Phosphorylation of Parathyroid Hormone by Human and Bovine Parathyroid Glands*

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The major glandular form of PTH is known to be an 84 amino acid straight chain peptide hormone in all species so far examined (1-4). The hormone is synthesized on the ribo-
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(Whatman CM-52, Reeve Angel Co.). After applying the lyophilized hormone from the P-100 chromatography step in the starting buffer, a linear conductivity gradient was developed, using 70 ml of 0.1 M ammonium acetate, conductivity 1.9 mS, pH 5.0, and 70 ml of 0.1 M ammonium acetate, conductivity 16.5 mS, pH 6.5. Fractions of 1 ml were collected and aliquots were taken for determination of 32P and 35S radioactivity by scintillation counting, or by using Cerenkov radiation (18). Other aliquots were employed for radioimmunoassay.

For chromatography of dansyl amino acids, the mixture of derivatized residues with standards was dissolved in 0.1% F3C3COOH, injected directly onto a C18 μBondapak column using a U6K (Waters) HPLC system consisting of two 6000-A pumps controlled by a M720 system controller and using C18 μBondapak columns. Samples for loading were diluted with 0.1% F3C3COOH or 0.13% heptafluorobutyric acid (F3CCOOH) and were generally pumped directly onto the HPLC column using one of the unused ports on the "aqueous" pump. Columns were then developed over 60-90 min at a flow rate of 1.5 ml/min, with linear gradients of acetonitrile containing 0.13% F3C3COOH or with linear gradients of acetonitrile containing 0.1% F3C3COOH. Column eluates were monitored for UV absorbance at 210 nm using a variable wavelength flow-through spectrophotometer (model M-480, Waters Associates) and a M730 data module (Waters). Radioactivity was determined in aliquots of eluted fractions either by scintillation counting or by using Cerenkov radiation (18). Other aliquots were employed for radioimmunoassay.

For chromatography of dansyl amino acids, the mixture of derivatized residues with standards was dissolved in 0.1% F3C3COOH, injected directly onto a C18 μBondapak column using a U6K (Waters) injector and then eluted with an isotonic system of 7.5% acetonitrile in 0.1% F3C3COOH (19).

Radioactivity Counting—Aqueous samples (0.05 to 0.1 ml) were counted in 10 ml of fluor (New England Nuclear, Formula 947) on a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co.) with window settings such that no correction for spillover into the 35S channel was needed. Alternatively, 32P in 1.5-ml fractions from HPLC was counted in minivials, without fluor, via Cerenkov radiation (18). All 32P data from a given incubation were corrected for decay and are reported as counts per min per total chromatographic fraction on the date of the incubation; analyses were completed within 15 days of the incubation.

Radioimmunoassay—Radioimmunoassays for PTH were carried out as previously described (16), employing as tracer [3H]labeled bovine PTH(1-84) and, as standard, purified bovine PTH(1-84) incubation; analyses were completed within 15 days of the incubation. In four experiments, as assessed by immunoreactivity, UV absorbance, and amino acid analysis, the approximate ratio of phosphorylated to unphosphorylated hormone was 1:6 (range 1:9 to 1:4).

Time Course of Phosphorylation—To assess the time course of phosphorylation, human parathyroid slices were incubated in vitro with 32P, and [35S]methionine for increasing time periods, following which hormone was extracted in 8 M urea, 0.1 N HCl and purified by gel filtration followed by ion exchange chromatography. At early incubation times, 35S was incorporated into a peak which eluted on ion exchange chromatography as a basic peptide in the elution position of ProPTH (Fig. 9, A and B). No co-elution of 35S with 32P was seen at the earliest time period (Fig. 34). With increasing...
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FIG. 1. Analysis of extracted \( {^{32}}P \), \( {^{36}}S \), and PTH immunoreactivity on gel filtration (A), ion exchange chromatography (B), and reversed phase HPLC (C) after incubation of human parathyroid tissue with \( {^{32}}P \) and \( [\text{a-}{^{35}}S] \) methionine for 4 h. An 8 M urea, 0.1 N HCl extract was initially gel filtered on Bio-Gel P-100 as described under "Experimental Procedures" (A). "PTH" indicates the elution position of standard PTH-(1-84), and \( V_0 \) and \( V_C \) indicate the position of the void volume and salt volume, respectively. PTH immunoreactivity in eluted fractions was determined with both NH\( _2 \)-directed (N, \( \text{---} \)) and COOH-directed (C, \( \text{===} \)) radioimmunoassays. Immunoreactivity is expressed as microgram equivalents (pg eqiu) of bovine PTH-(1-84) standard. The detection limit of the radioimmunoassays is represented by \( d \). The major region of immunoreactivity was pooled and analyzed by ion exchange chromatography on CM-cellulose, as described under "Experimental Procedures" (B). The concordant peak of NH\( _2 \) and COOH immunoreactivity and of \( ^{32}P \) and \( ^{35}S \) was then further analyzed by reversed phase HPLC eluting with a linear gradient of 28 to 48% ace- tonitrile in 0.1% FSCCOOH over 60 min on a C\text{18} Bondapak column, as described under "Experimental Procedures" (C). \( ^{32}P \) and \( ^{35}S \) were determined in all fractions in the experiment shown in C; however, no radioactivity was detected other than in the peaks shown.

Time of incubation, a second peak of \( ^{35}S \) appeared, in the elution position, on ion exchange chromatography, of PTH-(1-84) (Fig. 3, B and C; see also Fig. 1). Increasing \( ^{32}P \) was observed co-eluting with this material, with increasing time of incubation (Fig. 3; see also Fig. 1). When the total \( ^{36}S \) and \( ^{32}P \) radioactivity was determined in peaks eluting in the position of PTH-(1-84) and ProPTH, the overall time course suggested the early appearance and then disappearance of labeled amino acid in the peptide eluting in the position of ProPTH, the later appearance but then reduction of labeled amino acid in the peptide eluting in the position of PTH, and the progressive increase in the incorporation of \( ^{32}P \) into a peptide eluting in the position of PTH (Fig. 4).

The effect of preincubation of glandular tissue with \( ^{32}P \) was then determined. After a 4-h preincubation of human parathyroid tissue in RPMI 1640 medium with \( ^{32}P \), tissue slices were washed with medium and then reincubated with fresh RPMI 1640 medium containing \( [\text{a-}{^{35}}S] \) methionine but no unlabeled methionine. After 15 min of incubation, hormone was extracted in 8 M urea, 0.1 N HCl and purified by gel filtration followed by ion exchange chromatography as before. Two peaks of \( ^{35}S \) were now seen, one smaller one, eluting in the position of PTH-(1-84) and a more basic peak eluting later, in the position of ProPTH (Fig. 5). Additionally, two major peaks of \( ^{32}P \) were now seen as well, co-eluting with the two \( ^{35}S \) peaks (Fig. 5).

Site of Phosphorylation—To provide further evidence of the existence of phosphorylated hormone and to ascertain the nature of the phosphorylated residue, bovine parathyroid gland slices were incubated with \( [\text{a-}{^{3}H}] \) serine, and then labeled hormone was extracted and purified by HPLC as described above. An aliquot of the purified \( ^{3}H \)-labeled phosphorylated hormone was then subjected to mild acid hydrolysis, and the hydrolysate derivatized with dansyl chloride. The dansyl amino acids, with added standard dansyl serine and dansyl phosphoserine, were then injected onto a C\text{18} Bondapak column and eluted isocratically (Fig. 6). Two major peaks of \( ^{3}H \) radioactivity were observed, co-eluting with the derivatized standard phosphoserine and serine (Fig. 6).

Region of Phosphorylation—To determine the region of the molecule phosphorylated, HPLC-purified PTH, labeled with \( [\text{a-}{^{35}}S] \) methionine and containing \( ^{32}P \), was subjected to dilute acid hydrolysis. This procedure is known to cleave PTH at aspartic acid residues (22) generating only one methionine-
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FIG. 2. Analysis by reversed phase HPLC of 32P-labeled peptides extracted after incubation of bovine parathyroid tissue with 32P, for 4 h, as described under "Experimental Procedures." PTH immunoreactivity was determined in eluted fractions with both NH2-directed (N, O—O) and COOH-directed (C, C—O) assays and expressed as microgram equivalents (µg equiv) of bovine PTH-(1-84). The detection limit of the assays is represented by D. UV absorbance was determined at 210 nm (A210). Initial separation (upper left) was with a linear gradient of 24-48% acetonitrile (CH3CN) in 0.1% F3CCOOH over 60 min. The major peak (A) containing 32P (C—O), immunoreactivity, and UV absorbance was then rerun on the same C18 µBondapak column eluting with a linear (36-48%) gradient of acetonitrile in 0.13% F3CCOOH over 60 min (upper right). The peak (B) of co-eluting 32P, immunoreactivity, and UV absorbance was then rerun, eluting with a linear gradient of 28-40% acetonitrile 0.1% F3CCOOH over 60 min (lower left), resulting in an apparently homogeneous peak (B') which was then subjected to amino acid analysis. Similarly the peak (C) of co-eluting immunoreactivity and UV absorbance (upper right) was rerun eluting with a linear gradient of 28-40% acetonitrile in 0.1% F3CCOOH over 60 min (lower right) resulting in an apparently homogeneous peak (C') which was also subjected to amino acid analysis.

containing (35S-containing) fragment, PTH-(1-29), and several non-methionine-containing fragments. When the mixture of PTH fragments obtained by this procedure was resolved by HPLC, only a single peak of 32P was seen, co-migrating with the single peak of 35S (Fig. 7); consequently phosphorylated material co-chromatographed with an NH2-terminal fragment generated from purified PTH.

DISCUSSION

Our studies have demonstrated co-elution of incorporated 32P and PTH immunoreactivity from extracts of parathyroid tissue in multiple chromatographic systems. Thus phosphorylated material could not be distinguished from PTH-(1-84) in systems resolving on the basis of size (gel filtration), charge (ion exchange chromatography), or hydrophobicity (HPLC). Consequently the phosphorylated material appeared similar to native PTH-(1-84) in many of its chemical characteristics. Only when heptafluorobutyric acid, a stronger ion-pairing agent than trifluoroacetic acid, was employed in the solvent system was it possible to readily resolve the phosphorylated from the unphosphorylated entities (Fig. 2). NH2- and COOH-terminal immunoreactivity was retained in the phosphorylated as well as the nonphosphorylated peaks; amino acid analysis of each peak revealed, in each case, the composition of PTH-(1-84) and failed to disclose any contaminating entity in the phosphorylated peak. Finally, after dilute acid hydrolysis, the 32P co-migrated with an NH2-terminal fragment generated from phosphorylated PTH-(1-84) (Fig. 7). These results, therefore, provide strong evidence for the phosphorylation of PTH by parathyroid glands incubated in vitro.

The failure of progressive incorporation of radiolabeled amino acids into newly synthesized hormone during prolonged incubation (Fig. 4) suggests that with continued incubation either new biosynthesis was decreasing, or, more likely, hormonal degradation was increasing, or both events were occurring. Such an increase in proteolytic activity could include accelerated conversion of ProPTH to PTH, as suggested by the presence of radiolabeled ProPTH after a 20 min
incubation (Fig. 3) but not after 60 min. However, when tissues were rinsed after a 4-h incubation and then reincubated with fresh medium and radiolabeled amino acids for 15 min, proteolysis appeared to be slowed since both newly synthesized ProPTH and PTH were seen (Fig. 5). Of interest is the identification of $^{32}$P in a peak co-eluting with PTH (Fig. 3B) when only minimal $^{32}$P was seen in a ProPTH peak. This finding is consistent with phosphorylation of the hormone only after cleavage from the prohormone. However, complex in vitro kinetics involving accelerated conversion of ProPTH to PTH with increasing time of incubation, at which time phosphorylation with exogenous $^{32}$P may only have begun, could have obscured the capacity to demonstrate modest quantities of phosphorylated ProPTH prior to conversion. When a 4-h preincubation of parathyroid tissue with inorganic $^{32}$P was performed, the tissue washed and reincubated for 15 min, phosphorylation of both ProPTH and PTH was now clearly seen (Fig. 5). Whether this was due to reduced proteolysis, consequent to modification of the initial experimental protocol, requires further investigation. Nonetheless, phosphorylation of the hormone prior to cleavage from the prohormone was demonstrable. This, of course, does not exclude the possibility of phosphorylation of additional hormonal loci after cleavage.

Phosphorylation of serine, threonine, and tyrosine, and less frequently of histidine and lysine residues has been reported (10). Inasmuch as evidence for phosphorylation of both the bovine and human PTH molecules was obtained, but the bovine molecule lacks a threonine residue and the human is deficient in tyrosine, it was decided to explore the possibility that the site of phosphorylation would be serine residues. The results (Fig. 6) demonstrated clearly that a major site of hormonal phosphorylation appears to be on serine residues.

Multiple serine residues are present within the sequence of the PTH-(1-84) molecule (1, 2, 4). Our studies employing limited chemical cleavage (via dilute acid hydrolysis) of the molecule localize the phosphorylation site to residues within the NH$_2$-terminal 1 to 29 sequence of the molecule. Previous reports have suggested the possibility of co-translational phosphorylation of nascent peptides with phosphoseryl-tRNA (25). Additionally, post-translational modification (10) could account for the phosphorylation of PTH. In both bovine and human species the serine residue at position 17 is located within the sequence Ser-X-Glu which is a recognition site for the action of noncyclic AMP-dependent kinases ("physiological casein kinases") (24, 25), and therefore, this position could be a site of post-translational phosphorylation. As well, in both species of hormone the serine residue at position 3 could be a site for cyclic AMP-dependent phosphorylation, when located within the prohormone prior to cleavage inasmuch as the prohormone sequence (26) Lys-2-Arg"+-X+1-Y+2. Ser"+- constitutes a substrate-specific region for the cyclic AMP-dependent kinases. In view of the fact that the bioactivity of PTH is known to reside within the NH$_2$-terminal region of the molecule (27, 28), phosphorylation of serine residues within this region could be of particular significance in modulating hormonal action.

Further studies are, therefore, required to identify the precise site, mechanism, and extent of hormonal phosphorylation.

Table I

| Amino acid | Peak B | Peak C | Bovine PTH |
|------------|--------|--------|------------|
|            | Mol fraction | Mol | Residues/mol | Mol fraction | Mol | Residues/mol |
| Lys        | 0.632   | 9.02  | 3.488       | 9.22         | 9   |
| His        | 0.295   | 4.21  | 1.509       | 3.99         | 4   |
| Arg        | 0.368   | 5.25  | 1.889       | 5.02         | 5   |
| Asp        | 0.654   | 9.34  | 3.471       | 9.18         | 9   |
| Thr        | 0.014   | 0.20  | 0.058       | 0.15         | 0   |
| Ser        | 0.564   | 8.05  | 3.159       | 8.35         | 8   |
| Glu        | 0.776   | 11.08 | 4.075       | 10.78        | 11  |
| Pro        | 0.141   | 2.01  | 0.854       | 2.25         | 2   |
| Gly        | 0.277   | 3.95  | 1.599       | 4.23         | 4   |
| Ala        | 0.502   | 7.17  | 2.685       | 7.12         | 7   |
| Val        | 0.580   | 8.28  | 3.081       | 8.15         | 8   |
| Met        | 0.131   | 1.87  | 0.897       | 2.37         | 2   |
| Ile        | 0.230   | 3.23  | 1.111       | 2.93         | 3   |
| Leu        | 0.587   | 8.38  | 3.026       | 8.00         | 8   |
| Tyr        | 0.076   | 1.08  | 0.411       | 1.08         | 1   |
| Phe        | 0.145   | 2.07  | 0.761       | 2.01         | 2   |

* From HPLC profile shown in Fig. 2.
* Amide nitrogen and tryptophan not determined.

**Fig. 3.** Elution profiles from ion exchange chromatography of $^{32}$P (○) and $^{32}$S (○) extracted from human parathyroid tissue incubated for increasing time periods with $^{32}$P and $^{35}$S)methionine. Preliminary purification of the parathyroid extracts was by gel filtration, as described under "Experimental Procedures," and shown in Fig. 1. Pools from Bio-Gel P-100 columns were analyzed on CM-cellulose columns using an ammonium acetate buffer gradient, as described under "Experimental Procedures." PTH and ProPTH indicate the elution position of PTH-(1-84) and of the prohormone, respectively. Peaks of PTH immunoreactivity, determined in eluted fractions with COOH-directed assays coincided with the major peaks of $^{32}$P and $^{35}$S as shown in Fig. 1B.
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under various conditions of ambient calcium and the effect of hormonal phosphorylation on transport through the parathyroid cell and on bio- and immunoreactivity. Development of specific immunosassays for phosphorylated versus nonphosphorylated hormone could help clarify the in vivo significance of these findings which have been demonstrated by in vitro techniques. The identification of phosphorylated PTH may, therefore, be of major importance in further elucidating multiple aspects of the physiology of parathyroid hormone and of the parathyroid cell.

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