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Conformational studies of the synthetic precursor-specific region of preproparathyroid hormone

(leader sequence/signal sequence/NH_2-terminal extensions/parathyroid hormone)

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ABSTRACT **The secondary structure of a synthetic peptide representing the NH_2-terminal, precursor-specific extension sequence of preproparathyroid hormone was studied. NH_2-terminal extensions, or leader sequences, may serve a critical role in determining and facilitating the cellular secretion of proteins. These precursor regions, including the synthetic hormonal fragment studied, share common features of amino acid sequence and also may be similar in secondary structure. The secondary structure of the synthetic precursor peptide was predicted as described [Chou, P. Y. & Fasman, G. D. (1978) *Adv. Enzymol.* 47, 45–148]. The secondary structure was derived from circular dichroism spectra in both an aqueous buffer at physiological pH and in a nonpolar solvent selected to approximate the intramembranous environment. Two highly structured conformations were observed. In the aqueous buffer the secondary structure was 27% α-helix, 43% β-sheet, and 30% random coil. In the nonpolar solvent the secondary structure was 46% α-helix, 0% β-sheet, and 54% random coil. These findings correlated well with the two highest-probability structures predicted from the amino acid sequence. Both the relatively high content of secondary structure in a peptide of this size (30 amino acids) and the conformational transition observed in changing from aqueous to nonpolar environments may reflect structural properties critical to the physiological function of NH_2-terminal extension sequences, and both are consistent with current theories regarding the role of precursor regions in the intracellular transport and secretion of proteins.**

Parathyroid hormone (PTH), like other secreted proteins (1–14), is initially biosynthesized in a larger precursor form, preproparathyroid hormone (preproPTH), which contains an NH_2-terminal extension, or leader sequence (15). Although the biological role of precursor regions in the biosynthesis, post-translational modification, and intracellular transport of secreted proteins remains to be fully elucidated, it is thought that leader sequences promote the initial steps in protein secretion: the interaction of nascent peptide chains with intracellular membranes and the subsequent entry of protein into the cisternae of the rough endoplasmic reticulum (RER) (16–20).

Although the amino acid sequences of precursor regions from various preproteins differ widely, the NH_2-terminal extensions share common structural features. They are similar in length and contain a central highly hydrophobic sequence (1–15, 21, 22). Furthermore, predictions of the secondary structure of a number of these regions have demonstrated a high degree of conformational similarity among the sequences, despite differences in the physiological function and the species of origin of the proteins examined (23–26). Additional evidence for conformational similarity among leader sequences is suggested by the finding that related preproteins from one species may be accurately cleaved to their mature forms by microsomal membranes derived from another species (27, 28).

At present, quantities of native precursor-specific peptides sufficient to permit conformational studies are not available. *In vivo*, leader sequences are rapidly removed from nascent peptide chains before protein secretion, perhaps before biosynthesis of the entire preprotein is complete (29, 30), and thus far have not been subsequently identified and isolated (31–34). *In vitro*, the entire preprotein can be biosynthesized using cell-free translational systems. However, it is likely that the conformation adopted by the precursor-specific region, when it is contiguous with the complete mature protein sequence, may differ from the conformation it adopts early in protein biosynthesis. Finally, it is not yet practical to obtain large quantities of leader-sequence peptides by exposing preproteins to microsomal membranes *in vitro*.

To study the conformation of an isolated precursor sequence, we used a chemically synthesized peptide representing the NH_2-terminal extension of preproPTH (35). The 30-amino acid, single-chain peptide, [D-Tyr^{+1}]preproPTH-(−29 through +1) amide, contains the "pre" region and the prohormone-specific hexapeptide of PTH (see Fig. 1). For purposes of other biological studies, a D-tyrosine amide was placed at the COOH-terminus to provide a radioiodination site outside the precursor-specific sequence that would be resistant to enzymatic removal (35). The two NH_2-terminal methionines (residues −31 and −30), which are present when mRNA coding for preproPTH is translated in cell-free systems, were omitted because of the evidence that these "initiator" methionines are cleaved from NH_2 termini of nascent chains as they emerge from the large ribosomal subunits and because *in vitro* studies have indicated that both methionines are rapidly removed (36). Hence, it is unlikely that the two NH_2-terminal methionines play a role in the association of preproPTH with RER.

Recent investigations of the biological properties of this chemically synthesized precursor peptide have indicated that the synthetic peptide accurately represents the native precursor-specific sequence. The synthetic peptide can inhibit the membrane-mediated conversion of preproPTH to proPTH in a dose-dependent manner (37). These studies have been extended in our laboratory to preproteins unrelated to preproPTH. Preliminary results have indicated that the synthetic preproPTH peptide inhibits specifically and completely the conversion of three prehormones to their mature forms: prolactin, pre-growth hormone, and pre-placental lactogen (38). These studies have provided evidence that the synthetic precursor region of preproPTH is structurally similar to the precursor regions of native preproteins to the extent that it can compete with native preproteins for binding to a membrane-bound enzyme(s) or receptor.

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Abbreviations: CD, circular dichroism; HFP, 1,1,1,3,3,3-hexafluoro-2-propanol; PTH, parathyroid hormone; proPTH, proparathyroid hormone; preproPTH, preproparathyroid hormone; RER, rough endoplasmic reticulum.

Circular dichroism (CD) spectra of the synthetic precursor peptide were obtained in two different solvents. An aqueous phosphate buffer at physiological pH was used to approximate the cytosolic environment. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFP) was used as an organic nonpolar solvent that might approximate the intramembranous environment. Analyses of secondary structure derived from the CD spectra were compared with the predicted secondary structure obtained by using the Chou and Fasman parameters (39–46).

MATERIALS AND METHODS

The precursor-specific peptide, [D-Tyr^{+1}]preproPTH-(−29 through +1) amide was synthesized by the solid-phase method (47, 48) and purified as described (35). The purified peptide lacked detectable heterogeneity when analyzed by multiple criteria (35).

Secondary structure of the peptide was predicted from the amino acid sequence by using the protein conformational predictive method of Chou and Fasman based on a statistical analysis of 29 proteins with the x-ray atomic coordinates and the ϕ and ψ dihedral angles (39–46).

CD measurements were made with a Cary 60 recording spectropolarimeter equipped with a model 6001 CD accessory, as described (49), with the original photomultiplier tube replaced with an end-on Hamamatsu tube, #R375. All measurements were made at 23°C in a 0.2- to 0.5-mm (path length) cell (Optical Cell, Woodbine, MD) at a full-scale sensitivity of 0.04 deg. Peptide concentration was 0.167–0.606 mg/ml. Secondary structure (% α-helix, β-sheet, and random-coil conformation) was derived from the CD spectra by the method of Greenfield and Fasman (50) by using the α- and β-spectra of poly-L-lysine and the random-coil spectrum of the histones (51) in an iterative computer program to select a best-fit curve. Ellipticity, $[\theta]$, is expressed in deg cm^2/dmol of amino acid residue by using a mean residue molecular weight of 113.

The peptide was dissolved in either 2.5 mM sodium phosphate buffer (pH 7.4) or HFP (Eastman). Peptide concentration was determined on duplicate samples of the peptide-containing solutions by amino acid analysis. After lyophilization, aliquots were acid hydrolyzed (5.7 M HCl, 100°C, 24 hr) in the presence of 1:2000 mercaptoethanol in an evacuated desiccator. Amino acid analyses were performed using a Beckman model 121 MB analyzer. Amino acid content was determined by comparison of sample composition with mixtures of independently quantified standards. Mean peptide content was used to calculate peptide concentration in the samples used for CD analysis.

RESULTS

Use of Chou and Fasman parameters to predict secondary structure (39–46) yielded two highly probable conformations (Table 1). The majority of the leader sequence (positions −29 through −7) has high potential for either the α-helix or the β-sheet conformation, although the β-sheet is slightly favored. According to the criteria for nucleation sites and conformation elongation (39, 40), the α-helix conformation should terminate at −29 and −8, with $\langle P_\alpha \rangle = 1.14$ for this sequence length. (Symbols are explained in the Table 1 legend.) If the β-structure is adopted, it should terminate at residues −27 and −10, with $\langle P_\beta \rangle = 1.18$ for the sequence. However, β-turns must be predicted independently and removed from these regions, as described below.

A second region of interest, positions −10 through −4, includes residues surrounding the peptide bond (between positions −7 and −6) that is cleaved when the leader sequence is removed from preproPTH to yield proPTH (Fig. 1). This entire

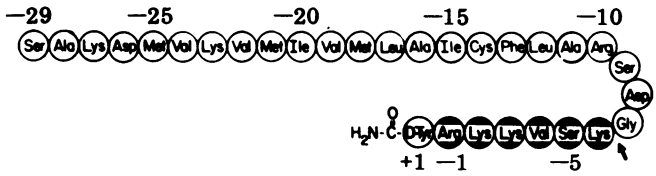


FIG. 1. The amino acid sequence of a synthetic peptide representing the NH_2-terminal, precursor-specific region of preproPTH, [D-Tyr^{+1}]preproPTH-(−29 through +1) amide. An arrow indicates the position of the peptide bond cleaved when the leader sequence is removed from the remaining "pre" sequence (35). The shaded residues indicate the prohormone-specific hexapeptide.

region has been predicted to contain β-turns (43, 45, 46). In particular, the tetrapeptide −10 through −7 has $p_t = 2.65 \times 10^{-4}$. The sequence −9 through −6 also has a high probability of forming a β-turn. Thus, the COOH-terminus of the leader sequence (position −7) is predicted to lie within or at the boundary of a β-turn. If positions −10 through −7 are taken to be in a β-turn (highest p_t value), then additional constraints are placed on the conformation of the NH_2-terminal portion of the leader sequence calculated above. The revised $\langle P_\alpha \rangle$ is 1.17 for the sequence −29 through −11, and the revised $\langle P_\beta \rangle$ is 1.19 for the sequence −27 through −11. Hence, both α-helix and β-sheet conformations are highly probable for the NH_2-terminal region.

The third conformationally discrete region is the prohormone-specific hexapeptide (positions −6 through −1). It has $\langle P_\alpha \rangle = 1.05$ and $\langle P_\beta \rangle = 0.93$. Inasmuch as several α-helix-promoting and β-sheet-breaking amino acids are present, this sequence is predicted to adopt an α-helical conformation.

The two predicted secondary structures are depicted in Fig. 2. The α-helix, β-sheet, and random-coil content for each are listed in Table 2. The predicted structural content of the peptide was found to correspond closely to the observed content derived from the CD spectra (Table 2).

Representative CD spectra of the peptide in phosphate buffer and HFP are in Fig. 3. In phosphate buffer, a single negative trough was observed at 214.5 nm, with $[\theta]_{214.5} = -18{,}800$ deg cm^2/dmol at a peptide concentration of 0.167 mg/ml. In HFP, ellipticity extrema were observed at 205 nm and 220 nm, with $[\theta]_{205} = -23{,}200$ deg cm^2/dmol and $[\theta]_{220} = -17{,}000$ deg cm^2/dmol at a peptide concentration of 0.606 mg/ml. The derived secondary structure of the peptide in each solvent is presented in Table 2. Two conformations of the preproPTH peptide were observed. In phosphate buffer, the peptide is highly structured, containing principally the β-sheet conformation as well as some α-helix. In HFP, a high α-helix content was found but no β-sheet conformation was detected. The higher content of β-sheet in the aqueous buffer cannot be attributed to differences in peptide concentration. In general, higher peptide concentrations promote β-sheet conformation; however, in this case, the peptide concentration in aqueous buffer was less than that in the nonpolar solvent.

DISCUSSION

NH_2-terminal extensions are a common structural feature of nearly all precursor forms of secreted proteins (1–15). These NH_2-terminal peptide segments may represent specialized sequences that serve an essential function in facilitating intracellular transport and processing of secreted proteins (16–20). There has been considerable interest and speculation regarding the relation of structure to function for these sequences (23–26).

By using the predictive method of Chou and Fasman (39–46),

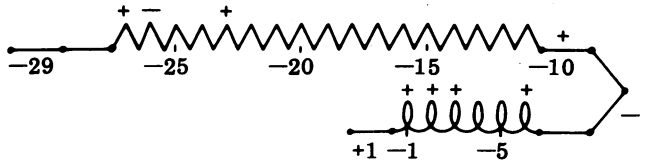
Table 1. Secondary structure parameters for preproPTH peptide predicted from amino acid sequence

Position number (i)	Residue	P_α	$\langle P_\alpha \rangle$	$i \rightarrow i+3$	P_β	$\langle P_\beta \rangle$	$i \rightarrow i+3$	$p_t \times 10^4$	$\langle P_t \rangle$	$i \rightarrow i+3$
−29	Ser	0.77 i		1.09	0.75 b		0.72	0.53		
−28	Ala	1.42 H		1.26	0.83 i		0.79	0.68		
−27	Lys	1.16 h		1.17	0.74 b		1.01	0.04		
−26	Asp	1.01 I		1.17	0.54 B		1.01	0.32		
−25	Met	1.45 H		1.18	1.05 h		1.30	0.12		
−24	Val	1.06 h		1.18	1.70 H		1.30	0.11		
−23	Lys	1.16 h		1.19	0.74 b		1.27	0.02		
−22	Val	1.06 h		1.16	1.70 H		1.51	0.03		
−21	Met	1.45 H		1.26	1.05 h		1.35	0.04		
−20	Ile	1.08 h		1.20	1.60 H		1.41	0.02		
−19	Val	1.06 h		1.28	1.70 H		1.22	0.11		
−18	Met	1.45 H		1.29	1.05 h		1.20	0.03		
−17	Leu	1.21 H		1.10	1.30 h		1.23	0.08		
−16	Ala	1.42 H		1.08	0.83 i		1.25	0.16		
−15	Ile	1.08 h		1.03	1.60 H		1.37	0.10		
−14	Cys	0.70 i		1.12	1.19 h		1.18	0.13		
−13	Phe	1.13 h		1.18	1.38 h		1.11	0.04		
−12	Leu	1.21 H		1.10	1.30 h		0.95	0.49		
−11	Ala	1.42 H		1.04	0.83 i		0.76	0.64		
−10	Arg	0.98 i		0.83	0.93 i		0.74	2.65	β-turn	1.35
−9	Ser	0.77 i		0.88	0.75 b		0.70	2.38		1.36
−8	Asp	1.01 I		0.88	0.54 B		0.70	0.95		1.36
−7 pre	Gly	0.57 B		0.89	0.75 b		0.98	0.78		1.12
−6 pro	Lys	1.16 h		1.04	0.74 b		0.98	0.20		
−5	Ser	0.77 i		1.04	0.75 b		0.98	0.39		
−4	Val	1.06 h		1.09	1.70 H		1.03	0.44		
−3	Lys	1.16 h			0.74 b					
−2	Lys	1.16 h			0.74 b					
−1	Arg	0.98 i			0.93 i					
+1	D-Tyr	—			—					

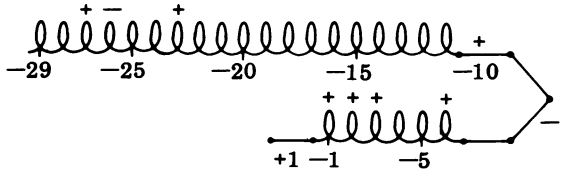
$\langle P_\alpha \rangle = 1.14$ (−29 to −8); $\langle P_\alpha \rangle = 1.05$ (−6 pro to −1); $\langle P_\beta \rangle = 1.18$ (−29 to −7 pre)

$P_\alpha = f_\alpha / \langle f_\alpha \rangle$ and $P_\beta = f_\beta / \langle f_\beta \rangle$ are the α-helix and β-sheet conformation parameters; f_α and f_β are the frequencies of a specific residue occurring in α-helix and β-sheet regions, respectively; $\langle f_\alpha \rangle$ and $\langle f_\beta \rangle$ are the average frequency of residues in the helix and β-regions, respectively; $\langle P_\alpha \rangle$ and $\langle P_\beta \rangle$ are the average helix and β-sheet conformational parameters, respectively, for any calculated sequence; $\langle P_\alpha \rangle_i \rightarrow i+3$ and $\langle P_\beta \rangle_i \rightarrow i+3$ are respectively the average helical and β-sheet potential of tetrapeptides extending from residue i to $i+3$ based on single residue information; p_t is the product of the bend frequencies in the four positions of the β-turn; $\langle P_t \rangle_i \rightarrow i+3$ is the average conformational parameter from residue i to $i+3$ for the β-turn, in which P_t is the average β-turn conformational parameter for a residue based on all four positions. Arrows indicate extent of either α- or β-elongation. However, the β-turns must be excluded from the evaluation of α- and β-regions.

two secondary structures were found to be highly probable for the precursor sequence of preproPTH: a form with high β-sheet content, corresponding to the aqueous conformation, and a form with high α-helix content and no β-sheet, corresponding to the observed conformation in a nonpolar environment.



A. 20% α, 57% β



B. 83% α, 0% β

FIG. 2. Predicted secondary structure of [D-Tyr^{+1}]preproPTH-(−29 through +1) amide. Conformation (*A*) has a high content of β-sheet (〰). Conformation (*B*) is predominantly α-helical (🌀) and devoid of β-sheet. Random coil is indicated by (●—●) The β-turn is indicated between residues −10 and −7.

Earlier predictions by other workers (23, 24, 26) depicted only a single high-probability structure, similar to the high β-sheet conformation depicted in this report (Fig. 2).

The predicted structures correspond to the observed structures of the precursor sequence in different solvents. This finding further supports the utility of the predictive method employed. Secondary structure of the NH_2-terminal peptide of preproPTH was examined by CD. In both the aqueous and the nonpolar solvent, the peptide was found to be highly structured for one of 30-amino acid length. However, the secondary structure differed markedly, depending on the solvent. In aqueous buffer at physiological pH, the precursor fragment assumed a predominant β-sheet structure (43%, Table 2) with

Table 2. Comparison of predicted and CD-derived secondary structures of preproPTH peptide

Structure predicted from sequence	Structure derived from CD
High β-sheet conformation:	In aqueous buffer:
α = 20%	α = 27%
β = 57%	β = 43%
Random + β-turn = 23%	Random = 30%
High α-helical conformation:	In HFP:
α = 83%	α = 46%
β = 0%	β = 0%
Random + β-turn = 17%	Random = 54%

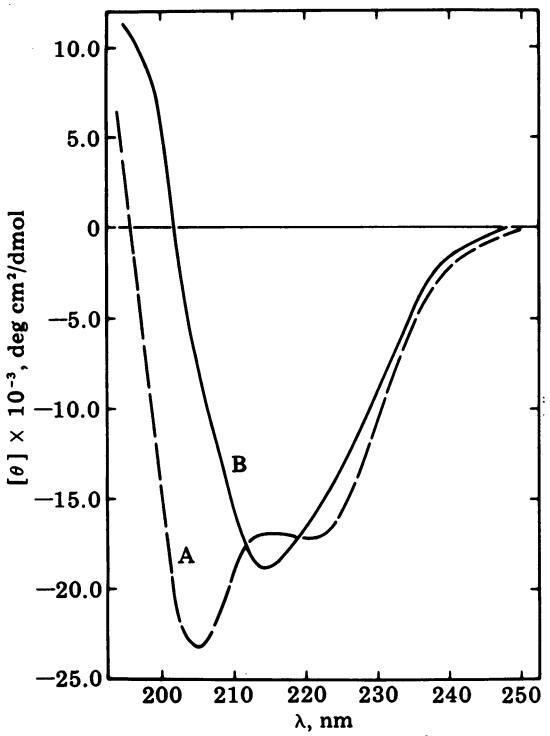


FIG. 3. CD spectra of [D-Tyr^{+1}]preproPTH-(−29 through +1) amide in two solvents: (*A*) HFP (---, 0.606 mg/ml); (*B*) 2.5 mM phosphate buffer (pH 7.4) (—, 0.167 mg/ml). Ellipticity, [θ], is calculated per mole of amino acid residue.

27% α-helix present. In the nonpolar solvent, no β-sheet structure was detected, and the α-helical structure increased to 46%.

Although marked qualitative changes in peptide conformation commonly occur as a result of changes in solvent, these conformation shifts may nevertheless reveal important physical properties essential to a peptide's biological function. The mechanism by which leader sequences may facilitate passage of nascent preproteins across the lipid bilayer of the RER and into the RER cisternal space may be in large part dependent on their conformation and has been visualized in several different manners (16–20, 23). According to Blobel and Sabatini (17) and Blobel and Dobberstein (19), one means by which the precursor segment may accomplish the physiologic role of permitting preproteins to traverse the RER may be that the leader sequence is a "signal" that interacts with a receptor or translocator within the RER. In this manner, the leader sequence may promote the attachment of ribosomes to the RER and the vectorial discharge of nascent protein into the RER cisternal space. According to the signal hypothesis, it is this interaction of the leader sequence with a specific membrane receptor that determines whether a nascent protein remains intracellular or is secreted. Competition experiments with a fragment of ovalbumin have suggested the existence of such a receptor or translocator apparatus (52). Other studies from the same laboratory have indicated that the receptor is distinct from the peptidase that removes the precursor segment (53). The presence of a conformation, either inherent or induced by the receptor and common to nearly all leader sequences, is an essential element of the signal hypothesis. Our conformational data are consistent with a high degree of inherent structure in the preproPTH precursor peptide.

An alternative model, termed the membrane-trigger hypothesis, has been proposed by Wickner (20). In this model, the leader sequence itself contains the physical properties necessary to initiate transmembrane passage of proteins without interacting with a specific membrane receptor. The interaction of the lipid bilayer with the nascent protein is visualized as triggering the folding of the preprotein into a conformation that spans the bilayer and ultimately leads to discharge of the protein into the RER cisternal space. The hydrophobic NH_2-terminal leader sequence interacts initially with the bilayer and then directs the folding of the protein. The energy for transmembrane passage is provided by the appropriate folding of the protein as it enters into the bilayer and, according to Wickner (20), the model is most easily visualized for proteins that can assume at least two different conformations: one in an aqueous environment and another in hydrophobic surroundings. The marked solvent-dependent alterations in conformation of the preproPTH precursor peptide is consistent with the membrane-trigger hypothesis. In the cytosol, the precursor peptide might assume an initial conformation similar to the high β-sheet form observed in aqueous buffer. Interaction with the RER might then cause a transition to a conformation high in α-helix content and devoid of β-sheet. As the α-helix forms, the span of the leader sequence would diminish. This constriction might facilitate or even initiate the entry of the protein into the lipid bilayer. Similarly, once protein traversed the bilayer, vectorial discharge on the cisternal side of the RER might be facilitated by the unfolding of the α-helix, when an aqueous environment was again encountered.

Lastly, leader sequences have been rapidly removed from preproteins by a protease(s) believed to be associated with the inner membrane surface of RER (54). The amino acids neighboring the cleavage site vary from preprotein to preprotein. Hence, it is likely that the specialized protease(s) that cleaves preproteins to yield more mature protein forms may recognize a common secondary structure present in all precursor sequence regions rather than specific amino acid residues present at the site of hydrolysis. Our conformational prediction, like that of others (23, 24), indicates that a β-turn may delineate the COOH-terminus and cleavage site of the leader sequence.

This study provides structural information about a single precursor-specific peptide. As other NH_2-terminal sequences are synthesized or native precursor fragments are isolated, the conformation of the precursor-specific region of preproPTH ultimately may prove representative of this entire class of peptide sequences, and the conformational data obtained may reveal an important relationship between structure and function for these specialized sequences.

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