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Interaction of Oxytocin with Ca^{2+}: I. CD and Fluorescence Spectral Characterization and Comparison with Vasopressin

Abstract: *Extracellular Ca^{2+} is required for the action of oxytocin and both the hormone and its receptor have binding sites for divalent metal cations. To characterize the cation-bound form of oxytocin, we monitored the binding of Ca^{2+} and Mg^{2+} to oxytocin as well as peptides representing its ring and tail regions in trifluoroethanol, a lipid-mimetic solvent, using CD and fluorescence spectroscopy. Binding Ca^{2+} ($K_d \sim 50$ μM) caused drastic CD and fluorescence changes leading to a helical conformation. Mg^{2+} caused CD changes smaller than and opposite to Ca^{2+}. However, the helical structure was enhanced when both Ca^{2+} and Mg^{2+} were present together. CD changes in the tail peptide of oxytocin showed its ability to bind Ca^{2+} and Mg^{2+} whereas the vasopressin tail peptide did not bind either cation. CD spectral changes on Ca^{2+} and Mg^{2+} binding to tocinoic acid (the ring moiety of oxytocin) were much smaller than those of oxytocin. These data suggest that the tail segment of oxytocin potentiates Ca^{2+} binding by the ring. While vasopressin displayed a CD spectrum similar to that of oxytocin, CD spectra of its cation-bound forms were markedly different from those of oxytocin; the Ca^{2+}-induced CD changes in vasopressin were very much smaller and of opposite sign, and Mg^{2+}-induced ones significantly larger than in oxytocin. Taken together, our observations bring out the structural differences between oxytocin and vasopressin in the context of their interaction with Ca^{2+} and Mg^{2+}. This may be relevant to understanding the differences in the bioactive conformations and receptor interactions of the two hormones.* © *1997 John Wiley & Sons, Inc. Biopoly* ***40:*** *433–443, 1996*

Keywords: *hormone–receptor interaction; Ca^{2+}–hormone interaction; bioactive conformation; structure–activity correlation*

INTRODUCTION

Oxytocin is a cyclic nanopeptide hormone [sequence: cyclo(Cys[1]-Tyr[2]-Ile[3]-Gln[4]-Asn[5]-Cys[6])-Pro[7]-Leu[8]-Gly[9]-NH_2] synthesized in the hypothalamus. It is involved primarily in uterine contraction and stimulation of milk release from the mammary tissue. Reported additional functions for oxytocin include an antiduretic effect[1] and blood vessel contraction.[2] Although the mechanism of oxytocin action has not been fully understood, there is evidence for receptor-mediated stimulation of inositol phosphate in a variety of systems.[3] The receptors for oxytocin[4] and vasopressin[5,6] have been recently cloned. Comparison of the derived amino acid sequences of these receptors reveals significant

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(36–45%) homology. This is not surprising since vasopressin, also a neurohypophyseal cyclic peptide hormone [sequence of arginine vasopressin: cyclo-(Cys[1]-Tyr[2]-Phe[3]-Gln[4]-Asn[5]-Cys[6])-Pro[7]-Arg[8]-Gly[9]], shares seven amino acids with oxytocin.

Divalent cations have long been known to be important for the contractile activities of neurohypophyseal hormones in many tissue types.[7,8] A great deal of effort has therefore gone toward understanding the role of various cations in the binding of oxytocin and vasopressin to their respective receptors. While monovalent cations have little effect, several divalent cations modulate the binding affinity of the hormone to the receptor and/or the concentration (i.e., availability) of receptors.[9] There is a consensus on the enhancement by Mg^{2+} of the receptor binding of oxytocin[3,9,10] and vasopressin.[3,9,11–14] The effect of Ca^{2+}, however, is less clear. Won et al.[15] and Tasaka et al.[16] have demonstrated that the increase in intracellular Ca^{2+} induced by oxytocin is dependent on extracellular Ca^{2+} and is suppressible by calcium channel blockers. In a detailed kinetic and equilibrium binding study, Pearlmutter and Soloff[9] showed that Ca^{2+} did not by itself potentiate the binding of oxytocin to the mammary gland receptor, but in the presence of 5 m*M* Mg^{2+}, Ca^{2+} decreased agonist binding at low concentrations (0.2–0.6 m*M*) but enhanced it at higher (>1 m*M*) concentrations. This dual effect was explained in terms of Ca^{2+} binding to one of the two sites in the receptor and causing a decrease in the concentration of the active receptor and, at the same time, increasing the affinity of the other receptor site to the hormone. As for vasopressin, Stubbs et al.[17] found Ca^{2+} to be essential for the hepatic glycogenolysis action of vasopressin. Pletscher et al.[12] found that Ca^{2+} at 1.5 m*M* increased vasopressin binding to platelet receptor, but was much less effective than Mg^{2+}. A slight increase in binding of vasopressin to hepatic V_1 receptors was also observed by Wang et al.[18] at Ca^{2+} concentrations between 2 and 10 m*M*.

Irrespective of the exact mode of its modulation of oxytocin action, the fact that extracellular Ca^{2+} is required for oxytocin's contractile activity[8,19,20] implies a role for this cation in oxytocin–receptor interaction. In a generally accepted model, Pearlmutter and Soloff[9] proposed two binding sites for metal ions in the oxytocin receptor. From oxytocin-cation binding data, these authors deduced that, under most experimental conditions, about 95% of oxytocin would be complexed with metal ion(s) including Ca^{2+} and Mg^{2+}. That oxytocin can bind various metal ions in water, albeit with low affinity ($K_d \sim 10^{-3}$–$10^{-4}M$), has also been demonstrated by others.[21,22] Other peptide hormones (gastrin[23]; glucagon[24]; GnRH[25]; insulin[26]) and their receptors[27] are also known to bind Ca^{2+}. These observations are consistent with a proposal that peptide hormones, in general, may bind Ca^{2+} (or Mg^{2+}) with enhanced affinity in the low-dielectric regions of the lipid/water interface and may interact with the receptor to form a ternary complex between the hormone, receptor, and the cation.[28,29] Our previous studies have shown that diverse hormones such as glucagon,[30] substance P,[31] bombesin,[32] and gonadotropin-releasing hormone[33] have the ability to bind divalent cations in lipid-mimetic solvents and to transport Ca^{2+} across the lipid bilayer of synthetic liposomes in aqueous buffers. In light of these observations, we decided to investigate the conformations of oxytocin in a lipid-mimetic solvent in the absence and presence of Ca^{2+} and Mg^{2+} using spectroscopic and computational techniques. The CD and fluorescence spectral results presented in this paper suggest that oxytocin undergoes a marked conformational change on binding either Ca^{2+} or Ca^{2+} and Mg^{2+} together, but only a moderate change on binding Mg^{2+} alone. Significantly different spectral data were obtained on vasopressin–cation interaction. An analysis of the conformation of Ca^{2+}-bound oxytocin using nmr and molecular modeling is presented in the accompanying paper.[34]

MATERIALS AND METHODS

Materials

Oxytocin and vasopressin (trifluoroacetate or acetate salts, > 98% pure by high performance liquid chromatography and amino acid composition) were obtained from either Bachem Inc. (Torrance, CA) or Sigma Chemical Co. (St. Louis, MO), and were dried under high vacuum before use. Sample-to-sample differences in CD were occasionally observed; the reported data are the results of several (>3) trials with each hormone-cation experiment. Tocinoic acid was purchased from Bachem, Inc. N-acetyl tyrosine methyl ester and trifluoroethanol (TFE) were from Sigma. L-proline-L-leucine-glycine-amide (Pro-Leu-Gly-NH_2), and L-proline-L-arginine-glycine-amide (Pro-Arg-Gly-NH_2) were bought from Bachem Bioscience, Inc. (Philadelphia, PA); NaH_2PO_4, $MgCl_2$, and $CaCl_2$ from Fisher Scientific Co. (Fairlawn, NJ); NaCl from BDH, Inc. (Toronto, Ont.); $Mg(ClO_4)_2$ and $Ca(ClO_4)_2$ from GFS Chemicals (Columbus, OH). TFE was further purified before use by distillation when the commercial sample was found to contain fluorescing impurities. The perchlorate salts were lyophilized and dried in high vacuum before use.

Methods

Concentrations of oxytocin and vasopressin were determined from absorbance using an extinction coefficient of 1200 $1M^{-1}$ cm^{-1} at 274 nm in TFE based on the absorbance of N-acetyl tyrosine methyl ester in TFE at this wavelength. Stock solutions of the hormone were prepared in TFE. Concentrations of the tail fragments of oxytocin and vasopressin were calculated by weighing and dissolving the peptides in measured volumes of TFE. All peptides were dried under high vacuum and/or by lyophilization before solutions were prepared.

Circular Dichroism. CD spectra were recorded at 22 ± 1°C on a computer-controlled Jasco J-600A spectropolarimeter using either a 5 or 10 mm quartz cell. The concentration of oxytocin was typically 35–50 μM (no concentration dependence was observed in the CD spectra up to 75 μM assuring lack of peptide aggregation) while the tail tripeptide concentrations were around 1 mM. Spectra were signal averages of 8 scans collected at a speed of 20 nm/min. Stock solutions of the metal perchlorate salts were prepared in TFE at concentrations of 5–10 mM. For metal ion titrations, aliquots of the stock solution were added to the peptide solution in the cell and mixed completely before spectral recording. Dilution of the peptide during titration was around 5–10% and was taken into account in calculating the mean-residue molar ellipticity $[\theta]$. The latter was expressed in deg cm^2 $dmol^{-1}$.

Fluorescence. Fluorescence spectra were recorded at 22 ± 1°C on a computer-controlled Perkin-Elmer LS-50 spectrofluorimeter. Peptide concentrations were typically 30 μM. Sample preparation and titrations were carried out as described above. The fluorescence excitation wavelength was set at 270 nm and emission was monitored between 285 and 400 nm. Slit widths for both excitation and emission were normally set at 5 nm. Spectra were corrected for dilution (<3% of total volume) and background fluorescence from the solvent. In cation titration experiments, aliquots of the stock salt solution were added to the peptide solution in a 5 or 10 mm quartz fluorescence cuvette and mixed magnetically. It was often possible to carry out the uv absorption, CD, and fluorescence measurements on a given solution using the rectangular 1 cm fluorescence cuvettes. These were CD transparent up to 195 nm and allowed the use of dilute hormone solutions without sacrificing the signal-to-noise ratio.

Estimates of cation binding constants of oxytocin were made by analyzing the binding isotherms to a curve-fitting program (GraFit, Erithacus Software Ltd., London, UK) using equations specifically derived to obtain the equilibrium concentrations of free and bound peptide rather than those of the cation.

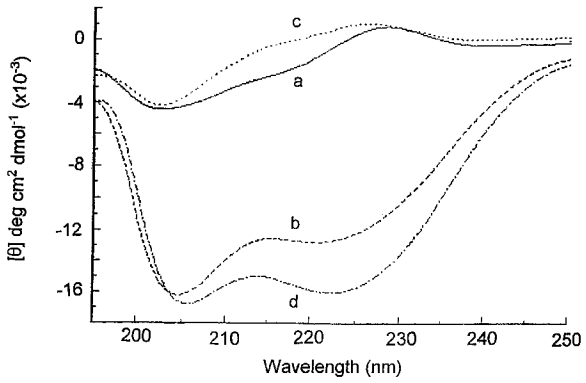

FIGURE 1 CD spectra of oxytocin (30 μM) in the far-uv region at 22 ± 1°C in TFE: (a) free oxytocin; (b) oxytocin + 2 molar equivalents of Ca^{2+}; (c) oxytocin + 2 molar equivalents of Mg^{2+}; (d) oxytocin + 2 molar equivalents each of Ca^{2+} and Mg^{2+}. Perchlorate salts of the cations in TFE were used.

RESULTS

Many earlier studies have examined the conformation of oxytocin and its analogues using CD spectroscopy in various solvents, including water[35–38] and TFE.[38] In all cases, oxytocin exhibits a positive CD band at about 225 nm and a negative band around 205 nm. In TFE an additional negative band around 215 nm is seen. The negative CD bands arise from the π-π^* transition of the peptide backbone amide, while the positive 225 nm peak presumably contains contributions from both the peptide backbone and from the π-π^* (B_{2u}) transition of the aromatic ring of the tyrosine residue.[35,39] As such, the 225 nm band will be affected by changes in the tyrosine residue (including ionization state of the phenolic group) as well as in the backbone conformation. CD studies of [2-cycloleucine]oxytocin, an analogue that does not contain an aromatic group,[38] indicated the contribution from tyrosine to be the more dominant of the two. The CD spectrum of oxytocin in TFE shown in Figure 1 is similar to that reported by Fric et al.[38] in this solvent showing the positive 225 nm band as well as two negative bands centered around 215 and 205 nm. The addition of Ca^{2+} caused a dramatic change in the spectrum of oxytocin (Figure 1). The positive CD band at 225 nm was changed into a large negative band, the negative band at 215 nm disappeared, and the negative band at 205 nm was considerably enhanced. Addition of Mg^{2+}, however, had a much different and smaller effect. The negative band at 215 nm became more positive and broader while changes

in ellipticities at 205 and 225 nm were minor (Figure 1). A similar broad peak in the 215 nm region was observed in the analogue [1-penicillamine, 2-cycloleucine]oxytocin,[38] and may be related to changes in the overlapping of the transitions of the amide and tyrosine bands. Addition of over 2 equivalents of $KClO_4$ had no effect on the oxytocin spectrum (data not shown), indicating that the observed CD spectral changes on Ca^{2+} and Mg^{2+} addition are not due to nonspecific ionic strength or solvent polarity effects. No significant CD changes were observable on adding any of the cations to oxytocin in water even up to 20 molar excess.

In an attempt to investigate the relative binding affinities of the two metal ions for oxytocin, competition experiments were carried out that yielded somewhat surprising results. Addition of 1 or 2 equivalents of Mg^{2+} to oxytocin in the presence, respectively, of one or 2 equivalents of Ca^{2+} increased the intensities of the negative CD bands at 225 and 205 nm beyond what was caused by the Ca^{2+} already present. The CD spectrum of oxytocin in the presence of two equivalents each of Ca^{2+} and Mg^{2+} is shown in Figure 1. Further addition of Mg^{2+} up to 10 molar excess caused a large decrease in both the negative peaks, suggesting the takeover by the Mg^{2+}-bound conformation of oxytocin (data not shown).

The Ca^{2+} binding isotherm of oxytocin derived from the CD spectral data is shown in Figure 2. The molar ellipticity at 225 nm continues to decrease until a cation : peptide ratio of about 1.5, beyond which there is a small increase that saturates around a ratio of 2.5. This (in conjunction with the cation competition experiments described above) would suggest the presence of two binding sites for Ca^{2+} in oxytocin, binding at the first higher affinity site causing a relatively larger CD change than binding at the second site. The Mg^{2+} titration curve (Figure 2) derived from CD changes at 216 nm suggests two binding sites for this cation as well but the spectral changes are very much smaller compared to the Ca^{2+}-induced changes.

CD studies were also carried out with oxytocin in TFE in the near-uv region (250–300 nm) where disulfide transitions predominate.[39] The spectrum in Figure 3 shows a positive peak at 250 nm and a negative peak around 280 nm as in previous studies.[35,38,40] The 250 nm CD band corresponds to conformers with C—S—S—C dihedral angles of ±90° and the longer wavelength band to the B_{2u} transition of the tyrosine ring[39,40] as well as to a transition involving the disulfide bond.[35,38,41] Addition of Ca^{2+} to oxytocin did not have an appreciable

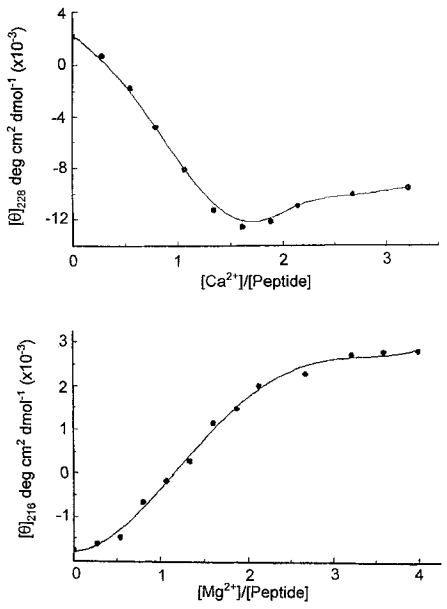

FIGURE 2 Binding isotherms of oxytocin–cation complexes at 22 ± 1°C in TFE: Ca^{2+} (top), Mg^{2+} (bottom) using, respectively, the molar residue ellipticites at 228 and 216 nm.

effect on the 280 nm peak but caused a substantial change in the 250 nm region coupled with a reversal in sign from positive to negative ellipticity. The change in the 250 nm band may represent a genuine alteration in disulfide orientation. However, it may also be interpreted as resulting from the influence of the relatively large CD change in the 220–230 nm (n-π^* transition) region of the peptide bond on the longer wavelength transitions as was earlier encountered in the study of oxytocin and its analogues.[35] Addition of Mg^{2+} induced rather different spectral changes (Figure 3). When compared to the free hormone, the intensity of the negative 280 nm CD band increased and the band blue shifted by about 5 to 275 nm, while the band at 250 nm became less positive. Assuming negligible influence from the lower wavelength bands, the near-uv data would suggest changes in the distribution of conformers for the disulfide bond as well as in the chiral environment of the tyrosine ring upon addition of Ca^{2+} and Mg^{2+}.

Fluorescence studies were carried out on oxytocin to investigate the effects of Ca^{2+} and Mg^{2+} on the ring moiety as deduced from the perturbations in the tyrosine chromophore. Upon addition of Ca^{2+}, there was a decrease in tyrosine fluorescence (Figure 4). Titration with Ca^{2+} indicated an apparent saturation at a Ca^{2+}: peptide ratio of 2 : 1 (Fig-

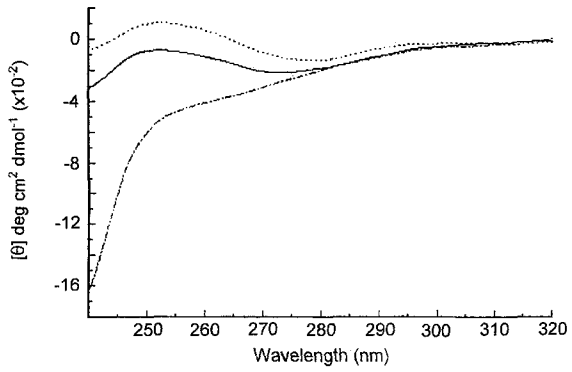

FIGURE 3 Near-uv CD spectra of oxytocin in the free state (dotted line) and in the presence of two molar equivalents of Ca^{2+} (dashed line) and Mg^{2+} (solid line).

ure 4, inset). There was a reversal in the fluorescence change beyond 2 : 1 Ca^{2+}: peptide, the emission intensity increasing with increasing Ca^{2+}. This trend did not saturate, however. Addition of Mg^{2+} to oxytocin also caused a reduction in fluorescence intensity as with Ca^{2+}, and saturation occurred at a Mg^{2+}: peptide ratio 2 : 1 (data not shown). Unlike Ca^{2+}, Mg^{2+} addition beyond this ratio caused no further fluorescence change. Also, a major part of the fluorescence change occurred at a 1 : 1 Ca^{2+}: peptide ratio.

An estimate of the binding constant for the high-affinity Ca^{2+} site in oxytocin was made by subjecting the data between zero and 1.5 Ca^{2+}: oxytocin in Figures 2 and 4 to a curve-fitting program. The values obtained ranged from 50 to 100 μM and may be regarded only as a rough estimate of the true binding constant, since analysis of the complete binding data extending up to a Ca^{2+}: oxytocin ratio of 2 or more was rendered difficult by the reversal in the sign of the spectral changes beyond a ratio of about 1.5.

For the sake of comparison, the Ca^{2+}- and Mg^{2+}-induced CD and fluorescence changes in vasopressin in TFE were also examined. The CD data (Figure 5) show that while the spectrum of free vasopressin is essentially similar to that of oxytocin, the spectral changes on cation addition were markedly different from those of oxytocin. Ca^{2+} diminished

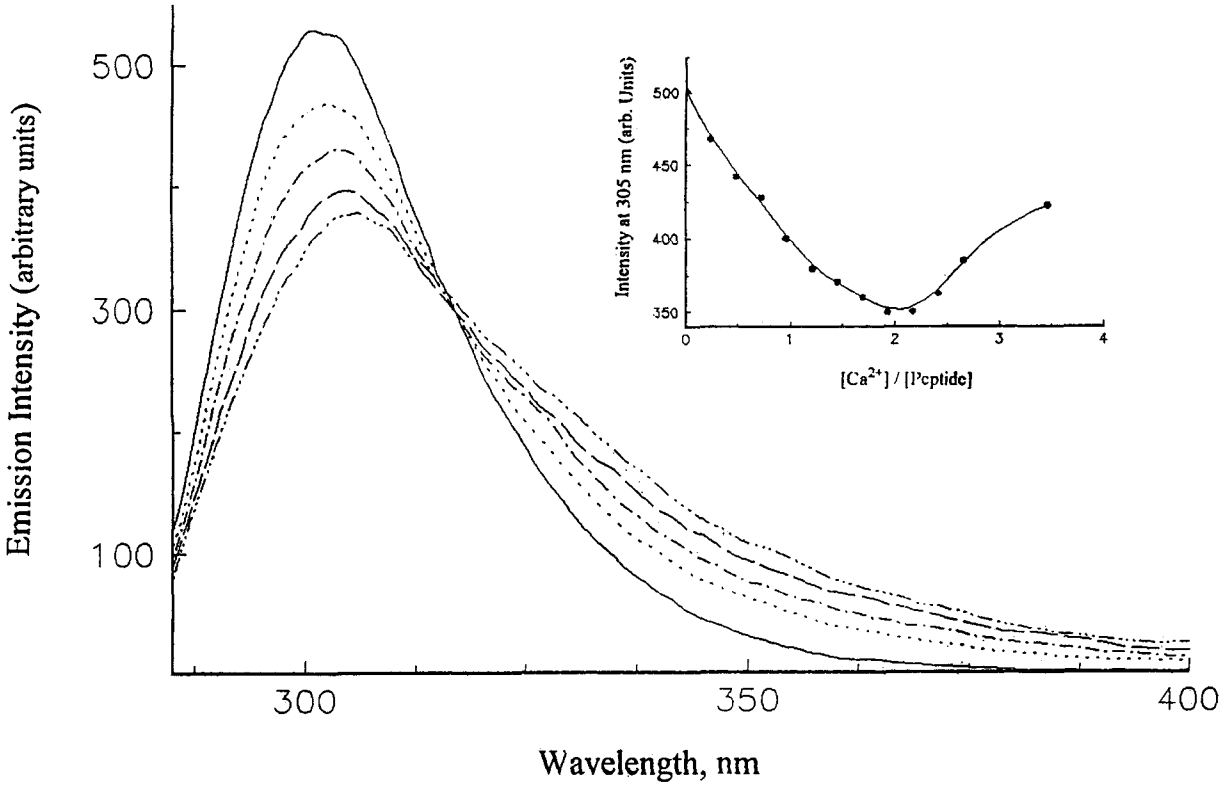

FIGURE 4 Fluorescence emission spectral changes at 22 ± 1°C in TFE of oxytocin (solid line) and increasing amounts of Ca^{2+} upto 2 molar equivalents (dotted lines). Inset: Plot of emission intensity at 305 nm against the molar ratios of Ca^{2+} to hormone.

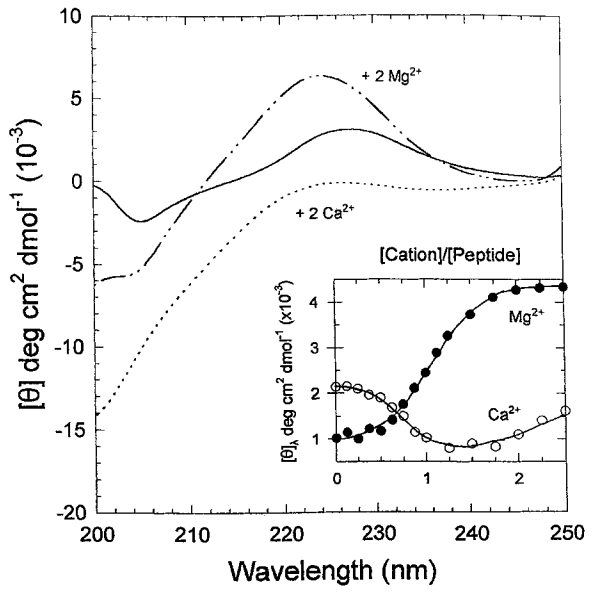

FIGURE 5 CD spectra of vasopressin (70 μM) in the far-uv region at 22 ± 1°C in TFE: (solid line) free vasopressin; (dash-double dotted line), vasopressin + 2 molar equivalents of Ca^{2+}; (dotted line) vasopressin + 2 molar equivalents of Mg^{2+}. Inset: Cation binding isotherms: Ca^{2+} (open circles, λ = 227 nm); Mg^{2+} (solid circles, λ = 221 nm).

the intensity of the positive CD band at 227 nm in vasopressin (Figure 5) and, at higher concentrations (>1 equivalent), blue shifted by about 6 nm. There was no reversal of the sign of this band unlike in oxytocin. Addition of Mg^{2+} to vasopressin led to a marked increase in the 227 nm band along with its blue shift to 220 nm. The CD spectra at different Ca^{2+} concentrations showed several crossover points, implying multiple conformers, while the Mg^{2+} spectra showed an isodichroic point at about 228 nm, suggesting a two-state transition (data not shown). The cation binding isotherms are shown in the inset of Figure 5. In spite of differences in magnitude and, in the case of Ca^{2+}, the sign of the ellipticity changes, these titration profiles are qualitatively similar to those of oxytocin shown in Figure 2. There is an initial lag, which is seen more clearly with Mg^{2+}, followed by a steeper change in the CD of both the hormones (Figures 2 and 5). With Ca^{2+}, there is a reversal in the ellipticity change above 1.5 mole ratio. Changes in vasopressin's fluorescence spectrum on adding either Ca^{2+} or Mg^{2+} were small (<10% of original emission intensity; Figure 6). With Mg^{2+}, there was an apparent saturation at about 1 equivalent while with Ca^{2+} saturation occurred at about 0.5 equivalent and a nonspecific fluorescence change persisted up to 2.5 molar equivalents of the cations (Figure 6). It is interesting that an indication for a 0.5 : 1 cation : hormone ("sandwich") complex is seen in almost all the cation-binding profiles for both the hormones (Figures 2, 5, and 6). This would suggest an intermediate in the cation binding process where two hormone molecules are brought together by one cation. Support for this intermediate is derived from the nmr data on the effect of Ca^{2+} titration on the chemical shifts of individual protons in oxytocin.[34]

To obtain a clue about the two cation binding sites in oxytocin and about the difference between the binding behavior between oxytocin and vasopressin, CD measurements were carried out on the tripeptides Pro-Leu-Gly-NH_2 and Pro-Arg-Gly-NH_2 that represent, respectively, the tail regions of these hormones and on tocinoic acid, which is the ring moiety of oxytocin. Figure 7 shows the CD spectra of Pro-Leu-Gly-NH_2 in TFE in the absence and presence of two equivalents of Ca^{2+}. Not surprisingly, these spectra are quite different from that of the complete hormone. The CD spectral features of the tripeptide (small negative band around 230 nm and larger positive band near 200 nm) are indicative of a β-turn structure,[42] which this peptide segment adopts in the crystal forms of deaminooxytocin.[43] Titration curves of Pro-Leu-Gly-NH_2 with Ca^{2+} and Mg^{2+} showed an end point at a peptide : cation ratio of 0.5 corresponding to a sandwich complex formed with two molecules of the peptide binding to one cation. Such sandwich complexes have been observed in other small peptides that do not have enough Ca^{2+} chelating groups in one molecule.[44] The cation binding isotherms are shown in the inset of Figure 7. The tripeptide representing the tail part of vasopressin, Pro-Arg-Gly-NH_2, showed a CD spectrum in TFE also suggestive of a β-turn, although the magnitude of the positive CD band was rather small. In contrast to the oxytocin tail peptide, titration of this tripeptide with either Mg^{2+} or Ca^{2+} had no appreciable effect on its CD spectrum (data not shown), indicating that the peptide, by itself, is unable to bind either cation. It would thus appear that the large difference in the CD spectra of free and Ca^{2+}-bound oxytocin (Figure 1) arises from concerted changes in the tail and ring regions of the molecule. This does not appear to happen in the case of vasopressin. These observations are further confirmed by the fact that Ca^{2+} and Mg^{2+} additions to tocinoic acid caused only a relatively small diminution of the 225 nm positive CD band with a small increase in the magnitude of the negative band at 205 nm (Figure 8). No definitive saturation was

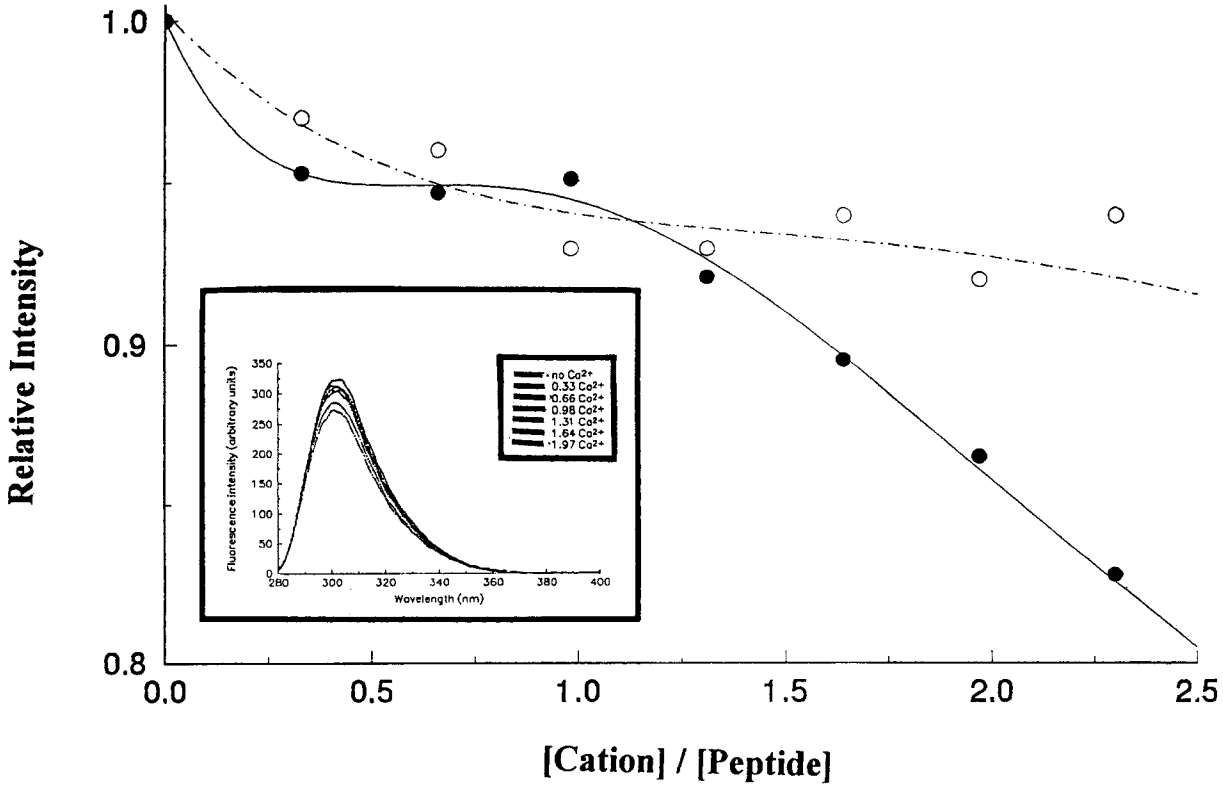

FIGURE 6 Changes in the fluorescence emission intensity of vasopressin at 305 nm as a function of cation-to-hormone molar ratios. The solid circles corresponds to Ca^{2+} and open circles to Mg^{2+}. Inset: Fluorescence spectra of vasopressin in the presence of indicated molar equivalents of Ca^{2+}.

observed except for an indication of a plateau around the 0.5 equivalent of the cation, suggesting a sandwich complex formation (data not shown).

DISCUSSION

The studies described in this and the accompanying paper[34] are aimed at understanding the role of Ca^{2+} in the biological action of oxytocin from the structural viewpoint. As mentioned in the Introduction, extracellular Ca^{2+} is essential for oxytocin activity. Biochemical studies on the effect of Ca^{2+} and Mg^{2+} on the *in vitro* action of the hormone on isolated tissue systems indicate that Ca^{2+} by itself has no appreciable effect on oxytocin binding to the receptor in contrast to Mg^{2+}. Interestingly, however, Ca^{2+} decreases the enhancement effect of Mg^{2+} at low (<1 mM) concentrations but increases it at higher concentrations (>2 mM) to a level higher than that reached by Mg^{2+} alone.[9] Since the levels of extracellular Ca^{2+} and Mg^{2+} are in the millimolar range, the observations at the higher concentrations of the two cations (>2 mM) may be expected to be more relevant. The exact molecular mechanism of how these cations promote the binding of the hormone to the receptor is largely unknown. Based on the nondependence of the forward rate constant on the concentration of the cation, Pearlmutter and Soloff [9] dismissed the possibility of hormone binding the cation first and then forming a ternary complex with the receptor (e.g., oxytocin–Ca^{2+} receptor). Instead, they suggested the formation of a receptor–cation complex prior to oxytocin binding. It may, however, be noted that their analysis of the kinetic data did not consider the possibility of an irreversible formation of the hormone–cation complex prior to binding to receptor (which might occur in the presence of millimolar Ca^{2+} and low-dielectric medium and could account for the observed cation concentration independence). Also, no kinetic data were obtained on their receptor preparation with Ca^{2+} and Mg^{2+} both present. The ability of oxytocin to bind these cations, weakly in aqueous

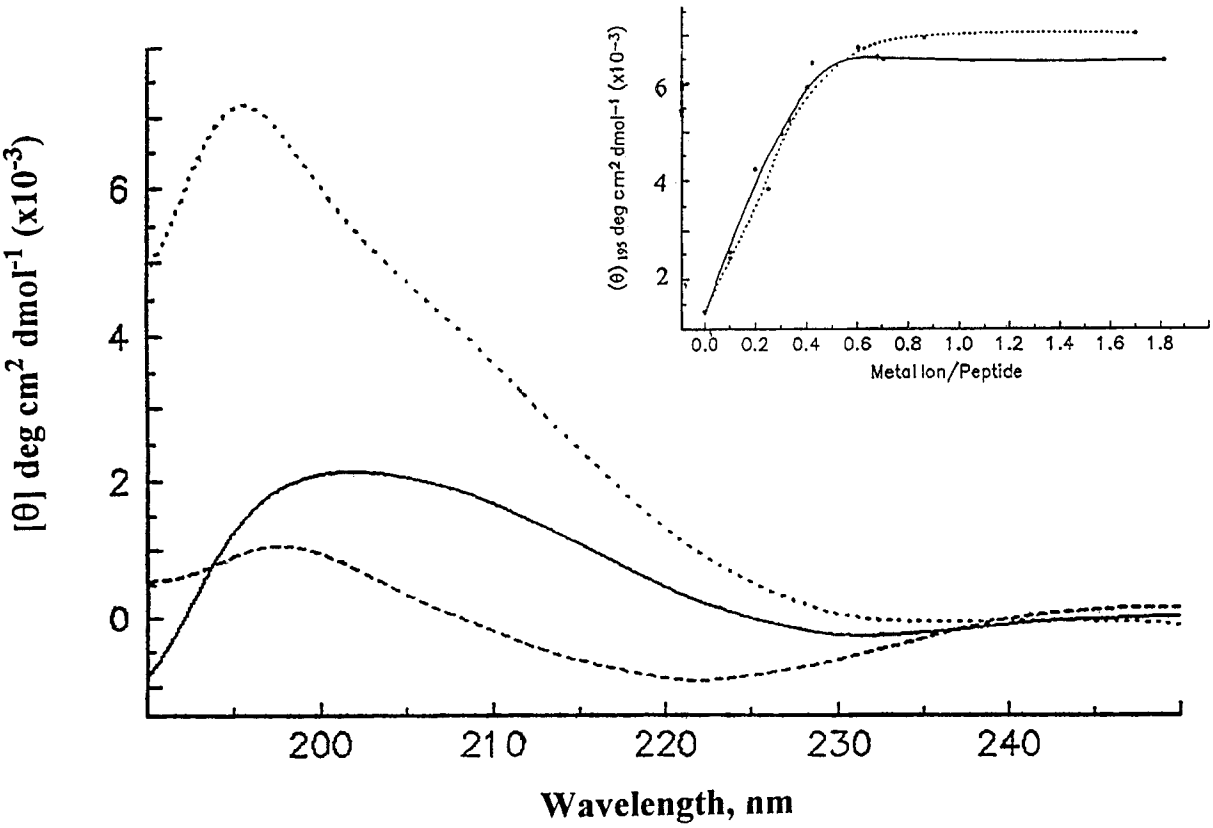

FIGURE 7 CD spectra at 22 ± 1°C in TFE of Pro-Leu-Gly-NH_2 in the free form (solid line) and in the presence of 1 molar equivalent of Ca^{2+} (dotted line), and Pro-Arg-Gly-NH_2 (dashed line). The inset shows the titration curves of Pro-Leu-Gly-NH_2 with Ca^{2+} (dotted line) and Mg^{2+} (solid line).

solution[9,21] and moderately in a low-dielectric lipid-mimetic solvent as shown in this study, makes it difficult to visualize the hormone in the uncomplexed state in the presence of millimolar levels of extracellular Ca^{2+} and Mg^{2+}. We had suggested[28] that the hormone–receptor interaction may involve a Ca^{2+}-bound rather than the free form of the hormone and would lead to the hormone–Ca^{2+}–receptor ternary complex as an intermediate in the signal transduction pathway. In this and the accompanying study,[34] we have attempted to characterize the cation-bound form of oxytocin in TFE assuming that such a structure would be prevalent in the lipid milieu (see, for example, studies on secretin by Gronenborn[45]); nmr data on bombesin[32] show that the conformation of the hormone bound to lipid bilayer vesicles is similar to that in TFE.

The CD and fluorescence results presented in this paper show that oxytocin is capable of binding two Ca^{2+} or Mg^{2+} ions in TFE. This is similar to our observations on other peptide hormones of comparable size.[31,32] However, unlike the case with the other hormones, there appears to be a significant difference in the modes of binding of Ca^{2+} and Mg^{2+} to oxytocin and hence in the conformation of the cation-bound hormone. A large CD spectral change and, arguably, a major conformational change take place on binding the first Ca^{2+} ion, which is followed by a smaller CD change. The CD spectral features of the 1 : 1 and 2 : 1 Ca^{2+}: oxytocin complexes suggest a significantly helical conformation (type III β-turn or 3_{10}-helix[42]). A similar structure was deduced for *t*Boc-Leu-Pro-Tyr-Ala-$NHCH_3$ based on CD in TFE.[46] In contrast, binding the Mg^{2+} ion causes relatively much smaller and qualitatively different CD changes in the hormone, implying a conformation for the Mg^{2+}: oxytocin complex that is different from that formed with Ca^{2+}. The fluorescence changes that accompany the binding of the two cations point to the perturbation of the electronic environment of the Tyr^2 residue in the cyclic part of the hormone. This, in conjunction with the

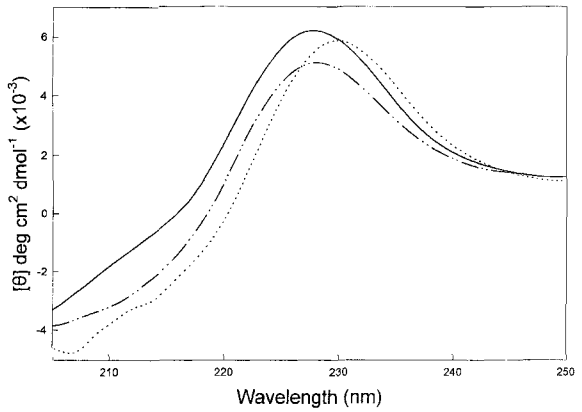

FIGURE 8 CD spectra of tocinoic acid (55 μM) in the far-uv region at 22 ± 1°C in TFE: (solid line) free tocinoic acid; (dash-double dotted line), tocinoic acid + 1 molar equivalent of Ca^{2+}; (dotted line), tocinoic acid + 1 molar equivalent of Mg^{2+}.

observation that the oxytocin tail tripeptide is capable of chelating Ca^{2+} (Figure 6), would suggest that both the ring and the tail region are involved in cation binding. The observed CD and fluorescence spectral changes may be explained by assuming that the high-affinity binding site for Ca^{2+} involves both the tail and ring regions while for Mg^{2+} it involves mainly the ring region. Large CD changes occur when both the ring and tail take part concertedly in binding Ca^{2+} in a 1 : 1 complex, or when both sites are filled (as seen in Figure 1). The largest change seems to occur when Ca^{2+} and Mg^{2+} are bound to their respective high-affinity sites (Figure 1). Binding of cations to the ring moiety alone seems to cause less drastic CD changes.

The conformational changes suggested by the spectral changes in oxytocin on Ca^{2+} binding may be understood in light of the nmr on free oxytocin and its 1 : 1 Ca^{2+}: hormone complex and computational data on 1 : 1 and 2 : 1 Ca^{2+}: hormone complexes presented in the following paper.[34] For our discussion here, these data may be summarized as follows. In the free hormone, the ring has a bowl-like shape with the tail pointing away from the ring as found in the x-ray structure of deamino oxytocin.[43] While the backbone carbonyl oxygens of Tyr^2 and Asn^5 are involved in intraring hydrogen bonds, several others are not and may be available for coordinating Ca^{2+}. In the 1 : 1 Ca^{2+} complex, the peptide carbonyls of Gln^4 and the peptide carbonyls of Cys^6 inside the bowl together with the peptide carbonyls of residues in the ring (Tyr^2, Ile^3, Gln^4, Asp^5, and Cys^6) and in the tail (Leu^8 and Gly^9) act as ligands for the Ca^{2+} ion. This results in a compact structure for the hormone (see figure in the accompanying paper) and involves major structural changes in both the ring and tail segments of the hormone on Ca^{2+} binding. Interestingly, the nmr and computational data show that the molecule adopts a more helical conformation in the Ca^{2+}-bound form than in the free form and this may account for the helical CD spectrum of the Ca^{2+}: oxytocin complex (Figure 1). A molecular model of the 2 : 1 Ca^{2+}: oxytocin complex obtained by energy minimization (see figure in the accompanying paper) shows that one Ca^{2+} ion is penta-coordinated to the peptide carbonyls of $Cys,^1$ $Tyr,^2$ $Ile,^3$ and Gln^4 and the side chain oxygen of $Asn,^5$ while the second Ca^{2+} ion is tetra-coordinated to the peptide carbonyls of $Cys,^6$ $Pro,^7$ $Leu,^8$ and $Gly.^9$ It is interesting to note from these data that the tail residues are reoriented from their positions in the 1 : 1 complex to enable them to bind the second Ca^{2+} ion. One also observes a conformational change in the ring when the second Ca^{2+} is added. The reorientation of the tail residues is likely to be responsible for the reversal of CD and fluorescence changes beyond the 1.6–2 Ca^{2+}: peptide ratios seen in Figures 2 and 4. In vasopressin, the presence of the positively charged arginine side chain at position 8 may be responsible for the poorer participation of the tail in chelating Ca^{2+} and for the resultant smaller changes in CD and fluorescence when compared to oxytocin. This would imply that the tail in oxytocin but not in vasopressin plays a major role in altering the structural parameters (e.g., dihedral angles) of the Ca^{2+}-bound hormone.

The near-uv CD data (Figure 3) suggest changes in the chirality of the disulphide bond on Ca^{2+} and Mg^{2+} binding. It is difficult, however, to be certain about this observation without additional supportive data. Data from the accompanying paper[34] show that on binding one Ca^{2+} ion, the dihedral angle of the C—S—S—C bond changes from +*gauche* to −*gauche*. This conformation may likely to persist in the 2 Ca^{2+} complex since the CD changes from the 1 to 2 Ca^{2+} complex are continuous and uniform.

The CD spectral data obtained in the competition experiments where Mg^{2+}-bound oxytocin was challenged with Ca^{2+} present an interesting case of a synergetic effect of the two cations. That Mg^{2+} is ineffective in bringing about an increase in the helical conformation of oxytocin may be due to its inability to force the tail residues to chelate it, in contrast to Ca^{2+}. (Ca^{2+} with a larger ionic radius can accommodate a larger number of chelating

groups to fulfill its octahedral or pentagonal bipyramidal coordination when compared to Mg^{2+}, which is smaller in size and usually has only six ligands.[47]) However, Mg^{2+} seems to augment the effect of Ca^{2+} once the first Ca^{2+} is bound to the peptide and the tail is brought closer to the ring. It would appear that when both Mg^{2+} and Ca^{2+} are present, the former would be bound to the ring and the latter to the tail of oxytocin. This conformation may have a bearing on the biochemical observation[9] of the enhancement of oxytocin binding to the receptor by Ca^{2+} in the presence of Mg^{2+}. We would like to suggest that, in oxytocin–receptor interaction, the effect of Ca^{2+} may arise mainly from the change in the structure of hormone, while the effect of Mg^{2+} may be mainly due to its binding to the receptor and enhancing the latter's affinity to the hormone. Both effects may be acting together in the presence of physiological amounts of Ca^{2+} and Mg^{2+}. It is, admittedly, premature to link the structural and biochemical data in the absence of direct structural studies on the receptor in the presence of the hormone and cations.

Although the tail peptide of oxytocin shows similar binding of Ca^{2+} and Mg^{2+} (Figure 6), it would appear that, in the intact hormone, Mg^{2+} does not bind the tail segment as effectively as Ca^{2+} does since Mg^{2+}-induced CD changes are smaller (Figures 1 and 2). A somewhat similar situation might exist for both the cations in the case of vasopressin, which shows relatively smaller CD spectral changes on binding these cations (Figure 5). The presence of the positively charged side chain of the Arg^8 residue may be the major cause for this. Due to its smaller effect on the conformation of the hormone compared to Mg^{2+}, it is likely that the observed biochemical effects of Ca^{2+} on vasopressin action may arise mainly from its effect on vasopressin receptor rather than on the hormone itself unlike the case of oxytocin. The differences between oxytocin and vasopressin in terms of their interaction with Ca^{2+} and Mg^{2+} and the resulting cation-bound conformations could provide a structure-based explanation for the functional differences between these two hormones that are closely related by their amino acid sequences. Such an explanation is not feasible by examining the closely similar experimentally determined conformations of these two hormones in the absence of the cations (compare, for example, the CD spectra in Figures 1 and 5[48,49]).

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