Characterization of Parathyroid Hormone Fragments Produced by Cathepsin D*

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Cleavage of parathyroid hormone by cathepsin D was studied. Four primary products were detected and separated by high performance liquid chromatography. Two of the fragments are fluorescent and therefore contain residue 23 (tryptophan). These fragments are NH₂-terminal in origin. The other two cross-react with antisera directed against COOH-terminal portions of the hormone; they are the complementary COOH-terminal fragments. Microsequencing and amino acid analysis showed that the two COOH-terminal fragments are 35–84 and 38–84 bovine parathyroid hormone. By CNBr cleavage and amino acid analysis, the two NH₂-terminal fragments were shown to be the complementary 1–37 and 1–34 fragments. The 1–37 fragment is transitory and is rapidly hydrolyzed to 1–34, so that only relatively small amounts are detected at any one time. However, 34–84 was not converted to 38–84, although cleavage at other sites in the COOH-terminal fragments was observed with more exhaustive digestion. The 1–34 fragment appears to be the final product of the action of cathepsin D on parathyroid hormone. Both enzymatically produced NH₂-terminal fragments were fully active in the renal membrane adenyl cyclase assay system.

Although the secreted form of parathyroid hormone (PTH') is an 84-residue polypeptide (see Fig. 1), substantial amounts of inactive COOH-terminal fragments of the hormone are found in the circulation (1–6). These fragments appear to be produced primarily in the Kupffer cells of the liver (7–9), but may also be generated in the kidney (10, 11) and in the parathyroid gland itself (12, 13). Since the known biological functions of PTH can be expressed by amino-terminal fragments (14), the production of complementary COOH-terminal fragments in vivo is an intriguing observation. However, the biological significance of PTH metabolism remains a subject for speculation since NH₂-terminal fragments are not normally found in the blood (15).

During studies of PTH receptors in bovine kidney, we found an enzyme generating PTH fragments very similar to those seen in vivo (16–18). This enzyme was ultimately shown to be cathepsin D (19). The major fragments were found to have residues 35 and 38 from the intact hormone as their NH₂ termini. A biologically active NH₂-terminal fragment was also detected (17). Similar results were obtained using rat kidney as the enzyme source, but in this case, the production of a fragment with residue 39 as its NH₂ terminus was observed (18). This fragment was identical to that found in the kidney in vivo (20). From these results it was proposed that cathepsin D is one of the enzymes responsible for PTH metabolism in vivo (19).

Hamilton et al. (21) obtained similar results in parathyroid gland tissue and further studied the nature of the PTH fragments generated by cathepsin D. However, they found that the enzyme cleaves PTH primarily at one position, producing fragments 1–34 and 35–84 as the dominant final products.

Here we describe further studies of PTH cleavage by cathepsin D. We confirm that this enzyme generates two major COOH-terminal products, 35–84 and 38–84. In addition, we also find the complementary NH₂-terminal fragments, 1–34 and 1–37, both of which are fully biologically active. The data show that 1–34 is particularly resistant to further hydrolysis and confirm the conclusion of Hamilton et al. (21) that the 1–34 fragment is a major product of the action of cathepsin D on this hormone.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine parathyroid hormone was purified from defatted glands (Sigma) as described elsewhere (22, 23). Oxidized forms of the hormone were removed in the final HPLC purification step (24). Hormone concentration was determined by UV absorption at 280 nm (ε = 7410 l/cm mol⁻¹) or by amino acid analysis.

Cathepsin D from bovine spleen was purchased from Sigma. This material was found to contain small amounts of apparently nonenzymatic protein and, consequently, it was usually finally purified by adsorption on peptatin-Sepharose at acid pH followed by elution at pH 8 (19).

An antiserum against the COOH-terminal region of PTH was kindly provided by Dr. B. Roos (Veterans Administration Hospital, University Circle, Cleveland, OH). Antiserum against CAMP and goat anti-rabbit antiserum were purchased from Shadrack Biologicals (Cleveland, OH).

**Cathepsin D Digestion of PTH**—Unless indicated otherwise in the individual experiments, 25–50 μg of PTH were added to a solution of cathepsin D (375 ng) in acetate buffer, pH 4.0, in a final volume of 300 μl. Incubation was at 37 °C for periods of time up to 90 min. The reactions were stopped by injection into the HPLC flow.

**HPLC**—All HPLC separations were done on C-18 μBondapak columns with acetonitrile/water solvent systems. The solvents all contained 0.1% trifluoroacetic acid. Detection of peptides was by UV (214 nm) and fluorescence (excitation 254 nm/emission 338 nm). Details of the various systems are given in the figure legends.

**Iodination and Microsequencing**—These procedures have all been described (17). Iodination was done by the Enzymebeam method and sequencing by manual Edman degradation.

**Amino Acid Analysis**—The o-phthalaldehyde method of Pfeifer et al. (25) was used in conjunction with automated sample mixing and injection on a HPLC as described in detail elsewhere (24). Lysine analysis was found not to be reliable with this method, and no lysine determinations are presented.

**CNBr Cleavage of PTH**—Cleavage was carried out in 70% formic acid.

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1 The abbreviations used are: PTH, parathyroid hormone; HPLC, high performance liquid chromatography.
Parathyroid Hormone Fragments Produced by Cathepsin D

Ala-Val-Ser-Glu-Ile-Gln-Phe-Met-His \(10\)

Gly-Leu

20 Arg-Glu-Met-Ser-Ser-Leu-His-Lys \(30\)

Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val

His

Asn

40 Ala-Ile-Ser-Ala-Gly-Leu-Ala-Val

Try

50 Gly-Ser-Ser-Gln-Arg-Pro-Arg-Lys

Asp

Glu

60 Gin-His-Ser-Gln-Val-Leu-Val-Asn

Asp

Leu

70 Gly-Glu-Ala-Asp-Lys-Ala-Asp-Val

Asp

Val

80 Gin-Pro-Lys-Ala-Lys-Ile

Fluorescence

Absorbance (214 nm)

Elution Time (min)

FIG. 1. The primary structure of bovine PTH.

FIG. 2. HPLC separation of the fragments of PTH produced by cathepsin D. PTH was digested for 90 min (A) or 30 min (B and C) as described under "Experimental Procedures." The HPLC gradient was a combination of a hyperbolic (curve 7 on Waters HPLC controller) and a linear gradient as follows: hyperbolic from 10 to 32% solvent B over 15 min and linear from 32 to 50% solvent B over 25 min. Solvent A was 12% acetonitrile and solvent B 60% acetonitrile. The flow rate was 1.5 ml/min.

RESULTS

Our earlier work utilized the technique of electrophoresis to demonstrate fragmentation of PTH. We have now developed an HPLC system for this purpose, and a characteristic elution pattern for the cathepsin D fragments of PTH is shown in the experiment illustrated in Fig. 2. Following the breakthrough peak, this system separates 4 peptides from the native hormone (arrow). Peaks 2–5 originate from PTH, but control experiments showed that peak 1 is not derived from the hormone and probably represents a small "self-digestion" fragment from cathepsin D. Panels A and B show the peaks detected by UV absorption (214 nm) following digestion for 90 and 30 min, respectively. Panel C shows the peaks detected by fluorescence. Since PTH has 1 Trp (residue 23; see Fig. 1), the fluorescent peptides must contain this residue and, based on our previous results, were thought to be NH2-terminal. Peaks 2 and 3 were presumed to be the COOH-terminal fragments found earlier, putatively 38–84 and 35–84. Analysis of the breakthrough peak showed the presence of only small amounts of poorly defined peptides, and cathepsin D eluted in the washout following the native hormone. Thus, 2–5 shown in Fig. 2 represent the primary fragments produced by cathepsin D action on PTH.

Prior to initiating purification and further characterization of the PTH fragments, we examined whether digestion with cathepsin D would destroy the biological activity of the hormone as expressed in the renal membrane adenylyl cyclase assay. The purpose of this experiment was to determine if the tryptophan-containing fragments are biologically active. The results are shown in Fig. 3. Nearly total hydrolysis of PTH produced no loss of biological activity; rather, the reaction mixture, containing primarily peak 4 material, appeared slightly more active than the native hormone (zero time), especially at low concentrations. Thus, it was clear that the fluorescent peaks must be of high biological activity, and their further characterization was of interest.

Confirmation that the nonfluorescent peaks originate from the COOH-terminal regions of PTH was obtained by use of a COOH-terminal-specific antiserum. As expected, only peaks...
Parathyroid Hormone Fragments Produced by Cathepsin D

FIG. 3. Effect of cathepsin D hydrolysis on the biological activity of PTH. 30 μg of PTH were digested with cathepsin D. At specified times during the incubation, aliquots of the reaction mixture were removed for bioassay and for HPLC analysis. The samples to be bioassayed were diluted into Tris buffer, pH 7.6, which inactivates cathepsin D. Panel A shows the bioassay results. Each point is the mean of three separate assays with cAMP analysis conducted in duplicate for each; error bars are the standard error from the mean. Times for digestion: W, 0 min; □, 15 min; △, 30 min; ●, 90 min. Panel B shows the HPLC profiles for each time sample. The samples were chromatographed on the gradient described in Fig. 2, but only the latter portion of the profile, where the hormone and the NH2-terminal fragments elute, is shown. The elution position of intact PTH is indicated by the arrow.

Confirmation of the chemical nature of the two COOH-terminal fragments was finally achieved by amino acid analysis. Table I indicates that the amino acid composition is in agreement with our identification of peak 2 as fragment 38-84 and peak 3 as fragment 35-84.

Identification of peaks 4 and 5 was accomplished by CNBr fragmentation and amino acid analysis. Residues 8 and 18 in PTH are methionine (Fig. 1), and CNBr generates the fragments 1-8 (homoserine), 9-18 (homoserine), and 19-84 (native hormone) or 19-34 (1-34 PTH). The CNBr fragments can be separated on HPLC, and their elution positions have been identified in other work (22). It was found (Fig. 5) that CNBr cleavage of peak 4 produces fragments identical to those generated from synthetic 1-34, while cleavage of peak 5 also produces 1-8 and 9-18. However, the third fragment from peak 5 is more hydrophobic than 19-34 and elutes in a later position. Amino acid analyses of the large CNBr fragment from 1-34, peak 4, and peak 5 are given in Table II. These data establish that peak 4 is indeed fragment 1-34, and peak 5 is fragment 1-37.

We investigated the probable precursor relationships of the four cathepsin D fragments by redigestion of each with cathepsin D (Fig. 6). It was found that 1-37 is rapidly and quantitatively converted to 1-34, presumably by release of the 35-37 tripeptide, Val-Ala-Leu. The 35-84 fragment is not converted to 38-84 by a similar cleavage. However, at high enzyme concentrations, it is slowly cleaved at another site in the chain to produce two new, presently unidentified fragments. These fragments were not detectable products of PTH cleavage under the conditions normally used. Likewise, 38-84 is cleaved, probably at the same locus, to generate two fragments. One of these is identical to that produced from 35-84, and the second is less hydrophobic than its companion frag-
Peaks associated with 1-8 and 9-18 are characteristic (24) and probably represent formylated forms produced by reaction of the solvent with PTH.

Solvent A was 1.6% acetonitrile and solvent B was 60% acetonitrile. The CNBr-generated fragments were cleaved by cathepsin D. This result is in agreement with the data in Fig. 3 which indicate that cathepsin D does not inactivate PTH even after prolonged digestion, since cleavage of 1-34 would produce fragments with reduced activity (14, 36).

**TABLE II**

<table>
<thead>
<tr>
<th>Residue</th>
<th>Experimental values for</th>
<th>Expected values for</th>
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<tr>
<td></td>
<td>Peak 4</td>
<td>Peak 5</td>
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<tr>
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</tr>
<tr>
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</table>

The biological activity of enzymatically produced 1-34 and the 1-37 fragment was assessed directly in the renal membrane adenylyl cyclase assay. As shown in Fig. 7, the enzymatically generated peptides did not differ from the recovered native hormone, either with regard to the amount required to produce half-maximal activation or the actual $V_{max}$ achieved.

**DISCUSSION**

These studies extend our previous work on the cleavage of PTH by cathepsin D which showed production of the COOH-terminal fragments, but did not fully identify them, and which indicated that 1-34 and 1-37 might also be produced (16-18). They also confirm an earlier report that 1-34 is produced by cathepsin D (21) and that enzymatically generated 1-34 is very similar to synthetic 1-34 in its biological properties.

Perhaps the most striking aspect of these findings is that 1-34 appears to be a final product of the action of cathepsin D. While the other fragments are further cleaved (Fig. 6), 1-34 is not. Cathepsin D has been reported to be specific for the Met sequence at residues 8 and 9, or the Leu-Trp sequence at 22-23, are present in the 1-34 region of the hormone (see Fig. 1). The fact that no further cleavage occurs probably reflects the nature of the folding of the peptide chain and is consistent with models for the structure of the hormone (30, 31). The potential biological result of this specificity is that an active fragment of the hormone important in its biological functions, as suggested by Martin et al. (32), can be generated by cathepsin D. The major source of such fragments in vivo could...
be the Kupffer cells (7, 9, 19) which are exceptionally rich in this enzyme (33).

A second observation of interest is the fact that the 37–38 bond can be cleaved in the intact hormone, but not in the 35–84 fragment. This suggests that some conformational rearrangement of the 35–37 region of PTH occurs following cleavage. It also helps establish the pathway by which the fragments are generated. Since 35–84 is not generated from 35–45, the only pathway by which both COOH-terminal fragments can be generated is one in which the intact hormone is cleaved at both the 34–35 and the 37–38 bonds. Thus, although 1–34 can result from a sequential pathway (i.e. intact PTH → 1–37 → 1–34), the converse (i.e. generation of 37–38 from 35–84) does not occur. Although the 34–35 bond appears to be the preferred site as evidenced by the apparently larger amounts of 35–84 produced during digestion, cleavage at the 37–38 bond is also substantial. This finding is in contrast to that of Hamilton et al. (21) who did not observe the production of either 1–37 or 38–84 by cathepsin D. However, a minor product was present in their preparations which was not further characterized and could represent the 38–84 fragment. The 1–37 fragment may have been completely converted to 1–34 under their conditions.

Finally, it is of interest to note our earlier suggestion that fragments such as 1–37 might actually be more biologically active than 1–34, based on structural considerations (30). As shown in Fig. 7, it has not been possible to confirm this idea in the assays we have conducted thus far. However, in our experience with the bioassay of this peptide, we occasionally observe that the 1–37 fragment does appear significantly more potent than either native hormone or 1–34. Thus, it is possible that this fragment may yet prove to be of interest in this regard. During purification, we have found it difficult to obtain quantitative recoveries of this fragment, which we attribute to adsorption losses due to its additional hydrophobicity (residues 35–37 are Ala-Val-Leu). Therefore, we are not confident that the dose-response curves accurately reflect the true concentration of this fragment in the assay, and further work is required before unequivocally ruling out the possibility that this fragment is more active than we can presently demonstrate. Furthermore, biological characterization must be conducted with assays other than the in vitro renal membrane system, since several cases are now known where adenylyl cyclase assay results are not consistent with those obtained in other systems. For example, 3–34 is a potent antagonist in the adenylyl cyclase assay (34), but a weak agonist in vivo (35), and 1–30 is of low potency in vitro, but of equal potency to 1–34 in perfused bone (36). Thus, further work will be necessary before the true biological properties of the 1–37 fragment are known.

REFERENCES