Chemical Synthesis of the Precursor-specific Region of Pre-Proparathyroid Hormone*

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Michael Rosenblatt, Joel F. Habener, George A. Tyler, Gary L. Shepard, and John T. Potts, Jr.

From the Department of Medicine, Harvard Medical School, and the Endocrine Unit, Massachusetts General Hospital, Boston, Massachusetts 02114

Parathyroid hormone (PTH), an 84-amino acid peptide, is initially biosynthesized intracellularly in a precursor form termed pre-proparathyroid hormone (Pre-ProPTH), which contains an NH₂-terminal extension, or leader sequence. Using the solid phase method, a 30-amino acid peptide representing the precursor-specific region of Pre-ProPTH was chemically synthesized. D-Tyrosine amide was placed at the COOH terminus to provide resistance to exopeptidase degradation and a site for radioiodination. [3H]Glycine and [14C]alanine were incorporated at positions near the COOH and NH₂ termini of the peptide, respectively, to monitor purification of the synthetic product. Purification of the synthetic peptide was performed by gel filtration and ion exchange chromatography under denaturing conditions. Homogeneity of the purified peptide was assessed by multiple criteria including amino acid analysis, Edman sequence analysis, sodium dodecyl sulfate-gel electrophoresis, isoelectric focusing, and thin layer chromatography, as well as by analyses of tryptic peptides prepared from the synthetic precursor-specific peptide. By these criteria, greater than 90% of the synthetic product consisted of a single homogeneous peptide representing the complete sequence of 30 amino acids.

Special difficulties encountered during synthesis and purification of this peptide seemed related to the marked hydrophobicity of the precursor-specific sequence; inasmuch as these issues may be pertinent to synthesis of leader sequences of other secreted proteins, the problems encountered and solutions chosen to overcome them are reviewed. Availability of the precursor-specific peptide of Pre-ProPTH should facilitate investigation of the mechanism of Pre-ProPTH attachment to, transport across, and proteolytic cleavage by rough endoplasmic reticulum, and generation of antisera for use in immunolocalization studies and immunoassays for detection of the precursor-related peptides, if present, in tissue and blood.

Studies of the biosynthesis of parathyroid hormone indicate that the hormone is formed initially as a larger precursor peptide that then undergoes two proteolytic cleavages to yield PTH, the principal form of the hormone stored in and secreted by the gland (1). The major product of translation of parathyroid mRNA in heterologous cell-free systems is a 115-amino acid peptide (Fig. 1), pre-proparathyroid hormone (1). The NH₂-terminal 25 amino acid sequence of this intracellular precursor is representative of a class of peptide sequences present in precursor forms of other polypeptide hormones (2-9), secreted proteins, and enzymes (10-17). These sequence regions have been termed variously leader sequences, signal regions, extra pieces, or pre segments. Leader sequences range in length from 18 to 26 amino acids and are composed principally of hydrophobic residues. The leader sequence is thought to promote the initial association of the nascent polypeptide chain with the rough endoplasmic reticulum and to promote entry of the protein into the cisternal space en route to intracellular transport and ultimate secretion by the cell (18, 19).

Pre-ProPTH is converted in vivo within seconds of its synthesis to an intermediate precursor (Fig. 1), proparathyroid hormone (20-25) by membrane-mediated removal of the leader sequence. ProPTH, which differs from PTH by a highly basic 6-amino acid NH₂-terminal sequence, is converted to PTH by removal of the prohormone-specific hexapeptide in the Golgi apparatus approximately 15 min after the synthesis of Pre-ProPTH (23-27).

In order to provide a means for investigation of the biological role of the precursor regions in hormone biosynthesis and intracellular transport, and for investigation of the mechanism of the successive proteolytic cleavages that follow Pro-ProPTH biosynthesis, we undertook chemical preparation of the precursor-specific region of the hormone. Several structural features were incorporated into the synthetic peptide to facilitate subsequent biological studies. A D-tyrosine amide was placed at the COOH terminus (position +1) to provide a site for radioiodination of the synthetic peptide. This position lies outside the precursor-specific sequence, and a COOH-terminal d-tyrosine amide is expected to be resistant to degradation by carboxypeptidase-like exopeptidases that are present in microsomal membrane preparations. We did not include in our synthesis of the precursor peptide the two NH₂-terminal methionines (positions -31 and -30) which are present when parathyroid mRNA is translated in membrane-free systems. The reasons for this are the evidence that the NH₂-terminal "initiator" methionines are cleaved from the NH₂ termini of nascent chains as they emerge from the large ribosomal subunits and that earlier studies in vitro indicated that both NH₂-terminal methionines are removed (28).

Therefore, it appeared unlikely that the NH₂-terminal two methionines would play a role in the association of Pre-ProPTH with RER.

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‡ Investigator, Howard Hughes Medical Institute.

§ The abbreviations used are: PTH, parathyroid hormone; ProPTH, preproparathyroid hormone; Boc, t-butoxycarbonyl; RER, rough endoplasmic reticulum; Me₂F, dimethyl formamide; SDS, sodium dodecyl sulfate.
leader sequence. Residues are specific to the prohormone. Residues in open circles and hatched circles constitute the NHS-terminal leader sequence. Shaded residues are specific to PTH, the principal secreted form of the hormone. The arrow indicates the peptide bond presumed to be cleaved in removal of the leader sequence.

We anticipated that certain sequence-dependent side reactions might occur during synthesis and that purification of the synthetic peptide might be particularly difficult and require special techniques. In order to minimize these side products, certain side chain protecting groups were selected as part of the synthetic approach, and to facilitate identification of the desired peptide during purification. [D-Tyr+]Pre-ProPTH-(-29--+1)amide was synthesized with incorporation of two radioactively labeled amino acids, one near each terminus of the synthetic peptide, with blocking groups depicted, is shown in Fig. 2.

MATERIALS AND METHODS

Synthesis of [D-Tyr+]Pre-ProPTH-(-29--+1)amide—A fragment of the precursor-specific region of Pre-ProPTH, [D-Tyr+]Pre-ProPTH-(-29--+1)amide was synthesized by a modification (29, 30) of the Merrifield solid phase method (31) using a Beckman model 880 automated synthesizer. Benzhydrylamine hydrochloride resin (poly-styrene, cross-linked with 1% divinylbenzene, Beckman) containing 0.51 nmol of NH₂/g served as the insoluble support. The sequence of the synthetic peptide, with blocking groups depicted, is shown in Fig. 2.

The chloroanisyl group was used to protect the α-amino group of each amino acid during coupling, except arginine, which was protected by the amoxybenzyl group. Side function protection was afforded as follows: (a) the hydroxyl group of serine was protected as the O-benzyl ether (Beckman); (b) the hydroxyl group of N-tyrosine as the O-2,6-dichlorobenzyl ether (Bachem); (c) the ε-amino function of lysine by the 2-chlorobenzyloxycarbonyl group (Bachem); (d) the guanidino function of arginine by the p-toluenesulfonfyl group (Peninsula). (e) the β-carboxyl function of aspartic acid at position −26 by the benzyl group (Beckman) and at position −8 by the phenacyl group (Bachem); and (f) the sulfhydryl function of cysteine by the acetamidomethyl group (Bachem). All other amino acids were obtained from Beckman Instruments and Peninsula Laboratories. Side chain protection for cysteine (position −14) and aspartic acid (position −8) was chosen to permit selective removal of the protecting group at a time other than treatment with hydrogen fluoride (HF).

The cycle of reactions for each amino acid incorporation was as follows: the NH₂-terminal NH₂ group of the peptide-copolymer resin complex (5 g, 2.55 mmol) was deprotected by two prewashes (1.5 min) with 30% (v/v) trifluoroacetic acid (Pierce) in methylene chloride, followed by treatment (30 min) with the same reagent. The peptide-copolymer resin complex was then washed (six times) with methylene chloride (freshly distilled over potassium carbonate). The resulting trifluoroacetate salt was neutralized by two prewashes (1.5 min), followed by treatment (10 min) with 10% (v/v) triethylamine in methylene chloride and followed by washing with methylene chloride four times. Amino acid incorporation was conducted by the addition of 6.4 mmol of Boc-amino acid (2.5 molar ratio) and 6.4 mmol of dicyclohexylcarbodimide in methylene chloride to the reaction vessel. After the mixture was stirred (2 h), the peptide-copolymer resin complex was washed with methylene chloride (six times) to remove the excess of coupling reagents. Completeness of coupling was assessed qualitatively by the fluorescamine test (32). Repeat coupling was necessary to complete incorporation of the following amino acids: serine at positions −6, −9, and −29; lysine at −6 and −23; arginine at −10; leucine at −12 and −17; phenylalanine at −13; cysteine at −14; isoleucine at −20; methionine at −18 and −21; and valine at −19 and −22. Triple coupling was required to obtain a negative fluorescamine test for incorporation of aspartic acid at position −8. After double coupling of isoleucine at position −15, an...
equivocal fluoroscence test was obtained. The peptide-copolymer resin complex was therefore acetylated by treatment with 2.5 ml of acetic anhydride and 0.75 ml of triethylamine in 25 ml of dimethylformamide (Me$_2$F, stored over molecular sieves) for 15 min. The fluoroscence test after acetylation was negative. Acetylation was also performed after single coupling of the first amino acid, d-tyrosine, to the solid support.

Because of the large number of repeat couplings required at many positions to obtain complete amino acid incorporation, another synthesis was attempted in which amino acids were coupled via symmetrical anhydrides (33, 34) instead of the standard carbodiimide-promoted procedure for amino acid coupling. No reduction in required repeat couplings was observed. Since the symmetrical anhydride method uses larger quantities of Boc-amino acid, requires an additional procedure (the preparation of each symmetrical anhydride), and cannot be employed with the synthesizer in fully automated mode, coupling via symmetrical anhydrides was not found to be advantageous for synthesis of the precursor-specific sequence.

Preparation of Boc-[3H]Glycine and Boc-[14C]Alanine—Radioactively labeled amino acids were incorporated at positions -7 and -28. [3H]Glycine and [14C]alanine were purchased from New England Nuclear. Using di-tert-butyl dicarbonate, radioactively labeled and adenine labeled (200-600 mCi), respectively, [3H]glycine and [14C]alanine were prepared by methods previously described (35). Completeness of reaction of Boc group introduction was assessed by thin layer chromatography in a solvent system of chloroform: methanol:acetic acid (85:10:5) using Silica Gel 60 precoated plastic sheets (E. Merck). Glycine and Boc-glycine, or alanine and Boc-alanine were used as markers in lanes adjacent to the extracted products. The radioactive band was quantitated after chromatography, the tlc sheet was exposed to fomograms of concentrated HCl in a covered glass tank for 1 h to remove the Boc group and permit staining with ninhydrin. No unprotected amino acid was detected after reaction. One hundred microcuries of Boc-[3H]glycine was added at the time of coupling to the peptide-copolymer resin and allowed to react for 1 h before the remaining nonradioactive glycine was readded. Boc-[14C]alanine was added by methods previously described (35).

Deprotection of Aspartic Acid at Position-8—After completion of the synthesis, the phenylacetic blocking group was removed by treatment with 1 M sodium thiophenoxide in Me$_2$F at 25°C for 8 h. Sodium thiophenoxide was prepared by cutting clean, shiny sodium metal into small chips and adding to 25 ml of Me$_2$F in an oven-dried, metal-lined plate, nitrogen line, drying tube, and dropping funnel. A slow flow of nitrogen was maintained while 5.5 g (5.13 ml) of thiophenol was added slowly dropwise, with continuous stirring. The mixture warmed to 280 nm was determined using a Beckman model 25 spectrophotometer, and aliquots of each fraction were taken for quantitation of radioactivity by liquid scintillation (Beckman model LS 9000).

After lyophilization of the selected fractions, the partially purified product was applied to a CM-Sepharose 4B (Pharmacia, Uppsala, Sweden) exchange column (1.5 x 20 cm). A Varigard apparatus was used to mix the eluting fractions. Two buffers containing ammonium acetate/5 M urea were used (8 M urea solutions were deionized to remove cyanates by passage through a Rexyn I-300 (Fisher) column immediately prior to addition of ammonium acetate): Buffer A had a conductivity of 8.0 mmho (pH 5.1) and Buffer B had a conductivity of 27 (pH 7.3). A shallow gradient was created by mixing two equal volumes of the selected two fractions of the Varigard apparatus with 100 ml each of Buffer A and the third compartment with 100 ml of Buffer B. Optical density and radioactivity of fractions of the column eluate were determined as described above.

Deprotection of Cysteine—After ion exchange chromatography, the copolymer fraction containing the cysteine residue was applied to a Bio-Gel P-6 (Bio-Rad) column (7.5 x 100 cm). Ultrafiltration absorption at 280 nm was determined using a Beckman model 25 spectrophotometer, and aliquots of each fraction were taken for quantitation of radioactivity by liquid scintillation (Beckman model LS 9000). After lyophilization of the selected fractions, the partially purified product was applied to a CM-Sepharose 4B (Pharmacia, Uppsala, Sweden) exchange column (1.5 x 20 cm). A Varigard apparatus was used to mix the eluting fractions. Two buffers containing ammonium acetate/5 M urea were used (8 M urea solutions were deionized to remove cyanates by passage through a Rexyn I-300 (Fisher) column immediately prior to addition of ammonium acetate): Buffer A had a conductivity of 8.0 mmho (pH 5.1) and Buffer B had a conductivity of 27 (pH 7.3). A shallow gradient was created by mixing two equal volumes of the selected two fractions of the Varigard apparatus with 100 ml each of Buffer A and the third compartment with 100 ml of Buffer B. Optical density and radioactivity of fractions of the column eluate were determined as described above.

Analytical Methods—Amino acid composition was determined after acid hydrolysis in 5.7 N HCl at 100°C in an evacuated desicator for 24, 48, 72, and 96 h in the presence of 1:2000 (v/v) mercaptoethanol. Amino acid analyses were carried out using a Beckman model 121 MB analyzer.

The tlc was performed using a solvent system of pyridine:1-butanol: acetic acid/water (10:15:3:12), and cellulose-coated plates (100 μm thickness, E. Merck) or Silica Gel 60 precoated plastic sheets (E. Merck) and ninhydrin staining. Thin layer electrophoresis was performed using a solvent system of pyridine:acetic acid:water (1:10:289), pH 3.5, and cellulose-coated plates. Tryptic digestion was performed at pH 8.7 by dissolving 500 μg of synthetic peptide in 0.5 ml of 0.1 M NaOAc bicarbonate. Five microliters of a trypsin solution (2 mg/ml, Worthington) was added and the mixture was held for 2 h at 37°C. The reaction was stopped by addition of one drop of glacial acetic acid, followed by lyophilization. Three repeat lyophilizations were performed after resuspending the mixture each time in 0.5 ml of water.

Isoelectric focusing was performed using polyacrylamide slab gels with gel concentration (r), 5%; cross-linked (C), 3%; pH 3.5 to 9.5; amphotere concentration, 2.4% (v/v), LKB Instruments. Bands were detected by 30 min of treatment of the gel with a solution of 150 ml of methanol, 350 ml of water, 17.2 g of sulfosalicylic acid, and 57.5 g of trichloroacetic acid followed by staining with Coomassie blue (0.115 g of Coomassie brilliant blue R250 in 100 ml of a solution of 500 ml of ethanol and 160 ml of acetic acid diluted to 2 liters with water).

Electrophoresis on SDS-polyacrylamide gels was performed using 12.5% labeled peptide (80) and gradient (10 to 20%) polyacrylamide slab gels (39). Automated Edman sequence analysis was performed to detect and quantitate contamination by error peptides containing deletions using a Beckman model 890 C Sequencer employing the single-coupling double cleavage method of Edman and Begg (40) and other previously described methods (41). Manual Edman degradations were performed as previously described (42). Phenylthiobenzyldienicromic (Worthington) was added and the mixture was held for 2 h at 37°C. The reaction was stopped by addition of one drop of glacial acetic acid, followed by lyophilization. Three repeat lyophilizations were performed after resuspending the mixture each time in 0.5 ml of water.

The first cycle of sequence analysis was performed using the Drabek reagent (46) in order to preserve positive charges on the side chains of the lysines near the COOH terminus and thereby decrease extractive losses during repetitive cycles of Edman degradation.

Synthesis of Pre-ProPTH-(29--39) —Synthesis of the leader sequence alone was performed as described above. Merrifield (polystyrene/1% cross-linked divinylbenzene copolymer) resin (Lab Systems), containing 0.75 mg of CICH$_2$/g served as the solid support.

Esterification of the first amino acid, glycine, to the copolymer resin was performed by adding 4.05 mmol of triethylamine to a mixture of 4.5 mmol of Boc-glycine and 6.0 g (0.75 meq of CICH$_2$/g) of ethyl (37).
Synthesis of Parathyroid Hormone Precursor Region

Results

Purification of [D-Tyr.sup.2]Pre-ProPTH(-29+1)amide—The chromatographic profiles for optical density, and 1H and 

\[ ^{14}C \] radioactivity obtained after application of 200 mg of crude peptide to a Bio-Gel P-6 column are co-plotted in Fig. 3. Fractions containing coincident \[ ^{14}C \] and \[ ^{1}H \] radioactivity in constant ratio (Peak 1) were pooled and lyophilized. A later-eluting peak (Peak 2) of presumed lower molecular weight was collected separately; this material also lacked \[ ^{14}C \] radioactivity. These findings suggest that the later-eluting material, deficient in \[ ^{14}C \] radioactivity, represents a peptide or group of peptides that had terminated prematurely during synthesis.

The yield of lyophilized material from Peak 1 (Fractions 50 to 70, Fig. 3) was 54 mg, corresponding to 23% of the material applied or a projected yield of 9.1% relative to the theoretical maximum of starting material. This material was applied to a CM-cellulose ion exchange column. The chromatographic profiles obtained for optical density and radioactivity are co-plotted in Fig. 4. The principal peak detected by optical density contained both \[ ^{14}C \] and \[ ^{1}H \] radioactivity in a constant ratio that was nearly identical to that found in the gel filtration chromatographic profile (Fig. 3). The selected fractions (Fractions 48 to 62, Fig. 4) were pooled and taken without lyophilization for deprotection of the sulfhydryl function of cysteine. After desalting on a Bio-Gel P-2 column, 13 mg of purified peptide was obtained, corresponding to 24% of the material applied or an overall projected yield of 2.1% of the theoretical maximum of starting material.

Chemical Characterization—Synthetic [D-Tyr.sup.2]Pre-ProPTH(-29+1) amide was analyzed for amino acid composition after acid hydrolysis (Table I). Hydrolysis was incomplete after acid treatment for 24 h. Therefore, compositional analyses after 24, 48, 72, and 96 h of acid hydrolysis were performed. The resultant mixture of peptide fragments was applied to a cellulose plate for TLC, followed by thin layer electrophoresis, as described above. A two-dimensional peptide map of the tryptic digest was obtained (Fig. 6). Four peptides were detected (Fig. 6). Although five fragments are expected in theory, the Lys.sup.2.2—Asp.sup.2.6 bond is probably not cleaved by trypsin because of the negatively charged side chain of aspartic acid. This procedure was particularly useful since tryptic fragments migrated by conventional fractionation procedures. Heterogeneity, if present, might have been

![Fig. 3. Elution profile obtained after gel filtration of crude synthetic [D-Tyr.sup.2]Pre-ProPTH(-29+1)amide on a Bio-Gel P-6 column. ————, optical density (280 nm); ————, \[ ^{1}H \] radioactivity; ————, \[ ^{14}C \] radioactivity. The fractions enclosed by arrows (Peak 1) were pooled and lyophilized for further purification. Peak 2 contained a synthetic side product.]

![Fig. 4. Elution profile of ion exchange chromatography using a CM-cellulose column. ————, optical density (280 nm); ————, \[ ^{1}H \] radioactivity; ————, \[ ^{14}C \] radioactivity; . . . . . . . , ionic strength gradient. Fractions from the principal peak (enclosed by arrows) were pooled for cysteine deprotection and subsequent desalting.]

Table I

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* Amino acid contents decreases with increasing exposure to acid. Values noted are the result of extrapolation to initiation of hydrolysis.
Synthesis of Parathyroid Hormone Precursor Region

FIG. 5. Photograph of cellulose-coated TLC plate using a pyridine:l-
butanol:acetic acid:water (10:15:3:12) solvent system. Lane a, crude
synthetic product before purification; Lane b, partially purified ma-
terial after gel-filtration; Lane c, purified peptide after ion exchange
chromatography, cysteine deprotection, and desalting.

FIG. 6. Two-dimensional peptide map of the mixture of tryptic
fragments of [d-Tyr']Pre-ProPTH-(-29-+1)amide obtained by TLC
followed by thin layer electrophoresis.

FIG. 7. a, polyacrylamide gel isoelectric focusing of purified [d-
Tyr']Pre-ProPTH-(-29-+1)amide (precursor-peptide lane). One
hundred fifty micrograms of material was loaded onto the slab gel in
an attempt to reveal the possible presence of minor components.
Lane Hgb contains a human hemoglobin marker. The pH gradient of
the slab gel is indicated. b, autoradiograph of SDS-gel electrophoresis
of 35S-labeled [d-Tyr']Pre-ProPTH-(-29-+1)amide.

detected by low levels of peptides in addition to those expected in theory. In some instances, detection of heterogeneity might be expected to be facilitated by use of the tryptic fragments because changes in a single amino acid might cause greater changes in the TLC and electrophoretic migratory properties of a fragment than of the intact peptide. Presence of such additional peptides would reflect errors in side chain groups; however, no additional peptides were detected.

The synthetic peptide appeared as a single band, pI 9.0, by polyacrylamide gel isoelectric focusing (Fig. 7a). An autoradiograph of 35S-labeled peptide analyzed by SDS-gel electrophoresis demonstrated a large single band, as well as two minor bands (Fig. 7b), which accounted for less than 5% of the total sample by densitometric methods; in fact, some of the apparent heterogeneity might be attributed to the iodination procedure per se.

The synthetic peptide was subjected to automated Edman sequence analysis for 26 repetitive cycles of degradation, to within four amino acids of the COOH terminus. The correct amino acid sequence was confirmed through valine at position -4. Accumulation of error peptides containing deletions was readily detected as “preview” (46, 47). Preview was determined at multiple steps by selecting cycles in which the yield of phenylthiohydantoin derivatives could be most accurately quantitated. At cycle 1, preview of alanine was <1%. At cycle 5, preview of valine was 3.5%. Preview accumulation continued at a rate of approximately 0.5 to 0.75% per cycle through cycle 18, where total preview of alanine was 10%. Hence, the purified synthetic peptide contains no more than 10% contamination by deletion-containing error peptides. These findings are also consistent with the known sensitivity of the monitoring technique for completeness of amino acid addition during synthesis (32). Fluorescamine testing detects an little as 1% of remaining unreacted NH2 groups during amino acid incorporation.

The synthetic side-product (Fig. 3, Peak 2, Fractions 73 to 78) collected after gel filtration that was deficient in 14C radioactivity and also of lower molecular size than the desired peptide was also chemically characterized. The findings suggest that the side product is a COOH-terminal fragment, [d-
Tyr']Pre-ProPTH-(-7-+1)amide, of the desired sequence. Amino acid composition of the peptide was: Gly, 0.8; Ser, 1.0; Tyr, 0.9; Lys, 3.2; and Arg, 1.2. Sequence analysis was consistent with premature chain termination caused by a blocked

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NH$_2$ terminus during synthesis as the mechanism responsible for formation of the side product. Sequence analysis yielded disproportionately small quantities of amino acid residues so that a single sequence could not be determined unambiguously. We have obtained similar results using sequence analysis when the NH$_2$ terminus of a side product peptide was blocked (30).

**Physical Properties of Synthetic Pre-ProPTH-(−29–−7)** — Purification of crude synthetic Pre-ProPTH-(−29–−7) has not been attained because of the poor solubility of the synthetic product in aqueous and organic solvents. Solubility was insufficient for column chromatography in the following solvents: 1 M acetic acid, glacial acetic acid, 8 M urea, 10 M guanidine/H$_2$O acetic acid (concentrated), ethanol, Me$_2$F, dimethyl sulfoxide, 70% propanol/30% ammonium hydroxide (concentrated), ethyl acetate, ethylene glycol, 1:10:289. The peptide was moderately soluble in 30% acetic acid/4 M urea, 30% formic acid/8% propanol, and the aqueous phase of a biphasic solvent system consisting of tetrahydrofuran, water, ethyl acetate, chloroform, tetrahydrofuran, and pyridine:acetic acid:water (1:1:1:1). Desalting was performed using a polystyrene SM-2 (Bio-Rad) column, but adequate purification of the crude peptide was not achieved.

Solubilization in detergents (10% ethanol, 2% Brij 35) followed by gel filtration was performed. As polyacrylamide gel supports of increasing exclusion limits were employed, up to Bio-Gel P-100 (exclusion limit ~100,000 daltons), the peptide (M, = 2762) consistently eluted at the void volume of the column, indicative of extensive aggregation. Similarly, high pressure liquid chromatography was not successful because the peptide did not adhere to CM-cellulose or sulfopropyl (SP)-Sephadex in the presence of detergents. High pressure liquid chromatography is also being tried as a technique for purification, thus far without success. It has, however, been possible to radioiodinate the peptide in dilute solutions (<1 µg/ml) and to use the radioactively labeled peptide in preliminary biological studies (48).

**Discussion**

Chemical synthesis by the solid phase method of a 30-amino acid precursor-specific fragment of Pre-ProPTH was difficult. Most of the amino acid incorporations required double or even triple coupling. In addition, the probable occurrence of adverse sequence-dependent side reactions necessitated the use of blocking groups, not frequently used, for the side chains of two amino acids. The protected β-carboxyl group of aspartic acid cyclizes during HF treatment to form an intrachain succinimide ring when glycine is adjacent and COOH-terminal to aspartic acid (36, 37). To prevent side chain cyclization, the phenylalanine group was used to protect the β-carboxyl group of aspartic acid and selectively removed prior to HF treatment (36, 37). Similarly, to prevent formation of a covalently linked dimer via disulfide bond formation, the acetamidomethyl group was used to protect the sulfhydryl function of cysteine. Acetamidomethyl group protection is resistant to removal by HF. The protecting group can be selectively removed after all purification steps have been performed with the peptide in monomeric form (49).

The purification and chemical characterization of the synthetic product was complicated by its hydrophobic character. Due to adherence to glassware and chromatography supports, yields of the product were substantially lower than has been our experience previously in the purification of peptide fragments representing other regions of PTH (29, 30, 50, 51). Compared to our previously synthesized PTH fragments, buffers of unusually high ionic strength and denaturing conditions were required to elute the peptide from CM-cellulose. A valuable aid to identification of the position of the desired peptide in the chromatographic eluant was the incorporation of two different radioisotopes into the peptide; one isotope was placed at a position near the COOH terminus and the other near the NH$_2$ terminus of the molecule. [H]H Glycine was added at position −7, the COOH-terminal residue of the leader sequence; [3H]alanine was added at position −28, 1 residue removed from the NH$_2$ terminus. Purification of the desired peptide was facilitated by monitoring column chromatography eluents for both $^3$H and $^{14}$C radioactivity; only fractions of the eluant containing both types of radioactivity were taken.

Several criteria indicated that the final isolated product was at least 90% homogeneous. During column chromatographic purification, a product with constant ratio of both types of radioactivity was found (Figs. 3 and 4). Analysis by CM-cellulose (Fig. 4), isoelectric focusing (Fig. 7a), and SDS-gel electrophoresis (Fig. 7b) of the product all showed a single, major component. Furthermore, treatment of the product with trypsin generated four peptide fragments without detectable quantities of other peptides (Fig. 6), providing additional evidence in support of the integrity of the amino acid side chain functions. Amino acid composition conformed to theoretical expectations and contamination as quantitated by Edman sequence analysis of the purified product by deletion-containing error peptides was less than 10%. The finding that preview during Edman sequence analysis accumulated at a near constant rate indicates that no single deletion-containing error peptide predominated among the contaminating peptides. Hence, there may be as many as 10 to 20 different peptides, accounting for a total contamination of 10%. Each of these peptides may be present in a quantity of 0.5 to 0.75% relative to the pure full length peptide, and each differs from the desired peptide by only the deletion of a single amino acid residue out of the total 30-amino-acid sequence. Such contaminants which are nearly identical to the desired peptide and which are present in such low quantities would not be expected to be detected by tlc of the intact peptide. Also, a precise value for total content of error peptides is difficult to estimate since, even in degradation of a totally homogeneous peptide from natural sources, non-specific cleavage results in the appearance of trace quantities of "background" phenylthiohydantoin-derivatives (47).

Premature cleavage can also occur during Edman degradation which in the case of synthetic peptides would be hard to distinguish from preview during Edman sequence analysis. The overall effect of such side reactions is to increase the apparent extent of preview. Thus, the purity of the synthetic peptide may be greater than the estimated 90%.

In addition to the synthesis of the "pre pro" peptide, [p-Tyr$^{38}$]Pre-ProPTH(−29–+1)amide, we also synthesized the leader sequence alone, Pre-ProPTH(−29–−7), in order to study the physicochemical properties of this precursor sequence. However, we encountered difficulties in the purification and characterization of the peptide as a result of unmitting the hydrophilic six-amino-acid prohormone-specific region. Except at low concentrations, the synthetic product is poorly soluble in most aqueous and organic solvents. Multicomponent solvent systems composed of mixtures of organic and aqueous solvents are now being tested to assist in purification and analysis of the peptide, but purification in these solvent systems requires specialized supports, such as polystyrenes, and peptide losses are great. We are now attempting column chromatographic purification in the presence of detergents. Thus far, although solubility is increased in the presence of detergents, the peptide appears to be either aggregated or sequestered within micelles. Therefore, successful fractiona-
tion by gel filtration or ion exchange chromatography has not yet been achieved, and we are, at present, unwilling to conclude that we have successfully synthesized and purified a homogeneous product representing the leader sequence

Precursor fragment of sequence -29—+1 include investigation by gel filtration or ion exchange chromatography has not involve interaction of the leader sequence with a specific receptor and thereby delineate the structural determinants required in the cleavage process. Other studies that are now feasible with the synthetic precursor fragment of sequence -29—+1 include investigation of the nature of precursor region attachment to RER during biosynthesis and the process of translocation. This attachment may be merely the result of nonspecific hydrophobic interactions between leader sequences and membranes or may involve interaction of the leader sequence with a specific receptor in the RER. If receptors are present on endoplasmic reticular membranes, competition by excess quantities of synthetic precursor fragment might inhibit the specific binding by Pre-ProPTH to RER or the cleavage of native Pre-ProPTH by such membranes. Finally, the synthetic precursor peptide will be used as an immunogen to generate antisera that react specifically with precursor sequences for potential use in immunoxytolocalization studies and radioimmunoassays. Such radioimmunoassays could be used for detection of release of precursor forms in vivo should the latter occur, particularly in states of abnormal parathyroid gland function.

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REFERENCES

Chemical synthesis of the precursor-specific region of pre-proparathyroid hormone.

M Rosenblatt, J F Habener, G A Tyler, G L Shepard and J T Potts, Jr