Thermodynamic and ^{1}H NMR Study of Proton Complex Formation of Histidine-containing Cyclodipeptides in Aqueous Solution

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A thermodynamic and ^{1}H NMR study of proton complex formation in aqueous solution of some L-histidine-containing cyclic L-dipeptides has been carried out. The enthalpic and entropic changes associated with protonation of the cyclodipeptides, obtained by potentiometric and calorimetric measurements, together with the ^{1}H NMR data and NOESY experiments, enable the role played by non-covalent interactions in proton complex formation to be assessed. In addition, a comparison with c(Glys–His) permits the influence of side-chain residues on the conformation of protonated species to be observed.

Weak chemical interactions such as hydrophobic, stacking and electrostatic forces are thought to be responsible for high specificity in interactions between macromolecules in biological systems.[1,2] These weak, non-covalent interactions explain both the origin of the folding tendency of groups (aryl–aryl, alkyl–aryl and alkyl–alkyl) in synthetic organic molecules[4–5] and the preferential disposition of nucleic bases in simple models of biological molecules.[6]

In recent years, cyclic dipeptides (3,6-disubstituted piperazine-2,5-diones) have drawn increasing attention as useful models of more complex biomolecules.[7–9]

The most interesting NMR results[10–15] and X-ray structural analyses[16,17] show that molecules containing aromatic side-chains (such as phenylalanyl, tyrosyl and histidyl residues) prefer a folded conformation with the aromatic ring facing the diketopiperazine ring which can adopt different conformations such as planar, chair and boat. Several hypotheses based on the above cited experimental results and potential-energy calculations[18] have been put forward in order to explain the interaction between diketopiperazine and the aromatic rings responsible for the stabilization of the folded conformation (π–π donor–acceptor type, intramolecular dipole-induced dipole, dispersion forces).

Studies of cyclodipeptides with two aromatic or pseudo-aromatic side-chains in solution indicate that the aromatic rings occupy 'face-to-face' the space over a planar diketopiperazine ring.[14] This conformation should permit both the interactions between the two aromatic rings and those between each side-chain residue and the diketopiperazine ring.[19,20] Furthermore, investigations of the folded–unfolded equilibria by means of NMR measurements show that the populations of preferred conformations decrease with increasing temperature.[10]

The folded form is favoured over other possible conformations of the side-chain residue by an enthalpy contribution averaging -3 kcal mol^{-1};* the pertinent entropy change is 3–4 cal mol^{-1} K^{-1}, unfavourable for the folded form.[10] On the basis of these results, it was reported that 'the folding of the aromatic ring against the diketopiperazine ring in these cyclic peptides is not the result of a hydrophobic or solvophobic interaction, if by this is meant an entropy-driven association of the two ring moieties resulting in the release of bound or ordered solvent molecules'.[10]

Thermodynamic data obtained by means of direct calorimetry show that solvophobic interactions,[21] weak non-covalent forces due to the stacking between aromatic or pseudoaromatic residues, or to the interaction of alkyl–aryl or alkyl–alkyl groups, are enthalpy driven.[22–24] In previous studies, we have interepreted the thermodynamic parameters of the proton complex formation of some cyclic L-dipeptides containing L-histidine, hypothesizing an equilibrium between the folded unprotonated and the unfolded protonated imidazole forms.[25,26] In addition, the comparison with the behaviour of cyclo-glycyl-L-histidyl c(Gly–His) showed the effects of side-chain groups in protonation. All our suggestions were based on the experimental results of Kopple [pertinent to c(Gly–His)[11]] and the theoretical calculations of Grebow [pertinent to c(Ala–His)[18]]; both suggested that the protonation of the imidazole ring hindered the interaction between the pseudoaromatic residue and the piperazinedione ring.

In order to obtain useful information on the conformational characteristics of the DKP ring (DKP = diketopiperazine), the imidazole residue and the substituted side-chain groups, we carried out a ^{1}H NMR investigation on the species which exist at different pHs of (cyclo-glycyl-L-histidyl, cyclo-L-alanyl-L-histidyl, cyclo-L-valyl-L-histidyl, cyclo-L-phenylalanyl-L-histidyl and cyclo-L-histidyl-L-histidyl) and correlated these results with the thermodynamic parameters previously determined.[26] Furthermore, the ΔG°, ΔH° and ΔS° values for protonation of cyclo-L-valyl-L-histidyl, cyclo-L-leucyl-L-histidyl and cyclo-L-prolyl-L-histidyl, here reported, provide a better understanding of the role of the side-chain in proton complex formation.

Experimental

Synthesis of Cyclic Dipeptides.—*N*-Benzyloxycarbonyl α-amino acids and L-histidine methyl esters were active-ester condensed to obtain linear dipeptides blocked at both terminals. The benzyloxycarbonyl group was removed by catalytic hydrogenation. The cyclization was carried out by heating under reflux in methanol. All the cyclic dipeptides were purified on a silica gel chromatographic column using $CHCl_3$–CH_3OH–H_2O (65:25:4) or $CHCl_3$–CH_3OH (5:1) as the eluent, and characterized by mass and NMR spectra.

Cyclo-(glycyl-L-histidyl), c(Gly–His). Triethylamine (100 mmol) was added to a stirred suspension of L-histidine methyl ester dihydrochloride (50 mmol) in dry chloroform (25 cm^3). The benzyloxycarbonylglycyl-4-nitrophenyl ester (46 mmol), obtained from benzyloxycarbonylglycine and 4-nitrophenol in

* 1 cal = 4.184 J.

the presence of dicyclohexylcarbodiimide, was added in several portions and the mixture was stirred at room temperature overnight. The reaction mixture was extracted with water (three times) then with aqueous ammonia (0.5 mol dm^{-3}) and finally with water until the rinse-water was practically neutral. The organic phase was dried over anhydrous Na_2SO_4, concentrated *in vacuo* and crystallized from ethyl acetate. The benzyloxycarbonyl-Gly-HisOMe (35 mmol) obtained was dissolved in absolute ethanol (40 cm^3) and hydrogenolysed over 10% palladium-on-charcoal catalyst (1.2 g) with a slow stream of hydrogen until CO_2 generation ceased. The catalyst was removed by filtration and the filtrate concentrated *in vacuo*. The N-deprotected dipeptide was then heated under reflux in anhydrous methanol (18 h) and the desired c(Gly–His) precipitated on cooling. Crystallization of the product from water–acetone gave 3 g (yield 33.6%) of colourless prisms with the following physical constants: m.p. 242–243 °C (lit.,[27] 242–243 °C); $[\alpha]_D^{25}$ +63° (*c* 2.6, water) [lit.,[28] $[\alpha]_D^{23}$ +65 (*c* 2.78, water)] (Found: C, 49.4; H, 5.25; N, 28.7. Calc. for $C_8H_{10}N_4O_2$: C, 49.48; H, 5.19; N, 28.85%); *m/z* 194 (M^+).

Cyclo-(L-*alanyl*-L-*histidyl*), *c*(*Ala–His*). c(Ala–His) (yield 34%) was obtained with the following physical constants: m.p. 252–253 °C (lit.,[29] 252–254 °C); $[\alpha]_D^{25}$ −10 (*c* 1, water) (Found: C, 51.8; H, 5.75; N, 26.95. Calc. for $C_9H_{12}N_4O_2$: C, 51.92; H, 5.81; N, 26.91%); *m/z* 208 (M^+).

Cyclo-(L-*valyl*-L-*histidyl*), *c*(*Val–His*). c(val–His) (yield 47%) was obtained with the following physical constants: m.p. 245–248 °C (lit.,[29] 220–224 °C); $[\alpha]_D^{25}$ −63 (*c* 1, water) (Found: C, 55.7; H, 6.8; N, 23.8. Calc. for $C_{11}H_{16}N_4O_3$: C, 55.92; H, 6.83; N, 23.71%); *m/z* 236 (M^+).

Cyclo-(L-*leucyl*-L-*histidyl*), *c*(*Leu–His*). c(Leu–His) (yield 63%) was obtained as a monohydrated compound with the following physical constants: m.p. 205–206 °C (lit.,[28] 204–206 °C); $[\alpha]_D^{25}$ −16.9 (*c* 1, water) [lit.,[28] $[\alpha]_D^{23}$ −17.2 (*c* 1.16, water)] (Found: C, 53.6; H, 7.5; N, 20.1. Calc. for $C_{12}H_{18}N_4O_2 \cdot H_2O$: C, 53.7; H, 7.51; N, 20.88%); *m/z* 250 (M^+).

Cyclo-(L-*prolyl*-L-*histidyl*), *c*(*Pro–His*). c(Pro–His) (yield 29%) was obtained as a monohydrate compound with the following physical constants: m.p. 167–169 °C (lit.,[30] 162–165 °C); $[\alpha]_D^{25}$ −119.8 (*c* 1, methanol) [lit.,[30] $[\alpha]_D^{25}$ −119.1 (*c* 1, methanol)] (Found: C, 53.0; H, 6.4; N, 22.9. Calc. for $C_{11}H_{14}N_4O_2 \cdot H_2O$: C, 52.37; H, 6.39; N, 22.20%); *m/z* 234 (M^+).

Cyclo-(L-*phenylalanyl*-L-*histidyl*), *c*(*Phe–His*). c(Phe–His) (yield 59%) presented the following physical constants: m.p. 263–264 °C (lit.,[29] 263 °C); $[\alpha]_D^{25}$ −69 (*c* 2, glacial acetic acid) [lit.,[28] $[\alpha]_D^{23}$ −72 (*c* 2, acetic glacial acid)] (Found: C, 63.4; H, 5.7; N, 19.3. Calc. for $C_{15}H_{16}N_4O_2$: C, 63.36; H, 5.67; N, 19.70%); *m/z* 284 (M^+).

Potentiometric Measurements.—Computer-controlled potentiometric titrations were performed with two distinct Metrohm Digital pH meters (Model 654) equipped with Metrohm 109 glass and Metrohm 404 saturated calomel electrodes. The titration cell was thermostatted at 25.0 ± 0.2 °C and all solutions were kept under an atmosphere of nitrogen, which was bubbled through a solution of the same ionic strength and temperature as that of the solutions under study. The electrode couples were standardized on the pH = $-\log C_H$ scale by titrating HNO_3 (0.01–0.005 mol dm^{-3}) with standard KOH (0.1 mol dm^{-3}) at 25 °C and I = 0.1 mol dm^{-3} (KNO_3). Solution aliquots of 25 cm^3 containing suitable amounts of cyclodipeptide and HNO_3 were titrated with standard KOH solutions. The ionic strength was kept at 0.1 mol dm^{-3} by adding KNO_3. The analytical concentrations of cyclodipeptides ranged from 0.004 to 0.008 mol dm^{-3}.

Calorimetric Measurements.—The calorimetric data were obtained by titration calorimetry using a Tronac Isoperibol apparatus (Model 450) equipped with a 25 cm^3 reaction dewar vessel. The calorimetric system was calibrated by titrating THAM with HCl according to Grenthe *et al.*[31] The cyclopeptide heats of protonation were determined by titrating solutions of the ligands with standard HNO_3 (0.2–0.4 mol dm^{-3}). The peptide concentrations ranged from 0.004 to 0.008 mol dm^{-3}. The heats of reaction, corrected for the heat of dilution, determined by separate experiments, were calculated by considering the calorie unit as equivalent to 4.184 J. Other experimental details were as previously reported.[26]

^1H *NMR Measurements.*—The ^1H NMR spectra and NOESY experiments were run in D_2O at 25 °C, with a Bruker AC-250 spectrometer. For NOESY spectra the mixing time was 0.600 s. Sample concentrations were close to 5×10^{-3} mol dm^{-3}. Chemical shifts were measured relative to TMA (tetramethylammonium chloride, 3.19 ppm). The pD of the D_2O solutions was adjusted by the addition of a small amount of DCl and NaOD to obtain completely protonated and completely unprotonated forms of the imidazole group.

Calculations.—Calculations concerning the electrodic system E° values, ligand purities and protonation constants were performed by the computer program SUPERQUAD.[32] The heats of protonation were calculated by the last-squares computer program DOEC.[33] Throughout, errors are expressed as three times the standard deviation (3σ) where σ is the standard deviation between observed and calculated values of all points used to obtain the reported thermodynamic parameters.

Results and Discussion

Thermodynamic Studies.—ΔG°, ΔH° and ΔS° values for protonation of the cyclodipeptides under study are reported in Table 1, together with data concerning the other dipeptides previously studied. The value of the protonation constant of the derivative containing the leucyl residue is significantly similar to that of the alanyl and glycyl derivatives and less than that of the valyl peptide. The protonation of imidazole for the prolyl-containing cyclodipeptide shows the highest ΔG° value of all the systems examined except the histidyl-histidyl derivative. As we expected for the protonation of imidazole nitrogen,[25] the protonation is mostly favoured by the enthalpy contribution with a small favourable entropy contribution. The various enthalpy and entropy values (Table 1) require, however, more consideration to identify the effect of the various side-chain groups. It may be opportune briefly to summarize the findings for the systems previously studied[26] starting with c(Gly–His). Considering that in the initial state of this molecule (non-protonated) the imidazolyl group is located over the DKP ring, and that in the final state the imidazolyl residue (now protonated) is located relatively far from the DKP system, the ΔH° value is the algebraic sum of the protonation process (exothermic contribution) and the unfolding process (endothermic contribution). In addition a smaller contribution may be given by a possible variation in the DKP ring conformation. A favourable or unfavourable contribution depends on the greater or lesser stability of the conformation assumed by the protonated or unprotonated state. ΔS° is the sum of the unfavourable contribution from the imidazole protonation and the favourable contribution produced by the elimination of the solvophobic interaction between the DKP ring and imidazolyl chain.

The presence of a substituent opposite the imidazole could necessitate the consideration of other contributions. In particular, if, in the initial state, the substituents interact with the DKP and/or the imidazole ring, the protonation ΔH°

Table 1 Thermodynamic parameters for the protonation of the imidazole ring in some histidine-containing cyclic-dipeptides at 25 °C and I = 0.10 mol dm^{-3} (KNO_3). The values in parentheses represent three times the standard deviation.

Cyclodipeptide	$-\Delta G^\circ$/kcal mol^{-1}	$-\Delta H^\circ$/kcal mol^{-1}	ΔS°/cal mol^{-1} K^{-1}	Ref.
c(Gly–His)	8.35	6.80	5.2	*a*
c(Ala–His)	8.31	6.75	5.2	*a*
c(Val–His)	8.61(1)	7.33(9)	4.3(3)	*b*
c(Leu–His)	8.29(1)	6.65(9)	5.5(3)	*b*
c(Pro–His)	8.71(1)	6.88(9)	2.8(3)	*b*
c(Phe–His)	8.56	7.69	2.9	*a*
c(His–His)	8.90	7.31	5.4	*a*
	7.49	6.78	2.0	

[a] Ref. 26. [b] This work.

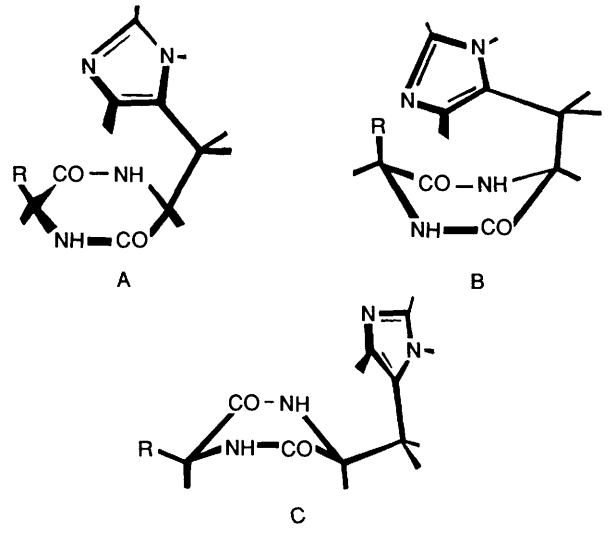


Fig. 1 Planar (A), flagpole (B) and bowsprit boat (C) conformations of the diketopiperazine ring in a cyclic dipeptide

should be influenced on one hand by an unfavourable contribution due to the breaking of the solvophobic interaction with the other substituent and, on the other hand, by a possible favourable contribution due to a greater interaction between the DKP ring and the R substituent, which is no longer sterically limited by the presence of the imidazole ring facing the DKP ring. The ΔS° contributions go in the opposite direction. This is based on the hypothesis that a rearrangement of the DKP ring occurs, for example from planar to flagpole boat (Fig. 1). Such a contribution, as reported by Kopple,[11] may seem of little importance, but could be accounted for by differing levels of interaction between the R substituent and the DKP ring. On this basis it was possible to interpret the ΔH° and ΔS° data for the valine and phenylalanine derivatives, where, in comparison to the alanine, either the bulkiest alkyl residues or the aromatic ring were able to interact significantly with the DKP ring when the imidazole was protonated. The exothermic contribution due to the interaction between the valine residue and the DKP ring proved to be greater than the endothermic contribution due to the breaking of the non-covalent interaction existing in the initial state between the same residue and the imidazole ring. Analogously, the same rationale was applied to the c(Phe–His). The highest protonation constant values for c(Val–His) and c(Phe–His) were therefore due to a more favourable enthalpy contribution generated by the interaction between the alkyl and aromatic substituents and the DKP in the final state. Obviously such an interaction is impossible in the case of the c(Gly–His) derivative and doesn't occur for the alanyl derivative because of its very short chain. The ΔH° and ΔS° values for the leucyl derivative seem to indicate no interaction between the isopropyl chain and the DKP ring, despite the dimensions of the alkyl residue. The c(Leu–His) dipeptide behaves like the alanyl residue derivative rather than one with a valine residue. This may be explained by steric factors. The protonation ΔH° of the derivative containing proline is the most exothermic of all those reported. According to the hypothesis above the proline residue ought to interact strongly with the DKP ring after protonation. However this seems very unlikely and the rationalization of these thermodynamic parameters must be considered separately from those of other cyclodipeptides.

The thermodynamic parameters for the first protonation of c(His–His) may be rationalized analogously to those for the phenylalanine derivative. As expected the second step is less exothermic and the ΔH° value is very similar to that of c(Gly–His). This may be explained since, the first imidazolyl group having already separated from the DPK ring, the protonation of the second group is no longer influenced by any eventual favourable enthalpy contributions and behaves as though there were no residue opposite.

^{1}H *NMR Investigations.*—In order to verify the hypotheses concerning the conformational preferences of unprotonated and protonated species, which represent both the initial and final states in the chemical equilibrium, ^{1}H NMR investigations were carried out at basic and acidic pH. Our first observation is that pH = 9 spectra and those corresponding to the pH of dissolution of the cyclodipeptide in water are identical. Substantial differences are observed, however, for the ^{1}H NMR data at pH = 3 (Table 2). While in all cases the pH = 3 downfield shift of the H_a and H_b imidazole protons and of the CH_{2His} and CH_{His} protons is caused by the electron-withdrawing effect of the protonated imidazole, the significant chemical shift differences of the R group protons (Table 2) are indicative of a conformational change which eliminates the stacking between imidazole and the DKP nucleus.

This is in agreement with previous semiempirical calculation studies[18] of conformational energies carried out on c(Ala–His) which showed destabilization of the folded conformation in an acidic medium. However in all the cases we studied, the two stereospecific coupling constants $J_{\alpha,\beta}$ between the α and β protons of the histidine residue (CH_{His} and CH_{2His}) are about equal and close to 4.5 Hz, thus showing[11,34] that among the possible conformations around the C_α–C_β bond of the histidine residue the folded one is favoured. In neutral and alkaline solutions the $J_{\alpha,\beta}$ coupling constant values are identical with those at pH = 3, clearly indicating that the imidazole protonation does not involve a significant rotation around the C_α–C_β bond. However the same cannot be said of the rotation around the C_β–C_γ bond, by which the DKP–imidazole interaction may be eliminated.

The ^{1}H NMR data obtained in D_2O do not permit us to identify which conformation is preferred by the DKP nucleus (Fig. 1) considering that it is not possible to obtain the coupling constants N–H, C_α–H.[11] Previous studies[19] have pointed out the relationship which exists between the DKP nucleus conformation and the amino acid sequence for cyclic dipeptides which contain aromatic and pseudo-aromatic nuclei. There are two factors which influence the conformational preferences: the maximization of the DKP–aromatic ring interaction and the avoidance of steric interaction between the side-chains. Thus, in the case of c(Gly–His) the flagpole boat conformation is preferred (Fig. 1, B) since the quasi-axial disposition of the histidyl residue is sterically possible and ensures maximum interaction with the DKP ring. In the other cases, in which the second amino acid residue is bulkier, steric interactions prevail, thus excluding the flagpole boat conformation. The quasi-planar conformation (Fig. 1, A) is then preferred since it

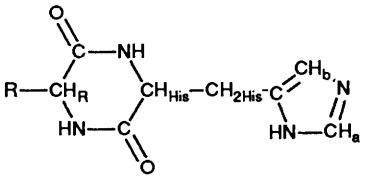
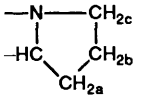
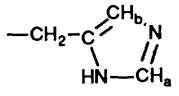
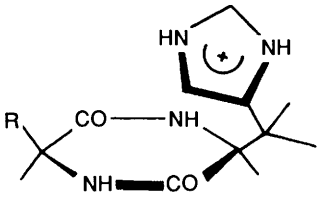


Table 2 Proton chemical shifts (δ, ppm) of the cyclodipeptides at pH = 3 (upper values) and at pH of cyclodipeptide dissolved in water (lower values)[a]

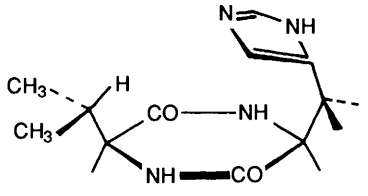
R	H_{1His}	H_R	H_b	H_a	H_{2His}			R group			
H	4.40	3.90	7.20	8.37	3.40/3.30			–H	3.62		
	4.35	3.70	6.97	7.70	3.30/3.10				3.20		
–CH_3	4.50	4.10	7.40	8.50	3.40/3.20			–CH_3	1.05		
	4.39	4.00	6.98	7.70	3.18/3.00				0.78		
CH_3–CH–CH_3	4.55	4.00	7.35	8.50	3.40/3.20	–CH	2.10	–CH_3	0.91/0.50		
	4.40	3.90	6.97	7.70	3.20/3.05		1.80		0.89/0.50		
CH_3–CH–CH_3 / CH_2	4.55	4.05	7.40	8.75	3.40/3.20	–CH	1.45	–CH_3	0.80/0.75	–CH_2	1.40/0.80
	4.40	3.90	6.97	7.70	3.20/3.05		1.40		0.80/0.75		1.10/0.30
	4.65	4.35	7.30	8.62	3.40/3.20	$–CH_{2a}$	2.30/1.70	$–CH_{2b}$	1.95	$–CH_{2c}$	3.50
	4.55	4.25	6.92	7.70	3.20/3.05		2.25/1.45		1.90		3.50
–CH_2–Ph	4.50	4.20	7.00	8.50	3.15/2.95	–CH_2	2.55/1.90	Ph-*o*	7.20	others	7.40
	4.40	4.15	6.83	7.86	2.95/2.75		2.60/1.80		7.20		7.40
	4.40	4.40	7.10	8.40	3.05/2.95	–CH_2	3.05/2.95	H_b	7.10	H_a	8.40
	4.25	4.25	6.90	7.70	2.85/2.35		2.85/2.35		6.90		7.70

[a] The chemical shifts of the cyclodipeptides in basic solution (pH = 9) are identical to those in water.



D

Fig. 2 Possible conformation of the studied cyclodipeptides at pH = 3



E

Fig. 3 Possible conformation of unprotonated c(Val–His)

maximises the imidazole–DKP ring interaction. However, not all previous studies agree on this as regards an aliphatic residue substituent.[29,35]

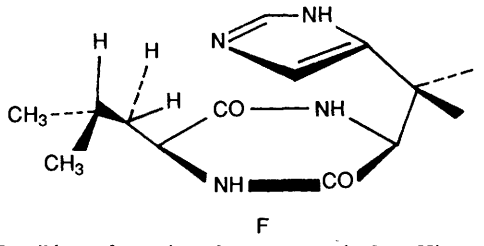
In the pH = 3 c(Ala–His) spectrum we observed that the methyl signal is significantly deshielded ($\Delta\delta$ = 0.27 ppm) with respect to the neutral solution. This may be explained by the separation of the imidazole moiety from the DKP ring by way of rotation around the C_β–C_γ bond which eliminates the shielding effect on the methyl group. This represents a change from conformation A (Fig. 1, R = CH_3), in which the imidazole, folded on the DKP nucleus, is facing the methyl, to conformation D (Fig. 2) in which the same imidazole nucleus, because of the rotation around the C_β–C_γ bond, can no longer shield the methyl group. The same deshielding effect is observed for the H_R proton, but more weakly because of the greater distance.

The same trend is noted for c(Gly–His) in which, notwithstanding less steric proton bulk (R = H) compared with the methyl, the deshielding is equally marked due to the flagpole boat conformation of the DKP nucleus. This, in neutral or basic medium, permits the imidazole to close in on the proton, which thus falls into the shielding zone of the imidazole anisotropy cone.

The spectroscopic data for c(Ala–His) and for c(Gly–His) protons which are not involved in group R, duplicates regularly for all the series of cyclodipeptides we studied (Table 2). The variations in the chemical shift of R group protons in the changeover from neutral to acid pH are useful indicators of the conformational preferences of individual cyclodipeptides.

In the neutral pH spectrum of c(Val–His) (Table 2) there are two distinct signals for the two diastereotopic methyl groups of the isopropyl group. No variation (Table 2) in the chemical shift values of these two diastereotopic methyls is caused by the protonation. On the contrary, the C–H signal of the isopropyl group is shifted downfield upon protonation. These results suggest that E (Fig. 3) is the favoured conformation. Only in this conformation, in fact, can the two methyl groups not be affected by the imidazole anisotropy because of the distance from the ring. However, the observed value (6.0 Hz) in both acid and neutral media, for the stereospecific coupling constant between H_R and the valine isopropyl proton may indicate[11] that both conformations in which one methyl group projects over the DKP ring are not extensively populated.

Molecular model inspection shows that in the E conformation the methyl over the C=O group is located inside the carbonyl anisotropy cone (shielding zone) and thus resonates at higher field than the other ($\Delta\delta \approx 0.4$) (Table 2).



F

Fig. 4 Possible conformation of unprotonated c(Leu–His)

In the case of c(Leu–His) the two methyls of the R group, although non-diastereotopic, are not equivalent, as shown by the two distinct peaks in the ^{1}H NMR spectrum. Molecular model investigation excludes a conformation in which the imidazole and the leucyl residue face each other on the DKP plane, because of steric hindrance. In a neutral environment therefore, F (Fig. 4) is the favoured conformation, as described previously in the literature.[14] The CH_2 peaks of the R group (Table 2), in neutral medium, give rise to an AXYZ system, because of spin–spin coupling with both the H_R and the isopropyl CH protons. In such a system, the AX part, corresponding to the CH_2 protons, resonates at unusually high fields, owing to the imidazole shielding, with a marked separation between the two diastereotopic A and X protons. The conformational changeover upon protonation, *i.e.* the imidazole rotation around the C_β–C_γ bond, is reflected by the two strong CH_2 peak downfield shifts. No effect is observed however for both the CH and CH_3 protons of the R group, which are too far from the imidazole to be in any way affected.

Analogously, in the case of c(Pro–His) the distancing effect of the imidazole, upon protonation, is very evident. In the change from neutral to acid medium only one proton of the fused ring of proline is deshielded, methylene *a* (Table 2), which points towards the imidazole nucleus. The opposite-facing proton of methylene *a* is not significantly affected, in the same way that no chemical shift difference is noted for all the other proline methylene protons.

There is no significant post-protonation shift of the CH_2 benzyl protons of c(Phe–His). This is in agreement with a conformation which places both the imidazole and phenyl nuclei over the DKP ring plane at a pH corresponding to the dissolution in water of the cyclodipeptide. Here the benzyl CH_2 is on the outside of the DKP ring and cannot be affected by the separation of the imidazole from the DKP which results from protonation. Little shielding (not negligible) is observed in neutral medium for the histidine methylene whose protons resonate at slightly higher values ($\Delta\delta \approx 0.3$ for both protons) than those observed for the same protons of all the other cyclodipeptides at neutral pH. Clearly this methylene, even external to the DKP ring, is shielded by the phenyl over the DKP plane since its π charge is greater than that of imidazole. Upon protonation and rotation of the imidazole, the CH_{2His} is still shielded, as may be appreciated by comparing the chemical shift values at pH = 3 with those of the same methylene at the same pH in all the other cyclodipeptides studied. After protonation the CH_{2His} occupies the same conformation position in all the cyclodipeptides we studied, and should therefore resonate at the same field values. The observed difference is therefore attributable to phenyl shielding. The downfield shift in acidic medium with respect to the CH_{2His} proton values of the same molecule in neutral medium is due to imidazole protonation. In the case of c(His–His) the chemical shifts at neutral pH of the protons of the histidine methylene groups occur at unusually high field values, which may be better appreciated by a comparison with the data for the same protons of all the cyclodipeptides studied ($\Delta\delta$ *ca.* 0.7 and 0.35, respectively, for each proton). This upfield shift partially disappears upon protonation. A significant downfield shift is observed for the two protons (respectively, $\Delta\delta = 0.6$ and 0.2), which indicates how histidine methylene groups in neutral pH conditions alternately face the opposite imidazole nucleus (rapidly with respect to the NMR time-scale) which alternately folds over the DKP ring. Consequently the preferred conformation places one and then the other imidazole nucleus over the DKP plane. Upon protonation of both the imidazole rings, the methylene proton chemical shift values do not entirely return to the 'normal' values observed for the cyclodipeptides we studied, there persisting a small but significant shielding effect which may only be interpreted on the grounds of a conformation in which the two imidazole nuclei alternately face the DKP ring, exercising a slight shielding effect on the 'opposite' methylene. Such an effect is weaker than that observed in neutral conditions because the π-electron clouds of the nuclei are only partially available owing to the protonation.

To confirm the hypothesis we advanced on the conformational preferences of the cyclodipeptides, we carried out a 2D NMR NOESY experiment at neutral and acid pH. We examined the valine derivative because we could better follow the NOESY experiments by means of the two diastereotopic methyl signals. In the neutral pH NOESY spectrum we observe that there is a cross correlation between the two valine methyls and the two imidazole protons. In particular we found:

(*a*) a stronger correlation between the higher field methyl group (the one over the DKP C=O group) and the H_a imidazole proton with respect to that observed between the same methyl group and the H_b proton.

(*b*) Cross peaks of the same intensity between the lower field methyl group and the two imidazole protons. Considering the asymmetry of the two imidazole protons with respect to DKP nucleus, the disposition of the imidazole ring in the conformation E is confirmed.

(*c*) No correlation between the imidazole protons and H_R or the valine isopropyl proton.

Conversely in the pH = 3 NOESY experiment of the valine derivative two cross peaks appear which show the correlation between H_b and, respectively, H_R and the valine isopropyl proton. No correlation exists between H_a and the same protons. This agrees with the above-cited rotation of the imidazole ring around the C_β–C_γ bond upon protonation. On the basis of these results we could then suggest that this rotation occurs by way of the approach of H_b to the DKP ring and the consequent distancing of H_a (Fig. 2, D).

In further support we noticed a correlation between H_b and the lower field methyl group, whereas we observed no cross peak between H_a and the same methyl group, in agreement with the hypothesis that upon rotation H_b approaches and H_a distances itself from the isopropyl group. The higher-field methyl group is still correlated more strongly with H_a than with H_b, which shows how, notwithstanding rotation, H_a is closer to the methyl group located over the C=O group of the DKP ring, as may be verified by careful inspection of molecular models.

Conclusions

The conformational characteristics of cyclodipeptides ascertained from ^{1}H NMR results back up the hypotheses based on thermodynamic protonation parameters, both as regards the systems already studied and those whose ΔH° and ΔS° values are cited here for the first time. In particular, it is found experimentally that the protonation of the imidazole ring results in its separation from the region above the DKP ring for all cyclodipeptides. Furthermore, in the presence of suitable substituent groups, such as the phenylalanine aromatic group, this separation facilitates a greater final-state interaction of the phenyl with the DKP ring with respect to the initial state, as the

shielding effect felt by the histidine methylene group shows. The position of the leucyl residue shown by ^{1}H NMR data agrees well with the similarity of the thermodynamic protonation data to those of the alanine derivative, rather than to those of the valine derivative, for which a solvophobic interaction has been confirmed. The only system for which no clear correlation exists between ^{1}H NMR and thermodynamic data seems to be that of the proline derivative. In fact, the deshielding due to protonation ascertained on the basis of spectroscopic measurements does not agree with the highest exothermic ΔH° value of all the systems studied. An explanation of this behaviour on the basis of our data is not possible. We may only suggest, together with Anteunis,[14] that the endothermic contribution due to separation of the imidazole is counterbalanced by the exothermic contribution due to the more energetically favourable configuration of the DKP ring-fused-proline system in the absence of constrictions imposed by the folding of the imidazole over the DKP ring. In conclusion, even if, in the presence of concomitant and diverse conformational effects, ^{1}H NMR data provides sufficiently detailed descriptions of the structural features of the species in the final and initial states, thermodynamic parameters are indicators once again of the presence of weak interactions in a given chemical equilibrium without which it would not be possible to gain an in-depth knowledge of bonding and conformation details.

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