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NON-COVALENT INTERACTIONS IN CYCLOPEPTIDE PROTON COMPLEX FORMATION IN AQUEOUS SOLUTION *

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ABSTRACT

$\Delta G^{\ominus}$, $\Delta H^{\ominus}$ and $\Delta S^{\ominus}$ protonation values of cyclic-L-dipeptides containing L-histidine (c-Gly-L-His, c-L-Ala-L-His, c-L-Val-L-His, c-L-Phe-L-His) were determined by potentiometry and calorimetry in aqueous solution at 25 °C and $I = 0.1$ mol dm^{-3} (KNO_3). The thermodynamic parameters were compared with those of imidazole, determined under the same experimental conditions, to assess the role played by non-covalent interactions in the proton complex formation. In addition, a comparison with c-Gly-L-His allowed an observation to be made of the effects of side-chain residues on conformations in protonation reactions.

INTRODUCTION

Although naturally occurring and synthetic cyclic dipeptides have been known since the beginning of the century [1,2], they have been the subject of intensive study only recently [3–7]. More than 100 cyclic dipeptides have been found to occur in nature. Diketopiperazine (DKP) derivatives as bicyclomycins or gancidin W [8,9] are powerful antibiotics.

In addition, c-L-His-D-Leu (His = histidine; Leu = leucine), unlike c-L-His-L-Leu, has been found to be a powerful hydrolytic catalyst [10]. The behaviour of the former, which has a His moiety (a well-recognized enzymatic entity) on one side and an apolar Leu side-chain on the other side of the nearly planar DKP ring, has been explained in terms of 'cooperativity' between the two substituents (i.e. His and Leu) in the DKP ring. DKP

* Dedicated to Professor James J. Christensen in memory of his contribution to innovation in calorimetry.

derivatives have also been used successfully as intermediates for carrying out stereoselective syntheses; good yields and purities as high as 90–95% have been obtained, especially when the DKP rings carry rather bulky R groups [11].

In the last decade c-His-Pro (pro = proline), a metabolite of thyrotropin releasing hormone (TRH), has been shown to have a number of actions [12], including inhibition of prolactin release from the pituitary [13], antagonism of ethanol sedation [14], inhibition of abstinence syndrome in opiate-dependent mice [15] and production of hypothermia [16].

Furthermore, cyclic peptides with amino acid residues containing complexing side-chains, such as imidazole (Im), carboxylate or thioether groups, can coordinate to metal ions in a way that mimics the coordination sites in enzymes [17]. In addition, such chains can 'encourage' the coordination of peptide nitrogens [18]; hence, the importance of this class of ligands for modelling protein conformation, membrane transport and other biological processes associated with metal binding [19–22]. The advantages of these model ligands over linear dipeptides are their 'constrained' geometry and the absence of free $-COO-$ and $-NH_2$ terminal groups.

Recent studies involving cyclopeptides have focused on the synthesis of their metal complexes and the subsequent characterization of these complexes in the solid state [23,24]. While characterization of metal complexes in solution is difficult, thermodynamic studies can give useful information [25–27].

Our aim was to study the thermodynamic behaviour of the complex formation of cyclic dipeptides containing complexing side-chain substituents. Obviously, before studying the complexation of such systems we needed to investigate the thermodynamics of the protonation.

A study, focusing mainly on the side-chain exchange action in histidine-containing cyclopeptides, has shown that the log K value of protonation of the imidazole residue is lower than the log K value of protonation of imidazole itself. This phenomenon is a peculiarity common to all systems that we found in the literature. All systems show a log K value for the protonation of the imidazole nitrogen of the histidine residue, which is lower than the log K value of protonation of imidazole itself. In addition, for c-L-His-L-His (Cyhis), a system studied previously [28], we found a log K value of protonation lower than the log K value of protonation of imidazole. This cannot be attributed to the presence of a second imidazole moiety in the DKP ring, since bis-imidazolylmethane (Bim), which contains two imidazole residues, shows a greater basicity when compared with imidazole itself [28]. On the basis of $\Delta H^{\ominus}$ and $\Delta S^{\ominus}$ values, the lower log K value has been attributed to the removal of the imidazole moiety from the space over the DKP ring, which results in the loss of a favourable non-covalent interaction [29]. It has also been shown that the thermodynamics of complexation with copper(II) depend mainly on the extent of intramolecular

interaction of the DKP ring with the imidazole residues and on the extent of intramolecular interaction of the side-chains with each other. To find out whether this is a feature commonly encountered in histidine-containing cyclic dipeptides, we investigated some histidine-containing cyclic peptides with alkyl residues of variable length (c-L-Ala-L-His, c-L-Val-L-His) (Ala = alanine, Val = valine) and with aromatic residues (c-L = Phe-L-His) (Phe = Phenylalanine). In addition to these systems, we also studied c-Gly-L-Hist (Gly = glycine), which served as a 'blank' system.

Since $\Delta G^{\ominus}$ alone gives little information, because the enthalpy and entropy changes may cancel out [30], we thought it would be interesting to determine the enthalpic and entropic contributions to the protonation equilibrium for the above systems at 25 °C and $I = 0.1$ mol dm^{-3} (KNO_3). The log K values of protonation for all the systems of interest were redetermined, under the same experimental conditions, in order to obtain homogeneous data.

EXPERIMENTAL

Ligand synthesis

Diketopiperazines were obtained according to the method outlined as follows for c-L-Phe-L-His. CBZ-L-Phe-*p*-nitrophenyl ester obtained from CBZ-L-Phe and *p*-nitrophenol, using dicyclohexylamine (DCC), was coupled with L-histidine methyl ester dihydrochloride in the presence of triethylamine. The carbobenzoxy (CBZ) group was then removed by hydrogenolysis using 10% palladium-on-charcoal as catalyst. The product so obtained was cyclized by refluxing in methanol. The diketopiperazine was characterized by means of mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy and elemental analysis. This procedure was followed for the synthesis of the other diketopiperazines.

Potentiometric titrations

Computer-controlled potentiometric titrations were performed with two distinct Metrohm digital pH meters (model 654) equipped with Metrohm 109 glass electrodes and Metrohm 404 saturated calomel electrodes. The titration cell was thermostated 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 the solution under study. Titrations of HNO_3 with KOH were performed before and after each set of experiments to convert readings to pH and to calculate log K values. The ionic strength was kept at 0.1 mol dm^{-3} (KNO_3). The analytical concentrations of cyclopeptides were in the range 3–7 mM.

Calorimetric titrations

The calorimetric data were obtained by titration calorimetry using a Tronac isoperibol apparatus (model 450) equipped with a 25 ml reaction dewar. The calorimetric system was calibrated by titrating trishydroxymethylaminomethene (THAM) with HCl according to Grenthe et al. [31]. The heats of protonation of the cyclopeptides were determined by titrating solutions of the ligands with standard HNO_3 (0.2 mol dm^{-3}). The concentrations were in the range 4.0–9 mM. Grade A glassware was used throughout. Other experimental details were as reported previously [32].

Calculations

Calculations concerning the electrode system $E^{\ominus}$ values, ligand purities and protonation constants were performed by the computer program SUPERQUAD [33]. The heats of protonation were calculated by the least-squares computer program DOEC [34]. Errors are expressed as three times the standard deviation.

RESULTS AND DISCUSSION

The log K values for the protonation of the cyclopeptides investigated in this study are reported in Table 1 together with those for the protonation of imidazole. These diketopiperazines have the general formula shown in Fig. 1 where R is H (Gly), $-CH_3$ (Ala), $CH_3-CH-CH_3$ (Val) or $C_6H_5-CH_2-$ (Phe). Previous data [36] are higher than the present results probably owing to the different experimental conditions used.

The log K values for the protonation of histidine-containing cyclopeptides are always lower than that of imidazole (Tables 1 and 2); neither polar

TABLE 1

Log K values for the protonation of the imidazole nitrogen in imidazole and in some histidine-containing cyclic dipeptides

Ligand [a]	log K [b]	Reference
Im	7.01	[35]
c-Gly-L-His	6.124 (2)	This work
c-L-Ala-L-His	6.094 (3)	This work
c-L-Val-L-His	6.294 (3)	This work
c-L-Phe-L-His	6.283 (3)	This work

[a] Im = imidazole; Gly = glycine; Ala = alanine; Val = valine; Leu = leucine; Phe = phenylalanine; His = histidine.

[b] Values in parentheses represent three times the standard deviation.

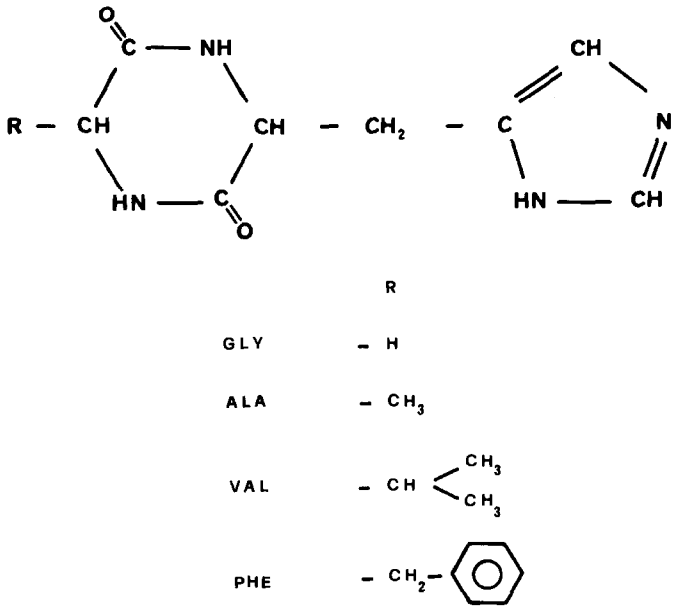

Fig. 1. Cyclic dipeptides investigated in this work.

(Asp) or non-polar (Phe, Val) residues alter this situation. The decrease in basicity is therefore a characteristic of the presence of the imidazole moiety in the DKP ring.

TABLE 2

Log K values for the protonation of the imidazole nitrogen in imidazole and in some histidine-containing cyclic dipeptides

Ligand [a]	log K	Reference
c-Gly-L-His	6.30	[36]
	6.3	[37]
c-L-Leu-L-His	6.40	[36]
c-L-Ser-L-His	6.27	[36]
	6.4	[38]
	6.55	[37]
c-L-Thr-L-His	6.59	[36]
	6.57	[38]
c-L-Tyr-L-His	6.48	[36]
c-L-Phe-L-His	6.50	[36]
c-L-Met-L-His	6.21	[36]
c-L-Asp-L-His	6.43	[36]
c-D-Thr-L-His	6.57	[38]
c-D-Ser-L-His	6.35	[38]

[a] His-histidine; Gly-glycine; Leu = leucine; Ser = serine; Thr = threonine; Tyr = tyrosine; Phe = phenylalanine; Met = methionine; Asp = aspartic acid.

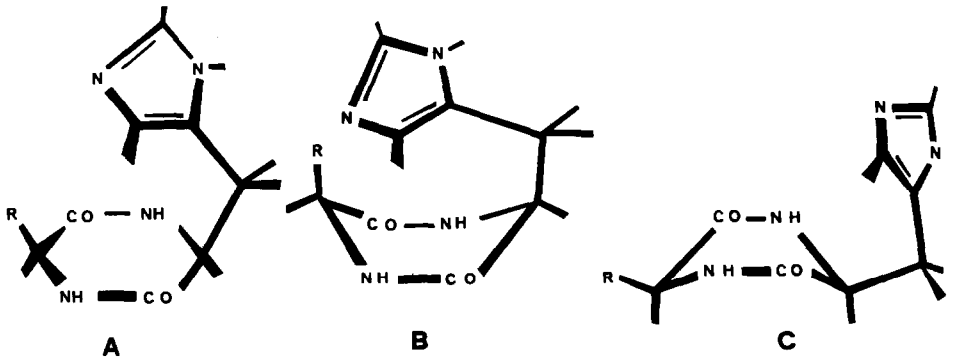

Fig. 2. Planar (A), flagpole (B) and bowsprit boat (C) conformations of the diketopiperazine ring in a cyclic dipeptide.

Kopple et al. [39,40] have shown that diketopiperazines with aromatic or pseudoaromatic substituents have conformational preferences in solution. In these cyclic peptides the favoured conformation in solution has the aromatic ring stacked against the DKP ring (see Fig. 2). These results are also substantiated by other findings [41]. The conformation shown in Fig. 2B is usual for monosubstituted cyclic peptides, whereas that shown in Fig. 2A is more common for cyclic peptides with two residues, particularly when one substituent is aromatic. In the latter case, if both amino acids have the same configuration, a near planar configuration of the DKP ring (Fig. 2A) removes side-chain interference and still maintains as much interaction as possible between the substituent and the DKP ring. A bowsprit boat conformation (Fig. 2C), on the other hand, would sacrifice most of this interaction [39].

Interestingly, some data [40–42] indicate that the protonation of the imidazole moiety, in some histidine-containing diketopiperazines, results in a change in the folded conformation. This conformational change and the consequent destabilization, due to the 'loss' of a stacking interaction, would be consistent with the decrease in the log K values of protonation of the imidazole residue in cyclic peptides with respect to that of imidazole itself. However, log K values are a function of both $\Delta H^{\ominus}$ and $\Delta S^{\ominus}$, and therefore a well-grounded interpretation requires a knowledge of these quantities. Table 3 shows enthalpy and entropy changes for the protonation of the investigated cyclic dipeptides; for comparison we also present the thermodynamic parameters for the protonation of imidazole [28].

First of all, we focus our attention on c-Gly-L-His. The protonation of the imidazole moiety in this diketopiperazine is less stabilized with respect to enthalpy and more stabilized with respect to entropy than the protonation of imidazole itself. This trend is consistent with the expected decreased interaction between the imidazole moiety of the cyclopeptide and the DKP ring on protonation of c-Gly-L-His. Proton magnetic resonance spectra performed at

TABLE 3

Thermodynamic parameters for the protonation of the imidazole nitrogen in imidazole and in some histidine-containing cyclic dipeptides

Ligand [a]	$\Delta G^{\ominus}$ (kcal mol^{-1})	$\Delta H^{\ominus}$ [b] (kcal mol^{-1})	$\Delta S^{\ominus}$ [b] (cal mol^{-1} K^{-1})
Im [c]	−9.56	−8.83	2.4
c-Gly-L-His [d]	−8.35	−6.80 (8)	5.2 (3)
c-L-Ala-L-His [d]	−8.31	−6.75 (6)	5.2 (2)
c-L-Val-L-His [d]	−8.58	−7.47 (8)	3.7 (3)
c-L-Phe-L-His [d]	−8.57	−7.69 (9)	2.9 (3)

[a] Im = imidazole; Gly-glycine; Ala = alanine; Val = valine; Leu = leucine; Phe = phenylalanine; His = histidine.

[b] The values in parentheses represent three times the standard deviation.

[c] Reference 35.

[d] This work.

different temperatures indicate that in cyclopeptides with an aromatic residue the folded conformer, in the unprotonated form, is favoured by about 3 kcal mol^{-1} in enthalpy, whereas it has a more negative entropy (about 4 cal mol^{-1} K^{-1}) [39]. Therefore the folded–unfolded equilibrium which occurs on protonation of c-Gly-L-His should result in an unfavourable enthalpic contribution. This is exactly what occurs. The protonation of imidazole in c-Gly-L-His is less enthalpically favourable than the protonation of the imidazole nitrogen (−6.80 compared with −8.83). The $\Delta S^{\ominus}$ value associated with the folded–unfolded equilibrium (approximately 4 cal mol^{-1} K^{-1}) corroborates this interpretation.

In addition to conformational energies associated with the protonation, the length (Gly, Ala, Val) and the nature (Phe, Im) of the side-chain must be considered in order to understand the differences observed within the cyclic peptides investigated. Dreiding models show that while no interaction between the short aliphatic side-chain and the DKP ring is possible in c-L-Ala-L-His, the hydrophobic valyl side-chain can occupy the space over the piperazinedione ring. We have shown that such a non-covalent [29] interaction is favourable with respect to enthalpy and unfavourable with respect to entropy [43]. $\Delta S^{\ominus}$ values (3.7 compared with 5.2 cal mol^{-1} K^{-1}) indicate that there is a higher degree of 'stiffening' in c-L-Val-L-His than in either c-Gly-L-His or c-L-Ala-L-His; this adds further support to the idea that in c-L-Val-L-His the hydrophobic side-chain 'lies' over the DKP ring. Accordingly, there are a whole series of reports which show that aliphatic side-chains in solution prefer the folded form [41]. The isopropyl group prefers an arrangement in which the aliphatic residue projects over the piperazinedione ring [40].

The thermodynamic parameters for c-L-Phe-L-His can be interpreted in a similar manner. The higher stability of the conformer in which the phenyl

side-chain projects over the DKP ring, is reflected in the $\Delta H^{\ominus}$ value, which is more favourable than those observed for c-Gly-L-His and c-L-Ala-L-His, where a non-covalent interaction cannot exist. Such an interpretation is also consistent with the $\Delta S^{\ominus}$ value, which reflects the 'stiffened' stereochemistry.

In conclusion, our thermodynamic data, together with those previously reported for the folded–unfolded equilibrium, help to elucidate the driving force which stabilizes the folded conformation, i.e. the interaction between the piperazinedione ring and the side-chains.

This problem has attracted the attention of many researchers in the past. Originally, the main driving force was thought to be of a π–π donor–acceptor type [39]. However, UV spectroscopy has shown that there is no indication of a charge-transfer absorption band in the spectra of cyclic peptides with aromatic substituents. Nitro groups, introduced into the aromatic side-chain, have also been used to investigate the problem [44]. If the aromatic ring acts as a donor of electron density in a donor–acceptor or charge-transfer complex, nitro substitution should destabilize the folded conformation [45,46]; if the aromatic ring acts as an acceptor, nitro substitution should increase the stability of the folded form [47]. Since neither effect is apparent, it must be concluded that donor–acceptor interactions are not a major factor in stabilizing the face-to-face arrangement of rings.

It has also been hypothesized that the folded form is stabilized by interaction of the two amide dipoles with the polarizable π-electron system of the aromatic side-chain [39]. However, this is not consistent with solvent effects. The folding energy is not greatly changed by the nature of the side-chain nor by the polarity of the solvent, even in an amide solvent [39,44,48,49]. Therefore, the nature of such an interaction must be short range and not dipolar in nature, e.g. as in a dipole-induced dipole [50]. It is concluded [39] that the folding of the aromatic ring against the diketopiperazine ring in cyclic peptides is not a result of a hydrophobic [51] or solvophobic [52] interaction, if an entropy-driven association is implied. We have recently demonstrated that the intramolecular solvophobic interaction which accompanies the protonation of some linear dipeptides with aliphatic and aromatic side-chains is enthalpically stabilized and entropically unfavourable [28,43]. Our experimental findings agree with Sinanoglou's [52] theoretical conclusions.

CONCLUDING REMARKS

Our thermodynamic data combined with spectroscopic data help to elucidate the driving force which stabilizes the folded conformation, i.e. the solvophobic interaction between the side-chains and the DKP ring. $\Delta H^{\ominus}$ and $\Delta S^{\ominus}$ values are diagnostic of the presence of a solvophobic interaction.

$\Delta H^{\ominus}$ and $\Delta S^{\ominus}$ values are also helpful in the interpretation of the low log K value of protonation of the imidazole residue in Cyhis. The thermodynamic parameters help us to show that, on protonation, the imidazole residue assumes a position away from the diketopiperazine ring in Cyhis.

It would be interesting to determine the $\Delta H^{\ominus}$ and $\Delta S^{\ominus}$ values for c-L-Leu-L-His, in which, as indicated by spectroscopic measurements, the hydrophobic leucyl side-chain does not compete effectively, even in water, for the space next to the diketopiperazine ring [39]. If this is the case we would expect an enthalpy contribution for this cyclic peptide which is comparable with that found for c-Gly-L-His or c-L-Ala-L-His. It would also be interesting to study systems in which the degree of rotational freedom is limited. In this context, studies are under way on systems such as c-L-Leu-L-His, c-L-Asp-L-His and c-L-Pro-L-His.

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