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Invited Review

D-Amino Acids in Animal Peptides

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Summary. Secreted peptides from diverse sources have been found to contain a D-amino acid. From the sequence of cloned m*RNA*s coding for the precursors of such peptides it could be deduced that in all cases tested so far the D-amino acid in the final product is derived from the corresponding L-amino acid present in the primary product of translation. Enzymes catalyzing such an L- to D-isomerization in peptide linkage have been isolated from the venom of a spider and the skin secretions of frogs. Even though these are completely different proteins, the reaction mechanism is the same, namely a deprotonation/re-protonation of the α-carbon of an amino acid with concomitant inversion of the chirality. Sequences potentially coding for homologues of the frog enzyme are present in the genome of different vertebrate species.

Keywords. Peptides; Enantiomerization; Isomerase; Chiral inversion.

Introduction

Proteins are synthesized on ribosomes as chains comprised of twenty different L-amino acids. Through a wide variety of post-translational and a few co-translational reactions, the primary products are modified in many different ways. In fact, every reactive group present in side chains of different amino acids can be modified by the addition of *e.g.* carbohydrates, hydroxyl-, acyl-, methyl-, or phosphoryl-groups *etc.* In particular, the reversible phosphorylation of the OH-groups of serine, threonine, and tyrosine residues plays a central role in cellular metabolism. Life as we know it would be unthinkable without protein modifications.

A most subtle type of a post-translational modification has been found in some secreted animal peptides of diverse origin, namely the conversion of certain amino acids from the L- to the D-configuration. This modification cannot be detected by standard methods for the determination of the amino acid sequences or the molecular mass. The first peptide where a D-amino acid was discovered was the heptapeptide dermorphin [1], a constituent of the skin secretions of South American tree frogs. After its amino acid sequence was elucidated, it was found that the synthetic product was devoid of biological activity. Through a variety of further analytical tests, including retention time on HPLC and enzymatic hydrolysis, it could finally be shown that in the natural product the second amino acid was a D-alanine (see Table 1). In the past two decades, a variety of peptides containing a D-residue were discovered. Moreover, the enzymatic mechanism of this L- to D-isomerization has been elucidated in some detail.

Peptides Containing a D-Amino Acid from Vertebrates

Dermorphin was discovered about 25 years ago in extracts from the skin of *Phyllomedusa sauvagei* [1]. This is a very potent opioid peptide which is, in different assays, on a molar basis about a thousand

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Table 1. Peptides from vertebrates containing a D-amino acid*

Phyllomedusinae	
Dermorphins	*Tyr*-D-*Ala-Phe-Gly-Tyr-Pro-Ser*>
	Tyr-D-*Ala-Phe-Gly-Tyr-Pro-Lys*
	Tyr-D-*Ala-Phe-Trp-Tyr-Pro-Asn*
Deltorphins	*Tyr*-D-*Ala-Phe-Asp-Val-Val-Gly*>
	Tyr-D-*Met-Phe-His-Leu-Met-Asp*>
	Tyr-D-*aIl-Phe-His-Leu-Met-Asp*>
Bombina species	
Bombinins H	*Ile*-D-*aIl-Gly-Pro-Val-Leu*-Gly . . .
Platypus venom	
Defensin-like	*Ile*-D-*Met-Phe-Phe-Glu-Met*-Gln . . .
CNP	*Leu*-D-*Leu-His-Asp-His-Pro-Asn* . . .

* *aIl*: *allo*-Isoleucine; >: carboxy-terminal amide; *CNP*: C-type natriuretic peptide

times more active than morphine [2]. Through a combination of c*DNA* cloning, pharmacological tests, and biochemical characterization, several additional opioid peptides were found in the skin of different frogs belonging to the sub-family *Phyllomedusinae* [3–5]. Besides homologues of dermorphin, a new group of peptides, the deltorphins were discovered. While dermorphins bind to μ-opioid receptors, the deltorphins have a high affinity and selectivity for delta-receptors [4–7]. Deltorphins have become an important tool in pharmacological research; using this term more than 500 publications are found in the Medline library. In all cases tested, the homologous peptides containing only L-amino acids were devoid of biological acticity.

Another group of peptides containing a D-amino acid was detected in skin secretions of *Bombina* species (fire-bellied toads). Back in the late sixties, the group of *H. Michl* in Vienna isolated from skin secretions of these frogs a peptide termed bombinin [8]. This was the first peptide with antimicrobial activity isolated from an animal source. Numerous examples of such peptides were subsequently found in different amphibia as well as in many other species. Using modern separation techniques and c*DNA* cloning, it was later shown that a family of bombinins with related sequences exists in skin secretions of these frogs [9, 10]. Moreover, in the sequence of the precursors of these bombinins deduced from cloned c*DNA*s, a second group of peptides could be predicted. These were then isolated and characterized from skin secretions and termed bombinins H as they have not only antimicrobial but, contrary to the bombinins, also hemolytic activity [11]. The analysis of these novel peptides also demonstrated, that some of them contained a D-amino acid, D-*allo*-isoleucine at the second position[1]. In these instances, the biological activities of the all-L-isomer *versus* the one with a D-residue were rather similar.

More recently, in the venom of the male platypus, two additional peptides with a D-residue at the second position were isolated and characterized (see Table 1). One is a member of the family of C-type natriuretic peptides [12], the other a homologue of a mammalian β-defensin [13].

Peptides Containing a D-Amino Acid from Invertebrates

Invertebrates produce a large variety of biologically active peptides acting as hormones, toxins, and antimicrobials, *etc.* A few of these were also found to contain a D-amino acid (see Table 2). The first examples were achatin I and fulicin, two neuropeptides from *Achatina fulica*, an African snail [14, 15], of which the latter one stimulated the contraction of the penis retractor muscle. A few additional examples were later found in other molluscs like *Mytilus* and *Aplysia*.

In all the examples mentioned so far, the D-amino acid was found at position 2 of the mature product. This is, however, not a general rule as such ''unnatural'' residues have also been detected at other places. For example, from different species of crustaceans, a hyperglycemic hormone regulating the

Table 2. Peptides with a D-amino acid* from invertebrates

Achatina fulica	
Achatin I	*Gly*-D-*Phe-Ala-Asp*
Fulicin	*Phe*-D-*Asn-Glu-Phe-Val*>
Fulyal	*Tyr*-D-*Ala-Glu-Phe-Leu*>
Mytilus edulis	*Ala*-D-*Leu-Ala-Gly-Asp-His* . . .
Aplysia kurodai	*Asn*-D-*Trp-Phe*>
Hyperglycemic H	<*Glu-Val*-D-*Phe-Asp-Gln-Ala-Cys* . . .
Bromocontryphan	*Gly-Cys-Hyp*-D-*Trp-Glu-Pro-Trp**-*Cys*>
ω-Agatoxin IVB	. . . *Leu-Gly-Leu*-D-*Ser-Phe-Ala*
Conotoxins	. . . *Ser-Ser-Phe*-D-*Phe-Lys-Ile*
	. . . *Gly-Gln-Phe*-D-*Met-Ala-Arg*

* <*Glu*: Pyroglutamic acid; *Hyp*: hydroxyproline; *Trp**: 6-*Br-Trp*; >: carboxy-terminal amide

[1] Isoleucine has two chiral centers. Chiral inversion at the α-carbon converts L-isoleucine to D-*allo*-isoleucine.

glycogen metabolism in these animals was isolated which was found to contain either L- or D-phenylalanine as the third residue [16]. From the venom of different *Conus* snails the group of *B. Olivera* isolated and characterized numerous peptides with very interesting biological properties [17]. These conotoxins were found to be modified by an astounding array of post-translational reactions. For example, the octapeptide contryphan from the venom of a fish-hunting snail contains tryptophan residues at positions 4 and 7, of which the first is in the D-configuration, while the second is the 6-bromo-derivative of this amino acid [18]. Two peptides, ω-agatoxins IVB and IVC, which block voltage-dependent Ca-channels, have been isolated from the venom of the funnel web spider *Agalychnis aperta* [19]. These contain either L- or D-serine at position 46 close to the carboxyl end. Similar cases were also found in some conotoxins [20] with a D-phenylalanine or D-methionine at position 3 from the COOH-terminus (see Table 2).

Biosynthesis of D-Amino Acids within the Peptide Linkage

From the examples mentioned earlier it is clear that several different amino acids can occur in the D-configuration. The origin of these residues could be deduced from the structure of the respective precursors obtained *via* c*DNA* cloning. In all instances it was found that at the position where a D-residue is present in the final product, a normal codon for the corresponding L-amino acid is present in the m*RNA*. This implies that at some stage during the maturation of the primary products of translation, the chiral inversion of a certain amino acid must take place (Fig. 1). This assumption is supported by the observation that in many cases, both diastereomers are present in the natural sources.

Such an inversion of an L- to a D-amino acid within a peptide linkage represents a rather unusual reaction for which no precedence existed. D-Amino acids can be formed from the corresponding L-isomer by the action of amino acid racemases. Most of these enzymes require pyridoxalphosphate as a cofactor and the reaction proceeds *via* a *Schiff* base as an intermediate. There exists, however, another class of racemases which operates without this cofactor. Examples are the proline, aspartate, and glutamate racemases from different bacteria [21–23]. The reaction catalyzed by these enzymes proceeds *via* deprotonation–protonation at the α-carbon of the substrate. Two cysteine residues act as proton acceptor and donor, respectively. As reviewed below, the isomerization of amino acids in peptides appears to proceed *via* a similar mechanism.

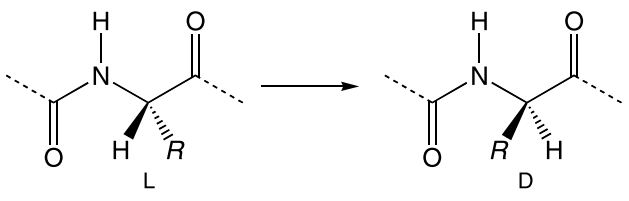

Fig. 1. Inversion reaction

An enzyme, which changes the chirality of amino acids within a peptide linkage (Fig. 1) could be termed peptidyl-aminoacyl-L-D-isomerase, similar to peptidyl-prolyl-*cis-trans*-isomerase. The first enzyme catalyzing such a reaction was isolated from the venom of the funnel web spider *Agalychnis aperta* [19]. It acts on serine-46 of the 48-residue peptide termed ω-agatoxin IV. Both forms with the L- or the D-isomer are present in this venom. Using different substrates, it was shown that the COOH-terminal tetrapeptide acetyl-*Leu-Ser-Phe-Ala* is the smallest substrate for this isomerase [24]. The position of the serine with respect to the carboxyl end can vary, as the above peptide terminating with two additional alanines is also a substrate. Isomerization also proceeds with peptides containing cysteine or alanine, while a model peptide containing dehydroalanine instead of serine was found to be a potent competitive inhibitor of the spider enzyme [25]. By carrying out the reaction in D_2O, it could be shown that the chiral inversion proceeds *via* an exchange of the proton on the α-carbon of serine-46. The residues in the active site of the isomerase, which act as proton acceptors and donors, respectively, have not yet been identified.

The amino acid sequence of this isomerase from spider venom has been determined [26]. It was found that this protein shows significant homology to serine proteases like trypsin or chymotrypsin. These hydrolytic enzymes operate *via* a ''catalytic triad'' whereby, through hydrogen bonding to a histidine and an aspartic acid, the hydroxyl group of the serine present in the active site is de-protonated and thus becomes a strong nucleophile binding to the carbonyl group of the scissile peptide bond. In case of the isomerase, through a similar triad, the serine oxy-

anion may act as a strong base abstracting the proton from the α-carbon of an amino acid generating most likely a planar anionic peptide enolate intermediate [25].

A second enzyme catalyzing the isomerization of an amino acid in the peptide linkage was recently purified from skin secretions of *Bombina* species (*B. bombina*, *B. variegate*, and *B. orientalis*). Through a combination of different methods, like partial amino acid sequences of the purified protein, isolation of c*DNA*, and genomic clones, the structure of this enzyme could be deduced [27]. This is a glycoprotein with a molecular mass of about 52 kDa with no discernible similarity to the isomerase from spider venom. The *Bombina* isomerase is derived from a polyprotein consisting of five copies of the enzyme with short spacer sequences in between. A search in the data banks has shown that sequences coding for homologues of the frog isomerase are present in the genome of different vertebrates including fish, chicken, and man [27]. For example, from human intestine, a protein binding the Fc-region of immunoglobulin G was characterized. Its amino-terminal domain H, a domain with no known function, is related to the frog skin isomerase [28]. It is, however, currently not known whether any of these genes code for an active isomerase.

The frog skin enzyme also acts on its substrate *via* a de-protonation/re-protonation reaction. This was demonstrated by carrying out the reaction in the presence of tritiated water. Radioactivity was found to be incorporated into the second amino acid of the substrate [27]. The reaction proceeds not only from the L- to the D-isomer but also in the reverse direction. The amino acid side chains, which are present in the active site of the enzyme are currently not known. A comparison of the putative isomerases from different species has shown that a central region, which includes two cysteine and two histidine residues, is most highly conserved. The catalytic activity is, however, insensitive to treatment with iodoacetamide; therefore, these cysteine residues can be excluded to act as catalytic bases. In contrast, incubation with diethylpyrocarbonate results in loss of activity [27] which indicates that at least one histidine residue participates in the catalytic reaction.

More recently, the substrate specificity of this isomerase has been investigated in some detail. The enzyme recognizes a variety of model peptides. In all cases, however, it acts exclusively on the second residue of substrates with a free α-amino group. This also shows that the L- to D-isomerization is a late reaction in the processing of *e.g.* the bombinin H precursors.

As mentioned earlier, the venom of the male platypus also contains two peptides with a D-amino acid at position 2 [12, 13]. From this source, an enzyme catalyzing the conversion of L- to D-amino acids could be isolated and characterized to some extent [29]. It is currently not known, whether this enzyme from a mammal is structurally related to the frog protein.

Conclusions

The occurrence of D-amino acids in animal peptides acting as hormones, antimicrobials, and toxins, *etc.* has been demonstrated in a variety of, but still isolated cases. Several invertebrate species, amphibian skin, and the venom of the male platypus are the only known sources for such peptides. The fact, however, that sequences potentially coding for homologues of a frog skin isomerase are present in the genome of several vertebrates raises the possibility that such enzymes are more widely distributed. If such enzymes really exist in other species as well, the intriguing question arises as to what the respective substrates might be. These could be novel peptides or else known ones where the presence of a D-amino acid has been overlooked. As already mentioned, the presence of a D-amino acid cannot be detected by usual sequencing techniques or mass spectroscopy. In the case of dermorphin, the first peptide of this group, the D-alanine was only found because the synthetic all-L-isomer was inactive. In other cases, however, only small differences in the biological function of the two isomers could be detected and the one with a D-amino acid may thus go unnoticed. Finally, it should be mentioned that D-amino acids have also been found in some proteins which are formed over many years in the process of aging. These include D-*Ser*^{26}-Aβ(25–35/40), a neurotoxic fragment of the *Alzheimer* peptide with the amino-terminal sequence *Gly*-D-Ser-*Asn-Lys* ... [30]. This fragment is suspected to be responsible for the loss of neurons during the progression of *Alzheimer*'s disease. In these cases the D-amino acids are apparently formed by slow non-enzymatic processes [31], but the possibility that isomerases as discussed in this review might also be involved should be kept in mind.

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