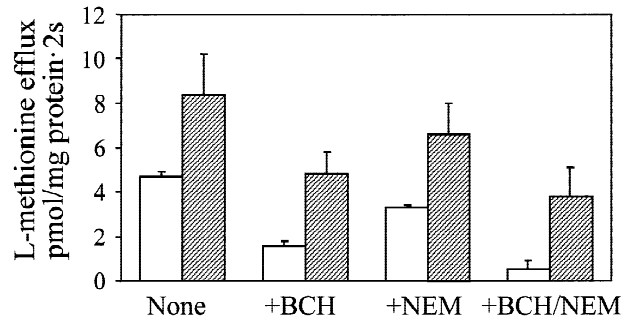

Fig. 1. Amino acid exchange across the $b^{0,+}$-like system. Efflux of L-[^{14}C]methionine (50 μM) in the absence (open bars) and presence (dashed bars) of 10 mM L-lysine in the *trans* side of the membrane. L-methionine transport rates (in pmol/mg protein · 2 sec) were measured under a *zero-trans* 100 mM KSCN gradient. Membranes were prepared in a medium containing 300 mM mannitol and 20 mM HEPES/Tris (pH 7.4), and pre-loaded with the appropriate amino acid concentration (15 min, 37°C). Incubation medium contained (mM) 100 KSCN, 100 mannitol, 20 HEPES/Tris (pH 7.4), and the appropriate concentration of amino acid. Vesicles were preloaded with 10 mM BCH (+BCH), 0.5 mM NEM (+NEM) or both (+BCH/NEM). Results are expressed as means ± SE ($n = 4$). In all conditions tested, amino acid transport was significantly higher in exchange conditions than in nonexchange conditions ($P < 0.001$).

Detection of rBAT Protein in BBMV

After the identification of the $b^{0,+}$-like exchange activity in jejunal BBM, the presence of the rBAT protein was tested. In rat kidney BBM, the heteromeric complexes of rBAT linked by sulfide bridges to the light subunit are better maintained when samples are prepared in the presence of NEM (Mosckovitz et al., 1994); for this reason, BBM of chicken intestine were also isolated by this method and then used for Western blot analysis, with an anti-rBAT specific antibody (Fig. 2). In the rat kidney, under nonreducing conditions, rBAT was mainly detected as complexes of 125 and >200 kDa (Fig. 2), as expected (Furriols et al., 1993), but when DTT was added, a single band of 95 kDa, corresponding to glycosylated rBAT, was observed. For the chicken intestine, too, a single band of 95 kDa was detected in reducing conditions. However, it was maintained in the absence of DTT. In these conditions, complexes of 130 and 156 kDa were also detected. These results support the hypothesis that the exchange activity of the intestinal $b^{0,+}$-

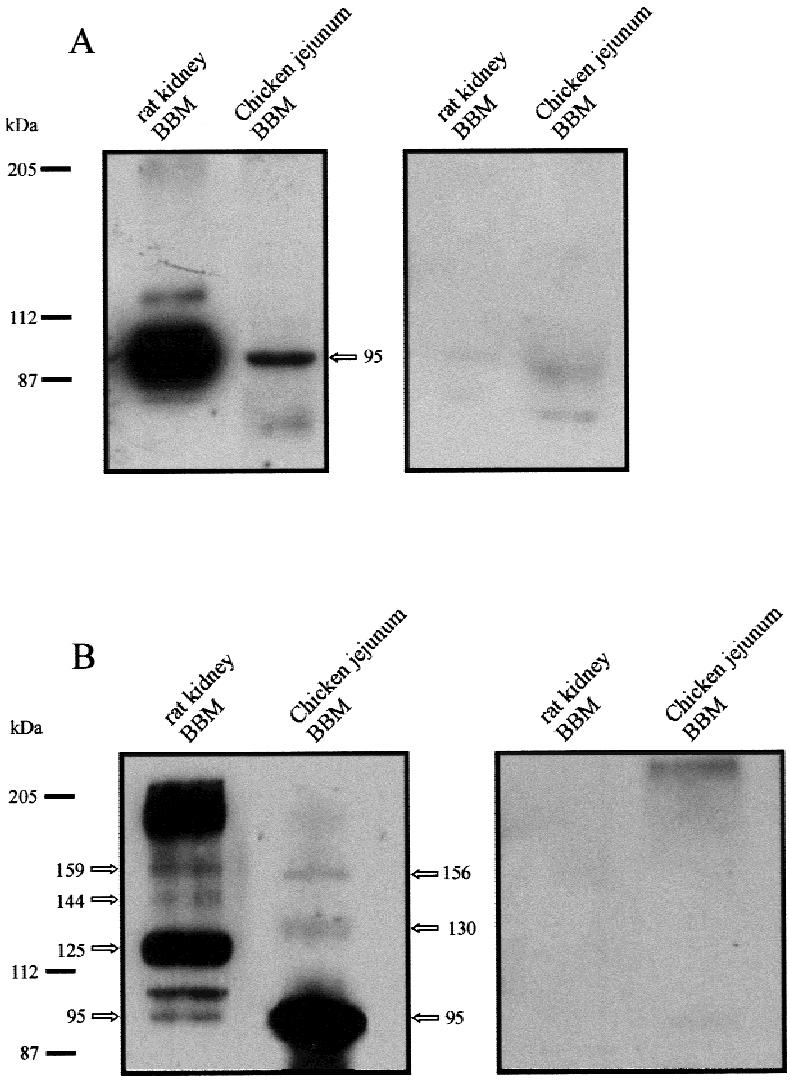

Fig. 2. Immunodetection of rBAT in membrane preparations from chicken jejunum. BBMV of chicken jejunum (50 μg protein per lane) and rat kidney (2 μg) were solubilized in sample buffer and resolved by 7.5% SDS-PAGE either in the absence or in the presence of 100 mM DTT (*A* and *B*, respectively). The polyclonal antibody MANRX raised against rBAT (Furriols et al., 1993) recognizes a protein band (≈95 kDa) in chicken purified BBM incubated with NEM, both in reducing (+DTT) and non-reducing conditions (−DTT). In the absence of DTT, higher molecular weight bands are detected in the membranes from both species (rat preparation used as positive control). None of these bands was observed when detection was performed with the corresponding pre-immune serum (right panels).

like system is associated with the presence of rBAT in the apical membrane of the chicken jejunum. To further characterize this transport system, both the specificity and the kinetic mechanism of exchange were subsequently analyzed.

Specificity of $b^{0,+}$-like Exchange Activity

Efflux of L-arginine (5 μM) was measured in the presence of 5 mM neutral and cationic amino acids or the analogue AIB (aminoisobutyric acid) in the extravesicular medium. L-arginine and L-leucine are the best inducers of the exchange activity, although homoexchange is slightly higher than heteroexchange (Fig. 3*A*). In contrast, L-alanine, L-glycine and L-proline do not stimulate L-arginine efflux. This indicates that only amino acids that are substrates of the $b^{0,+}$-like system increase efflux rates. In addition, the presence of AIB in the extravesicular medium had no effect on L-arginine efflux (Fig. 3*A*) or L-arginine influx (Fig. 3*B*) in homoexchange conditions.

Kinetics of the $b^{0,+}$-like Exchange Activity

Homoexchange velocity (Fig. 4) was calculated by measuring L-arginine efflux within 5 sec. Internal L-arginine concentration (Arg_{in}) increased from 0.5 to 100 μM and external L-arginine (Arg_{out}) from 0 (non-*trans*-stimulated condition) to 100 μM using vesicles pretreated with 0.5 mM NEM. The results show an increase in L-arginine efflux while increasing the *trans*-side amino acid concentration. In the absence of Arg_{out}, a low amino acid efflux was detected, which can be fitted by nonlinear regression to one transport system ($K_m = 1.63 \pm 2.1$ μM; $V_{max} = 1.63 \pm 0.4$ pmol/mg · 5 sec) plus diffusion ($K_D = 74 \pm 14$ nl/mg · 5 sec). In order to study the kinetic

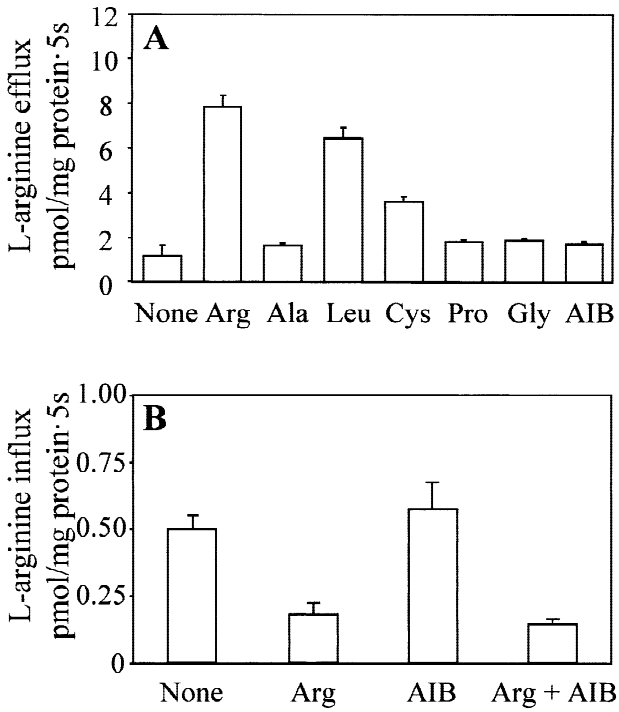

Fig. 3. L-arginine exchange across the $b^{0,+}$-like system. Vesicles were prepared as described in legend of Fig. 1, pre-incubated with 0.5 mM NEM (15 min, 25°C) and incubated under *zero-trans* 100 mM KSCN gradient. *A*) The suspension was preloaded (15 min, 37°C) with 5 μM L-arginine (0.5 μM L-[^{3}H]-arginine) and incubated in a medium containing no amino acid (*None*) or 5 mM L-amino acid or the amino acid analogue *AIB*. *B*) L-arginine influx (0.5 μM L-[^{3}H]-arginine) was determined in vesicles preloaded with 0.5 μM unlabeled L-arginine and incubated in the presence of no amino acid (*None*) or 5 mM L-arginine and/or *AIB*. Results are means ± SE ($n = 4$). L-arginine, L-leucine and L-cysteine significantly induced L-[^{3}H]arginine efflux and L-arginine and L-arginine plus AIB significantly reduce L-[^{3}H]arginine influx ($P < 0.001$).

properties of system $b^{0,+}$-like as an amino acid exchanger, the non-*trans*-stimulated efflux was subtracted from total L-arginine efflux (Fig. 5*A* and 5*B*) and the results were analyzed both by linear (Lineweaver-Burk plots) and nonlinear regression analysis (*see* METHODS). For all kinetic curves, the Hill coefficient (comprised between 0.8 and 1.2) did not differ statistically from 1, suggesting that the homoexchange of Arg_{out}/Arg_{in} has a 1:1 stoichiometry.

Lineweaver-Burk plots (Fig. 5*C* and 5*D*) show straight lines with different slopes. This kinetic behavior can only be attributed to a sequential mechanism and rules out a *ping-pong* mechanism, which is characterized by a nonintersecting pattern (Cleland, 1970). To determine to what extent a sequential mechanism could account for the exchange rates measured, Arg_{in} efflux velocities, considering system $b^{0,+}$-like an obligatory exchanger with a 1:1 stoichiometry, were simulated (*see* METHODS). Sequential mechanisms (ordered or random)

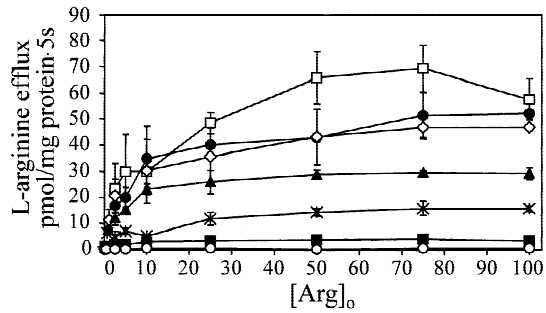

Fig. 4. Arg_{out}/Arg_{in} homoexchange velocities across the $b^{0,+}$-like system. NEM-treated vesicles were preloaded with increasing L-arginine concentrations (μM): 0.5 (○), 5 (■), 25 (✳), 35 (▲), 50 (◇), 75 (●) and 100 (□) (containing 0.5 μM L-[^{3}H]arginine), and incubated under a *zero-trans* 100 mM KSCN gradient in a medium containing increasing concentrations of unlabeled L-arginine (0–100 μM). Results are expressed as means ± SE ($n = 4$).

reproduce the experimental results (Fig. *A* and 5*B*), but the *ping-pong* mechanism does not (*data not shown*). Therefore, these results do not discriminate between ordered and random binding of substrates to the transporter, and for this reason the kinetic parameters were calculated considering both types of sequential mechanisms. The kinetic values obtained by nonlinear regression analysis indicate that the binding affinity of Arg_{out} is higher than that observed for Arg_{in}, suggesting an order in the binding of the substrates (Table). The V_{max} exchange values (202–330 pmol/mg · 5 sec) were significantly higher than the values calculated in non-exchange conditions (1.6 pmol/mg · 5 sec).

Discussion

The present study deals with the properties of the $b^{0,+}$ transport system present in the brush border membrane of jejunal chicken enterocytes as an amino acid exchanger. We used specific inhibitors of Na^+-independent neutral and cationic amino acid transport systems to separate $b^{0,+}$ transport activity from that of other transport systems present in the apical membrane (Torras-Llort et al., 1996; Soriano-García et al., 1998). With this strategy, the efflux of L-methionine in the presence or absence of L-lysine on the *trans* side of the membrane was studied, using vesicles preloaded with BCH and pre-incubated with NEM to ensure that the flux of L-methionine across the membrane was restricted to the $b^{0,+}$ pathway. The results show a dramatic *trans*-stimulation of $b^{0,+}$-like system, which is shown for the first time in the apical membrane of the intestinal epithelium, thus indicating that this system mainly behaves as an amino acid exchanger. These results agree with evidence obtained with the heterologous expression system, like rBAT-expressing *Xenopus* oocytes and OK

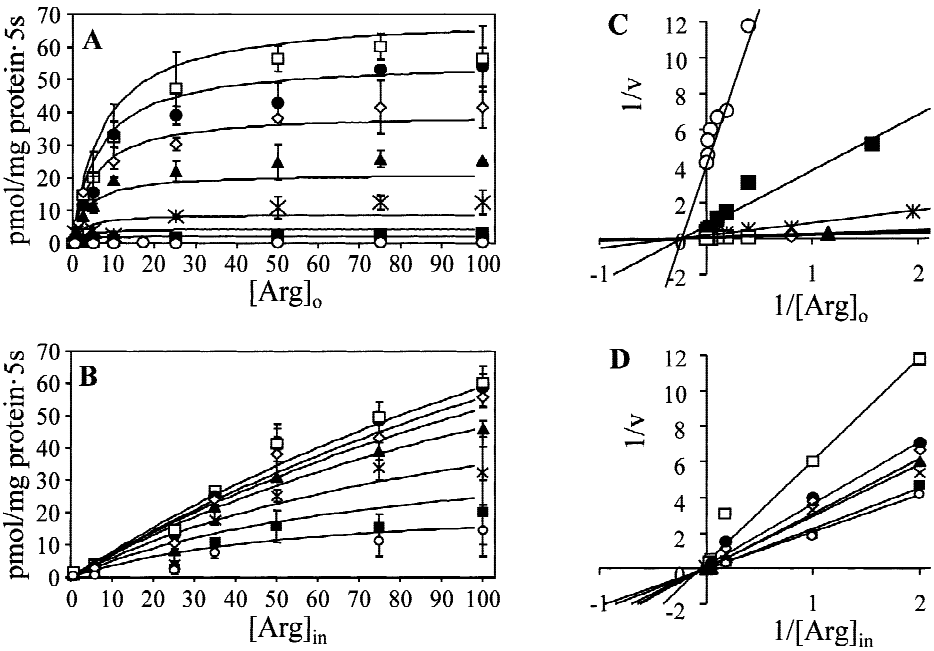

Fig. 5. *A* and *B*, comparison of L-arginine efflux, after subtracting the amino acid efflux in nonexchange conditions (symbols), with the efflux velocities obtained by the simulation (lines) of the $b^{0,+}$-like homoexchange (*see* METHODS). Results were expressed as pmol/mg · 5 sec and referred to the extravesicular (*A*) or the intravesicular (*B*) L-arginine concentrations. *C* and *D*, Lineweaver-Burk plots. The concentrations of internal L-arginine were (μM): 0.5 (○), 5 (■), 25 (✳), 35 (▲), 50 (◇), 75 (●) and 100 (□), in *A* and *C;* external L-arginine concentrations were (μM): 0.5 (○), 5 (■), 10 (✳), 25 (▲), 50 (◇), 75 (●) and 100 (□), in *B* and *D*. Results are means ± SE ($n = 4$).

Table. Kinetic constants of the $b^{0,+}$-like exchanger

	Ordered mechanism	Random mechanism
V_{max}	202 ± 8	330 ± 2.4
$K(Arg_o)$	8.5 ± 0.3	2.0 ± 0.3
$K(Arg_{in})$	177 ± 10	28 ± 6.0
α	–	11.8 ± 3.3

The kinetic values were calculated by nonlinear regression analysis, using the strategy described by Cleland (1970). V_{max} values are expressed as pmol/mg · 5 sec; $K(Arg_{out})$ and $K(Arg_{in})$, are the dissociation constants (expressed in μM) for extravesicular and intravesicular L-arginine, respectively; α is a constant denoting the influence of one substrate on the binding affinity of the other substrate in a random mechanism.

cells (Bush et al., 1994; Coady et al., 1994; Ahmed et al., 1995; Chillarón et al., 1996; Mora et al., 1996).

Since the rBAT protein is involved in the $b^{0,+}$ activity, we examined whether rBAT was present in the apical membrane of the chicken jejunum. This was confirmed by Western blot analysis, albeit with a lower signal than in the rat renal preparations, revealing lower expression of rBAT in the chicken intestine or lower affinity of the antibody. In nonreducing conditions, the presence of the heteromeric complex of rBAT and the light subunit (the $b^{0,+}$-AT) in these preparations is not guaranteed, since the mobilities of the higher molecular weight bands in rat kidney and chicken intestine membranes may be different. In the rat kidney, the rBAT protein-light $b^{0,+}$ subunit complex appears as a 125 kDa band together with other forms of aggregation (Wang & Tate, 1995; Palacín, Chillarón & Mora, 1996). In the intestine, no information is available on the mobility of such complexes; however, the 130 and 156 kDa bands appearing in our preparations may correspond to these complexes but in different forms of aggregation. In nonreducing conditions, the 95 kDa band is the most intense, suggesting that many of the complexes are lost during the purification procedure.

The study of the $b^{0,+}$ like exchange specificity shows that the system behaves as an amino acid exchanger for neutral and cationic amino acids. In addition, amino acid efflux (and, therefore, exchange rates) was slightly higher ($P < 0.05$) in homoexchange (Arg_{in}/Arg_{out}) than in heteroexchange (Arg_{in}/Leu_{out}) conditions. These differences could be attributed to the nature of the extravesicular substrate. In the case of a cationic amino acid such as arginine, the effect of membrane potential across the vesicles (negative inside) would be more advantageous than for a dipolar substrate such as leucine. However, previous results obtained in rBAT-expressing oocytes showed lower homoexchange rates for Leu_{in}/Leu_{out} than for Arg_{in}/Arg_{out} (Chillarón et al., 1996), suggesting that the carrier reorientates more slowly when loaded with leucine.

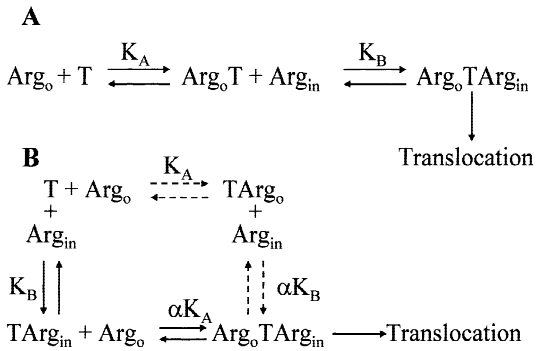

Fig. 6. Kinetic models for an ordered (*A*) and a random sequential mechanism (*B*) of L-arginine homoexchange (Arg_{in}/Arg_{out}) across the $b^{0,+}$-like transport system (*T*). K_A and K_B are the dissociation constants for the formation of the binary and the ternary complex, respectively; α is a constant denoting the influence of one substrate to the binding affinity of the other substrate in a random mechanism.

The results of the kinetic analysis of countertransport via system $b^{0,+}$-like clearly rule out a model of exchange based on a *ping-pong* mechanism. The intersecting pattern obtained is consistent with a sequential type mechanism involving the formation of a ternary complex of the carrier with two substrate molecules. When considering a sequential model, several alternatives can describe the order of substrate binding for the formation of the ternary complex. However, neither the kinetic experiments nor the simulation of the $b^{0,+}$-like exchange activity discriminate between ordered and random binding of the substrates. For this reason, both mechanisms were formulated for the interpretation of the kinetic results. The kinetic values obtained considering both mechanisms show that the binding affinity for the external amino acid is always higher than for the internal substrate. An *ordered* mechanism, in which the free carrier first binds to the external amino acid, followed by the binding of the internal one, may account for these results (Fig. 6*A*). However, since the binding affinity for the internal amino acid is not very low (in the micromolar range), these results could also be explained by a *random* mechanism with a "preferential" route (Fig. 6*B*). In this last model, the free carrier binds both substrates but tends to bind the external amino acid first. Such "preferential" behavior may be due to the negative membrane potential, which would favor the binding of cationic amino acids to the transporter from the external side rather than from the internal one.

The $b^{0,+}$-like system present in the chicken jejunum showed very low amino acid efflux and influx rates in nonexchange conditions, which is consistent with the tightness of the exchange activity reported for system $b^{0,+}$ (Bush et al., 1994; Coady et al., 1994; Chillarón et al., 1996). The kinetic properties of the intestinal transporter are similar to those described in nonexchange conditions, i.e., high affinity and extremely low transport capacity (Torras-Llort et al., 1996; Soriano-García al., 1998). However, in exchange conditions, the kinetic profile shows a dramatic increase in its maximum transport capacity (100–200 fold higher), confirming the obligatory nature of amino acid exchange across system $b^{0,+}$-like and the key role of this transporter in the intestinal absorption of amino acids. Taking into account the characteristics of a sequential obligatory exchanger, the kinetic behavior described here for system $b^{0,+}$-like could be interpreted as a highly efficient way to avoid the free translocation of the empty transporter thus ensuring the exchange of both substrates.

A functional model for system $b^{0,+}$ exchange activity was proposed by Coady et al. (1996), from results obtained using cut-open oocytes expressing rabbit rBAT. These authors observed that AIB induced amino acid currents across this pathway without being transported itself, thus suggesting variable stoichiometry of exchange. In order to explain these results, a "double-gated" pore model with a binding site accessible at each side of the membrane was proposed. However, our results show no effect of AIB on L-arginine efflux or influx (Fig. 3*A* and *B*), thus excluding variable stoichiometry for the exchange via system $b^{0,+}$-like in the chicken intestine. Although the "double-gated" pore also involves the formation of a ternary complex, our results are more compatible with a double pathway exchanger with one binding site at either side of the membrane. A similar model was proposed by Dierks et al. (1988) for the aspartate/glutamate antiporter, which includes two functional "subunits" or pathways with binding sites alternating at each membrane domain in which the translocation step is under membrane potential control.

This is the first evidence for the rBAT/$b^{0,+}$-like exchange mechanism in a freshly isolated tissue. In the chicken jejunum, this transport system behaves as an obligatory amino acid exchanger and involves the formation of the ternary complex during the exchange cycle. Moreover, the functional model proposed here could help to clarify the defective amino acid transport described in aminoacidurias associated with rBAT mutations.

This work was supported by grants ALI96-910 and PM99/0172 from Dirección General de Investigación Científica y Técnica (Ministerio de Educación y Cultura, Spain) and grants 1995SGR537 and 1999GR271 from the Generalitat de Catalunya. M.T.-L. was a research fellow of the Recerca i Docència program (Universitat de Barcelona). J.F.S.-G. and R.E. were recipients of a predoctoral fellowship from CIRIT (Generalitat de Catalunya). D.T. was a recipient of a grant from the Ministerio de Educación y Cultura. We thank Robin Rycroft for editorial help.

References

Ahmed, A., Peter, G.J., Taylor, P.M., Harper, A.A., Rennie, M.J. 1995. Sodium-independent currents of opposite polarity evoked by neu-

tral and cationic amino acids in neutral and basic amino acids transporter cRNA-injected oocytes. *J. Biol. Chem.* **270:**8482–8486

Bush, A.E., Herzer, T., Waldegger, S., Schmidt, F., Palacín, M., Biber, J., Markovich, D., Murer, H., Lang, F. 1994. Opposite directed currents induced by the transport of dibasic and neutral amino acids in *Xenopus* oocytes expressing the protein rBAT. *J. Biol. Chem.* **269:**25581–25586

Cleland, W.W. 1970. *In:* The Enzymes. P.P. Boyer, editor. vol. 2 pp. 1–65. Academic Press, New York

Coady, M.J., Jalal, F., Chen, X-Z., Lemay, G., Berteloot, A., Lapointe, J.-Y. 1994. Electrogenic amino acid exchange via the rBAT transporter. *FEBS Lett.* **356:**174–178

Coady, M.J., Chen, X-Z., Lapointe, J.-Y. 1996. rBAT is an amino acid exchanger with a variable stoichiometry. *J. Membrane Biol.* **150:**1–8

Chairoungdua, A., Segawa, H., Kim, J.Y., Miyamoto, K., Haga, H., Fukui, Y., Mizoguchi, K., Ito, H., Takeda, E., Endou, H., Kanai, Y. 1999. Identification of an amino acid transporter associated with the cystinuria-related type II membrane glycoprotein. *J. Biol. Chem.* **274:**28845–28848

Chillarón, J., Estévez, R., Mora, C., Wagner, C.A., Suessbrich, H., Lang, F., Gelpí, J.L., Testar, X., Busch, A.E., Zorzano, A., Palacín, M. 1996. Obligatory amino acid exchange via systems $b^{0,+}$ and y^+L-like. A tertiary active transport mechanism for renal reabsorption of cystine and dibasic amino acids. *J. Biol. Chem.* **271:**17761–17770

Dierks, T., Riemer, E., Kramer, R. 1988. Reaction of the reconstituted aspartate/glutamate carrier from bovine heart mitochondria. *Biochim. Biophys. Acta* **943:**231–244

Eleno, N. Devés, R., Boyd, C.A.R. 1994. Membrane potential dependence of the kinetics of amino acid transport systems in human placenta. *J. Physiol.* (*London*) **479:**291–300

International Cystinuria Consortium 1998. Nontype I cystinuria caused by mutations in *SLC7A9* encoding a subunit ($b^{0,+}$AT) of rBAT. *Nature Genet.* **23:**52–57

Furriols, M., Chillarón, J., Mora, C., Castelló, A., Bertrán, J., Camps, M., Testar, X., Villaró, S., Zorzano, A., Palacín, M. 1993. rBAT, related to L-cystine transport is localized to the microvilli of proximal straight tubules, and its expression is regulated in kidney by development. *J. Biol. Chem.* **268:**27060–27068

Kanai, Y., Segawa, H., Miyamoto, K., Uchino, H., Takeda, E., Endou, H. 1998. Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 antigen (CD98). *J. Biol. Chem.* **273:**23629–23632

Kessler, M., Acuto, O., Storelli, C., Murer, H., Müller, M., Semenza, G. 1978. A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Their use in investigating some properties of D-glucose and choline transport system. *Biochim. Biophys. Acta* **506:**136–154

Malathi, P., Preiser, H., Fairclough, P., Malett, P., Crane, R.K. 1979. A rapid method for the isolation of kidney brush border membranes. *Biochim. Biophys. Acta* **554:**259–263

Mastroberardino, L., Spindler, B., Pfeiffer, R., Skelly, P.J., Loffing, J., Shoemaker, C.B., Verrey, F. 1998. Amino acid transport by heterodimers of 4F2hc/CD98 and members of a permease family. *Nature* **395:**288–291

Mora, C., Chillarón, J., Calonge, M.J., Forgo, J., Testar, X., Nunes, V., Murer, H., Zorzano, A., Palacín, M. 1996. The rBAT gene is responsible for L-cystine uptake via the $b^{0,+}$-like amino acid transporter system in a "renal proximal tubular" cell line (OK cells). *J. Biol. Chem.* **271:**10569–10576

Mosckovitz, R., Undenfriend, S., Felix, A., Heimer, E., Tate, S.S. 1994. Membrane topology of the rat kidney neutral and basic amino acid transporter. *FASEB J.* **8:**1069–1074

Palacín, M., Bertran, J., Zorzano, A. 2000. Heteromeric amino acid transporters explain inherited aminoacidurias. *Curr. Opin. Nephrol. Hypertens.* **9:**547–553

Palacín, M., Chillarón, J., Mora, C. 1996. Role of the $b^{0,+}$-like amino acid transport system in the renal reabsorption of cystine and dibasic amino acids. *Biochem. Soc. Trans.* **24:**856–863

Palacín, M., Estévez, R., Bertrán, J., Zorzano, A. 1998. Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol. Rev.* **78:**969–1054

Pineda, M., Fernández, E., Torrents, D., Estévez, R., López, C., Camps, M., Lloberas, J., Zorzano, A., Palacín, M. 1999. Identification of a membrane protein, LAT-2, that co-expresses with 4F2 heavy chain, an L-type amino acid transport activity with broad specificity for small and large zwitterionic amino acids. *J. Biol. Chem.* **274:**19738–19744

Sato, H., Tamba, M., Ishii, T., Bannai, S. 1999. Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. *J. Biol. Chem.* **274:**11455–11458

Soriano-García, J.F., Torras-Llort, M., Ferrer, R., Moretó, M. 1998. Multiple pathways for L-methionine transport in brush-border membrane vesicles from chicken jejunum. *J. Physiol.* (*London*) **509:**527–539

Torras-Llort, M., Ferrer, R., Soriano-García, J.F., Moretó, M. 1996. L-Lysine transport in chicken jejunal brush border membrane vesicles. *J. Membrane Biol.* **152:**183–193

Torras-Llort, M., Soriano-García, J.F., Ferrer, R., Moretó, M. 1998. Effect of a lysine-enriched diet on L-lysine transport by the brush-border membrane of the chicken jejunum. *Am. J. Physiol.* **274:**R69–R75

Torrents, D., Estévez, R., Pineda, M., Fernández, E., Lloberas, J., Shi, Y-B., Zorzano, A., Palacín, M. 1998. Identification and characterization of a membrane protein (y^+L Amino Acid Transporter-1) that associates with 4F2hc to encode the amino acid transport activity y^+L. A candidate gene for lysinuric protein intolerance. *J. Biol. Chem.* **273:**32437–32445

Verrey, F., Jack, D.L., Paulsen, L.T., Saier, M.H., Jr., Pfeiffer, R. 1999. New glycoprotein-associated amino acid transporters. *J. Membrane Biol.* **172:**181–192

Wang, Y., Tate, S.S. 1995. Oligomeric structure of a renal cystine transporter: implications in cystinuria. *FEBS Lett.* **368:**389–392