Conversion of Proparathyroid Hormone to Parathyroid Hormone by a Particulate Enzyme of the Parathyroid Gland*

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The conversion of proparathyroid hormone (proparathormone) to parathyroid hormone (parathormone) by subcellular fractions of the bovine parathyroid has been investigated. The identification of the conversion product as parathormone was established by its elution position during ion exchange chromatography and gel filtration, and by partial amino acid sequence analysis of its NH₂-terminal region. Total homogenates and derived subcellular fractions (600 x g pellet, 5,000 x g pellet, 20,000 x g pellet, 190,000 x g pellet, and 190,000 x g supernatant) all catalyzed the conversion of exogenous [³H]- or [¹⁴C]prohormone. Over 60% of the converting activity was in the particulate fractions; the 190,000 x g particulate fraction contained the highest specific converting activity. The converting activity appeared to be an integral component of the membranes since it could only be partially removed by extraction with Triton X-100. The production of parathormone by the particulate converting enzyme increased with time and the concentration of enzyme protein. The optimum pH range was between 7 and 9, and the enzyme was inactive below pH 6. Conversion by the particulate enzyme was inhibited by benzamidine or chloroquine, but not by pancreatic trypsin inhibitor, indicating its dissimilarity to trypsin. When a mixture of [¹⁴C]proparathormone and [³H]parathormone was used as substrate, the particulate enzyme did not metabolize the hormone despite over 70% conversion of the prohormone to hormone and other peptides. There was a close correlation between the subcellular distribution of converting activity and that of newly formed parathormone found in the membrane fraction. These data suggest that the particulate converting activity is that concerned with the formation of parathormone in vivo.

Parathormone is formed in vivo by proteolytic cleavage of its precursor, proparathormone (1, 2). The prohormone contains a basic hexapeptide on the NH₂ terminus (3) that precedes the 84-amino acid sequence of the hormone and may also contain a peptide segment on the COOH terminus which is as yet not fully characterized (4). The conversion of proparathormone to parathormone involves, therefore, the removal of both the NH₂-terminal hexapeptide and the putative carboxyl adduct.

Virtually all of the hormone and prohormone of the parathyroid is associated with membrane-limited structures (5). Several lines of evidence indicate that newly formed proparathormone is transported through the cisternal space of the endoplasmic reticulum to the Golgi region of the cell where the conversion to parathormone takes place (6, 7). In addition, there may also occur along the same pathway complete degradation of the prohormone or hormone which may constitute a mechanism for the short or long term control of hormone production (8, 9). Based on these considerations, it seems likely that the physiological enzymes responsible for the intracellular metabolism of the prohormone and hormone are also membrane-associated. Until now, however, little has been reported on the capacity of membranes of the parathyroid tissue to metabolize proparathormone or parathormone.

In this report, we describe studies on the in vitro conversion of the prohormone to hormone catalyzed by a particulate-associated enzymatic converting system. This activity appears to represent that which forms the hormone from the prohormone in the intact cell.

EXPERIMENTAL PROCEDURE

Tissue Processing—Bovine parathyroid glands were obtained from the abattoir and trimmed to remove fat. From 5 to 15 g of tissue were cut into 0.3 to 0.5-mm thick slices using a Stadie-Riggs apparatus, then "conditioned" for 1 h in Krebs bicarbonate buffer containing 5% fetal calf serum at 37°C. For the incorporation of radioactive amino acids into tissue proteins, slices were transferred from the initial incubation buffer to the same type medium containing in addition [¹⁴C] or [³H]-amino acids (New England Nuclear Corp., Boston, Mass.). Following incubation, the slices were separated from the medium and...
homogenized in 6 volumes of SHM buffer at 4°C using a Willems polytron (Brinkmann Instruments Inc., Des Plains, Ill.) at low speed.

To study the subcellular distribution of converting activity, the homogenates were fractionated by differential centrifugation using Spinco J-21B and Spinco L2-65 centrifuges (Beckman Instruments, Spinco Division, Palo Alto, Calif.). The homogenate was centrifuged at 20,000 x g for 15 min. The resultant pellet was resuspended in 1 tissue volume of SHM buffer and centrifuged as before. The combined supernatant fluids were centrifuged at 5,000 x g for 15 min. The resultant pellet was centrifuged at 190,000 x g for 90 min. When only the 100,000 x g pellet was desired, homogenates in 6 volumes of SHM buffer were centrifuged at 20,000 x g for 15 min, and the resulting supernatants were then centrifuged at 100,000 x g for 1 to 2 h.

Preparation of Carrier Hormonal Protein and Radioactive Hormonal Peptides—Carrier protein was prepared by extracting bovine parathyroid glands with 0.1 M urea, 0.2 M HCl, 0.1 M cysteine, and processing to the trichloroacetic acid powder stage as described previously to yield a crude hormone preparation (10, 11). Radioactive parathyroid gland proparathormone standards and proparathormone substrate for in vitro conversion studies were prepared from tissue slices incubated with radioactive amino acids. The tissue was processed to yield the trichloroacetic acid powder preparation and then chromatographed through columns of Sephadex G-100 and carboxymethylcellulose (12). One preparation of [3H]proparathormone labeled with [3H]serine and [3H]leucine (11,800 dpm per mg) served as substrate for most of the studies of prohormone conversion and was also used in the study involving sequence analysis.

Results

Homogenates of bovine parathyroid glands converted radioactive proparathormone substrate to a peptide which, based on its co-migration with authentic hormone during ion exchange chromatography, was tentatively identified as parathormone (Fig. 1). The production of the hormone was linear with time for up to 2 h, and corresponded to a decrease in the amount of the prohormone substrate. The converting activity was destroyed by incubation in a boiling water bath for 10 min.

In order to determine the subcellular distribution of the converting activity, experiments were performed in which tissue homogenates were separated by differential centrifugation into several fractions (Table I). Recovery of the converting activity in the fractions was 80 to 90% of that in the tissue homogenate. Of the activity recovered, 60 to 70% was associated with the particulate fractions. The 190,000 x g pellet contained about 30% of the activity and had a specific converting activity about twice that of the other fractions. In order to test the possibility that differences in converting activity among the fractions, experiments were performed in which tissue homogenates were separated by differential centrifugation into several fractions (Table I). Recovery of the converting activity in the fractions was 80 to 90% of that in the tissue homogenate. Of the activity recovered, 60 to 70% was associated with the particulate fractions. The 190,000 x g pellet contained about 30% of the activity and had a specific converting activity about twice that of the other fractions. In order to test the possibility that differences in converting activity among the fractions, experiments were performed in which tissue homogenates were separated by differential centrifugation into several fractions (Table I). Recovery of the converting activity in the fractions was 80 to 90% of that in the tissue homogenate. Of the activity recovered, 60 to 70% was associated with the particulate fractions. The 190,000 x g pellet contained about 30% of the activity and had a specific converting activity about twice that of the other fractions.
activity might be due to dilution of the radioactive prohormone substrate by nonradioactive endogenous parathormone in the fractions, tissue slices were incubated 2 h in the presence of [3H]prohormone in order that the distribution of [3H]prohormone after tissue fractionation might approximate that of total prohormone. The results showed that approximately equal amounts of [3H]prohormone were in each of the particulate subcellular fractions, with about one-half that level in the supernatant. Based on previous estimates of total tissue parathormone content (1), the amount in any fraction was estimated to be less than 5% of that added as substrate. Thus, the amount of radioactive parathormone formed by the subcellular fractions (Table I) was not appreciably influenced by isotope dilution of the radioactive prohormone substrate.

Effect of Triton X-100 on Particulate Converting Activity—The 190,000 x g particulate fraction was washed with buffer containing Triton X-100 in an attempt to solubilize the converting activity. In three experiments at low detergent to protein ratios (2/1 or less, w/w), more than 75% (range 78 to 93%) of the activity remained insoluble (i.e. sedimented at 190,000 x g). At higher ratios of detergent to protein (8 to 16/1), about 50% (range 48 to 65%) of the total activity remained associated with the particulate fraction. In all cases the specific activity of the insoluble enzyme was slightly higher than that of the control fractions (washed with buffer alone). In one study, the particulate enzyme (190,000 x g pellet) was subjected to three repetitive extractions with Triton X-100 at a detergent to protein ratio of 8/1. Under these conditions, 70% of the converting activity was finally solubilized, 49% during the first extraction, 13% during the second, and 8% during the third. The specific activities of each of the extracts and residue fractions were about the same. These results indicate that the converting activity is an integral component of the membranes which can be released when the membranes are disrupted.

Fig. 2 illustrates the conversion of proparathyroid hormone to parathormone by a particulate preparation before and after extraction with Triton X-100. In comparison to conversion by the starting fraction (190,000 x g pellet), the extracted fraction produced more parathormone and less metabolic side products per unit of prohormone metabolized. This Triton-washed particulate preparation was stable after repeated thawing and refreezing over a 10-month period and was the type used in all subsequent experiments unless otherwise stated.

Confirmation That Major Conversion Product Is Parathormone—After chromatography on carboxymethylcellulose, the putative [3H]parathormone was chromatographed on Sephadex G-100. It co-eluted exactly with authentic [14C]parathormone, which indicated that their hydrodynamic radii were identical.

The partial amino acid sequence of the putative hormone product was examined by Edman degradation (Fig. 3). Radioactive parathormone and proparathormone were isolated from gland slices which had been incubated in the presence of [3H]serine and [3H]leucine. A portion of the radioactive proparathormone was subjected to conversion by the particulate enzyme fraction. When the positions of the [3H]serine and [3H]leucine residues in the radioactive product were compared with those in the authentic [3H]parathormone they corresponded exactly; when compared to the starting [3H]proparathormone substrate they also corresponded exactly after shifting the prohormone 6 residues to the left to account for the NH2-terminal hexapeptide.

By these criteria we conclude that the product of the converting activity is parathormone.

Characterization of Converting Activity—The conversion rate increased with increasing enzyme concentration, and the optimum pH range for the converting activity was between pH 7 and 9. Below pH 6 the formation of parathormone was negligible and the prohormone substrate was not otherwise metabolized. CaCl₂ at a concentration of 10⁻⁴ M stimulated by about 20% the conversion of proparathormone to parathormone at pH 7.4; EGTA at 10⁻³ M inhibited conversion by the same percentage.

Since it has been reported that trypsin can convert proparathyroid hormone to parathormone (21), we compared the effect of various protease inhibitors on the conversion by the particulate enzyme fraction to the production of a parathormone-like peptide by mild trypsin digestion. Both activities were inhibited by...
detected metabolic products, even when higher levels of radioactivity were utilized.

Substrate might have been due to adsorptive effects and produced no hormone (Fig. 4). The initial loss of 10 to 12% of the hormonal incubation was demonstrated by the continuing conversion of proparathyroid hormone fragment (1-34) did not affect the conversion of the hormone. Only 0.3 pg of this hormone was soluble, however, and the remainder sedimented with the membranes and presumably did not equilibrate with the radioactive substrate. Based on these results, the two converting activities exhibited different spectra of sensitivity to the protease inhibitors.

Specificity of Converting Activity—The converting activity did not appear to metabolize parathormone appreciably when the latter was added as substrate. Fig. 4 illustrates the results of incubating a mixture of [3H]parathormone and [14C]proparathormone. After 3 h of incubation, 75% of the proparathormone had been metabolized and parathormone was the major radioactive product. Small amounts of a variety of other fragments were also formed. In contrast, the bulk of the added parathormone was still present in this reaction mixture and only trace amounts of metabolic products could be observed. In a separate experiment designed to quantify the recovery of hormone following incubation with the particulate enzyme, 88% of the [3H]parathormone substrate was recovered after 1 h and 86% after 3 h. No discrete metabolic products could be discerned in the elution profiles. Hormone recovery was 97% after 1-h incubation with particulate enzyme which had been heated to 100°C for 10 min.

Experiments were performed to test whether or not other peptides would compete with radioactive proparathormone during conversion. A 200-fold molar excess (100 µg) of parathormone fragment (1-34) did not affect the conversion of the radioactive proparathormone. A large excess of bovine serum albumin (1.5 to 2 mg) inhibited the conversion by only 25 to 45%.

Physiological Correlates of Converting Activity—We compared the distribution of converting activity within the particulate fractions of gland homogenates to those of newly synthesized (i.e. radioactive) proparathormone, newly formed parathormone, and total hormone content. Gland slices were incubated with [3H]leucine for 15 min, at which time the formation of [3H]parathormone had just begun (7). Accordingly, the newly formed parathormone should be still in or near its site of conversion. Table II lists the results of one such experiment. The largest single portions of converting activity and newly formed (i.e. radioactive) parathormone were in the 190,000 × g pellet. In contrast, [3H]parathormone was distributed evenly in all of the fractions, and total parathormone (by radioimmunoassay) was highest in the 20,000 × g pellet fraction. There was a close correlation (r > 0.9) in the fractions between the distributions of converting activity and [3H]parathormone.

Our earlier studies had established that the bulk of the newly synthesized parathormone was associated with particulate structures of the parathyroid cell (5). We suggested that the formation of parathormone occurred within a membrane-limited organelle, presumably in the Golgi zone (7). Moreover, conversion of proinsulin to insulin (24-26) and of β-lipotropin to β-melanocyte-stimulating hormone (27) have been reported to be catalyzed by membrane-associated enzyme activities. These considerations made it reasonable to suppose that the physiological proparathormone-to-parathormone "convertase" similarly would be associated with particulate structures.

Our present results support this postulate. They establish that subcellular particulate fractions of the gland can efficiently convert added proparathormone to parathormone at physiological pH and with the production of relatively few side products. The distribution of the converting activity, moreover, correlates well with the presence of newly formed parathormone in these fractions, in keeping with the idea that this activity in fact represents that which functions in situ.

The particulate converting activity exhibited a unique selectivity for proparathormone as a substrate. This is shown...
by the studies in which exogenous parathormone remained essentially unmetabolized despite a substantial conversion of the prohormone when both substrates were present in the same incubation mixture (Fig. 4). Also, pointing to a high degree of substrate selectivity by this enzyme preparation was that large amounts of parathormone fragment (1-34) did not affect the rate of conversion of the proparathormone to the hormone. The membrane-associated converting system therefore appeared to recognize the NH₂-terminal hexapeptide region of proparathormone and not to recognize parathormone.

The specific mechanism by which the convertase produces parathormone from proparathormone substrate is unclear. The evidence that is available—namely the inhibition of parathormone production by benzamidine—suggests that the system includes an endopeptidase with trypsin-like specificity. In concert with this suggestion, Goltzman et al. (21) recently reported that the—arginine—alanine—peptide bond of proparathormone is extremely sensitive to cleavage by trypsin. On the other hand, the inhibition of activity by chloroquine, the lack of substantial inhibition by specific trypsin inhibitors, and the failure of the convertase to metabolize parathormone indicate that the convertase is different from trypsin. The effectiveness of the inhibitory agents might of course be modulated by restrictions imposed by the membrane in which the convertase is situated.

The convertase described here is clearly not the same as the parathormone peptidase activity reported by Fischer et al. (28) in porcine parathyroid gland extracts. The latter preparation rapidly cleaved bovine parathormone (proparathormone was not tested) whereas our proparathormone convertase did not. Moreover, the parathormone peptidase was active at pH 6 in which range the proparathormone convertase was inactive. Finally, Fischer et al. (28) found that calcium altered substantially the patterns of hormone degradation, whereas we found little effect of either calcium or of calcium-chelating agents on the convertase. It is conceivable that both types of enzymatic activity play physiological roles in the parathyroid cell—one for the production of parathormone from its precursor and the other for its further intracellular metabolism. In regard to the latter possibility, Chu et al. (9) and Habener et al. (9) have proposed that intracellular parathormone levels are controlled by a calcium-regulated degradation step.

A question of prime importance with regard to paraparathormone specifically, and other precursor peptides in general, is the physiological significance of the peptide adducts which are removed during production of native peptides. Although our knowledge is incomplete, there appears to be at least two types of precursor peptides. The first type appears to be the primary product of mRNA translation and contains hydrophobic peptide adducts on the NH₂ terminus. Blobel and associates in the "signal hypothesis" (29) proposed that the function of this adduct is to direct the nascent peptide into the cisternal space of the endoplasmic reticulum, after which this "signal" peptide is removed. Probable examples of these "prepeptides" are the precursor to immunoglobulin light chain (30); the precursor to paraparathormone (proparaparathormone) (31); and precursors of pancreatic enzymes (32). The second type of precursor peptide results from the cleavage of the "signal sequence" from the first type. These prepeptides have a longer half-life and can be isolated from the cell. Examples of these are paraparathormone (1, 2), proalbumin (33-35), and proinsulin (24).

As an extension of the concept for the function of peptide adducts of the first type, we speculate that peptide adducts of the second type of precursor also serve as processing signals. These would direct the molecules to appropriate sites within the cell where they are packaged into a variety of subcellular organelles including secretory granules and lysosomes. The processing signal would permit entry of the prepeptide into the appropriate pathway or site. The importance of this type of signal is underscored by the discovery of similar basic hexapeptide adducts on proparathormone and proalbumin (3, 35), two entirely different proteins from different organs, both of which appear to be processed intracellularly in the same fashion. Secondly, the unique capacity of the membrane fraction of the parathyroid to metabolize proparathormone but not parathormone itself could mean that the strongly basic adduct on the NH₂ terminus allows the prepeptide access to a specific metabolic compartment where further processing and routing could occur. Peptides inaccessible to this compartment by virtue of lacking the basic peptide adduct would be processed at other sites in the Golgi or endoplasmic reticulum. Further study of the converting system for proparathormone in the parathyroid cell in terms of precise subcellular location and specificity may provide substantiation of this proposal and otherwise aid in the interpretation of these intracellular events.

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