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Conformational role of His-12 in C-peptide of ribonuclease A

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Possible interactions of the His-12 ring with other side chain and backbone groups of C-peptide lactone (CPL) are discussed. The works published so far are critically reviewed and compared with the latest results obtained by the authors. The main new conclusion is that in the helical conformation of CPL, the Phe-8 and His-12 rings are clustered together. Studies of Phe-8 → Ala analogs of CPL and calculations of ring current effects satisfactorily explain the observed environmental shifts of Phe-8 and His-12 protons in NMR spectra of CPL. Interaction between both rings is favorable for α-helix formation, but cannot explain an increase in helix stability related with protonation of His-12. This effect arises from favorable interactions of the charged His^+-12 ring with the helix backbone.

1. Introduction

N-terminal fragments of RNase A provide an excellent model for studies of various sequence-determined effects on stability of α-helical conformations of polypeptide chains in aqueous solution. In recent years, the conformational properties of these peptides, as well as their synthetic analogs, have been studied extensively [1–4] (for a review of earlier works, see ref. 5). We concentrate here on one of these fragments – the lactone form of C-peptide (CPL). This is a 13-residue-long fragment of RNase, with the sequence KETAAAK-FERQHHse-lactone, obtained by CNBr cleavage of the protein [6].

The helix-coil transition in CPL molecules is a strongly cooperative, all-or-none process [7–9]. one of its most intriguing features is a characteristic pH dependence. CD data show [7] that the helix content is greatest at a pH of about 5 and decreases markedly in more acidic and basic solu-

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Table 1

Helix fraction (f) and chemical shifts (δ, in ppm) of NMR signals of His and Phe rings in CPL and M1 at low, medium and high pH (3° C, 0.1 M NaCl)

f has been calculated as explained in ref. 10 from CD data taken from refs. 7 and 25. The δ values for His-12 and Phe-8 in CPL have been taken from refs. 10 and 7, respectively, and extrapolated from 4.5 to 3° C. Those of His-12 in M1 are from ref. 14.

	pH 2	pH 5	pH 8
M1 (AcKETAAAKAERQHMGG)			
f	0.32	0.39	0.31
δ His (C2H)	8.65	8.65	7.73
δ His (C4H)	7.33	7.35	7.01
CPL (KETAAAKFERQHHse-lactone)			
f	0.20	0.34	0.22
δ His (C2H)	8.59	8.53	7.63
δ His (C4H)	7.25	7.15	6.82
δ Phe (*ortho*)	7.24	7.23	7.24
δ Phe (*meta*)	7.31	7.27	7.30

tions (see table 1). The apparent pK values of helix titration are 3.4 and 6.6 [10]. The latter value corresponds strictly to the pK of the His-12 imidazole ring, determined from NMR measurements of CPL [10]. It is clear, then, that protonation of this group leads to increased stability of the helix. This conclusion has been checked by comparative studies of synthetic peptides with sequences similar to that of CPL. After His-12 was substituted with alanine, the helix content dropped considerably and remained unchanged within the range pH 5–8 [3].

Hypotheses have been proposed involving interactions of the His^+-12 ring with other CPL residues which could affect the α-helix stability of CPL. We shall discuss these proposals in detail in the present paper and examine them in the light of new results reported here.

2. Glu^--9–His^+-12 salt bridge

An early hypothesis that the helical conformation of CPL is stabilized by a salt bridge between Glu^--9 and His^+-12 residues has been proved false by experiments with model peptides [11] and Glu-9 → Leu C-peptide analogs [12]. An analysis of CD and NMR data performed by Bierzyński [10] has shown that the shape of the pH-titration curve of the CPL helix can be understood only with the assumption that deprotonation of Glu-9 leads to a substantial drop (~ 30%) in the helix content. This effect can be related, at least in part, to the Glu^--9–His^+-12 interaction. In all peptides studied so far, with His and Glu at positions i and $i-3$, respectively, the NMR signals of the glutamate γ-protons are sensitive to histidine protonation [10,11] and, in some cases, the histidine ring proton signals are sensitive to glutamate protonation [10]. The observed shifts do not seem to be related to any detectable changes in backbone conformation.

The existence of the Glu^--9–His^+-12 salt bridge in CPL is, therefore, quite probable, although it is unfavorable for the α-helical conformation. A simple rationalization of this conclusion has been proposed by Bierzyński et al. [11]. This is based on general estimates of the contribution of side chain-side chain interactions to helix-coil transition entropy. Basically, an interaction between the long chains of residues i and $i \pm 3$ is rather unfavorable for a helical conformation, whereas that between the short, bulky groups of residues i and $i \pm 4$ is more likely to stabilize it [11].

3. His-12–Phe-8 ring interactions

The most striking feature of the ^{1}H-NMR spectra of CPL are the strong environmental shifts of a number of side chain proton signals induced by an increase in pH from pH 2 to 5. Of these, the upfield shifts of the His-12 ring protons are the most pronounced. These shifts are consistent with α-helix formation. They closely follow the pH-titration curve of the helix content (pK 3.4) and not those of either Glu-2 (pK 4.0) or Glu-9 (pK 4.3) [10] deprotonation. As shown in table 1, at 3° C the signals shift by 0.06 (C2H) and 0.1 (C4H) ppm on helix content increasing with pH from 20 to 33%. From these data, including small corrections for the effects of the Glu^--9–His^+-12 interaction [10], the signals of both protons in the helical (δ_h) and nonhelical (δ_n) conformations of CPL can be readily calculated (see table 2).

The secondary shifts of the His-12 ring protons resulting from α-helix formation (Δ_h) are very

Table 2

Chemical shifts (in ppm) of the NMR ring proton signals of His-12 and Phe-8 in CPL molecules in the α-helical (δ_h) and nonhelical (δ_n) conformations at low (pH 2–5) and high (pH 8) values of pH (3° C, 0.1 M NaCl), calculated from the data given in table 1

Small corrections for His^+-12 signals due to the effect of Glu-9 deprotonation have been introduced as described in ref. 10. Δ_h is the secondary shift related to α-helix formation, assumed to be the same before and after deprotonation of His-12. Positive values of Δ_h are upfield.

	pH 2–5		Δ_h	pH 8	
	δ_h	δ_n		δ_h	δ_n
His (C2H)	8.32	8.67	0.35	7.36	7.71
His (C4H)	6.59	7.41	0.82	6.18	7.00
Phe (*ortho*)	7.21	7.24	0.04	7.21	7.25
Phe (*meta*)	7.08	7.37	0.29	7.08	7.37

large: only Van der Waals contacts and aromatic ring current effects could be responsible for shifts of such magnitude [13]. The former have been observed in excessively packed regions within proteins, but never in flexible peptide molecules. Furthermore, they are always manifested by downfield shifts [13]. Therefore, the only explanation for the observed His-12 ring proton shifts in CPL is that this residue interacts, when in the helical conformation, with an aromatic ring 2. The unique, and most likely, candidate is the ring of Phe-8 which can be close to His-12 in the α-helical structure.

If this supposition is correct, the Phe-8 ring proton signals should also be shifted in the α-helix, because of the proximitiy of the His-12 ring: this is indeed the case [7]. At 3°C, on helix content increasing with changing pH from pH 2 to 5, the Phe-8 *meta* proton signals shift by 0.04 ppm (see table 1).

To investigate further the effect of Phe-8 on His-12, we have synthesized [14] a model peptide (M1) with the sequence AcKETAAAKAER-QHMGG. In M1, the whole of segment 1–12 is the same as in CPL with the exception of a Phe-8 → Ala substitution. At pH 5 and 3°C, the peptide is even more helical than CPL. Nevertheless, its His-12 ring proton signals occur at much lower fields (table 1), adjacent to the positions calculated for nonhelical CPL molecules (cf. δ_n values in table 2). Moreover, although the helix content in M1 changes within the region pH 2–5, as it does in CPL, the histidine signals show much smaller shifts which are also opposite in direction.

Another interesting observation concerns the nonequivalence of the His^+-12 β-protons in both peptides [14]. With a temperature decrease from 21 to 4°C, the difference between the His^+-12 β-proton shifts in CPL increases more than 2-fold, from 0.13 to 0.28 ppm. Evidently, conformational freedom of the side chain of this residue is reduced [26]. No such effect is observed in M1. The splitting between the His^+-12 β-protons is much smaller (0.08 ppm) and does not change when the temperature is reduced from 30 to 3°C, in spite of a substantial increase in helix content, quite similar to that observed in CPL (see table 3). Therefore, immobilization of the His^+-12 ring in CPL does not result directly from the backbone helical conformation, but arises from an interaction with Phe-8 accompanying helix formation.

Table 3

Helix fractions (f) and splittings (B) of the His-12 β-proton signals in M1 and CPL at room temperature and low temperature (pH 4.3, 0.1 M NaCl)

Data taken from refs. 10 and 14.

Temperature (°C)	f	B (ppm)
M1		
30	0.22	0.08
3	0.39	0.08
CPL		
21	0.17	0.13
4	0.33	0.28

Analysis of the possible mutual orientation of His(i) and Phe ($i-4$) rings in the α-helix is greatly facilitated by the low number of sterically allowed conformers of the aromatic side chains. In the random coil, there are six conformers allowable for each residue, namely, three for the C_α–C_β bond ($\chi_1 = 60$, 180 and $-60°$) and two for the C_β–C_γ bond ($\chi_2 = 90$ and 270°) according to the IUPAC-IUB nomenclature [15]. In the α-helix, one of the C_α–C_β rotamers ($\chi_1 = 60°$) is excluded for bulky side chain groups because of steric hindrance caused by the neighboring helix turn [16]. Therefore, there remain four conformers for each side chain, giving a total of 16 various mutual orientations. Because of symmetry of the Phe-8 ring, they reduce to eight different, 2-fold degenerate conformers. For all of these, the distance between both rings has been estimated from computer-simulated conformations generated in the model sequence AAAAAAAFAAAHA with standard amino acid coordinates [17]. Only for two conformers is the calculated distance small enough to give significant ring current shifts.

The precise mutual orientations of the Phe-8 and His^+-12 rings in these two conformers have been determined using an energy minimization procedure (see fig. 1 and table 4). Using these orientations, the secondary shifts of Phe-8 and His^+-12 ring proton signals resulting from ring current effects have been estimated for both con-

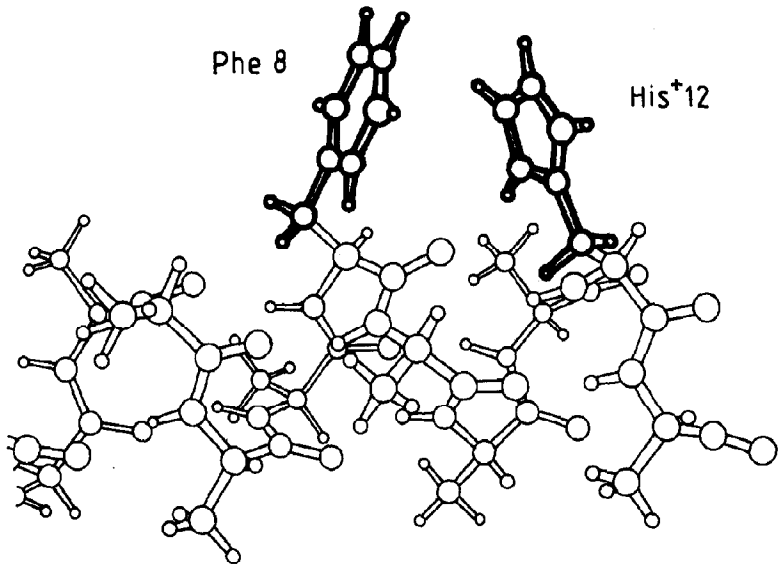

Fig. 1. Conformation of the model peptide $(Ala)_7$-Phe-Ala-Ala-Ala-His-Ala in the α-helical conformation corresponding to conformer I in table 4. In conformer II (table 4) the histidine ring is rotated around its C_β–C_γ bond by about 180°. The geometry of both conformations was determined using the energy minimization program of Nemethy et al. [17], starting from the ideal α-helical conformation of the backbone [16] and the following side chain orientations: $\chi_1^{Phe} = 180°$, $\chi_2^{Phe} = 90°$, $\chi_1^{His} = -60°$ and $\chi_2^{His} = 90°$ for conformer I and $-90°$ for conformer II. Standard amino acid geometry and energy parameters are used in this algorithm [17] and energy is given as a sum of torsional, Lennard-Jones and electrostatic terms. Minimization has been performed with the conjugated gradient method.

Table 4

Calculated and experimental (Δ_h) secondary shifts of His-12 and Phe-8 ring proton signals (in ppm) related to α-helix formation in CPL

Two ring conformers (I, II) have been found as described in the legend to fig. 1. Calculations of ring current shifts have been performed using the Johnson-Bovey equation (see text). Upfield shifts are positive.

	Calculated shifts		Averaged for conformers I and II	Experimental Δ_h for 100% helix [c]
	Conformer I [a]	Conformer II [b]		
His (C2H)	0.07	0.80	0.43	0.35
His (C4H)	1.41	0.03	0.72	0.82
Phe (*ortho*)	0.06	0.06	0.06	0.04
Phe (*meta*)	0.15	0.15	0.15	0.29 (0.21)
Phe (*para*)	0.00	0.00	0.00	(0.22)

[a] $\chi_1^{Phe} = -179°$, $\chi_2^{Phe} = 89°$, $\chi_1^{His} = -76°$, $\chi_2^{His} = 53°$.
[b] $\chi_1^{Phe} = -179°$, $\chi_2^{Phe} = 89°$, $\chi_1^{His} = -77°$, $\chi_2^{His} = -149°$.
[c] Δ_h values in parentheses are taken from ref. 4. The others are from table 2 in this work.

formers according to the Johnson-Bovey equation [18]. The parameters of the ring current intensity given in ref. 13 (p. 231) have been used. This procedure has been proven to yield good results, at least for shifts caused by the phenylalanine ring [19]. For histidine, they are less reliable [13]. In table 4, the calculated shifts for both conformers are compared with the Δ_h values determined from the experiment. It is evident that the shifts calculated for each conformer separately are different from those obtained experimentally. Secondary chemical shifts arising from neighboring carbonyl groups, estimated with the approximation proposed in ref. 20 (data not shown), are small and cannot account for this discrepancy. The averaged shifts for both conformations (column 3 in table 4) are reasonably close to the Δ_h values. Only for the Phe-8 *para* proton is the calculated shift different from that reported by Rico et al. [4] as being characteristic of the α-helix in CPL.

These results suggest that both conformers in which Phe-8 and His^+-12 rings are clustered together are densely and equally populated in the α-helical conformation of CPL. The mutual ring orientations can differ somewhat in aqueous solutions, from those obtained in our calculations. We have found that ring rotations within the range of $\pm 15°$ are quite possible. These deviations do not change significantly the calculated values for the secondary shifts (data not shown). More drastic forms of reorientation are not feasible due to steric hindrance.

4. Helix-stabilizing effect of Phe-8–His-12 ring clusters

The chemical shifts of Phe-8 and His-12 ring proton signals in a nonhelical conformation of CPL (δ_n in table 2) are close to those measured in M1 – a peptide devoid of a Phe residue. Both rings appear close to each other only when the helix is formed. This suggests that ring clustering can be favorable for the α-helical conformation. Indeed, substitution of Phe-8 by Ala in CPL leads to an approx. 2-fold drop in helix content at pH 5 [14].

The effect of His^+-12 deprotonation on helix stability is not straightforward. At high pH, the signals of the His-12 ring protons in CPL are also shifted upfield compared with their positions in M1 while those of the Phe-8 *meta* protons lie upfield from the δ_n calculated for low pH (cf. tables 1 and 2). With helix unfolding at higher temperatures, all these signals shift strongly downfield. The δ_n and δ_h values at high pH, calculated assuming that the helix-induced secondary shift $\Delta_h = \delta_n - \delta_h$ is the same as that in acidic solutions, are quite reasonable. For His-12, they are close to those measured in M1 and for Phe-8 exactly the same as those in the acidic region. It appears that neither the population nor the geometry of ring clusters in the helical CPL molecules changes perceptibly after deprotonation of His^+-12.

The following interpretation seems to be the most plausible to us. Even in the case where His-12 is uncharged, the free energy of Phe-His ring clustering in the α-helix (ΔG) is sufficiently low such that the clusters are fully populated when the helix is formed. Histidine protonation leads to a further decrease in ΔG. Although there is no visible change in ring cluster population, the total free energy of α-helix formation, including ΔG for ring clustering, is considerably reduced.

This hypothesis is compatible with the calculations of the ring current shifts for the signals of CPL protons. These indicate that the clustered conformation of Phe-8 and His-12 rings is densely populated in the helical molecules. Moreover, the temperature shifts of the proton signals for both rings follow exactly the helix unfolding [14]. Within the error limits of NMR and CD measurements, the secondary shifts related with the helical conformation are, therefore, temperature-independent. Thus, either the enthalpy of the ring clustering is close to zero or ΔG is always low and the equilibrium shifted strongly in favor of the clustered conformation. We believe the latter explanation to be correct.

Our energy calculations suggest the possible source of the decrease in ΔG related with protonation of His-12. Although the interaction energy of the Phe-8–His-12 rings does not depend perceptibly on His-12 protonation, in the clustered conformation interactions of the His^+ ring with negative charges of the C = O backbone groups are much stronger.

5. His^+-12–helix dipole interactions

The NMR data obtained thus far do not provide evidence for any interactions of the His^+-12 ring in CPL other than those with Glu^--9 and Phe-8, discussed above. Nevertheless, investigations of CPL analogs indicate that, at least in these peptides, if not in CPL itself, His^+-12 must be involved in yet another conformationally important interaction. In M1, as well as in the Phe-8 → Ala C-peptide analog studied by Shoemaker et al. [1], the basic limb of the helix titration is still noticeable, although less pronounced than in CPL. Evidently, the protonated histidine can stabilize the helix not only by an interaction with Phe-8 but also in some other manner.

One possibility could be via an interaction with the negative pole of the aggregated helix dipole [21]. This hypothesis has been proposed by Shoemaker et al. [3], mainly by analogy to a much better documented helix-stabilizing effect of negatively charged groups situated at the positively charged N-terminus of a helix [12]. The only objection that could be formulated against this hypothesis is as follows. The helix-dipole-related phenomena studied thus far [21–23] could be described successfully by the simple model of an aggregated dipole, with one positive and one negative charge situated at the respective termini of the helix. According to this model, for an effective interaction with the negative charge, the His^+-12 ring should be oriented in quite a definite way. The NMR data do not support this contention. The splitting of His^+-12 β-proton signals in M1 (table 3) is not affected by the helical conformation. Evidently, the conformational freedom of this residue is not significantly reduced when the helix is formed.

Closer inspection of the α-helix dipole electrostatic field given in refs. 21 and 23 and the calculations performed recently by Godzik and Wesołowski [24] lead to a somewhat different conclusion. They indicate that the total sum of electrostatic energies, arising from interactions of His^+-12 with the peptide groups of the CPL helix, is practically independent of the conformation of the histidine side chain. The energy thus calculated includes what, in a macroscopic approach, appears as an effect of the 'aggregated helix dipole'.

Therefore, the conformational flexibility of the His^+-12 side chain in M1 is fully compatible with its favorable interaction with the C-terminus of the helix backbone. Moreover, the orientation of the His^+-12 ring towards Phe-8 in CPL does not prevent it from a similar interaction. This can be responsible, in part, for the increased stability of the CPL helix after protonation of His-12. Nevertheless, this effect alone cannot explain the increase in amplitude of the helix pH-titration in the presence of Phe-8, unless an interaction of His^+-12 with the helix terminus is actually more favorable when the Phe-8–His^+-12 ring cluster is formed. This may be so in aqueous solutions. In any case, the increase in helix stability of CPL observed after protonation of His-12 seems to arise from favorable interactions of the charged imidazole ring with the helix backbone, and not with any side chain group of the peptide.

6. Concluding remarks

Even a small, 13-amino-acid-residue peptide, such as CPL, is a very complicated molecule, the conformation of which depends on many interrelated side chain–side chain and side chain–backbone interactions. It should be borne in mind that none of these interactions is independent of the others and treating them separately is a crude, though necessary, simplification. All interactions discussed in this article can be, and no doubt are, modified by other types which have not been taken into account. Until the point is reached where a total description of the CPL molecule is feasible, all conclusions as to the conformational importance of the various individual interactions must be accepted with skepticism. In particular, the results obtained with CPL analogs and model peptides, although extremely valuable, should be used with great caution in the interpretation of the conformational properties of C-peptide itself.

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