CHEMCOMM
Communication
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Design of histidine-Zn^{2+} binding sites within a β-hairpin peptide: enhancement of β-sheet stability through metal complexation

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Received (in Cambridge, UK) 18th January 2001, Accepted 9th May 2001
First published as an Advance Article on the web 8th June 2001

We describe the design and characterisation of two simple 'metalloproteins' based on Zn^{2+} co-ordination sites involving histidine residues close to the N- and C-termini of two β-hairpin peptides (His_2-β, KHYTVSINGKKITVHI and His_3-β, HKHYTV-SINGKKITVHI); we show by NMR and circular dichroism spectroscopy that Zn^{2+} complexation co-operatively enhances the stability of these partially pre-organised β-sheet peptides.

Metals play a pivotal structural and catalytic role in numerous protein and enzyme scaffolds.[1] Metal complexation stabilises some of the smallest structural motifs known (zinc finger domains),[2] while changes in metal co-ordination shell and ligand lability are central to catalytic turnover in enzymatic processes.[3–5] Previously, metal binding sites have been designed into proteins and short peptides, through the incorporation of both natural and non-natural amino acids, to study the effects on protein stability and peptide secondary structure.[6–9] While effects on structural enhancement of α-helical peptides have been described,[7] the extension of these principles to the templating of β-sheet structures[10] for subsequent use as novel molecular architectures for molecular recognition or catalysis, has been rather limited. Previously, the His_3-Zn^{2+} co-ordination site within carbonic anhydrase, which employs residues within β-sheet and β-turn,[11] has been used successfully to design a zinc binding site on one face of an octapeptide incorporating a type II (Pro-^{D}Ser) turn.[9] Here, we investigate by NMR and CD spectroscopy the effect of zinc co-ordination on the stability of β-sheet secondary structure in a partially pre-organised β-hairpin peptide. We show that engineering a metal binding site between the termini of two β-strands, resulting in both His_2-Zn^{2+} and His_3-Zn^{2+} co-ordination shells, co-operatively enhances β-sheet secondary structure in a β-hairpin system.

In previous studies, we have described the folding of a model 16-residue β-hairpin system (KKYTVSINGKKITVSI) that is ~50% populated in aqueous solution.[12] We have replaced a Lys-Ser cross-strand pair in the β-hairpin sequence (underlined) with a His-His pair to facilitate cross-strand metal binding initially through a His_2-Zn^{2+} co-ordination site (peptide **His_2**-β; residues Lys2 to Ile17 in Fig. 1(a)). In a second peptide, we have extended the N-terminal sequence by an additional overhanging His residue to generate a potential His_3-Zn^{2+} co-ordination site (peptide **His_3**-β). We envisaged that the greater conformational flexibility of the terminal His (which is not part of the β-sheet), should greatly facilitate Zn^{2+} co-ordination, with the final tetrahedral site occupied by either the C-terminal carboxylate group or a solvent molecule [Fig. 1(b)] in an analogous fashion to the carbonic anhydrase catalytic site.

NMR analysis of Hα chemical shift deviations from random coil values[13] provides a useful handle on the extent of folding prior to metal complexation, showing that the two β-hairpins (**His_2**-β and **His_3**-β) are similarly populated in aqueous solution at pH 7.0 and that the additional N-terminal His residue has little effect on β-hairpin folding. A number of cross-strand Hα↔Hα NOEs, in particular between Ser 7↔Lys12 and Thr5↔Thr14, together with long range NH↔NH (Val6↔Ile13) and NH↔Hα NOEs (Ser7↔Ile13) establish that the peptides fold with the proposed strand alignment shown in Fig. 1(a), with stabilising interactions between side chains consistent with this fold (for example Tyr4↔Val15). The similarity of the Hα shifts for His3 and His16 precludes the observation of an Hα↔Hα NOE between these residues, but Hα chemical shift perturbations are consistent with cross-strand interactions close to the N- and C-termini. Calculation of His pK_a values from pH titration analysis of His Hε chemical shifts, shows them to fall in the range 5.73 to 6.36 suggesting that metal complexation will be optimum around pH 7 where all imidazole rings are unprotonated. The pH-dependent stability profile determined from changes in CD ellipticity and Hα chemical shifts shows that the population of folded hairpin decreases below pH 5.5, consistent primarily with the formation of a $HisH^+$-$HisH^+$ pair between opposing β-strands that has a significant destabilising effect on β-hairpin structure. The difference in Hα chemical shift deviations from random coil values at pH 3.5 and 7.2 are shown in Fig. 2 for **His_2**-β. Interestingly, all shifts are perturbed at low pH suggesting that an electrostatic repulsion between $HisH^+$ residues (His3 and His16) close to the N- and C-termini, as well as protonation of the C-terminal carboxylate group, has a co-operative destabilising effect on the hairpin that is sensed by all residues, including those in the distant NG turn sequence. Similar effects are observed for both peptides **His_2**-β and **His_3**-β. Thus, the data indicate that at pH 7 the two peptides are already populating a partially pre-organised β-hairpin conformation.[12]

The uncomplexed peptide shows a very weak band at 216 nm in the CD spectrum due to β-sheet and β-turn conformation in equilibrium with random coil, the latter resulting in a strong negative ellipticity at 200 nm (Fig. 3).[14] CD data are also shown

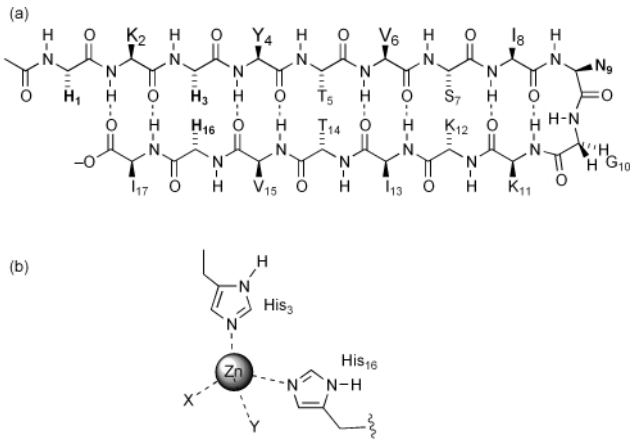

Fig. 1 (a) Schematic representation of peptide **His_3**-β (1–17) showing the peptide main chain alignment; residues are identified using the single letter amino acid code. **His_2**-β (2–17) lacks the N-terminal His (Lys2-*N*-acetylated), the same numbering scheme is used for each peptide; (b) proposed zinc co-ordination site; for **His_3**-β X = His1, Y = H_2O or the C-terminal carboxylate group of Ile17, for **His_2**-β X = H_2O, Y = H_2O or CO_2^-.

 DOI: 10.1039/b100702p

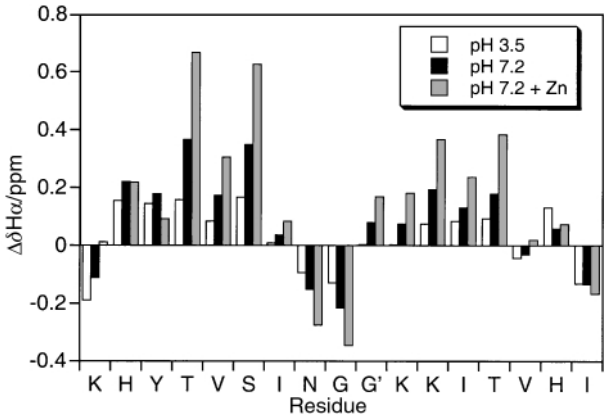

Fig. 2 Deviations of Hα chemical shifts from random coil values ($\Delta\delta$Hα, ppm) for **His$_2$**-β in water at pH 3.5 and 7.2 (298 K), and in aqueous solution (pH 7.2) and 1 equiv. of $ZnCl_2$. All $\Delta\delta$Hα values were determined after full assignment of NOESY and TOCSY data collected at 500 MHz.

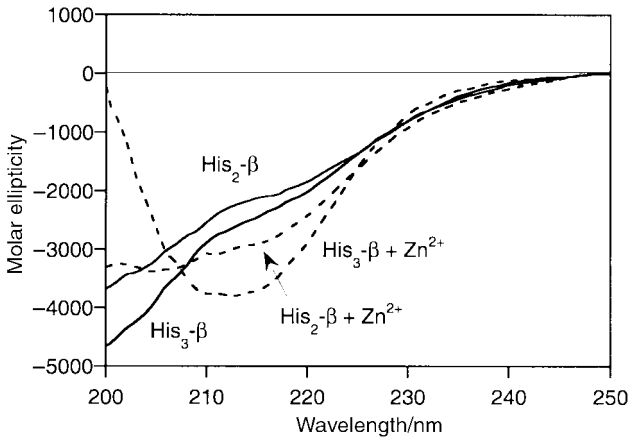

Fig. 3 Far UV-CD spectra of **His$_2$**-β and **His$_3$**-β in aqueous solution (pH 7.2) in the absence of zinc and in the presence of 10 equiv. of $ZnCl_2$. All spectra were recorded on an Aviv 62DS spectrophotometer at 293 K, as previously described.[12]

for the two peptides in the presence of excess $ZnCl_2$ at pH 7.0. In both cases the negative ellipticity at 216 nm becomes more pronounced, however, the effect is greatest for **His$_3$**-β, with the ellipticity also approaching zero at 200 nm. The data imply that Zn^{2+} chelation with **His$_3$**-β induces a greater degree of β-sheet secondary structure.

Significant perturbations to Hα chemical shifts are also consistent with further induction of β-sheet secondary structure by metal binding. In particular, Thr5 and Ser7 are shifted further downfield by > 0.2 ppm ($\Delta\delta$Hα values of 0.67 and 0.63 ppm, respectively for **His$_2$**-β; see Fig. 2); secondly, His Hε resonances are seen to broaden considerably, consistent with metal complexation. The effects of zinc titration on the folding of peptide **His$_3$**-β are equally pronounced but more significant at low concentrations of the metal ion. The Gly residue in the turn sequence has previously proved to be a good indicator of the extent of folding, since the two Hα resonances experience a large difference in chemical shift in the folded state due to the anisotropic effects of neighbouring carbonyl groups.[15] In this case the Gly residue in the turn is well removed from the direct effects of zinc binding. In the presence of Zn^{2+} the Gly Hα splitting for **His$_3$**-β is much larger than for the free peptide (300 *versus* 141 Hz), indicating that chelation of zinc by residues close to the peptide N- and C-termini co-operatively stabilises β-sheet secondary structure along the full length of the hairpin. **His$_2$**-β achieves the same magnitude of Gly Hα splitting but only at much higher metal ion concentrations (>10 equiv.). Using the quantitative methods previously described to estimate hairpin stability from the Gly Hα splitting,[15] we conclude that zinc binding enhances the stability of each hairpin by up to 3 kJ mol^{-1} at 298 K, reflecting the net balance between favourable electrostatic interactions with the metal and opposing entropic effects of organising the peptide backbone and His side chains. Although we cannot state unambiguously that all three His residues of **His$_3$**-β are simultaneously involved in complexation, the observed differences between the two peptides suggest that the overhanging His residues plays some role (dynamic or otherwise) in metal complexation.

In native proteins where Zn^{2+} is used solely in a structural capacity, His_3-Zn^{2+} is less common in the stabilisation of β-sheet structure than alternative His_2-Cys_2 or His-Cys_3 motifs. A recent report of a redesigned zinc-finger motif has shown that His_4-Zn^{2+} co-ordination can be accommodated with two His residues located in the β-sheet,[16] although structural analysis suggests that the short β-hairpin component (only three residues per strand) is distorted by the tetrahedral co-ordination geometry around the Zn^{2+} ion. Here we have used a significantly larger element of β-sheet secondary structure than previously reported with the metal co-ordination site incorporated close to the N- and C-termini to minimise distortions. Our preliminary data from NMR and CD indicate that the integrity of the β-sheet is enhanced although tetrahedral co-ordination around zinc must produce some local distortion.

We thank the EPSRC and Glaxo-SmithKline for financial support through a CASE collaboration, and John Keyte in the School of Biomedical Sciences for peptide synthesis. We are grateful to S. R. Griffiths-Jones and M. Jourdan for helpful discussions.

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