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SPECIAL TRANSPORT AND NEUROLOGICAL SIGNIFICANCE OF TWO AMINO ACIDS IN A CONFIGURATION CONVENTIONALLY DESIGNATED AS D

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Summary

We point out an ability of certain amino acids to be recognized at a biological receptor site as though their amino group bore, instead of an α relationship to a carboxylate group, a β, γ or δ relationship to the same or a second carboxylate group. For aspartate, the unbalanced position of its amino group between a pair of carboxylates allows its occasional biorecognition as a β- rather than as an α-amino acid, whereas for proline and its homologs, their cyclic arrangement may allow the imino group, without its being replicated, to be sensed analogously as falling at either of two distances from the single carboxylate group. The greater separation might allow proline to be seen as biologically analogous to γ-aminobutyric acid. This more remote positioning of the imino group would allow the D-form of both amino acids to present its amino group in the orientation characteristic of the natural L-form. The dual modes of recognition should accordingly be signalled by what appears to be low stereospecificity, actually due to a distinction in the enantiorecognition of the two isomers. Competing recognition for transport between their respective D- and L-forms, although it does not prove that phenomenon, has been shown for proline and, significantly, even more strongly for its lower homolog, 2-azetidine carboxylate. Such indications have so far revealed themselves rather inconspicuously for the central nervous system binding of proline, reviewed here as a possible feature of a role suspected for proline in neurotransmission.

Introduction

Traditionally, we tend to count too strongly on a sharp discrimination between the two enantiomorphs of each amino acid in favor of what we see by convention as its L-isomer. In doing so, a few cases of presumably unrelated recognition of the two isomers may be overlooked. For example, D-aspartate residues enter the stable proteins of tooth enamel and cerebral white matter, and proline is one of the D-amino acids found in kidney and serum extracts of mutant mice lacking D-amino acid oxidase (see Christensen, 1992). Furthermore, unexpectedly weak or even reversed discrimination between the two isomers may be overlooked. A relative weakness in stereoselection is rare in enzyme

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action, but is much more typical for membrane transport of amino acids (Oxender and Christensen, 1963).

In this paper, we review results accumulated over the past several decades together with some speculation that may stimulate investigations of a possible biological role for D-forms of amino acids. Elsewhere in this volume (Christensen *et al.* 1994; MacLeod *et al*. 1994), details of the properties of amino acid transport systems are presented. Several reviews and recent books describe the characteristics of a variety of transport systems in some detail (Kilberg and Häussinger, 1992).

In general, transport systems do not destabilize their substrates, a defining difference from enzymes, and they show a lower substrate specificity than do enzymes. The acceptance of D-isomers may not, however, reflect a weakness of stereoselection, but instead two concurrent modes of enantiorecognition. In the recognition process, the amino group of the D-isomer may be seen not as to the usual carboxylate group, but as β, γ or δ to a carboxylate group situated as a structural feature of that amino acid. Examples of that viewpoint are discussed in the following sections.

Competition of D- and L-aspartate in transport

Aspartate illustrates this idea through its possession of two nearby carboxylate groups, the amino group lying α to the first, and β to the second, carboxylate group. For example, transport system X_{AG}^{-}, the Na^{+}-dependent transport system for aspartate and glutamate (Fig. 1), can bind aspartate using the β about as often as the α carboxylate group (Gazzola *et al.* 1981). To assist the reader in visualizing the probable structural explanation for this behavior, see Fig. 2 and select a viewpoint first at one and then at the other carboxylate group of aspartate. One then sees the amino group shift its protrusion, from left to right, or *vice versa*, according to which carboxylate group was selected as the viewpoint.

Suppose that we momentarily identify aspartate as a β-amino acid as defined by transporter acceptance of the carboxylate group lying β to the amino group (Figs 1 and 2). Then the form conventionally designated as D can be considered, for the moment, to have an L configuration and hence to serve logically as a substrate for that site. Accordingly, D-aspartate competes strongly with L-aspartate for transport (Gazzola *et al.* 1981), as it does in certain other biological events. For glutamate transport *by that same system*, the larger γ-separation of its distal carboxyl group from the amino group precludes competition by the D-isomer with the L-isomer. The prochiral glutamate analog 3-aminoglutarate also competes for acceptance by the same site, confirming that the β separation is indeed acceptable (Gazzola *et al.* 1981). When the distal anionic group is the sulfonate or sulfinate group, as for the analogous cysteate or cysteine sulfinate, the D-isomers are not inhibitors. This result shows that the transport recognition site does not recognize the sulfonate group at the β-position as though it were a carboxylate and, hence, does not recognize the two anionic groups of aspartate by precisely the same chemistry (Gazzola *et al.* 1981).

Pall (1970) had earlier found that the uptake of L-cysteate was inhibited more strongly by D-aspartate than by L-aspartate in *Neurospora crassa*. His classical experiments were made at pH 6, which ensures that the anionic forms were the actual reactants.

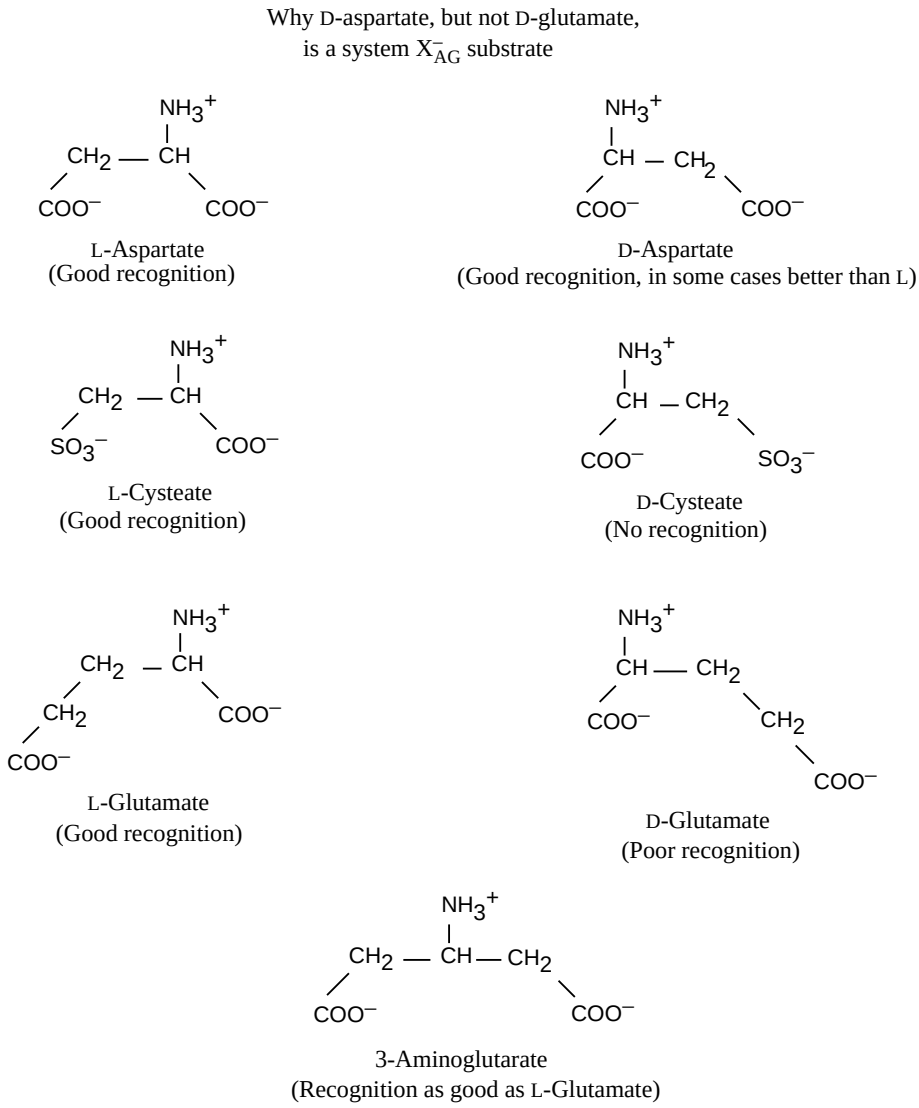

Fig. 1. Both the enantiomorphs of aspartate are accepted by transport system X^-_{AG}, whereas only the L-form of glutamate is accepted. The acceptance of 3-aminoglutarate reveals that the separation shown by a β-carboxyl group is acceptable, whereas that for the carboxyl group of glutamate is too great. L-Cysteate has been shown to inhibit aspartate uptake, although D-cysteate does not. Reproduced with permission from Gazzola *et al.* (1981).

Furthermore, the high affinity shown by the strongly acidic cysteate supported the same conclusion. D-Glutamate was less inhibitory than its L-enantiomorph, but only moderately so.

In a strongly contrasting study, Garcia-Sancho *et al.* (1977) discovered a related anomaly in the Ehrlich cell at pH 4.2, but one applying to the transport by system L of

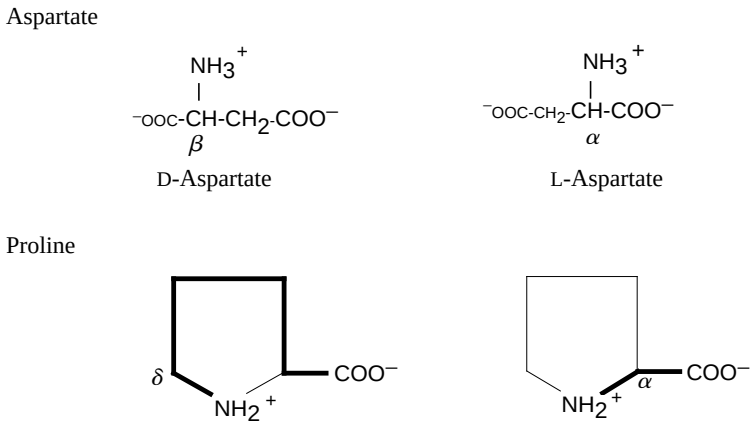

Fig. 2. Consequences proposed for the biorecognition of alternative intervals of separation of two critical groups on certain amino acid molecules. If the separation marked leads to the usual preference for the L-isomer, then the one marked with one of the subsequent Greek letters β, γ or δ should lead to a preference for the D-isomer, given that no change occurs in the geometry of the receptor site that recognizes the relationship between these two groups. Note that, at least for proline, a third intermediate point of recognition, presumably apolar bonding to more than one methylene group, may be necessary to the sensing of a preferred greater separation between the two charged groups.

aspartic acid, not aspartate, and to the D-isomer as well as the L-isomer. The discrimination against D-glutamate was much stronger. It is striking that the same explanation can serve for the stereoselective anomaly in two dissimilar transport systems. Parallel differences in the biological recognition of the two enantiomorphic pairs, aspartate and glutamate, are important in neurology. The acceptance of D-glutamate, even if rarer, is surely also important.

The case illustrated for aspartate transport may lead the reader to consider the parallel importance of a γ position for the amino group with respect to the carboxylate. This is illustrated in neurochemistry by the biological functions of γ-aminobutyric acid (GABA), by studies of the natural and artificial analogs of GABA and their biorecognition and potential pharmacological action. When these analogs are chiral, optical configuration again plays a role, although of course not for the prochiral GABA molecule itself.

Proline and its homologs

Heterocyclic amino acids, such as proline, might also be recognized in either of two ways, according to which of the two separations between the single imino group and the single carboxylate group is selected biologically. The imino group of proline would present two different recognition signals through these two different distances of separation from the single carboxylate group (Fig. 2). By changing our perspective, we see the chain of carbon atoms wending its longer, alternative course from the carboxylate group to the nitrogen atom. Thus, when the unchanged L-proline is pictured as a δ- rather

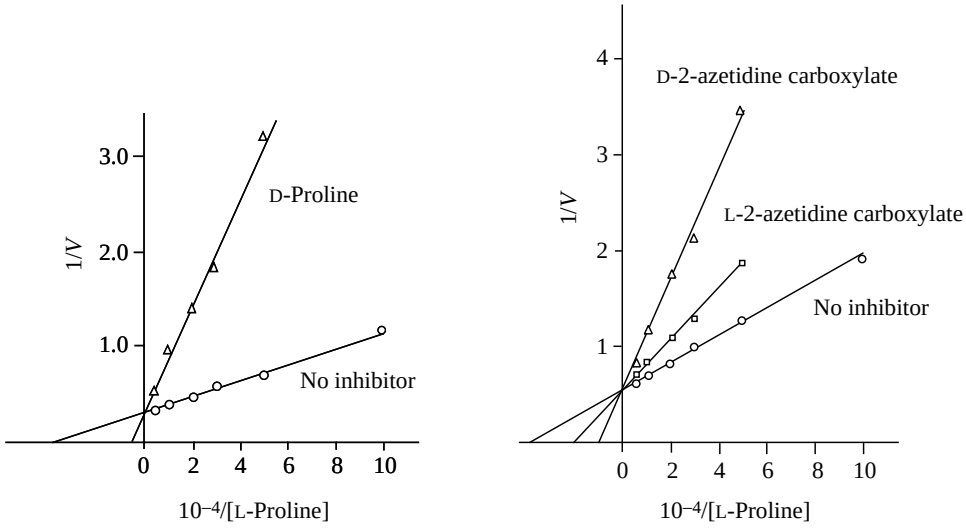

Fig. 3. Proline transport into the yeast *Saccharomyces chevalieri* at 30 °C from a galactose/potassium phosphate medium, pH 5.5. Left, effect of external L-proline concentration on inhibition of 5 min L-proline uptake (V), apparent K_m 25 μmol l^{-1}, by D-proline, K_i=89 μmol l^{-1}. Right, comparison of the competitive inhibition of the 5 min uptake of L-proline by D- and L-azetidine carboxylate. The results correspond to K_i values of 200 μmol l^{-1} for the L-and 77 μmol l^{-1} for the D-analog. See text for discussion. Adapted from Figs 5 and 8 of Magaña-Schwencke and Schwencke (1969).

than as an α-imino acid, in that unfamiliar and totally inappropriate frame of reference, it might be seen as a D-imino acid. Ambiguity can be minimized by adhering to L-glyceraldehyde as our standard for assigning the D-configuration.

Proline, perceived as a δ-amino acid, may therefore bear a structural relationship to GABA and its homologs. The four-carbon 2-azetidine carboxylate is one step closer to GABA (see Fig. 4). These features of the proline molecule may prove pertinent to the problem of a suspected function for proline in neurotransmission, a problem now accepted as an important challenge of neurobiology (see Bennett *et al.* 1976). The evidence for such a role has been vigorously reviewed by Fremeau *et al.* (1992), who noted that exogenously loaded L-proline is released in a Ca^{2+}-dependent manner following K^+-induced depolarization. Numerous ordinary amino acids do not share in this behavior.

A 4.0 kb cDNA construct was isolated from putative glutamatergic neurons derived from several regions of the rat brain (Fremeau *et al.* 1992). Transient expression of the cognate cDNA unexpectedly conferred to HeLa cells the properties of a *de novo* high-affinity Na^+-dependent L-proline transporter. This cDNA predicts a 637-residue protein with 12 putative transmembrane domains and exhibits 44–45 % sequence identity with other neurotransmitter transporters. The expression of this transporter in subpopulations of putative glutamatergic pathways was taken to 'support a specific role for L-proline in specific excitatory pathways in the CNS', one not yet understood. The expression of this

transporter in the selected central nervous system (CNS) tissues gives special significance to cases showing exceptionally low biological stereospecificity to proline. Fig. 3 recalls a notable case from a quarter of a century ago in *Saccharomyces chevalieri*, which shows a strong competitive inhibition of L-proline uptake by D-proline with 28% of the affinity shown for the L-isomer (Magaña-Schwencke and Schwencke, 1969).

Admittedly, such low transport stereospecifity is not unusual. For example, the Ehrlich cell shows only a 4:1 preference for uptake of methionine in its L-form over the D-form (Oxender, 1965). Indeed, preferences not exceeding a single order of magnitude for mediated transport of ordinary dipolar L-amino acids over that of their D-isomers are quite usual in mammalian tissue (Oxender and Christensen, 1963). No basis has been noted for suspecting that the two enantiomorphs are transported by different means.

Our conclusion that the yeast transport system has highly unusual stereospecificity for the transport of proline is further justified by the results with its lower homolog, the α-imino acid 2-azetidine carboxylate (Fig. 3). The D-isomer proved to be a much stronger competitive inhibitor of proline transport than the L-isomer, a remarkable and unexplained finding highly supportive to our hypothesis. Fig. 3 also indicates that the greater separation of the imino from the carboxylate group is critical to the degree of stereospecificity, lending support to this argument. It seems unlikely that this flexible recognition of the imino acid stereoisomers is exclusively a property of yeast. 2-Azetidine carboxylate is widely distributed among plants and it is not toxic to mammals, so it may have a biological function (Romeo, 1989).

Proline might be viewed as a potential biological analog to GABA, and to GABA's lower homolog, β-alanine. Interest in the membrane transport of β-alanine has intensified greatly, particularly within the CNS and the intestine (Thwaites *et al.* 1993; Munck and Munck, 1992). *N*-Monomethyl derivatives of GABA and β-alanine inhibit GABA functions (Curtis *et al.* 1961), suggesting that these artificial alkylimino acids have some GABA-like neurological properties. For the intestinal imino acid transport system, the imino acid 2-(methylamino)isobutyrate serves as a model substrate in the rabbit (Munck, 1993). Note that the N-methylation to convert an amino acid analog into its imino form has been useful for limiting its transport to a selected system (Christensen *et al.* 1965).

A finding that L-proline is, as anticipated, the form accepted by a given receptor site does not prove whether that site makes this choice by recognizing the α-imino or instead the δ-imino feature or even by recognizing the two together. Instead, a choice for D-proline could arise from receptor possession of the opposite pair of alternative capabilities, and the degree to which one of these isomers is preferred, as in Fig. 3, may point quantitatively to the dominant basis of biorecognition of each substrate or substrate analog. Note, however, that if two separate enantioselectivities serve the two proposed receptor positions, they cannot be occupied simultaneously by two enantiomers. Therefore, the usual signal for shared mediation of transport by enantiomers, namely competition between them, will persist and the two will compete for binding at a receptor site rendered common by overlap of the two substrates noted previously. This means that the usual kinetic test for differentiating the two modes of stereoisomer recognition are not available. Their discrimination should, however, prove sensitive to differences in the

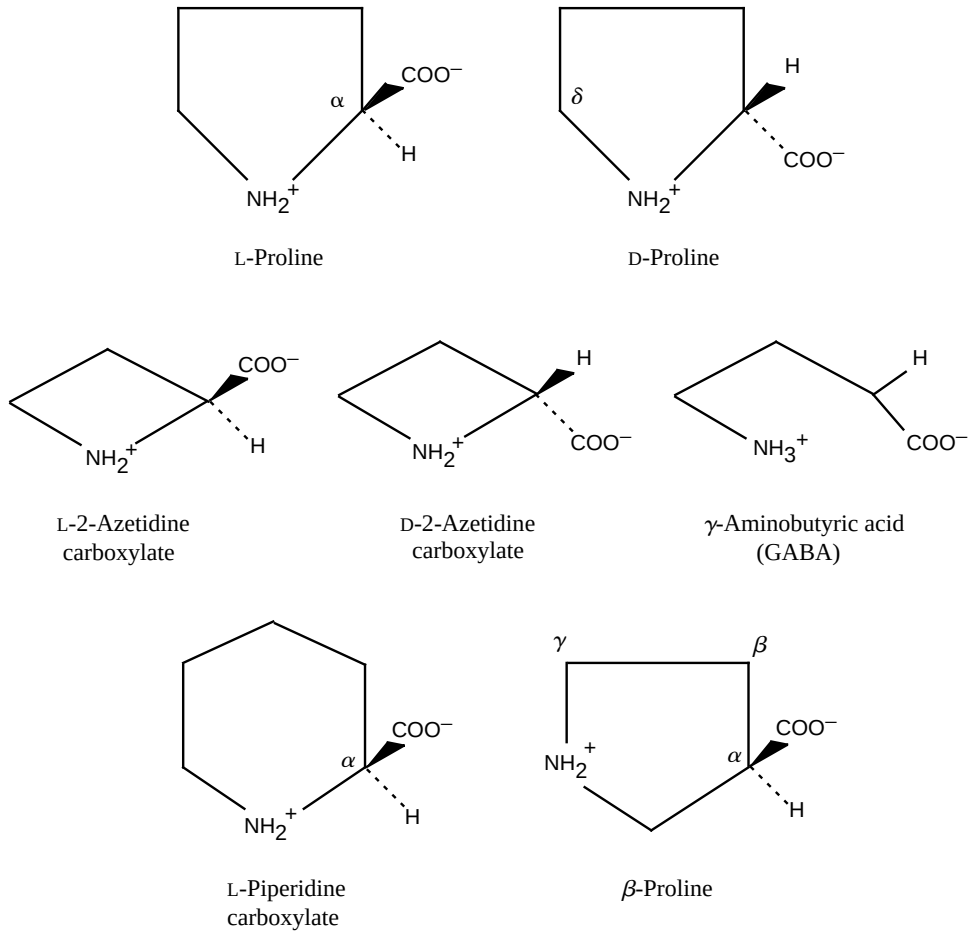

Fig. 4. Structural representations of proline and some of its close analogs. Being limited to two dimensions, these diagrams seek only in an arbitrary manner to contrast the enantiomorphs.

span of carbon atoms recognized as separating the charged groups. Here, the difference in span is three carbon atoms for proline (Fig. 2) and only two carbon atoms for 2-azetidine carboxylate.

Dual enantioselectivity in CNS proline recognition?

An important advantage in the recommended search for possible dual enantioselectivity in the biorecognition of the proline isomers may arise from the contrasting ranges of specificity shown among transport systems for this amino acid, as illustrated in Table 1. These comparisons among transport systems point to strong, possibly associated differences in the mode of recognition of proline, its homologs and their stereoisomers. For example, the highly restricted structural requirements for proline recognition by the IMINO system, accepting its higher but not its lower homolog, is associated with a very strong

Table 1. *Variety in substrate affinity ranges among several proline transport systems*

System and source	Na^+-dependency	Stronger competitors
IMINO, intestinal brush-border, rabbit; Stevens and Wright (1985)	Yes	Only L-pipecolate, not AZTC
Yeast; Mangaña-Schwencke (1969)	No; hardly likely for yeast	D-AZTC>L-AZTC, not pipecolate; L-Pro⩾D-Pro
Human enterocyte, $Caco_2$ in culture; Nicklin *et al.* (1992)	Yes	% inhibition L-Pro uptake: L-Pro 68%, D-Pro 37%
Synaptic membrane; cf. Greene *et al.* (1986); Fremeau *et al.* (1992)	No	L-pipecolate; L-AZTC>D-AZTC, but both weak

AZTC, 2-azetidine carboxylate, 4-carbon homolog of proline.

preference for the L-isomer of proline. In contrast, relatively lower general specificity of the yeast system, accompanied with remarkably high acceptance of the D-isomers, is associated with the opposite strong preference for the lower homolog of proline. The contrasts illustrated in Table 1 show that searchers are likely to encounter in the CNS not just one but a number of chemically distinct proline binding sites at which to look for enantioselectivity. Furthermore, associated clues may guide them towards sites more likely to show this trait. Analogs not included in Table 1, such as β-proline (DeFeudis *et al.* 1980; Fles and Ghyczy, 1964) might offer further clues as to whether and where this trait occurs.

Table 1 suggests also that the search should not exclude transport systems that are independent of any inorganic ion, whether of high or intermediate affinity, nor those expressed predominantly in non-neural tissues. For example, an elegant study of glutamate transport into various fibroblasts in primary culture surprised its neurobiologist authors with high-affinity K_m values of 5–20 μmol l^{-1} (Balcar *et al.* 1994). The search should take advantage of all the ingenuity so far attained in discriminating even minor components of amino acid binding.

Discussion

An unanticipated alternative frame of biological reference can alter or reverse our judgement as to the mode in which transport systems recognize some amino acids. Perhaps this paper will stimulate investigations of proline function for neurotransmission, centering on unusual modes of amino acid recognition under a fresh three-dimensional view of these molecules and the differences among them.

Even though lexicographers point out that to be ambidextrous means literally that one is 'right-handed on both sides', we may be wise not to insist on the latinate opposite 'ambisinistral'. Instead, one may inquire whether proline molecules can sometimes receive a left-handed recognition from the alternative approach and, thus, present, on occasion, a biologically significant dual enantioselectivity, rather than merely a weak ability to sense configuration.

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