DALTON COMMUNICATION

Metal ion and proton stabilisation of turn motif in the synthetic octapeptide histidyltris(glycylhistidyl)glycine

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Protonation and metal ion co-ordination can induce α- and γ-turns respectively, of the linear octapeptide His-(Gly-His)$_3$-Gly (His = histidine, Gly = glycine) and in both cases the imidazole groups of appropriately positioned His residues have been shown to be essential in the stabilisation of the folded structure; divalent metal ions exhibit different folding inducing ability of the octapeptide with Ni^{2+} having the greatest ability at physiological pH.

Among the available methodologies for the stabilisation of well defined conformations in linear oligopeptides,[1] the approach using metal ions as peptide side chain 'cross-linking' agents[2] is particularly promising in view of the inherent potential and simplicity of the principle on which it is based. The folded conformation of the oligopeptide is in fact stabilised by metal ion co-ordination to the side chain donor atoms of appropriately positioned residues. Stabilisation is thought to result from a decrease in the entropy of the unfolded form pertinent to the metal-bound form.[3] While most of the previously reported metallo-peptides contain α-helices as the secondary structure elements,[2a–c] systems containing reverse turns have appeared only recently.[2d] We report here a new metal-based strategy to stabilise turn motifs in a synthetic peptide. Reverse turns play an important structural role in the molecular architecture of globular proteins,[4] but their actual contribution to the folding process is not well understood,[5] probably due to the low energy contribution that each of these relatively small motifs provide to the stabilisation of the folded protein structures. Only a limited number of synthetic oligo- or glyco-peptides, which generally contain a proline residue, have been shown to adopt a significant amount of reverse turn in solution.[6] Newer methods to control and fine tune the structure of oligopeptides can be of some importance in the understanding of the process of protein folding and for the progress in the *de novo* design and synthesis of proteins,[7] and peptide-based therapeutic agents.[8]

We have synthesised the linear octapeptide H-His^1-Gly^2-His^3-Gly^4-His^5-Gly^6-His^7-Gly^8-OH (octaHG), containing alternate histidine (His) and glycine (Gly) residues.† Its circular dichroism (CD) spectrum undergoes a marked change in the range pH 2–8, where the growth of a positive band at 193 nm and a decrease in CD activity above 205 nm parallel the protonation of the peptide [Fig. 1(*a*)]. These spectral changes are indicative of the propensity of octaHG to assume an ordered structure on protonation and imply that protons can also act as 'cross-linking' agents through the formation of hydrogen bonds. Mechanics and dynamics calculations show that the addition of three protons, to the peptide amino group and two imidazoles, strongly stabilises a folded structure characterised by hydrogen bonding between the couples of His residues 1–5 and 3–7 [Fig. 2(*a*)]. In addition, this folding promotes an intramolecular hydrogen bond between the C=O of the fourth residue and the N–H of the eighth residue, leading to the formation of a 13-membered ring corresponding to an α-turn motif. Proton NMR studies (500 MHz) carried out in 90% H_2O–D_2O and at different pH values did not give exhaustive information about the conformational changes observed in the CD experiments, due to the high degree of overlap of the signals, which did not allow a complete assignment of the peptide resonances. However, from the combined analysis of one- and two-dimensional TOCSY and NOESY experiments at pH 3.9, the following assignments have been made: δ 8.79 (NH-Gly^8); 8.58 (imidazole CH-2 of His^1, His^3, His^5, His^7); the remaining amide protons of glycine were at 8.5, 8.28 and 8.24, the amide protons of histidine at 8.45, 8.24 and the sequence specific assignment was not obtained. Other assignments were as follows: δ 7.41–7.29 (imidazole CH-4 of His^1, His^3, His^5, His^7); 4.74–4.62 (CH-α of His^3, His^5,

† The peptide was synthesised on a Milligen/Biosearch 9050 peptide synthesiser using *N*-fluorenylmethoxycarbonyl (Fmoc) amino acid pentafluorophenyl esters (Millipore). The peptide was assembled starting from Fmoc-Gly-Pepsyn KA resin (Millipore). The peptide was cleaved from the resin with a mixture of trifluoroacetic acid and water (95:5 v/v). The crude peptide was purified by ion exchange chromatography on a CM-Sephadex C-25 (NH_4^+ form) column. The FAB mass spectrum of the purified peptide afforded the predicted molecular ion ($M\,H^+$ 795) (Found: C, 48.36; H, 5.35; N, 28.31. Calc. for $C_{32}H_{42}N_{16}O_9$: C, 48.36; H, 5.33; N, 28.20%). The CD measurements were performed on a Jasco J-600 spectropolarimeter; UV/VIS on a Hewlett-Packard HP-8452A spectrophotometer; NMR measurements were carried out on 1.5 mM sample solutions at 500.13 MHz on a DMX-Bruker spectrometer. One-dimensional spectra were collected using 16 K data points over a spectral width of 5000 Hz, collecting 64 scans. The NOESY and TOCSY spectra were acquired over 4 K data points and 500 t_1 increments in the absorption mode with time-proportional phase incrementation (TPPI) for quadrature detection in the t_1 dimension. Water saturation was achieved by low-power irradiation during the relaxation delay introduced between scans. A total of 64 transients were collected for each t_1 increment. Mixing times of 50 and 200 ms were employed for the total correlation (TOCSY) and nuclear Overhauser enhancement (NOESY) experiments, respectively. Two-dimensional spectra were processed with Xwinnmr on an INDY workstation.

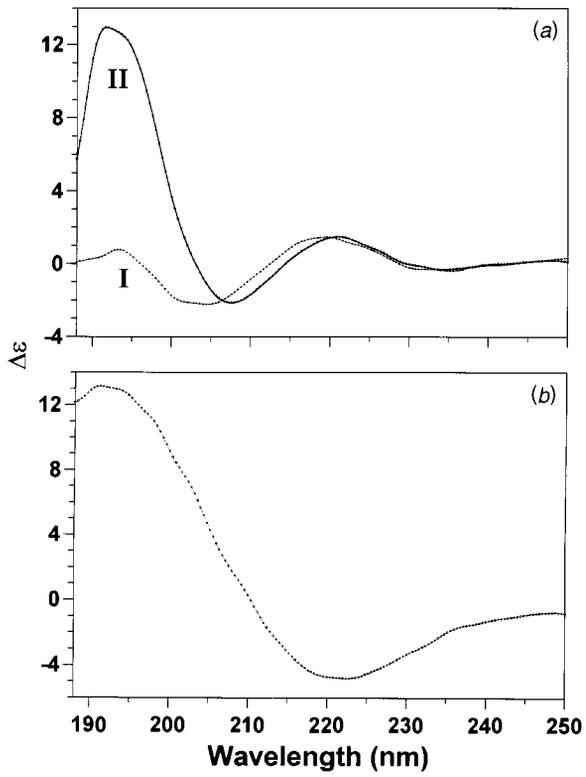

Fig. 1 The CD spectra of (*a*) octaHG (2×10^{-4} M) in 10 mM phosphate buffer at pH 6.8 (**I**) and 10 mM acetate buffer at pH 5 (**II**); and (*b*) octaHG (1×10^{-4} M) in the presence of an equimolar concentration of Ni^{2+} at pH 7

His[7]); 4.34 (CH-α of His[1]); 4.00–3.80 (CH_2 of Gly[2], Gly[4], Gly[6], Gly[8]); 3.40 (CH_2-β of His[1]); 3.30–3.18 (CH_2-β of His[3], His[5] His[7]). It is apparent, on the basis of these assignments, that there is a certain dispersion of the amide protons. The α-protons of all the residues are slightly different. These observations suggest the presence of a structured peptide.

Changes of the CD spectra similar to those induced by protonation of octaHG can be observed upon addition of metal ions to the peptide, but the optical activity is often much stronger and shows, along with the positive peak at 193 nm, a well defined negative band near 220 nm. These CD features resemble those of type II β-turns,[9] but here the positive CD peak occurs at higher energy. It is worth noting that the presence of the band at 193 nm in the CD spectra of the protonated peptide rules out a possible assignment to a charge-transfer transition. As shown in Fig. 1(*b*), the effect of the addition of Ni^{2+} to octaHG (1:1 ratio) at pH 7.0 is already remarkable. Additional ^{1}H NMR investigations of the 1:1 complex of Ni^{2+} with octaHG showed the loss of degeneracy of the imidazole CH-2 of the four histidine residues, while the chemical shifts of the remaining signals were essentially unchanged. The NOESY experiments were of little help for a conformational study, due to the high degeneracy of the system, even in the presence of the metal ion.

The influence of different metal ions on the conformation of octaHG has also been investigated. An excess of Cd^{2+} (10:1), at pH 7.0, produces CD changes comparable to those caused by protonation of the peptide. In the presence of Zn^{2+} [at high metal to ligand ratio (*e.g.* >30:1)], at pH 6, CD spectra also show an increase in the intensity of the 193 nm band. With Cu^{2+} the CD features attributable to the structured peptide can be observed at pH 5.0 (as a strong enhancement of the effect already observed for protonation) in a metal to ligand ratio 1:1, while at pH 7.0 the CD spectrum is very different and does not show any feature of ordered peptide structure. This behaviour is readily explained by the known ability of Cu^{2+} to induce deprotonation in the peptide backbone in neutral medium, with formation of chelates including peptide nitrogen donors, particularly with histidine-containing peptides.[10] This prevents the formation of the folded peptide conformation. Plots of CD spectra intensity at 193 nm *vs.* metal to ligand ratio for Ni^{2+} and Cu^{2+} at pH 7 and 5, respectively, reach a maximum at a metal to ligand ratio of 1:1, indicating that the ML species is formed. Preliminary UV resonance-Raman studies of the Ni^{2+} and Cu^{2+} complexes of octaHG have been performed using a 210 nm excitation wavelength. At this wavelength the spectra are dominated by the histidine side chain vibrations. A 3–10 cm^{-1} up-field shift observed for some ring mode frequencies confirms the co-ordination of the imidazole nitrogen to the metal ions.[11] The visible absorption spectra of the yellow Ni^{2+}–octaHG complex shows a band at 422 nm ($\varepsilon = 100$ M^{-1} cm^{-1}) indicating

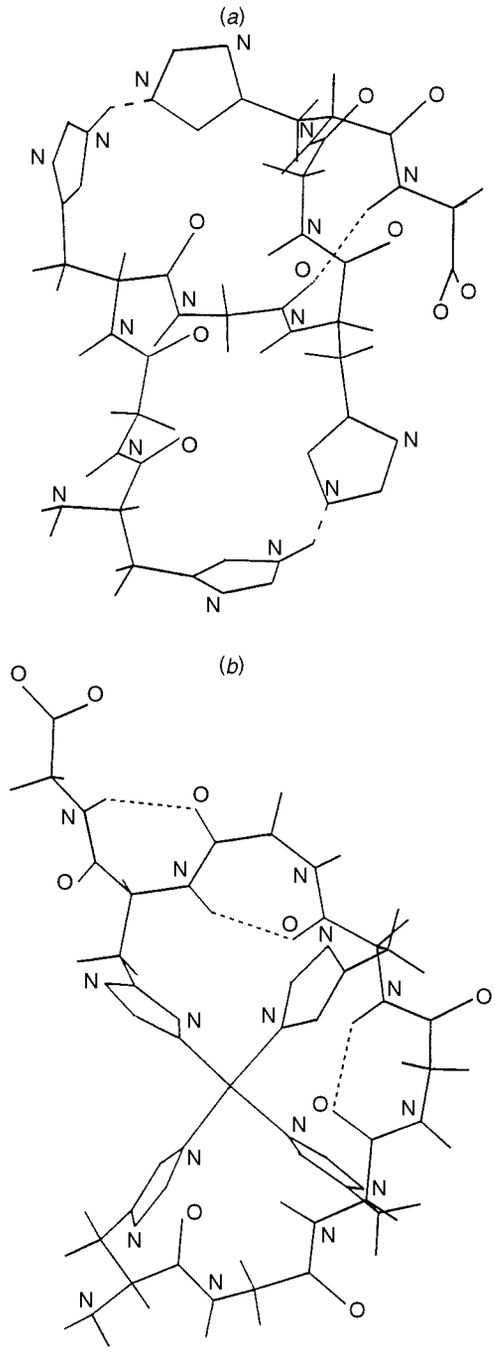

Fig. 2 The (*a*) molecular mechanics optimized structure of octaHG protonated at peptide amino group and two imidazoles. Imidazole hydrogens not involved in hydrogen bonding are not shown for the sake of clarity. (*b*) Molecular mechanics optimized structure of octaHG copper(II) complex. Imidazole hydrogens are not shown for the sake of clarity

that the metal ion is co-ordinated to the four histidines in a square-planar geometry. The frozen-solution EPR spectrum of the $^{63}Cu^{2+}$–octaHG complex at pH 5 exhibits $g_{\|} = 2.254$ and $A_{\|} = 184 \times 10^{-4}$ cm^{-1} values. These are almost coincident with the values determined for the $[Cu(Him)_4]^{2+}$ (Him = imidazole) species.[12] By contrast, the EPR parameters determined for this system at pH 7 ($g_{\|} = 2.178$ and $A_{\|} = 205 \times 10^{-4}$ cm^{-1}) show that Cu^{2+} experiences a stronger equatorial field, most likely due to the in-plane co-ordination of a deprotonated peptide nitrogen. Therefore, the folding of octaHG is induced by intramolecular binding of the metal ion to the side chain imidazole group of the histidine residues. The CD spectra of metallo–octaHG are, in fact, also concentration independent. Molecular mechanics and dynamics calculations‡ show that the folded conformation of the peptide strongly stabilised by such a co-ordination arrangement is a triple γ-turn motif [Fig. 2(*b*)]. This structure is further stabilised by two internal amide hydrogen bonds, while an additional NH···O=C interaction induces a fourth γ-turn fold involving the C-terminal glycine residue of the peptide.

The relative intensity of the CD spectra of metallo–octaHG complexes reflects the folding inducing ability of the metal ions. This ability is apparently correlated with the imidazole binding strength and the tendency to assume square-planar co-ordination by the metal ion, showing the trend $Cu^{2+} \cong Ni^{2+} > Cd^{2+} > Zn^{2+}$. Moreover, the selectivity with which Ni^{2+} effectively induces folding of the octapeptide at physiological pH could be of particular interest for the *de novo* design of proteins.

‡ Molecular mechanics and dynamics calculations were performed on a Silicon Graphics Indigo R3000 workstation, using the Insight and Discover software packages (Byosim Technologies Inc.). Molecular mechanics geometry optimisations were performed using the conjugate gradient algorithm requiring that the residual gradient of energy did not exceed 0.01 kcal mol^{-1} A^{-1} (cal = 4.184 J). Molecular dynamics calculations were performed under vacuum over a time period of 110 ps (10 ps of thermal equilibration followed by 100 ps of dynamics) at a constant temperature of 300 K, using a time step of 1 fs. The consistent valence force field (CVFF) was used in all mechanics and dynamics calculations, but the metal–peptide simulations were performed using suitable modifications of the CVFF to describe properly metal ions in a square-planar environment. The following steps were performed to investigate the conformational characteristics of the peptide in different protonation states or co-ordination environments: (*i*) the peptide was graphically composed using Insight software, imposing dihedral angles to obtain a completely extended conformation of the backbone, (*ii*) the molecule was energy minimised and then submitted to 110 ps of molecular dynamics at a constant temperature of 300 K under vacuum, (*iii*) conformations saved every 5 ps were energy minimised and compared with each other. The structure featuring the lowest energy value was considered the absolute energy minimum of the molecule.

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