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Aspartate buffer and divalent metal ions affect oxytocin in aqueous solution and protect it from degradation

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ABSTRACT

Oxytocin is a peptide drug used to induce labor and prevent bleeding after childbirth. Due to its instability, transport and storage of oxytocin formulations under tropical conditions is problematic. In a previous study, we have found that the stability of oxytocin in aspartate buffered formulation is improved by the addition of divalent metal ions (unpublished results). The stabilizing effect of Zn^{2+} was by far superior compared to that of Mg^{2+}. In addition, it was found that stabilization correlated well with the ability of the divalent metal ions to interact with oxytocin in aspartate buffer. Furthermore, LC–MS (MS) measurements indicated that the combination of aspartate buffer and Zn^{2+} in particular suppressed intermolecular degradation reactions near the $Cys^{1,6}$ disulfide bridge. These results lead to the hypothesis that in aspartate buffer, Zn^{2+} changes the conformation of oxytocin in such a way that the $Cys^{1,6}$ disulfide bridge is shielded from its environment thereby suppressing intermolecular reactions involving this region of the molecule. To verify this hypothesis, we investigate here the conformation of oxytocin in aspartate buffer in the presence of Mg^{2+} or Zn^{2+}, using 2D NOESY, TOCSY, ^{1}H–^{13}C HSQC and ^{1}H–^{15}N HSQC NMR spectroscopy. Almost all ^{1}H, ^{13}C and ^{15}N resonances of oxytocin could be assigned using HSQC spectroscopy, without the need for ^{13}C or ^{15}N enrichment. ^{1}H–^{13}C and ^{1}H–^{15}N HSQC spectra showed that aspartate buffer alone induces minor changes in oxytocin in D_2O, with the largest chemical shift changes observed for Cys^{1}. Zn^{2+} causes more extensive changes in oxytocin in aqueous solution than Mg^{2+}. Our findings suggest that the carboxylate group of aspartate neutralizes the positive charge of the N-terminus of Cys^{1}, allowing the interactions with Zn^{2+} to become more favorable. These interactions may explain the protection of the disulfide bridge against intermolecular reactions that lead to dimerization.

1. Introduction

Oxytocin is a nonapeptide hormone secreted by the posterior lobe of the pituitary gland, and is involved in the control of labor and bleeding cessation after child birth (Maughan et al., 2006). The peptide consists of nine amino acids (Cys–Tyr–Ile–Gln–Asn–Cys–Pro–Leu–Gly–NH_2) with an internal disulfide bridge, and an amidated C-terminus (du Vigneaud et al., 1953). Oxytocin is the preferred drug to prevent postpartum hemorrhage and is commonly formulated in aqueous solution for parenteral administration (Gard et al., 2002). The instability of oxytocin in aqueous solution under harsh circumstances, particularly under tropical conditions, presents a significant challenge to pharmaceutical scientists (Hawe et al., 2009). The instability of oxytocin in aqueous solution has been reported in several studies (Hogerzeil et al., 1993; Trissel et al., 2006). It has been found that the degradation rate strongly depends on the pH of the formulation, with the highest stability reported at pH 4.5 (Hawe et al., 2009). Several studies have been aimed at the improvement of the stability of oxytocin in aqueous solution (Avanti et al., 2011; Hawe et al., 2009). The most recent finding is that the use of divalent metal ions, in combination with certain buffers, strongly increases the stability of oxytocin in aqueous solution (Avanti et al., 2012).

Previously, we have shown that Zn^{2+} in combination with aspartate buffer strongly stabilizes oxytocin in aqueous solutions, whereas Ca^{2+} and Mg^{2+} only have minor effects. The stabilization

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Table 1
Information about the NMR experiments.

Experiment	Correlations provided	Number of scans	Nuclei	Spectral width (Hz)	Carrier (ppm)	Maximum evolution time (ms)	Reference
2D ^{15}N–^{1}H HSQC	^{15}N and ^{1}H separated by one bond (N^{H}–H^{N} Nε–Hε for Gln, Nδ–Hδ for Asn, N–H for amide of Gly[9])	512	^{15}N ^{1}H	1944 8000	120.39 5.03	20.5 85	Kay et al., 1992; Mulder et al., 2011
2D ^{13}C–^{1}H HSQC	^{13}C and ^{1}H separated by one bond (Hα–Cα, Hβ–Cβ, Hγ–Cγ, etc.)	40	^{13}C ^{1}H	8000 8000	48.191 5.04	16 100	Kay et al., 1992; John et al., 1992
2D ^{1}H–^{1}H TOCSY	Correlates all protons in a J-coupled spin system	20	^{1}H ^{1}H	8000 8000	5.04 5.04	16 85	Davis and Bax, 1985
2D ^{1}H–^{1}H NOESY	Correlates all protons which are close in space (<0.5 nm)	16	^{1}H ^{1}H	8000 8000	5.04 5.04	25 160	Boyd et al., 1984
1D ^{1}H		128	^{1}H	8000	5.04	2000	

occurred as a result of complex formation of Zn^{2+} ions and aspartate with oxytocin, which suppressed dimerization by protecting the Cys[1,6] disulfide bridge. In line with those results, ITC data demonstrate that, among the tested divalent metal ions (Zn^{2+}, Ca^{2+}, and Mg^{2+}), only Zn^{2+} is able to strongly interact with oxytocin in the formulation conditions (unpublished results). These results lead to the hypothesis that Zn^{2+} induces a conformational change, thereby stabilizing oxytocin in aspartate buffer. Aspartic acid is one of the non-essential amino acids that is normally synthesized in the body. It consists of two carboxylate groups with pKa_1 and pKa_2 of 2.1 and 3.9, and one amine group (pKa_3 of 9.8). Aspartate is a commonly used buffer in parenteral products approved by the FDA for formulation purposes (Jurgens et al., 1982). The aim of this study was to investigate the conformation of oxytocin in aspartate buffer in the presence of divalent metal ions (Zn^{2+} and Mg^{2+}) by using two-dimensional (2D) Nuclear Magnetic Resonance (NMR) spectroscopy.

Nuclear Magnetic Resonance spectroscopy is the most suited technique to study the conformational details of proteins or peptides in solutions. Several one-dimensional NMR studies of oxytocin and vasopressin analogs have previously been published, studying the peptide hormones in various solvents, including deuterated dimethylsulfoxide (Sikorska et al., 2006; Glickson et al., 1976; Johnson et al., 1969), deuterated trifuoroethanol (TFE) (Ananthanarayanan et al., 1996; Rholam et al., 1990), and aqueous solutions (Glickson et al., 1976; Sikorska and Rodziewicz-Motowidlo, 2008; Smith et al., 1973). Since resonance overlap is much reduced in 2D NMR spectra in comparison with one-dimensional NMR, we use here 2D NMR spectroscopy. In addition, natural abundance 2D HSQC spectroscopy provides access to backbone ^{13}C and ^{15}N chemical shifts that are highly sensitive reporters of peptide conformation.

Oxytocin solutions are commonly formulated at a concentration of 5 IE/mL which corresponds to approximately 0.01 mM. The concentration used in this study (10 mM) was higher, since it enables NMR measurements without ^{13}C or ^{15}N enrichment, relying only on the low natural abundance of these isotopes.

A complete NMR analysis of oxytocin in phosphate buffer has been reported (Ohno et al., 2010). However, no reports are available on the ^{1}H, ^{13}C, and ^{15}N resonance assignments of oxytocin in aspartate buffer in the absence and presence of divalent metal ions. In this study, we used 1D and 2D NMR spectroscopy to investigate the conformation of oxytocin in the presence of Zn^{2+} or Mg^{2+} in aspartate buffer at pH 4.5.

2. Materials and methods

2.1. Chemicals and reagents

Oxytocin monoacetate powder (Diosynth. Oss, The Netherlands) was kindly provided by MSD, Oss, The Netherlands. Deuterium oxide (D_2O, isotopic purity 99.9 atom % D) containing 0.75% TSP (3-(trimethylsilyl) propionic-2,2,3,3,-*d*4 acid, sodium salt) was purchased from Aldrich, Steinheim, Germany and deuterated L-aspartic acid-2,3,3-*d*3 was purchased from Medical Isotope, Inc, NH. Zinc chloride was purchased from Fluka, Steinheim, Germany. All reagents used for the NMR experiments were of analytical grade (purity > 99%), and were used without further purification.

2.2. Sample preparation

Two different types of NMR samples were prepared with the following compositions:

1. Samples used for 2D ^{13}C–^{1}H HSQC NMR10 mM oxytocin (OT) in 10 mM deuterated aspartate buffer (pH 4.5) in D_2O containing 0.75% TSP in the absence (OT–AP) and presence of 100 mM $ZnCl_2$ (OT–AP–Zn) or 100 mM $MgCl_2$ (OT–AP–Mg). The reference solution was oxytocin in D_2O.
2. Samples used for 1D ^{1}H NMR, 2D ^{15}N–^{1}H HSQC, ^{1}H–^{1}H TOCSY, and ^{1}H–^{1}H NOESY.10 mM oxytocin in 10 mM aspartate buffer (pH 4.5) in 90%/10% (v/v) H_2O/D_2O containing 0.75% TSP in the absence and presence of 100 mM $ZnCl_2$ or 100 mM $MgCl_2$. The reference solution was oxytocin in a 90%/10% (v/v) H_2O/D_2O.

2.3. NMR spectroscopy

NMR Spectra were recorded using a Varian Unity INOVA 600 MHz NMR spectrometer equipped with pulsed field-gradient probes. The spectra were recorded at 278 K, processed using NMRPipe (Delaglio et al., 1995) and analyzed using Sparky (Goddard and Kneller, 2003). $^{3}J_{HNH\alpha}$ coupling constants were determined by recording 1D ^{1}H NMR spectra. All information about the NMR measurements is summarized in Table 1. TSP was used as an internal standard having a chemical shift (δ) of 0.0 ppm. Chemical shift referencing for ^{13}C and ^{15}N was done following the IUPAC recommendations of Markley et al. (1998) (for indirect referencing).

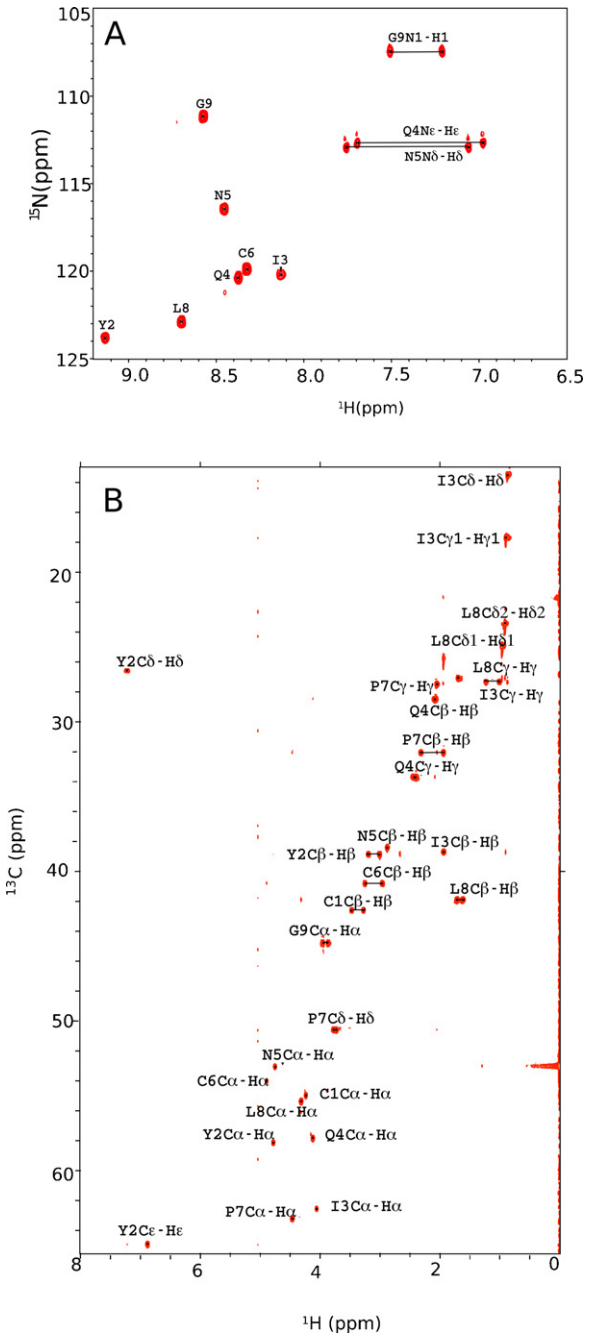

Fig. 1. 2D NMR spectra of oxytocin in aspartate buffer. (A) ^{15}N–^{1}H HSQC spectrum, which shows the correlation between amide protons and amide nitrogens in the backbone of the peptide. The correlation between amide protons and amide nitrogens in the side chain of Gln[4], Asn[5] are indicated, as well as the C terminal Gly[9] (which has a carboxy-amide group instead of a regular carboxylate, marked N1–H11 and N1–H12); (B) ^{13}C–^{1}H HSQC spectrum, showing correlations between side chain carbons and their attached protons. The Tyr[2] Cδ and Cε signals are aliased (i.e. folded in) in this spectrum. The true Tyr[2] Cδ and Cε chemical shifts are 132.69 and 117.95 ppm, respectively.

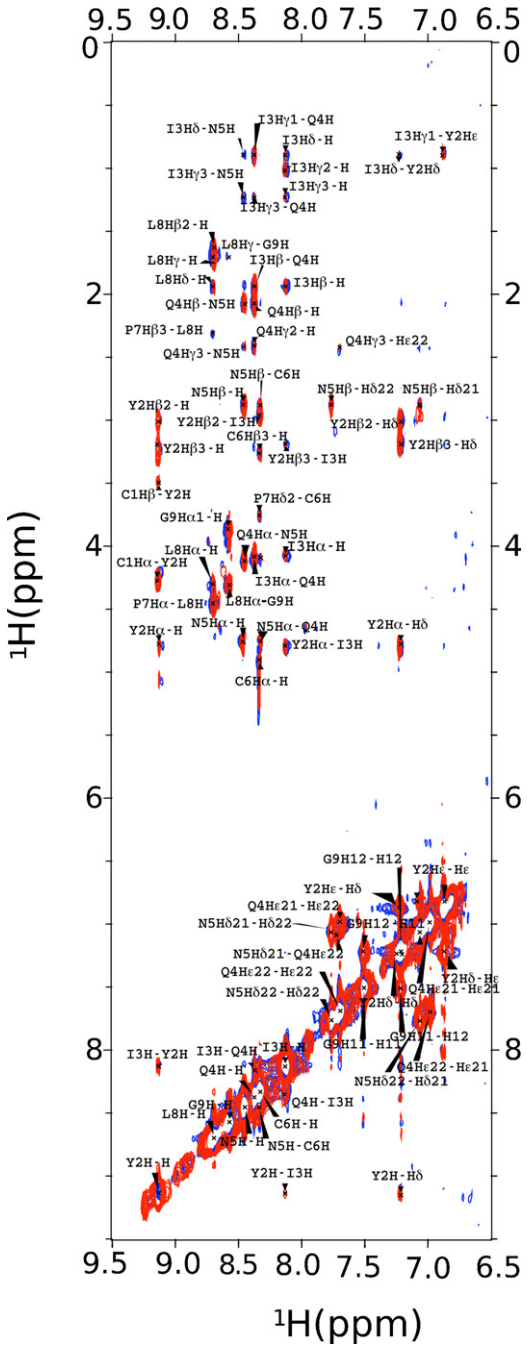

Fig. 2. Overlay of the 2D ^{1}H–^{1}H NOESY spectrum of oxytocin in aspartate buffer in the absence and presence of Zn^{2+}.

3. Results

3.1. Assignment and data deposition of backbone and aliphatic side chain resonances of oxytocin in aspartic acid buffer

The assignment of proton, carbon-13 and nitrogen-15 resonance frequencies was accomplished using 2D ^{13}C–^{1}H HSQC (Kay et al., 1992; John et al., 1992), ^{15}N–^{1}H HSQC (Kay et al., 1992; Mulder et al., 2011) and ^{1}H–^{1}H TOCSY (Davis and Bax, 1985) NMR data. Since the peptide consists of only 9 amino acids, the assignment of ^{13}C–^{1}H HSQC spectra can be easily achieved based on the uniqueness of carbon and proton resonance of each amino acid (Cavanagh et al., 2006), To avoid disturbance of Hα signals by the strong signal of H_2O protons in ^{13}C–^{1}H HSQC experiments, D_2O was chosen as solvent. No attempt was made to correct for the small isotope shift arising from the backbone amide deuteron.

Amide proton and amide nitrogen resonances in the ^{15}N–^{1}H HSQC were assigned by means of ^{1}H–^{1}H correlations visible in a ^{1}H–^{1}H TOCSY spectrum. Fig. 1 summarizes the assignment of most of the oxytocin resonances. All peaks observed in ^{1}H–^{15}N (A) and ^{1}H–^{13}C HSQC spectra (B) are annotated with the one letter amino acid symbol and its position in the sequence.

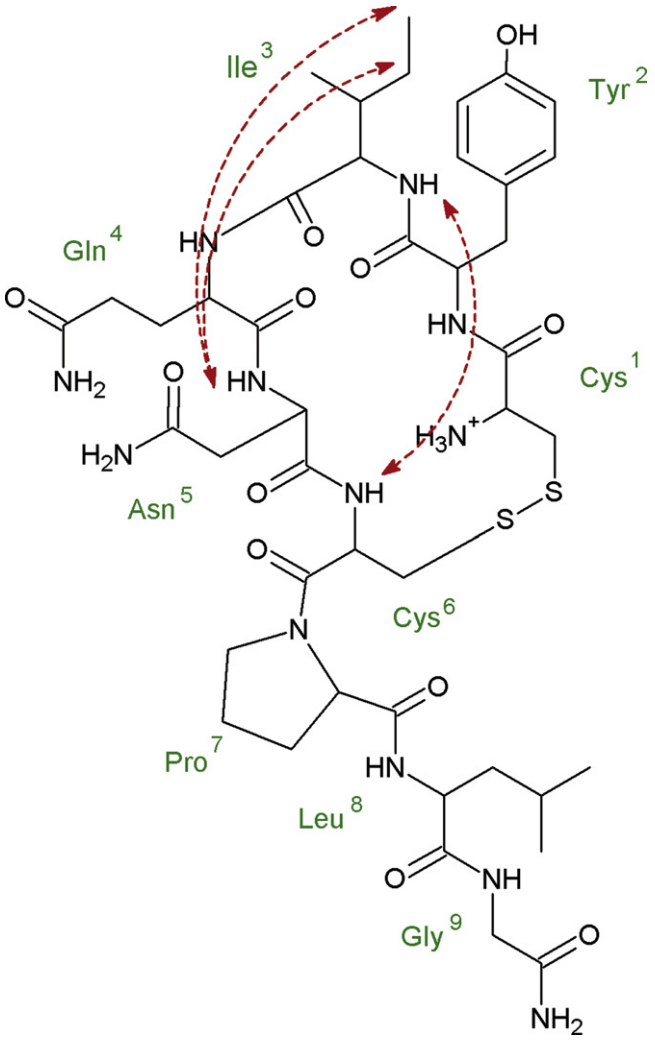

Fig. 3. Summary of short non-trivial distances observed in the 2D ^{1}H–^{1}H NOESY spectrum of oxytocin in aspartate buffer.

3.2. NOE analysis

To obtain information about pairs of protons which are close in space, 2D ^{1}H–^{1}H NOESY (Boyd et al., 1984) spectra were recorded for oxytocin in aspartate buffer in the absence and in the presence of Zn^{2+}. From the NOESY spectrum (Fig. 2), it was found that in aspartate buffer residue Ile^{3} is close to Cys^{6} and to Asn^{5}. A virtually identical NOESY spectrum was obtained in the presence of Zn^{2+} (see Supporting information). The short distances from NOESY experiments are schematically presented in Fig. 3.

3.3. Chemical shift difference ($\Delta\delta$)

The chemical shift differences induced by divalent metal ions upon complexation with oxytocin in aspartate buffer were analyzed to get information about which residues are involved in binding of the divalent metal ions to oxytocin and to learn about the extent of the perturbation caused by these ions.

3.3.1. Influence of aspartate buffer on the conformation of oxytocin in water

Aspartate buffer is one of the buffers known to stabilize oxytocin if divalent metal ions are present in the liquid formulation. The effects of Zn^{2+} and Mg^{2+} ions on the Cα and Hα chemical shifts of oxytocin in the presence of aspartate are displayed in Fig. 4. To investigate the influence of aspartate buffer on the conformation of oxytocin, the chemical shift (δ) of Cα and Hα backbone resonances of oxytocin in deuterated aspartate buffer was compared with the chemical shift of Cα and Hα resonances of oxytocin in D_2O. The difference between those chemical shifts was expressed as chemical shift difference ($\Delta\delta$) in ppm.

Fig. 5A shows that aspartate buffer induces minor chemical shift changes for oxytocin in D_2O. The largest chemical shift changes were observed for the Cα and Hα resonances of Cys^{1}. ^{13}Cα chemical shifts of Cys^{1} and Cys^{6} were more shielded and Cα's of Tyr^{2} and Ile^{3} were more deshielded in the presence of aspartate buffer. The ^{13}Cα chemical shifts of Gln^{4}, Asn^{5}, Pro^{7} and Leu^{8} were not affected by the presence of aspartate buffer. As shown by black bars in Fig. 5A, the Hα's of Cys^{1}, Ile^{3} and Cys^{6} were also affected. The strong effects of aspartate on the Cα and Hα chemical shifts of Cys^{1} could be due to an electrostatic interaction between the positive charge of the N-terminus at Cys^{1} and the negative charge of the carboxylate group of the aspartate at pH 4.5 In order to test this hypothesis, we recorded 2D ^{13}C–^{1}H HSQC of oxytocin in the presence and absence of aspartate buffer at pH 1.6. Highly similar chemical shifts were measured from the two spectra (see Supporting information). These results show that at a very low pH, where the carboxylate groups of aspartate are protonated, there is no effect at Cys^{1}. This observation confirms that at higher pH, when most of the carboxylate groups of aspartate are deprotonated and thus negatively charged, there is indeed an electrostatic interaction between the aspartate and the N-terminus of Cys^{1}.

3.3.2. Influence of zinc ions on the conformation of oxytocin in water

Fig. 5B shows that Cα and Hα chemical shifts of almost all amino acid residues of oxytocin are shifted in the presence of Zn^{2+}. The largest chemical shift changes are observed for Cα of Tyr^{2}. In Tyr^{2}, Gln^{4}, Cys^{6}, Pro^{7}, and Gly^{9} the Cα nuclei are more deshielded in the presence of Zn^{2+}. In contrast, in Ile^{3} and Asn^{5} the Cα nuclei are more shielded in the presence of Zn^{2+}. The presence of Zn^{2+} does not cause changes in the Cα and Hα chemical shifts of Leu^{8}. The largest $\Delta\delta$ is observed for the Hα of Cys^{1}. Interestingly, similar to the effect observed for aspartate buffer on oxytocin, most chemical shift changes for Cα induced by Zn^{2+} are of opposite sign when compared to those for Hα of the same residue.

3.3.3. Influence of divalent metal ions on oxytocin in aspartate buffer

As shown in Fig. 5C, the effects of Zn^{2+} on the chemical shifts of oxytocin in aspartate are very similar to the effects observed in D_2O (Fig. 4B), suggesting that a similar change is induced in both circumstances. A small chemical shift change was observed only in the Cα of Cys^{1}.

The effects of Mg^{2+} on the chemical shifts of Cα and Hα resonances of oxytocin in aspartate buffer are much smaller than those observed in the presence of Zn^{2+} ions. Although smaller, also in the case of Mg^{2+}, the effects on Cα resonances are generally opposite to those seen for the Hα resonance of the same residue (Fig. 5D).

3.4. $^{3}J_{HNH\alpha}$ coupling constants of OT in aspartate buffer in the absence and presence of metal ions

To obtain information about $^{3}J_{HNH\alpha}$ coupling constants, 1D proton NMR spectra were recorded for OT in aspartate buffer in the absence and presence of Zn^{2+} and Mg^{2+}. From the results displayed in Table 2, we have found that $^{3}J_{HNH\alpha}$ coupling constants of oxytocin in all conditions are relatively similar.

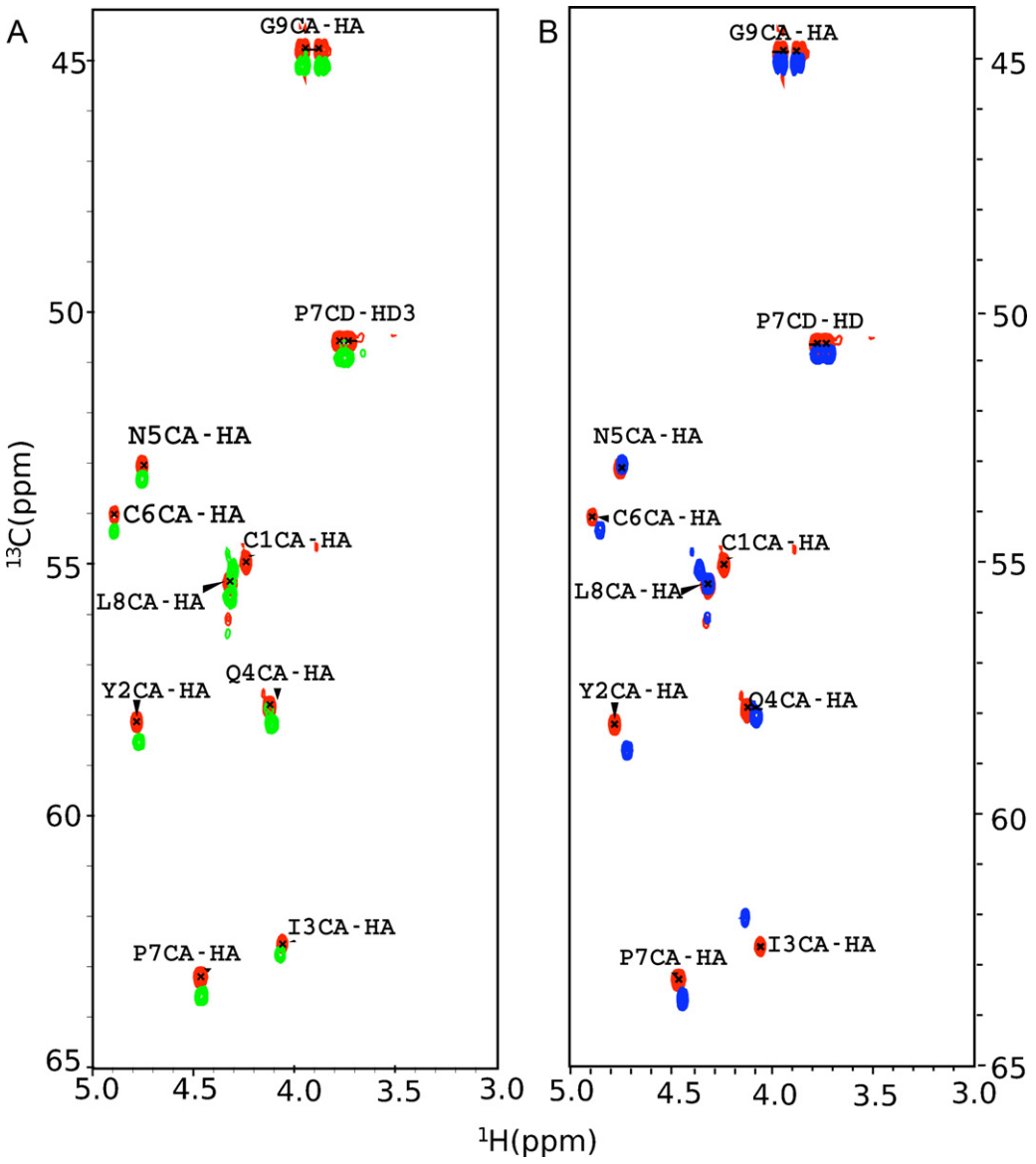

Fig. 4. Overlay of Cα and Hα signals from ^{13}C–^{1}H HSQC spectra of oxytocin in deuterated aspartate buffer without (red) and with Mg^{2+} (green, A) or Zn^{2+} (blue, B). The Pro^{7} Cα–Hα correlation is also shown in these spectra.

4. Discussion

Our study presents nearly complete NMR assignment of oxytocin in aspartate buffer in the presence and absence of divalent metal ions, Zn^{2+} and Mg^{2+}. 2D ^{1}H–^{1}H NOESY spectra of oxytocin in aspartate buffer clearly demonstrate that the residue pairs Ile^{3}–Asn^{5} and Ile^{3}–Cys^{6} are in close proximity. These NOEs are also present in a 2D ^{1}H–^{1}H NOESY spectrum of oxytocin in water. Molecular dynamic studies by Wyttenbach et al. (Wyttenbach et al., 2008) suggest an interaction between Hα of Tyr^{2} and the Gly^{9} amide group of oxytocin in water, but we did not find evidence to support this interaction.

Table 2
^{3}J HnHα coupling constants of oxytocin in aspartate buffer in the absence and presence of divalent metal ions (Hz).

Residue	Oxytocin in aspartate buffer	Oxytocin and $MgCl_2$ in aspartate buffer	Oxytocin and $ZnCl_2$ in aspartate buffer
Y2	7.46	7.46	7.3
L8	6.24	6.24	6.29
G9	5.88	5.88	5.67
	6.13	6.21	6.14
N5	8.05	8	8.04
Q4	3.71	3.99	3.47
C6	6.4	6.27	6.12
I3	n.d	n.d	n.d

n.d: not determined.

This study clearly shows that Zn^{2+} and aspartate buffer interact with oxytocin in solution at pH 4.5. Aspartate buffer induces a minor change in the NMR spectrum oxytocin in D_2O with the largest chemical shift changes are observed in Cys^{1}. We suggest that there is an electrostatic interaction between the positively charged N-terminus of Cys^{1} and the negatively charged carboxylate groups of aspartate. This hypothesis is supported by the fact that at pH 1.6, when the most carboxylate groups of aspartate are protonated, no changes in chemical shifts are found. Zn^{2+} induces strong and similar effects on oxytocin in aspartate buffer and in D_2O, suggesting that a similar change is induced in both circumstances. In our previous study (unpublished results), Zn^{2+} was shown to improve oxytocin stability only slightly in water, but much more in

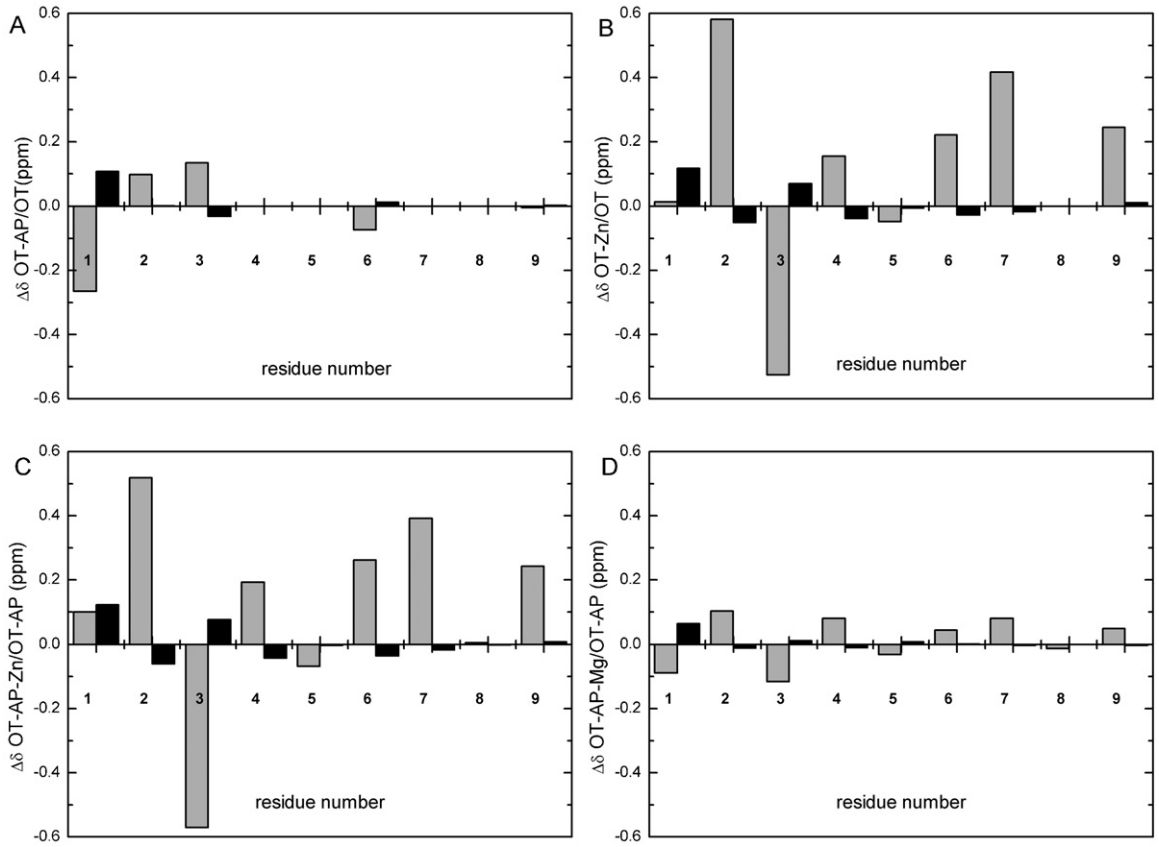

Fig. 5. Chemical shift difference ($\Delta\delta$) of Cα (light gray bars) and Hα (black bars) for (A) oxytocin in deuterated aspartate buffer (pH 4.5) and (B) oxytocin in D_2O in the presence of Zn^{2+} relative to the chemical shifts of the same nuclei measured in D_2O. Oxytocin in deuterated aspartate buffer (pH 4.5) in the presence of (C) Zn^{2+} and (D) Mg^{2+}, relative to the chemical shifts of the same spins measured in deuterated aspartate buffer pH 4.5, analyzed by 2D ^{13}C–^{1}H HSQC spectroscopy.

the presence of aspartate. The small effect of Zn^{2+} on the chemical shift of the Cα of Cys^1, which was observed only in the presence of aspartate, may be relevant in this respect. We suggest that the negatively charged carboxylate groups of aspartate neutralize the positive charge of the N-terminus of Cys^1, allowing the interactions with Zn^{2+} to become more favorable. It is well-known that Zn^{2+} interact favorably and specifically with nitrogen lone pair donors in proteins, whereas Ca^{2+} and Mg^{2+} more generally bind oxygen atoms to complex carboxylates (Glusker et al., 1999). The arrangement of carbonyl/carboxyl groups around the Zn^{2+} might also play a role and may explain the observed chemical-shift changes and the protection of the disulfide bridge against intermolecular reactions that lead to dimerization. This result was confirmed experimentally from the degradation pattern of oxytocin in the presence of divalent metal ions measured by LC/MS(MS). It was found that divalent metal ions, particularly Zn^{2+}, strongly reduced reactions of the cysteine groups leading to the formation of oxytocin dimers (unpublished result).

A modeling study of oxytocin–zinc complex by Liu et al. (2005) demonstrated the ability of Zn^{2+} to coordinate with oxytocin by which the structural conformation of oxytocin in a physiological environment was strongly affected. The observation of chemical-shift changes for the Cα and Hα resonances suggest that small changes in backbone dihedral angles may occur to accommodate Zn^{2+}, although the effects may also arise from a direct influence on the chemical shifts through electric field effects. Drastic changes in the overall structure of the molecule can be ruled out, since the values of $^{3}J_{HNH\alpha}$ coupling constants of the oxytocin in the absence and presence of metal ions are quite similar (Table 2) and no changes were observed in the pattern of NOEs upon addition of Zn^{2+} (Fig. 2). Previous conformational studies of oxytocin by nuclear magnetic resonance (NMR) have been performed in phosphate buffer (Ohno et al., 2010). It was also studied the conformation of vasopressin and its derivates in aqueous solution (Trzepalka et al., 2004), the backbone conformation of the two related peptides is expected to be very similar.

Given the very similar coupling constants, increased propensities for the formation of canonical helical or extended secondary-structure elements are not likely, despite the observation of rather significant and anti-correlated changes in Cα and Hα chemical shifts (Wishart, 2011). Rather, the interaction with Zn^{2+} appears directly responsible for the observed spectral changes, which are most likely due to polarization changes of nearby chemical bonds. The effects of Mg^{2+} on the chemical shifts of Cα and Hα resonances of oxytocin in aspartate buffer are much smaller than those of Zn^{2+}. This result is in agreement with our finding from ITC that Mg^{2+} show only very weak heat effects when added to solutions of oxytocin (unpublished results). The small chemical-shift changes in the backbone of residues Cys^1, Tyr^2 and Ile^3 in the presence of Mg^{2+} in aspartate buffer may be due to a cation-pi interaction between Mg^{2+} and the aromatic side chain of Tyr^2. However, the interactions between Mg^{2+} and oxytocin in aspartate buffer are too weak to cause protection against degradation. We have found that there were no changes in NOESY spectrum of oxytocin upon addition of 100 mM of Zn^{2+}. Also backbone $^{3}J_{HNH\alpha}$ couplings are very similar for all studied samples. It means that there are no such drastic changes in oxytocin structure. The only large changes observed are the differences in Cα–Hα secondary chemical shifts, and these are attributed to a direct effect of zinc ions in the adduct. Moreover, binding of oxytocin–Zn complex in aspartate buffer is

very weak, as shown by the small K_d values obtained from the ITC data (0.16 mM).

5. Conclusion

In conclusion, our NMR studies reveal that Zn^{2+} significantly affects oxytocin in aqueous solution at the molecular level. Zn^{2+} causes changes in the chemical shifts of almost all residues of oxytocin while Mg^{2+} only induces very modest chemical-shift changes in some residues. In contrast, analysis of the NOESY spectra in the presence and absence of Zn^{2+} showed that the same NOEs are present under both circumstances, albeit with slightly altered intensities. The presence of a positive charged of Zn^{2+} per se will cause polarization of nearby chemical bonds and thus may explain the observed chemical shift changes. The differences in ligand coordination preferences of Zn^{2+} and Mg^{2+} are likely responsible for the observed differences, whereas the pH-dependent stability may be related to the ability of aspartate buffer to assist this process.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2013.01.051.

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