*Proc. Nat. Acad. Sci. USA*
Vol. 71, No. 3, pp. 653–656, March 1974

# The N-Terminal Amino-Acid Sequence of Bovine Proparathyroid Hormone

**(parathyroid hormone/protein structure/prohormone)**

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*Communicated by E. R. Sears, October 12, 1973.*

**ABSTRACT** **Proparathyroid hormone (calcemic fraction-A) is a biosynthetic precursor of parathyroid hormone in bovine glands. Limited amounts of the isolated prohormone have been obtained for the purpose of initial structural studies. The results of automated sequence analysis on two separate preparations indicate that the N-terminal region of the prohormone consists of the sequence Lys-Ser-Val-Lys-Lys-Arg followed by a sequence exactly corresponding to residues 1 to 34 of bovine parathyroid hormone. Also observed was a minor sequence (about 12% of the total) in which the N-terminal lysine was absent. These data suggest that more than one species of prohormone may exist. Due to the small quantities of sample available, the analyses were restricted to the N-terminal portion of the prohormone only. However, because the amino-acid composition of the prohormone indicates it to be a molecule containing more than 100 amino acids, the possibility remains that additional residues occur at the C-terminus. Thus, the prohormone structure based on these data is believed to consist of the hexapeptide sequence above, followed by the known sequence of the 84 residues in parathyroid hormone, possibly followed by an additional sequence of 10–15 residues.**

Several recent reports indicate that parathyroid hormone (PTH) is synthesized in the bovine gland via a larger precursor molecule, proparathyroid hormone (ProPTH) (termed earlier calcemic fraction-A, CF-A)§ (1–4). Similar findings have also been reported with normal human (5) and human adenoma tissue (6), and with chicken (7) and rat (8) glands. The bovine prohormone has been purified and partially characterized (4). The amino-acid composition indicates the presence of additional amino acids compatible with the polypeptide being a larger, precursor form of parathyroid hormone. Because the gland contains only about 3% as much ProPTH as PTH, only very small quantities of prohormone have been available for initial structural studies. Despite this limitation, the sensitivity of currently used methods and efficiency of the automated sequencing procedure have permitted us to perform N-terminal sequence studies on sub-milligram quantities of two preparations of bovine prohormone. This report presents the results of these studies.

Abbreviations: TCA, trichloroacetic acid; PTH, parathyroid hormone; ProPTH, proparathyroid hormone.

§ The terms calcemic fraction-A, CF-A, or ProPTH used to designate the peptide precursor are synonymous. The latter term is used in the present report to simplify nomenclature in the literature.

## METHODS

*Isolation of Prohormone.* The two preparations of prohormone used in this study were purified according to procedures already reported (1). Briefly, the method involves extraction of kilogram quantities of bovine glands with 8 M urea-0.2 M HCl, organic solvent and salt fractionation, and finally, precipitation with trichloroacetic acid (TCA) to yield a powder (TCA powder) containing both the hormone and prohormone. This powder is then mixed with smaller quantities of a similar TCA powder prepared from slices of fresh glands which had been incubated with radioactive amino acids in order to label tissue proteins. This mixture of TCA powders is then processed through several steps of gel filtration and ion exchange chromatography. The radioactivity is utilized for location of the prohormone fraction, since it is the most highly labeled peptide in the gland following short incubations with radioactive amino acids.

*Sequence Methodology.* The samples were transferred to a Beckman model 890C sequencer in 20% acetic acid. An analysis program consisting of a single coupling step with phenylisothiocyanate and a double cleavage step was used (9). Reagents were Beckman, sequenator grade. [$^{35}$S]Phenylisothiocyanate was obtained from Amersham/Searle Corp.

*Conversion and Identification.* Conversion of the thiazolinones to the phenylthiohydantoin derivatives was performed in 0.2 ml of 1 N HCl for 10 min at 80°. A combination of gas chromatography and thin-layer chromatography with radioautography was used for identification and quantitation of the phenylthiohydantoin amino acids. The gas chromatographic procedures have already been fully described (10, 11). For quantitative determination of a number of amino acids by thin-layer chromatography, the method of Jacobs *et al.* (12) was employed. This procedure involves the use of high specific activity [$^{35}$S]phenylisothiocyanate at the coupling step. The resulting [$^{35}$S]phenylthiohydantoin derivatives were then separated using two-dimensional thin-layer chromatography. Radioautographs of the plates were made by exposing them overnight to x-ray film (Eastman Kodak type NS). Identification of the resulting spots was accomplished by chromatographing mixtures of the standard phenylthiohydantoin derivatives in the same fashion. Once located, the equivalent areas of each plate were scraped and the radioactivity of the scrapings was determined in a Tri-Carb liquid scintillation counter.

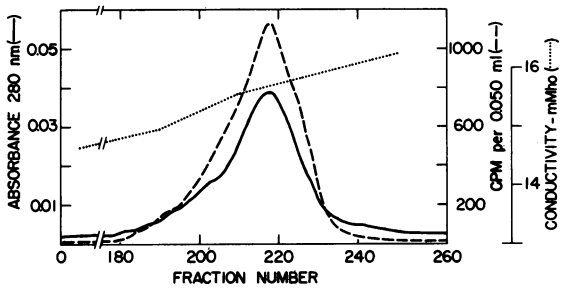


FIG. 1. Elution of ProPTH from carboxymethyl-cellulose. Material comprising the ProPTH–PTH peak which was eluted from a large Sephadex G-100 column was concentrated and applied to a carboxymethyl-cellulose column (0.9 cm × 12 cm) and eluted with a linear gradient of 0.01 M ammonium acetate, pH 5, to 0.33 M ammonium acetate, pH 7. The fractions comprising the prohormone peak were pooled, lyophilized, and reapplied to a 0.9 cm × 33 cm carboxymethyl-cellulose column. The sample was eluted with a gradient between 0.05 M ammonium acetate, pH 5.0, and 0.16 M ammonium acetate, pH 7.0. Fractions 210 to 225 were pooled, lyophilized, and used as *preparation 1*. The yield was 950 μg. A similar pattern was obtained with *preparation 2* in which the yield was 600 μg.

## RESULTS

The prohormone used in the study was obtained from two separate preparations of parathyroid glands (preparations 1 and 2). Fig. 1 illustrates the elution profile obtained in the final carboxymethyl-cellulose column step in the purification of preparation 1. A major peak of radioactivity and a corresponding peak of absorbancy were eluted. There appeared to be some heterogeneity of these peaks at both the leading and trailing edges. This pattern is typical for this stage of the procedure and is similar to that already reported (1). The prohormone eluted at a conductivity of 15 to 16 mmho–about twice that observed for the hormone (not shown in the figure). N-terminal amino-acid analyses through the prohormone peak, carried out by the dansyl method of Gray (13), revealed lysine to be the major N-terminal residue.

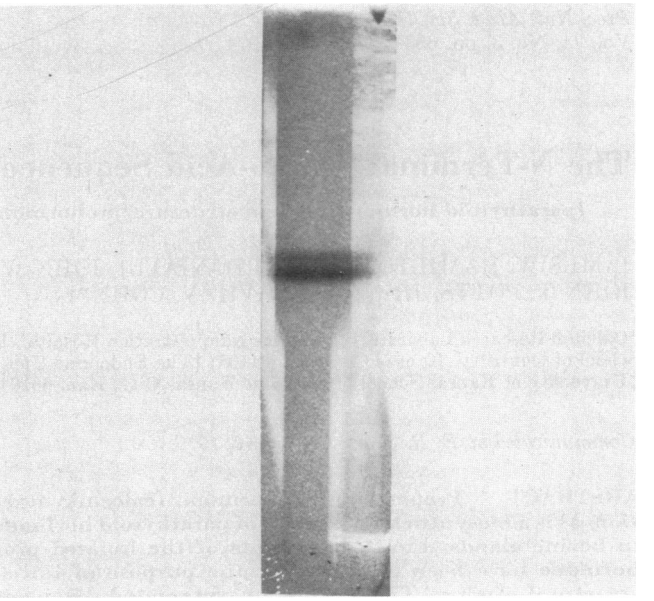

FIG. 2. Results of analytic gel electrophoresis of ProPTH (*preparation 1*). Samples of about 20 μg of protein were electrophoresed for 100 min at pH 4.0 on 15% acrylamide gels. Each of the two bands was sliced from the gel, dissolved in $H_2O_2$, and assayed for radioactivity in a liquid scintillation counter. The faster band contained about 4-fold more radioactivity than the slower.

Also consistently observed was the presence of a small amount of N-terminal serine. Analytical gel electrophoresis was carried out on aliquots across the peak. These results indicated that a major and minor component was present throughout the peak. The two peptide bands were sliced from the gels, dissolved in $H_2O_2$ and counted in toluene phosphor. Both bands were radioactive, with the slower band containing about four times the activity of the faster band in all gels (Fig. 2). Specific immunoreactivity to anti-PTH-antiserum of samples from the leading and trailing edges and center

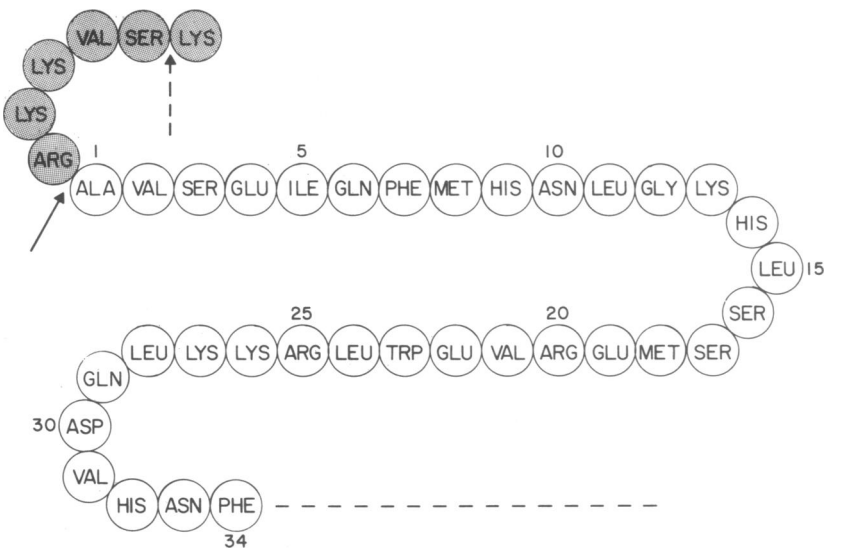


FIG. 3. Partial amino-acid sequence of ProPTH, showing the amino-terminal hexapeptide (*shaded residues*) followed by the first 34 amino acids of the 84-residue native hormone molecule. Final cleavage of prohormone into hormone occurs at a trypsin-sensitive lysine residue (*solid arrow*). The minor sequence, found on analysis of one preparation of the prohormone, was identical except for lack of the amino-terminal lysine (*dotted arrow*).

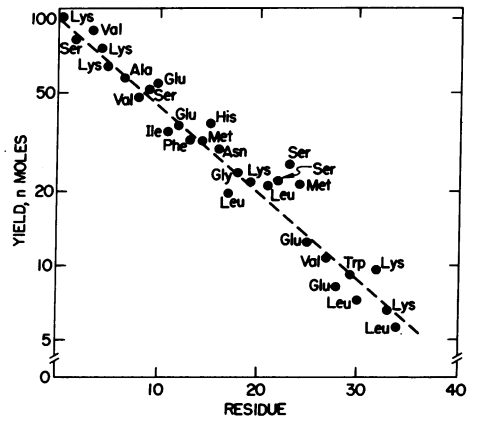


FIG. 4. Yield curve of 36 cycles of automated sequencing of *preparation 1*. 900 $\mu$g of sample were analyzed. Not all residues are shown.

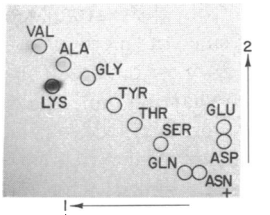


FIG. 5. Radioautograph of thin-layer plate. An aliquot of the phenylthiohydantoin from cycle 1 (from analysis of preparation 2) was applied to a standard silica gel plate (Analtech). Dimension 1. Chloroform:methanol, 90:10. Dimension 2. Ethylene dichloride: glacial acetic acid, 30:2. The plate was developed 15 cm in both directions. After drying, the plate was exposed overnight to Kodak type NS x-ray film.

of the peak was also relatively constant for the three fractions. These studies indicated that the two components present were rather evenly distributed throughout the peak. Amino-acid analysis of the pooled peak agreed well with that already reported (4).

Preparation 2 appeared to be similar to preparation 1 but was judged to be somewhat less pure. This conclusion was based on analytical gel electrophoresis which showed that several additional minor components were present in addition to the major prohormone band.

A single sequence analysis performed on each of the two preparations gave an identical N-terminal sequence for the prohormone. This is shown in Fig. 3. The N-terminal region of the prohormone consisted of the hexapeptide shown, added to the N-terminal alanine of the previously established (14, 15) PTH sequence. In the analysis of preparation 1, a minor sequence was observed, as well. This sequence was identical to the major one except that it lacked the initial lysine residue (Fig. 3). Analysis of the ratio of the valine at step 3 of the major sequence to the valine at step 2 of the minor sequence (chosen for reliable quantitative recovery of the phenylthiohydantoin derivative) indicated the minor peptide comprised about 12% of the total sample. The yield curve for the analysis of preparation 1 is shown in Fig. 4. The repetitive yield for this analysis was 92%.

The sequence analysis of preparation 2 was performed in an identical manner except that [$^{35}$S]phenylisothiocyanate was incorporated into the coupling reagent. The increased sensitivity of this method was particularly useful in the analysis of this sample since the amount of starting material was less than that used in the analysis of preparation 1. A typical radioautograph is shown in Fig. 5. Although some very faint spots were found on the radioautograph, the major spot was readily identified by its intensity relative to the others. By scraping the adsorbent from the plate and counting in a liquid scintillation counter, those residues which were not readily quantified on the gas chromatograph were measured quantitatively. Only one sequence was observed in the analysis of preparation 2; the minor sequence found in preparation 1 was not detected in this degradation.

## DISCUSSION

The present results are limited in that they deal only with the structure of the N-terminal portion of the prohormone. None the less, the hypothesis that ProPTH is a precursor of PTH is supported by the structural data presented here. The existence of a hexapeptide sequence followed by the known N-terminal sequence of PTH is consistent with an intracellular conversion of the prohormone to the hormone by enzymatic removal of the hexapeptide. In particular, the cluster of basic residues immediately preceding the N-terminal alanine of the PTH sequence would provide a suitable site for cleavage by enzymes with trypsin-like specificity. This structure undoubtedly explains the observation that an increased immunoreactivity to anti-PTH-antiserum occurred in the incubation solution when trypsin was incubated *in vitro* with ProPTH (4).

The presence of two components in the purified ProPTH (preparation 1) coupled with the finding of a minor sequence comprising 12% of the total sample which lacked the amino-terminal lysine of the hexapeptide sequence, suggests that more than one species of prohormone may exist. One possible interpretation of these data is that more than one cleavage step at the amino-terminal end of the molecule is involved in the physiological conversion of the prohormone to the hormone. This type of conversion then would be similar to that found by Steiner and coworkers in the study of proinsulin in which several intermediate precursors exist in the tissue in addition to the complete proinsulin (16). An alternative explanation is that a cleavage occurs at the amino-terminal end of the molecule which is related to extraction of the tissue and is unrelated to physiological processes. In terms of either explanation, however, it would be possible that there exists a yet larger prohormone species than the one described in this report.

The present data do not allow us to draw direct conclusions about the C-terminal nature of the ProPTH. The estimation of minimum molecular weight from the amino-acid composition of the two prohormone batches, a well as estimates of molecular weight based on SDS-gel electrophoresis, are consistent with a molecule containing more than 100 amino acids and a molecular weight of about 12,000 (4). If only six residues are present at the N-terminus, it would seem reasonable that additional residues are on the C-terminus. This conclusion appears in contrast to the report by Habener *et al.* (17) that the C-terminus of ProPTH is identical to PTH. If the latter situation is in fact the case, then we must conclude that the present ProPTH preparations are contaminated with a peptide or peptides which lack a free amino group at the amino-terminal residue(s) which prevent reaction during Edman degradation. An alternate interpretation is that the prohormone preparation analyzed by Habener *et al.* (17) is

chemically different from those studied there. This situation is also quite possible since the methods used in the two studies differ substantially. In view of these considerations, the possibility remains open that the molecule described here is but one of a family of prohormone species which exists in the parathyroid gland, any member of which could have longer additions at the N-terminus than the hexapeptide sequence and might have additional amino acids at the COOH-terminus, or both.

The important conclusion that can be drawn from the present studies, even at this stage, is that the hexapeptide amino-terminal sequence reported here is at least a part of the bovine prohormone sequence. This information has permitted the synthesis of several peptides containing a specific prohormone sequence such as −6 to 12 as well as −6 to 34 (18).‡ Immunizations with these peptides has led to development of antisera that are useful for detection of multiple preparations containing native prohormone (19). One antiserum is highly selective for measurement of prohormone; the native prohormone as well as synthetic subfragments of prohormone are detected, whereas native 1–84 bovine hormone is not detected. This antiserum, as well as others of similar specificity, should make possible the estimation of the concentration of prohormone in gland extracts and in tissue fluids. This may allow one to test whether prohormone is secreted *in vivo* in many situations. Furthermore, the synthetic peptides −6 to 12, −6 to 34, and related peptides should provide useful substrates for analysis of enzymatic activity directed at the immediate amino terminus of proparathyroid hormone during the process of intracellular conversion to hormone.

This work was supported in part by Grants DE 01523 and AM 15951, National Institutes of Health, to the Calcium Research Laboratory at Kansas City and by Grants AM 05401 and AM 11794, National Institute of Arthritis and Metabolic Diseases, to the Endocrine Unit at Massachusetts General Hospital.

‡ In this terminology, —6 refers to the N-terminal lysine of ProPTH and 12 and 34 refer to residues 12 and 34, respectively, of bovine PTH. Accordingly —6 to 12 denotes residues 1 to 18 of the bovine prohormone.

1. Hamilton, J. W., MacGregor, R. R., Chu, L. L. H. & Cohn, D. V. (1971) *Endocrinology* **81**, 1440–1447.
2. Cohn, D. V., MacGregor, R. R., Chu, L. L. H. & Hamilton, J. W. (1972) in *Calcium, Parathyroid Hormone and the Calcitonins*, eds. Talmage, R. V. & Munson, P. L. (Excerpta Medica, Amsterdam), pp. 173–182.
3. Kemper, B., Habener, J. F., Potts, J. T., Jr., & Rich, A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 643–647.
4. Cohn, D. V., MacGregor, R. R., Chu, L. L. H., Kimmel, J. R. & Hamilton, J. W. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1521–1525.
5. Chu, L. L. H., MacGregor, R. R., Liu, P. I., Hamilton, J. W. & Cohn D. V. (1973) *J. Clin. Invest.* **52**, 3089–3094.
6. Habener, J. F., Kemper, B., Potts, J. T., Jr. & Rich, A. (1973) *Science* **178**, 630–633.
7. MacGregor, R. R., Chu, L. L. H., Hamilton, J. W. & Cohn, D. V. (1973) *Endocrinology* **92**, 1312–1317.
8. Chu, L. L. H., MacGregor, R. R., Anast, C. S., Hamilton, J. W. & Cohn, D. V. (1973) *Endocrinology* **93**, 915–924.
9. Edman, P. & Begg, G. (1967) *Eur. J. Biochem.* **1**, 80–91.
10. Pisano, J. & Bronzert, T. J. (1969) *J. Biol. Chem.* **244**, 5597–5607.
11. Niall, H. D., *Methods in Enzymology* (Academic Press, New York), in press.
12. Jacobs, J. W., Sauer, R. T., Niall, H. D., Keutmann, H. T., O'Riordan, J. L. H., Aurbach, G. D. & Potts, J. T., Jr. (1973) *Fed. Proc.* **32**, 648 Abstr.
13. Gray, W. R. (1967) in *Methods in Enzymology*, ed. Hirs, W. H. W. (Academic Press, New York), Vol. XI, pp. 139–151.
14. Niall, H. D., Keutmann, H., Sauer, R., Hogan, M., Dawson, B., Aurbach, G. & Potts, J. T., Jr. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 1586–1588.
15. Brewer, H. B., Jr. & Ronan, R. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 1862–1869.
16. Clark, J. F. & Steiner, D. F. (1969) *Proc. Nat. Acad. Sci. USA* **62**, 278–285.
17. Habener, J. F., Kemper, B., Potts, J. T., Jr. & Rich, A. (1973) *Endocrinology* **92**, 219–226.
18. Tregear, G. W., manuscript in preparation.
19. Habener, J. F., Tregear, G. W., van Rietschoten, J., Hamilton, J. W., Cohn, D. V. & Potts, J. T., Jr. (1973) *Clin. Res.*, in press.