Pre-proparathyroid Hormone: Analysis of Radioactive Tryptic Peptides and Amino Acid Sequence†

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ABSTRACT: The amino acid sequence of bovine pre-proparathyroid hormone has been partially determined by analysis of the polypeptide labeled selectively with radioactive amino acids. Analysis of tryptic peptides containing methionine or lysine indicated that parathyroid hormone, proparathyroid hormone, and pre-proparathyroid hormone had several common peptides. Two lysine-containing peptides present in proparathyroid hormone but not in parathyroid hormone were also present in pre-proparathyroid hormone. In addition, pre-proparathyroid hormone contained several additional lysine- and methionine-containing peptides not present in parathyroid hormone or proparathyroid hormone. Analysis by repetitive Edman degradation of the polypeptide labeled with lysine, methionine, and other amino acids indicated that pre-proparathyroid hormone contained 25 additional amino acids at the amino terminus of proparathyroid hormone; the identities of 17 of the 25 amino acids have been established. An unusual feature found was the presence of methionyl-methionyl at the amino terminus and the presence of 5 methionines within the first 14 amino acids.

Translation in cell-free systems of mRNA from eukaryotes and from viruses that infect eukaryotes has revealed the existence of several proteins not observed previously in studies with intact cells. The products of the translation of mRNAs from poliovirus (Jacobson et al., 1970) and encephalomyocarditis virus (Boime and Leder, 1972) are single large precursor proteins that, in vivo, are apparently rapidly cleaved into several specific viral proteins. Larger forms of myeloma light chains (Milstein et al., 1972) and human placental lactogen (Boime et al., 1975) have also been observed when their respective mRNAs were translated in cell-free extracts. Recently, we translated RNAs from both bovine and human parathyroid tissue in a wheat-germ cell-free system. The major protein synthesized, pre-proparathyroid hormone (Pre-ProPTH),[1] is larger than both parathyroid hormone (PTH) and proparathyroid hormone (ProPTH) (Kemper et al., 1974; Habener et al., 1975).

Some understanding of the biological function, now mostly unknown, of these larger proteins may be afforded by determination of their structure. The amino acid sequence unique to the premyeloma light chain contains a large proportion of leucines (Schechter, 1973), indicating that this region may be quite hydrophobic. These chemical data are in agreement with the hypothesis that this region may interact with the membranes of the endoplasmic reticulum (Milstein et al., 1972). Structural analysis is also an important factor in determining whether these larger proteins are physiological precursors, as we have suggested, for Pre-ProPTH (Kemper et al., 1974; Habener et al., 1975) inasmuch as the precursor must contain the sequence of the final mature protein species, as well as an additional sequence of amino acids. In this report, we compare the tryptic peptides containing methionine and lysine of Pre-ProPTH with those of PTH and ProPTH, and report the partial sequence analysis of the amino terminus of Pre-ProPTH. Pre-ProPTH contains 115 amino acids, i.e., 31 more than PTH, and 25 more than ProPTH.

Experimental Procedures

Materials. [^{3}H]Leucine (31 Ci/mmol), [2,3-^{3}H]alanine (53 Ci/mmol), [3-^{3}H]arginine (27.3 Ci/mmol), [2,3-^{3}H]aspartic acid (24 Ci/mmol), [3,4-^{3}H]proline (34 Ci/mmol), [2,6-^{3}H]tyrosine (45 Ci/mmol), [4,5-^{3}H]isoleucine (65 Ci/mmol), and [3-^{3}H]glutamic acid (16.2 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.). [4,5-^{3}H]Lysine (60 Ci/mmol) and [2,3-^{3}H]phenylalanine (40 Ci/mmol) were obtained from Schwarz/Mann (Orangeburg, N.Y.), and [2,5-^{3}H]histidine (55 Ci/mmol) and [^{35}S]methionine (200–330 Ci/mmol) were obtained from Amersham/Searle (Arlington Heights, Ill.).

Methods. Preparation of wheat-germ extracts, conditions of protein synthesis, isolation of PTH, ProPTH, and Pre-ProPTH, tryptic digestion of the proteins, and paper chromatography and electrophoresis of tryptic peptides are described in the accompanying paper (Kemper et al., 1976). Individual sequence analyses were made on Pre-ProPTH labeled with combinations of amino acids, as indicated in the legends to the figures. Isolated radioactive Pre-ProPTH was subjected to automated repetitive Edman degradation in a Beckman 890 C sequenator. At each cycle, total radioactivity was determined in the butyl chloride extract and the radioactive amino acids released were identified by thin-layer chromatography as described previously (Jacobs et al., 1974).

Results

Two tryptic peptides in PTH corresponding to residues 1–13 and 14–20 contain methionine (Potts et al., 1968;

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[1] Abbreviations used are: PTH, parathyroid hormone; ProPTH, proparathyroid hormone; Pre-ProPTH, pre-proparathyroid hormone; [^{35}S]Met-tRNA^{fMet}, initiator transfer RNA from wheat germ charged with [^{35}S]methionine.

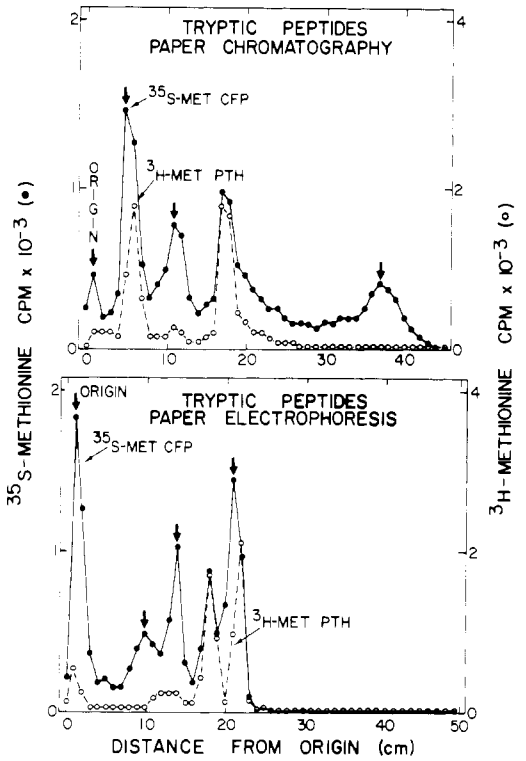

FIGURE 1: Analysis of the methionine-containing tryptic peptides of PTH and Pre-ProPTH. To obtain [*methyl*-^{3}H]methionine-labeled PTH, slices of parathyroid tissue were incubated with 100 μCi/ml of [^{3}H]methionine (10.6 Ci/mmol) in Dulbecco's medium without methionine supplemented with 5% fetal bovine serum, and PTH was isolated from acrylamide gels (pH 4) after electrophoresis, as described previously (Kemper et al., 1972). PTH and Pre-ProPTH were combined before the Bio-Gel P-2 filtration (see Experimental Procedures). Descending paper chromatography was developed for 18 hr, and electrophoresis was carried out for 2 hr at 3000 kV. The relative migrations of the PTH peptides are consistent with previous studies (Potts et al., 1968). Arrows mark the positions of additional peptides in Pre-ProPTH not corresponding to those in PTH. (●—●) [^{3}H]Methionine-labeled peptides of Pre-ProPTH; (○- - -○) [^{3}H]methionine-labeled peptides of PTH.

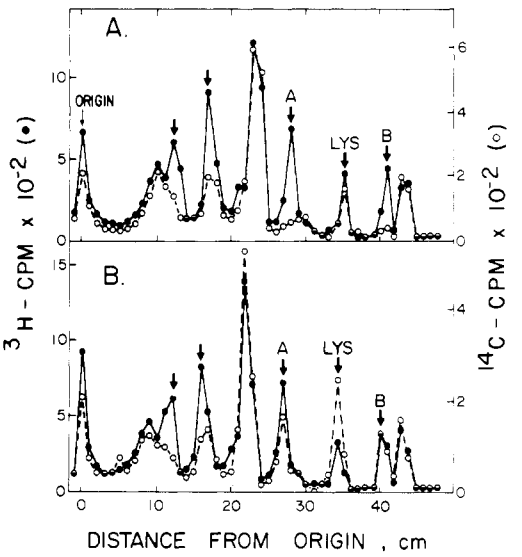

FIGURE 2: Coelectrophoresis on paper of the lysine-containing tryptic peptides of (A) PTH and Pre-ProPTH; and (B) ProPTH and Pre-ProPTH. To obtain [^{14}C]lysine-labeled PTH and ProPTH slices of parathyroid tissue were incubated with 20 μCi/ml of [^{14}C]lysine (0.30 Ci/mmol) in Earle's balanced salt solution with 5% fetal bovine serum for 25 min (ProPTH) or 75 min (PTH). Pre-ProPTH, ProPTH, and PTH were isolated from sodium dodecyl sulfate–acrylamide gels as described in Experimental Procedures. Pre-ProPTH and PTH or Pre-ProPTH and ProPTH were combined before the Bio-Gel-P-2 step. Electrophoresis was for 2 hr at 3000 kV, and lysine was electrophoresed in parallel as a marker. Arrows A and B indicate the peaks present in Pre-ProPTH and ProPTH but not PTH, and the unlabeled arrows indicate the extra peaks in Pre-ProPTH compared with ProPTH. (●—●) [^{3}H]Lysine-labeled peptides of Pre-ProPTH; (○- - -○) [^{14}C]lysine-labeled peptides of PTH (upper panel) or ProPTH (lower panel).

Brewer and Ronan, 1970; Niall et al., 1970). Figure 1 shows a comparative analysis by paper electrophoresis and chromatography of [^{35}S]methionine-labeled peptides from Pre-ProPTH and of [^{3}H]methionine-labeled peptides from PTH. One peptide of Pre-ProPTH corresponds to the 1–13 peptide of PTH, and a second peptide probably corresponds to 14–20, although this comigration is complicated by the apparent presence of a second additional peptide from Pre-ProPTH. There are at least two additional peaks of [^{35}S]methionine that do not have corresponding [^{3}H]methionine peaks. This observation suggests that there are several additional methionines in Pre-ProPTH, and conflicts with our earlier data indicating that there were no additional methionines in Pre-ProPTH (Kemper et al., 1974). We believe that the reason for this conflict may be that, in the earlier studies, the isolated Pre-ProPTH and carrier proteins were difficult to dissolve, possibly because some sodium dodecyl sulfate remained associated with them. Altered chromatographic mobilities of the two methionine-containing tryptic peptides of PTH were observed when comparisons were made with studies in which PTH was isolated without use of sodium dodecyl sulfate–acrylamide gels (Habener et al., 1973). In the present studies, a different procedure was used to remove residual sodium dodecyl sulfate. The proteins were redissolved in 4 *M* urea, passed through a Bio-Gel-P2 column and then oxidized with performic acid before digestion with trypsin (Kemper et al., 1976). The tryptic peptides from PTH isolated in this manner had mobilities similar to the peptides from PTH not exposed to sodium dodecyl sulfate (Habener et al., 1973), and the additional methionine-containing peptides of Pre-ProPTH were then observed. It is clear that our earlier data (Kemper et al., 1974) indicating no additional methionines were in error.

A comparison of the lysine-containing tryptic peptides of Pre-ProPTH with those of either PTH or ProPTH demonstrates that every peak of radioactivity present in PTH or ProPTH has a corresponding peak present in Pre-ProPTH (Figure 2). Two of the peaks (A and B) are unique to ProPTH when compared with PTH (Kemper et al., 1972) and are also present in Pre-ProPTH. Two peaks, marked by arrows at 12 and 16–17 cm, appear to be unique to Pre-ProPTH, and there may be a third unique peak at the origin. Thus, Pre-ProPTH contains the structure of ProPTH and PTH and also has some additional amino acid sequence, as would be expected of a precursor.

Because our studies have indicated that there is no additional sequence at the carboxyl terminus of Pre-ProPTH (Kemper et al., 1974, 1976), the extra sequence is probably at the amino terminus, which would permit direct analysis of the amino acid sequence by automated Edman degradation. The release of ^{35}S radioactivity from isolated Pre-ProPTH labeled with [^{35}S]methionine at each cycle of degradation is shown in Figure 3. Methionine residues are released at cycles 1, 2, 7, 11, 14, 39, and 49. Although the amount of radioactivity released at position 49 is small, it is

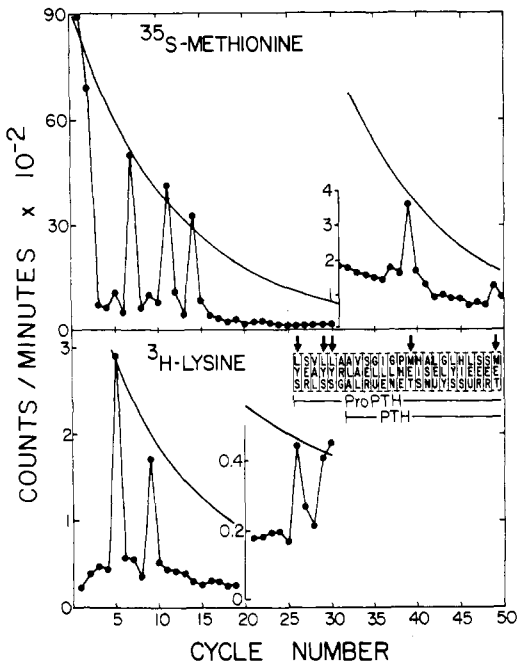

FIGURE 3: Sequence analysis of Pre-ProPTH labeled with methionine or lysine. The methionine data are from Pre-ProPTH labeled with 400000 cpm of [^{35}S]methionine (293 Ci/mmol). The lysine data are from Pre-ProPTH labeled only with 50000 cpm of [^{3}H]lysine. An aliquot of 1/3 of the total radioactivity released at each degradation cycle of automated Edman degradation was assayed for radioactivity. The identity of the amino acid was confirmed by thin-layer chromatography. The continuous curved line represents the theoretical recoveries of methionine or lysine at each cycle determined from a least-squares analyses of the log of the [^{35}S]methionine, or [^{3}H]lysine radioactivity recovered plotted against the number of cycles. The continuation of the sequential degradation into the amino acid sequences of ProPTH and PTH is indicated by the insert.

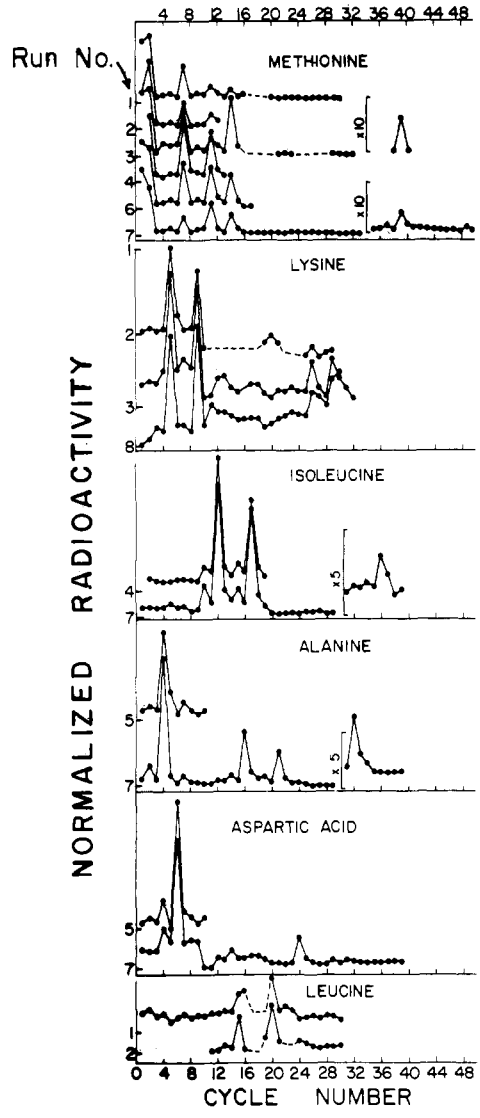

FIGURE 4: Thin-layer chromatography of amino acids removed during sequence analysis of Pre-ProPTH labeled with various radioactive amino acids. Pre-ProPTH for each sequenator run from wheat-germ cell-free reactions containing the following combinations of radioactive amino acids: analysis 1, methionine and leucine; analysis 2, methionine, lysine, and leucine; analysis 3, methionine, lysine, and arginine; analysis 4, methionine, proline, isoleucine, tyrosine, and glutamic acid; analysis 5, histidine, phenylalanine, alanine, and aspartic acid; analysis 6, methionine; analysis 7, methionine, aspartic acid, arginine, alanine, and isoleucine; analysis 8, lysine. The radioactivity plotted represents the relative amount of radioactivity that comigrated on thin-layer chromatography with the indicated amino acid at each cycle of degradation. The amount of radioactivity in each peak for an individual amino acid has been plotted relative to the amount of radioactivity in the peak with maximum radioactivity that has been arbitrarily assigned the value 1 to permit coplotting of the individual analyses. The actual amounts of radioactivity in counts/min present in the maximal peaks are as follows: Methionine, run 1, 400; 2, 1680; 3, 2950; 4, 1465; 6, 30,400; 7, 20,270; Lysine, run 2, 650; 3, 124; 8, 330; Leucine, run 1, 46; 2, 550; Alanine, run 5, 33; 7, 1290; Aspartic acid, run 5, 33; 7, 620; Isoleucine, run 4, 230; 7, 1530. For a particular amino acid, the base line of each successive analysis is lowered slightly to improve clarity. Occasionally, if the total ^{3}H radioactivity released at a cycle was not greater than the background, thin-layer chromatography was not performed at that cycle. Radioactivities in these cycles are plotted as background and are connected by dotted lines. The numbers on ordinate scale refer to the individual sequence analyses, and the numbers on abscissa scale refer to the number of cycles of degradation.

about the amount expected on the basis of the repetitive yield of each cycle. PTH has a methionine at positions 8 and 18 (Figure 5) that could correspond to the methionines released at cycles 39 and 49. If the methionines do correspond, then lysine residues present in ProPTH at positions −6, −3, and −2 (Figure 5) should be released at cycles 26, 29, and 30. In Figure 3, the release of ^{3}H radioactivity at each cycle from Pre-ProPTH labeled with [^{3}H]lysine is shown. Lysine residues, as expected, are released at cycles 26, 29, and 30, as well as at cycles 5 and 9.

To obtain additional information about the amino acid sequence of Pre-ProPTH, combinations of different ^{3}H-labeled amino acids were incorporated into Pre-ProPTH, and sequential Edman degradation of the isolated protein was performed as before. Figure 4 shows the amount of radioactivity at each cycle of the degradation that comigrated on thin-layer chromatography with the indicated amino acid. For each analysis the radioactivity is normalized to the cycle with the greatest radioactivity. The actual amount of radioactivity contained in the maximum peak is indicated in the legend to Figure 4. Seven separate sequential degradations were done, and the results with methionine and lysine confirm the positions assigned to them from the data in Figure 3. In addition, four other amino acids were detected in one or more determinations: leucine at 15 and 20; alanine at 4, 16, 21, and 32; aspartic acid at 6 and 24; and isoleucine at 12, 17, and 36. Additional support for the belief that the structure of ProPTH begins at cycle 26 is provided in run 6, in which alanine at 32 and isoleucine at 36, as expected, were detected. The partial structure of Pre-ProPTH, based on our studies, is shown in Figure 5. In addition to the amino acids assigned by detection on thin-layer chromatography, we have assigned arginine to position 22. Although

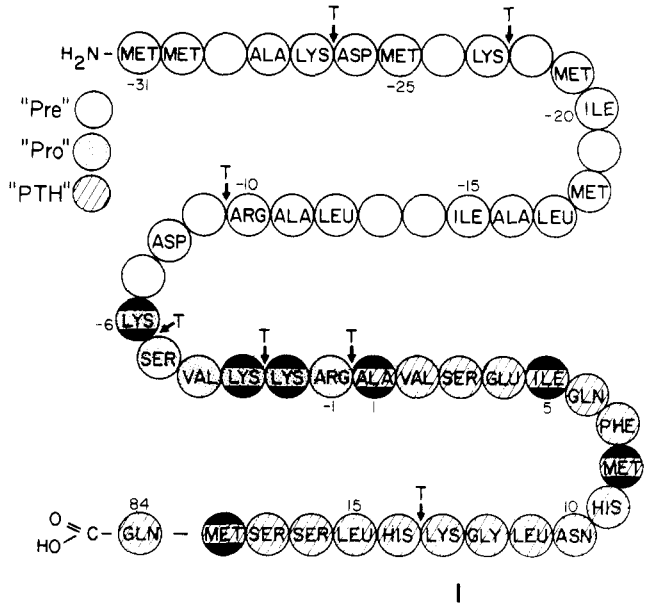

FIGURE 5: Partial amino acid sequence of Pre-ProPTH. The numbers refer to the polypeptide numbering system, beginning with −31 at the amino terminus of Pre-ProPTH. The sequence beginning at the amino terminus of ProPTH (lysine, −6) is taken from previous analyses of the structures of ProPTH (Hamilton et al., 1974; Jacobs et al., 1974) and PTH (Brewer and Ronan, 1970; Niall et al., 1970). The shaded residues in this region represent overlapping residues detected in the present analysis of Pre-ProPTH.

^{3}H was released in analyses 3 and 6 at cycle 22, technical problems prevented the detection of arginine by thin-layer chromatography. None of the other ^{3}H-labeled amino acids present in these two runs could account for the released ^{3}H at position 22.

Discussion

We have demonstrated that Pre-ProPTH contains tryptic peptides in common with ProPTH and PTH and contains extra peptides corresponding to an additional sequence, as well. Repetitive analyses by Edman degradation of Pre-ProPTH proved helpful in establishing the structural criteria for a precursor/product relationship between Pre-ProPTH and PTH, as well as in defining certain features of the Pre-ProPTH structure itself. The determination that methionine was present at cycles 39 and 49, alanine at cycle 32, and isoleucine at cycle 36 corresponds to the presence of methionine at positions 8 and 18, of alanine at position 1, and of isoleucine at position 5 in the known sequence of bovine PTH. The detection of lysine at cycles 26, 29, and 30 corresponds to the known sequence of lysine at positions −6, −3, and −2 in the prohormone-specific sequence of ProPTH (Figure 5). The fact that 25 cycles of degradation were required before detection of the first residue of this series of amino acids in the sequence of ProPTH established that 25 residues are added to the structure of Pre-ProPTH at the amino terminus of the ProPTH molecule.

Seventeen of the 25 residues of the Pre-ProPTH sequence have been established (Figure 5). In addition to lysine and methionine, five other amino acids were determined at one or more sequence positions. Because the amount of radioactivity incorporated was not large using three of these five amino acids (arginine, leucine, and aspartic acid), it cannot be stated with certainty whether these amino acids that were detected at certain cycles are not present at additional later positions. The detection of methionine, lysine, alanine, and isoleucine, however, within the ProPTH and PTH sequence seems to establish the fact that these residues do not occupy any of the eight sequence positions within Pre-ProPTH in which the amino acid present has not yet been identified. It was not possible to test for the presence of additional amino acids, inasmuch as they are not available as preparations with specific activity that is sufficiently high for adequate incorporation of radioactivity into each amino acid residue with the quantities of parathyroid mRNA presently available. The structure of Pre-ProPTH, insofar as it has been determined, is shown in Figure 5.

The sequence shown is consistent with the tryptic-peptide studies. According to the sequence, three extra methionine-containing peptides are expected in Pre-ProPTH, and three major peaks are seen (Figure 1). Likewise, three new lysine-containing peptides are expected, and two major new peaks are seen, with a possible third at the origin (Figure 2).

The question of whether the structure of Pre-ProPTH corresponds to all of the "structural" codons of PTH mRNA that are translated into protein in vivo is not yet definitely answered, but what seems certain is that something larger than ProPTH is initially synthesized. Methionine, although readily detected at cycles 39 and 49 deep within the PTH sequence, was not found at cycle 25 (position −7, Figure 5), the position immediately adjacent to the amino-terminal lysine residue at position −6 in the ProPTH sequence. The absence of a methionine residue (the initial residue believed to be involved in translation in eukaryotic systems (see Kemper et al., 1976)) preceding the amino terminus of ProPTH makes it extremely unlikely that initiation of polypeptide chain growth occurs with the synthesis of a molecule as small as ProPTH. On the other hand, it also is possible that precursors of PTH larger than Pre-ProPTH exist that are synthesized and secreted into the circulation under abnormal conditions (Wong and Lindall, 1973; Benson et al., 1974).

Pre-ProPTH has not been detected thus far in studies using intact cells. Pre-ProPTH thus seems to differ from precursors like proinsulin and ProPTH, which are relatively stable in the cell and do not begin to be converted to their respective proteins for at least 15–20 min after their synthesis (Steiner et al., 1967; Cohn et al., 1972; Kemper et al., 1972). Pre-ProPTH is apparently converted very rapidly at its site of synthesis, perhaps even before the synthesis of the carboxyl terminus is completed. This method of post-translational cleavage may also occur with larger precursor viral proteins (Jacobson et al., 1970; Boime and Leder, 1972) and the possible precursors for the myeloma light chain (Milstein et al., 1972) and human placental lactogen (Boime et al., 1975). The conversion of Pre-ProPTH to ProPTH or PTH has not been demonstrated in vitro under conditions that allow the conversion of ProPTH to PTH (Habener et al., 1975). The initial cleavage of the Pre-ProPTH specific sequence is apparently necessary before the Arg-Ala bond, which is normally very sensitive to tryptic cleavage (Cohn et al., 1972), is cleaved, resulting in the conversion of ProPTH to PTH. The additional sequence in Pre-ProPTH may alter the conformation of the amino terminus in a way that blocks the cleavage of this bond; the mechanism by which this occurs is not known as yet.

The structure of Pre-ProPTH is unusual, with 5 methionines present in the first 14 amino acid residues. The amino-terminal methionine has been shown to be derived from wheat-germ initiator Met-tRNA$_F$ (Kemper et al., 1976) and thus may be initially cleaved from Pre-ProPTH in vivo

although it is not removed in vitro in the wheat-germ extract. Although the structure of Pre-ProPTH is not yet completely known, the 5 methionines, 2 leucines, 2 isoleucines, and 3 alanines detected, a total of 12 residues of the 17 so far identified, suggest that the structure is hydrophobic. This is consistent with the presumption that the pre-myeloma light chain is hydrophobic because of the high proportion of leucines found in the amino terminus (Schechter, 1973). It has been suggested that the function of the premyeloma protein is to interact with the membranes of the endoplasmic reticulum to initiate the binding of ribosomes synthesizing myeloma proteins to the endoplasmic reticulum and to facilitate the transport of the newly synthesized protein through the membrane (Milstein et al., 1972). The rapid cleavage of Pre-ProPTH is consistent with this model, inasmuch as the unique sequence would function at the site of synthesis and, for instance, could be removed either in the membrane or after it had penetrated through the membrane before synthesis of the carboxyl terminus was completed.

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