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L to D Amino Acid Isomerization in a Peptide Hormone Is a Late Post-translational Event Occurring in Specialized Neurosecretory Cells*

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Modification of the chirality of a single amino acid residue within a peptide chain appears to be novel additional mechanism leading to structural and functional diversification of eukaryotic bioactive peptides. This phenomenon has been studied at the cellular level in a neuroendocrine organ which elaborates a mixture of diastereoisomers of a 72-residue neuropeptide, crustacean hyperglycemic hormone. For the first time, amino acid isomerization has been shown to occur in the perikarya of fully specialized neurosecretory cells, as a late step of the maturation of the hyperglycemic hormone precursor and after propeptide cleavage. The specificity and efficiency of this phenomenon indicates the existence of a new enzyme family involved in the biogenesis of peptide hormones.

As a general rule in living cells, the native conformation of a peptide or a protein chain is dictated by its amino acid sequence, which is itself strictly encoded at the genome level. Conformational changes associated with cellular stress or with mutations of the gene coding for the protein are, under physiological conditions, strictly controlled by complex mechanisms leading to repair or degradation of the modified protein (1). In the last few years, evidence has accumulated for the existence of a mechanism which modifies peptide structure by selectively inverting the configuration of a single aminoacyl residue from the natural L- to the D-form (2). This is in contrast to the widely accepted paradigm concerning the presence of only L-aminoacyl residues in proteins and peptides from eukaryotic organisms. Such a phenomenon, though crucial for the bioactivity of the molecule because it introduces a major stereochemical change, has probably been largely underestimated in eukaryotic peptides (and proteins) because it remains undetectable by routine physicochemical or molecular analytical methods.

The presence of a D-aminoacyl residue in a eukaryotic peptide was first demonstrated in small (4–8 residue) peptides isolated from different animal sources such as frog skin (opioid peptides dermorphins, dermenkephalins, or antimicrobial peptides bombinins) (3) or mollusc nervous tissues (neuroexcitatory peptides from different species) (4). In each case, the

D-amino acid residue is located at the second position of the sequence. The situation is different in the octapeptide contryphan, isolated from the venom of the marine gastropod *Conus radiatus*, where a D-tryptophan constitutes the fourth residue of the sequence (5). The presence of a D-amino acid residue (D-phenylalanine) was also detected in position 3 of the sequence of a 72-amino acid crustacean neurohormone (crustacean hyperglycemic hormone, CHH)¹ (6, 7). This neurohormone is largely synthesized in the major crustacean neurosecretory system, the X-organ-sinus gland complex, located in the eye-stalks. The coexistence of CHH and [D-Phe³]CHH within sinus glands was described recently for the crayfish *Orconectes limosus*, the biological model used in the present study (8).

The only case where a D residue (D-serine) has been found near the C terminus (at position 46) is a 48-amino acid spider toxin isolated from the venom of the funnel web spider *Ageles nopsis aperta* (ω -agatoxin 4B) (9), also named ω -agatoxin TK D (10).

Changing an L to a D residue has major functional consequences. For example, the all-L residue counterparts of frog skin opioid peptides and molluscan fulcin and fulyal were shown to be totally devoid of biological activity (2, 11). The D residue-containing isoform of agatoxin demonstrated increased potency against P-type calcium channels and enhanced stability in the venom (9). The crustacean [D-Phe³]CHH displays different kinetics and increased potency in its hyperglycemic effect and, in addition, exhibits a potent inhibitory activity upon molting gland ecdysteroidogenesis (6, 12).

In every case where the mRNA encoding the D-amino acid containing peptide has been cloned, a typical codon was found corresponding to the modified residue. This indicates that amino acid epimerization should occur as a post-translational event. This is in agreement with studies where the stereoinversion of the serine 46 of ω -agatoxin 4C (the all-L residue equivalent to the ω -agatoxin 4B) was obtained *in vitro* after incubation with a venom fraction (although with a rather low yield and slow kinetics) (9). Isomerization of agatoxin Ser⁴⁶ in the venom gland may therefore be considered as a late, post-secretory and reversible event. Nevertheless, such a mechanism could hardly be applied to a cellular system, as suggested by the few data available on the biosynthesis of the frog skin peptides (13).

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¹ The abbreviations used are: CHH, crustacean hyperglycemic hormone; ELISA, enzyme-linked immunosorbent assay; MALDI, matrix-assisted laser desorption ionization; RP-HPLC, reversed-phase high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SG, sinus gland; TOF, time-of-flight; TPCK, tosylamido-phenylethyl chloromethyl ketone; XO, X-organ; tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

This paucity of information on the timing and mechanism of the isomerization process in peptide-secreting cells urged us to use the crustacean X-organ-sinus gland as a model to investigate this phenomenon. Because we knew that a 3:1 mixture of L-/D-isoforms was produced by the crayfish neuroendocrine system (8), we decided as a first step to determine at which biosynthetic intermediate the D-phenylalanyl residue could be identified. In parallel, we undertook a structural exploration of the CHH-producing system to elucidate to what extent the neurosecretory cells were specialized in the production of the CHH isomers.

EXPERIMENTAL PROCEDURES

Experimental Animals and Tissue Dissection—Crayfish *O. limosus* were collected by a commercial fisherman in the river Saône (France) and kept at 11–14 °C in the laboratory in filtered recirculating freshwater under natural photoperiod. The animals (20–30 g) were fed once a week with catfood pellets, sampling of the eyestalks being made 3–4 days after feeding. Both male and female crayfish were used at different periods of the year including the breeding season. Though molt stage of the animals was not precisely determined, external morphological parameters indicated if the animals had molted recently or not. Before sampling the eyestalks, the animals were anesthetized by immersion in ice-cold water for 15 min. For immunohistochemistry and labeling experiments, eyestalk tissues were dissected to separate the ganglia containing the XO-SG complex from the surrounding tissues (epidermis, muscle, and retina).

Incubations and Labeling Experiments—Immediately after dissection, pulse incubations were performed on watch-glasses (10 organs/200 μ l of medium) at room temperature in an orbital shaker in modified Eagle's medium with Earle's salts (Life Technologies, Inc.), depleted in leucine and cysteine, diluted 1:1 with freshwater crustacean saline (14) (osmolality: 420 mosM) as described in Ref. 15. L-[³H]leucine (1.6 MBq) and L-[³⁵S]cysteine (1.6 MBq) were added just prior to incubation. Chase incubations were performed in incubation medium enriched with L-leucine (0.125 g/L), L-cysteine (0.12 g/L), and antibiotics (0.10 mg/L streptomycin and 0.16 mg/L tetracycline). In preliminary experiments, organs were directly put into electrophoresis sample buffer (1 organ/15 μ l) after incubation (see below) and disrupted by sonication before heating (95 °C, 10 min) and electrophoresis. Some incubations included monensin sodium salt (70 nM, Sigma) in the medium as prohormone maturation inhibitor (16).

Before RP-HPLC, labeled organs were homogenized in 15% trifluoroacetic acid (1 ml/10 organs). After centrifugation, the protein pellet was rinsed six times with 15% TFA prior to extractions with 10% acetic acid, first at 80 °C for 5 min and then twice at room temperature. The supernatant containing the peptides was injected into the HPLC column.

Reversed-phase High-performance Liquid Chromatography and Mass Spectrometry—Separation of peptides by RP-HPLC was performed essentially as described in Ref. 8. Fractionation of the X-organ-sinus gland extracts was performed on a Nucleosil C₁₈ column (Macherey-Nagel, 5- μ m particle size, 250-mm long and 4.0-mm internal diameter). The peptides were eluted from the column with a gradient of acetonitrile in water (both eluents containing 0.1% trifluoroacetic acid), at a flow rate of 0.75 ml/min. UV absorbance of the eluent was monitored at 220 nm. Fractions of thirty seconds duration were collected. Separation of tryptic fragments was performed on a microbore (250 mm \times 2.0 mm internal diameter) column, filled with the same stationary phase as above. Radioactivity in aliquots of each chromatographic fraction was determined after mixing with 1 ml of biodegradable scintillation solution (Ecoscint A, National Diagnostics).

Matrix-assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry was performed on a Voyager Elite instrument (Perseptive Biosystem, Inc., Framingham, MA). The molecular mass of peptides present in HPLC fractions was determined as follows. Dried aliquots of fractions were redissolved in 2 μ l of 30% formic acid. One-half microliter was deposited on the MALDI stainless steel target and immediately mixed with 0.5 μ l of matrix (a saturated solution of sinapinic acid in acetonitrile/water 30:70 (v/v), 0.1% trifluoroacetic acid). After drying, the target was introduced into the mass spectrometer and the spectra recorded.

Polyacrylamide Gel Electrophoresis and Autoradiography—SDS-PAGE was performed with a MiniProtein system (Bio-Rad) with tricine (Sigma-Aldrich, St Louis, MO) in the buffers instead of glycine, according to Ref. 17. HPLC fractions were dried with a Speed-Vac (Savant

Instruments Inc., Holbrook, NY) before addition of a 20- μ l sample buffer (4% SDS, 12% glycerol (w/v), 50 mM Tris-HCl, 2% mercaptoethanol (v/v), 0.01% bromphenol blue, pH 6). Molecular weight markers were from the "Low molecular weight range kit" from Amersham Pharmacia Biotech with aprotinin (6.5 kDa) added. After migration in a 10% acrylamide gel, proteins were stained with Coomassie Blue to visualize the markers. The gel was destained and then dried on a Whatman paper sheet with a gel-dryer (Bio-Rad) before autoradiography with Hyperfilm β Max (Amersham Pharmacia Biotech) x-ray film or with a combination of Biomax MS film with an intensifying screen system (Biomax Tanscreen-LE), both from Kodak.

Primary Antisera—Different antisera were used for immunoassays and immunohistochemistry. Two rabbit antisera, named *r*-anti-pQL and *r*-anti-pQD in this article, were raised against the synthetic pQL and pQD octapeptides described below, respectively. The production and preliminary characterization of these antisera have been described earlier (8). For double labeling experiments, an additional antiserum (named *gp*-anti-pQL) was produced in a guinea pig against pQL conjugated to bovine thyroglobulin.

Immunohistochemistry—After removal, eyestalks were dissected and the medulla terminalis together with the X-organ and sinus gland were fixed overnight at 4 °C in a 4% paraformaldehyde solution in 0.01 M, pH 7.2 phosphate-buffered saline. For double immunostaining, antisera from two species were used, *r*-anti-pQD and *gp*-anti-pQL. After washing in phosphate-buffered saline, medullae were incubated with both primary antibodies diluted (*r*-anti-pQD at 1:400 and *gp*-anti-pQL at 1:200) in PBS to 0.01 M containing 0.5% Triton X-100 and 5% normal goat serum (Sigma-Aldrich) for 18 h at room temperature.

After several washes in phosphate-buffered saline/normal goat serum/Triton, a mixture of secondary antibodies coupled to fluorochromes was added for 18 h at room temperature under darkness. Secondary antibodies solutions were anti-rabbit conjugated with rhodamine (Roche Molecular Biochemicals) at 1:200 and anti-guinea pig coupled with fluorescein isothiocyanate (Sigma-Aldrich) at 1:100, diluted in the same buffer as the primary antibodies. After washings, tissue fragments were mounted in Vectashield H-1000 (Vector Lab., Burlingame, CA) on microscope slides under glass coverslips and kept at 4 °C until use.

Confocal Microscopy—Confocal microscopy was carried out using the TCS 4D confocal imaging system (Leica, Heidelberg, Germany), equipped with a \times 63 objective (plan apo, NA = 1.4). For fluorescein isothiocyanate and rhodamine, an Argon-Krypton ion laser adjusted to 488 nm and 568 nm was used. For each optical section, double fluorescence images were acquired in sequential mode. The signal was processed to integrate 8 lines to reduce noise. The focus step between sections was generally 1 μ m. A focal series was collected for each specimen and eventually processed to produce single composite images (extended focus), combining a high spatial resolution with an increased field depth. Micrographs were processed and assembled with Adobe Photoshop 5.0. (Adobe Systems Inc, San José, CA).

As a specificity control for the observed fluorescence, single labeling experiments were performed with either of the antiserum, and the preparation was examined at both excitation wavelengths. No overlapping of the emission fluorescence could be detected.

Synthetic Peptides—The following synthetic peptides were used in this study: two octapeptides with a sequence identical to the N-terminal part of the isoforms of the crayfish CHH (pGlu-Val-Phe-Asp-Gln-Ala-Cys-Lys (octapeptide pQL) and pGlu-Val-D-Phe-Asp-Gln-Ala-Cys-Lys (octapeptide pQD); the corresponding peptides with a glutamine instead of a pyroglutamate at the N terminus (octapeptides QL and QD, respectively); the octapeptide pGlu-D-Val-Phe-Asp-Gln-Ala-Cys-Lys; and a 15-mer peptide with the sequence Asp-Gln-Ser-Val-Ser-Lys-Arg-Gln-Val-Phe-Asp-Gln-Ala-Cys-Lys (corresponding to residues 29–42 of the prepro-CHHA according to Ref. 18). These peptides were synthesized by Neosystem Lab (Strasbourg, France). After deblocking and a first step of purification, the cysteinyl residues were reduced by dithiothreitol under argon. The reduced peptides were further purified by RP-HPLC.

Enzyme-linked Immunosorbent Assay—Direct ELISA was performed on aliquots of HPLC fractions as described in Ref. 8. Briefly, 5 or 10 μ l of each fraction were deposited in the wells of the microtiter plate and dried under vacuum. After a 2-h incubation with coating buffer (0.1 M sodium carbonate, pH 9.6), the immunological procedure included 3 incubation steps: first with the primary antibody (*r*-anti-pQL and *r*-anti-pQD) diluted 1:500 and 1:1000, respectively, in PBS buffer containing Tween 20 and sodium azide (both from Prolabo, France); then with the secondary antibody (goat anti-rabbit IgGs conjugated to alkaline phosphatase, Sigma-Aldrich) diluted 1:2000 in phosphate-buffered

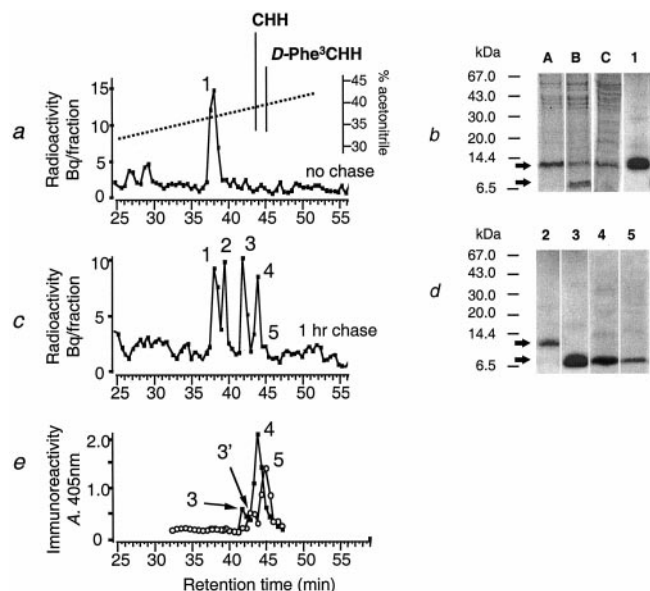


FIG. 1. Identification by RP-HPLC and SDS-PAGE of CHH biosynthetic intermediates after radioactive labeling of 10 X-organ-sinus gland complexes. Only the relevant part of the chromatograms are shown. *a*, radioactivity profile after a 2-h pulse and no chase. The elution gradient (acetonitrile, 0.1% trifluoroacetic acid in water, 0.1% trifluoroacetic acid) is indicated by the dotted line. The vertical lines indicate the elution time of CHH and [D-Phe³]CHH marked on the UV trace (data not shown). Fractions from peak 1 were analyzed in *b*, *b*, composite autoradiogram after SDS/PAGE of crude extract of [³⁵S]cysteine-labeled XO-GS complexes with no chase (lane A) and chased for 2 h (lane B), showing the ~12-kDa pro-CHH band and the ~7.7-kDa CHH band (arrows). Lane C is the same as lane B except that 70 nM monensin was included in both pulse and chase medium. One organ equivalent was applied. Lane 1, material from peak 1 in *a*. Molecular mass markers (kDa) are indicated on the left. *c*, radioactivity profile after a 2-h pulse and 1 h chase. Numbers correspond to radiolabeled peptides analyzed by SDS/PAGE as shown in *d*. *d*, composite autoradiogram after SDS/PAGE of peaks from *c*. *e*, immunological detection of the different CHH forms. Direct ELISA was performed on 10- μ l aliquots of HPLC fractions from *c* using the specific *r*-anti-pQL (filled squares) or *r*-anti-pQD (open circles) antisera. Absorbance at 405 nm was measured 60 min after addition of the phosphatase substrate. Peak 3' marks *r*-anti-pQD immunoreactive fractions, which almost co-eluted with peak 4 (CHH).

saline-Tween-sodium azide; and finally with the phosphatase substrate solution (*p*-nitrophenyl phosphate disodium salt dissolved in coating buffer). The two first steps lasted 1.5 h at 37 °C. Readings of the absorbance were performed every 15 min.

Tryptic Digestions—Before use, 10 to 20 μ l of the stock enzyme slurry (TPCK-treated enzyme (EC 3.4.21.4, Sigma-Aldrich) attached to agarose beads in 1 M acetic acid) was vortexed with 2 ml of the digestion buffer (10 mM Tris-HCl, pH 8 containing 5 mM EDTA and 10 mM dithiothreitol to reduce disulfide bonds). After centrifugation (2000 \times *g*, 10 min), the supernatant was discarded and the gel resuspended in 20 μ l of digestion buffer. Pro-CHH digestions were performed as follows. The dry peptide was redissolved in 5 μ l of acetonitrile/water (1:1). Then 45 μ l of digestion buffer and 2 μ l of enzyme suspension were added to the tube. After 3 h at 37 °C, the digestion mixture was diluted to 200 μ l with 10% acetic acid and then filtered on a Millex GV4 Millipore filter (0.2- μ m pore size). Before HPLC, internal standards (2 μ g of each synthetic peptide, octapeptides pQL, pQD, QL, QD) were added to the hydrolysate.

RESULTS AND DISCUSSION

At Which Step of Peptide Biogenesis Does Isomerization Occur?—CHH is synthesized in the ribosome-associated endoplasmic reticulum of crayfish neurosecretory perikarya as a ~14-kDa prohormone (18). The deduced ~12-kDa CHH precursor was characterized in the present study by *in vitro* radioactive labeling of X-organ-sinus gland complexes followed by chromatographic and electrophoretic analysis. After a 2-hour

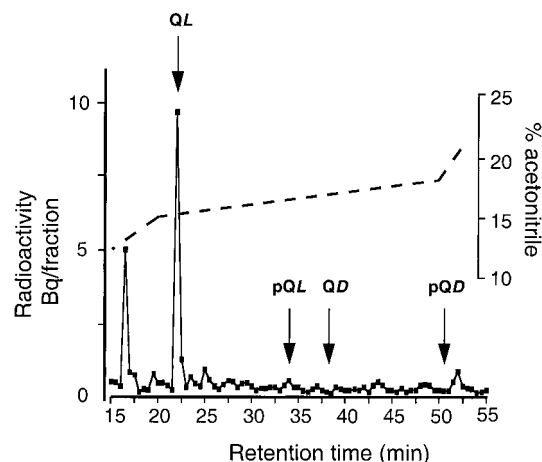


FIG. 2. Identification of the N-terminal octapeptide of labeled pro-CHH by tryptic digestion and HPLC separation of the fragments. Dried fractions containing labeled pro-CHH (Fig. 1, peak 1) from several experiments were hydrolyzed to completion with trypsin and analyzed by RP-HPLC using synthetic octapeptides as internal standards. A peak of radioactivity which comigrated with QL was obtained, whereas no radioactive peak was observed in the QD elution position (arrows). Such proteolytic cleavage was reproduced with different preparations of peaks 1 and 2 (Fig. 1) with a similar result. Chromatographic analysis was performed on Nucleosil C₁₈, 5- μ m particle size of dimensions 2.0 \times 250 mm, and flow rate 0.2 ml/min. Fractions were collected every 30 s. The elution gradient (acetonitrile/0.1% trifluoroacetic acid in water/0.1% trifluoroacetic acid) is represented by the dotted line. Elution positions of the synthetic peptides are shown by arrows.

labeling period with [³⁵S]cysteine, a prominent radioactive peak was eluted at 38 min, a few minutes earlier than the UV-absorbant CHH peaks (Fig. 1a).

The corresponding autoradiogram shows a peptide with an apparent molecular mass close to 12 kDa, identical to the mass of the labeled prohormone visualized on autoradiograms of crude extract from labeled X-organ-sinus gland complexes (Fig. 1b, lane A). When organs were rinsed for one hour in cold medium after labeling, a lighter band was present on the autoradiogram of crude extracts, with an apparent molecular weight close to 7.7 kDa (Fig. 1b, lane B) matching CHH visible as the major Coomassie Blue-stained band after electrophoresis of a sinus gland extract (data not shown). The possible precursor to product relationship between the 12-kDa and 7.7-kDa bands was confirmed by pulse-chase incubations in the presence of monensin (a compound known to inhibit propeptide processing, Ref. 16) where, even after a 1-h chase, only the 12-kDa band was visible (Fig. 1b, lane C).

After RP-HPLC of extracts from labeled organs chased for 1 h, radioactive mature CHH and [D-Phe³]CHH were characterized (Fig. 1, *c* and *d*, peaks 4 and 5). In addition, several radioactive biosynthetic intermediates were identified, including two forms of prohormones (Fig. 1c, peaks 1 and 2). These peaks have been shown in another study to correspond to the molecule with the native and amidated C terminus, respectively (15). Peptide mapping following tryptic digestion realized on larger scale preparations of these two compounds demonstrated that no fragment corresponding to the CHH N-terminal octapeptide with a D-phenylalanyl residue could be detected in the hydrolysate (Fig. 2).

After radioactive labeling, the percentage of radioactive [D-Phe³]CHH was calculated to be close to 16% (15). If a comparable percentage of a putative D-Phe-containing pro-CHH was present in our preparations, the corresponding D-Phe-containing fragment would have been clearly identifiable after RP-HPLC.

During pulse-chase experiments, another radiolabeled inter-

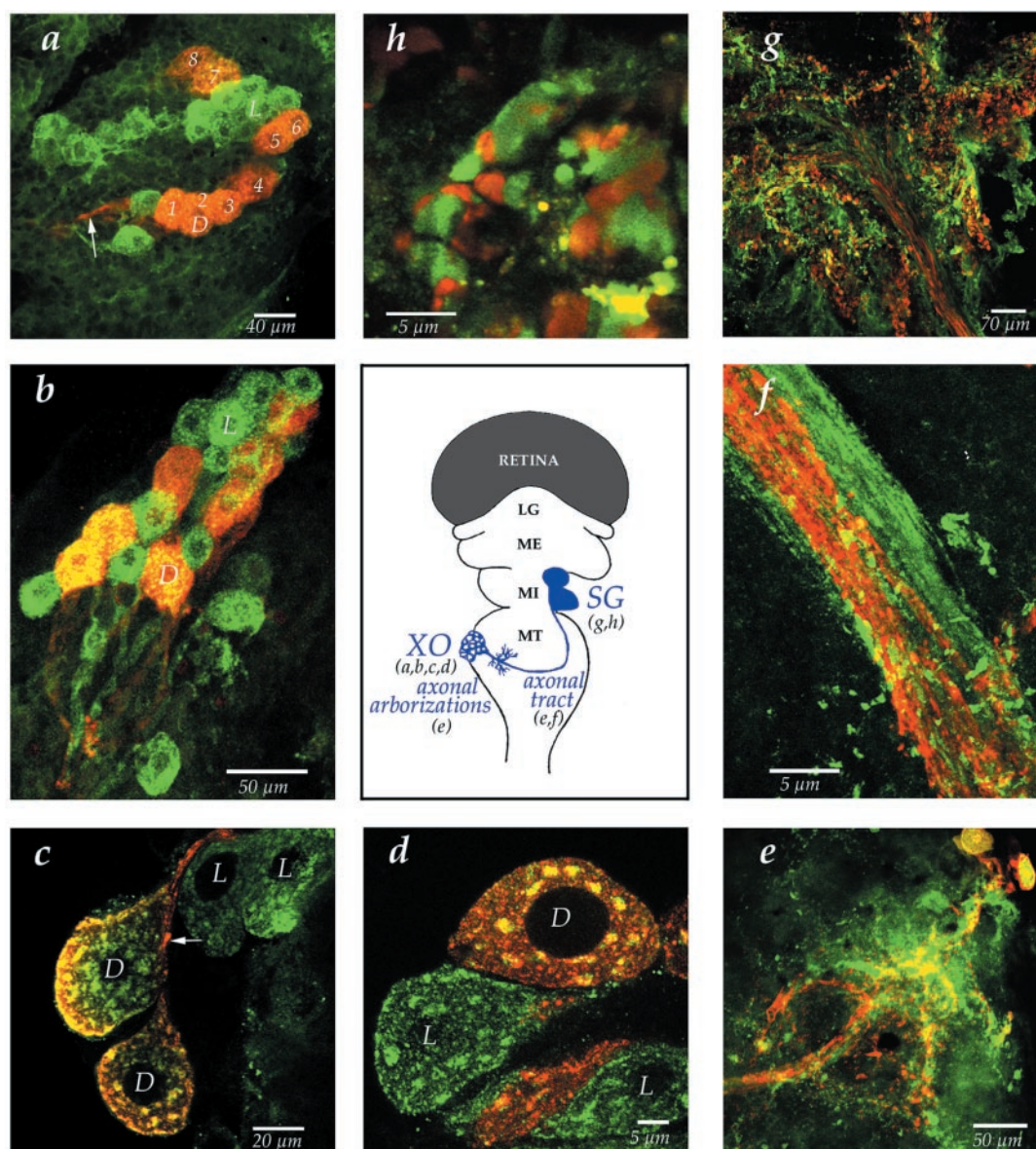


FIG. 3. Multiple confocal micrographs of double immunolabeled crayfish X-organ-sinus gland *in toto*. *gp*-anti-pQL and *r*-anti-pQD antisera were used at dilutions 1:200 and 1:400, respectively. Central drawing: schematic representation of the crayfish eyestalk nervous structures. LG, lamina ganglionaris; ME, medulla externa; MI, medulla interna; MT, medulla terminalis; SG, sinus gland; XO, X-organ. *a*, confocal views of the X-organ showing orange and green cell bodies and red axon (arrow). *b*, view of another organ where the eight orange D-cells are visible. *c*, enlarged view of D-cells showing the different granule cluster colorations and the axon appearing in red (arrow). *d*, cells from another organ with a reduced amount of green granules in the D-cell. *e*, axonal arborization with the X-organ in the upper right corner and both L- and D-arborization types. *f*, both types of axons in the axonal tract. *g*, general view of the sinus gland with both terminal types. *h*, enlargement of axon terminals in the sinus gland. Images *a*, *b*, *e*, *f*, and *g* were collected as a focal series and then processed to give single composite images (extended focus). Images *c*, *d*, and *h* are single optical sections.

mediate was identified as immature CHH form with unblocked N-terminal (glutamyl residue) (Fig. 1, *c* and *d*, peak 3). This assumption was based on the following grounds: MALDI-TOF mass spectrometry showed the presence in corresponding fractions of an ion with a 8421-Da mass versus 8404 Da for the mature CHHs (spectra not shown). The 17-Da difference may be attributed to cyclization of the glutamyl residue present as the N-terminal residue after cleavage of the pro-CHH. This was confirmed by the presence of a radioactive fragment comigrating with the octapeptide with the sequence Glu-Val-Phe-Asp-Glu-Ala-Cys-Lys after trypsinization of peak 3 followed by RP-HPLC (data not shown). Accordingly, peak 3 showed in direct ELISA a clear immunoreactivity against an anti-pQL antiserum (Fig. 1*e*). When ELISA was performed on aliquots of fractions from the same analysis with an anti-pQD antiserum, a minor immunoreactive zone was obtained, in the ascending

front of the major CHH peak (Fig. 1*e*, peak 3'). As for peak 3, MALDI-TOF mass spectrometry of peak 3' fractions demonstrated the presence of an 8421-Da ion, in addition to the 8404-Da CHH ion (data not shown). This indicates the presence in the extracts of an N-terminally unblocked form of [D-Phe³]CHH. The storage of unblocked forms of CHH in crab sinus gland has already been reported (19, 20).

From our data, we conclude that tissue extracts contain immature forms of CHH and [D-Phe³]CHH with unblocked N termini. Therefore, isomerization of the Phe³ residue should occur before cyclization of the N-terminal glutamyl residue of the CHH. Besides, the *in vitro* proteolytic cleavage experiments show that the D-phenylalanyl residue is not present at the prohormone level. Taken together, our results show that isomerization is realized shortly after (or even simultaneously with) propeptide cleavage.

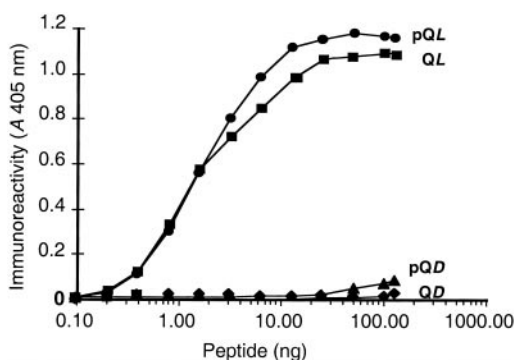


FIG. 4. Specificity of the *r*-anti-pQL antiserum versus synthetic peptides mimicking different N-terminal extremities of CHH or putative CHH intermediates. pQL, pGlu-Val-Phe-Asp-Glu-Ala-Cys-Lys (●); QL, Glu-Val-Phe-Asp-Glu-Ala-Cys-Lys (■); pQD, pGlu-Val-D-Phe-Asp-Glu-Ala-Cys-Lys (▲); QD, Glu-Val-D-Phe-Asp-Glu-Ala-Cys-Lys (◆). Peptides with the sequence pGlu-D-Val-Phe-Asp-Gln-Ala-Cys-Lys and Asp-Gln-Ser-Val-Ser-Lys-Arg-Gln-Val-Phe-Asp-Glu-Ala-Cys-Lys were also included in the assay; these results are not visible because values were always at background level. Absorbance at 405 nm was measured 20 min after addition of the phosphatase substrate.

What Is the Cellular Specificity of the Isomerization Process?—Confocal analysis of whole-mounts of X-organ-sinus gland complexes was performed after double immunofluorescent labeling using specific antisera *gp*-anti-pQL and *r*-anti-pQD. Two distinct cell types were observed, either green fluorescent, indicative of a labeling by the anti-pQL antiserum exclusively (“L-cells”), or orange from the superposition of fluorescein isothiocyanate and rhodamine fluorescence, attesting a labeling by both antisera (for the sake of clarity these cells will be subsequently called “D-cells”, although L- and D-immunoreactivity are both present in their perikarya) (Fig. 3, *a* and *b*).

Organs from animals of both sexes and different physiological status (in terms of molt and reproductive stage) were processed for immunohistochemistry and examined. A constant overall pattern of fluorescence was observed, with the orange D-cells arranged mostly in a horseshoe-shaped configuration at the surface of the X-organ (Fig. 3*a*), except when the organ was accidentally stretched during dissection (Fig. 3*b*). The number of the different CHH cells was also constant with a figure of 8 D-cells over a total of 30 CHH cells. Variations in the coloration (from yellow to dark orange) was observed between different organs or different cells in the same organ (Fig. 3*b*). Nevertheless, the distinction between L- and D-cells was never ambiguous.

In an earlier immunohistochemical study (8), the presence in crayfish and lobster neurosecretory systems of cells labeled either exclusively by the *r*-anti-pQL antiserum or both by the *r*-anti-pQL and *r*-anti-pQD antiserum was described, which suggested that D-cells were actually producing a mixture of both isoforms. Here it was observed that, in the D-cells, secretory granules with different labeling were not evenly distributed in the cytoplasm (Fig. 3, *c* and *d*); *gp*-anti-pQL labeled (green) granules clusters being generally in the perinuclear region whereas the peripheral part of the cytoplasm was enriched with mixed (yellow/orange) or *r*-anti-pQD labeled (red) clusters. A major observation was that in every case, green fluorescence was no longer detectable as the granules enter the axon of these cells (Fig. 3*c*, arrow).

Interpretation of the colocalization of anti-pQL and -pQD immunoreactivity in the perikarya of D-cells required a precise determination of the specificity of the antisera versus the different molecules studied. It was established by direct ELISA on pro-CHH preparations that the specific antisera did not recog-

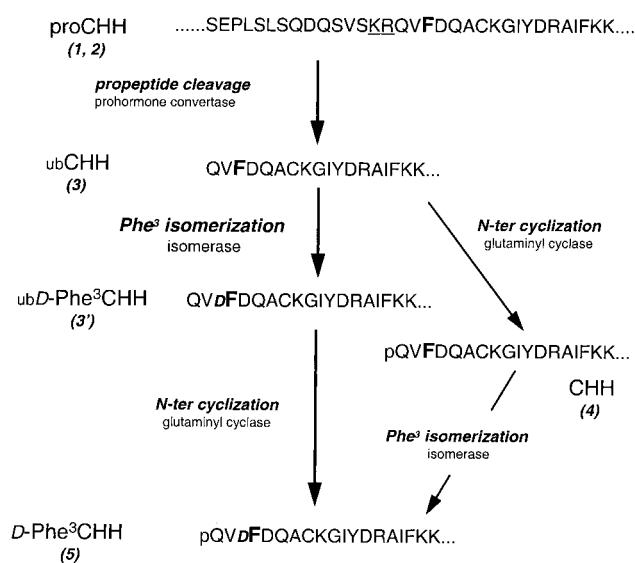


FIG. 5. Proposed sequence of the different steps in the maturation of the CHH precursor leading to [D-Phe³]CHH in the specialized neurosecretory cells. Only part of the prohormone sequence surrounding the isomerization site is shown with the dibasic cleavage site underlined. Sequences are given in one letter code. The phenylalanyl residue subjected to epimerization is in bold. *ub*, N-terminally unblocked. Numbers in parentheses refer to the peaks marked in Fig. 1. The putative processing enzymes are indicated below the different maturation steps.

nize significantly the precursor molecule (data not shown), as it was suggested by the assay depicted in Fig. 1*e*. This may be explained by steric hindrance of the binding of the specific IgGs to the pro-CHH, because of the extension of the molecule on the N-terminal side of the peptide chain. This hypothesis was demonstrated by immunoassays with synthetic peptides (Fig. 4), which showed that the *gp*-anti-pQL antiserum recognized specifically the Glu and pGlu N-terminally ending octapeptides containing a phenylalanyl residue in the L-configuration exclusively.

Consequently, the *gp*-anti-pQL antiserum labeling in the D-cells very likely visualizes the unblocked form of CHH generated by prohormone cleavage, before inversion of the chirality of the Phe³ residue, which fully agrees with biochemical data. The isomerization process is clearly evidenced by the reactivity of the secretory granules with the *r*-anti-pQD antiserum.

Variations in the relative amounts of L and D immunoreactive material observed between different D-cells (Fig. 3, *c* and *d*) may be explained if prohormone cleavage and Phe³ isomerization are occurring with different kinetics. In this context, it is interesting to note that the only peptide isomerase characterized to date, that from *A. aperta* venom, inverts the Ser⁴⁶ of agatoxin with a rather low efficiency, because only 65% of the L-Ser-containing toxin may be isomerized, even after several days of incubation with enzyme preparation (9), probably as a result of reaction reversibility. The situation is very different in crustacean neurosecretory cells producing the D-isomer of CHH, where all of the [L-Phe³]CHH seems isomerized during the maturation process, before the secretory granules leave the perikaryon to migrate within the axon to the neurohemal organ. Although a precise timing of the different events has not been made, it was established in another study (15) that mature radiolabeled CHH and [D-Phe³]CHH are already present at the X-organ level 3 h after the beginning of the experiment.

In the medulla terminalis containing the X-organ-sinus gland complex, axonal arborizations from the two CHH cell types were clearly segregated (Fig. 3*e*). These structures being

classically considered as important regulatory input areas (21), their spatial organizations suggest a differential nervous regulation of the two cell types. This is in agreement with physiological data indicating distinct functions for CHH and D-[Phe³]CHH (12). In the axonal tract, axons from D-cells were grouped together (Fig. 3f), but in the sinus gland L- and D-axon terminals were not segregated but rather tightly intermingled (Fig. 3, g and h).

We have demonstrated in the present work that the isomerization process is an integral part of the cascade of prohormone processing events. A comprehensive maturation scheme of the CHH precursor in the D-cells is proposed Fig. 5.

Although our results indicate that isomerization is realized before CHH N-terminal cyclization, the possibility that this process occurs to some extent on mature (blocked) CHH cannot be excluded, depending upon the substrate specificity of the putative enzyme, which is not known at the moment.

A intriguing aspect of the post-translational isomerization of peptides is the strict positional specificity. This process affects only one given residue near the extremity of the molecule. For example, Phe³ of lobster CHH is epimerized, but Phe¹⁷ is not (6). This may be explained by an optimal accessibility of the peptide termini to a putative modifying enzyme; however, the existence of site-specific recognition, well known for other enzymes such as prohormone convertases (22) cannot be ruled out. To date, this cannot be yet demonstrated when considering the wide variation of the amino acid motifs surrounding the isomerization site in the few D-amino acid containing peptides described so far. Moreover, the modified residue differs according to the peptide considered (*i.e.* alanyl in dermorphin, methionyl in dermenkephalin, asparagyl in fulcin, phenylalanyl in CHH, etc.). This strongly suggests the existence of a family of peptide isomerases with different substrate specificities. The nature and specificity of the members of this putative enzyme family remains to be elucidated. To date, the only enzyme with a proposed peptide isomerase activity isolated so far belongs to the widely distributed subtilisin-like serine protease family (23), like the prohormone convertases.

The modification of the chirality of a given residue in a peptide chain constitutes an elegant way to increase structural diversity and hence to diversify properties and functions from a given genomic motif. Considering the broad range of animal groups where D-amino acid containing peptides have been described (molluscs, arachnids, crustaceans, amphibians), it is tempting to speculate that such a phenomenon should exist in

other groups as well, including higher organisms like mammals and humans. The demonstration of this hypothesis as well as further investigations into the occurrence and physiological significance of such a mechanism is a fascinating research goal.

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