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The Isolation of Identical Thyroxine Containing Amino Acid Sequences from Bovine, Ovine and Porcine Thyroglobulins

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SUMMARY. Identical, thyroxine containing tryptic peptides have been isolated from digests of bovine, ovine and porcine thyroglobulins. This 19 residue hormone containing sequence, NH_2-Asn-Ile-Phe-Glu-T4-Gln-Val-Asp-Ala-Gln-Pro-Leu-Arg-Pro-Cys-Glu-Leu-Gln-Arg-COOH, is completely conserved across these three species, and it represents a principal site of thyroxine synthesis. HPLC maps of tryptic digests of the thyroglobulins have been monitored at several wavelengths and suggest that, in each case, only a small number of tryptic peptides are iodinated *in vivo* and that an even smaller number of tryptic peptides contain thyroid hormone. These data are consistent with a high degree of selectivity in iodination of tyrosines within thyroglobulin and the subsequent coupling of these selected tyrosines to form thyroid hormone.

Recently, we described the amino acid sequence of a tryptic peptide containing a principal thyroxine forming site in bovine thyroglobulin (1). This peptide was derived from a naturally occurring 10,000 dalton fragment obtained upon reduction of the native protein (2). We have now isolated similar low molecular weight polypeptide components from ovine and porcine thyroglobulins and prepared thyroxine-rich tryptic peptides which have been subjected to amino acid sequence analysis. Identical amino acid sequences were obtained with the thyroxine found at residue position 5 in each case. The coding for this sequence has been located at the 5' end of the bovine thyroglobulin messenger RNA corresponding to the amino terminus of thyroglobulin (3). In view of the discussion in the literature concerning the origin and significance of these naturally occurring fragments

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(4-7), these observations should help to clarify their role in the structure and function of thyroglobulin.

MATERIALS AND METHODS

Bovine, ovine and porcine thyroglobulins were prepared from fresh or frozen thyroids as previously described (8). In each case, 19S thyroglobulin was reduced and alkylated (2) and applied to a column of Sepharose CL-4B gel in 6 M urea, pH 7.0, 0.1 M sodium phosphate. The column effluent was pooled as six fractions, A through F, as previously described (2). The lowest molecular weight fraction, F, was further purified on a column of Biogel-P60 in 0.1% NH_4HCO_3 and the peak corresponding to the 10kDa region of the pattern pooled and lyophilized. The homogeneity of the pooled fractions was confirmed by SDS gel electrophoresis (2). Tryptic digests of the 10kDa fragments were carried out as previously described (1). The tryptic peptide mixtures were lyophilized and redissolved in 1.0% NH_4HCO_3 containing 5% acetonitrile and subjected to HPLC on an Ultrasphere C-18 reverse-phase column (Beckman Instruments). The HPLC elutions were carried out on a Beckman Model 332 Gradient HPLC System equipped with a Model 420 Pump Controller and a variable wavelength UV-visible detector. The elution program for collecting peptides was as previously described (1). Peptides resolved by HPLC were collected and the solvent removed under a stream of nitrogen. Aliquots were hydrolyzed and then analyzed on a microbore amino acid analyzer. Amino acid sequence determinations were by Edman degradation using a Beckman 890C sequenator (1). PTH derivatives were identified by HPLC and residues were confirmed by back hydrolysis in HCl followed by amino acid analysis. HPLC profiles of whole thyroglobulins were obtained using a starting buffer of 5% acetonitrile in 1% NH_4HCO_3 and a linear gradient of acetonitrile to 50% in 100 minutes. Iodoamino acids were assayed by HPLC using an Altex C-8 column; elution was isocratic with acetonitrile:acetic acid:water (100:5:95 by volume). Peptides were digested with Pronase (Calbiochem) for 48 hours prior to HPLC analysis to free the iodoamino acids.

RESULTS

SDS-urea gel electrophoresis following the reduction and alkylation of 19S thyroglobulins from bovine, ovine and porcine thyroids revealed a homologous series of polypeptides in each species, ranging in size from the parent peptide or gene product of 330kDa (9, 10) to approximately 10kDa (12) (Figure 1). In view of the apparent homology between the natural fragmentation pattern of the three species, tryptic digests of the 19S thyroglobulins were carried out and peptide maps were prepared by HPLC (Figure 2). As may be seen in Figure 2A, the peptide maps were very complex when monitored at 230 nm. This was expected in view of the large size of thyroglobulin. When the elution profiles were examined at 325 nm, however, where peptides containing both iodotyrosine derivatives (MIT and DIT) as well as thyroxine absorb (11), a more simple elution pattern was observed (Figure 2B). From 4 to 7 peaks with significant absorption at 325 nm were seen in each species. When the elution profiles were examined at 350 nm, where thyroxine alone shows a significant absorption (11), a single late eluting region was observed (Figure 2C). This region consisted of a doublet in the case of bovine and ovine proteins and a broad single peak in the case of porcine thyroglobulin. These data

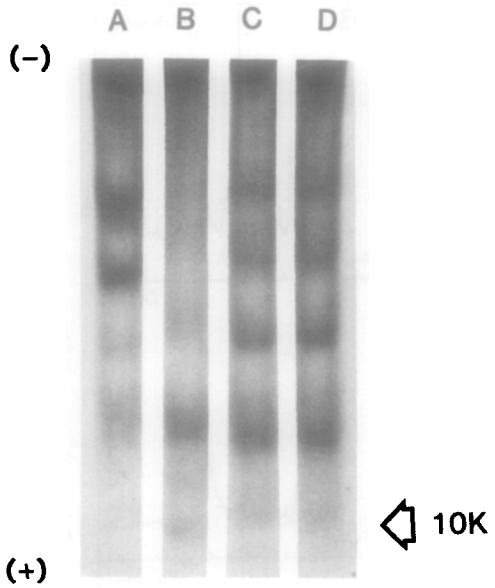

Figure 1. Five percent SDS-urea polyacrylamide slab gel electrophoresis of reduced and alkylated bovine, ovine and porcine 19S thyroglobulins. Gels contained acrylamide (5% T, 3.6% C) and 6 M urea in 0.1 M sodium phosphate buffer, pH 7.2. Samples applied were ~ 250 μ g of protein. Lanes A, B and C contained ovine, porcine and bovine thyroglobulins, respectively. Lane D is a duplicate of lane C.

strongly suggest that thyroxine occurs within only a few tryptic peptides with similar elution properties in HPLC in all three of these species.

Purification of the naturally occurring 10kDa fragment from reduced and alkylated thyroglobulin of each species was accomplished using gel chromatography (2). Samples were checked for homogeneity by gel electrophoresis on 10% acrylamide in the presence of 0.1% SDS (Figure 3). HPLC of the tryptic digests of ovine and porcine 10kDa fragments revealed a single, late eluting peak with absorbance at 350 nm. This peak was collected in each case and its amino acid composition and sequence established as described earlier for the bovine fragment (2). The amino acid sequence found in the porcine and ovine tryptic peptides was identical to that reported earlier for bovine thyroglobulin. In each case, iodine analysis of both the aqueous and organic phases of each sequenator cycle located the iodinated amino acid solely at residue position five. Assay of Pronase digests of each species' 10kDa fragment determined that the iodinated residue was predominantly thyroxine, with a small amount of mono- and diiodotyrosine also present. The sequence determined in each species was: Asn-Ile-Phe-Glu-(T4)-Gln-Val-Asp-Ala-Gln-Pro-Leu-Arg-Pro-Cys-Glu-Leu-Gln-Arg.

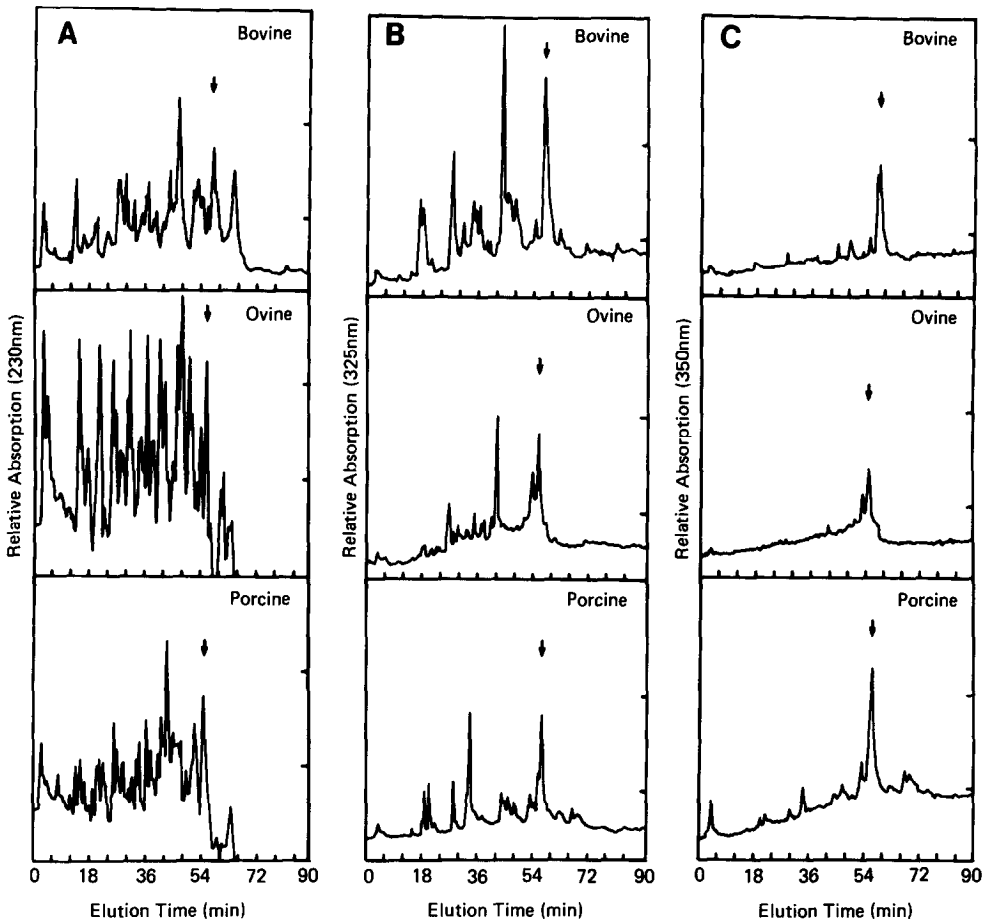

Figure 2. HPLC tryptic peptide maps of bovine, ovine and porcine thyroglobulins. Profiles in A were monitored at 230 nm, profiles in B were monitored at 325 nm and profiles in C were monitored at 350 nm. Samples of ~ 1 mg were injected in the starting buffer (5% acetonitrile in 1% NH_4HCO_3). A linear gradient was employed increasing to 50% acetonitrile in 100 minutes at a flow rate of 1 ml per min.

DISCUSSION

The naturally occurring, low molecular weight peptides which may be observed in virtually all preparations of thyroglobulin have been the focus of a number of reports in the past (4-7). Lissitzky and coworkers (12) have suggested that the low molecular weight peptides seen in reduced and alkylated thyroglobulins may be an artifact of isolation due to in vitro proteolysis during isolation of the protein from thyroid tissue. This suggestion should be examined in light of the recent observations by several groups that the low molecular weight fractions are enriched in thyroid hormone (2, 6, 7) and occur with similar size patterns in most species. It is likely that these peptides reflect a

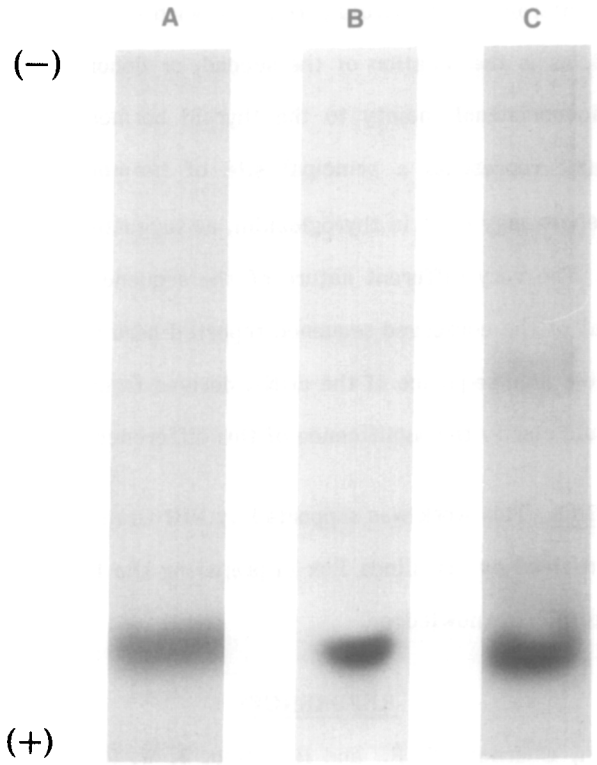

Figure 3. SDS polyacrylamide slab gel electrophoresis of purified 10kDa fragments. Samples applied were ∿ 50 μ g of protein in each case. Lanes A, B and C contained bovine, ovine and porcine 10kDa fragments, respectively.

complex post-translational processing of thyroglobulin by as yet uncharacterized enzymes (13, 14).

The data presented here define a 19 residue, hormone-containing amino acid sequence derived from one of these low molecular weight (10kDa) fragments which has been shown to be completely conserved across three vertebrate species. Since iodine analyses and iodoamino acid analyses of the intact 10kDa fragments indicated thyroxine was the predominant unique iodoamino acid in each case and there was a single iodine-containing tryptic peptide observed in each digest, we have placed the thyroxine at the single iodine containing residue in these tryptic peptides. In each case, hormone formation involves a tyrosine residue at position 5 of the peptide and presumably at residue position 5 of the intact or parent thyroglobulin chain (3).

This conserved amino acid sequence strongly suggests that the amino terminal region of the intact thyroglobulin chain (MW ∿ 330,000) is important in hormone formation since it provides one of the two tyrosines necessary for thyroxine biosynthesis. The

exact nature of the interaction of thyroid peroxidase with thyroglobulin (15) is yet to be completely defined, as is the location of the second, or donor, tyrosine residue which contributes the diiodophenenol moiety to the thyroid hormone. While the sequence reported here clearly represents a principal site of hormone formation, additional hormonogenic sequences may exist in thyroglobulin, as suggested by the recent report of Marriq et al. (16). The very different nature of the sequence reported by Marriq and coworkers from that of the conserved sequence reported here is of interest. Continuing studies on the nucleic acid sequence of the cDNA derived from thyroglobulin messenger RNA (3, 17, 18) should clarify the significance of this difference.

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