

AMINO ACID SEQUENCE OF BOVINE THYROCALCITONIN

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Abstract.—Bovine thyrocalcitonin has been isolated in homogeneous form, and its complete amino acid sequence determined. The bovine hormone is a single-chain 32 amino acid polypeptide. It contains a 1-7 amino terminal disulfide bridge, and the carboxyl terminal amino acid is prolinamide. The bovine thyrocalcitonin sequence differs from the porcine sequence by three amino acid residues and from the human sequence by 18 amino acid residues.

Two species of thyrocalcitonin, porcine and human, have been isolated in homogeneous form¹⁻⁶ and their complete amino acid sequences determined.⁷⁻¹⁰ These two hormones have nearly equal biological activity but differ in their amino acid sequences by 18 amino acids out of a total of 32. These strikingly different sequences suggest that amino acid substitution or modification of individual amino acid residues in the thyrocalcitonin molecule might be tolerated without loss of biological activity. Initial studies relating chemical structure to biological function with porcine thyrocalcitonin have shown that reduction and alkylation of the cystine disulfide bridge results in complete loss of biological activity.¹¹ Oxidation of the single tyrosine residue with tyrosinase, or alkylation of the single tryptophan residue with 2-hydroxy-5-nitrobenzylbromide, results in greater than 90 per cent loss of biological activity.¹² In contrast, the methionine residue of porcine thyrocalcitonin may be oxidized to the sulfoxide form or alkylated with iodoacetic acid without significant loss of biological activity.¹¹ On the other hand, the methionine sulfoxide form of human thyrocalcitonin has a very low biological activity.⁶ It therefore appears that certain amino acid residues in both porcine and human thyrocalcitonin cannot be modified without loss of biological activity, despite the marked differences in the primary amino acid sequences of the two molecules. The biological effect of isosteric substitution of individual amino acid residues in either the porcine or human hormone has not as yet been reported. The primary amino acid sequence of bovine thyrocalcitonin described in this communication permits further insight into the evolution of the structure of the thyrocalcitonin molecule.

Materials and Methods.—The procedures used in the isolation and characterization of bovine thyrocalcitonin are described in detail in a separate report.¹³ The starting material utilized in these studies was obtained from Armour Pharmaceutical Company (Kankakee, Illinois). The methods employed in the purification were similar to those developed for the isolation of porcine thyrocalcitonin and involved fractionation of acid-extracted thyroid powder by two consecutive gel filtration steps (Sephadex G-50 and G-25) followed by ion exchange chromatography on carboxymethylcellulose.⁵ The isolated peptide was shown to be homogeneous by disc gel electrophoresis, thin-layer chromatography, and Edman amino terminal analysis.

Amino acid analysis, total enzymic digestion with papain and aminopeptidase-M, reduction and alkylation, thin-layer chromatography, and disc gel electrophoresis were per-

formed as previously reported.⁵ Glutamic acid, aspartic acid, and asparagine were resolved by use of the lithium citrate buffer system on the amino acid analyzer.¹⁴ Enzymatic digestion of thyrocalcitonin with trypsin (TPCK, Worthington, molar ratio of peptide to enzyme, 100:1) was performed at pH 8.0 in 0.1 *M* trimethylamine acetate buffer at 37°C. The digestion, monitored in a pH state (Radiometer type TTTle) was complete in 45 to 50 min. Cleavage of thyrocalcitonin with a 50 *M* excess of cyanogen bromide (Eastman) was performed in 70% formic acid for 24 hr at 25°C.¹⁵ *N*-bromosuccinimide (Fisher) cleavage was conducted with a 5 *M* excess of reagent in 70% acetic acid for 45 min at 25°C.¹⁶ Cleavage with sodium metal in liquid ammonia was performed at -77°C for 30 min in 15 ml of liquid ammonia.¹⁷ Limited hydrolysis in dilute acid was performed by heating the peptide (1 mg/ml) in 0.03 *N* HCl for 9 hr at 110°C in a clamped sealed desiccator which had been repeatedly flushed with oxygen-free nitrogen.¹⁸ Peptide fragments resulting from the various methods of cleavage were isolated by preparative thin-layer chromatography on Brinkman cellulose F plates (butanol-pyridine-acetic acid-water 30/20/6/24), gel filtration on Sephadex G-10, G-15, and G-25, or ion exchange chromatography on carboxymethylcellulose⁵ or Dowex 50 (linear gradient: 0.05 *M* sodium acetate, pH 3.43, to 0.5 *M* sodium acetate, pH 4.87).¹⁹ Isolated peptides were assayed for purity by chromatography on thin-layer plates and by Edman amino terminal analysis. Hydrazinolysis was conducted for 18 hr at 80°C in 0.5 ml of 97% hydrazine/2 mg of protein. The carboxyl terminal residue was identified by high-voltage electrophoresis in formic acid.²⁰

Phenylisothiocyanate degradations were performed by the three-stage procedure of Edman.^{21, 22} The phenylthiohydantoin (PTH) amino acids were identified by the gas-liquid chromatographic technique of Pisano.²³⁻²⁶ In addition, an aliquot of residual peptide was obtained for "subtractive" amino acid analysis following selected steps of the degradation.

Results and Discussion.—Amino acid composition: Bovine thyrocalcitonin has been isolated in homogeneous form after a 40,000-fold purification.¹³ The purified peptide was resolved into two components by chromatography on cellulose thin-layer plates and carboxymethylcellulose. One component contained methionine sulfoxide, the other methionine. Both components had a biological activity of 200 MRC units/mg by rat bioassay.¹³ The amino acid composition of the isolated hormone, determined by a combination of acid hydrolysis and total enzymic digestion, is as follows: Arg₁, Asp₁, Asn₃, His₁, Thr₂, Ser₄, Glu₁, Ala₁, Val₁, Lys₁, Phe₂, Tyr₂, Gly₃, Pro₂, Met₁, Trp₁, Leu₃, 1/2 Cys₂ (analyzed as *S*-carboxymethylcysteine). Amino terminal analysis on reduced and alkylated thyrocalcitonin by the Edman procedure revealed 1/2 cystine (analyzed as PTH-*S*-carboxymethylcysteine). Enzymic digestion, alkylation both before and after reduction, and titration with Ellman's reagent for free thiols indicated that the two half-cystine residues in the molecule were present as a disulfide bridge.

Tryptic digestion: Tryptic digestion of the native thyrocalcitonin produced three major peptides separable by thin-layer chromatography. The individual peptides were obtained in homogeneous form by chromatography on preparative thin-layer plates or Dowex-50. The amino acid composition of hydrolysates of these three peptides is shown in Table 1. One peptide (T₁) contained 14 amino acid residues including the two half-cystines. Since the Edman amino terminal analysis of the intact molecule had revealed half-cystine, this peptide (T₁) was designated the amino terminal tryptic peptide. Carboxyl terminal analysis of T₁ by hydrazinolysis yielded lysine and suggested that this residue was at position

TABLE 1. Peptide fragments of bovine thyrocalcitonin.

Amino Acid Residue	TRYPSIN						CYANOGEN BROMIDE				N-BROMO-SUCCINIMIDE				DILUTE ACID					
	T ₁		T ₂		T ₃		CNBr-1		CNBr-2		NBS-1		NBS-2		DA-1		DA-2		DA-3	
	Mole fract.	Res./mole	Mole fract.	Res./mole	Mole fract.	Res./mole	Mole fract.	Res./mole	Mole fract.	Res./mole	Mole fract.	Res./mole	Mole fract.	Res./mole	Mole fract.	Res./mole	Mole fract.	Res./mole	Mole fract.	Res./mole
Asp	.102	1.06	.418	2.88	---	---	.178	3.71	---	---	.125	1.07	.183	3.30	.183	2.16	.091	1.03	.118	1.97
Thr	.093	.97	---	---	.095	1.05	.054	1.13	.147	.99	.107	.92	.062	1.16	.085	1.00	.089	1.01	.074	1.23
Ser	.241	2.60	---	---	.093	1.03	.165	3.54	.149	1.00	.312	2.76	.048	.90	.230	2.72	.274	3.11	.067	1.12
Glu	---	---	---	---	.084	.93	---	---	---	---	---	---	---	---	---	---	---	---	.057	.95
Pro	---	---	---	---	.169	1.87	---	---	.268	1.80	---	---	.098	1.83	---	---	---	---	.113	1.88
Gly	---	---	---	---	.272	3.00	.072	1.49	.287	1.93	---	---	.170	3.17	---	---	---	---	.169	2.81
Ala	.092	.95	---	---	---	---	.050	1.04	---	---	.125	1.07	---	---	.084	.99	.092	1.04	---	---
Val	.100	1.03	---	---	---	---	.053	1.10	---	---	.112	.96	---	---	.092	1.08	.089	1.01	---	---
Met	---	---	---	---	.090	1.00	---	---	---	---	---	---	.051	.95	---	---	---	---	.056	.93
Leu	.189	1.96	.153	1.06	---	---	.141	2.94	---	---	.221	1.89	.070	1.31	.172	2.03	.194	2.20	.066	1.10
Tyr	.088	.91	.147	1.01	---	---	.085	1.77	---	---	.100	.88	.049	.91	.079	.93	.079	.89	.047	.78
Phe	---	---	---	---	.198	2.19	.064	1.33	.150	1.01	---	---	.093	1.73	---	---	---	---	.119	1.98
Arg	---	---	.150	1.03	---	---	.043	.90	---	---	---	---	.055	1.03	---	---	---	---	.063	1.05
Lys	.095	.99	---	---	---	---	.048	1.00	---	---	---	---	.058	1.08	.090	1.00	.090	1.01	---	---
His	---	---	.133	.92	---	---	.048	1.00	---	---	---	---	.052	.97	---	---	---	---	.051	.85
Trp*	---	1.0	---	---	---	---	---	1.0	---	---	---	---	---	---	---	---	---	---	---	---
1/2 Cys*	---	2.0	---	---	---	---	---	2.0	---	---	---	2.0	---	---	---	2.0	---	---	---	---

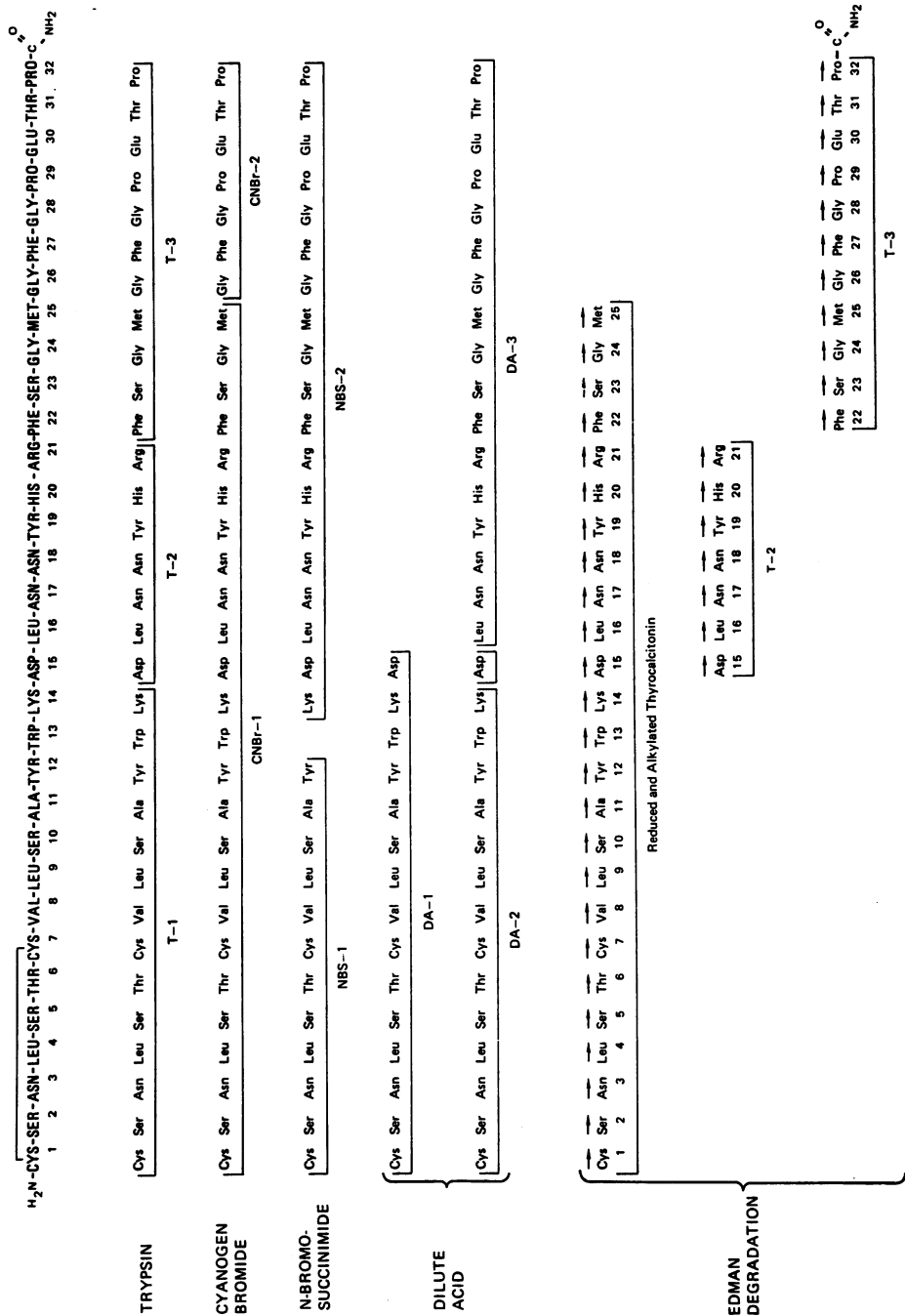
*Residue per mole determined by enzymatic digestion.

14 in the amino acid sequence of the intact molecule. Total enzymic digestion of T₁ revealed one residue each of tryptophan and asparagine per mole of peptide. The second peptide (T₂) contained seven residues. Enzymic digestion of this peptide showed one residue of aspartic acid and two residues of asparagine per mole of peptide. The third peptide (T₃) contained eleven residues. Enzymatic digestion of T₃ revealed glutamic acid to be present as the dicarboxylic acid rather than the amide.

Cyanogen bromide cleavage: Cleavage of native thyrocalcitonin with cyanogen bromide produced two major peptides. The two peptides were isolated in homogeneous form by preparative thin-layer chromatography. The amino acid composition of hydrolysates of these peptides is shown in Table 1. The larger peptide (CNBr-1) included the twenty-one amino acid residues in the T₁ and T₂ peptides plus four additional residues: Phe, Ser, Gly, and homoserine lactone (Fig. 1). The smaller peptide (CNBr-2) was a heptapeptide containing the amino acid residues present in T₃ minus the four additional residues which were present in CNBr-1. These results indicated that the methionine (analyzed as homoserine lactone) was at position 25 and aligned the tryptic peptides as T₁, T₂, T₃, starting at the amino terminal end of the molecule (Fig. 1).

N-Bromosuccinimide cleavage: Two major peptides were obtained on cleavage of the intact molecule with N-bromosuccinimide. The amino acid composition of these two peptides is shown in Table 1. The smaller peptide (NBS-1) contained the amino acids present in T₁ except for tryptophan (which was oxidized and further cleaved) and lysine. The larger peptide (NBS-2) contained lysine and the amino acid residues in T₂ and T₃. These results confirmed that T₂ and T₃ were the middle and carboxyl terminal peptides and that lysine (the carboxyl residue of T₁) was at position 14 in the amino acid sequence. Furthermore, tryptophan, which was specifically cleaved by N-bromosuccinimide, was at position 13 (Fig. 1).

FIG. 1.



Dilute acid cleavage: Thyrocalcitonin was cleaved with dilute acid (0.03 N HCl) in order to cleave preferentially the molecule at the single aspartic acid

residue and to determine the position of this residue in the amino acid sequence. The peptides were isolated in homogeneous form by chromatography on Dowex-50 or preparative thin-layer chromatography. Three major peptides were obtained in addition to free aspartic acid. Their amino acid composition is shown in Table 1. One peptide (DA-1) contained the 14 amino acid residues in T₁ plus one residue of aspartic acid per mole of peptide. Enzymatic digestion of DA-1 revealed one residue each of aspartic acid and asparagine per mole of peptide. A second peptide (DA-2) had the same amino acid composition as DA-1 minus aspartic acid. One residue of asparagine per mole of peptide was obtained on enzymatic digestion of DA-2. These findings indicated that only the carboxyl terminus of the aspartic acid residue in peptide DA-1 had been cleaved, while in the second peptide, DA-2, cleavage of both the amino and carboxyl ends of aspartic acid had occurred, resulting in the loss of this residue. Thus, aspartic acid was located on the carboxyl end of the first peptide, DA-1.

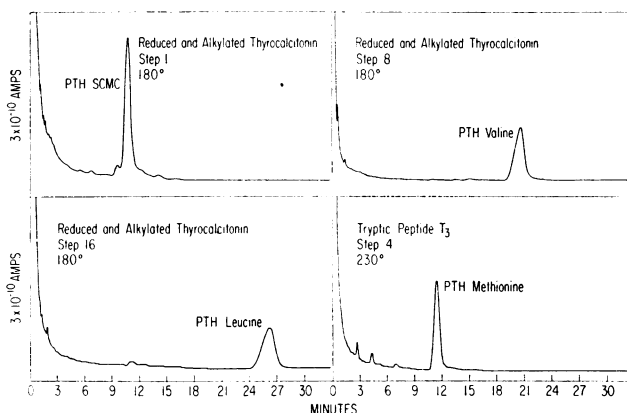
The third peptide (DA-3) contained the amino acid residues in the tryptic peptides T₂ and T₃ minus aspartic acid. This indicated that peptide DA-3 contained the amino acids following the aspartic acid of DA-1. Two residues of asparagine per mole of peptide were present on enzymatic digestion of DA-3. The results obtained on enzymatic digestion of peptides DA-2 and DA-3 indicated that no deamidation of asparagine residues had occurred prior to cleavage at the aspartic acid. These combined results assign position 15 to the aspartic acid (Fig. 1).

Edman degradations: The complete amino acid sequence of bovine thyrocalcitonin was determined by sequential Edman degradations. Degradations were performed on the following peptides: (1) reduced and alkylated thyrocalcitonin, (2) peptide T₂, and (3) peptide T₃ (Fig. 1). Two degradations were performed on the reduced and alkylated molecule. One series involved 25 cycles (shown in Fig. 1), the other 20 cycles. Subtractive analysis of the residual peptide after selected steps in the degradations revealed a high efficiency of cleavage with no significant overlap at each degradative step. In addition, the subtractive analysis (94% cleavage) obtained after the first step in the degradation of reduced and alkylated thyrocalcitonin indicated that no significant cyclization of the amino terminal *S*-carboxymethylcysteine had occurred.²⁷

The phenylthiohydantoin derivatives were directly identified by gas-liquid chromatography without significant overlap or contaminant peaks, thus permitting a definitive identification of each amino acid in the sequence (Fig. 2). Amino acid analysis of the residual peptide after ten cycles with peptide T₃ revealed prolinamide as the terminal amino acid residue. The carboxyl terminal prolinamide was confirmed by cleavage of thyrocalcitonin with sodium metal in liquid ammonia.

Summary.—The complete amino acid sequence of bovine thyrocalcitonin has been determined (Fig. 1). The covalent structure was determined by chemical and enzymatic cleavages and sequential Edman degradations. The bovine hormone is a 32 amino acid polypeptide with a 1–7 amino terminal disulfide bridge. The carboxyl terminal amino acid is prolinamide. The prolinamide and the amino terminal disulfide bridge are analogous to porcine and human thyro-

FIG. 2.—Gas-liquid chromatograms of the phenylthiohydantoin derivatives (PTH amino acids) obtained during Edman degradations of bovine thyrocalcitonin. Column packing—10% DC560, size—2 mm × 4 ft, gas—argon, flow rate—50 ml/min.



calcitonin (Fig. 3). The bovine differs from the porcine hormone by only three amino acid residues. These differences involve only a single substitution in the genetic code and include a lysine substituted for an arginine at position 14, an

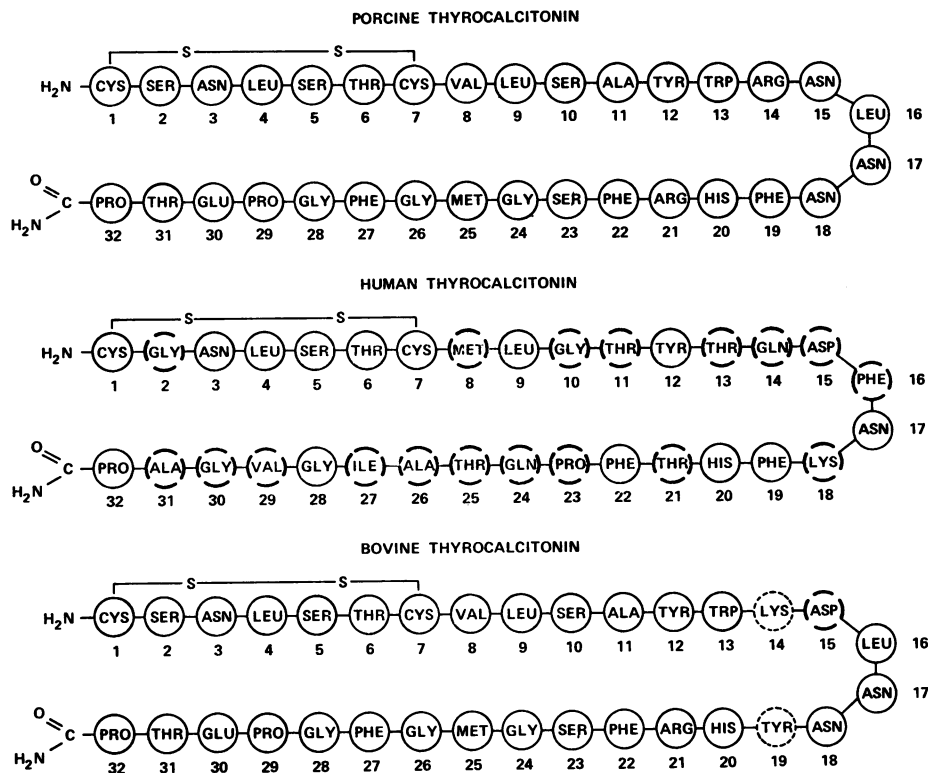


FIG. 3.—Comparison of the amino acid sequences of porcine, human, and bovine thyrocalcitonin. The heavy, broken circles shown under human thyrocalcitonin and the dotted circles used under bovine thyrocalcitonin represent amino acid sequence differences when compared to porcine thyrocalcitonin. The amino acid substitution at position 15, aspartic acid, is the only amino acid substitution which is shared by the bovine and human hormones.

aspartic acid substituted at position 15 for an asparagine, and a tyrosine substituted for a phenylalanine at position 19. Both the bovine and porcine hormone differ from the human sequence by 18 of 32 amino acid residues (Fig. 3). Approximately one third of these different amino acids involve two base substitutions in the genetic code. In comparison to porcine thyrocalcitonin, the substitution of aspartic acid at position 15 is the only substitution which is shared by the bovine and human hormones. All three hormones, despite their variability in sequence, have been shown to have nearly equal biological activity (125 to 200 MRC units/mg) by the rat bioassay.^{1-6, 13} The ultimate appreciation of the structural evolution of the thyrocalcitonin molecule and the role of individual amino acid residues in the biological and immunological activity of the hormone will await the isolation, characterization, and sequence of thyrocalcitonin from a variety of invertebrate and vertebrate species.

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Abbreviations: PTH, phenylthiohydantoin; MRC, Medical Research Council.

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