# Complete Amino Acid Sequence of Human Parathyroid Hormone†

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ABSTRACT: Structural analysis of human parathyroid hormone has been hindered by the scarce supplies of available starting material, tissue pooled during surgery for hyperparathyroidism. Using successive lots of hormone extracted and purified from this source, we have completed the amino acid sequence of the 84-residue molecule by means of Edman degradation of tryptic peptides and their subfragments. The technique of biosynthetic labeling with specific radioactive amino acids was extensively used to complement the analyses of extracted hormone. The human parathyroid hormone molecule differs at 11 sequence positions from the bovine and from the porcine hormones. The carboxyl terminus, while highly conserved overall, contains changes at positions 79 and 83 which may be sufficient to effect observed alterations in immunological cross-reactivity with antibovine antisera directed toward this region. The most marked sequence differences occur through the middle portion of the molecule, where six of the eight residues in the segment 40–47 differ from either or both of the other species. Proline residues are especially abundant in this region of the human hormone. These changes could confer significant conformational differences to the human molecule which would be expected to further influence immunoreactivity and perhaps also the nature of enzymatic cleavages occurring during in vivo metabolic degradation of the hormone.

The complete sequences of parathyroid hormone from two species, bovine (Brewer and Ronan, 1970; Niall et al., 1970) and porcine (Sauer et al., 1974), have been established using hormone obtained from glands available as a byproduct of meat processing, but progress in the structural analysis of the human hormone has been restricted by the need to accumulate human tissue gradually during surgery in patients with hyperparathyroidism.

Several years ago, the sequence of the biologically active amino-terminal one-third of the molecule was reported by Brewer and co-workers (1972) and by ourselves (Niall et al., 1974). The sequence of this region was determined first because of its accessibility to automated Edman degradation. There were, however, discrepancies between the two proposals involving residues 22, 28, and 30; reexamination of the amino-terminal region by the respective laboratories has so far failed to reconcile these differences (Brewer et al., 1975; Keutmann et al., 1975). Nevertheless, a variety of physiological and immunological studies have ensued using synthetic peptides comprising the active region (Tregear et al., 1974).

There has been a clear need for structural information concerning the remainder of the molecule, in light of the numerous ongoing investigations concerning structure–function relations (Rosenblatt et al., 1976), conformation (Fiskin et al., 1977), peripheral metabolism (Segre et al., 1977), and radioimmunoassay of the hormone in blood for physiological and clinical studies, since there is extensive evidence that the predominant circulating form of hormone is a carboxyl-terminal fragment [see Habener and Potts (1976) for a review].

We, therefore, prepared successive lots of purified human parathyroid hormone (hPTH)[1] as sufficient quantities of adenoma tissue were accumulated. In the course of this, we were able to further define conditions for isolation and to obtain definitive compositional analysis of the molecule (Keutmann et al., 1978). These preparations were then employed in structural analysis of the middle and carboxyl-terminal regions, in conjunction with microsequencing techniques utilizing hormone biosynthetically labeled with radioactive amino acids.

As a result of these combined analytical approaches, the sequence of the entire 84-residue molecule has been completed. This report provides a detailed description of these structural studies as well as an assessment of how sequence differences from the animal hormones might influence the comparative physiological and immunological properties of human parathyroid hormone.

## Materials and Methods

*Hormone Preparations.* Successive lots of human parathyroid hormone were extracted from pooled adenomatous and hyperplastic tissue by means of phenol, processed into a trichloroacetic acid precipitate, and purified by gel filtration on Bio-Gel P-100 and ion-exchange chromatography on carboxymethylcellulose. Details of these procedures have been published previously (Keutmann et al., 1974, 1978).

*Biosynthetic Labeling.* Human parathyroid adenoma tissue, freshly obtained from surgery, was sliced and incubated with the appropriate radioactive amino acids in Earl's balanced salt solution (Gibco) as previously described (Keutmann et al., 1975). Labeled amino acids, obtained from New England Nuclear (Ala, Pro, Gln, Lys, Leu, and Thr) or Amersham-Searle (Phe) were used in quantities of 25 $\mu$Ci/mL of medium for $^{14}C$ and 150 $\mu$Ci/mL for $^{3}H$. The remaining, unlabeled amino acids were added to a concentration of $5 \times 10^{-4}$ M. The tissue was extracted with 8 M urea/0.2 N HCl, combined with the medium, and precipitated with $Cl_3AcOH$ as described by Kemper et al. (1972). The pooled $Cl_3AcOH$ preparations from multiple incubations with a given set of amino acids were

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[1] Abbreviations used: hPTH, human parathyroid hormone; Tos-PheCH$_2$Cl, (1-tosylamido-2-phenyl)ethyl chloromethyl ketone; TLC, thin-layer chromatography; TLE, thin-layer electrophoresis; $Cl_3AcOH$, trichloroacetic acid.

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further purified by gel filtration and ion-exchange chromatography (Keutmann et al., 1975) in the presence of bovine parathyroid hormone as carrier.

*Enzymatic Cleavage Procedures.* Tos-PheCH$_2$Cl-trypsin and chymotrypsin were each obtained from Worthington, and thermolysin was obtained from Calbiochem. In each case, the digestions were carried out at an enzyme/substrate ratio of 1:100 (M/M) in 0.1 M ammonium bicarbonate buffer (pH 8.7), for 1 h at 37 °C. Staphylococcal protease (Miles Laboratories) was used at a molar ratio of 1:50 in the same buffer at 37 °C for a 22-h time period.

*ε-Amino Group of Lysine Blockade.* Lysine residues were blocked in order to limit tryptic cleavage to arginine residues. Parathyroid hormone was treated with a 40-fold molar excess of maleic anhydride for 20 min at pH 9.0, 20 °C (Klotz, 1967), and separated from the reagents by gel filtration on Sephadex G-25.

To reexpose lysines for further tryptic digestion, maleoyl groups were removed by treatment with 0.1 N formic acid for 45 min at 80 °C.

*Edman Degradation.* All degradations were done by the three-stage manual Edman procedure (Edman, 1960; Niall and Potts, 1970). Radioactive phenyl [$^{35}$S]isothiocyanate (Amersham-Searle) was used as a coupling reagent for increased sensitivity (Jacobs and Niall, 1975). Phenylthiohydantoins were identified by thin-layer chromatography (Edman and Begg, 1967) (followed by autoradiography) or by gas chromatography (Pisano and Bronzert, 1969). Yields of phenylthiohydantoins at successive cycles were quantitated either from the gas chromatographs or, in the case of radioactive derivatives identified by TLC, by counting after scraping from the thin-layer plate.

Edman degradations of biosynthetically labeled peptides were done under the same conditions, except for the use of unlabeled phenyl isothiocyanate. Aliquots of the ethyl acetate phase containing the radioactive phenylthiohydantoin were counted for $^{3}$H and $^{14}$C activity by scintillation counting. Whenever possible, the identification of the radioactive phenylthiohydantoin was confirmed by counting after thin-layer chromatography.

*Amino Acid Analysis of Peptide Fragments.* Acid hydrolysis was carried out in 5.7 N HCl at 110 °C for 24 h in vacuo, in the presence of 1:2000 (v/v) mercaptoethanol. Analyses were performed using the Beckman Model 121 MB automatic analyzer. Amino acids were normalized by best fit based upon recovery of all stable residues.

To establish the carboxyl-terminal residue surviving after Edman degradation carried to the penultimate residue of a fragment, the final reaction mixture was dissolved in analyzer buffer and applied directly to the column without hydrolysis.

*Column Procedures.* Column chromatography employed Sephadex (Pharmacia) or Bio-Gel (Bio-Rad) resins eluted with ammonium acetate buffer (0.14 M, pH 5.0). Ammonium bicarbonate buffer (0.1 M, pH 8.7) was used for elution of maleoylated peptides. All columns were 0.9 × 70 cm in size, run at 20 °C. Specific column chromatographic separations are described further under Results. Column elution was monitored by optical density at 206 and 280 nm by use of the LKB "Uvicord" Model 2089 continuous monitor. Biosynthetically labeled preparations were also monitored by scintillation counting of aliquots (usually 25 μL) from successive eluate tubes.

*Thin-Layer Techniques.* Thin-layer chromatography (TLC) was done in the solvent system butanol–water–pyridine–acetic acid (150:120:50:30) using Merck 100-μm glass-backed cellulose plates, with 20–30 μg of sample applied to the plates for analytical runs. For preparative scale chromatography, 100 μg of sample was striped along 1.5 cm of the origin, with guide spots at either side for identification.

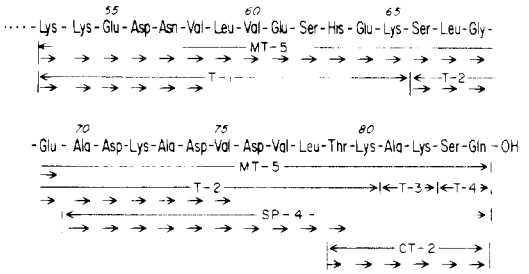


FIGURE 1: Sequence analysis of the 53–84 fragment (peptide MT-5) of hPTH. Peptide subfragments employed in the analysis are designated beneath the sequence by the following code: MT, maleoylated tryptic; T, tryptic (after removal of maleoyl groups from lysines); SP, staphylococcal protease; CT, chymotryptic. Arrows denote residues identified by manual Edman degradation.

Thin-layer electrophoresis (TLE) was done using the same plates in a buffer system butanol–pyridine–water (1:30:270), pH 6.5. Plates were run for 40 min at 4 °C, 600 V, 11 mA, using the Camag electrode system. All plates were stained with ninhydrin.

*Synthesis of Fragment 38–44.* The fragment (38–44) used in enzymatic cleavage studies was prepared by the solid-phase technique (Merrifield, 1967) as described in detail by Tregear et al. (1974). The peptide was purified by gel filtration on Bio-Gel P-2 in 1 N acetic acid and adsorption chromatography on a column of Merck silica gel 60 using the same buffer system as that employed for TLC (above).

## Results

In undertaking the structural analysis, knowledge of the tactics useful in sequence analysis of the homologous bovine and porcine hormones suggested that the human hormone was most readily analyzed by separate regions: the amino terminus, previously completed by automated Edman degradation of the intact hormone; the carboxyl terminus, comprising residues 53–84; and the middle region, commencing in the vicinity of residue 35 (end point of the earlier amino-terminal analyses) and extending through residue 52. The carboxyl-terminal and middle regions coincided with the pattern of cleavage obtained from tryptic cleavage of maleic-blocked hormone. This had been shown earlier (Keutmann et al., 1975, 1978) to take place at arginine residues positioned identically to the bovine and porcine hormones.

Multiple peptide subfragments were isolated, analyzed for composition, and degraded by the Edman technique as extensively as possible to provide sequence overlap. Biosynthetic labeling was used to permit high-sensitivity microsequencing of peptide fragments, especially in difficult regions of sequence analysis. Mixture degradations—analyses of several peptides simultaneously without separation of component fragments (Gray, 1968)—were done as a confirmation of most sequence positions. The combined results are presented first for the carboxyl and then the middle region of the molecule.

*The Carboxyl Terminus (53–84).* Residues 53–69. The overall strategy for analysis of the carboxyl-terminal region is outlined in Figure 1. It was based on the fragment 53–84 prepared by tryptic digestion of maleoylated hPTH and separated by gel filtration on a column of Sephadex G-50 (Figure 2). The fragment prepared from 1.5 mg (150 nmol) of hPTH was degraded for 17 cycles, through residue 69, by the manual Edman procedure. Location of histidine at position 63 had been

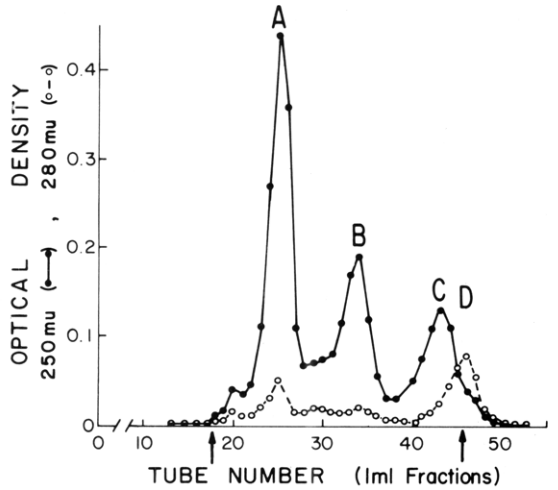


FIGURE 2: Sephadex G-50 gel filtration of a tryptic digest of human parathyroid hormone after maleic blockade of lysine residues. Cleavage of arginine residues at positions 20, 25, 44, and 52 produced fragments 53–84 (A), 1–20 and 26–44 (coeluting as B), 45–52 (C), and 21–25 (D). Fraction size was 1-mL. Arrows denote void and salt volumes of column, respectively.

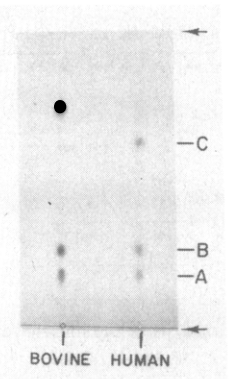


FIGURE 3: Thin-layer chromatography of tryptic subdigests of fragment 53–84 (MT-5) from bovine and human parathyroid hormone. Component A represented fragment 53–65 and component C fragment 66–80. Component B proved to represent the two dipeptides 81–82 and 83–84 (see text).

separately identified in other studies (Segre et al., 1977) using automated degradation of the peptide isolated after labeling with radioiodine.

Residues 70–75. The sequence was continued toward the carboxyl terminus by use of peptides prepared by tryptic subdigestion of another preparation of the 53–84 fragment, after reexposure of lysine residues by treatment with 0.1 N formic acid. Thin-layer chromatography of the tryptic digest (Figure 3) showed three components. After elution from a preparative-scale plate, the following compositional analyses were obtained: component A: $Asp_2$, $Ser_1$, $Glu_3$, $Val_2$, $Leu_1$, $His_1$, $Lys_3$; component B: Ser, Glu, Ala, Lys; component C: $Asp_3$, $Thr_1$, $Ser_1$, $Glu_1$, $Gly_1$, $Ala_2$, $Val_2$, $Leu_2$, $Lys_2$. When the preparation was subjected to electrophoresis following chromatography, component B resolved into two peptides: Ala, Lys and Ser, Glu. This indicated a total of four tryptic peptides from the human 53–84 fragment, compared with three from the bovine. Based upon the compositions and the results of the initial Edman degradation (above), these were identified as residues 53–65 (T-1, Figure 1), 66–80 (T-2), 81–82 (T-3), and 83–84 (T-4). The tryptic fragments were separated by gel filtration on Sephadex G-25, and T-1 (53–65) was subjected to six cycles of degradation (Figure 1) in order to confirm the sequence of residues 53–58 established by degradation of the intact 53–84 peptide. Peptide T-2 (66–80) was degraded for ten cycles, extending the sequence through residue 75 (Figure 1).

Residues 79–84. Analysis of the extreme carboxyl terminus was done next, using products of chymotryptic digestion of 50 nmol of maleoylated 53–84 peptide. Only a single cleavage, between residues 78 and 79, was obtained under the relatively mild digestion conditions employed. The carboxyl-terminal hexapeptide 79–84 was separated from the longer fragment 53–78 by Sephadex G-25 gel filtration and found by composition to contain two residues of lysine and one each of threonine, serine, alanine, and glutamic acid (or glutamine). The sequence Thr-Lys-Ala-Lys-Ser– was established in five cycles of Edman degradation, and the final glutamine residue was identified directly by amino acid analysis. This sequence was found again in a second degradation of the unfractionated chymotryptic peptide mixture from digestion of another preparation of 53–84 peptide.

Since identification of the phenylthiohydantoin of maleoylated lysine is difficult to achieve unequivocally by thin-layer chromatography, the assignment of lysines was confirmed by degradation of the chymotryptic fragment (79–84) from a human hormone preparation biosynthetically labeled with [$^{14}$C]lysine. Counts from labeled lysines were seen at cycles 2 and 4, consistent with location of this residue at positions 80 and 82.

In the bovine hormone, the presence of proline at position 83 limited tryptic cleavage to the first of these two lysines (position 80), yielding the tetrapeptide Ala-Lys-Pro-Gln. The substitution in the human hormone of serine for proline at position 83 rendered both lysines susceptible to cleavage, producing the two fragments Ala-Lys (positions 81 and 82) and Ser-Gln (positions 83 and 84), consistent with the sequence assigned by degradation of the carboxyl-terminal chymotryptic fragment.

The glutamine at position 84 was the only glutamine residue identified during Edman degradation of the entire 53–84 region. Residue 64, glutamine in both the porcine and bovine hormone, was identified as glutamic acid in the human molecule. To confirm this finding, the biosynthetic labeling technique was used to rule out the unlikely possibility of selective deamidation. A preparation of human hormone labeled with [$^{14}$C]glutamine was treated as described above to produce the 53–84 peptide, which was subdigested with trypsin after removal of maleoyl groups. The peptide subfragments were then fractionated by thin-layer chromatography and electrophoresis. Glutamine counts were associated only with the 83–84 fragment derived by electrophoretic separation of component B (Figure 3), independently confirming the absence of glutamine from position 64 as well as sites other than residue 84 within the carboxyl-terminal region.

Residues 76–78. To complete the remaining portion of the 53–84 region, staphylococcal protease was used to cleave the glutamic acid residue at position 69, as done previously in structural analysis of porcine parathyroid hormone (Sauer et al., 1974). Edman analysis of an aliquot of the product from digestion of 40 nmol of the 53–84 fragment showed cleavages at residues 55 and 61, as well as 69. The carboxyl-terminal fragment 70–84 (SP-4, Figure 1) was separated by Sephadex G-25 gel filtration and subjected to ten cycles of manual degradation, through the threonine residue at position 79. This confirmed the earlier results from degradation of tryptic fragment T-2 and provided the sequence of positions 76–78.

*The Middle Region (Residues 38–52).* Residues 45–52

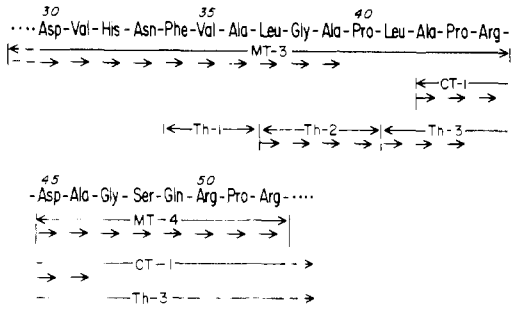


FIGURE 4: Structural analysis of the middle region of hPTH. Peptides MT-3 (which originates at residue 26) and MT-4 are obtained from tryptic digestion of maleoylated hormone (see Figure 2). Other fragments isolated and used in analysis include chymotryptic (CT) and thermolytic (Th). Arrows denote residues identified by Edman degradation of the purified peptide.

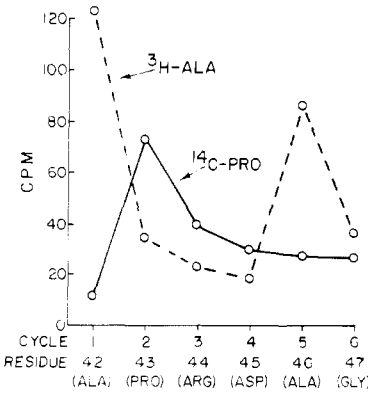


FIGURE 5: Identification of residues 42 and 43 by Edman degradation of chymotryptic fragment 42–78 from human hormone biosynthetically labeled with [$^3$H]alanine and [$^{14}$C]proline. Alanine counts were found at cycles 1 (position 42) and 5 (known to be the alanine at position 46). Proline radioactivity was found at cycle 2, corresponding to position 43. This position is occupied by tyrosine in the bovine hormone.

were determined by Edman degradation of fragment MT-4 from tryptic cleavage of maleoylated hPTH. This peptide (150 nmol) was obtained from the same digest that produced the initial 53–84 preparation described above (Figure 2). The pooled 45–52 product from Sephadex G-50 was rechromatographed on Sephadex G-25 to eliminate any small amounts of fragment 21–25 remaining after the Sephadex G-50 step. The following amino acid composition was obtained: Asp, Ser, Glu, Pro, Gly, Ala, $Arg_2$. Seven cycles provided the sequence Asp-Ala-Gly-Ser-Gln-Arg-Pro-, and the terminal arginine was identified after direct application to the amino acid analyzer.

Residues 38–39. Sequence analysis of the amino terminus, using automated degradation of intact and cyanogen bromide cleaved hPTH, had extended from residues 1 through 37 (Niall et al., 1974). Initial efforts to extend the sequence employed fragment 26–44 from a tryptic digest of maleoylated hormone. This fragment (MT-3) coeluted from Sephadex G-50 with fragment 1–20 (Figure 2). Since the terminal $\alpha$-amino group of the hormone molecule is blocked along with the $\epsilon$-amino groups after maleoylation, the 1–20 fragment does not react in the Edman degradation, permitting analysis of the 26–44 fragment without separation of the two peptides. This fragment was initially employed in studies (Keutmann et al., 1975) reconfirming the sequence of residues 28 and 30. Extended degradation of a preparation of 26–44 fragment reached residue 39 (Figure 4), but extractive losses due to its markedly hydrophobic nature prevented sequence determination from progressing further.

Residues 40–44. To approach the structure of the remaining residues 40–44, products of chymotryptic and thermolytic digestion of native hormone were used (Figure 4), taking advantage of the abundance of susceptible neutral residues in this region of the molecule. At this nearly complete stage of sequence analysis, two residues of proline and one each of leucine and alanine from the compositional analysis remained to be assigned.

Pilot scale digestions of native hPTH had indicated the presence of a chymotrypsin-sensitive residue at position 41. An aliquot of 40 nmol of native hPTH was digested, and the long fragment CT-1 (residues 42–78) was separated by gel filtration on Sephadex G-50. By Edman degradation, alanine and proline were found at cycles 1 and 2 (residues 42 and 43). Arginine at cycle 3 confirmed the presence of this residue at position 44, as concluded earlier from compositional studies and the cleavage at this position by trypsin after maleic blockade. Aspartic acid and alanine at cycles 4 and 5 (positions 45 and 46) corresponded to those previously found in degradation of the 45–52 fragment.

Thermolysin, which cleaves the peptide bond amino terminal to neutral and aromatic residues, was next used to prepare a fragment (Th-3, Figure 4) similar to the chymotryptic peptide just described, with the addition of residue 41 at the amino terminus. This peptide extended through residue 58 and was separated best by gel filtration on Bio-Gel P-30. Leucine was found at cycle 1 of Edman degradation, followed by alanine and proline as assigned (above) to positions 42 and 43.

These results left proline as the most probable residue for the final position 40. Knowing that the already established sequence for residues 37–39 indicated exact homology between the bovine and human hormones, advantage was taken of the cleavage pattern of thermolysin to identify the peptide corresponding to this region in the bovine molecule. In preliminary studies with the bovine hormone, fragment 37–40 (Leu-Gly-Ala-Ser) was isolated from thermolysin digests of the intact molecule, eluting from Bio-Gel P-30 at a $K_d$ of 0.8 and from subsequent TLC at an $R_f$ of 0.62. When a comparable P-30 eluate fraction of thermolysin-cleaved human hormone was screened by TLC, a tetrapeptide with the composition (Gly, Ala, Leu, Pro) was located with a similar chromatographic mobility ($R_f$ 0.64). This fragment (Th-2, Figure 4) was isolated by preparative-scale TLC. Three cycles of Edman degradation yielded the sequence Leu-Gly-Ala- and the final cycle, corresponding to residue 40, was identified as proline by amino acid analysis.

It was possible to rely extensively on biosynthetic labeling for separate verification of the sequence Pro-Leu-Ala-Pro thus found for residues 40–43. Residue 41 was confirmed by thermolysin digestion of a [$^3$H]leucine-labeled human hormone preparation. Leucine radioactivity eluted with the fragment 41–58 (Th-3), and these counts were localized to cycle 1 by Edman degradation.

The assignment of residues 42 and 43 was confirmed by means of the biosynthetic study illustrated in Figure 5. A preparation of hPTH internally labeled with [$^{14}$C]proline and [$^3$H]alanine was digested with chymotrypsin. The 42–78 fragment (CT-1, Figure 4) was isolated as before and subjected to six cycles of Edman degradation. Alanine and proline counts were found at cycles 1 and 2, respectively, representing positions 42 and 43. At cycle 5, a second rise in tritium counts marked the alanine known to be at position 46.

In addition, it was deemed essential to eliminate directly and independently the presence of phenylalanine and threonine from this sequence region, since the content of these two amino acids found during compositional analysis of the entire mole-

cule (Keutmann et al., 1978) was somewhat in excess of the single residue of each already located elsewhere. Thus, two biosynthetic labeling experiments were carried out using combinations of these residues.

An hPTH preparation labeled with [$^{14}$C]phenylalanine and [$^{3}$H]alanine was digested with thermolysin and subjected to Bio-Gel P-30 gel filtration (Figure 6). Phenylalanine counts were found to elute exclusively with fragment 34–36 (Th-1, Figure 4) with no counts in the 41–58 fragment. Phenylalanine was thus eliminated from all positions except 34.

The presence of a threonine residue in the middle region was ruled out definitively by tryptic cleavage of maleic-blocked [$^{14}$C]threonine and [$^{3}$H]alanine-labeled hPTH. After Sephadex G-50 gel filtration, no threonine counts were found in the 26–44 region of the column, as marked by the alanine counts; all $^{14}$C radioactivity eluted with the 53–84 fragment containing threonine at position 79.

The ease with which residue 41 (leucine) was cleaved by both chymotrypsin and thermolysin was surprising to us, in view of the two nearby prolines at residues 40 and 43 which might have been expected to inhibit cleavage. This prompted us to confirm this finding by studies using a synthetic peptide comprising residues 38–44 (Gly-Ala-Pro-Leu-Ala-Pro-Arg). Products of cleavage, under conditions of enzyme treatment similar to those used earlier, were assessed by TLC, amino acid analysis, and Edman degradation. Both enzymes were found to cleave at residue 41 in a manner identical to that observed in our study of the native hormone.

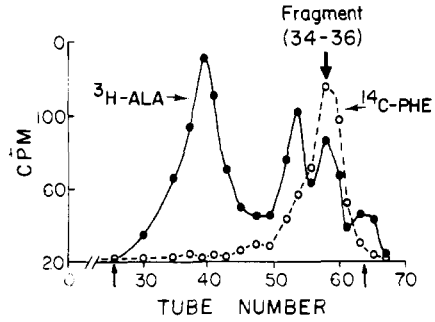


FIGURE 6: Use of a thermolysin digest of [$^{14}$C]phenylalanine and [$^{3}$H]-alanine-labeled hPTH to localize phenylalanine. When passed over Bio-Gel P-30, all phenylalanine counts eluted with fragment 34–36 (Th-1), known to contain Phe at position 34. This and other peptides were marked by alanine counts (closed circles). Arrows denote void and salt volume of column, respectively.

## Discussion

The difficulties in isolating sufficient human parathyroid hormone for structural studies, which have prevented completion of the sequence analysis until now, stem from several factors. Not only is the source of adenoma tissue for extraction extremely limited, but the yield of hormone is low—approximately 0.5–1 mg/100 g of tumor tissue. Large quantities of immunoreactive hormone are lost at various steps in the extraction procedure. At least part of this represents fragments, some of which can be recovered for immunological studies (Keutmann et al., 1978; diBella et al., 1978), but it would appear that extensive fragmentation of the hormone may occur in the tissue prior to extraction. In some preparations, heterogeneity has been observed at the final ion-exchange purification step (Keutmann et al., 1978); this further reduces the yield, as separate evaluation of the different fractions is necessary. There is no evidence, however, that these fractions represent separated isohormones analogous to those demonstrated in the bovine species (Keutmann et al., 1971).

The strategy employed in our structural analysis of the overall molecule was markedly influenced by the scarcity of hormone supply. Efficient use of the extracted hormone was afforded by a high-sensitivity methodology, especially the use of radioactive phenyl isothiocyanate as coupling reagent in the Edman degradation. The conjoint use of biosynthetic labeling as a sequencing technique became particularly important as structural analysis progressed: (a) the later regions to be analyzed (such as the 40–43 segment), being the most difficult, placed extra demands on supplies of extracted hormone; (b) in later stages, the options for residues likely to occupy a given position were reduced, permitting more directed choices of labeled amino acids for incorporation; (c) the biosynthetic method provided an accurate alternative means to confirm certain residues that had proven difficult to identify by conventional methods, and to eliminate possible ambiguities due to selective deamidation.

The last named of these was especially relevant to the identification of glutamic acid at position 64, which could have arisen through loss of the side-chain amide of a glutamine initially present at this position. This possibility was examined by biosynthetic experiments employing [$^{14}$C]glutamine which avoid the potential problem of deamidation (Keutmann et al., 1975); the results showed glutamine to be absent from tryptic fragment 53–65. Despite this further evidence favoring glutamic acid at position 64, we plan to undertake studies using products of cell-free translation of human parathyroid mRNA (which permit equivalent incorporation of Asn, Asp, Glu, and Gln) with the aim of checking all assignments of side-chain amidated residues.

Limited tryptic digestion of maleic-blocked human parathyroid hormone was initially used in our reexamination of portions of the amino-terminal sequence (Keutmann et al., 1975, 1978) and has proven particularly appropriate in providing useful fragments in high yield for the remainder of the structure.

The entire 45–52 region and an extensive segment of the carboxyl terminus could be sequenced by degradation of these tryptic peptides. The carboxyl-terminal 53–84 fragment was completed using subdigestions with trypsin, chymotrypsin, and staphylococcal protease. The hydrophobic nature of the middle portion of the molecule called for alternative cleavage procedures. After chymotrypsin and thermolysin proved promising in pilot studies with intact bovine hormone, they were employed successfully with both extracted and biosynthetically labeled human preparations for analysis of the final 40–43 segment. Our recently reported composition of the human hormone based on multiple hydrolysates of the purified peptide (Keutmann et al., 1978) corresponds appropriately to the amino acid composition of hPTH calculated from the sequence presented here. The molecular weight of the human hormone as computed from the structure is 9427.

In the course of these studies, we have had further opportunity to examine the still disputed residues at positions 22, 28, and 30 in the amino-terminal region and have repeatedly confirmed our original findings as reported by Niall et al. (1974). Despite the obvious confusion persisting due to the unresolved differences between our results and the report of Brewer and associates (1972, 1975) concerning the nature of these three residues, we conclude from our earlier studies (Niall et al., 1974; Keutmann et al., 1975, 1978) and present findings that the structure presented here for the human hormone (Figure 7) is the correct sequence of the 84 amino acids comprising the principal, if not sole, form of parathyroid hormone obtainable from adenomatous or hyperplastic human tissue.

Figure 7 compares the sequences of the human, bovine, and

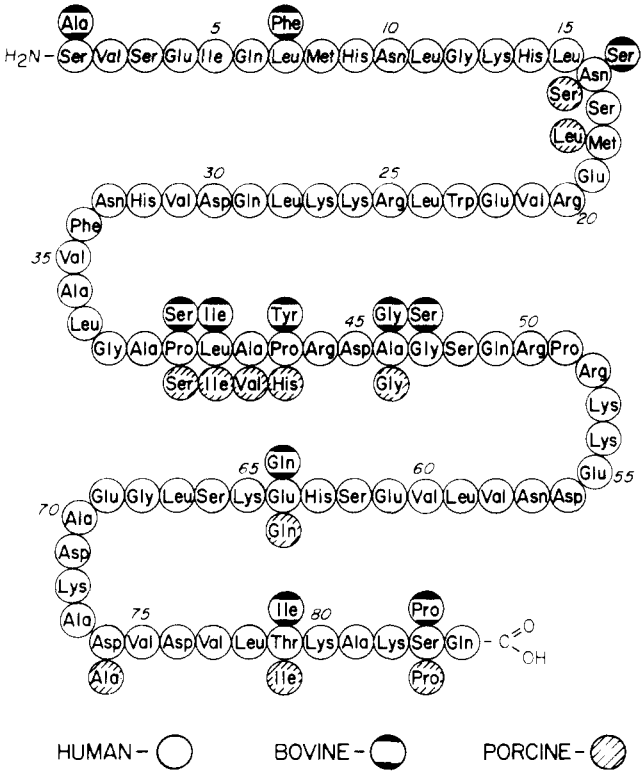


FIGURE 7: Comparison of the amino acid sequences of human, bovine, and porcine parathyroid hormone. The human hormone is shown by the backbone sequence; substitutions found in the bovine and porcine hormones are indicated by circles alongside.

porcine molecules. The human hormone differs from each of the other species at 11 positions. Eight of these represent residues unique to the human molecule. All substitutions represent single-step mutations in the triplet-base codon, except for the substitution of proline (human) for tyrosine (bovine) at position 43. This position is unique in containing a different amino acid for each of the three species; the tyrosine found here in the bovine is the only occurrence of this residue thus far known in a parathyroid hormone molecule. Nonetheless, the human hormone, like the porcine, has proven to be a satisfactory tracer after labeling with radioiodine, which must occur predominantly at the histidine residues.

The most abundant substitutions among the three hormones occur in the midportion of the molecule: through the region 40–47, six of the eight residues in hPTH differ from either or both of the other species. Especially noteworthy is the high content of proline in one relatively short segment of the human sequence. Three residues of proline out of the 12 between positions 40 and 51 could impart marked conformational differences to this part of the molecule. This may, in turn, influence the immunological cross-reactivity between human and other species of hormone, especially with the numerous antisera currently in use that appear to be directed toward the middle region. The rate or extent of enzymatic cleavages during peripheral metabolic breakdown of the hormone, studied earlier with the bovine hormone (Segre et al., 1977), might also be affected. Although the sequences found in the human hormone at the principal sites of cleavage identified in the bovine (residues 33–34 and, secondarily, 36–37) are identical in the two molecules, the extensive substitutions in the human commencing at nearby residue 40 could influence the ease with which such cleavages take place.

Although the carboxyl-terminal region is generally well conserved, the changes that are seen at positions 79 and 83 may be sufficient to impair immunological cross-reactivity, as observed with antibovine antisera directed toward this region of the molecule (Hendy et al., 1974; Murray et al., 1975). Replacement of proline at position 83, for example, may favor extension of a region of $\alpha$-helical structure (Fiskin et al., 1977) further toward the carboxyl terminus.

Our analyses show the location of charged residues throughout the human hormone molecule to be identical with the bovine, with the exception of the substitution of glutamic acid for glutamine at position 64. This difference of a single negative charge may be reflected in the finding that hPTH elutes slightly earlier than bPTH from a carboxymethylcellulose ion-exchange column (Keutmann et al., 1975). There is an additional negative charge difference between human and porcine hormone; aspartic acid, present at position 74 in hPTH and bPTH, is replaced by alanine in pPTH.

In addition to fragments and analogues based on the active amino-terminal region for use in a wide range of structure–function studies, synthetic peptides from other regions of the molecule have recently been prepared as the sequence studies have progressed, among them the 44–68 and 53–84 sequences (Rosenblatt et al., 1977, 1978) along with a number of shorter fragments. The use of one of these in supporting the current sequence analysis is described under Results. These peptides are now being widely applied to the development and characterization of antisera, in further immunological comparison between discrete regions of hPTH and bPTH, and for studies designed to discern any potential biological effects not previously recognized outside the amino-terminal region. The complete sequence of human parathyroid hormone should now permit the synthesis of additional peptides, containing the middle portion with its distinct differences from other species, for continued definition of the physiological and immunological properties of the human hormone.

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# Thyroxine-Induced Conformational Changes in Prealbumin†

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ABSTRACT: The effects of thyroxine binding on the conformation of human prealbumin and bovine serum albumin have been examined. A blue shift in protein absorption was observed with prealbumin, whereas a red shift was observed with bovine serum albumin. In the case of prealbumin, where the two binding sites are identical, the total absorption change was confined to the binding of the first ligand and has been interpreted as resulting from a conformational change. A blue shift observed in the absorption spectrum of thyroxine, however, was the same for the first and second bound molecules. These data have been interpreted in terms of two identical and interacting sites on prealbumin and explain the origin of the difference in binding affinities between the first and second sites. Fluorescence quenching by thyroxine and thyroxine effects on tryptic hydrolysis of prealbumin are in accord with the above interpretation.

Prealbumin binds 2 mol of thyroxine ($T_4$)[1] or $T_3$ with binding constants that are two orders of magnitude different for each mole (Ferguson et al., 1975; Cheng et al., 1977). The negative cooperativity observed in the binding of the two hormone molecules is not due to heterogeneity in the binding sites, since these are identical as indicated from the X-ray analysis of Blake et al. (1971, 1974). Moreover, the closest approach of the two sites is almost 10 Å (Blake and Oatley, 1977), so that a steric mechanism cannot explain the interaction between the two sites. There are binding data at several pH values with the analogue DIPA, which suggests that an electrostatic interaction may contribute to the negative cooperativity (Cheng et al., 1977). A common mechanism of developing cooperativity in subunit proteins involves a conformational change on binding a ligand on one subunit, which then alters the interactions between subunits and, consequently, the binding affinity for subsequent ligands. We have examined the binding of $T_4$ and DIPA to PA in order to determine whether conformational changes are produced. For comparison, the binding of thyroxine to another thyroid hormone transport protein, i.e., bovine serum albumin, was also evaluated.

## Materials and Methods

Human serum prealbumin (PA), obtained from Behring Diagnostics, was purified further by gel electrophoresis, as

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[1] Abbreviations used: PA, human serum prealbumin; BSA, bovine serum albumin; $T_4$, L-thyroxine; $T_3$, 3,5,3′-triiodo-L-thyronine; DIPA, 3-(4-hydroxy-3,5′-diiodophenyl)propionic acid; ANS, 8-anilino-1-naphthalenesulfonate; Tris, tris(hydroxymethyl)aminomethane.