The Mode of Conversion of Proparathormone to Parathormone by a Particulate Converting Enzymic Activity of the Parathyroid Gland*

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The cleavage products from the conversion of proparathormone to parathormone by a bovine and porcine parathyroid microsomal converting activity have been analyzed. In the conversion reaction, the first 6 amino acid residues of the prohormone (Lys-Ser-Val-Lys-Lys-Arg) are released as an intact hexapeptide. This is rapidly converted to a pentapeptide by removal of the NH₂-terminal lysine and then to a tetrapeptide by removal of the COOH-terminal arginine.

In order to test for the presence of a postulated COOH-terminal extension of the parathormone sequence in proparathormone, mixtures of ¹⁴C-proparathormone and ³⁵S-parathormone were subjected to digestion by trypsin or Staphylococcus aureus protease. The resulting radioactive peptides from the hormone and its precursor were compared. There was no evidence that any fragments different from those except those accounted for by the NH₂-terminal hexapeptide adduct on proparathormone. Thus, the conversion of the prohormone to the hormone catalyzed by the microsomal membrane activity requires only the cleavage of this hexapeptide.

The formation of parathormone within the parathyroid gland involves the proteolytic cleavage of the hormone precursor proparathormone (1–3) in or near the Golgi region of the cell (4). Proparathormone includes within its structure the 84-amino acid sequence of parathormone preceded by a basic hexapeptide at the NH₂ terminus (5). In addition, we have raised the possibility that an additional small peptide sequence might be present at the carboxyl end of the hormone sequence (6). Thus, the conversion of the prohormone to the hormone must involve, at the minimum, the removal of 6 amino acid residues at its NH₂ terminus and perhaps an additional sequence at its COOH terminus.

We reported earlier (7) that cell-free particulate and soluble fractions of bovine parathyroid catalyzed the formation of parathormone when incubated in vitro with exogenous radioactive proparathormone, but they did not metabolize parathormone itself. The most active converting activity was associated with the microsomal fraction of the gland homogenate. The activity was trypsin-like in terms of its presumptive cleavage of the arginine–alanine peptide bond of proparathormone, its alkaline pH optimum, and inhibition by the trypsin inhibitor benzamidine. It was dissimilar from trypsin, however, in its inhibition by chloroquine, a cathespin B, inhibitor that does not block the action of trypsin, and by its lack of inhibition by pancreatic trypsin inhibitor. We presented evidence to suggest that this membrane activity represented that which forms parathormone in situ. Some of these results have been independently confirmed (8).

In order to determine the mode of proteolytic cleavage catalyzed by the microsomal converting activity, we have now examined the amino acids and peptides released from radioactive proparathormone substrate in in vitro experiments. We conclude that the hormone is formed by removal of the intact NH₂-terminal hexapeptide segment of proparathormone.

**EXPERIMENTAL PROCEDURES**

Preparation of Radioactive Proparathormone and Parathormone Substrates—Fresh bovine parathyroid glands were trimmed, sliced, and incubated in Krebs-Ringer bicarbonate buffer for 1 to 2 h as described previously (7). The slices were transferred to fresh buffer containing a mixture of 15 ³⁵S-labeled amino acids (New England Nuclear) and incubated 1 h to allow incorporation of the radioactive amino acids into newly formed proparathormone and hormone. Batches of the incubated tissue (about 60 g) were homogenized with 18 U.S.C. Section 1734 solely to indicate this fact.
in liquid scintillation vials contained in a fraction collector set to change every 20 or 60 s. The amount of each amino acid was determined by integration of the area under ninhydrin peaks. Their yield of each amino acid in picomoles was calculated by dividing the number of reaction products. If a peak contained more than one amino acid, the radioactivity was determined by multiplying the disintegrations per min in the amino acid by its specific radioactivity based on an average of the lowest picomole yields of amino acids, excluding those which were abnormally low. For example, a peak with a composition in picomoles of Ala, 20; Val, 18; Ile, 2; Lys, 45 (1.5 to 3 ml) was collected and assayed for radioactivity. Fractions 1751, there was a substantial contamination due to isoleucine. Those peaks that were major markers. The pH was adjusted to 2.8 with formic acid and the fraction was diluted with distilled water to lower its conductivity, adjusted to pH 2.5 to 2.8, and applied to a column (6 x 55 cm) of SP-Sephadex C-25 (Pharmacia). Radioactive peptides and amino acids were eluted with a linear gradient from 0.01 M ammonium acetate to 0.5 M ammonium acetate, pH 6.8, at a flow rate of 6 to 8 ml/h. Fractions of 1.5 to 3 ml were collected and assayed for radioactivity. Fractions from radioactive peaks were lyophilized and analyzed for amino acid composition or sequence.

When the total incubation mixture was analyzed, the mixture was diluted with 4 ml of ice cold 6 M urea containing crude parathyromone (trichloroacetic acid powder preparation) as carrier and purified 14C-labeled proparathormone as marker. The pH was adjusted to 2.8 with formic acid. The solution was chromatographed on SP-Sephadex as described above. The composition of each chromatographic component was determined as described above for the analysis of proparathormone. The yield of each amino acid in picomoles was calculated by dividing the disintegrations per min in the peak by its specific radioactivity in the prohormone substrate. If a peak contained more than one radioactive amino acid a minimal peptide composition was estimated based on an average of the lowest picomole yields of amino acids, excluding those which were abnormally low. For example, a peak with a composition in picomoles of Ala, 20; Val, 18; Ile, 2; Lys, 45 would be estimated as 20 pmol of Ala, Val, Lys, with a slight contamination due to isoleucine. Those peaks that were major products of the conversion of proparathormone were analyzed further by sequence analysis.

The specific radioactivities (disintegrations per min per nmol) of the amino acid residues appreciably labeled in the 14H-proparathormone that was used as substrate for conversion in this report were: Ser, 7,200 dpm/nmol; Gly, 2,700; Ala, 1,110; Val, 23,000; Ile, 6,650; Leu, 13,600; Phe, 26,100; Lys, 20,200; His, 8,600; Arg, 28,800. The overall specific radioactivity of the prohormone was estimated at 921,000 dpm/nmol based on the above data and by assuming an amino acid composition for proparathormone of parathormone plus that of the NH2-terminal hexapeptide. The results of the total amino acid analysis of the converting activity were suspended in the homogenizing buffer at 5 to 10 mg of protein/ml and stored at -20°. The converting activity was stable indefinitely under these conditions. The conversion of proparathormone to parathormone was measured in the analyzer effluent by scintillation spectrometry in a Packard model 3375 scintillation spectrometer. Quenching was corrected by use of external standards.

The trichloroacetic acid supernatant of each reaction mixture was diluted with 4 ml of ice cold 6 M urea containing 0.1% SnCl2. Following removal of the acid by vacuum desiccation, the sample was applied to the amino acid analyzer as described above to determine the identity and amount of radioactivity in the residue. The trichloroacetic acid-soluble and -insoluble peptides were chromatographed on carboxymethylcellulose columns as described previously (7). The amount of parathormone formed during each conversion reaction was corrected for recovery of the hormone residue, a portion of the phenylthiohydantoin or the unconverted trichloroacetic acid supernatant of each reaction mixture was applied to the amino acid analyzer as described above to determine the identity and amount of radioactivity in the residue. The specific radioactivity of the parathormone isolated from the same batch of tissue as the prohormone was 190,000 dpm/nmol. The specific radioactivities of the parathormone isolated from the same batch of tissue as the prohormone were 190,000 dpm/nmol. The specific radioactivity of the parathormone isolated from the same batch of tissue as the prohormone was 190,000 dpm/nmol. The specific radioactivity of the parathormone isolated from the same batch of tissue as the prohormone was 190,000 dpm/nmol. The specific radioactivity of the parathormone isolated from the same batch of tissue as the prohormone was 190,000 dpm/nmol. The specific radioactivity of the parathormone isolated from the same batch of tissue as the prohormone was 190,000 dpm/nmol.
Conversion of Proparathormone to Parathormone

FIG. 2. Elution patterns of radioactive products formed during a 30-min incubation of 3H-proparathormone with bovine parathyroid membrane converting enzyme. Of prohormone, 835,000 dpm (907 pmol) were incubated with 180 μg of membrane protein. A, trichloroacetic acid-insoluble products separated by chromatography on carboxymethylcellulose. The peak of Fraction 21 is parathormone formed during the incubation, and that of Fraction 44 is unconverted proparathormone substrate. B, trichloroacetic acid-soluble fraction separated by chromatography on SP-Sephadex. The gradient was from 0.01 to 0.4 M ammonium acetate, and 3 ml/fraction were collected. Peaks B, F, J, and K represent fragments from the NH₂-terminal region of proparathormone (see Table I and text).

FIG. 3. Elution patterns of radioactive products formed during 5 min (A and D), 10 min (B and E), and 20 min (C and F) of incubation of 3H-proparathormone with bovine parathyroid membrane converting enzyme. Of substrate, 2.3 nmol (2.1 x 10⁶ dpm) were incubated in a total volume of 900 μl. Three hundred microliters were removed at the end of each incubation period and separated into trichloroacetic acid-precipitable and trichloroacetic acid-soluble fractions prior to chromatography. A, B, C, carboxymethylcellulose elution profiles of trichloroacetic acid-insoluble fractions; D, E, F, SP-Sephadex elution profiles of the trichloroacetic acid-soluble fractions. The salt gradient for the SP-Sephadex columns was from 0.01 to 0.5 M ammonium acetate, and 1.5 ml/fraction were collected. As described in the text, Peak K contains the NH₂-terminal hexapeptide derived from proparathormone. Peaks B, F, and J are derived from this hexapeptide by subsequent degradation.

The multiplicity of peptide peaks observed after 30 to 90 min of incubation (Figs. 1 and 2) suggested that in addition to the conversion of the prohormone to the hormone, some extraneous proteolytic degradation of the prohormone was occurring. In order to more precisely follow the generation of products related specifically to the formation of the hormone, a series of short term incubations was performed. Fig. 3 shows the results of one such experiment. The prohormone was incubated with the bovine membrane fraction for periods of 5, 10, and 20 min. Only two prominent peaks of radioactivity were observed in the trichloroacetic acid precipitate fraction (Fig. 3, A to C), that of the newly formed hormone peak that increased in amount during incubation, and the unreacted prohormone that decreased during incubation. In the trichloroacetic acid-soluble portion of the reaction mixture (Fig. 3, D to F) the SP-Sephadex elution profiles contained four major peaks of radioactivity at each time period (Peaks, B, F, J, and K), in addition to one (Fractions 5 to 12) that was not adsorbed to the column.

In order to determine the mode of cleavage of the prohormone substrate by the membrane-converting activity, each of the major peaks in the trichloroacetic acid-soluble fraction of the reaction mixture (Fig. 3, D to F) was analyzed for its content and sequence of radioactive amino acids and from these data its yield in picomoles was calculated. Table I summarizes the results of analyses of the fractions obtained from the experiment shown in Fig. 3 and from similar incubations that were performed to obtain enough of each peptide sample to analyze repetitively. The bovine mem-

* As reported previously [7], the hormone itself is not metabolized to any appreciable extent by the membrane activity even upon prolonged incubation.
brane activity was used in all of these experiments to obtain the peaks for amino acid compositional studies and for most of the studies to determine sequence of the peptides. As mentioned above, however, porcine membranes generated more Peak K than did bovine membranes. Hence, we used the porcine membranes in order to obtain an adequate supply of this peak. Peaks F, J, and K were all small peptides that appeared to have been derived from the NH₂-terminal region of the proparathormone sequence. Based on amino acid composition and sequence analysis, Peak K proved to represent the intact NH₂-terminal hexapeptide of the prohormone sequence. In the sequence analysis of this and the other peptide peaks, serine was not specifically identified because of destruction upon back hydrolysis (15) and its relatively low specific radioactivity. It was placed in position 2 since each of the other residues was unambiguously identified. Peak J was identified as the pentapeptide Ser-Val-Lys-Lys-Arg by its minimum amino acid composition and partial sequence determination. Although in the latter analysis, neither serine nor other residues was unambiguously identified. Peak J was based on the following logic: (A) the radioactivity in residue 1 (assayed in the thiazolinone derivative prior to back hydrolysis) was appropriate for a serine residue (7,200 dpm/nmol) but was too low for arginine (28,800 dpm/nmol); (b) the pattern of isotope yield per amino acid residue in the analysis of sequences corresponded to that obtained for Component F that contained serine but not arginine; and (c) as shown subsequently, Component K is a precursor of this component and since K contains arginine at residue 4, its desysine derivative would contain arginine at residue 5. Peak F was based on compositional and sequence analysis unambiguously identified as the tetrapeptide Ser-Val-Lys-Lys. Finally, Peak B was identified as free lysine by its elution position from SP-Sephadex that corresponded to authentic lysine and by automated amino acid analysis of the sample both before and after acid hydrolysis. Its trailing edge likely contained a rising level of arginine since in somewhat longer incubations, free arginine could be distinctly detected as a separate peak of radioactivity that eluted slightly after the lysine peak (for example, Fraction 41, Fig. 2).

The time course for the production from proparathormone of parathormone and the three major peptide products observed in the experiment illustrated in Fig. 3 are plotted in Fig. 4. The amount of parathormone increased throughout the experiment. The hexapeptide appeared at the earliest time examined and remained at a fixed level thereafter. The pentapeptide was also present after 5 min of incubation but increased in amount during incubation. The tetrapeptide slowly increased in amount after an initial lag. When summed, the molar yield of the three peptides was only slightly less than that of the hormone that was formed at each time period. These data suggest that the NH₂-terminal hexapeptide was liberated intact. By removal of its NH₂-terminal lysine it was rapidly converted to the pentapeptide and then by slower removal of its COOH-terminal arginine to the tetrapeptide. Based on elution positions and compositional analyses the major peaks of Fig. 2 that correspond to those of Fig. 3 are indicated by the same lettering. In addition, as mentioned above, free arginine is the peak at Fraction 41.

Analysis of several other SP-Sephadex peaks from the longer term incubation experiments shown in Fig. 2 (for example, peaks centered at Fractions 44, 82, and 95) and from other similar experiments indicated that they were primarily peptides or amino acid mixtures that apparently originated from the hormone sequence of the proparathormone substrate. For example, the peak at Fraction 44 (Fig. 2) always contained Pro, Val, Leu, Lys, and in some experiments contained in addition Ser, Gly or Ala, and Ile, and could represent a fragment from the COOH-terminal region of the hormone sequence. The molar yields of the minor peptide peaks.

In evaluating amino acid compositional data based on radioactivity of the individual residues, only those residues that are substantially labeled with radioisotope can be detected.

### Table I

<table>
<thead>
<tr>
<th>Peak</th>
<th>Relative (^{14} )Amino-acid composition</th>
<th>(^{14} )Amino-acid sequence analysis</th>
<th>Structure assumed</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>Lys</td>
<td>Val</td>
<td>Lys</td>
</tr>
<tr>
<td>F</td>
<td>Ser (0.94), Val (1), Lys (2.47)</td>
<td>Val</td>
<td>Lys</td>
</tr>
<tr>
<td>J</td>
<td>Ser (0.99), Val (1), Lys (2.41), Arg (1.13)</td>
<td>Val</td>
<td>Lys</td>
</tr>
<tr>
<td>K</td>
<td>Ser (1.07), Val (1), Lys (3.09), Arg (1.0)</td>
<td>Lys</td>
<td>Val</td>
</tr>
</tbody>
</table>

\(^{a}\) See Fig. 3 for typical location of these peaks derived by SP-Sephadex chromatography.

\(^{b}\) Average of two to three analyses for Peaks B to J and one analysis for Peak K. The values were normalized to valine.

\(^{c}\) Amino acid residues were identified after back hydrolysis of thiazolinones obtained by automated Edman degradation.
assuming that each peak was a single peptide, were small when compared to the level of the hormone formed during the incubation, and even if peptide peaks with similar amino acid compositions were combined never exceeded 15% of the yield of parathormone in the 1st hour of incubation.

Examination of Structure of Proparathormone — The results described thus far indicated that during the conversion of proparathormone to parathormone, the only major products formed in addition to the hormone itself resulted from the liberation and subsequent metabolism of the NH$_2$-terminal hexapeptide of the prohormone. In view of our earlier studies that suggested that an additional COOH-terminal peptide sequence might be present in proparathormone (6), an examination of the structure of the radioactive prohormone was performed in order to aid in the interpretation of the conversion data. Mixtures of $^3$H-parathormone and $^{14}$C-proparathormone were subjected to hydrolysis by either trypsin or by Staphylococcus aureus protease, an endopeptidase specific for cleavage on the carboxyl side of glutamic acid residues (16).

Digests were chromatographed on SP-Sephadex columns using the same conditions as for conversion digests. The elution profiles were analyzed in order to detect the presence of a peptide or peptides that was not present in parathormone, and which might originate from the COOH terminus of proparathormone. Fig. 5 shows that the only unique peak resulting from the digestion of the prohormone with trypsin (Fraction 127) had the composition Lys, Arg.. This peak could have represented the Lys-Arg sequence in positions 5 and 6 of the proparathormone substrate. The tetrapeptide representing the prohormone residues 1 to 4 (Lys-Ser-Val-Lys) was not detected, however, possibly because it co-eluted with another peptide peak.

In the profiles resulting from the S. aureus protease digestion (Fig. 5B), two unique peptide peaks were observed. The first of these (Fraction 26) contained $^3$H and hence was derived from parathormone. Amino acid analysis gave the composition Ala, Val, Ser, Glu, which is that of the first 4 NH$_2$-terminal residues of the hormone. The second peak (Fraction 204) contained $^{14}$C and hence was derived from the proparathormone substrate. It had a composition of Lys, Ser, Val, Glu, Ala, Arg., and was identified as the NH$_2$-terminal decapeptide resulting from cleavage at residue 10 of proparathormone.

These results combined with those described in the previous section provide convincing evidence that the sole difference in structure between parathormone and its precursor is the presence of the hexapeptide adduct on the NH$_2$-terminus of the latter.
like attack could explain why the intact hexapeptide has not been detected within the parathyroid gland (8).

It must yet be determined whether the converting activity associated with the parathyroid membranes is a single enzyme complex combining both endo- and exopeptidase as a functional system, or whether the membrane fraction used in these studies expressed some proteolytic activity unrelated to conversion in vivo. Purification of the converting system and comparisons between conversion products formed in vitro and in situ should allow resolution of this question. These studies are in progress. It would be of interest to determine whether metabolism of the hexapeptide occurs within the membrane system of the cell, or whether it is transferred to the cytosol prior to its degradation. At this point, the means by which the hexapeptide is metabolized within the cell are not known. Our preliminary studies in this area have indicated, however, that the process is quite rapid in situ.

The present data also clarify the question of the structure of the COOH-terminal region of proparathormone. We previously summarized evidence in support of a view that a peptide sequence existed on the COOH-terminus of prohormone which was not present in the structure of parathormone (6). The most compelling evidence includes (a) amino acid composition of the prohormone, which is not in accord with the concept that proparathormone consists of 90 amino acid residues (i.e. the 84-amino acid parathormone structure and the NH₂-terminal hexapeptide), and (b) migration rates upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration that suggest that the prohormone has a molecular weight of about 12,500. Since the NH₂-terminal hexapeptide that proparathormone consists of 90 amino acid residues (i.e. the 84-amino acid parathormone structure and the NH₂-terminal hexapeptide), and (b) migration rates upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration that suggest that the prohormone has a molecular weight of about 12,500. Since the NH₂-terminal hexapeptide that proparathormone consists solely of a go-amino acid peptide moiety, and (6) migration rates upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration that suggest that the prohormone has a molecular weight of about 12,500. Since the NH₂-terminal hexapeptide adduct sequence was well established, this evidence might be interpreted to mean that the prohormone has a COOH-terminal adduct of as many as 15 additional amino acid residues. Following proteolytic digestion of the prohormone with either pure enzymes or the parathyroid particulate converting activity in the current study, however, we found no peptides that could not be ascribed to the NH₂-terminal region of the prohormone. These results agree with the earlier results of Habener and his co-workers (11, 20). Based on these experiments, we concur with the latter workers that the structure of proparathormone consists solely of a 90-amino acid peptide chain containing an NH₂-terminal hexapeptide followed by the 84-amino acid sequence of parathormone itself. The conversion of the prohormone to the hormone, therefore, requires only the removal of the NH₂-terminal hexapeptide. The reason for the anomalous migration of the prohormone and its apparent excess content of amino acids must yet be explained.

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