Biosynthesis of a D-amino acid in peptide linkage by an enzyme from frog skin secretions

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D-amino acids are present in some peptides from amphibian skin. These residues are derived from the corresponding L-amino acids present in the respective precursors. From skin secretions of *Bombina*, we have isolated an enzyme that catalyzes the isomerization of an L-Ile in position 2 of a model peptide to D-allo-Ile. In the course of this reaction, which proceeds without the addition of a cofactor, radioactivity from tritiated water is incorporated into the second position of the product. The amino acid sequence of this isomerase could be deduced from cloned cDNA and genomic DNA. After expression of this cDNA in oocytes of *Xenopus laevis*, isomerase activity could be detected. Polypeptides related to the frog skin enzyme are present in several vertebrate species, including humans.

amphibia | bombinin H | isomerase | chirality

From amphibian skin, a wide variety of biologically active peptides have been isolated (1). One of these compounds, the heptapeptide dermorphin (present in the skin of the South American tree frog *Phyllomedusa sauvagei*), is a potent opiate that binds with high affinity and selectivity to μ -opiate receptors (2). Quite unexpectedly, dermorphin was found to contain D-Ala at position 2, and this residue is essential for binding to the receptor. Subsequently, several additional opiate peptides containing a D-amino acid were found in the skin of other *Phyllomedusinae* (3). The bombinins H, antibacterial and hemolytic peptides isolated from skin secretions of the frog *Bombina variegata*, contain either L-Ile or D-allo-Ile (D-allo) as the second amino acid (4). Peptides with D-amino acids in their sequences were also found in different invertebrate species, such as snails (5–7), the venom of a spider (8), and crustaceans (9). The most recent addition to this group was a homologue of C-type natriuretic peptides isolated from the venom of the male platypus (10).

In principle, the biosynthesis of these D-amino acids in animal peptides has been clarified. As shown for dermorphin (11) and subsequently for several cases of vertebrate and invertebrate peptides, the secreted products are derived from larger precursors encoded by mRNAs. At the positions where a D-amino acid is present in the end product, a codon for the corresponding L-isomer has been found (reviewed in refs. 12 and 13). This finding implies that, at some stage in the processing of the precursors, a conversion of certain L-amino acids to the D-form takes place. Indeed, in many cases, the L and D forms of the mature peptides coexist in the respective secretory glands and secretions, which can be interpreted as incomplete isomerization. An enzyme that catalyzes such an unusual reaction has been characterized from the venom of a funnel web spider (8). In this case, the chirality of a Ser close to the C terminus of a peptide termed ω -agatoxin IV is inverted. This “isomerase” is related to Ser proteases and functions without any added cofactors (14, 15).

As mentioned above, skin secretions of *Bombina* species contain a group of hemolytic peptides that contain either L-Ile or D-allo as the second amino acid (4). We hypothesized that an

enzyme catalyzing the posttranslational L-D-isomerization should also be present in these secretions. In this article, we describe the isolation and partial characterization of this enzyme.

Materials and Methods

Enzyme Purification. Skin secretions from *Bombina* (those from the closely related species *B. variegata* and *Bombina bombina* were mixed, whereas those from *Bombina orientalis* were studied separately) were collected and processed as described (16, 17). The glycoprotein fraction eluted from ConA-Sepharose with methylmannoside was passed over a Sephacryl S-300 column (20 mM Hepes buffer, pH 7.6, containing 1 mM EDTA). Individual fractions were tested for isomerase activity by using an enzyme immunoassay (see below). Active fractions were concentrated, rechromatographed under the same conditions, and subsequently passed over SP-Sepharose. The flow-through was adjusted to pH 8.6 and fractionated over a Q-Sepharose column. The enzyme could be eluted with a gradient of 1–2.5 mM NaCl. Active fractions were concentrated with Centricon filters retaining only proteins with a mass of 30–100 kDa. These fractions were again chromatographed over SP- and Q-Sepharose, which yielded considerable narrower peaks of activity. Individual fractions were then once more carried through the same protocol. Last, an apparently homogeneous protein with an apparent molecular mass of 52 kDa (as judged by SDS/PAGE) was obtained.

Assays for Enzymatic Activity. The peptide Ile-D-allo-Gly-Pro-Val-Leu-Gly-Cys-amide, which contains the N-terminal heptapeptide sequence of bombinin H, was coupled via the SH group to keyhole limpet hemocyanin. An antibody against this construct was generated in rabbits. For the competitive immunoassay, this peptide was coupled to acetylcholinesterase, and the amount of this enzyme bound to the antibody was measured by using acetylthiocholine as substrate and the Ellmann reagent to measure the amount of free thiocholine (see refs. 18 and 19 for details).

For partly purified enzyme preparations, an assay with a fluorescent peptide was used. The peptide Ile-Ile-Gly-Pro-Val-Leu-Gly-Cys-amide was coupled via the SH-group to the fluorophore Bodipy FL IA [(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-S-indacene-3-propionyl)-N'-iodoacetylenediamine; Molecular Probes]. Similar derivatives were prepared for dermorphin, Ala- and Met-deltorphins, and several other peptides, each containing a Cys inserted after the last amino acid. Fluorescent peptides were separated by reverse-phase HPLC, and

Abbreviation: D-allo, D-allo-Ile.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY916557, AY916558, and AY916559).

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Fig. 1. Characterization of the reaction product. (A) Separation of substrate and product by reverse-phase HPLC on an Aquapore RP300 column (see ref. 17 for details). Substrate (15 μ g in 50 μ l of buffer) and $\approx$ 1 μ g of crude enzyme were incubated for 8 h at 37°C. The arrow indicates the position in the gradient where synthetic Ile-D-allo-Gly-Pro-Val-Leu-Gly-Ser-amide is eluted from the column (at $\approx$ 25.9% acetonitrile). Product yield was $\approx$ 8%. (B) Substrate (15 μ g) and enzyme were incubated in 50 μ l of tritiated water (specific activity, 5 Ci/ml (1 Ci = 37 GBq); American Radiolabeled Chemicals, St. Louis) for 8 h at 37°C. Subsequently, the sample was dried, redissolved several times in water, and dried again. Manual Edman degradation was performed by using standard conditions. After each step, radioactivity was extracted into *n*-butyl acetate and measured in a scintillation counter. Radioactivity (counts per min $\times$ 10⁻³) is plotted against the step of Edman degradation. In the control incubated in the absence of enzyme, <300 counts per min could be extracted after each Edman cycle.

their mass was determined by MALDI MS. Enzyme samples were incubated with 2 nmol of substrate in 100 mM phosphate buffer/5 mM EDTA, and the pH was adjusted to 6.5. Aliquots of the reaction mixture were injected onto a 218TP C-18 column (Vydac, Hesperia, CA) with a gradient of acetonitrile (solvent A, 0.1% TFA; solvent B, 80% acetonitrile in solvent A). Fluorescence emission was recorded at 514 nm (excitation at 480 nm).

In some experiments that required larger amounts of enzyme, the peptide Ile-Ile-Gly-Pro-Val-Leu-Gly-Ser-amide was used as substrate. The product could be separated from this substrate by reverse-phase HPLC (Fig. 1A). Chiral Edman degradation to identify the D-allo in the product was performed by following the procedure given in ref. 20. The mixtures of substrate and product were incubated with Leu aminopeptidase (sequencing grade; Sigma) by following the recommended conditions.

Cloning Experiments and Sequence Analysis. Total RNA from skin of *B. variegata* was prepared as described (16). cDNA was synthesized with either random or sequence-specific primers with SuperScript II (GIBCO/BRL) according to the manufacturer's instructions. PCR (Pwo polymerase, Boehringer) was performed with the following degenerate primers deduced from sequences of the cyanogen bromide-generated fragments: AAY-CAYGAYCCNAARGARACNCC (forward) and ATYTC-

NGTRTCYTTNGCNGG (reverse). The amplified fragment was used to screen a genomic library from *B. orientalis* (21) constructed in Lambda FIX II (Stratagene).

Enzyme Expression in *Xenopus* Oocytes. cDNA coding for amino acids 4–413 of the mature *Bombina* isomerase was fused at the 5' end to a cDNA coding for the signal peptide of human plasminogen activator. The construct was then cloned into the translation plasmid T7TS (22), which contains the 5' and 3' untranslated regions of the β -globin mRNA of *Xenopus laevis* as well as a multiple cloning site with stop codons in all reading frames in between. From the linearized vector, RNA was transcribed *in vitro* (mMessage Machine T7, Ambion, Austin, TX) and injected into *Xenopus* oocytes. Injected and control oocytes were incubated for 3 days at 20–25°C by using the conditions as described in ref. 23. Supernatants were collected, adjusted to 5 mM EDTA, and concentrated on a 3kD Centricon filter to a volume of $\approx$ 1 μ l per two oocytes. Isomerase activity was determined with the enzyme immunoassay (30 pmol of peptide; incubation time, 8 h).

Results

Isolation and Partial Characterization of the Isomerase. From skin secretions of *B. variegata* and *B. bombina*, we purified a glycoprotein with a molecular mass of $\approx$ 52 kDa moving as a single band on an SDS/PAGE (data not shown). In three different assays, it could be shown that this protein catalyzes the conversion of L-Ile to D-allo at position 2 of a model peptide with the N-terminal sequence of bombinin H (4). Such an experiment with the substrate Ile-Ile-Gly-Pro-Val-Leu-Gly-Ser-amide is shown in Fig. 1A. The product could be separated from the substrate by reverse-phase HPLC and it coeluted with the synthetic peptide containing the D-amino acid. The product was found to be resistant to the action of Leu aminopeptidase, as was demonstrated earlier for other peptides with a D-amino acid at position 2 (C.M. and G.K., unpublished data). Moreover, by using chiral Edman degradation (20), the presence of D-allo could be corroborated (data not shown). By analogy with peptidylprolyl-cis/transisomerases, this enzyme could be termed peptidylaminoacyl-L/D-isomerase, hereafter referred to as the isomerase, for brevity.

Next, we incubated this substrate and the enzyme in tritiated water. This incubation resulted in the incorporation of radioactivity into the product, selectively into position 2 as revealed by manual Edman degradation (Fig. 1B). Similar results were obtained by using the synthetic peptide containing D-allo at position 2 (data not shown). This finding implies that the change of chirality proceeds in both directions via a deprotonation/protonation reaction at the α -carbon of the second amino acid. Such a mechanism has been demonstrated for the isomerase from spider venom (14).

By using the peptide Ile-Ile-Gly-Pro-Val-Leu-Gly-Cys-amide (coupled to a fluorophore via the SH-group) as substrate (see *Materials and Methods*), a time-course experiment showed that at equilibrium, the concentration of the product, a diastereomer of the substrate, was slightly less than 50% (Fig. 2). The reaction catalyzed by the frog skin isomerase was sensitive to diethyl pyrocarbonate (Fig. 2), but it was not inhibited by iodoacetamide (data not shown). This result indicates that a His residue is part of the active site, whereas free Cys residues can be excluded. Moreover, treatment with 2.5 mM hydroxylamine had no effect (data not shown), which argues against the presence of pyridoxal phosphate, a cofactor of many amino acid racemases. The enzyme was also not inhibited by phenylmethylsulfonyl fluoride, which blocks the isomerase from spider venom (13).

In this assay for isomerase activity, the pH optimum was found to be pH $\approx$ 6.5, with a steep decline of activity at pH <5.5. At pH

Fig. 2. Time course of the conversion of the peptide Ile-Ile-Gly-Pro-Val-Leu-Gly-Cys-amide, labeled with a fluorophor (see *Materials and Methods*), to the product containing D-alle as the second residue. Reaction conditions were 100 mM phosphate buffer (pH 6.5) and 5 mM EDTA (●). In a parallel sample, the enzyme was preincubated in the presence of 1 mM diethyl pyrocarbonate for 16 h on ice (■). Aliquots removed at different times were analyzed by reverse-phase HPLC.

7.3–7.5 used for the enzyme immunoassays, the activity was $\approx 40\%$ of the maximum (Fig. 3).

longed incubation, no trace of the product with a D-alle could be detected by reverse-phase HPLC.

Amino Acid Sequence of the Isomerase. From skin secretions of *B. variegata* collected from ≈ 500 "milkings" of frogs, enough purified isomerase could be obtained to determine the N-terminal sequence of the protein and of some of its cyanogen bromide fragments by automated Edman degradation (see Fig. 4). Oligonucleotides deduced from these sequences were used for PCR experiments with total mRNA isolated from Bombina skin (16). This amplification yielded a cDNA fragment representing about the first half of the isomerase. With Northern blot analyses, it could be demonstrated that the isomerase was encoded by a large mRNA containing $>8,000$ nucleotides (data not shown). All attempts to amplify the rest of the isomerase with 5'- and 3'-RACE failed. In a different approach, we screened a genomic library prepared from the related species *B. orientalis* (21). We could isolate two clones that contained the sequence of an exon with an ORF for 409 aa, starting with residue 4 of the mature isomerase and part of an intron. The first 3 aa of the enzyme and a preceding pro-sequence or spacer sequence are encoded by a separate small exon. These two genomic fragments were assembled to yield a *B. orientalis* cDNA, which can be translated into a polypeptide comprising 412 aa (see Fig. 4). This cDNA codes for a protein with a calculated mass of 45.0 kDa. A single *N*-glycosylation site is present close to the amino end. The cDNA and genomic fragments containing the genetic information for this protein do not code for a signal sequence and its ORF does not terminate with a stop codon.

Starting with the sequence of the *B. orientalis* isomerase, we screened a cDNA library from skin of *B. variegata* by using PCR and suitable primers. Five different cDNAs, termed *va* to *ve*, could be amplified and were sequenced fully or in part. All of these cDNAs code for proteins that are >90% identical to the *B. orientalis* isomerase (see Fig. 7, which is published in the supporting information on the PNAS web site). These homologous proteins are linked by spacer segments in the following order: —*va*—(33)—*vb*— and —*vc*—(10)—*vd*—(48)—*ve*— (numbers in parentheses indicate the length of the spacers). The spacer sequences are relatively rich in Pro and charged amino acids. Individual isomerase domains are apparently excised from this polypeptide by unknown proteases.

Expression of the Isomerase cDNA in *Xenopus* Oocytes. For the expression of the cloned isomerase cDNA, we used *Xenopus* oocytes. Because the C-terminus of the enzyme present in skin secretions is not known, we decided to construct a cDNA encoding amino acids 4–413 of the mature *Bombina* isomerase, followed by a stop codon. At the 5' end, this fragment was fused to a cDNA coding for the signal peptide of human plasminogen activator. The construct was then cloned into the translation plasmid T7TS (22), between the 5' and 3' untranslated regions of the β -globin mRNA of *X. laevis*. From this vector, RNA was

Fig. 5. Expression of isomerase cRNA in *Xenopus* oocytes. Product yield in pmol is plotted against the number of injected oocytes from which supernatants were collected (■). As controls, supernatants from mock-injected oocytes were analyzed in parallel (●).

transcribed *in vitro* and injected into oocytes. Media from injected and control oocytes were collected, concentrated, and assayed for isomerase activity by using the enzyme immunoassay. In these experiments, formation of the product containing D-allo could be demonstrated (Fig. 5).

Homologues of the Frog Isomerase in Other Species. A search in the data banks for polypeptides related to the *Bombina* isomerase originally yielded only one major hit, namely, the N-terminal H-domain of the human IgG-Fc binding protein (25). This domain comprises ≈ 450 aa and has no known function. Subsequently, ESTs coding for fragments of related proteins from *X. laevis* and chicken were deposited in the data banks. Last, in the genome of the fugu fish, three genes potentially coding for homologous proteins are present (FuguGenscan.26525, FuguGenscan.28866, and FuguGenscan.28655). A comparison of the amino acid sequences of the frog isomerase and some of its homologues from other vertebrates are shown in Fig. 6.

Discussion

From skin secretions of *Bombinae*, we have isolated an enzyme that catalyzes the conversion of the second amino acid of a model peptide from the L- to the D-configuration. The change in chirality probably proceeds by means of a deprotonation/protonation mechanism at the α -carbon of the second residue. Such a reaction mechanism has been demonstrated for the isomerase from spider venom (14) as well as for some bacterial

racemases (26, 27). In these cases, different amino acids (e.g., Cys, Lys, or His) can function as proton donors and acceptors. In the comparison of related genes from other species (Fig. 6), two His residues are present in the most highly conserved segment in all four species. Because the isomerization reaction is inhibited by diethyl pyrocarbonate, it seems to be likely that one or both of these residues is part of the active site. The pH dependence (Fig. 3) also suggests that isomerase activity is blocked if one or more His residues carries a positive charge.

For the tests of isomerase activity, we used a peptide with the N-terminal sequence Ile-Ile-Gly... of bombinin H. The only other substrate for the enzyme was a peptide derived from the sequence of C-type natriuretic peptide from the frog *Rana catesbeiana* (24) starting with the sequence Leu-Leu-Asn... In this case, the second Leu was converted to D-Leu. With a few other peptides that we tested, L-D-isomerization could not be detected (see *Results*). Many more experiments will be necessary to determine the substrate specificity of the isomerase.

Enzymes catalyzing the posttranslational maturation and modification of biologically active peptides are usually highly conserved in evolution. For example, the enzyme from *Conus* snails that catalyzes the vitamin K-dependent formation of the γ -carboxy glutamic acids present in some venom peptides is highly homologous to the corresponding carboxylase from bovine liver (28, 29). Thus, it seems to be surprising that structurally unrelated enzymes catalyze the L-D-isomerization of amino acids in peptide linkage in spider venom (15) and frogs. Evidence indicates that, in both cases, the catalytic mechanism is basically the same (namely, removal of a proton from the α -carbon and concomitant addition from the opposite side in an acid-base type of catalysis). However, one must bear in mind that the amino acids that are subject to this isomerization reaction reside at very different positions in the respective substrates. In case of the spider enzyme, it is the third residue from the carboxyl end, both in the ω -agatoxins IVB and IVC as well as in the C-terminal fragments that were tested as substrates (8, 14). Conversely, results presented here indicate that the frog isomerase acts exclusively on the second amino acid from the N terminus. Evidence also indicates that the active-site residues involved in proton abstraction and addition are different. The enzyme from spider venom has a catalytic triad characteristic for Ser proteases, and it is indeed inhibited by phenylmethylsulfonylfluoride (13), an inhibitor of this type of enzymes. The activity of the frog skin enzyme is blocked by diethyl pyrocarbonate, which reacts with His residues, whereas phenylmethylsulfonylfluoride has no effect. These results indicate that two different classes of en-

Fig. 6. Comparison of amino acid sequences of the *Bombina* skin isomerase with other vertebrate sequences deduced from cDNA or genomic DNA. Man, domain H of the human IgG-Fc binding protein (25); Bird, chicken EST BM488067; Fish, FuguGenscan.28655. Asterisks indicate conserved His and Cys residues.

zymes catalyzing such a reaction exist. According to this hypothesis, one type would act on the variety of peptides containing a D-residue at position 2, which have been isolated from vertebrate and invertebrate species (5, 6, 10, 12). The other class would target residue 3 from the carboxyl end, such as in the ω -agatoxins IV. Another potential substrate for an enzyme of this group would be a recently characterized peptide from the venom of a *Conus* snail (30), which contains a D-Phe at position 3 from the C terminus. Still other isomerases may catalyze the formation of a D-amino acid at other positions in the respective peptide chains (7, 9). Both the spider and the frog isomerase act on small peptides corresponding to the C- or N-terminal sequences, respectively, of the mature products. These findings imply that *in vivo* as well, formation of the D-amino acid is a late reaction in the processing of the precursors. Indeed, a peptide containing the last five amino acids of the pro-region linked to the N-terminal hexapeptide of bombinin H is not a substrate. However, in case of the more complex precursors of the amphibian opioid peptides, evidence for a different mechanism has been presented (31, 32). From skin of *P. sauvagei*, a precursor protein has been isolated that, upon digestion with trypsin and carboxypeptidase, yielded the processing intermediates of dermorphin and deltorphin (also termed dermenkephalin) predicted from the cDNA sequence (11). By using specific antibodies, it could be shown that D-Ala was already present in these fragments (31). This result suggests that in the skin of these frogs, L-D-isomerization already takes place at an early stage of processing, before the action of prohormone convertases.

Based on the sequence comparison of homologues from different species (see Fig. 6), we consider it to be likely that enzymes related to the frog isomerase are widely distributed among vertebrate species. This finding raises the intriguing possibility that peptides containing a D-amino acid are also present in these species. These may be unknown peptides that have not been isolated. In addition, the presence of D-isomers may have been overlooked in hormones, antibacterial or modulatory peptides of the immune system, or neuropeptides that have earlier been characterized. Indeed, D-amino acids cannot be detected with the usual sequencing techniques and by MS. In case of dermorphin, the first animal peptide that was found to contain a D-amino acid, this residue was discovered only because the synthetic all-L-isomer was biologically inactive (2). In other cases, both naturally occurring isomers are active, albeit with subtle differences. For example, such differences have been demonstrated for the two forms of ω -agatoxin IV from spider venom (8), the hypoglycemic hormone of lobsters (9), as well as the bombinins H (33). Similar cases may exist, but have not been noted, in peptides isolated from different vertebrate species.

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