A Conformational Study of Porcine Thyrocalcitonin

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SUMMARY

The structure of porcine thyrocalcitonin has been evaluated by circular dichroism, optical rotatory dispersion, infrared spectroscopy, and fluorescence. The degree of helical structure was estimated by circular dichroism (222 nm) and optical rotatory dispersion (233 nm) at the n → π* transition of the α helix. The optical activity at this transition suggests that thyrocalcitonin contains approximately 10% α-helical structure in aqueous solution. The spectrum of thyrocalcitonin in 6 M guanidine was similar to that of a randomly coiled polypeptide. In 2-chloroethanol, thyrocalcitonin formed a structure containing approximately 50% α helix. The near ultraviolet circular dichroic spectrum of thyrocalcitonin revealed a major band at 288 nm, indicating that the tryptophanyl chromophore had restricted rotational freedom. Reduction and alkylation of the amino-terminal heptapeptide ring of thyrocalcitonin produced no significant change in the circular dichroic or optical rotatory spectra. In addition, the effects of alkaline pH and temperature on tryptophanyl emission were similar to the unmodified hormone. These findings preclude a high degree of organized structure in the heptapeptide ring, which is in marked contrast to the amino-terminal hexapeptide ring of oxytocin.

These studies indicate that porcine thyrocalcitonin exists predominately in a random coil in aqueous solution. The polypeptide, however, does not appear to possess a rigid conformation and a coil → helix equilibrium may exist in water with guanidine shifting the equilibrium in favor of the coil and 2-chloroethanol in favor of the helix. The latter type of transition may occur at the receptor site of the hormone in lipid layers or cellular membranes where the water concentration is significantly reduced.

Porcine thyrocalcitonin is a single chain 32 amino acid polypeptide hormone of known amino acid sequence (1–3). The peptide contains a 1-7 amino-terminal disulfide bridge, carboxyl-terminal residue of prolineamide, and a single tyrosine and tryptophan residue, which are adjacent in the amino acid sequence. The solubility of the hormone is small since there are relatively few charged residues. Initial studies relating chemical structure to biological function have revealed that chemical modification of the single cystine, tryptophan, or tyrosine residue results in almost complete loss of biological, but in little loss of immunological activity (4, 5). Modification of the single methionine residue, however, produces no significant loss of either biological or immunological reactivity (4, 5). Previous studies have also indicated that purified TC may lose biological activity at alkaline pH (6), and with storage at 0° (7).

The purpose of this communication is to evaluate the secondary and tertiary structure of porcine thyrocalcitonin, and the role that its conformation may play in the biological and immunological activity of the hormone.

EXPERIMENTAL PROCEDURE

Porcine Thyrocalcitonin Preparation—Porcine TC was isolated in a homogeneous form as previously reported (8). Biological activity was 200 MRC units2 per mg as measured by rat bioassay (9). Protein concentrations were determined by absorbance measurements at 280 nm 0.1 M acetic acid (ε280 = 7570, E1%1 cm = 2.1) or by amino acid analysis. Reduction of TC followed by alkylation with iodoacetic acid was performed as outlined previously (8).

Circular Dichroism—The Cary model 60 spectropolarimeter equipped with a Pockels cell was used. The optical density of all solutions was close to 2 at 280 nm. Determinations were made in 1-cm cells in the near ultraviolet region, and with 0.5- to 2-mm cells in the far ultraviolet region. A spectral curve was obtained on each solvent either before or after each sample. The temperature of solutions was maintained at 25.0° unless otherwise stated. A thermistor probe was used to monitor the temperature of the cell in studies involving the temperature dependence of ellipticity. When the effect of temperature was studied, measurements were made 30 min after the equilibrium temperature had been obtained. The reduced mean residue ellipticity was obtained from the following equation:

\[ \theta_2 = \frac{3}{(n^2 + 2)} \frac{(100 \theta) \lambda}{d} \]

where θ is in degrees, l in centimeters, and c in moles of residue per liter. Measurements were corrected for refractive index at n20°, but not for dispersion.

Optical Rotatory Dispersion—The Cary model 60 spectropolarimeter was used. The optical density of all solutions was close to 2 at 280 nm, and determinations were made in 0.5- to 2-mm
cells. The temperature of all solutions was 25.0°C. The reduced mean residue rotation was obtained from the following equation:

$$\frac{1}{\mu_{\text{h}}} = \frac{3}{(n^2 + 2)} \frac{100}{(100 \alpha \gamma)}$$

where $\alpha$ is in degrees, $l$ in centimeters, and $c$ in moles of residue per liter. Measurements were corrected for refractive index at $n^2$, but not for dispersion.

Infrared Spectroscopy—A Beckman model IR 7 infrared spectrometer calibrated against water was used. Determinations were made on 0.1 mg of TC in a potassium bromide pellet.

Fluorescence Measurements—Emission spectra were obtained with a Turner model 210 recording spectrophotofluorometer (10), with 1-cm$^2$ cells. Solutions had optical densities of less than 0.02 at the wave length of excitation (278 mp). The sample compartment was equipped with a water jacket, and was maintained at 25°C unless otherwise indicated. In experiments where the temperature was varied, the fluorescence measurements were determined 15 to 30 min after the equilibrium temperature had been reached. Fluorimetric pH curves were obtained by titrating TC with small amounts of 0.5 M KOH or HCl from an agla syringe, while the solution was stirred magnetically. pH determinations were made on a Radiometer model 25 pH meter. Excitation was at 278 mp, and emission was observed at 350 mp. The absorbance of TC solutions remains constant with pH since 278 mp is an isosbestic point of tyrosyl ionization.

Polarization of Fluorescence—Fluorescence polarization was determined in an Amino-Bowman spectrophotofluorometer as described by Chen and Bowman (11). Polarization, $P_c$, is defined as $(I_V - G I_H)/(I_V + G I_H)$ where $I_V$ and $I_H$ represent the intensity of vertically and horizontally polarized emission, excitation with vertically polarized light. $G$ is a grating correction factor defined as $I_V/I_H$ utilizing horizontally polarized exciting light.

Excitation was at 270 mp, and emission intensities were measured at 350 mp. Polarization of samples was determined in 95% glycerol as a function of temperature (10–40°C), and as a function of glycerol concentration (75 to 96%) at 25°C (12). In the experiments where the temperature was varied, the fluorescence measurements were made 15 to 30 min after the temperature of the solution became constant.

The Perrin equation for rigid spherical molecules is as follows:

$$\frac{1}{P - \frac{1}{3}} = \frac{1}{P_0 - \frac{1}{3}} \left(1 + \frac{RT\tau}{\eta V}ight)$$

where $P_0$ is the polarization at $T/\eta \to 0$; $\eta$, the viscosity in poise; $RT$, the thermal energy; $\tau$, the excited lifetime of the molecule; and $V$ the molecular volume. The relaxation time ($\tau$) was determined from the slope and intercept of a plot of $(I/\mu) - 100\alpha\gamma$ versus $(T\tau/\eta)$, in accord with the Perrin equation:

$$\mu_{\text{h}} = \frac{1}{\text{slope}} \frac{\text{intercept}}{3} \frac{\rho_{\text{H}_2\text{O}}}{\rho_{\text{H}_2\text{O}}}$$

Fluorescence lifetimes were measured by the TRW Instruments (El Segundo, California) nanosecond flash apparatus (13) on solutions containing 75, 85, and 95% glycerol while the values for 80 and 90% glycerol solutions were obtained by interpolation.

Materials—Guaniidine hydrochloride was obtained from Mann, and had little absorption or fluorescence in 6 M solution. 2-Chloroethanol was obtained from The Matheson Company, Inc., East Rutherford, New Jersey, and was redistilled. The distilled product had little absorbance in the 200 to 260 mp range. Glycerol was spectroquality, and was obtained from Coleman and Bell. Other compounds were reagent grade, and glass distilled water was used throughout.

RESULTS
Spectral Studies

Circular Dichroism

Far Ultraviolet—The CD spectrum of native thyrocalcitonin in the wavelength region of 200 to 260 mp is reproduced in Fig. 1. The major dichroic activity is centered near 200 mp ($[\theta]_{200} = -9,700$) the region of the ellipticity band of the peptide bond in an unorganized polypeptide (14). There is also a second, weaker dichroic band near 222 mp ($[\theta]_{222} = -3,300$), the wave length of the $n \to \pi^*$ transition of the $\alpha$ helix, which forms a shoulder on the stronger band. The CD spectrum of reduced and alkylated TC was measured in order to determine whether the disulfide group of the heptapeptide ring contributed to the spectrum of the native hormone. The spectra of native and reduced and alkylated TC were indistinguishable (Fig. 1). Since the minor band at 222 mp was unaffected by reduction of the disulfide bond, its origin was investigated by measuring the CD spectrum in 6 M guanidine, a solvent in which little or no organized structure remains in proteins (15). In 6 M guanidine the shoulder disappeared and the ellipticity at 222 mp decreased to $-1,100$, while the major band appears to maintain its rotational strength (Fig. 1). In order to see if the TC molecule is capable of forming a helical structure, CD measurements were performed in 2-chloroethanol, an $\alpha$ helix inducing solvent. A completely new spectrum was observed (Fig. 1) with two major negative

![Fig. 1. The far ultraviolet CD spectra of TC, reduced and alkylated TC, and TC in 6 M guanidine and 2-chloroethanol (pH = 3.1, 0.005 M acetic acid).](http://www.jbc.org/)}
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The near ultraviolet CD spectra of TC, reduced and alkylated TC, and TC in 6 M guanidine (pH = 3.1, 0.005 M acetic acid).

Near Ultraviolet—In Fig. 2 is shown the CD spectra of TC in the near ultraviolet region. The spectrum of native TC in aqueous solution, pH 3.1, has a major positive peak at 288 μm ([θ]288 = 1800) and a minor one at 280 μm which is about half as strong. This spectrum corresponds to that of the indole chromophore in diketopiperazine model compounds (16). In addition, there are two weak positive dichroic peaks at 258 ([θ]258 = 700) and 264 μm ([θ]264 = 750) (inset, Fig. 2). A similar spectrum has been obtained with phenylalanine diketopiperazine model compounds. There was almost no dichroic activity at 275 μm, the region of tyrosyl activity. There was negligible optical activity between 240 and 255 μm. Reduced and alkylated TC gave a CD spectrum ([θ]288 = 1600) similar to that of the unmodified hormone (Fig. 2). The CD spectrum of TC in 6 M guanidine was similar to that in water, although there was an approximately 50% reduction in the ellipticites due to the tryptophanyl and phenylalanyl residues.

The dependence of the tryptophanyl CD spectrum on temperature is illustrated in Fig. 3. There is a progressive decrease in ellipticity at 288 μm as the temperature is increased from 30–45°. Further increase in temperature produced only trivial changes in the spectrum.

Optical Rotatory Dispersion

The ORD of native TC in the wave length region of 200 to 260 μm is shown in Fig. 4. The major trough occurs near 230 μm with a cross over at 205 μm. The [m'] at 233 μm, the peak of
Fig. 6. The fluorescence emission spectra of native TC and TC in 6 M guanidine (pH = 3.1, 0.005 M acetic acid, O.D. = 0.02).

The n → π* transition of the α helix (17), was -2600. No significant change was found in the ORD of the reduced and alkylated hormone (Fig. 4). The ORD spectrum of TC in 6 M guanidine (Fig. 4) resembled that of a random chain polypeptide (17). Its optical rotation at 233 μm was -1800, the cross over disappeared and the major negative band now occurred below 210 μm. The spectrum of TC in 2-chloroethanol is also included in Fig. 4. An appreciable increase was found in the major band centered near 233 μm ([m] 233 = -7500), and the cross over was at 222 μm.

Infrared Spectroscopy

The infrared spectrum of native thyrocalcitonin in the region of the amide I band is illustrated in Fig. 5. The amide I band is near 1658 cm⁻¹, the frequency associated with polypeptides predominately in unordered conformations (18). No significant bands were present at 1632 and 1685 cm⁻¹, the frequencies characteristic of β structure (pleated sheet).

Fluorescence

Emission Spectra

The emission spectrum of native TC at pH 3.1 revealed a maximum at 348 μm (Fig. 6), which is characteristic of the indole spectrum of tryptophan or simple tryptophanyl peptides in aqueous solution (19). There was no significant fluorescence at 300 μm, the region of tyrosyl emission. This result was not unexpected since the tyrosyl (Residue 12) and tryptophanyl (Residue 13) chromophores are neighbors and energy transfer can occur from the former to the latter with a high efficiency as has been found in the dipeptide, tryptophanyl tyrosine (20). There were only minor, insignificant changes in the emission spectrum or quantum yield of TC in 6 M guanidine when compared with the data in water (Fig. 6).

Effect of pH

Acid—The fluorescence intensity of native TC, measured at 348 μm, decreased uniformly by about 25% when acidic solutions of TC were neutralized to pH 7.5 (Fig. 7). It is uncertain whether this decrease represents an intramolecular change or is the result of molecular association even though a similar titration curve was obtained in a solution containing twice the concentration of TC. When solutions were back-titrated from neutrality to pH 2.5 the fluorescence intensity returned to its initial value. The loss in fluorescence with neutralization suggests that a pH-dependent modification in the behavior of the tryptophanyl residue occurs since titration of other polypeptide hormones, which appear to be predominately random chains, revealed a small increase in fluorescent intensity in the same pH region (21). The pH-dependent decrease in fluorescence disappeared in reduced and alkylated TC.

In solutions of TC containing 3 M urea or 6 M guanidine there were only trivial changes in emission intensity between pH 2.5 and 7.5 (Fig. 7). These results may reflect either a loss of weak intrachain tryptophanyl interactions in the molecule or an increase in TC solubility.

Alkali—The loss of tryptophanyl emission by radiationless
energy transfer to ionized tyrosine has been studied in synthetic peptides, polypeptide hormones, and in proteins (20–23). The degree of quenching was found to decrease with the distance between the chromophores in a homologous series of peptides of tryptophan and tyrosine (20). The quenching of tryptophanyl emission with tyrosyl ionization in TC is illustrated in Fig. 8. The high degree of alkaline quenching is in accord with the evidence of efficient transfer from tyrosine to tryptophan suggested by the complete absence of tyrosyl emission at acid pH in both water and guanidine solutions (Fig. 6). No significant difference was observed in the pH-fluorescence behavior in alkali between solutions of native TC in water and 6 M guanidine or aqueous solutions of reduced and alkylated TC.

The pK of the tyrosyl residue determined fluorometrically by quenching of tryptophanyl fluorescence was near 10 (Fig. 8), which is in agreement with the pK of the tyrosyl group determined by spectrophotometric titration at 245 nm (8). The normal value of phenolic ionization precludes strong interactions of this residue with other residues in the polypeptide chain.

Effect of Temperature

The thermal dependence of fluorescence emission has been studied in tryptophan and tyrosine, synthetic polypeptides of tryptophan and tyrosine, polypeptide hormones, and proteins (21, 22, 24). Any deviation from a monotonic decline in emission intensity with increasing temperature implies a structural alteration. The temperature-fluorescence profile of tryptophanyl emission of TC at pH 3.1 is shown in Fig. 9. A monotonic decline was observed over the temperature range of 25–65°C. Reduced and alkylated TC showed a similar decline in emission intensity with increasing temperature (Fig. 9).

Polarization of Fluorescence

The variation of polarization of native and reduced and alkylated TC as a function of glycerol concentration is shown in Fig. 10. The data for the 2 molecules are indistinguishable, indicating that cleavage of the heptapeptide ring has no effect on the rotational properties of the indole chromophore. The lifetimes of the native hormone in 95, 85, and 75% glycerol were 4.6, 4.1, and 3.6 <i>x</i> 10<sup>-8</sup> sec, respectively. The intercept ((1/<i>I</i>0) – (1/3)), is larger than that found in model peptides (12). A relaxation time of 8.4 <i>x</i> 10<sup>-10</sup> sec, was obtained by the Perrin equation. This value is larger than that suggested as the maximum for the indole group in a structureless polypeptide chain (12).

Measurements of the variation of polarization of TC in 95% (w/w) glycerol with temperature gave an intercept similar to the one reported above although the relaxation time was slightly smaller.

DISCUSSION

Secondary Structure—It is of interest in understanding the biological and chemical behavior of porcine TC to know if it possesses organized structure. It is not immediately apparent whether a molecule of 32 amino acid residues will form an ordered structure in solution. Measurements of ORD, CD, infrared spectroscopy, and fluorescence can indicate whether the polypeptide possesses hydrogen-bonded or other interactions. These parameters, therefore, have been utilized in order to gain some appreciation of the extent of organization in porcine TC.

The degree of secondary structure in TC was assessed by CD, ORD, and infrared spectroscopy. The principal CD band of TC in the far ultraviolet is centered near 200 nm which is the wave length corresponding to the <i>π → π</i> transition of the random form of the peptide bond. A weaker dichroic band is present at 222 nm, the region of the <i>n → π</i> transition of the peptide bond in its α-helical form. This degree of ellipticity, if caused by helical structure, would indicate that TC contains approximately 10% α helix if one accepts a [α] value of −30,400 for this transition in an α helix (14). It should be pointed out that the aromatic chromophores also have transitions in this wave length region and may also contribute to the ellipticity (95). The CD spectrum of TC is normalized in 6 M guanidine and approaches that of unordered polypeptides. Similar results were obtained when the degree of helical structure was evaluated by ORD. The mean molar residue rotation at 233 nm was
-2,400; if caused by α-helical structure, this would indicate that TC contains about 10% helix assuming a [α] value of -13,000 and -1,800 for the transition between an α helix and random chain (17). The band at 233 nm was essentially eliminated in 6 M guanidine and there was a spectral shift at lower wave lengths from a positive to a negative band. The spectrum in 6 M guanidine is characteristic of the peptide chromophore when in a randomly coiled polypeptide (17).

The TC polypeptide chain is capable of forming a high degree of α-helical structure as indicated by the CD and ORD spectra in 2-chloroethanol. The ellipticity at 222 nm, and the optical rotation at 233 nm indicated that the molecule contained about 50% α helix based on values of [α] and [α] of synthetic polypeptides in their helical and random forms.

The combined results suggest that TC in acid solution contains a very small amount of α helix, which may correspond to about one turn for a polypeptide of 32 residues.

Tertiary Structure—The tertiary interactions of TC were also evaluated by the behavior of its chromophoric residues. The CD spectrum in the near ultraviolet region was determined since this region is free of contributions from the peptide chromophore which absorbs in the same ultraviolet region as the second electronic transition in the aromatic groups. The positive CD spectrum, at 288 nm, is clearly caused only by the tryptophanyl residue in TC. The rotational strength of the band implies that the indole residue lacks some degree of rotational freedom with respect to the asymmetric carbon of the tryptophanyl or to some other neighboring asymmetric center. An indole heptapeptide ring interaction can be excluded since the magnitude of the molar ellipticity was not significantly affected by reduction of the disulfide bond. The dichroic activity in 6 M guanidine and in water at 45° approaches values in linear dipeptides and may represent its limiting value in this polypeptide (16). The positive CD spectrum from 255 to 265 nm can be assigned to the phenylalanine residue or residues in TC (phenylalanine residues are present at positions 19, 22, and 27 in the amino acid sequence (1-3)). The presence of a phenylalanine dichroic band, which is significantly reduced in 6 M guanidine, indicates that one or more of these residues lacks some rotational freedom.

The fluorescence properties of tyrosine and tryptophan have recently been employed as an indicator of tertiary structure in polypeptide hormones (21). In TC the wave length maximum of the indole fluorescence was 348 nm, which is very close to that of model tryptophanyl peptides of low molecular weight and indicates that the fluorochrome is essentially in an aqueous environment (19). The difference in the emission spectrum to 6 M guanidine and the monotonic decline in tryptophanyl emission with increasing temperature are in accord with this point of view. Consequently, it is unlikely that the indole residue is part of a cluster of groups stabilized by hydrophobic interactions. The only fluorimetric evidence suggesting a perturbation of the indole group in TC is the acid titration curve in water. The quenching of indole emission with neutralization is the reverse of the changes observed in other polypeptide hormones and in several stable proteins (21). Moreover, the pH dependence of the fluorescence of TC between pH 2.5 and 7.5 was normalized in 6 M guanidine. The change in fluorescence may originate from the rupture of a weak interaction between the indole group and the side chain of a neighboring residue.

It should be emphasized that the CD data are much more convincing than the fluorescence data in showing an important interaction of the indole moiety with other parts of the hormone. This result is not unusual since the two types of data represent different electronic processes with minor differences in energies and major differences in lifetimes. The important role played by the electronic configuration in the interaction of the molecule with the solvent has been reported recently in the case of two aromatic diketopiperazines. Nuclear magnetic resonance spectra indicate that the diketopiperazines Gly-L-Tyr and Gly-L-Trp in dimethyl sulfoxide have a folded configuration in their ground state whereas they have unfolded forms in their excited states, as revealed by fluorescence measurements (26).

Energy transfer between the indole and phenol chromophores has been proposed to explain the lack of tyrosyl fluorescence in TC and the quenching of tryptophanyl fluorescence in alkaline solution. The neighboring positions of the 2 aromatic residues can readily account for the failure to observe tyrosyl fluorescence since it was not observed in Trp-Tyr whereas emission was observed in Trp-Gly-Trp which becomes stronger in Trp-Gly-Y Trp (20). The quenching of tryptophanyl fluorescence by phenolate by energy transfer is a more efficient process and has been reported in numerous polypeptides and proteins (21-23).

The large value of the intercept in the Perrin plot of the polarization data presumably arises from energy transfer between indole groups. TC should therefore be associated in glycerol solutions since it contains only a single tryptophanyl residue.

The relaxation time calculated for TC is considerably greater than expected for a structureless molecule of 32 residues. A value which is half as large has been reported for adrenocorticotropic hormone which contains 39 residues (12). It is not clear, however, whether the large value represents organized structure arising from the association of TC molecules or whether it exists in its free state. It should also be noted that TC could acquire structure in the concentrated glycerol solutions used in the polarization experiments, as was observed in solutions of TC in 2-chloroethanol.

There was no significant change in either the CD or ORD spectral patterns, or in the temperature-fluorescence profile with reduced and alkylated TC when compared to the native hormone. These results suggest that the disulfide bond does not contribute measurably to the optical activity of TC, and that there is little, if any, organized structure in the heptapeptide ring. Oxytocin has a similar amino-terminal disulfide bridge which is composed of 6 amino acid residues. CD measurement on oxytocin and various analogues have revealed significant optical activity in the ultraviolet region which have been attributed to the disulfide chromophore (27, 28). In oxytocin there is a marked change in ellipticity with reduction of the six-member disulfide ring. The failure to observe any dichroic activity in TC in the region of the disulfide bond transition in both the 210 and 250 nm regions (27, 28) also suggests that the heptapeptide ring does not interact with other residues of the hormone. The marked difference in CD activity between TC and oxytocin should reflect differences in rigidity between the two rings. The smaller ring size or stronger residue interactions in oxytocin may account for its greater optical activity.

In summary, it appears that porcine TC exists predominately in an unordered conformation in aqueous solutions. The lack of a high degree of ordered structure in porcine TC is similar to the results obtained with glucagon (29, 30), parathyroid hormone.
(21), and adrenocorticotropic hormone (21). The polypeptide fragments of the S-peptide of ribonuclease (31), and the tetra-decapeptide from myoglobin (32) have also been shown to contain little helical structure in aqueous solution. TC, however, does not appear to possess a rigid conformation and a coil ⇄ helix equilibrium may exist in water with guanidine shifting the equilibrium in favor of the coil and 2-chloroethanol in favor of the helical form. Similarly, the structure of glucagon has been shown to vary depending on its environment, and is helical in the crystalline state (29, 30, 33). These results suggest that TC, as well as other polypeptide hormones, do not exist in rigid structures in nonpolar solvents.

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Recently the amino acid sequences of human (34), bovine (35, 36), and salmon TC (37) have been deduced. Both human and salmon TC are significantly different in sequence and have a longer biological half-life when compared to the porcine hormone. It will be of interest to compare the physicochemical properties of TC from a variety of species in order to gain a greater appreciation of the relationships between protein structure and functional physiology.

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