Total Solid-Phase Synthesis, Purification, and Characterization of Human Parathyroid Hormone-(1–84)†

T. Fairwell,* A. V. Hospattankar, R. Ronan, H. B. Brewer, Jr., J. K. Chang, M. Shimizu, L. Zitzner, and C. D. Arnaud

ABSTRACT: The complete sequence of human parathyroid hormone [Keutmann, H. T., Sauer, M. M., Hendy, G. N., O'Riordan, J. L. H., & Potts, J. T., Jr. (1978) *Biochemistry 17*, 5723] has been synthesized by the solid-phase method employing the phenylacetamidomethyl resin. Glutamine and asparagine were attached to the peptide chain by using active ester coupling, and all other amino acids were coupled by using dicyclohexylcarbodiimide. Double coupling was employed at every cycle of the synthesis, and both deprotection and coupling were monitored qualitatively. The peptide was cleaved from the resin along with the majority of the side-chain protecting groups by anhydrous hydrogen fluoride, and the formyl group from tryptophan was removed by treatment with 1 M piperidine in 8 M urea. The synthetic protein was purified by ion-exchange chromatography on CM-Sephadex, and the fraction eluting at the position of the native hormone under identical conditions was found to have 100% of the biological activity of the native hormone. This fraction was subjected to further CM-Sephadex chromatography under identical conditions, and the final product was found to be homogeneous by amino-terminal analysis, column chromatography, disc gel electrophoresis, and isoelectric focusing. The overall yield of the pure isolated protein was 12%. The structure of the synthetic protein was verified by (1) sequence analysis using the Edman degradation procedure, (2) amino acid composition, (3) comparison of the biological activity in vitro by the adenylate cyclase assay using dog and rat renal plasma membranes, and (4) comparison of the immunological activity specific to the intact molecule as well as an assay specific for the "mid region" of the molecule with the corresponding native human and bovine hormones.

Parathyroid hormone has a major role in the modulation of the calcium concentration in blood, and the primary amino acid sequence of bovine (Brewer & Ronan, 1970; Naill et al., 1970) and human parathyroid hormone (bPTH and hPTH, respectively)[1] (Keutmann et al., 1978) has been reported. The majority of the biological and physiological studies of hPTH have been performed by utilizing synthetic fragments of the hormone, and fragments covering the complete sequence have been synthesized (Tregear et al., 1974; Rosenblatt et al., 1978). However, the total synthesis of this 84 amino acid peptide by using the solid-phase method was considered difficult because of the limitations associated with the synthesis of large peptides or proteins using the conventional Merrifield resin. A major problem with solid-phase synthesis techniques is the complexity of the final product, especially during the syntheses of long peptides (Merrifield, 1969). In addition to the incomplete reactions at various steps during the synthesis, the peptide can rearrange or cleave off from the solid support. The conventional Merrifield resin, 1% or 2% cross-linked poly(styrene-*co*-divinylbenzene) resin, undergoes extensive acidolysis during long peptide synthesis which reduces the overall maximum yield. As a result, attempts to synthesize larger peptides like ribonuclease A (Gutte & Merrifield, 1971) and human leukocyte interferon (Smith et al., 1981) resulted in relatively low yields. In addition to the yield, the heterogeneity of the final product frequently is significant, making it difficult to isolate the protein to homogeneity. Thus, the stability of the peptide–resin bond to acidolysis is of paramount importance in solid-phase peptide synthesis.

The [[[4-(oxymethyl)phenyl]acetamido]methyl]poly(styrene-*co*-divinylbenzene) resin (PAM resin) introduced by Merrifield and his co-workers (Mitchell et al., 1976) possesses increased acid stability. The electron-withdrawing phenylacetamidomethyl (PAM) group inserted between the peptide and the polystyrene matrix increases the acid stability (e.g., 50% trifluoroacetic acid in methylene chloride) of the peptide ester by 100-fold relative to the peptide ester from the conventional Merrifield resin. Use of this resin not only increases the yield but also has the added benefit of greatly reducing trifluoroacetylation, which results in chain termination. As a result, the PAM resin enhances the applicability of the solid-phase method for the synthesis of longer peptides.

We undertook the chemical synthesis of human parathyroid hormone in order to prove the stability and usefulness of the PAM resin. hPTH was selected because of its size, because it is a well-characterized protein which has been studied in some detail, and, most importantly, because it is in extremely short supply from the native source. We report here in detail the first successful solid-phase synthesis of a long polypeptide, hPTH, by using the PAM resin and its physical characteristics, biological activity, and immunological properties. In addition, the physiochemical properties of synthetic hPTH are compared to those of the native human and bovine parathyroid hormones.

Materials and Methods

The published procedures (Barany & Merrifield, 1979; Merrifield, 1969; Erickson & Merrifield, 1976) for the automated solid-phase method were followed with minor modifications. The PAM resin was prepared according to the

† From the Molecular Disease Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205 (T.F., A.V.H., R.R., and H.B.B.), Peninsula Laboratories, Belmont, California 94002 (J.K.C. and M.S.), and the Endocrinology Section, Medical Service, Veterans Administration Hospital, and the Department of Medicine and Physiology, University of California, San Francisco, California 94121 (L.Z. and C.D.A.). *Received October 25, 1982; revised manuscript received February 4, 1983.* A preliminary account of this work was presented at the 7th American Peptide Symposium, Madison, WI, June 1981.

[1] Abbreviations: hPTH, human parathyroid hormone; bPTH, bovine parathyroid hormone; HF, hydrogen fluoride; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; DMF, dimethylformamide; Cl-Z, α-Boc-ε-*o*-chlorobenzyloxycarbonyl-L-lysine.

0006-2960/83/0422-2691$01.50/0

modified procedure of Tam et al. (1979). The 4-[[[[(*tert*-butyloxycarbonyl)glutaminyl]aminoacyl]oxy]methyl]-benzeneacetic acid was prepared first in excellent yield (40%), as well as purity, and was coupled to the aminomethyl resin (1% cross-linked, 100–200 mesh) by using DCC. A relatively low substitution level of 0.2 mM/g of resin was used in this synthesis. The excess free amino groups of the resin were blocked by acetylation by using *N*-acetylimidazole. A sample of the resin was deprotected with 50% TFA–CH_2Cl_2 and hydrolyzed in HCl–propionic acid, and the glutamic acid was quantitated by amino acid analysis to be 0.197 mmol/g of resin.

The Boc-Gln-PAM resin (3.5 g, 0.69 mM Gln) was placed in the reaction vessel of a Beckman 990B peptide synthesizer, and the instrument was programmed to perform the synthesis automatically. However, a more controlled synthesis with frequent monitoring was performed which, for all practical purposes, was performed semiautomatically. All amino acids were protected at the α-amino position with the *tert*-butyloxycarbonyl group (Boc), a mild acid-labile protecting group, except for arginine, where the more soluble amyloxycarbonyl (Aoc) derivative was employed. The following side-chain protecting groups were used: Asp (OBzl), Glu (OBzl), Ser (Bzl), Thr (Bzl), Lys (Cl-Z), Arg (Tos), His (Tos), and Trp (CHO). The side chain of methionine was left unprotected. Boc or Aoc groups were removed at each cycle of the synthesis by treatment with TFA–CH_2Cl_2. Initially, a 25% solution of TFA was used, and the acid concentration was increased to 30% by step 15 and to 35% by step 25, and a concentration of 40% was used from step 40 to the end of the synthesis. A reaction time of 30 min was found to be sufficient for complete deprotection of even the sterically hindered amino acid residues in this protein.

All couplings, except for asparagine and glutamine, were performed by the dicyclohexylcarbodiimide (DCC) method, which has been used successfully for the synthesis of numerous peptides. A 4.2-mmol sample of Boc-amino acid, a 6-fold excess, in methylene chloride was added to the peptide resin followed by an equimolar amount of DCC in CH_2Cl_2. Because of their low solubility, Aoc-Arg (Tos) and Boc-His (Tos) were dissolved in a 1:1 mixture of CH_2Cl_2–DMF. The reaction time for all DCC couplings was 2 h, and double coupling was employed at every cycle. Both deprotection and coupling were qualitatively monitored by the Kaiser ninhydrin test (Kaiser et al., 1970). Boc-Asn and Boc-Gln were coupled as their *p*-nitrophenyl esters in DMF, employing a 10-fold excess of the Boc-amino acid and a reaction time of 16 h. Double coupling was also employed in these reactions, and after every second active ester coupling, the peptide resin was treated with *N*-acetylimidazole (Markley & Dorman, 1970) to block any unreacted amino group. 1,2-Ethanedithiol (1%) was added to TFA in CH_2Cl_2 during all cleavages after the first methionine was attached to the peptide resin to protect the methionine residues from any acid-catalyzed oxidation. Boc-N^{in}-formyltryptophan was used in order to protect the indole nucleus of tryptophan from oxidation (Ohno et al., 1973). To avoid dilution of the acid and to swell the resin properly, we included a preliminary 2-min wash with acid before the deprotection step. The trifluoroacetate salt was neutralized by a prewash with 10% triethylamine in CH_2Cl_2 followed by two 8-min treatments with the same reagent.

After the complete primary sequence of hPTH was synthesized, the last Boc group was removed with TFA prior to the HF cleavage. The peptide was cleaved from the resin with the simultaneous removal of the side-chain protecting groups by treatment with anhydrous HF. One gram of the protected peptide resin was treated with anhydrous HF (10 mL) in the presence of anisole (2 mL) and methyl ethyl sulfide (0.25 mL) as scavengers at −20 °C for 15 min and at 0 °C for 60 min as previously described (Sakakibara et al., 1967). Excess HF was removed under high vacuum, and the residue was washed 3 times with anhydrous ether. The peptide was extracted with aqueous acetic acid (10%) and lyophilized. The crude peptide was treated with 50 mL of 5% sodium bicarbonate in order to reverse any N → O acyl shift, which might have occurred during the HF cleavage (Sakakibara et al., 1962). After 2 h at pH 7.5, the synthesized polypeptide was dialyzed at 4 °C against distilled water and lyophilized. The N^{in}-formyl group from the tryptophan was removed by treating the protein with 1 M piperidine in 8 M urea at 0 °C for 45 min (Ohno et al., 1973).

Purification. The crude peptide was initially desalted by gel filtration on a Bio-Gel P-2 column in 0.1 M acetic acid. The fractions corresponding to the major peptides were pooled and lyophilized, and the protein was chromatographed on a CM-Sephadex ion-exchange column (13 × 0.5 cm). The column was equilibrated with 0.05 M ammonium acetate in 6 M urea at pH 5.6 (buffer A). Crude synthetic hPTH was dissolved in 0.025 M ammonium acetate in 6 M urea and applied to the column. Initially, 30 mL of buffer A was passed through the column, followed by a gradient of 0.11–0.25 M ammonium acetate–6 M urea at a flow rate of 6 mL/h. All fractions were collected and tested for the biological activity in vitro by the activation of renal adenylate cyclase. Fractions showing maximal activity (90–100%) were pooled and desalted on a Bio-Gel P2 column in 0.1 M acetic acid. The synthetic peptide was further purified by two additional fractionations under similar experimental conditions which yielded a single component on CM-Sephadex C-25 chromatography. The final yield of the purified synthetic hPTH was 12%. Native human and bovine parathyroid hormones were purified by gel filtration and ion-exchange chromatography as described earlier (Brewer & Ronan, 1970; Brewer et al., 1972).

Purity Assessment. Amino acid sequence analysis of the synthesized peptide was carried out in a modified Beckman 890B peptide sequencer by using the procedure described by Fairwell & Brewer (1979). Approximately 600 nmol of the synthetic peptide was used, and the degradations were carried out for 60 cycles. The phenylthiohydantoin amino acids were identified quantitatively by high-performance liquid chromatography (HPLC) (Zimmerman et al., 1977) as well as by chemical ionization mass spectrometry (Fairwell & Brewer, 1980). A large quantity (5–10%) of the derivative of each step in the sequence was used for the HPLC analysis, which permitted the detection of a preview of the next residue of as little as 0.01%. The amino acid composition of the crude peptide as well as the purified material from the CM-Sephadex column was determined on a Beckman 120 analyzer after acid hydrolysis in constant-boiling HCl at 110 °C for 24 h in the presence of mercaptoethanol.

Polyacrylamide gel electrophoresis in the presence of 8 M urea was performed at pH 4.4 (0.6 × 8.5 cm glass tubes) by utilizing a stacking gel (2.5% acrylamide with 7.4% cross-linkage) and a running gel (10% acrylamide with 2% cross-linkage). The anodic buffer (pH 5.3) contained 0.05 M KOH–0.06 M acetic acid in 2 M urea, and the cathodic buffer (pH 6.1) was 0.05 M β-alanine and 0.045 N acetic acid in 2 M urea. Protein samples (10–15 μg) were dissolved in 50 μL of the cathodic buffer containing 25% sucrose. Methyl green was used as the tracking dye. Electrophoresis was performed

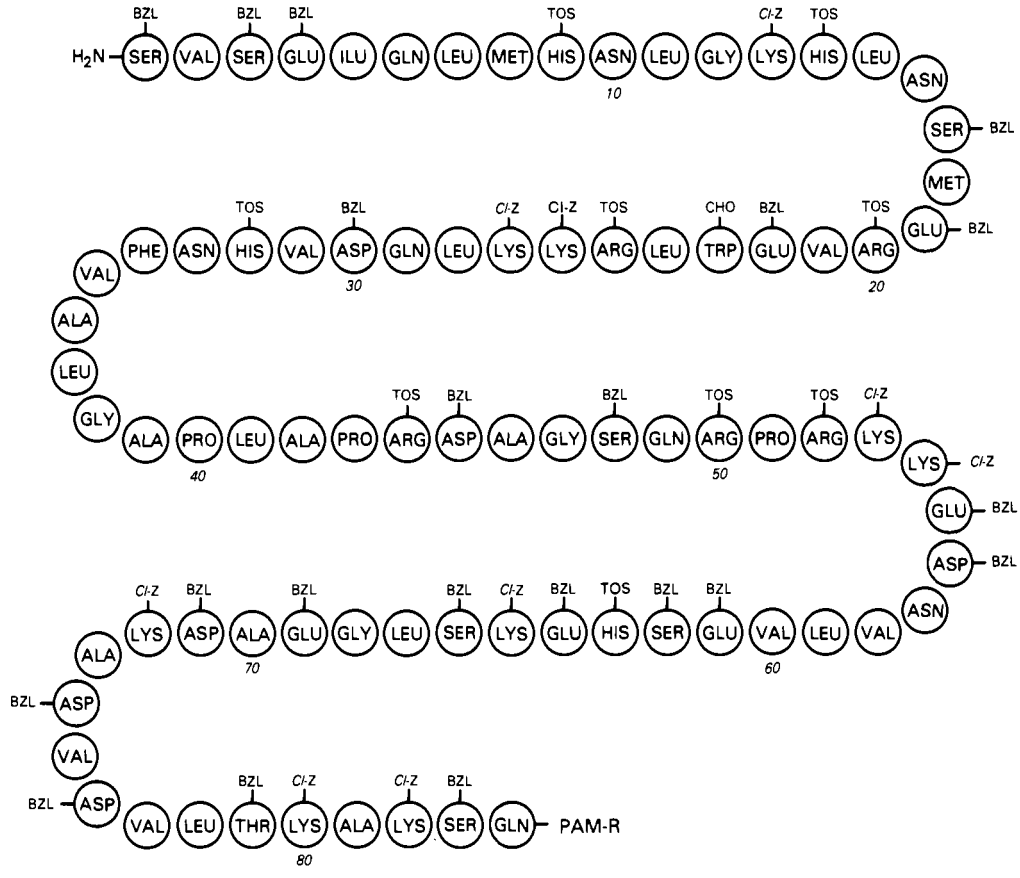

FIGURE 1: Amino acid sequence of hPTH showing the side-chain protecting groups used during the synthesis. The synthesis was started from the glutamine at the C terminal.

at 2–5 mA/tube for 2 h, and the gels were fixed with 20% trichloroacetic acid for 16 h followed by staining with 1% Coomassie blue for 6 h. Destaining was carried out with 7.5% acetic acid.

Polyacrylamide gel isoelectrofocusing was performed at a pH range of 3–10. Protein samples were isoelectrofocused in a 7.5% acrylamide disc gel (0.6 × 7.5 cm glass tubes) containing 8 M urea and 3% ampholine for 21 h by applying a constant voltage (250 V). Gels were stained with 0.2% Coomassie brilliant blue G-250 in 12% trichloroacetic acid for 16 h and destained with 7.5% acetic acid.

Bioassay and Immunoassay. Biological activity of the synthetic peptide in vitro was performed in two systems by using rat renal and dog renal plasma membranes (Krishna et al., 1968; Marcus & Aurbach, 1971; Nissensen & Arnaud, 1979). The dose–response curve of the peptide was determined in triplicate within the linear portion of the curve. An MRC standard, bovine native parathyroid hormone (MRC 72/286), with a potency of 2600 IU/mg, was used as the standard.

The immunological assay for hPTH was performed according to the procedure described by Arnaud et al. (1971). "Intact" PTH was assayed by using antiserum CM-12M. Midregion-specific immunoreactivity was assessed by using antiserum GPIM (Gallagher et al., 1980).

Results

The amino acid sequence of human parathyroid hormone with the side-chain protecting groups assembled on the PAM resin is illustrated in Figure 1. The total weight of the fully protected peptide–resin was 85% of the calculated theoretical amount, based on the amount of glutamine initially attached to the resin. This can be considered as a nearly quantitative yield, considering the fact that small aliquots of sample were removed twice at each cycle for the Kaiser ninhydrin test. Amino acid analysis of the crude peptide showed a 64% yield following HF cleavage.

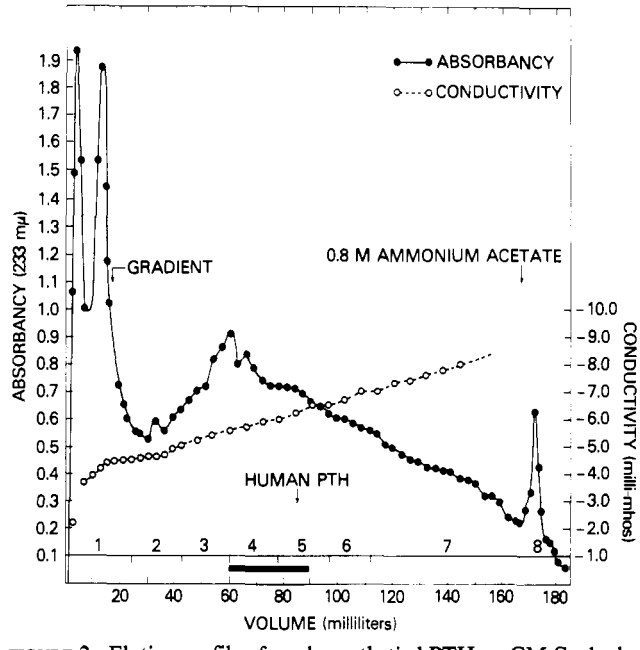

FIGURE 2: Elution profile of crude synthetic hPTH on CM-Sephadex C-25 using a linear gradient of ammonium acetate in 6 M urea. Bioassay using the in vitro dog renal adenylate cyclase assay system indicated that pools 4 and 5 contained maximal biologically active peptide (shaded area). Fractions contained in this area were pooled for further purification (see Figure 3).

The crude peptide was initially desalted by gel chromatography on Bio-Gel P-2. The major fraction was purified by

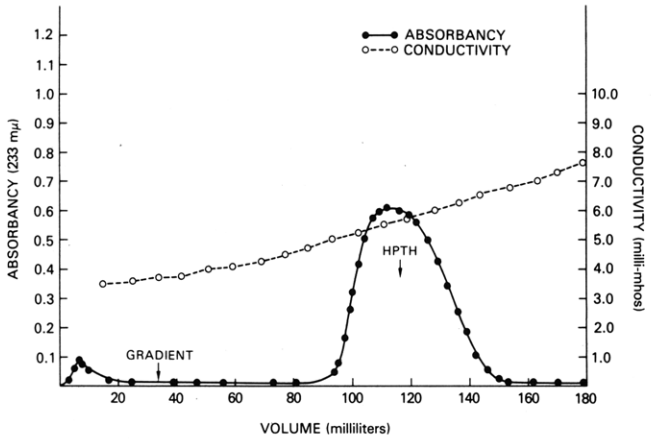

FIGURE 3: Rechromatography of biologically active synthetic hPTH on CM-Sephadex C-25. Fractions corresponding to the major peptide (100–140 mL) were pooled and desalted.

Table I: Amino Acid Composition of Purified hPTH[a]

amino acid	theoretical composition	synthetic hPTH[b]
aspartic acid	10	10.2
threonine	1	0.8
serine	7	6.5
glutamic acid	11	11.1
proline	3	3.4
glycine	4	4.4
alanine	7	7.1
valine	8	7.3
methionine	2	2.0
isoleucine	1	1.0
leucine	10	10.5
phenylalanine	1	1.0
histidine	4	4.0
lysine	9	9.0
arginine	5	5.2

[a] Moles of amino acid per mole of protein. [b] Duplicate values obtained from 24-h hydrolysis in 5.7 M HCl at 110 °C.

ion-exchange chromatography on a CM-Sephadex C-25 column (Figure 2). Amino acid analysis of samples taken from different fractions in the region of elution of native hPTH indicated that the desired peptide was present in the middle region of the major peak. Native human PTH eluted at a conductivity of 6.5 $m\Omega^{-1}$ under the same chromatographic conditions. The amino acid compositions of pools 4 and 5 were in excellent agreement with the theoretical values for human PTH. Fractions 4 and 5 were pooled and rechromatographed twice on a CM-Sephadex column under identical conditions, and the final chromatographic product was a single component eluting at the same position as the native hormone under identical conditions (Figure 3). Amino acid analysis of the final product was in excellent agreement with theoretical values for the hPTH native peptide (Table I). The final yield of the purified synthetic hPTH was 12%.

Automated Edman sequence analysis of the CM-Sephadex-purified peptide for 60 cycles revealed the desired sequence with only 5–6% preview of the next residue, resulting from incomplete cleavage or coupling during the synthesis. Estimation of the deletion at any point in the desired sequence was performed as described by Tregear et al. (1974). The percentage of preview sequences observed at selected steps is shown in Table II. As is well-known in solid-phase synthesis, the deletion sequences were found after isoleucine at step 5 and at various prolines in the middle of the sequence. An additional difficult residue to couple in the synthesis is the glutamine at step 29, as reported by Tregear et al. (1974).

Table II: Preview of Amino Acid Derivatives Observed during Edman Degradation of Synthetic hPTH

sequence step no.	residue[a]	preview residue	% of preview residue
1	Ser	Val	0.0
5	Ile	Gln	0.61
10	Asn	Leu	0.85
15	Leu	Asn	0.32
20	Arg	Val	1.4
25	Arg	Lys	2.4
30	Asp	Val	3.15
35	Val	Ala	5.8
40	Pro	Leu	8.2
45	Asp	Ala	11.6
50	Arg	Pro	8.2
55	Glu	Asp	8.3
60	Val	Glu	14

[a] No corrections were made for the background peaks. Previews were observed mostly after the isoleucine at step 5 and the prolines at steps 40, 43, and 51.

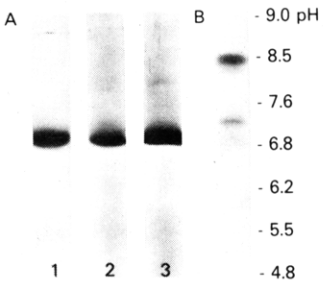

FIGURE 4: (A) Polyacrylamide gel electrophoresis (pH 4.4) of purified (1) synthetic hPTH, (2) native hPTH, and (3) native bPTH. (B) Polyacrylamide gel isoelectrofocusing of purified synthetic hPTH at a pH range of 3–10.

However, we have blocked any unreacted amino group by acetylation at this step. The preview of glutamic acid at step 60 was only approximately 7% after correction for the Glu from background peaks. This is performed by subtracting an average peak area that can be attributed to Glu from the background (as a result of problems associated with the Edman sequence analysis). In the sequence analysis, the amino acids were found to be in the correct sequence for 60 cycles, and no incomplete deprotection of the side-chain protecting groups or acylation of anisole by the carboxyl groups was observed in the mass spectra of the phenylthiohydantoin derivatives.

Polyacrylamide gel electrophoresis in 8 M urea at pH 4.4 of the synthetic peptide showed a component which was similar to the mobility of reference samples of native human and bovine parathyroid hormone (Figure 4A). Analytical isoelectric focusing revealed one major and one minor component (Figure 4B). The p*I* of the major component was 8.5, the same as that for the native hormone.

The approximate potencies (dog renal plasma membrane adenylate cyclase assay) of the crude peptide and the various pools from the CM-Sephadex chromatography fractionation (Figure 2) are shown in Table III. These were derived from parallel dose–response curves and are expressed as the dose of peptide producing half-maximal stimulation of adenylate cyclase in the assay. The native human hormone used in these assays was equivalent in activity to the native bovine hormone (MRC standard 72/286; potency 2600 IU/mg), and the synthetic peptide isolated in pools 4 and 5 demonstrated the greatest activity and was 95% and 100% as active, respectively, as the preparation of native hPTH used as standard in all assays. There were no differences in the potencies of native hPTH, native bPTH, and synthetic hPTH by using dog renal

Table III: Biological Activity of Synthetic hPTH[a]

sample		ng of peptide for half-max activity	% of native hPTH-(1-84)
native hPTH-(1-84)		100	
crude synthetic hormone		400	24
CM-Sephadex:	pool 1[b]	2000	5
	pool 2	200	50
	pool 3	180	60
	pool 4	110	95
	pool 5	100	100
	pool 6	150	75
	pool 7	200	50
	pool 8	300	33

[a] Biological activity determined by adenylate cyclase assay in dog renal plasma membranes. [b] See Figure 2 for elution positions of various pools.

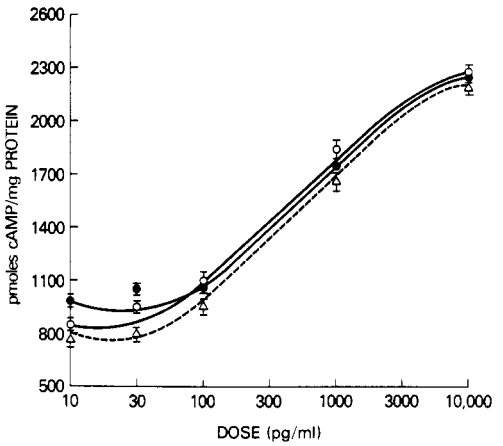

FIGURE 5: Comparison of dose–response curves for purified native hPTH (●), native bPTH (○), and synthetic hPTH (△) in dog renal adenyl cyclase assay systems. Incubation time was 30 min at 30 °C. (The bovine native PTH employed is an MRC standard with a potency of 2600 IU/mg.) Each point is the mean ± SE of triplicate determinations.

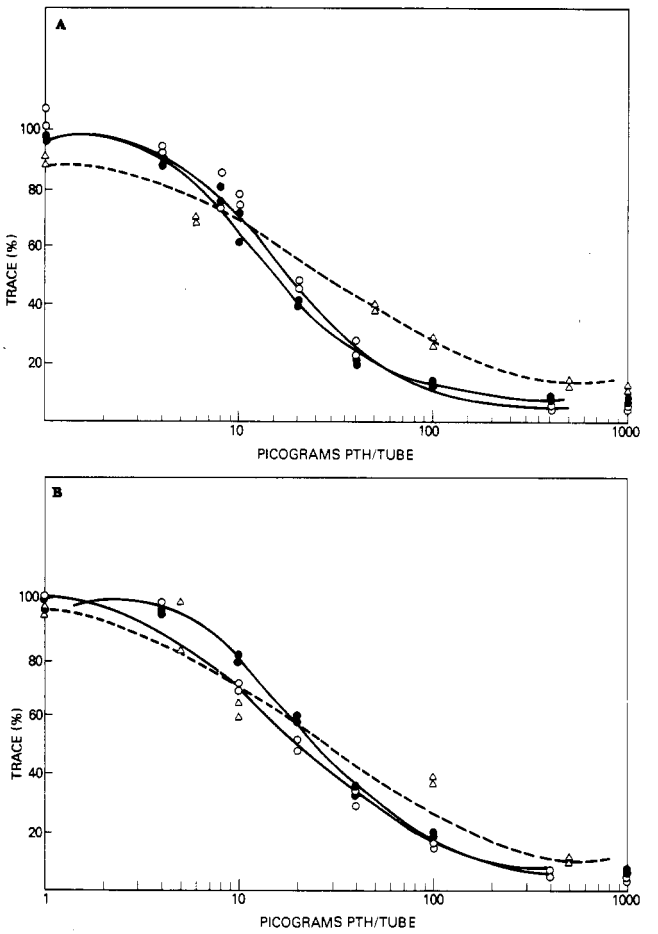

FIGURE 6: Radioimmunoassay dose–response curves for bPTH (○), native hPTH (●), and synthetic hPTH (△) using CH 12M antiserum (A) (specific for intact hPTH) and GP 1M antiserum (B) (specific for mid-region hPTH).

plasma membranes (Figure 5). However, the potencies of native and synthetic hPTH's were approximately 3 times lower than that for native bPTH by using rat renal cortical membranes (data not shown).

Comparison of the immunologic reactivity of our synthetic preparation of hPTH with those of native bPTH and hPTH in radioimmunoassays specific for intact hPTH and mid-region hPTH is shown in panels A and B, respectively, of Figure 6. Although the immunopotency of the synthetic peptide is similar to that of the native peptides in both assay systems, the slopes and configurations of the dose–response curves for the synthetic peptide appear to differ slightly from those of the native peptides.

Discussion

The successful synthesis of human parathyroid hormone-(1-84) by the solid-phase technique required that the resin used as the solid support be stable during the acidolytic deprotection of Boc groups from the amino terminus of the assembled peptide. For this reason, the PAM resin was chosen with a low substitution level. The synthesis procedure described by Tam et al. (1979) allows exact control of the extent of amino acid substitution, and the free amino groups of the resin can be blocked by acetylation, preventing any undesirable side reactions. With the growing chain length of the peptide, the volume of the resin increased; however, its physical characteristics were not noticeably changed. The stability and the behavior of this solid support were clearly of great importance in the solid-phase peptide synthesis. Side-chain protecting groups for the individual amino acid chains were selected so that they were relatively stable toward the reagents used and at the same time could be removed in one step together with the cleavage of the peptide from the solid support, except for tryptophan, where the formyl group from the indole nucleus was removed by piperidine.

The reaction conditions, 6-fold excess of the amino acid and DCC, and the double coupling at every cycle were all chosen for this synthesis in order to achieve the maximum yield and reduce the quantity of other incomplete peptides. The individual deprotection and coupling steps were qualitatively monitored even though it necessitated the use of the synthesizer in a semiautomatic manner. An initial acid concentration of 25% TFA was used to deprotect the α-amino groups, and the acid concentration was raised to 40% by step 40. Even though the ester bond attaching the peptide chain to the resin was extremely stable toward 50% TFA in CH_2Cl_2 as reported by Merrifield and his co-workers (Mitchell et al., 1976), it has been proposed that the benzyl ester group used to protect the side-chain functions of various amino acid derivatives can be better protected by reducing the acid concentration. As the peptide grows longer in length, steric factors can influence the extent of deprotection at every stage, and, therefore, the acid concentration was increased as the synthesis progressed. A final concentration of 40% TFA was used from cycle 40 to the completion of the synthesis. It is difficult to completely ascertain the effectiveness of this procedure, and it is probably

not required when small peptides are synthesized. Even at the low acid concentration (25%), the 30 min used for deprotection should be more than adequate to deprotect even the sterically hindered amino acid derivatives. There is, however, no definite data to demonstrate that these changes in the acid concentration reduced the heterogeneity of the final product. Methionine-containing peptides are known to undergo *tert*-butylation, during HF cleavage, when the peptides have been protected with Boc groups, and in this synthesis, the last Boc group was removed before HF cleavage. Edman sequence analysis has been reported to be a very sensitive and convenient method for the detection of deletion sequences (Tregear et al., 1974). In our opinion, it is desirable to use a large quantity of the peptide and identify the amino acid derivatives representing the deletion peptides. In most cases, by overloading the HPLC column, it is possible to detect as low as 0.01% of a deletion sequence. However, it is difficult to estimate the exact percentage of preview toward the end of a long sequence run, because of the fact that the number and quantity of background peaks due to nonspecific cleavage of the peptide bonds as well as the incomplete coupling and cleavage during the sequence analysis increase, making it difficult to ascertain what percentage of a particular peak is due to preview. However, a significant quantity of a deletion sequence at any step can be easily detected. Mass spectral analysis of these derivatives can be used to detect incomplete removal of side-chain protecting groups as well as acylation of anisole by the carboxyl groups of the peptide. The limitations of each of these methods are due to inherent problems in the chemistry of the Edman reaction itself, and the analysis of derivatives near the end of a 60-cycle sequence run may only be useful for determining the primary sequence of the peptide. The purified synthetic peptide had the desired sequence for the first 60 residues from the amino terminus. Since the 53–84 region of this peptide has been synthesized without any difficulty and no serious problems were noticed, it is only logical to assume that any deletion or chain termination which occurred during the synthesis could easily be detected by the sequence analysis of the amino-terminal end. The overall amino acid composition of the synthetic material was identical with that of the native hormone. The synthetic peptide chromatographed in the position of the native human hormone on CM-Sephadex, and the purified synthetic hormone possessed nearly 100% biological activity in adenyl cyclase assays when compared with native hPTH. The difference in the potency of the native human and bovine hormones in the rat assay may be attributed to the specificity of the two membrane species used in the assays. Previous studies in several laboratories have shown that deletions in the amino-terminal sequence of PTH are associated with reductions in biological activity.

The only evidence that we have been able to obtain to date suggesting that the hPTH we have synthesized might be different from the native hPTH is immunologic. The shapes and slopes of the dose–response curves produced by the synthetic peptide in two radioimmunoassays with different specificities for hPTH demonstrate minor differences when compared with those of native bPTH and hPTH. Whether these differences reflect an artifact of our radioimmunoassay systems or an alteration from the true structure of the native hormones is conjectural at present, and the problem requires further study. One possible explanation is that the peptide we have synthesized contains an Asp residue in position 76 as first reported by Keutmann et al. (1978) whereas this position has been reported to be Asn by Hendy et al. (1981), who determined the structure by nucleotide sequencing. Similar immunoassay results were reported by Manning et al. (1981) using human PTH antiserum. The synthetic C-terminal peptide (53–84 with Asp at position 76) was reported to be less reactive than the corresponding native hormone. We are currently synthesizing hPTH with Asn at position 76, and it may be possible to clarify this apparent immunological difference by investigating the immunological behaviors of the Asp- and Asn-containing peptides as compared with native hPTH.

The yield of 12% hPTH in the present synthesis was excellent, considering the size of this protein. Moreover, it is reasonable that, with optimization of reaction conditions, the yield will improve and the time required for the synthesis will be shortened.

Clearly, the scarcity of hPTH obtained from natural sources has been an important impediment to progress in the study of the physiology and pathophysiology of mineral and bone metabolism in humans. The availability of synthetic hPTH should now permit detailed clinical and biochemical studies of the role of this important hormone in calcium metabolism.

Registry No. Human parathyroid hormone-(1–84), 68893-82-3.

References

Arnaud, C. D., Tsao, H. S., & Littledike, E. T. (1971) *J. Clin. Invest. 50*, 21.

Barany, G., & Merrifield, R. B. (1979) in *The Peptides* (Gross, E., & Meienhofer, J., Eds.) Vol. 2, pp 1–208, Academic Press, New York.

Brewer, H. B., Jr., & Ronan, R. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1862.

Brewer, J. B., Jr., Fairwell, T., Ronan, R., Sizemore, G. W., & Arnaud, C. D. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3585.

Erickson, B. W., & Merrifield, R. B. (1976) *Proteins (3rd Ed.) 2*, 255–527.

Fairwell, T., & Brewer, H. B., Jr. (1979) *Anal. Biochem. 99*, 242.

Fairwell, T., & Brewer, H. B., Jr. (1980) *Anal. Biochem. 107*, 140.

Gallagher, B., Riggs, B. L., Jerpbak, C. M., & Arnaud, C. D. (1980) *J. Lab. Clin. Med. 95*, 373.

Gutte, B., & Merrifield, R. B. (1971) *J. Biol. Chem. 246*, 1922.

Hendy, G. W., Kronenberg, H. M., Potts, J. T., Jr., & Rich, A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7365.

Kaiser, E., Colescott, R. L., Bossinger, C. D., & Cook, P. I. (1970) *Anal. Biochem. 34*, 595.

Keutmann, H. T., Sauer, M. M., Hendy, G. M., O'Riordan, J. L. H., & Potts, J. T., Jr. (1978) *Biochemistry 17*, 5723.

Krishna, G., Weiss, B., & Brodie, B. B. (1968) *J. Pharmacol. Exp. Ther. 163*, 379.

Manning, R. M., Adami, S., Papapoulos, S. E., Gleed, J. H., Hendy, G. N., Rosenblatt, M., & O'Riordan, J. L. H. (1981) *Clin. Endocrinol. (Oxford) 15*, 439.

Marcus, R., & Aurbach, G. D. (1971) *Biochim. Biophys. Acta 242*, 410.

Markley, L. D., & Dorman, L. C. (1970) *Tetrahedron Lett.*, 1787.

Merrifield, R. B. (1969) *Adv. Enzymol. Relat. Areas Mol. Biol. 32*, 221.

Mitchell, A. R., Erickson, B. W., Ryabsfer, N. N., Hodges, R. S., & Merrifield, R. B. (1976) *J. Am. Chem. Soc. 98*, 7357.

Niall, H. D., Keutmann, H. T., Sauer, R. T., Hogan, M. L., Dawson, B. F., Aurbach, G. D., & Potts, J. T., Jr. (1970) *Hoppe-Seyler's Z. Physiol. Chem. 351*, 1586.

Nissensen, R. A., & Arnaud, C. D. (1979) *J. Biol. Chem. 254*, 1669.
Ohno, M., Tsukamoto, S., Makisumi, S., & Izumiya, N. (1973) *Bull. Chem. Soc. Jpn. 46*, 3280.
Rosenblatt, M., Segre, G. V., Tregear, G. W., Shephard, G. L., Tyler, G. A., & Potts, J. T., Jr. (1978) *Endocrinology (Philadelphia) 103*, 978.
Sakakibara, S., Shin, K. H., & Hess, G. P. (1962) *J. Am. Chem. Soc. 84*, 4921.
Sakakibara, S., Shiminoshi, Y., Kishida, Y., Okada, M., & Sugihara, H. (1967) *Bull. Chem. Soc. Jpn. 40*, 2164.
Smith, M. E., Komoriya, A., & Anfinsen, C. B. (1981) *Biology of Interferon Systems* (Demeyer, E., Ed.) p 39, Elsevier, Amsterdam.
Tam, J. P., Kent, S. B. H., Wong, T. W., & Merrifield, R. B. (1979) *Synthesis 12*, 955.
Tregear, G. W., van Rietschoten, J., Greene, E., Niall, H. D., Keutmann, H. T., Parsons, J. A., O'Riordan, J. L. H., & Potts, J. T., Jr. (1974) *Hoppe-Seyler's Z. Physiol. Chem. 355*, 415.
Zimmerman, C., Pisano, J. J., & Appella, E. (1977) *Anal. Biochem. 77*, 569.

Reactions of Spinach Ribulose-1,5-bisphosphate Carboxylase with Tetranitromethane†

Gary Barnard, Harry Roy,* and Yash P. Myer

ABSTRACT: Tetranitromethane [TNM, $C(NO_2)_4$] rapidly inactivated spinach ribulosebisphosphate carboxylase (RuBPCase). The extent of inactivation was increased by preincubation of the enzyme with Mg^{2+} and bicarbonate. Activity was substantially protected by 6-phosphogluconate, a competitive inhibitor of carboxylation, by ribulose 1,5-bisphosphate, a substrate, and by *p*-(chloromercuri)benzoate, which was used to covalently block enzyme sulfhydryl groups. Sulfhydryl titration with Ellman's reagent showed that over one-third of the titratable cysteine residues were lost upon complete inactivation. The losses of activity and of sulfhydryl groups titratable in nondenatured enzyme occurred with similar kinetics. Approximately half the sulfhydryl groups that were lost upon TNM-induced inactivation were restored by mercaptoethanol and dithiothreitol (DTT). High concentrations of DTT, however, did not restore more than 20% of the activity lost. Amino acid analysis revealed that about one-third of the modified sulfhydryls had been irreversibly oxidized. Both amino acid analysis and difference spectroscopy showed that little or no tyrosine modification occurred. These results suggest that TNM inactivates spinach RuBPCase by modification of cysteine sulfhydryls. This indicates that the inactivation of RuBPCase associated with SH modification does not depend solely on the presence of bulky groups attached to the modified sulfur. This conclusion differs from that obtained in similar studies using the RuBPCase of *Rhodospirillum rubrum*, in which TNM modified a single tyrosine per dimer of catalytic subunits [Robison, P. D., & Tabita, F. R. (1979) *Biochem. Biophys. Res. Commun. 88*, 85].

The enzyme D-ribulose-1,5-biphosphate carboxylase/oxygenase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] (RuBPCase[1]) is bifunctional. It catalyzes the CO_2-fixation step in the C-3 photosynthetic carbon-reduction cycle, which converts a molecule each of D-ribulose 1,5-bisphosphate (RuBP) and CO_2 to two molecules of D-3-phosphoglycerate (3-PGA) (Calvin et al., 1955). The enzyme also catalyzes the reaction of RuBP with O_2 to yield a molecule each of 3-PGA and 2-phosphoglycolate, a substrate of photosynthetic carbon oxidation (Ogren & Bowes, 1971).

In all higher plants, the enzyme is composed of nonidentical large (M_r 56 000) and small (M_r 14 000) subunits (Siegel et al., 1972). Small subunits are not found in RuBPCase from the purple, nonsulfur bacterium *Rhodospirillum rubrum*. This enzyme consists of a dimer of large (M_r 56 000) subunits. RuBPCases in higher plants show great structural homology, especially in the large subunits, which bear the catalytic site (McIntosh et al., 1980; Zurawski et al., 1981). Amino acid composition data (Takabe & Akazawa, 1975a) and partial sequence data (Stringer et al., 1981; Herndon et al., 1982) suggest that only limited sequence homology exists between RuBP carboxylase from a higher plant, spinach, and that from *R. rubrum*.

Nevertheless, affinity-labeling techniques have indicated conservation of primary structure in the active sites of carboxylases from these two sources (Herndon et al., 1982). In both enzymes, lysine residues are essential for catalysis (Paech & Tolbert, 1978; Schloss et al., 1978a; Robison et al., 1980; Lorimer, 1981b) and for the binding of activating CO_2 and Mg^{2+} (O'Leary et al., 1979; Lorimer, 1981a). Arginine (Schloss et al., 1978b; Chollet, 1981) and histidine (Saluja & McFadden, 1982) may also be important in catalysis by the spinach enzyme. Recently, Fraij & Hartman (1982) suggested that methionine is located at the active site of the *R. rubrum* enzyme. Numerous studies have shown sulfhydryl

† From the Biology Department, Rensselaer Polytechnic Institute, Troy, New York 12181 (G.B. and H.R.), and the Chemistry Department, State University of New York, Albany, New York 12222 (Y.P.M.). *Received January 10, 1983.*

[1] Abbreviations: RuBP, D-ribulose 1,5-bisphosphate; RuBPCase, ribulosebisphosphate carboxylase; PCMB, *p*-(chloromercuri)benzoate; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; PGA, 3-phosphoglycerate; TNM, tetranitromethane, $C(NO_2)_4$; 6-PGluA, 6-phosphogluconate; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SCM-cysteine, *S*-(carboxymethyl)cysteine; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

0006-2960/83/0422-2697$01.50/0