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Evidence for the presence of a secondary structure at the dibasic processing site of prohormone: the pro-ocytocin model

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Bioactivation of pro-proteins by limited proteolysis is a general mechanism in the biosynthesis of hormones, receptors and viral protein precursors. This proceeds by cleavage of peptide bonds at the level of single or pairs of basic residues in the proforms. Examination of a number of cleavage loci in various precursors failed to reveal any consensus primary sequence around the dibasic cleavage sites. Thus it has been proposed, on the basis of secondary structure predictions [Rholam,M., Nicolas,P. and Cohen,P. (1986) *FEBS Lett.*, 207, 1-6], that those basic residues which operate as signal loci for the proteolytic enzyme machinery are situated in, or next to, privileged precursor regions most often constituted by flexible and exposed motifs, e.g. β-turns and/or loops. Peptides reproducing the N-terminal processing domain of the hormone precursor, pro-ocytocin-neurophysin, were examined by a combination of spectroscopical techniques including circular dichroism, infrared Fourier transform and one- and two-dimensional proton NMR. The results indicate that: (i) the region situated on the N terminus of the Lys-Arg doublet is organized as a β-turn in solution; (ii) the sequential organization of the residues participating in the β-turn determines the privileged relative orientation of the basic amino acid side chains and the subtype of turn; (iii) the peptide segment situated on the C-terminal side of the dibasic, corresponding to the N-terminal octapeptide of neurophysin, is organized as an α-helix. It is concluded that the β-turn situated on the N terminus of the dibasic cleavage site is an important feature for processing enzyme recognition and that the respective structures situated on both sides of the β-turn determine the degree of exposure and accessibility of the processing site. Furthermore the data suggest that the subtype of turn and the relative orientation of the Lys and Arg side chains do not play a direct role in substrate recognition.

Key words: β-turn/hormone biosynthesis/ocytocin/prohormone/proteolytic processing

Introduction

Proteolytic activation of macromolecular precursors is a general process frequently encountered in peptide hormones, receptors and viral protein biosynthesis (Darby and Smyth, 1990). The detailed mechanism of substrate recognition by the enzyme machinery and the nature of the proteases participating in this maturation have not yet been fully deciphered. Few endoproteases have been completely purified and proposed as candidate process enzymes (Mizuno and Matsuo, 1984; Gluschankof *et al.*, 1987; Gomez *et al.*, 1988; Maret and Fauchère, 1988; Fuller *et al.*, 1989; Kuks *et al.*, 1989; Plevrakis *et al.*, 1989; Darby and Smyth, 1990; Bourdais *et al.*, 1991). Although cDNAs encoding subtilisin-like protease sequences have been identified as inducers of various pro-proteins in *in vivo* processing (Thomas *et al.*, 1988; Wise *et al.*, 1990; Zollinger *et al.*, 1990), so far little is known about the corresponding enzyme proteins and their mechanism of action.

Examination of pro-protein sequences in databases reveals a certain number of features about the sites where these processing proteases may act. In summary: (i) basic amino acids function as recognition signals; (ii) only a proportion of the dibasic amino acid cleavage sites are indeed cleaved both *in vivo* and *in vitro*, whereas 90% of the monobasic sites remain unprocessed (Devi, 1991; Kuks and Cohen, 1992; Rholam and Cohen, 1992; Bourdais and Cohen, 1991); (iii) secondary structure predictions strongly suggest that, in the case of dibasic cleavage sites, those doublets of basic amino acids are generally situated in exposed and flexible regions of the precursor molecules, i.e. in β-turns or loops (Geisow and Smyth, 1980; Rholam *et al.*, 1987; Bek and Berry, 1990).

The model of the common precursor for ocytocin and neurophysin (pro-OT/Np) is particularly suitable for studying the structural parameters which govern enzyme-substrate recognition and for understanding the mechanisms conferring selectivity to these endoproteolytic reactions. Indeed in this relatively simple prohormone, ocytocin occupies the N-terminal segment of the precursor whereas neurophysin is C-terminal. The Gly-Lys-Arg processing sequence connects the two domains and constitutes the site at which endoproteolytic cleavage occurs both *in vivo* and *in vitro* (Clamagirand *et al.*, 1986, 1987a; Créminon *et al.*, 1988; Brakch *et al.*, 1989).

By using a series of peptides reproducing, or mimicking, various segments of the pro-ocytocin processing domain and a convertase isolated from bovine tissues, we were able to show the importance of certain residues in conferring substrate recognition by the processing protease (Créminon *et al.*, 1988; Brakch *et al.*, 1989). Site directed mutagenesis performed on human pro-somatostatin also demonstrated the key role of proline residues in favouring the adequate conformation for *in vivo* processing in transfected mammalian cells (Gomez *et al.*, 1989; Brakch *et al.*, 1991).

We have now undertaken an extensive study of the solution conformation of given pro-ocytocin-related peptides by a combination of spectroscopical techniques including circular dichroism (CD), infrared–Fourier transform (IR–FT), and one- and two-dimensional proton NMR at 400 MHz. The results point to the existence of privileged secondary structures and suggest the key role of β-turn on the N-terminal side of the Lys–Arg cleavage site in the endoproteolytic reaction.

Results

Pro-ocytocin convertase is a divalent cation-dependent endoprotease identified in endocrine tissues from bovine species (Clamagirand *et al.*, 1986, 1987a; Plevrakis *et al.*, 1989). It produces selective cleavage of the Arg12–Ala13 bond in semi-synthetic pro-OT/Np leading to stoichiometric amounts of OT–Gly–Lys–Arg12 and neurophysin (Brakch *et al.*, 1989). Previous observations demonstrated that, *in vivo*, the Arg12–Ala13 bond cleavage is the first event of a cascade of reactions leading to mature, C-terminally amidated, ocytocin (Clamagirand *et al.*, 1986, 1987a, 1991). By using a series of synthetic substrates reproducing the N-terminal processing domain of pro-ocytocin, we were able to establish a certain number of rules governing substrate recognition by the processing enzyme (Brakch *et al.*, 1989). The aim of the present work is to demonstrate, by using combined spectroscopical methods, the existence of privileged secondary structures at, and around, the cleavage site of pro-OT/Np. Four model peptides representing N- and C-terminal extensions of the [Leu8→Leu15] processing motif of pro-OT/Np have been studied, i.e. [Pro7]-XXIII, Pro7-Leu-Gly-Gly-Lys-Arg-Ala-Val-Leu-Asp-Leu-Asp-Val-Arg20; XXIII, Leu8-Gly-Gly-Lys-Arg-Ala-Val-Leu-Asp-Leu-Asp-Val-Arg20; XXV, Pro7-Leu-Gly-Gly-Lys-Arg-Ala-Val-Leu15 and XXIV, Leu8-Gly-Gly-Lys-Arg-Ala-Val-Leu15.

CD and FT–IR analyses

The CD spectra of XXIV, XXV, XXIII and [Pro7]-XXIII in 2,2,2-trifluoroethanol (TFE)/H_2O are shown in Figure 1. The CD patterns of the two shorter fragments (peptides XXIV and XXV) are characterized by two minima at 199–200 and 218–224 nm, whereas the spectra of the two larger fragments (peptides XXIII and [Pro7]-XXIII) are characterized by a red shift and by an enhanced intensity of the negative dichroism above 200 nm. In addition, the spectrum of [Pro7]-XXIII shows a positive band below 200 nm. These features indicate the existence in solution of: (i) a conformational equilibrium between aperiodic structures and folded conformations (Rholam *et al.*, 1990) and (ii) a higher contribution of folded conformations in the two larger peptides.

The FT–IR spectra of XXIV and XXIII in TFE and TFE/D_2O respectively exhibit maxima in the 1657–1670 cm^{-1} region (Figure 2). The computed second derivative patterns of these spectra show intense bands in the 1660–1678 cm^{-1} range for XXIV and in the 1657–1680 cm^{-1} domain for XXIII. Since bands located between 1665 and 1685 cm^{-1} are generally assigned to different types of β-turns (Byler and Susi, 1986; Bushnell *et al.*, 1990), the above features may be attributed to the existence, in solution, of a population of folded peptide molecules containing in their backbone one, or more, β-turn(s), whereas the band located at 1657 cm^{-1} is attributed to α-helical structure (Dong *et al.*, 1990).

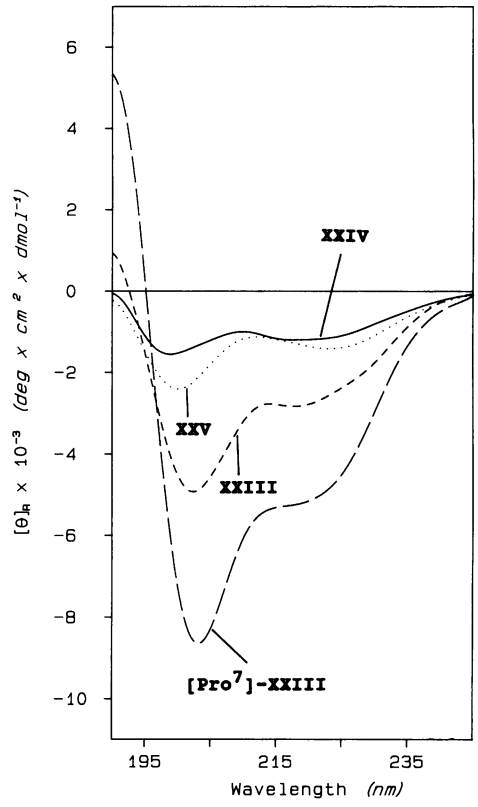


Fig. 1. CD spectra of peptides XXIV, XXV, XXIII and [Pro7]-XXIII in TFE/H_2O (95:5, v/v).

NMR analysis

NMR parameters, i.e. chemical shifts, coupling constants, temperature coefficients of amide protons and intramolecular NOE effects, have been measured for all peptides in the following solvents: DMSO-d_6, DMSO-d_6/H_2O, H_2O/D_2O.

Peptides XXIV and XXV. In all solvents except DMSO/H_2O, the NMR parameters of peptide XXIV point to a disordered structure in solution. In fact, backbone coupling constants are in the range of values typical of random structures. In addition, relatively high temperature coefficients of amide protons as well as the existence of backbone NOE effects only of sequential type support this conclusion. In DMSO/H_2O, on the contrary, weak NH–NH interactions appear in the NOESY two-dimensional spectrum. These interactions (Figure 3b) involve the two peptide fragments that include Gly9, Gly10 and Lys11 residues on one side and Ala13, Val14 and Leu15 on the other. A possible structure based on these data points to two β-turns involving the Leu8–Lys11 and Arg12–Leu15 segments.

Unlike the previous case, NH–NH NOE interactions that are relevant for secondary structure implications can be determined in peptide XXV not only in DMSO/H_2O but also in pure DMSO. Measurements carried out at two different temperatures always show the same weak intramolecular NOE effects between Gly9 and Gly10 and between

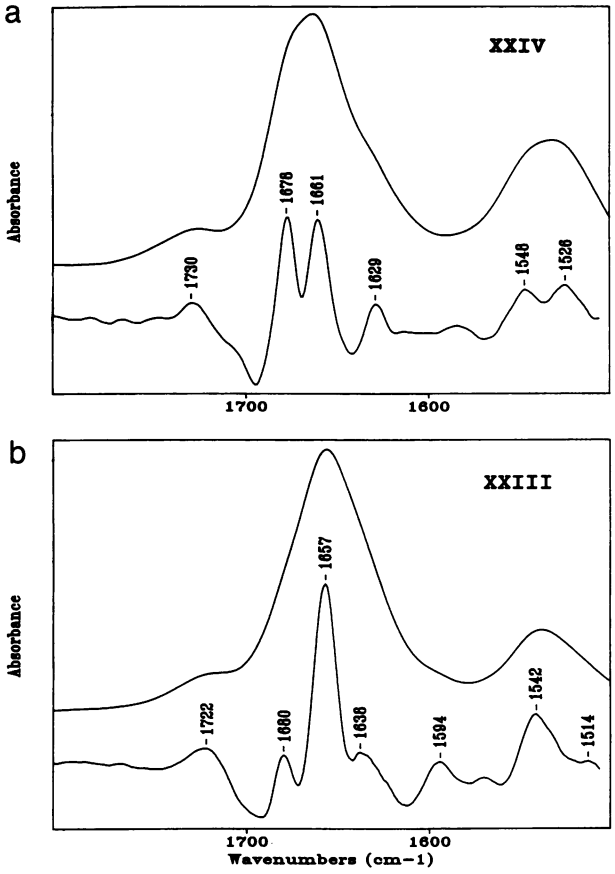


Fig. 2. Infrared spectra in the amide I region of peptide XXIV in TFE and XXIII in TFE/D_2O (90:10, v/v). In each panel the upper line represents the original spectrum while the lower line refers to the second derivative.

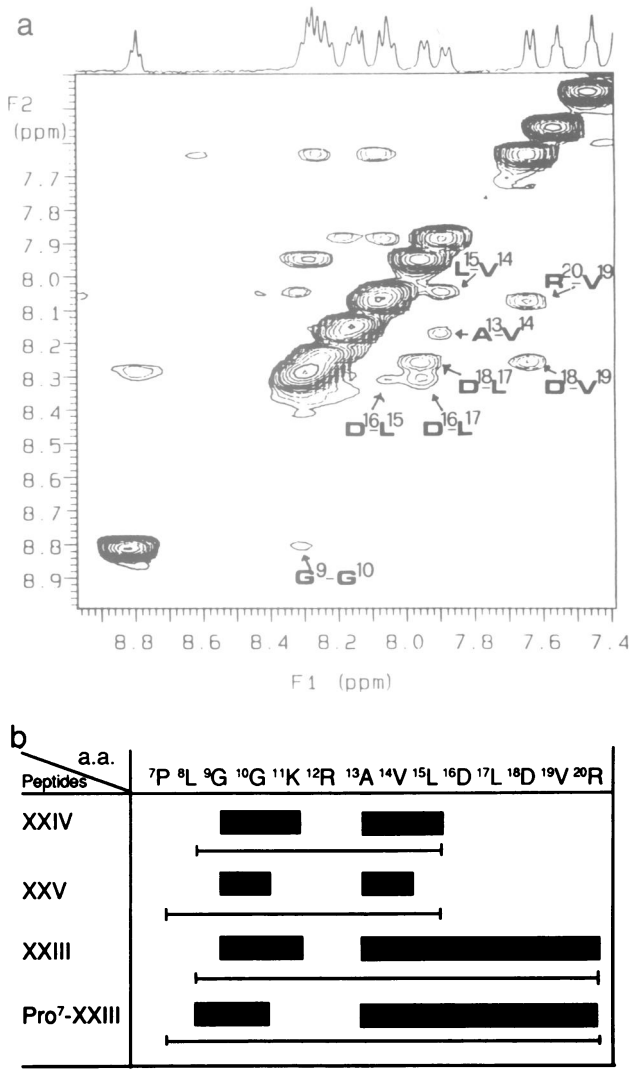


Fig. 3. (a) 400 MHz two-dimensional NOESY map of peptide XXIII. Only the region of the NH resonances where relevant NH−NH cross peaks appear is shown. Experimental details: time domain matrix of 512 × 2048 complex data points mixing, time 200 ms zero filling in F1 before transformation, Gaussian resolution in both dimensions. (b) Schematic representation of NH−NH contacts for the four peptides analysed.

Ala13 and Val14 (Figure 3b). These data point to the existence of two β-turns within the peptide segments Pro7−Gly10 and Lys11−Val14. By simple comparison of the NOE effects with peptide XXV, it appears very likely that the two β-turns are architecturally different.

Peptides XXIII and [Pro7]-XXIII. The proton chemical shifts of these peptides were found to be similar to those reported for the shorter fragments. Backbone coupling constants reveal instead a trend to lower values in going from DMSO to DMSO/H_2O. This fact can be interpreted as indicative of a higher population of folded structures in the latter solvent. Non-sequential backbone NOE effects both in DMSO and DMSO/H_2O (Figure 3a and b) are observed for these two peptides. The effects are stronger than in previous cases and include more peptide units. In particular NOEs between consecutive peptide NHs along the C-terminal segment starting from the Ala13 to the last Arg20 residue, have been measured and strongly support the possibility of a helical-type structure in the C-terminal sequence.

More structurally informative NOE effects are found in the DMSO/H_2O system. They include the NH resonances in the peptide segment Leu8−Gly10. These are quite similar to the effects found for the shorter peptides in the same solvent system. On the basis of the reported NOE effects, it is possible to postulate that a folded region appears in the N-terminal part of these peptides and it is compatible with a β-turn involving the segments Leu8−Lys11 for peptide XXIII and Pro7−Gly10 for the [Pro7]-XXIII peptide.

Molecular models. Energy minimizations were performed in order to build plausible molecular models based on the NOE data determined experimentally for all peptides analysed.

The NH−NH interactions suggest for peptide XXIV a possible structure that involves two β-turns of type I: the first starting from Leu8 residue, the latter from Arg12 residue. These turns are stabilized by intramolecular hydrogen bonding between Leu8 CO and Lys11 NH and between Arg12 CO and Leu15 NH, respectively (Figure 4a). The analysis of the H-bond pattern shows also the existence of additional H-bonds between Leu8 NH and Lys11 CO and between Leu8 NH and the nitrogen atom of the Arg12 side chain (Figure 5). These data underline the existence of successive hydrophobic and hydrophilic domains with Lys

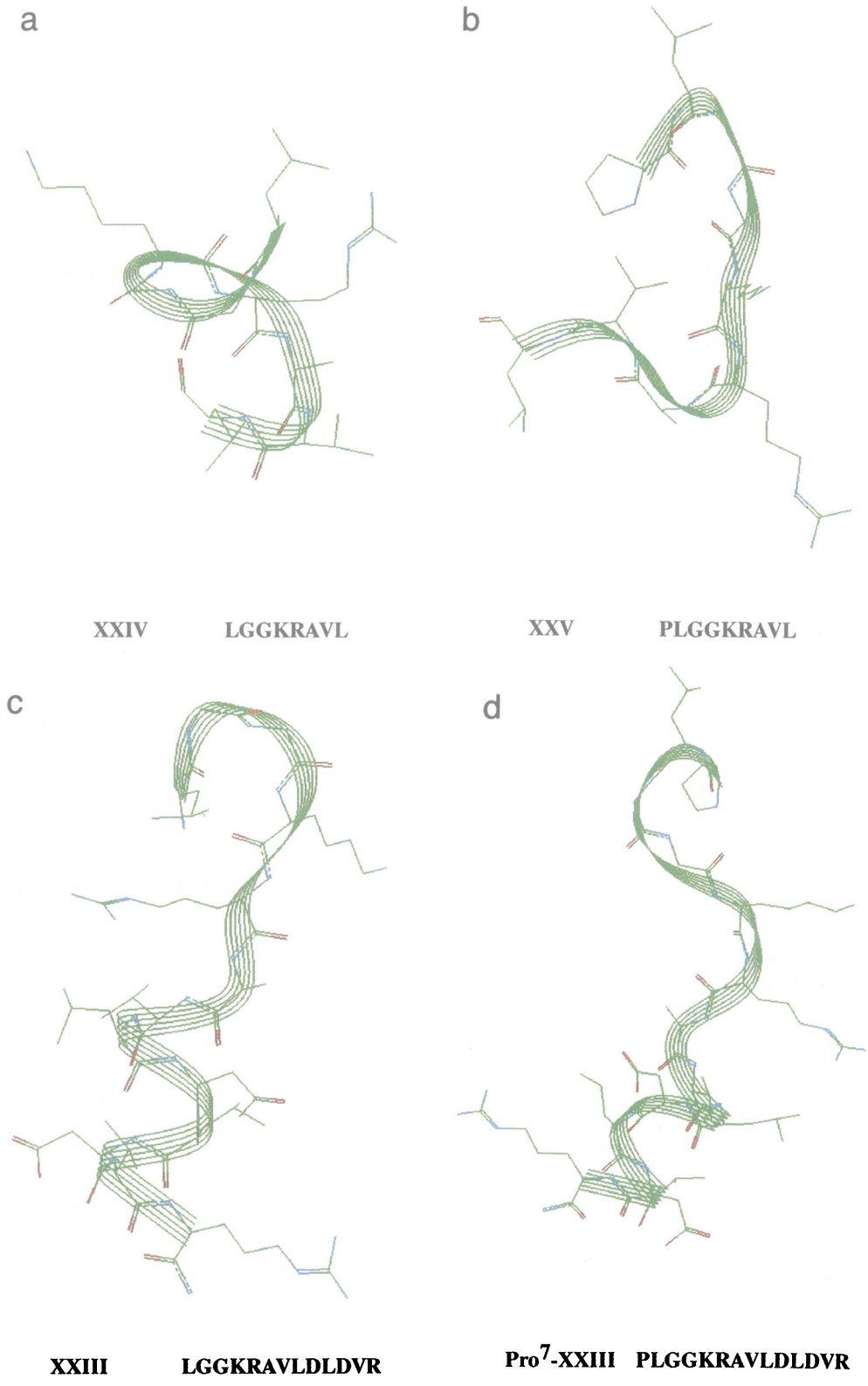
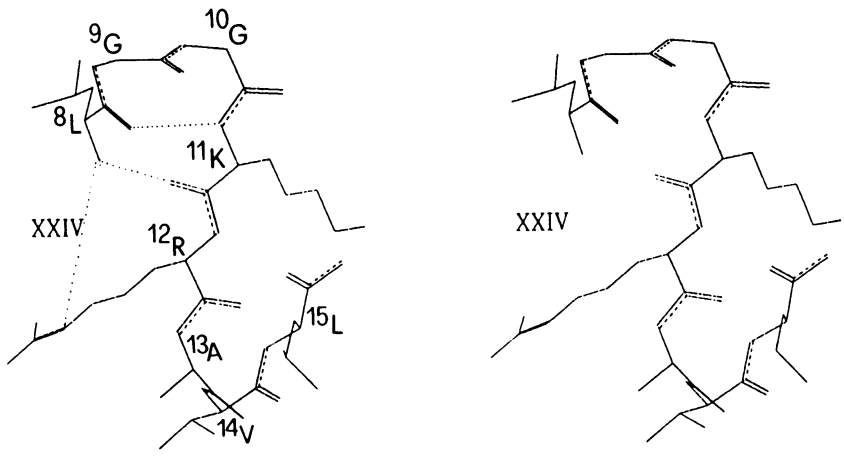


Fig. 4. Stereo views of the molecular models of peptides XXIV (**a**), XXV (**b**), XXIII (**c**) and [Pro]-XXIII (**d**). Molecular models are the result of the minimization procedure carried out with the molecular restraints derived from the measured NOE effects.

and Arg side chains pointing toward opposite directions (Figure 4a).

In the case of peptide XXV, in which a Pro residue has been added to the N terminus, the NOE data suggest that there are two type II β-turns, starting from Pro7 and from Lys11 residues. Four different models were built with all possible combinations of type I and type II β-turns in the peptide segments (7−10 and 11−14). Each model was tested with the minimization procedure. The most stable structure is the one suggested by the NOE data. The analysis of the



PEPTIDE **XXIV** : $^{8}L\text{-}^{9}G\text{-}^{10}G\text{-}^{11}K\text{-}^{12}R\text{-}^{13}A\text{-}^{14}V\text{-}^{15}L$

Fig. 5. Stereo view of the molecular model of peptide XXIV in which the inter-residue hydrogen bonds in the N terminus are shown.

H-bond pattern shows H-bonds between Pro7 CO and Gly10 NH, between Lys11 CO and Val14 NH and an extra bond between Pro7 NH and Gly10 CO. This structure (Figure 4b) is different from that of peptide XXIV: the Lys and Arg side chains are now situated on the same side of the molecule and determine two well defined hydrophobic and hydrophilic domains.

Molecular models of peptides XXIII and [Pro7]-XXIII were built with the same energy minimization procedures (Figure 4c and d). The results yield two slightly different structures. In the N-terminal segment, the two peptides share the same type of structured element analogous to that which were found for shorter peptides (type I β-turn for peptide XXIII and type II β-turn for peptide [Pro7]-XXIII). In the C-terminal part, the two peptides are characterized by a common helical structure starting from the Ala13 residue.

Kinetic data

Table I shows kinetic data obtained with each peptide using the purified pro-ocytocin convertase. They clearly show that whereas the smaller motif (peptide XXIV) was not cleaved as previously shown (Brakch *et al.*, 1989), extension on the N-terminal side by a Pro residue (peptide XXV) led to a good substrate for the enzyme. Similarly the C-terminally extended form of peptide XXIV, i.e. [Leu8→Arg20] pro-OT/Np (peptide XXIII) was cleaved by the enzyme, while Pro extension of the sequence in [Pro7→Arg20] pro-OT/Np (peptide [Pro7]-XXIII) led to a substrate which was cleaved with the same efficiency by the convertase (Table I).

Table I. Kinetic parameters for the reaction of various peptides with the putative pro-ocytocin converting endoprotease

Peptide	V_{max} (pmol/min^{-1})	K_m (μM)
[Pro7]-XXIII	9	290
XXIII	12	400
XXV	13	320
XXIV	–	–

Discussion

The results presented in this work come from different spectroscopical methods including CD, FT−IR and NMR. Each technique complements a piece of information as far as the structural features of the peptides are concerned. Indeed, CD and FT−IR data reveal the existence of conformational equilibria between aperiodic and folded structures in TFE/H_2O solutions. This supports the hypothesis of a minor folded population for those peptides in both organic and aqueous solutions. These findings are confirmed by the rather weak NOE effects observed in DMSO/H_2O and are in agreement with coupling constants and temperature coefficient data that are, instead, typical of averaged structures.

The above results indicate that the folded conformers of the N-terminal domain of pro-OT/Np are organized in such a way that the Lys11−Arg12 cleavage doublet is situated immediately next to β-turns. Therefore, these structural determinations can give wealth to a rationale for an understanding of the behaviour of the peptides studied with regard to their cleavage by a putative pro-ocytocin-converting enzyme (Brakch *et al.*, 1989). In addition, the NOE effects are very revealing from a structural point of view. In combination with computer modelling techniques the following conclusions can be drawn: when the peptide is formed of eight amino acids reproducing the segment 8−15 of the natural prohormone (peptide XXIV), two type I β-turns appear to characterize a folded structure. In this structure the two polar Arg and Lys side chains lie on opposite sides (Figure 4a). N-terminal extension by a Pro residue (peptide XXV) causes a structural modification. The two type I β-turns are then converted into two type II β-turns, the first one including the Pro residue. As a consequence the two Arg and Lys side chains are located on the same side of the molecule thus forming a hydrophilic environment (Figure 4b). Interestingly, similar structural data were obtained with peptides derived respectively from the [Leu8→Leu15] and [Pro7→Leu15] sequences by C-terminal extension (peptides XXIII and [Pro7]-XXIII) (Figure 4c and d).

In the light of these data, it can be inferred that neither the sub-type of β-turn (type I versus type II) nor the

respective orientation of the basic amino acid side chains (either *in trans* as in peptides XXIII and XXIV or *in cis* as for peptide XXIV and [Pro7]-XXIII) plays a key role in enzyme recognition since essentially similar kinetic values were obtained with peptides cleaved by the pro-ocytocin convertase (Brakch *et al.*, 1989).

Peptide XXIV, which is not cleaved by the endoprotease, possesses a compact structure in which one of the two β-turns situated on the N-terminal side of the doublet Lys−Arg is stabilized by an additional H-bond involving the guanidyl residue of Arg12 and the NH moiety of Leu8. Since both N- and C-terminal elongations of this peptide suffice to restore the substrate character of this sequence, it can be hypothesized that this extra H-bond could explain its behaviour relative to cleavage by the putative pro-ocytocin convertase (Brakch *et al.*, 1989). Therefore, these data strongly suggest that the secondary structures organized by the sequences situated around the cleavage site play a critical role in the accessibility to the basic residues. They also provide a rational explanation for previous data showing that Lys11 and Arg12 substitutions by either Nle11 or Nle12 or by D-Lys11 abolished the substrate character of the reference pro-OT/Np [1−20] peptide (Créminon *et al.*, 1988; Plevrakis *et al.*, 1989).

Another straightforward conclusion from the spectroscopy data concerns the peptide segment situated on the C terminus of the Lys11−Arg12 doublet, which, because of its typical succession of polar and hydrophobic residues, tends to organize as an α-helix. This finding, in keeping with previous predictions (Créminon *et al.*, 1988), reinforces the proposal that the neurophysin domain of pro-OT/Np plays a critical role in the maturation of the precursor by participating in the adequate conformation for correct exposure of the restriction sequence. Site directed mutagenesis on cDNA encoding this prohormone will establish the biological relevance of these structural data (G.Boileau *et al.*, in preparation).

Interestingly, reports by others indicated no ordered secondary structure for the N-terminal segment of mature neurophysin in neurophysin−dipeptide complex crystals (Benatan *et al.*, 1991). This may indicate that the stability of this particular segment is directly related to its covalent connexion to the N-terminal [1−12] segment of the unprocessed precursor.

Altogether, the present observations provide the first physico-chemical evidence for the existence of ordered secondary structures at the proteolytic processing site of a simple prohormone. A comparable feature was previously observed in the case of oxyntomodulin, an active fragment of pro-glucagon (Aumelas *et al.*, 1989). It is noteworthy that similar secondary structures were reported at the protein sites undergoing post-translational modifications, as for example phosphorylation (Tinker *et al.*, 1988). It is tempting to hypothesize that such organization may be found as a key feature in different prohormones and pro-proteins (M. Rholam *et al.*, in preparation).

Materials and methods

Peptides

Peptides were synthesized by the solid phase method (Tinker *et al.*, 1988) using either an NPS 4000 semi-automated multisynthesizer (Neosystem, Strasbourg, France) or an Applied Biosystems machine. Peptide purification and analysis were conducted using a set of analytical techniques including HPLC, amino acid composition, N-terminal sequencing and fast atomic bombardment mass spectrometry as in Merrifield (1963). Four pro-OT/Np related peptides were used and are numbered as in Brakch *et al.* (1991) (see Results section for sequences of peptides).

Enzyme assay

Each of the peptides described above was tested for its ability to be a substrate using a purified preparation of the pro-ocytocin convertase isolated from bovine neurohypophysis (Plevrakis *et al.*, 1989). For K_m and V_{max} determinations the standard assay was as follows: 5−25 nmol of peptide were incubated with an aliquot of purified enzyme (0.8 μg of protein) in a final volume of 50 μl of 0.1 M ammonium acetate, pH 7.0, for 5 h. At the end of the incubation period, the reaction was stopped with 0.1 N HCl (10 μl) and the entire sample was subjected to HPLC analysis on a C_{18} column (ultrabase: SFCC-Shandon) eluted with a gradient of 5−40% acetonitrile in 0.05% TFA in 30 min at a flow rate of 0.5 ml/min.

Each of the fragments was identified by reference to standards and by analysis of amino acid composition using a Waters Picotag station.

CD measurements

CD spectra were performed at room temperature (25°C) using a Jasco model J-500 automatic recording circular dichrograph equipped with a Jasco 500 N data processor. Cylindrical fused quartz cells of 0.1 cm path length were used. The CD instrument was standardized with D-10 camphorsulphonic acid and epiandrosterone. Spectra are reported in units of mean residue ellipticity (peptide molecular weight/number of amino acids) $[\Xi]_R$ ($deg \cdot cm^2 \cdot dmol^{-1}$). Samples for all experiments were prepared by dissolving weighed quantities of the peptide in a minimum amount of water then by adding TFE up to a final content of 95% (v/v). Peptide concentration ranged from 1.7×10^{-4} to 4.8×10^{-5} M.

Infrared−Fourier transform spectroscopy

FT−IR spectra were recorded at room temperature using a Perkin−Elmer 1720 Infrared−Fourier transform spectrophotometer, nitrogen flushed at 2 cm^{-1} nominal resolution. Cells with a CaF_2 window and a 0.1 cm path length were used for all experiments. In order to obtain spectra with a satisfactory signal-to-noise ratio, up to 100 scans were collected for each experiment. Solvent baseline spectra were recorded under identical conditions. Data treatment (including apodization, solvent subtraction and derivatization) was performed on an IBM PS/2 model 50-2 personal computer with Spectra Calc. software (Galactic Ind. Corp., Salem, NH, USA). Samples for all measurements were prepared by dissolving weighed quantities of the peptide in the minimum amount of D_2O and then by adding TFE to 90% (v/v). The peptide concentrations in the experiments ranged from 5.4×10^{-3} to 8.7×10^{-3} M.

NMR measurements

NMR experiments were carried out on a Varian Unity 400 spectrometer and on a Bruker AM 400 spectrometer. Solutions were prepared by dissolving 4−6 mg of each peptide in the following solvents: DMSO-d_6 (99.99% isotopic purity, Aldrich), DMSO-d_6/H_2O (70:30, v/v and 90:10 v/v); TFE-d/H_2O (70:30 v/v); H_2O/D_2O (90:10 v/v). D_2O and TFE-d (Aldrich) were 99.9% and 99% isotopic purity, respectively.

NMR chemical shifts were referred to internal tetramethylsilane (TMS) in the case of DMSO and TFE solutions and to internal 3-(trimethylsilyl)-propionic acid−2,2,3,3,4,4-d_4 in the case of water solutions.

Proton chemical shift assignments were made using techniques such as correlated spectroscopy (COSY), homonuclear Hartman−Hahn experiments (HOHAHA), nuclear Overhauser enhancement spectroscopy (NOESY) and rotating frame nuclear Overhauser enhancement spectroscopy (ROESY).

Computational details

The starting models for energy minimization were derived from NOE data. Energy minimizations were performed with the Silicon Graphics IRIS 4D25GT Turbo computer, using the DISCOVER program from the Discover package of Biosym Technologies with the '*ab initio* potentials' of the CVFF (covalent valence force field). The conjugate gradient method was used for all minimizations. Several runs of minimization were performed until the maximum derivative was <0.01 K_{cal}/mol. The solvent-accessible surfaces were computed with the Connolly algorithm.

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