INDICATIONS OF SPATIAL RELATIONS AMONG STRUCTURES RECOGNIZING AMINO ACIDS AND Na^+ AT A TRANSPORT RECEPTOR SITE*

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SUMMARY: The orientation of the hydroxyl group on carbons 3 or 4 of proline, whether *cis* or *trans*, is decisive to the interaction between the amino acid and Na^+ for a Na^+-linked transport system of the pigeon erythrocyte and the rabbit reticulocyte. Consideration of this response in relation to other strong effects of the position of the hydroxyl group in two transport systems suggests that at the transport receptor site under study, the two substrates bind in juxtaposition, Na^+ *trans* to the carboxyl group of the amino acid.

Recently we described for 3 cell types how structural features of amino acid sidechains report their nearness to the point of Na^+-binding, both when the amino acid and Na^+ react together with System *Ly*$^+$ for cationic amino acids[1-4], and when they act as cosubstrates for transport by System *ASC*[2,5], which serves for neutral amino acids[6,7]. The first case contributes the following points to the argument:

1. A structure recognizing the distal cationic group of basic amino acids is demonstrated by the substrate specificity of System *Ly*$^+$ [8,9].

2. Neutral amino acids strongly inhibit uptake of cationic amino acids only when a monovalent cation such as Na^+ or Li^+ is present; external Na^+ and the neutral amino acid exchange together for the previously accumulated cationic amino acid.

3. As the chain length of the neutral amino acid is varied, the interaction with the monovalent cation passes through an optimum at 5 carbons. The interaction is strongly enhanced by introduction of a terminal hydroxyl or sulfhydryl group, or if the terminal carbon is part of a carboxamide group. The appropriately positioned groups enhance Na^+ reactivity, and Na^+ in turn enhances reactivity of the amino acid bearing them as expressed by K_i values.

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The action of a hydroxyl group is negligible when it is on carbon 3, and small when it is on carbon 6[4].

These spatial requirements for the reactivity of the combination of a neutral amino acid and a monovalent cation with System L^{+}_{y} led us to conclude that Na^{+} occupies the position otherwise taken by the distal cationic group of the basic amino acid[1-4]. Similar behavior was then observed for System *ASC*:

1. L-Arginine shows a strong Na^{+}-independent inhibition of the linked uptake of neutral amino acid and Na^{+} (Table I). The action of lysine is weaker but definite. The cationic amino acids have no detectable uptake by way of System *ASC*, and they do not stimulate alanine exodus.

2. Guanidine and methylguanidine cations compete with Na^{+} in both systems. These cations are more effective than ammonium ion, consistent with the contrast between arginine and lysine, as shown in Table I. The inhibitory action shown by the cationic amino acids (left) is quite unlike that shown by the monovalent cations (right), in that the K_i values of only the latter depend on the Na^{+} concentration used to generate the inhibitable rate of serine uptake. None of these cations replaces Na^{+} in stimulating serine uptake.

3. Mutual enhancement of transport reactivity between neutral amino acid and Na^{+} is observed to depend on chain length and the position of polar groups. The optimal chain length for the neutral amino acid in the 3 cell-types is one carbon less than that for System L^{+}_{y} of the Ehrlich cell. Enhancement of reactivity by the hydroxyl group is optimal when the hydroxyl group is positioned on carbon 4, and slightly weaker when on carbon 3. (The optimal distance for System L^{+}_{y} is not so distinctly greater than that for *ASC* in the rabbit reticulocyte or the pigeon erythrocyte[2,5].) As chain length is increased, a transition occurs from an increasing, Na^{+}-dependent substrate action to a Na^{+}-independent inhibition of transport[5,6].

These surprising parallelisms between Systems *ASC* and L^{+}_{y} seem to us to mean that a cation-binding component of both receptor sites can be occupied alternatively by the distal cationic group of a basic amino acid, the α amino and

Table I. *BASIC AMINO ACIDS AND OTHER CATIONS AS INHIBITORS OF SYSTEM ASC IN THE PIGEON RED BLOOD CELL.* On the left, the uptake of serine, 0.02 to 0.8 mM, was observed from Krebs-Ringer phosphate solution, $[Na^+]$ = 93 or 118 mN. On the right, the uptake of serine, 0.05 mM, was observed at $[Na^+]$ = 10 mN and [choline$^+$] = 145 or 70 mN. L Isomers were used except where indicated to the contrary. See text for caution about the difference in the nature of the action of simple cations and that of cationic amino acids.

Basic amino acid	K_i, mM	Monovalent cation (75 mN)	% Inhibition
α-amino-β-guanidino-propionic acid	8	Li^+	48
arginine	8	K^+	14
canavanine	5-6	NH_4^+	21
homoarginine	9-10	guanidinium$^+$	41*
ornithine	85	methylguanidinium$^+$	30
lysine	85		
D-arginine	18		

* The constrast between NH_4^+ and the guanidinium ion was even more pronounced at higher levels of Na^+. For example at 75 mM of these two cations, inhibition was 1% and 30%, respectively, $[Na^+]$ being 25 mN and [choline$^+$], 55 mN.

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carboxyl groups being fixed in a normal manner, or by a suitable simple cation. When that cation is Na^+, and a neutral amino acid completes occupation of the site, transport of both takes place. Accordingly we suppose that the sharp specificity as to the position of the hydroxyl or similar group on the sidechain serves *in both systems* to tell us where the cation-recognizing component of the site lies in space, and therefore where the Na^+ is bound. The Na^+-bonding possibilities of the unshared electrons of oxygen or sulfur lead us to picture not only juxtaposition but bonding between the two bound cosubstrates.

We are now able to report further instances where mutual interaction with Na^+ is enhanced in clear response to the positioning in space of the hydroxyl group. These results persuade us that Na^+ takes a position *trans* to that taken by the carboxyl group at Site *ASC* of the pigeon erythrocyte and the rabbit reticulocyte. Table II shows two measures of substrate action, 1) stimulation of

Table II. *COMPARISON OF REACTIVITIES OF ALLO AND THREO AMINO ACIDS WITH SYSTEM ASC**. The procedures have been described elsewhere[4,7,8]. The cells were brought to apparent internal levels of ^{14}C-alanine of 0.7 to 1.4 mM per kg cell water by 30-min incubation. Decline of ^{14}C content was then observed during 5 (for threonine) or 10 min at 37° at 20 mM external levels of the test amino acids as listed. Inhibition of uptake of ^{14}C-serine (0.02 - 0.8 mM) from the suspending medium was observed during 3-min intervals. The K_i values for DL amino acids were halved to obtain approximate values for the L isomers.

	Pigeon Red Blood Cells				Rabbit Reticulocytes
Amino or inimo acid	Stimulation of alanine exodus $[Na^+]_o$=118 mN	K_i at $[Na^+]_o$= 25 mN	K_i at $[Na^+]_o$= 118 mN	$K_{i_{25}}/K_{i_{118}}$	Stimulation of alanine exodus $[Na^+]_o$=118 mN
	%	mM	mM		%
L-proline	15.7	2.0	1.8	1.1	8.2
DL-3-*cis* hydroxyproline+	0.6	11	11	1.0	**
DL-3-*trans* hydroxyproline+	14.9	0.08	0.07	1.1	**
L-4-*cis* hydroxyproline	3.8	2.0	2.0	1.0	2.0
L-4-*trans* hydroxyproline	30.7	0.27	0.16	1.7	13.3
L-*allo*-free threonine	24.1	0.22	0.085	2.6	**
DL-*allo*-free threonine	22.6	0.27	0.10	2.7	**
DL-*allo* threonine	22.1	0.24	0.10	2.4	**

* The external presence of L-pipecolic acid stimulated alanine exodus during 10 min by only 1%, whereas L-5-*trans* hydroxypipecolic acid caused a stimulation of 4%. + Generou provided by Dr. James Ogle, Department of Biochemistry, University of Cincinnati-Colleg of Medicine. ** Not determined.

exodus of previously accumulated alanine, and 2) decrease of K_m for uptake (or of K_i, measuring inhibition of serine uptake) produced by increasing external $[Na^+]$ from 25 to 118 mN. In addition the value of K_i *per se* indicates the apparent affinity of the amino acid for Site *ASC*.

Comparison with earlier results[5] shows that proline (Table II) is a poor substrate compared with the analogous norvaline, probably in part because of crowding by the C-5-methylene group attached to the imino N. We may recall that the N-methyl amino acids tested do not react with System *ASC*[6]. Removal of one or more methylene groups from access to optimal apolar bonding as a result of

the cyclic character of proline may also contribute. Introduction of a hydroxyl group at C3 or C4 (particularly the former) decreases reactivity, but only if the orientation is *cis*. We may consider two possible origins of the large effect of the orientation of the hydroxyl group on the proline molecule:

1. A *cis* orientation may prevent apolar bonding to the *cis* aspect of the carbon atom concerned. Consider that alanine, like serine, is an enormously better substrate for System *ASC* than glycine; the high Na^+-sensitivity of its uptake (similar to that of threonine in Table II) suggests that a response to the α-methyl group, perhaps apolar bonding, assists in preparing the recognition site for Na^+. Conversely, the fixation of Na^+ appears to enhance alanine binding despite the absence of any structure on the alanine sidechain likely to react with Na^+. Similar interactions might attend apolar bonding to the *cis* aspect of carbon 3 of proline, unless prevented by a 3-*cis* hydroxyl group, which according to both our tests does lower the reactivity of proline (Table II).

That explanation does not, however, appear sufficient. First of all, the reactivity of proline in contrast to that of alanine is only moderately increased by elevating $[Na^+]$. Secondly, the introduction of the 3-*trans* hydroxyl group does enhance proline reactivity as measured by K_i values in Table II. Finally, introduction of the 4-*trans* hydroxyl group strongly enhances proline reactivity as measured by both our tests.

2. A *trans* orientation may place the hydroxyl group in juxtaposition to a structure to which it bonds. By the evidence traced already, this structure, associated spatially with the cation-recognizing locus, may well be Na^+.

For threonine, in contrast, the transport system discriminates little if at all as to the configuration at C3, presumably because the hydroxyl group of either isomer is free to take a *trans* position.

Figure 1 shows a diagrammatic visualization suggested for the *ASC* site. Four recognition components are included: 1) one marked +, for the α-carboxyl group, 2) another marked -, for the α-amino or -imino group, 3) a third for apolar mass, evidently exerted on most of the methylene and methyl groups of the

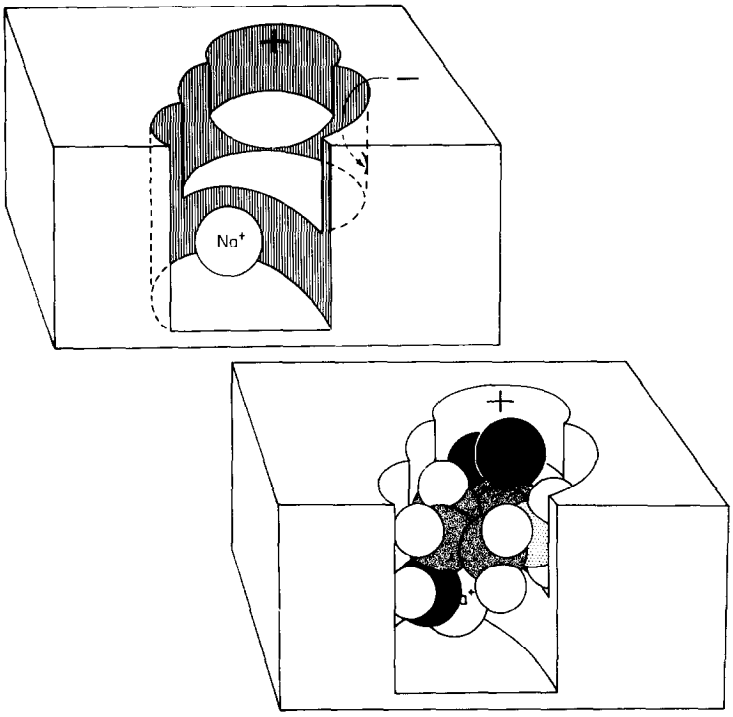

Fig. 1. *Diagrammatic representation proposed for transport site ASC, showing Na^+ (top) and both Na^+ and hydroxyproline (bottom) in suggested relative positions.* Undoubtedly the receptor structure to some degree mediates the interaction between the two cosubstrates. Structural considerations persuade us, however, to propose juxtaposition and thus a direct interaction between the two, as suggested by the lower sketch. No implication is intended that Na^+ must enter the site first. See text for further discussion.

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usual substrates, and 4) a fourth, for Na^+, whereby mutual interaction occurs favorable to the reactivity both of Na^+ and the amino acid as expressed by their K_m or K_i values. Features that the model seeks to illustrate besides access to apolar bonding and the *trans* relationship between the points recognizing the carboxyl group and Na^+ are: insufficiency of vertical space for an α-methyl group and also of space for an N-methyl group; and sufficiency of space for the sidechains of valine and leucine, the ß-methyl group of the former presumably taking a position unfavorable to Na^+ binding. Not shown is a feature causing methionine and norleucine to exert a Na^+-independent inhibition of *ASC* transport, not associated with their own transport[5]. We suppose that additional apolar bonding in a region distal to the Na^+-recognizing area (perhaps as re-

presented by the floor in the immediate foreground) causes these amino acids to be bound more stably *without* than *with* Na^+ included in the complex. Also not shown is an obstacle causing a much higher K_i when the chain is longer than those of methionine and norleucine.

We must recognize that an entirely static description of this site is not fully justified by the above results, since the transport system does not meet the equilibrium assumption. The coupling ratio between the fluxes of Na^+ and the amino acid usually exceeds one in this system, and depends on substrate structure[10], rising on introduction of a hydroxyl group on the carbon atoms already designated. For example, introducing the 4-*trans* hydroxyl group raises this ratio for proline from 0.2 to 3.0, a finding that also supports interaction between the hydroxyl group and Na^+. V_{max} also depends on amino acid structure[6]. Hence the geometry of the sites may already have begun to respond to the substrates before the associative steps are completed, as suggested above for alanine.

Besides suggesting the position in space taken by the cosubstrate Na^+, these results are, we believe, the first demonstration of the way in which a second center of asymmetry, other than the α-carbon, may determine reactivity of an amino acid with a specific transport system. Alkali-metal binding may perhaps assist discrimination of the position of the hydroxyl group at enzymatic as well as at transport-mediating sites, although we do not see such discriminations as the principal meaning of the widespread participation of Na^+ in transport.

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