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NMR Studies on Ni(II) Induced Cyclization of a Histidine-Tagged Peptide

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Abstract: A linear decapeptide, HGASYQDLGH, was synthesized and used as a model to evaluate the effect of nickel addition upon non-covalent backbone cyclization. The NMR data, obtained for the peptide in the presence of the metal ion, support the existence of predominant folded structures in solution, where the two His residues are maintained close to each other. These results suggest that insertion of even a single His residue at each peptide terminus can be used efficiently to reduce peptide flexibility without any backbone modification.

Keywords: nickel complex; NMR; peptide folding; peptide-metal complex; solution structure

INTRODUCTION

Synthetic peptides are in principle good candidates for the development of new drugs and diagnostic tools, because of their favourable chemical and physical properties such as low toxicity and easy handling and storage [1]. Methods for their large-scale synthesis are well known and several steps are fully automated [2]. In addition, it has been widely reported that short fragments are in some cases able to maintain the biological properties of the whole cognate protein with high sensitivity and specificity [3–7].

Unfortunately, the extensive conformational flexibility that these molecules exhibit in solution represents a major problem in designing efficient protein-mimicking linear peptides. This intrinsic flexibility can be modulated by changing solution properties such as ionic strength, viscosity, pH and polarity of the solvent, but the conformational bias obtained via these changes is usually small. A design of predetermined conformations can also be pursued by modifying the peptide covalent framework with cystine bridge formation in cyclic analogues or with the insertion in the sequence of templates or modified amino acids [8]. However, some of conformational preferences induced by cyclization or by insertion of non-protein residues are often too drastic and prevent a proper induced fit of the peptide on the receptor. It would be desirable to induce only a mild propensity in peptides to adopt a folded conformation, notably a pseudocyclic one in which the two termini occupy close spatial positions, while retaining a general ability to assume multiple conformations.

The fact that linear peptides exhibit, at the same time, low folding propensities and a high capability of metal ion chelation [9] makes alternative approaches possible. The affinity of His residues

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for Ni(II) is well known [10] and documented by the widespread use in protein purification of His tags composed of six His residues placed at either terminus of a protein sequence. We have recently shown that a mimic of classical His tags, obtained by placing three His residues at each end of a peptide with low propensity to assume independent secondary structure organization, can indeed induce ordered structure [11]. Several structural studies are available, showing that model peptides reproducing Ni(II)–protein coordination sites dramatically change their conformation upon metal ion complexation [12–14]. It is tempting to check whether even simply flanking a given peptide sequence with a single His residue at each terminus could be sufficient to induce an ordered secondary structure. This type of Ni(II)-histidine affinity is investigated here on the linear decapeptide HGASYQDLGH (henceforth called HPH).

MATERIALS AND METHODS

Amino acid derivatives and the resin for the peptide synthesis were obtained from Novabiochem (Laufelfingen, Switzerland) and all the other reagents and solvents were purchased from Applied Biosystems Italia (Monza, Italy), Sigma Aldrich Italia (Milano, Italy), Pierce Science France Europe (Bezons, France) and Fluka (Buchs, Switzerland). Solid-phase peptide synthesis was carried out on a model 350 multiple peptide synthesizer (Syro, MultiSynTech, Bochum, Germany) using standard reaction cycles with Fmoc (9-fluorenylmethoxycarbonyl) chemistry and Dic (1,3-diisopropylcarbodiimide) HoBt (1-hydroxybenzotriazole) activation [15]. The amino acid side-chain protecting groups used were: test-butyl (tBu) group for Ser and Tyr, trityl (Trt) for Gln and His, *tert*-butyl (otBu) for Asp. The Fmoc group was cleaved with a 40% (v/v) piperidine solution in *N,N*-dimethylformamide (DMF). HPH was cleaved from the resin and deblocked using a cocktail consisting of 88% (v/v) trifluoracetic acid (TFA), 6% phenol, 2% triisopropylsilane and 4% water, then purified by gradient reversed-phase high pressure liquid chromatography (RP-HPLC) on a Vydac semi-prep C_{18} column (1.0×25cm). The mobile phase solvents were water containing 0.1% (v/v) TFA and methanol. A linear gradient from 0% to 100% of methanol in 30 min was applied. The yield measured by HPLC analysis of the crude peptide was 80%. HPH identity was confirmed by amino acid analysis using the PICO TAG model 510 analyser (Waters, Milford, MA): H(2) 2.02, G(2) 2.11, A(1) 0.96, S(1) 0.85, Y(1) 1.15, Q(1) 1.00, D(1) 1.02, L(1) 0.95.

HPH, lyophilized from a pH 5.6 aqueous solution, was dissolved in 0.5 ml of DMSO-d_6 to yield a 4 mM solution. Proton chemical shifts were referred to the solvent isotopic impurity (2.5 ppm). Additions of Ni(II) were carried out by mixing variable volumes of a 1 M solution of $NiCl_2$ in DMSO-d_6 to the peptide samples up to a molar peptide:nickel ratio of 1 : 5.

The structural investigation of the peptide was performed by 1D and 2D COSY, TOCSY and NOESY 1H experiments. All NMR spectra were acquired at 300 K using a Bruker DRX 600 spectrometer equipped with a SGI workstation. All data were processed and analysed with the SwaNMR software [16], version 3.4.815. A 1D proton NMR spectrum was recorded using a spectral width of 6001.22 Hz, corresponding to 10 ppm and 32 K data points. The TOCSY experiment was employed to assign the resonances within each spin system. On the same sample a NOESY spectrum was acquired with a mixing time of 120 ms, to detect NOE connectivities suitable for sequence specific assignment and structure determination.

2D COSY spectra, obtained in the range of 293–318 K with 5 K increments, were used to calculate the temperature coefficients of amide protons of HPH in the presence of 20 mM Ni(II). In all 2D spectra a total of 512 blocks was collected in t_1 with 1024 data points and 64 scans in t_2, over a spectral width of 7 kHz. A 90-degree shifted sinebell function was applied in the t_2 dimension. The same function was imposed in t_1 on 512 points with a shift of 90 degrees. Zero filling was applied before the 2D Fourier transform to end up with a final matrix size of 2048×2048 real points. Distance restraints for structural calculation were derived from NOE intensities referring the intra-residue H_β-H_β correlation of the Asp residue to the geminal inter-proton distance of 1.8 Å.

Ten different initial random structures were generated for restrained torsional angle dynamics calculations by using DYANA 1.5 distance geometry software [17] running on a Silicon Grafics workstation R4400. The imposition of the experimental constraints yielded 10 sets of structural results, whose lowest energy 100 conformers were analysed. Each conformer was refined by 10 000 cycles of energy minimization with AMBER force field [18] following the conjugate gradient algorithm. For each set of calculations, a final mean solution structure was

produced from a group of 50 refined conformers, none of which had NOE violations higher than 0.5 Å. The obtained 10 mean structures converge so much that no cluster analysis was performed. All displayed structures were generated with the program MOLMOL 2.5.1 software [19]. The same software was used for accessible surface area calculations.

RESULTS AND DISCUSSION

The sequence of HPH was selected according to secondary structure predictions, performed on a remote server [20], such that the peptide does not exhibit any specific folding trend. Furthermore, a search for homologies of HPH against the protein sequence data base SWISSPROT [21] did not give any positive result, indicating that no specific function is associated with the peptide.

Thus, HPH can be considered as a model peptide to test if and how His tags can activate Ni(II)-induced backbone folding. DMSO was chosen as the solvent, since it could dissolve equally well both the uncomplexed peptide and its Ni(II) adduct. In addition, unlike alcohol mixtures that are often used for conformational studies of small peptides, DMSO has no intrinsic propensity to induce any specific conformation.

Even a simple visual inspection during the NMR sample preparation yielded preliminary information on the complex formation between Ni(II) ions and the peptide. Indeed, the presence of the metal ion gives the DMSO solution a pale green colour, which can be attributed to the presence of residual water molecules in the organic solvent, coordinating Ni(II) in an octahedral complex. Addition of nickel to the sample of HPH in the DMSO solution induced a transition from a colourless to yellow coloured solution. This change could be ascribed to the formation of a planar complex of Ni(II) with the peptide, as suggested by Gasmi *et al.* for a related Ni(II)–peptide complex [13].

NMR spectra of HPH were recorded in DMSO-d_6, in the presence and absence of Ni(II) ions. Proton chemical shifts are reported in Table 1.

Proton NOESY spectra, shown in Figure 1, were recorded for HPH at Ni(II) molar ratios of 0, 1, 2 and 5. Structurally relevant medium- and long-range NOEs could be observed only in the presence of the metal ion and the NOESY cross-peak

Table 1 ^{1}H Chemical Shifts (ppm) of HGASYQDLGH Measured at 600 MHz in DMSO and at 300 K in the Absence (in Parentheses) and in the Presence of a Fivefold Excess of Nickel(II)

Residue	NH	αH	βH	Others	NHΔδ/ΔT(*ppb*)
H1	8.47	4.18	3.19	εH 7.48 δH 8.95	
	(8.47)	(4.16)	(3.16)	(εH 7.40 δH 8.96)	
G2	8.78	3.81, 3.9			4.7
	(8.88)	(3.69, 3.81)			
A3	8.36	4.38	1.18		4.2
	(8.28)	(4.28)	(1.09)		
S4	8.05	4.26	3.45, 3.53		6.3
	(8.05)	(4.14)	(3.44, 3.53)		
Y5	7.85	4.43	2.69, 2.92	2.6H 6.98 3.5H 6.61	2.7
	(7.81)	(4.29)	(2.61, 2.89)	(2.6H 6.88 3.5H 6.52)	
Q6	8.14	4.22	1.72, 1.85	γCH 2.08, 2.08	6.0
				εNH2 6.79, 7.23	
	(8.13)	(4.10)	(1.64, 1.76)	(γCH 2.06, 2.06)	
				(εNH2 6.71, 7.18)	
D7	8.27	4.56	2.52, 2.74		6.7
	(8.29)	(4.44)	(2.44, 2.63)		
L8	7.88	4.22	1.45, 1.60	γH 1.45 δCH3 0.81, 0.85	5.7
	(7.81)	(4.10)	(1.36, 1.49)	(γH 1.36 δCH3 0.69, 0.74)	
G9	8.18	3.65, 3.72			5.3
	(8.18)	(3.54, 3.62)			
H10	8.04	4.48	2.92, 3.14	εH 7.30 δH 8.95	
	(8.05)	(4.38)	(2.86, 3.06)	(εH 7.16 δH 8.87)	

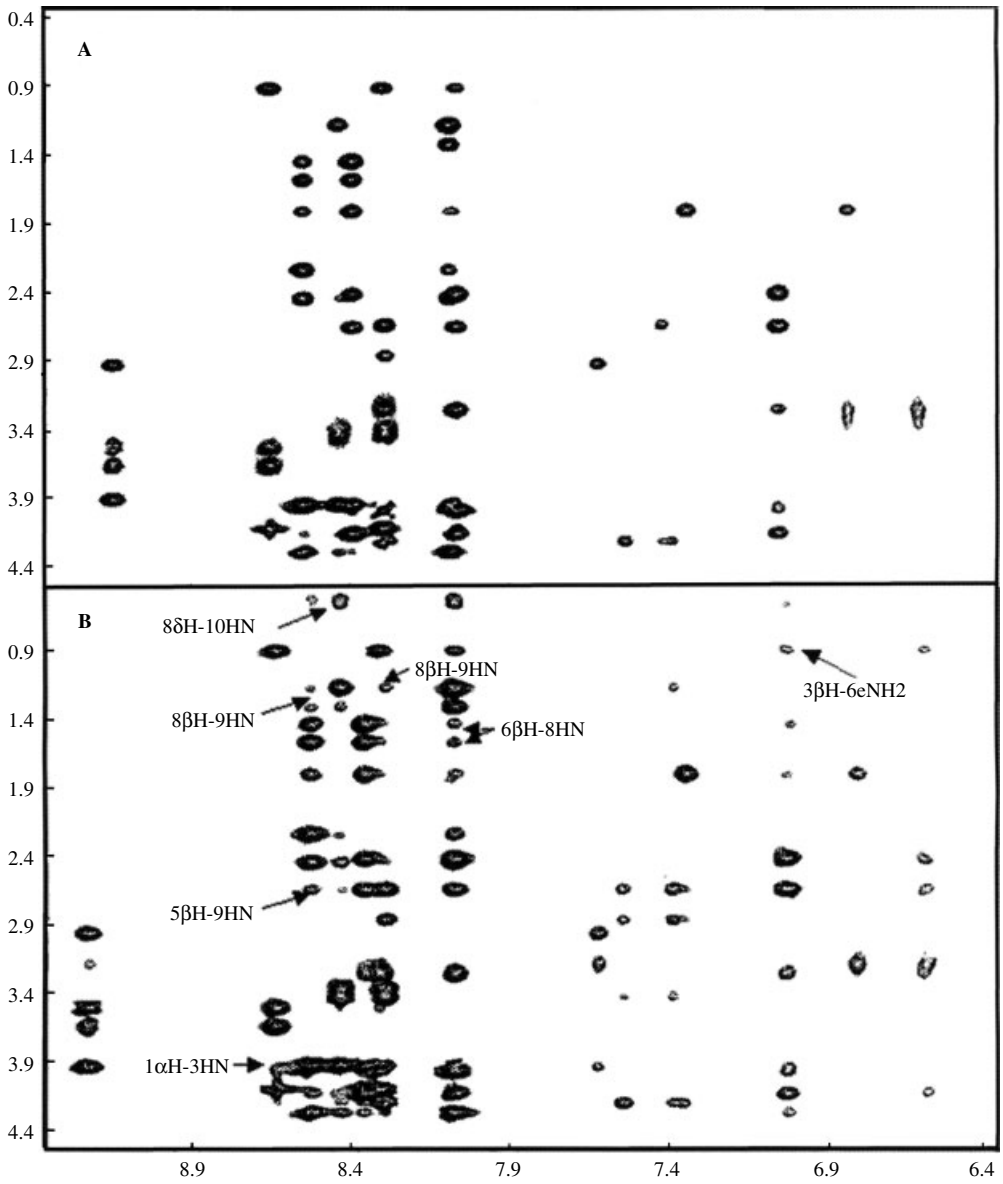

Figure 1 Comparison of NOESY spectra of HPH recorded in the absence (A) and in the presence of Ni(II) in a fivefold excess (B). Some of the structurally relevant NOEs, detectable only upon the addition of the metal ion, are highlighted.

intensities reach a maximum at the highest metal ion concentration. Accordingly, all the NOE data discussed below, shown in Figure 2, were collected in the presence of 20 mM Ni(II). It should be noted that these findings imply a rather weak Ni(II) complexation of the peptide in DMSO solution. In any case, the *ex novo* appearance of NOEs upon addition of the metal ion is diagnostic of a Ni(II)-induced peptide folding.

The structural dynamics suggested above are supported also by the observation that temperature coefficients of amide proton chemical shifts, measured in the presence of the metal ion (Table 1), do not suggest a unique hydrogen-bonding pattern, consistent with a single and stable peptide conformation, whilst addition of Ni(II) induces a set of new HPH NOEs, as illustrated in Figure 2.

Hence, a panel of 176 NOEs, derived from the analysis of the NOESY spectra obtained for HPH in the presence of the metal ion, were used as distance constraints in a molecular dynamics calculation to elucidate the structure of the peptide. It is important to note that, among the detected NOEs, 57 were (i,

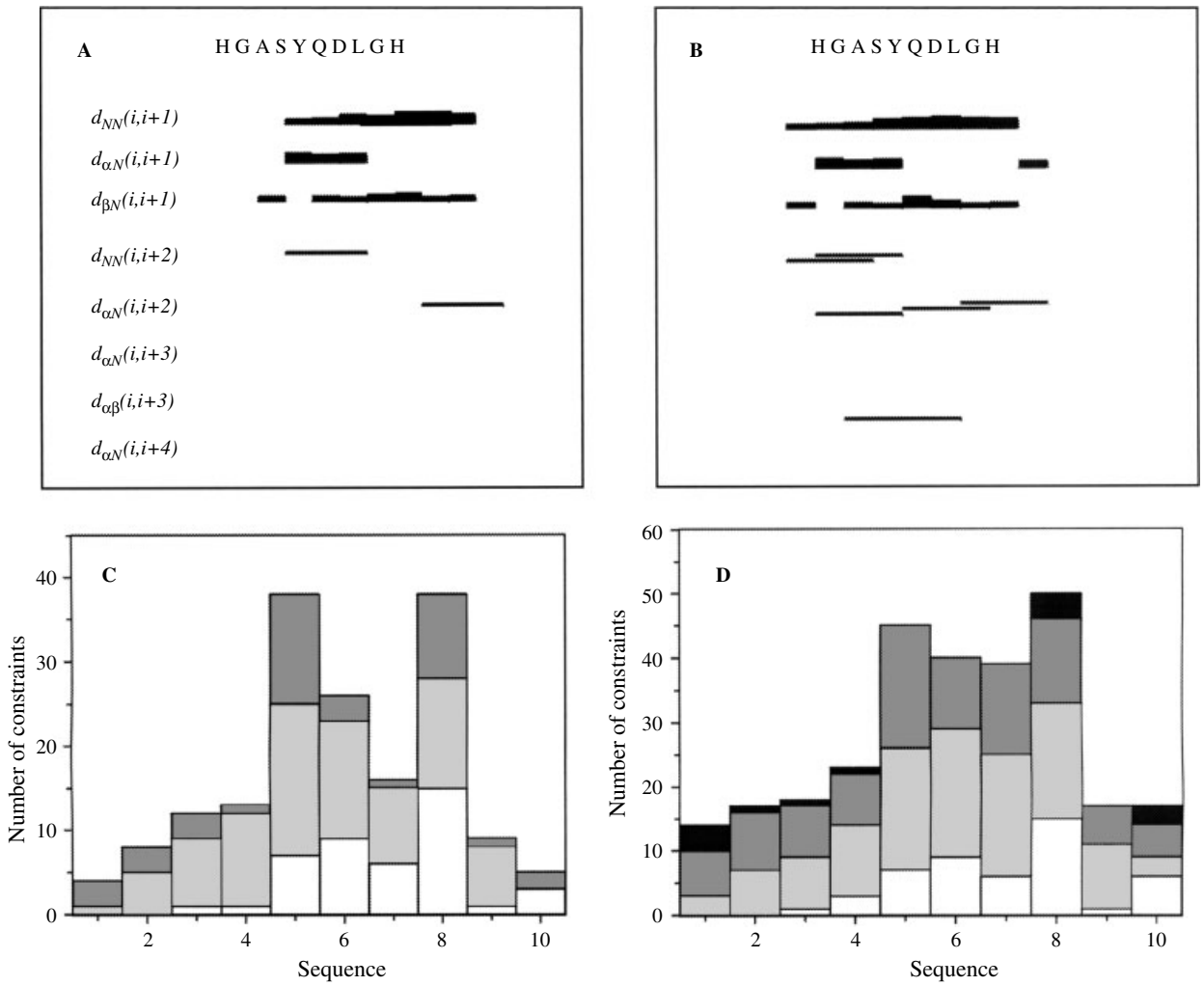

Figure 2 Comparison of panels of NOE connectivities diagnostic for secondary structure observed for HPH in DMSO-d_6 solution (A) in the absence and (B) in the presence of Ni(II) in a fivefold excess. Intensities are reflected by line thickness. The number of intra-residue (white), short (grey), medium (dark grey) and long-range (black) NOEs are also given for each residue (C) in the absence and (D) in the presence of a fivefold excess of Ni(II).

i + 1), 44 (i, i + 2; i, i + 3) and 2 (i, i + 4), a remarkably high number for such a small linear peptide.

In order to avoid artifacts in the restrained torsion angle dynamics calculations, all the intra-residue and (i, i + 1) NOEs have not been considered. Furthermore, 10 different initial random structures were used and a total of 500 low-energy conformers, 50 from each set of calculations, were analysed.

None of these structures had distance violations greater than 0.78. The relatively low number of NOE restraints is not consistent with a single ordered structure but, at low resolution, it can be said that all NMR data hint to a consensus backbone structure consistent with complexation of Ni(II) at the two terminal His residues. The distribution of the ϕ, ψ angles in the Ramachandran plot for all the 500 analysed conformations indicates their reliability (Table 2). The variability of the torsion angles calculated for the HPH residues also indicates that additional flexibility is present not only, as expected, for the external G2 and A3 residues, but also for the inner Q6 and D7 residues. This feature is consistent with the presence of a multiple conformational exchange along all the peptide backbone.

The virtual absence of diagnostic NOEs at both amino and carboxyl termini, due to the less crowded proton environment in addition to some local flexibility, prevents a detailed structural analysis of the geometry of the Ni(II) complex.

Table 2 Averaged Values of ϕ and ψ Dihedral Angles of HPH Decapeptide Obtained for the 50 Lowest Energy Conformers of Each of the 10 Calculations. Each Residue Position in the Ramachandran Plot (Rpp) is Reported as Most Favoured Region (MFR), Additional Allowed Region (AAR) and Generously Allowed Region (GAR)

Residue	ψ	ϕ	Rpp
H1	-118.6 ± 37.4	—	—
G2	-38.9 ± 24.5	42.6 ± 48.1	—
A3	50.1 ± 5.8	-133.5 ± 22.1	AAR
S4	-80.8 ± 4.6	171.9 ± 6.6	GAR
Y5	-8.3 ± 3.3	-76.6 ± 6.2	MFR
Q6	49.8 ± 15.0	-53.5 ± 4.1	AAR
D7	-25.0 ± 1.1	94.2 ± 13.4	GAR
L8	21.1 ± 8.8	-80.4 ± 2.6	MFR
G9	-2.0 ± 8.2	148.8 ± 7.9	—
H10	—	5.1 ± 9.2	—

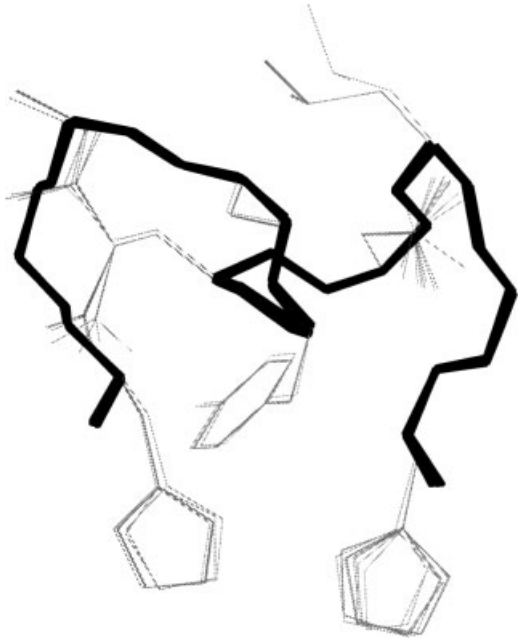

Figure 3 Overlay of the lowest energy conformers obtained for each of the 10 independent structure calculations performed for HPH in the presence of Ni(II).

The lowest energy structures of each independent calculation are shown in Figure 3. The close proximity of the two His side chain is apparent. The Y5 side chain constantly points towards a position which could be occupied by the metal ion, while the Q6 and D7 side chains, possibly involved in the complex formation, are in opposite directions. In the large assembly of HPH conformers, which has been analysed here, a hydrogen bond between D7 amide proton and Y5 carbonyl group was found 431 times. An H1 carbonyl oxygen is bound to Q6 or A3 amide protons, respectively, in 39% and 61% conformers of a total of 468 cases where this His hydrogen bond is present. In 86 conformers an additional hydrogen bond between G2 amide proton and the carbonyl of the Glu side chain is found. These results, analysed in the light of the observed temperature dependence of amide proton chemical shifts, $\Delta\delta/\Delta T$, suggest that the life-time of these hydrogen bonds has to be short enough to yield average values. Furthermore, the small $\Delta\delta/\Delta T$ observed for Y5 amide proton (Table 1) is consistent with the high solvent shielding that this proton experiences in all the 500 conformers with an accessible surface area, ASA, always lower than 1.6 $Å^2$. This exposure corresponds to 5.9% of the amount that can be calculated for a totally exposed Y amide. The average ASA value, calculated for the latter proton in all the conformers, is 0.9%, indicating that structures with a folded backbone are predominant in the conformational equilibrium. The presence of two γ-turns in the conformational equilibrium, involving residues 1–3 and 5–7, can be inferred by the ϕ and ψ torsion angles of residues G2 and Q6, consistently with the H1 $\leftarrow$ A3 and Y5 $\leftarrow$ D7 hydrogen-bonding pattern observed in the calculated conformers [23].

CONCLUSIONS

The observation that under our experimental conditions a diamagnetic complex is obtained from the linear decapeptide and Ni(II) ions indicates the low-spin character of Ni(II) which adopts a square planar geometry [14,22]. As suggested by Figure 3, the His and Tyr side chains are most likely involved in the Ni(II) complexation, leaving the fourth position free for a solvent molecule.

Our data can be summarized in the following way: (i) extensive conformational equilibrium may be suggested for HGASYQDLGH in the absence of the metal ion, as largely predictable for linear peptides of this length and sequence; (ii) the possibility of a stable ion complex with the Asp side chain, as well as with the backbone carboxyl group, which have been demonstrated to be potential donors in Ni(II)–peptide interaction studies [11,22], can be ruled out for this peptide; (iii) the conformation of the His-tagged peptide is dramatically influenced by the formation of the Ni(II) complex with the

Table 3 Residue by Residue Comparison of Secondary Structures Calculated for the Lowest Energy Conformers of HPH and H_3PH_3 Peptides

HPH	H_3PH_3
	H1 coil
	H2 coil
H1 coil	H3 coil
G2 coil	G4 bend
A3 bend	A5 bend
S4 bend	S6 bend
Y5 bend	Y7 bend
N6 bend	N8 bend
D7 bend	D9 bend
L8 bend	L10 bend
G9 coil	G11 helix
H10 coil	H12 helix
	H13 helix
	H14 coil

onset of backbone folding, even though the usually observed strong Ni(II)/imidazole interaction is partially quenched by unfavourable entropic effects.

A comparison of the lowest energy structures of HPH with those obtained for a similar peptide having three His residues at each terminus [11], H_3PH_3, indicates that different conformations are reached by the inner sequence ASYQDL in the two molecules (Table 3). A folding motif is centred in the middle of HPH, while a more complex sequence of secondary structure elements is observed for H_3PH_3.

The present data suggest that Ni(II) chelation can be used to mildly constrain flexible peptides, thereby enhancing biological properties such as immunochemical reactivity, particularly useful for the development of diagnostic tools. Furthermore, the insertion of only one His residue at each end of short bioactive peptides seems to cause a tighter and, hence, more predictable folding.

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