Amino Acid Transport in Kidney

HETEROGENEITY OF α-AMINOISOBUTYRIC UPTAKE*

CHARLES R. SCRIVER AND FAZL MOHYUDDIN

From The deBelle Laboratory for Biochemical Genetics, The McGill University-Montreal Children's Hospital Research Institute, Montreal 25, Canada

SUMMARY

1. More than one mode of transport can be observed for concentrative uptake of α-aminoisobutyric acid (AIB) by rat kidney cortex slices when the initial concentration of AIB in the medium is varied between 0.2 and 16 mM.

2. The relative contribution from both systems to the total observed uptake at various concentrations of AIB was calculated. The $K_v$ values for the two systems were 3.87 mM and 24.8 mM.

3. Sodium-dependent inhibitory reactions influence the uptake of 0.8 mM AIB more than of 8.0 mM AIB; inhibitors affecting —SH groups have the opposite effect.

4. L-Alanine is an effective competitive inhibitor of AIB transport at high substrate concentrations; more than one $K_i$ value for L-alanine can be derived depending on the concentration of AIB. External L-proline enhances the affinity of AIB for uptake at low substrate concentrations; an inhibitory effect is observed at high substrate concentrations. Although omission of sodium from the medium abolishes concentrative uptake of AIB at all concentrations, it is restored at low substrate concentration by the addition of 4 mM L-proline to the external sodium-free medium. L-Proline placed internally stimulates AIB accumulation at low substrate concentrations only in the presence of sodium.

5. A site at which a ternary complex between proline or sodium and the AIB-carrier complex may be formed is proposed for AIB transport at low concentrations; another system with different properties is used predominantly for AIB transport at high concentrations.

Membrane transport of amino acids is mediated by a number of different agencies in mammalian kidney (1). At least five systems with selective specificity for certain groups of amino acids have been recognized. Furthermore, a single mediation does not account for all of the uptake of individual amino acids in some of the groups (2, 3). More than one agency is apparently available for the uptake of the imino acids and glycine (4) and for lysine (5) in human kidney. The kinetics of uptake of the imino acids and glycine, in vivo in man, and the genetic regulation of this uptake, indicate that transport is achieved by a group-specific agency with high capacity and low affinity for the substrates, and by two or more substrate-specific mediations with relatively low capacity, but high affinity (4). Amino acid uptake by other tissues has been studied extensively (6), and evidence for the transport of single amino acids by more than one agency has also been documented in the mouse Ehrlich ascites tumor cell for methionine (7-9), β-alanine (10), phenylalanine (11), lysine (11), and α,γ-diaminobutyric acid (12) in rat kidney for lysine (13), and in bone for α-aminoisobutyric acid and glycine (14). Christensen has also discussed (15) the criteria by which one can distinguish heterogeneity of amino acid transport into a tissue.

Previous investigations of AIB uptake by rat kidney cortex slices (16, 17) have shown no evidence for heterogeneity of its concentrative uptake. When we observed this phenomenon, an effort was made to confirm it by quantitative and qualitative means. The initial impression has not been altered by any of the tests reported below. A stimulative effect of imino acids upon AIB accumulation, which is influenced by Na⁺ and which occurs exclusively at low substrate concentrations, proved to be a particularly interesting observation. A ternary complex involving the imino acid, sodium, and the AIB site complex, may be formed during uptake of AIB at low concentrations on a site related to, but not identical with, the mediation for proline uptake; this was not a characteristic of AIB uptake at high concentrations.

METHODS

Kidney cortex slices, obtained from Long-Evans hooded rats, were incubated in the manner described previously (3) with the following modifications. Single slices weighing approximately 10 mg were placed in each incubation flask; only one rat was used for each experiment.
for each experiment Tris(hydroxymethyl)aminomethane-Ringer glucose buffer (total osmolality, 300 mosmols), pH 7.4, at 37°, was substituted for Krebs-Ringer bicarbonate buffer for greater stability of pH under certain conditions used in the experiments. No difference in uptake was observed with Tris buffer under steady conditions when compared with the results obtained with Krebs-Ringer bicarbonate buffer (3). Estimation of total tissue water, determination of extracellular space with 14C-inulin, calculation of intracellular and medium concentration of 14C-labeled amino acid, and the use of Michaelis-Menten kinetics to describe the uptake of amino acids under steady state conditions (18) were performed as described previously (3, 16).

Results

Inhibition of Preloading with Amino Acids on the IO-min uptake of AIB was observed after preincubating slices for 40 min at 37° in the presence of unlabeled amino acid and then transferring the slices to flasks containing the labeled AIB. The influence of preloading with amino acids on the 10-min uptake of AIB was observed after preincubating slices for 40 min at 37° in the presence of unlabeled amino acid and then transferring the slices to flasks containing the labeled AIB. In studies of efflux, slices were first preincubated for 40 min in medium containing label, and then transferred to flasks containing Tris-Ringer buffer at 17°. The slices were removed at intervals and the retained radioactivity of the slice measured.

Materials

α-Aminoisobutyric acid-1-14C (specific activity, 4 mC per mmole) and L-proline-14C (uniformly labeled) (specific activity, 200 mC per mmole) were obtained from New England Nuclear. Radiochemical purity was confirmed by one-dimensional ascending chromatography in each of the following solvent systems: butanol-acetic acid-water (12:3:5 by volume); water-saturated phenol; and 2,6-lutidine-water (2:2:1 by volume). Tissue extracts were recovered after incubation with AIB-14C and chromatographed, revealing a single peak for all of the radioactivity in the RF corresponding to AIB. Unlabeled amino acids were obtained from Mann Research Laboratories (chemically pure grade), and their purity confirmed by chromatographic methods.

Uptake of AIB at Low and High Concentrations

The time course for AIB uptake by rat kidney cortex slices was similar to that published by previous authors (16, 17). Ninety per cent or more of total net accumulation of AIB was achieved after 40 min of incubation at initial concentrations of 0.1, 1.0, and 10 mM; this time interval was therefore chosen for all experiments done under steady state conditions. After accounting for uptake by the nonsaturable portion (19), two different transformations of the Michaelis equation were employed to describe the mode of AIB uptake. The customary double reciprocal Lineweaver-Burk analysis (1/u versus 1/[S]) and the Eadie transformation (u versus u/[S]) (Fig. 1) both clearly showed that more than one mode of uptake was used for AIB accumulation. The uncorrected value (1.5 mM) for uptake at low substrate concentrations closely approximated the value obtained by other workers who used similar concentrations of AIB (16, 17). Corrections can be made as usual for the relative contributions to the observed uptake from each of the two systems at any of the given substrate concentrations. Thus

\[ u_{\text{observed}} = u_1 + u_2 \]  

\[ u_1 = \frac{V_{\text{max},1}[S]}{K_{m,1} + S} \]

\[ u_2 = \frac{V_{\text{max},2}[S]}{K_{m,2} + S} \]

The appropriate values were solved by a numerical analysis of the least squares method with the use of 12 sets of experimental values for \( u_{\text{observed}} \) and \( S^2 \). The accuracy of \( K_m \) and \( V_{\text{max}} \) estimates was then verified by substitution of the corrected values into the equations (Table I). It is apparent that the first approximations of \( K_m \) and \( V_{\text{max}} \) values were erroneous (Table II); the corrected values were each higher than the first approximations, but significant differences still existed between them.

The Fortran II program for the analysis of

\[ u_{\text{observed}} = \frac{V_{\text{max},1}[S]}{K_{m,1} + S} + \frac{V_{\text{max},2}[S]}{K_{m,2} + S} \]

was written by Mr. David Slaughter, Med. II, McGill University School of Medicine, and the analysis was performed on an IBM 7044 computer at the McGill Computing Centre.
Inhibition of AIB Uptake by Physical and Chemical Agents

Boiling, freezing plus thawing, and cyanide, each inhibited AIB uptake equally at both low and high concentrations (Table III). Fluoride, dinitrophenol, ouabain, and omission of sodium from the medium, each produced a significantly greater inhibition of AIB accumulation at low concentrations.

Recrystallized iodoacetamide and sodium iodoacetate buffered to pH 7.4, each inhibited AIB uptake more at high concentration than at 0.8 mM.

AIB Uptake in Presence of Other Amino Acids

A second amino acid was added to the incubation medium at 5 times the initial AIB concentration and, after achieving steady state conditions, the uptake of AIB at 0.8 mM or 8.0 mM was compared with uptake by control slices taken from the same rat and incubated for the same period without the second amino acid present at 5 times the concentration of substrate. Values shown are average of three or more determinations.
alanine was about 9 mM when the observed $K_m$ for AIB was 2.8 mM, and 27 mM when the observed $K_m$ value for AIB was 16.5 mM; this dissimilarity would indicate that a single site was not used for AIB uptake (2, 3, 15, 19). L-Leucine and L-lysine were both ineffective inhibitors of AIB.

The imino acid, l-proline, stimulated the uptake of AIB at low concentrations (Table IV and Fig. 3). The effect was characteristic of competitive stimulation (Fig. 3), since the $K_m$ for AIB uptake was decreased without an alteration in the maximum rate of uptake. A second imino acid, 4-hydroxy-l-proline also stimulated net uptake of AIB at low concentrations (Table IV). Both imino acids modestly inhibited the uptake of 8 mM AIB. A time course study indicated that the stimulative effect was present after short term incubation as well as after 40 min of incubation. This suggests that metabolites of proline were not responsible for the stimulation of AIB uptake.

**Interrelations of AIB and L-Proline Uptakes**

**Competition**—Whereas l-proline stimulated the uptake of AIB at low concentrations, AIB was a competitive inhibitor of l-proline uptake at equivalent concentrations (Fig. 4). The $K_m$ for AIB uptake was 1.8 mM, while its $K_i$ value on the l-proline site was 3.3 mM; the $K_m$ value for l-proline uptake on its own site was 2.8 mM, while its $K_i$ value on the AIB system was 4 mM. These values suggest that uptake of proline and AIB is not achieved on a common or identical site.

**Preloading**—Slices were first incubated for 40 min in the presence of unlabeled amino acid (20 mM), then removed, blotted, and transferred to fresh medium containing the labeled substrate amino acid. Accumulation was measured after 10 min of incubation at 37° in Tris-electrolyte buffer, pH 7.4. Control (unloaded) tissues were first incubated in amino acid-free medium.

**Table V**

<table>
<thead>
<tr>
<th>Substrate amino acid and preloaded amino acid</th>
<th>Distribution ratio $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIB (0.8 mM)</td>
<td>Unloaded</td>
</tr>
<tr>
<td>AIB</td>
<td>1.70</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.70</td>
</tr>
<tr>
<td>L-Proline</td>
<td>1.70</td>
</tr>
<tr>
<td>AIB (8.0 mM)</td>
<td>1.50</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.50</td>
</tr>
<tr>
<td>L-Proline</td>
<td>1.50</td>
</tr>
<tr>
<td>l-Proline (0.8 mM)</td>
<td>1.92</td>
</tr>
<tr>
<td>l-Proline (8.0 mM)</td>
<td>1.70</td>
</tr>
</tbody>
</table>

$^a$ Mean of triplicate observations.

$^b$ p value <0.01 when preloaded ratio compared with that for unloaded control slices.

$^c$ Accumulation above control level can be accounted for by net uptake occurring on low $K_m$ system.
loading, the 10-min uptake of the second amino acid was then measured. L-Proline enhanced the uptake of AIB at low concentrations only (Table V); preloading with AIB slightly inhibited L-proline uptake. There was no enhancement of AIB uptake from the medium following preloading with glycine or AIB itself (Table V).

Efflux—Slices were preloaded by incubation for 40 min with AIB at initial concentration of 0.2 and 8 mM. Efflux of AIB into medium with or without added L-proline was then measured; efflux of AIB was also measured when the slices were simultaneously preloaded with L-proline. The addition of L-proline to the medium did not enhance exodus of AIB at either internal concentration of AIB. Simultaneous preloading with L-proline did not inhibit 5 min exodus of AIB, and there was no significant difference in the relative amount of AIB retained after 15 min of efflux at low or high intracellular concentrations in the presence or absence of the preloaded amino acid (Fig. 5). From this one can conclude that L-proline does not produce its stimulative effect on net uptake of AIB at low concentrations by a selective block of AIB at such concentrations.

Effect of Na⁺ on Interaction of L-Proline with AIB Uptake

Omission of sodium from the medium caused a decrease in the rate of AIB uptake (Table III) and concentrative uptake was not observed at initial concentrations of 0.2 to 16 mM (Fig. 6). Addition of L-proline to the sodium depleted medium re-

stored concentrative uptake of low concentrations of AIB (Fig. 6); high concentrations of AIB were not similarly influenced. Hydroxyproline had a similar effect, but glycine or alanine did not (Table VI).

When the cell was first loaded with 20 mM L-proline in the presence of sodium for 40 min, the 10-min uptake of 0.8 mM AIB at 37°C in Tris-electrolyte buffer in which the sodium was replaced by choline and compared with uptake in the presence of other amino acids (4.0 and 40 mM, respectively).

<table>
<thead>
<tr>
<th>Amino acid added to medium</th>
<th>Uptake⁵</th>
<th>0.8 mM AIB</th>
<th>8.0 mM AIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nothing*</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>L-Proline</td>
<td>115⁶</td>
<td>87⁴</td>
<td></td>
</tr>
<tr>
<td>Hydroxy-L-proline</td>
<td>146⁶</td>
<td>107⁴</td>
<td></td>
</tr>
<tr>
<td>L-Alanine</td>
<td>94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>93</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

⁵ Mean of three or more paired observations.
⁶ Effect of withdrawal of Na⁺ is itself significant (Table II). Results are expressed in relation to uptake from sodium depleted medium without second amino acid.

p < 0.01 when compared with uptake by control slices.

p < 0.01 when compared with uptake on low K₉ system.

Fig. 6. The effect of omission of Na⁺ from the medium on saturation kinetics for AIB uptake in the absence (●) and presence (○) of L-proline at low (left) and high (right) initial medium concentrations of AIB. Removal of Na⁺ (Na⁺O) results in nonconcentrative uptake of AIB by both systems. Concentrative entry of AIB is restored on the low K₉ system by addition of 4 mM L-proline to the initial medium; the resultant K₉ for AIB uptake (first approximation, 1.5 mM) is comparable to that obtained in the presence of the normal medium Na⁺ concentration without added L-proline (see Fig. 1). u and S are as defined in legend to Fig. 1.
DISCUSSION

The net uptake of AIB by rat kidney cortex slices is concentration-dependent. Time course studies indicate that steady state conditions are established in about 40 min. Both observations confirm earlier work (16, 17). The need for more than one $K_m$ value to describe the uptake of AIB is a new finding. The first approximation of the lower $K_m$ value corresponds closely to previous estimates of that value for this tissue (16, 17); the first approximation of the second $K_m$ value describes another mode for AIB transport which is operative at higher concentrations. A more accurate estimate of the $K_m$ values was obtained by accounting for the relative contribution of each system, independent of the other, to the total observed uptake.

The $K_m$ value obtained under steady state conditions is thought to be valid also for uptake under initial rate conditions (18). In the present investigation, the estimations of the $K_m$ values under both types of incubation condition compared favorably. Thus, there are consistent indications for heterogeneity of AIB uptake, while remaining aware that more than one $K_m$ value is not in itself proof of such heterogeneity (9, 15).

Green and Rosenberg (7) assigned AIB uptake to the alanine-preferring mediation of the mouse Ehrlich ascites tumor cell, and Inui and Christensen (9) further confirmed the existence of only one apparent mode for mediated uptake of AIB in that tissue over a concentration range from 1 to 32 mM. Finerman and Rosenberg (14) found more than one mode of concentrative transport for AIB uptake in bone; that study and the present one illustrate that characteristics of transport may apparently be specific for the tissue as well as for the conditions of the experiment.

Quantitative and qualitative differences in the transport of the substrate at widely differing concentrations should be identifiable if more than one mode of uptake is used (15). We found that inhibitors acting predominantly through sodium-dependent mechanisms were more effective against uptake at low AIB concentrations, while an inhibitor of —SH-dependent mechanisms impaired AIB uptake more at high substrate concentrations. We also observed that L-alanine interacts with AIB during uptake in a manner suggesting that a portion of AIB accumulation is achieved in kidney by an alanine-preferring system (7). However, the interaction between L-alanine and AIB over a wide concentration range was such as to indicate that more than one agency serves AIB transport in this tissue.

The effect of L-proline on AIB accumulation provided strong qualitative evidence for a heterogeneous mode of AIB uptake. This imino acid over a wide range of concentration, as well as its 4-hydroxy analogue, enhanced the net uptake of AIB at low substrate concentrations, whereas it was an inhibitor of AIB uptake at higher substrate concentrations. The initial uptake of low external concentrations of AIB was also enhanced if L-proline was first placed internally. The stimulatory effect of proline could not be attributed to selective impairment of AIB-exodus from the cell. Omission of sodium from the external medium, a procedure which impairs influx of AIB into kidney slices by a saturable mediation (17), inhibited AIB transport; this effect was, however, offset by the addition of L-proline externally to the Na⁺-free incubation medium, but not if the imino acid was first placed internally. It is thus implied that the interaction of proline with AIB during uptake at low concentrations occurs at the exterior of the cell and in association with influx of substrate.

It has been proposed that Na⁺ participates in the transport process as the ternary complex Na⁺ES (9), where E represents the carrier, and ES is the transient carrier-substrate complex. Although the Michaelis constants indicate that proline and AIB do not share an identical site during uptake at low solute concentrations, L-proline may have a relationship to AIB binding during influx analogous to the relationship proposed for sodium ion. In this sense, AIB may use a site at low concentrations which is part of, or adjacent to, but not identical with, the reactive site for transport of proline. Hydroxy-L-proline can substitute for L-proline in the stimulation of AIB uptake, whereas glycine cannot, even though the imino acids and glycine are believed to share a selective common transport mediation in kidney (1–3, 20, 21). This apparent discrepancy can be explained if there is more than one component of uptake for the imino acids and glycine (4). At high concentrations the three substrates share a common mediation, whereas at low concentrations glycine is apparently transported by a system which can be discriminated from another agency serving the imino acids (4). Thus imino acid-AIB interaction need not involve glycine when an agency predominantly operative at low substrate concentrations is being tested.

Combination of two molecules of amino acid with a single molecule of the transport agency has been suggested to explain certain peculiarities of proline uptake in pancreas (29), and of tryptophan uptake by ascites tumor cells (23). A 1:2 carrier-amino acid combination has been considered (22, 23), and also an exchange mechanism (7, 24), in explanation of the phenomenon of competitive stimulation. For instance, the stimulatory effect of methionine on tryptophan transport (24) in Ehrlich cells could be accounted for by rapid uptake of methionine by one system and exchange counterflow with tryptophan on another system. Under the conditions of the present investigation, “exchange” of internal proline with external AIB is inhibited when Na⁺ is omitted from the medium, but stimulation of AIB accumulation by external proline is not. The loss of exchange was not an artifact of the prior loading of L-proline since this was performed in the presence of sodium and only the exchange reaction was performed in a sodium-free medium. Moreover, it has been shown that true exchange systems for neutral amino acids function independent of the sodium concentration in the medium (25, 26). Thus we concluded that the stimulatory effect of proline on AIB uptake is unlikely to occur by an exchange mechanism. In addition, uptake of imino acids is apparently Na⁺ sensitive (14), and, thus, as postulated (7, 24), omission of Na⁺ should inhibit stimulatory phenomena if they are dependent on an exchange process. Thus the relationship of sodium and cosubstrate, with respect to AIB transport at low concentrations, suggested to us the explanation which we have advanced concerning an interaction at the outer surface of the membrane. Apparently this proposed phenomenon is characteristic of only certain membrane sites, and the other mode of AIB uptake, which was postulated to account for the accumulation of AIB at higher concentrations, does not subscribe to these particular conditions. In this sense these descriptions of AIB uptake in kidney confirm the impression which has been continuously sustained by others (27) that the macromolecular basis of membrane transport is highly selective and elaborately organized.

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