Synthesis of a Fragment of Human Parathyroid Hormone, hPTH-(44–68)

Michael Rosenblatt, Henry T. Keutmann, Geoffrey W. Tregear, and John T. Potts, Jr.*

The Endocrine Unit, Massachusetts General Hospital, and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02114. *Received February 7, 1977*

A 25 amino acid peptide representing an internal region of human parathyroid hormone, hPTH-(44–68), was synthesized by the solid-phase method. This region was synthesized to obtain antisera against the carboxy-terminal two-thirds of PTH and to determine whether portions of the hormone other than the amino-terminal one-third participate in biologic effects resident in the intact hormone. Because human parathyroid hormone differs from the bovine homologue at three positions, the human fragment was prepared. Detailed chemical analysis of the synthetic peptide as well as the side products was performed. Homogeneity was evaluated by amino acid composition, thin-layer chromatography, thin-layer electrophoresis, and polyacrylamide-gel isoelectric focusing. Sequence analysis was performed to detect and quantitate deletion-containing error peptides. Several shorter chain length peptides formed during synthesis. Among the mechanisms responsible for generation of side products are premature chain termination due to steric inaccessibility and cyclization of glutamic acid to form pyroglutamyl peptides. Formation of side products by these mechanisms may be sequence dependent. Synthetic hPTH-(44–68) lacked agonist activity in vitro and in vivo and does not inhibit native parathyroid hormone in vitro, indicating lack of receptor binding by this region of the molecule.

Determination of a large portion of the sequence of human parathyroid hormone (hPTH),[1-4] an 84 amino acid peptide, has made possible the synthesis of a fragment representing the midregion of the hormone. Structural analysis of the region 44–68[5] showed the human differs from bovine hormone (bPTH) at positions 46, 47, and 64, where a substitution of alanine for glycine, glycine for serine, and glutamic acid for glutamine occurs at each of these respective sites (Figure 1). Such a synthetic fragment could be used in an attempt to generate region-specific antisera for the human hormone. The solid-phase method was used to synthesize hPTH-(44–68). The desired peptide was purified to homogeneity as assessed by thin-layer chromatography, thin-layer electrophoresis, amino acid composition, polyacrylamide-gel isoelectric focusing, and Edman sequence analysis for detection of deletion-containing error peptides. The synthetic process also produced shorter chain length side products, separately isolated and identified, that indicate several discrete regions of the molecule where side reactions occur. The synthetic hPTH-(44–68) was evaluated for biologic activity in vitro and in vivo and for possible hormonal antagonism.

Experimental Section

Synthesis and Purification. A fragment of human parathyroid hormone representing the sequence region 44–68 was synthesized by a modification of the Merrifield solid-phase method.[6,7] Synthesis was performed using a Beckman Model 990 automated synthesizer. Merrifield (polystyrene–1% divinylbenzene copolymer) resin, containing 0.75 mequiv of $ClCH_2$/g (Lab Systems, Inc.), served as the insoluble support.

The *tert*-butyloxycarbonyl (*tert*-Boc) group was used to protect the α-amino group of each amino acid during coupling, except arginine, which was protected by the amyloxycarbonyl group. Side-function protection was afforded as follows: (a) the hydroxyl group of serine was protected as the *O*-benzyl ether; (b) the carboxyl group of aspartic acid and glutamic acid was protected as the benzyl ester; (c) the imidazole nitrogen of histidine and the guanidine function of arginine were protected by the *p*-toluenesulfonyl group (protected histidine was stored as the dicyclohexylamine salt and desalted immediately before use); and (d) the ϵ-amino function of lysine was protected by the 2-chlorobenzyloxycarbonyl group. All amino acids were obtained from Beckman Instruments, except α-*tert*-butyloxycarbonyl-ϵ-2-chlorobenzyloxycarbonyl-L-lysine, which was obtained from Bachem Chemicals.

Esterification of the first amino acid, glycine, to the copolymer resin was performed by adding 4.05 mmol of triethylamine (Pierce) to a mixture of 4.5 mmol of *tert*-butyloxycarbonylglycine and 6.0 g (4.5 mequiv of $ClCH_2$–) of copolymer resin in 25 mL of absolute ethanol and refluxing at 80–85 °C for 24 h. Esterification efficiency was approximately 20% based on weight analysis of the reaction products. The *tert*-butyloxycarbonylamino acid–resin complex was washed with ethanol (six times), water (six times), and methylene chloride (six times) and then allowed to swell for 2 h in methylene chloride.

The α-amino group was deprotected by pretreating the peptide–copolymer resin complex (1.5 min) with 30% trifluoroacetic acid (Pierce) in methylene chloride (v/v) followed by treatment for 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% triethylamine in methylene chloride (v/v) and followed by washing with methylene chloride (four times). The peptide bond was formed by the addition of 4.5 mmol of *tert*-butyloxycarbonylamino acid and 4.5 mmol of dicyclohexylcarbodiimide 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 free it of the reagents of coupling. Asparagine and glutamine were incorporated by the "active ester" method. For these amino acids, 9 mmol of the *p*-nitrophenyl "active ester" in dimethylformamide was added and allowed to react overnight. Completeness of coupling was assessed qualitatively by the fluorescamine test.[8] Repeat coupling was necessary to complete incorporation of histidine at position 63, serine at 62, valine at 60, asparagine at 57, aspartic acid at 56, glutamic acid at 55, lysine at 53, and glutamine at 49.

The peptide was cleaved from the copolymer resin with simultaneous removal of the side-chain protecting groups using an excess of doubly distilled, anhydrous hydrogen fluoride at 0 °C for 1 h in the presence of 3.5 mL of distilled anisole per 2.5 g of peptide–resin. Hydrogen fluoride was then removed by distillation under reduced pressure. The peptide–resin mixture was washed with anhydrous ether to remove anisole. The peptide was then extracted by alternate washes of glacial acetic acid and water (four times each). The washes were combined and lyophilized to yield 1.34 g of crude peptide.

Crude peptide was initially purified by gel filtration. Crude hPTH-(44–68), 300 mg, was dissolved in 25 mL of 1 M acetic acid. The cloudy white suspension was centrifuged to remove insoluble material; the supernatant was applied to a Bio-Gel P-6 column (5.0 × 100 cm, Bio-Rad Laboratories). UV absorption at 280 nm was determined using a Beckman Model DB-G grating spectrophotometer. After lyophilization, the partially purified peptide was applied to a CM-cellulose (CM-52, Whatman) ion-exchange column (2.0 × 20 cm). A Varigrad apparatus was used to mix the eluting buffers. Two ammonium acetate buffers were used: buffer A had conductivity 1.5 mmho (pH 5.1) and buffer B had conductivity 20 mmho (pH 6.2). A shallow-sloped conductivity gradient was created by filling the first two compartments of the

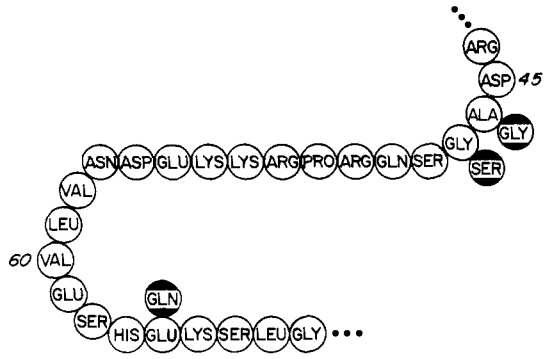

Figure 1. The sequence of the synthetic peptide fragment hPTH-(44–68). Beside positions 46, 47, and 64 are depicted the amino acids that occur in the bovine homologue.

Varigrad apparatus with 100 mL each of buffer A and the third compartment with 100 mL of buffer B.

Later eluting, lower molecular weight side products separated by gel filtration were applied to, but did not adhere to, a CM-cellulose column (1.2 × 15 cm) using a conductivity gradient identical with that described above. This material was then applied to an ion-exchange column (1.2 × 15 cm) of DEAE-cellulose (DE-52, Whatman) and eluted using a conductivity gradient created as described above with a total of 300 mL of ammonium bicarbonate buffer from conductivity 1.0 (pH 8.9) to 20 mmho (pH 7.5).

Analytical Methods. Amino acid composition was determined after acid hydrolysis in 5.7 N HCl at 110 °C in an evacuated desiccator for 24 h in the presence of $^1/_{2000}$ (v/v) mercaptoethanol. Total enzymatic digestion using papain followed by aminopeptidase M and prolidase was performed as previously described.[9] Amino acid analyses were carried out using a Beckman Model 121M analyzer.

Two thin-layer chromatography systems were employed using cellulose-coated plates (100 μ, Brinkmann Instruments) and ninhydrin staining: (A) pyridine–acetic acid–water (30:1:270) and (B) pyridine–1-butanol–acetic acid–water (10:15:3:12).

Two thin-layer electrophoresis systems were employed using cellulose-coated plates and ninhydrin staining: (A) 2% formic acid and 8% acetic acid, pH 2 (600 V, 5–8 mA, 45 min), and (B) pyridine–acetic acid–water (30:1:270), pH 6.5 (600 V, 11 mA, 1.5 h).

Polyacrylamide-gel isoelectric focusing was performed using prepared slab gels: gel concentration T = 5%, cross linkage C = 3%, pH 3.5–9.5, ampholine 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. The precipitated peptide bands appear white and can be readily visualized against a black background.

Automated Edman sequence analysis was also performed, using a Beckman 890C sequencer employing the single-coupling, double-cleavage method of Edman and Begg,[10] and other previously described methods.[11] Manual Edman degradations were performed as previously described.[12] Phenylthiohydantoin derivatives of amino acids were identified by thin-layer chromatography on silica-gel plates (Analtech)[10,13] and by gas–liquid chromatography using a two-column system (10% DC-560 and 1.5% AN-600).[14]

Bioassay. The synthetic hormone fragment hPTH-(44–68) was assessed for agonist activity in vitro by its ability to stimulate cAMP formation in a modification[15] of the rat renal cortical adenylyl cyclase assay of Marcus and Aurbach.[16–18] Parathyroid hormone-like activity in vivo was assessed in the chick hypercalcemia assay.[19] The fragment was also assessed for ability to inhibit the action of purified native bovine PTH[9] in stimulating adenylyl cyclase activity in vitro, by methods previously described for evaluating PTH antagonists.[20]

Results

Purification of the Synthetic Products. The chromatographic profile (UV absorption at 280 nm) of 300 mg of crude synthetic peptide applied to the above-described gel-filtration column is depicted in Figure 2. After amino acid analysis of the material in each peak, peak 1, which eluted at a volume calculated to correspond to a molecular weight of 2300 and which contained 70 mg of fluffy white powder, was selected for further purification by CM-cellulose ion-exchange chromatography. The resulting chromatographic profile is depicted in Figure 3. Purified hPTH-(44–68), 16.4 mg, was obtained in peak B. Analytical data for this material are presented in the next section. Although the other peaks were high in UV absorption, they contained a relatively small amount of peptide (combined total = 10.2 mg). Anomalously high UV absorption and loss of anionic groups can occur as the result of failure to completely remove amino acid side-chain protecting groups or due to the formation of anisole adducts at the time of HF cleavage.[21]

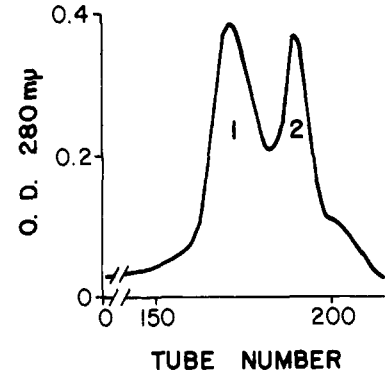

Figure 2. Elution profile of hPTH-(44–68) on polyacrylamide-gel (Bio-Gel P-6) column. Peak 1 contains the desired peptide, hPTH-(44–68). Peak 2 represents lower molecular weight side products.

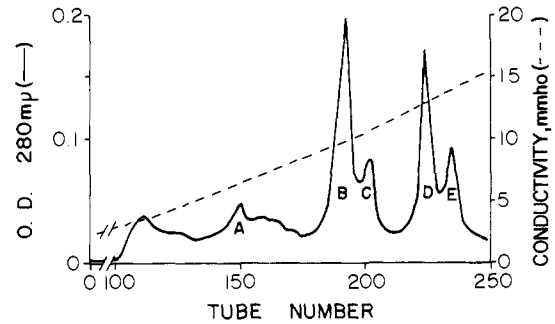

Figure 3. Elution profile of hPTH-(44–68) on a CM-cellulose (CM-52) ion-exchange column employing a conductivity gradient of ammonium acetate. Peak B represents the desired peptide.

Forty-five milligrams of later eluting, lower molecular weight products, represented by peak 2 of the gel-filtration profile (Figure 2), was obtained; 33 mg of this material was separately purified by DEAE-cellulose ion-exchange chromatography, yielding the chromatographic profile of Figure 4.

Analysis of Purified hPTH-(44–68). Synthetic hPTH-(44–68) (CM-cellulose purified peak B, Figure 3) was analyzed for amino acid composition after acid hydrolysis and enzymatic digestion. Composition of the purified synthetic peptide, CM-cellulose peak B (Figure 3), conformed closely to that expected for hPTH-(44–68) (Table I). The peptide was a single component in the two thin-layer chromatography systems described above: R_f = 0.92 in system A and R_f = 0.1 in system B (Figure 5). Thin-layer electrophoresis also demonstrated only one component in the two systems employed; electrophoretic mobility relative to leucine was 1.2 in system A and 1.3 in system B (Figure 5).

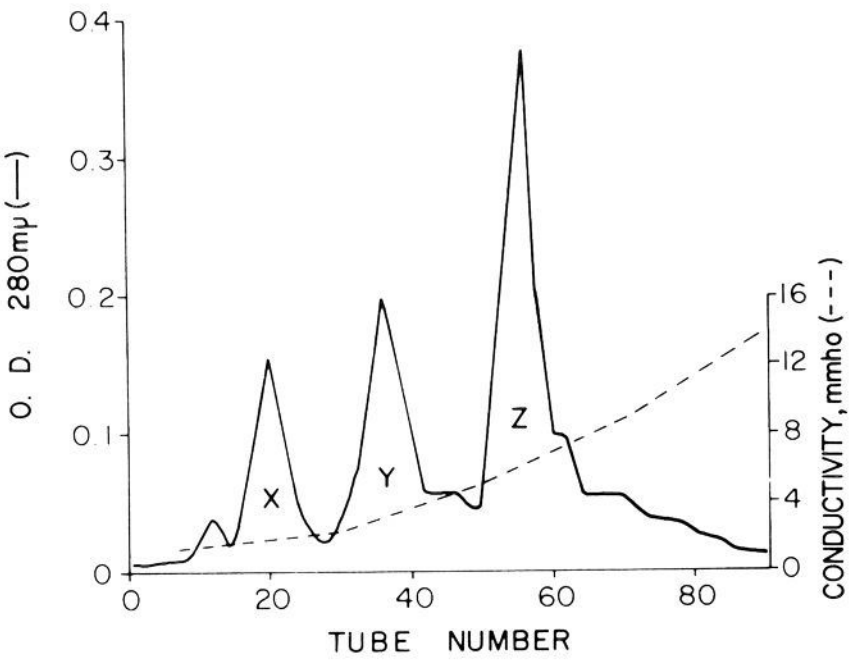

Figure 4. Elution profile of late-eluting synthetic side products (Bio-Gel P-6, peak 2, Figure 2) on a DEAE-cellulose (DE-52) ion-exchange column employing a conductivity gradient. Peaks X, Y, and Z represent COOH-terminal fragments of the desired product.

Table I. Amino Acid Content of Synthetic hPTH-(44–68)[a]

Amino acid	Expected	Obtained
Histidine	1	0.94
Arginine	3	2.99
Aspartic acid[b]	3	3.09
Serine	3	2.78
Glutamic acid[b]	4	4.10
Proline	1	1.03
Glycine	2	2.04
Alanine	1	1.00
Valine	2	2.08
Leucine	2	2.00
Lysine	3	3.19

[a] All values represent the average of four separate aliquots of the peptide after acid hydrolyses for 24 h, expressed as moles of amino acid per mole of peptide. [b] Total enzymatic digestion was performed to evaluate the content of glutamine and asparagine. Three separate digests were averaged: asparagine content was 1.0 (1 expected); glutamine content was 0.8 (1 expected). No side-chain protected amino acid derivatives were detected.

The synthetic hPTH-(44–68) appeared as a single band at pH 8.6 by polyacrylamide-gel isoelectric focusing.

Automated Edman sequence analysis was also performed through approximately three-quarters of the sequence, but, after that point, extractive losses prevented further analysis. Accumulation of deletion-containing error peptides is readily detected as "preview" because of the amplification effect inherent in the Edman method. Preview was determined at all steps in which the yield of phenylthiohydantoin derivatives could be quantitated. The ratio of the yield of phenylthiohydantoin derivative detected one cycle prematurely to that detected at the appropriate cycle of Edman degradation, multiplied by 100%, is the percent preview.

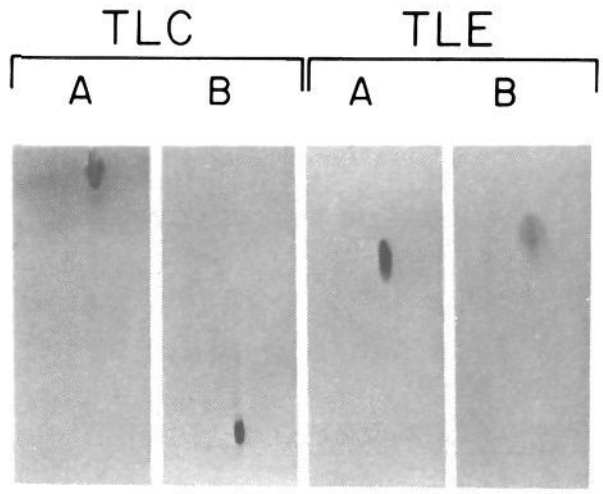

Figure 5. Chromatographic analysis of hPTH-(44–68). Thin-layer chromatography (TLC) systems (A) and (B) shown on left; thin-layer electrophoresis (TLE) systems (A) and (B) shown on right. Systems, R_f values, and electrophoretic mobility are provided in the text.

At cycle 1, 77 nmol of aspartic acid was detected one cycle prematurely; 1930 nmol was detected appropriately at cycle 2. Hence, preview of aspartic acid at cycle 1 is 0.4%. Similarly, the preview of alanine at cycle 2 was 0.7%; 1260 nmol was detected at cycle 3. Glycine preview at cycle 3 was 1%; 740 nmol was detected at cycle 4. Proline preview at cycle 7 was 2.9%; 290 nmol was detected at cycle 8. Leucine preview at cycle 15 was 5.5%; 58 nmol was detected at cycle 16. Hence, the purified synthetic peptide may contain no more than 6% contamination by deletion-containing error peptides.

Analysis of Synthetic Side Products. Edman sequence analysis and amino acid compositional data were used to identify the lower molecular weight side-product peptides of peaks X, Y, and Z of DEAE-cellulose chromatography (Figure 4). Amino acid analyses are given in Table II. These indicate that these fractions are peptide fragments shortened, by various degrees, at the NH_2 terminus. For peak Y, the first cycle of Edman degradation yielded mixtures high in content of valine and leucine and low in asparagine. Subsequent cycles of Edman degradation continued to yield mixtures of amino acids consistent with starting material composed of multiple peptides. Together with the amino acid analysis, Edman sequence analysis suggests that this material represents a family of peptides that cochromatograph in

Table II. Amino Acid Content after Acid Hydrolysis of Side Products of the Synthesis of hPTH-(44–68)[a]

	Peak X, hPTH-(61–68)		Peak Y,[b] mixture		Peak Z, hPTH-(55–68)	
Amino acid	Expected	Obtained	Expected	Obtained	Expected	Obtained
Histidine	1	1.0	1	1.0	1	1.0
Arginine	0	<0.1	0	<0.1	0	<0.1
Aspartic acid	0	0.2	<1	0.4	2	2.1
Serine	2	1.8	2	1.9	2	1.9
Glutamic acid	2	1.9	2	2.1	3	3.0
Glycine	1	1.1	1	1.0	1	0.9
Alanine	0	0.1	0	0.1	0	0.1
Valine	0	0.1	<2	1.2	2	2.2
Leucine	1	1.1	<2	1.6	2	2.0
Lysine	1	1.1	1	1.1	1	1.2

[a] All values expressed as moles of amino acid per mole of peptide. [b] Peak Y represents a mixture of peptides whose NH_2 termini are in the region of 56–60; therefore, aspartic acid, valine, and leucine, which are present at positions 56, 57, 58 and 60, and 59, respectively, are not present in integral quantities.

both purification systems employed. At minimum, the fragment sequences 56–68, 57–68, 58–68, 59–68, and 60–68 are present. This finding is further substantiated by the multiple components (at least six) seen in the several thin-layer chromatographic and electrophoretic systems applied.

Amino acid composition of peak X and peak Z is also given in Table II. However, by Edman degradation, only trace quantities of phenylthiohydantoin derivative could be detected despite subjecting milligram quantities of peptide to Edman sequence analysis, which suggests a blocked NH_2 terminus. Amino acid analysis for peak X is consistent with the fragment sequence hPTH-(61–68) and hPTH-(55–68) for peak Z. In each case, the NH_2-terminal amino acid is glutamic acid. Glutamic acid has been previously suspected of undergoing internal cyclization to the pyrrolidone form during solid-phase synthesis.[22,23] With glutamic acid in this form, the NH_2 terminus would not be degraded by the Edman reaction.

To further test this hypothesis, the peptides of peak X and peak Z were both subjected to three cycles of Edman degradation and then acid hydrolyzed to determine amino acid content. Amino acid composition was identical both before and after three cycles of Edman degradation, indicating blockage of the NH_2 terminus. To determine if NH_2-terminal blockage was due to cyclization of glutamic acid to the pyroglutamyl form, the peptides of peak X and peak Z were treated with the enzyme pyroglutamyl aminopeptidase (Boehringer Mannheim) by dissolving 300 μg of peptide in a solution containing 350 μL of H_2O, 600 μL of 0.1 M ammonium bicarbonate, 50 μL of mercaptoethanol–H_2O (1:32, v/v), 20 μL of 0.05 M EDTA, and 10 μL of pyroglutamyl aminopeptidase (5 mg/mL) and reacting for 3 h at 37 °C. The peptides were then sequenced by the Edman method. Although prior to treatment with pyroglutamyl aminopeptidase, the amino terminus appeared to be blocked, the first four cycles of degradation yielded the sequence Ser-His-Glu-Lys for peak X and Asp-Asn-Val-Leu for peak Z, confirming that peak X is the pyroglutamyl peptide hPTH-(61–68) and peak Z is the pyroglutamyl peptide hPTH-(55–68). In our experience, the efficiency of pyroglutamyl peptidase is approximately 75% under the conditions employed. The yields of the phenylthiohydantoin derivatives in nanomoles were serine = 62, aspartic acid = 45, and glutamic acid = 64 for peak X and aspartic acid = 56, asparagine = 57, and valine = 58 for peak Z. Adjusting for incomplete coupling and recoveries during Edman degradation, pyroglutamyl peptidase deblocked 37% of peak X and 57% of peak Z.

Bioactivity. hPTH-(44–68) demonstrated no agonist activity in the adenylyl cyclase assay system in vitro at all concentrations tested to a maximum of 1.35×10^{-4} M, a concentration 1000-fold higher than that of effective doses of bPTH- or hPTH-(1–84) or -(1–34). The synthetic fragment also failed to demonstrate PTH-like activity in vivo when administered intravenously in the chick hypercalcemic assay. hPTH-(44–68) also failed to inhibit native bPTH-stimulated adenylyl cyclase activity in vitro at all doses tested up to a molar ratio of hPTH-(44–68) to native hormone of 500:1.

Discussion

Synthesis of a human parathyroid hormone fragment, hPTH-(44–68), was undertaken for purposes of evaluating the biologic properties of this region of the hormone, as well as producing region-specific antisera. Although our laboratory has considerable experience in the synthesis of the native sequence, fragments, and analogues of the NH_2-terminal region of parathyroid hormone, this mid-region fragment had not been previously synthesized. Therefore, anticipating that chemical problems different in nature from those previously encountered[24] might arise during synthesis, we undertook detailed chemical and analytical evaluation of the side products as well as the desired hPTH-(44–68) to provide further evaluation of the solid-phase technique.

The major synthetic product after purification not only yielded the expected theoretical amino acid composition but also was found to be homogeneous in several analytical systems of high resolution, including polyacrylamide-gel isoelectric focusing and Edman sequence analysis, the latter performed to quantitate the extent of contamination, if any, by deletion-containing error peptides at each cycle of Edman degradation; if a fraction of the synthetic product did contain a deletional error, such an error would be detected and quantitated as a "preview" of the amino acid expected at the next cycle of degradation.[24] When the NH_2-terminal three-quarters of the synthetic peptide was so evaluated, the cumulative preview was only 6%. Hence, hPTH-(44–68) could be as pure as 94%.

However, a considerable portion of the synthetic product represented peptides of lower molecular weight than the desired hPTH-(44–68), a result not found during synthesis of the NH_2-terminal region of PTH.[24] These side products were isolated and several were identified as COOH-terminal fragments of hPTH-(44–68). At least two separate mechanisms appear responsible for formation of the observed side products.

The first side reaction involves two of the glutamic acids of the sequence, which are present at positions 61 and 55. At each of these positions, some of the glutamic acid γ-benzyl ester appears to cyclize to form the pyroglutamyl peptide. This side reaction has not been observed for other regions of PTH, such as hPTH-(1–34), despite the presence of three glutamic acids in the sequence. Pyroglutamyl acid formation does occur to a small extent at position 6 in the synthesis of hPTH-(1–34), but the cyclization involves glutamine rather than glutamic acid.[24] The variable occurrence of a side reaction involving the same amino acid when it occurs in different regions of the hormone supports the premise that such side reactions are sequence dependent.

The second mechanism responsible for generating truncated sequences appears to be dependent on steric factors such as peptide chain length and peptide–resin interactions.[25,26] Again, this type of premature chain termination appears to be sequence dependent. For hPTH-(44–68), the termination occurs in the region 56–60 and results in formation of a group of prematurely terminated peptides. As expected, early chain termination was not detected during synthesis, despite fluorescamine monitoring for completeness of amino acid addition. Fluorescamine testing only detects one type of synthetic failure, namely, incomplete coupling of an amino acid to the free NH_2-terminal amino function of the growing peptide chain. Peptide chains whose length or conformation makes them sterically inaccessible, or whose NH_2 terminus cannot be successfully deprotected, are not detected by fluorescamine monitoring.

The possibility that such COOH-terminal peptides result from fragmentation of the completed peptide at the time of hydrogen fluoride cleavage is unlikely because complementary NH_2-terminal peptides were not found; however, this possibility cannot be excluded because not all of the side products of synthesis could be definitively identified. Discovery and identification of these peptide side products emphasize the need to purify and thoroughly

chemically evaluate by multiple techniques any synthetic peptide before it is used in a biological application.

The availability of antisera capable of identifying exclusively the COOH-terminal region of hPTH might enhance the clinical efficacy of the PTH radioimmunoassay. Although an immunization program has produced antisera to synthetic hPTH-(44–68), the titers obtained thus far have not been sufficiently high to make practical a COOH region directed radioimmunoassay of requisite sensitivity to detect PTH in blood. Although further immunization programs continue, now that the primary structure of the COOH-terminal region of the hormone recently has been completely elucidated, the establishment of a COOH terminal specific radioimmunoassay can be approached through the synthesis of longer COOH-terminal regions of the hormone, such as hPTH-(53–84) as well as the present peptide.

Although some sequence homology exists between the region 44–68 and the known biologically active NH_2-terminal region 1–34 of the hormone, hPTH-(44–68) lacked both hormone agonist and antagonist activity in the assay systems used in vitro and in vivo. This complete lack of biological activity in several assay systems, as well as the lack of inhibitory effects, suggests that the middle portion of the hormone does not bind to receptors. Thus, we have tested directly earlier assumptions that full agonist activity and receptor-binding requirements for the interaction of PTH with renal and bone receptors reside within the NH_2-terminal one-third of the hormone sequence.[27,28]

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References and Notes

(1) Nonstandard abbreviations: hPTH, parathyroid hormone (human); bPTH, parathyroid hormone (bovine); *tert*-Boc, *tert*-butyloxycarbonyl.
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