Conformational Study of Histidine- and Tryptophan-Containing Peptides in Solution Based upon the Combination of Nuclear Magnetic Resonance, Circular Dichroism, and Fluorescence Measurements. 13

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Oligopeptides containing both histidine and tryptophan of the series H-Gly-His-$(Gly)_n$-Trp-Gly-OH (n = 0, 1, 2), together with the dipeptide H-His-Trp-OH and the tripeptide H-His-Trp-Gly-OH, have been prepared and characterized, and their conformational behaviors in dilute solution have been investigated. In particular, our study was aimed at clarifying whether and under which conditions intramolecular interactions between the two aromatic rings are present, and how these affect the behavior of the whole molecule. Our strategy consisted of the simultaneous study by several spectroscopic techniques (UV absorption, fluorescence, circular dichroism (CD), ^{1}H NMR analysis) of the compounds in their different ionization states (pH 2–11) and over a wide temperature range (−70 to +50 °C). This approach permits reasonable guesses about some of the stablest conformers in solution, in particular, for $^+H_2$-His$^+$-Trp-O$^-$ and H-Gly-His-Trp-Gly-O$^-$. In the first case the two aromatic rings are relatively close to each other and form an angle of ca. 45° with respect to one another. In the latter case they are almost perpendicular to each other, the $H_{\delta'}$ proton of His being the closest to the indole ring; the main chain rather closely follows a β-turn. In no case did we find evidence of parallel stacking between the two rings. We also considered in detail the question of charge-transfer (CT) complexes between histidine and indole, and on the basis of the pH dependence of both UV-absorption and NMR data we could rule out the existence of CT complexes in the ground state. The marked quenching of the indole fluorescence upon protonation of the histidine ring for both H-Gly-His-Trp-Gly-OH and H-Gly-His-Gly-Gly-Trp-Gly-OH is ascribed to a dynamic process. More generally, the data and the strategy presented in this paper are used to critically outline the potential and limits of the various spectroscopic techniques to the problem of conformation of flexible molecules in solution.

Introduction

The study of conformational equilibria of flexible peptides in solution is still a task of considerable difficulty. Contrary to the case of proteins, the crystalline conformation, as obtained from X-ray, is of little significance for the conformational equilibria in solution, as generally it represents only one of the very many conformers which are present. Conformational energy calculations can certainly be useful, but their reliability, although very much increased in the last few years, cannot as yet be taken for granted, particularly when solvent or temperature effects are being questioned; also, it is not possible to correlate readily this calculated potential energy with thermodynamic energy parameters.

Thus, the experimental approach to determine the conformational equilibria in solution of small peptides is still necessary and this can only be done by using spectroscopic techniques. The best way to proceed is to apply various spectroscopic methods, and possibly to compare a series of structurally closely related compounds, thereby eliciting a conformational picture which is consistent simultaneously with all the methods used and with the various structures.

In this paper, pursuing such a strategy on peptides containing two aromatic residues spaced by glycine units,[1-4] we will analyze the case of H-His-Trp-OH, H-His-Trp-Gly-OH, H-Gly-His-Trp-Gly-OH, H-Gly-His-Gly-Trp-Gly-OH, and H-Gly-His-Gly-Gly-Trp-Gly-OH, as investigated by ^{1}H NMR, circular dichroism, UV absorption, and fluorescence techniques, at different temperatures (in the range +50 to −70 °C) in water and methanol solutions, and for different ionization states (in the pH range ca. 2–11). In this analysis, we will address the particular issue of the charge-transfer (CT) complexes between tryptophan and histidine. The question of whether and to what extent CT complexes involving indole, and in particular the pair indole–imidazolium, really do exist, has been extensively debated in the chemical literature[5-10] and has not been definitely resolved. In the present paper we show that on the basis of UV absorption and NMR techniques CT complexes in the ground state can be ruled out at least in peptides of the type H-Gly-His-$(Gly)_n$-Trp-Gly-OH with both n = 0 and n = 2.

Experimental Section

Materials and Methods. The amino acid derivatives, except the *N*-hydroxysuccinimide esters, were purchased from Fluka. The dipeptide H-His-Trp-OH was a Vega product and H-Gly-Trp-OH was a Serva product.

The synthesis of the histidine-containing oligopeptides is outlined in Schemes I and II. The Z-protected *N*-hydroxysuccinimide esters (I and IV) and the tryptophan peptides H-Trp-Gly-OMe (III) and Z-Gly-Trp-Gly-OMe (IX) were prepared in our laboratory and described in the first paper of this series.[11] The hydrazide derivatives Z-His-$NHNH_2$ (VIII) and Z-Gly-His-$NHNH_2$ (VI) were prepared according to the method of Holley and Sondheimer.[12] The fully protected tri- and tetrapeptides (XI, XIV) and the precursors of the penta- and hexapeptides (XIX, XXIV) were synthesized by Dr. R. Guarnaccia in our laboratory using a classical stepwise procedure. They were characterized by melting point and microanalysis (Table I).

Thin-layer chromatography was performed on silica gel HF_{254} precoated plates. Solvent mixtures and developing agents were the same as those used in the case of the tryptophan family.[11] The

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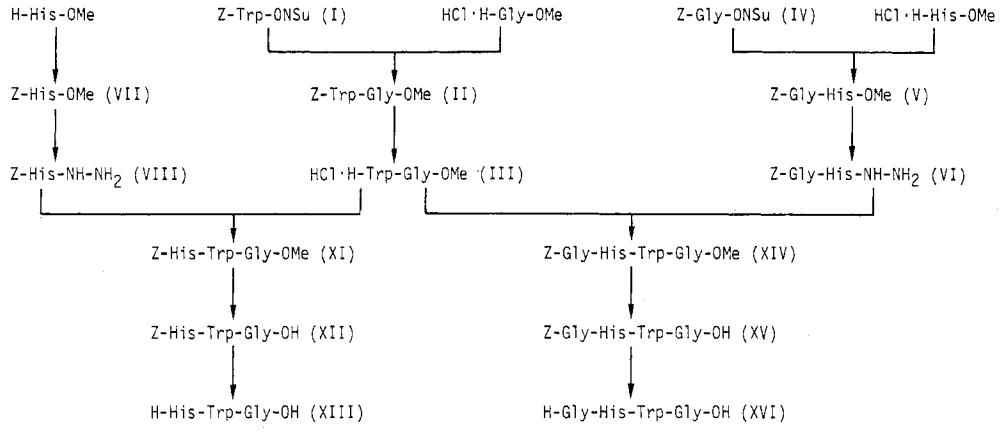
Scheme I. Synthesis of the Tri- and Tetrapeptides



Scheme II. Synthesis of the Penta- and Hexapeptides

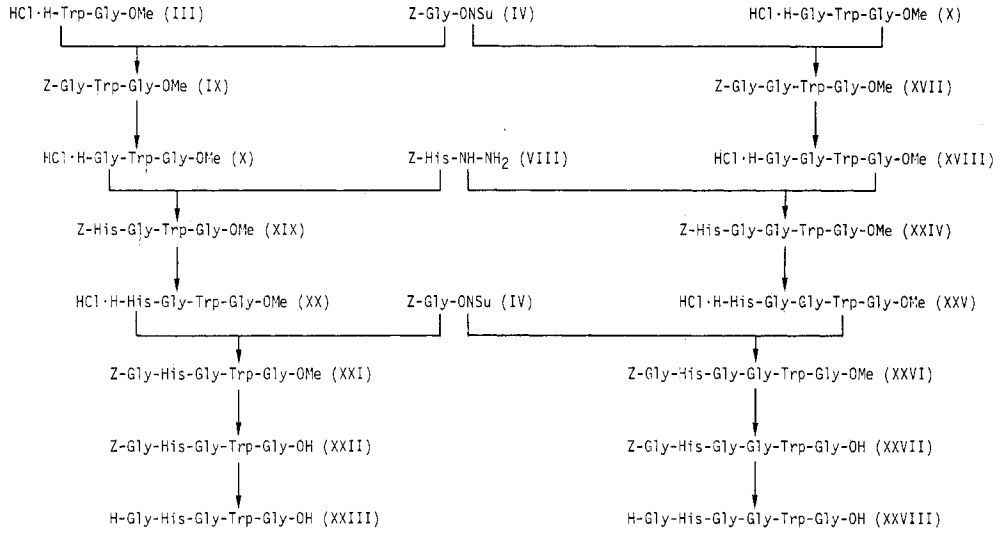


TABLE I: Characterization of Intermediate Products

compd	mp, °C	calcd			found		
		C	H	N	C	H	N
Z-His-Trp-Gly-OCH_3	207–212	61.53	5.53	15.38	61.49	5.68	15.57
Z-Gly-His-Trp-Gly-OCH_3	192–193	59.69	5.51	16.24	59.69	5.57	16.49
Z-His-Gly-Trp-Gly-OCH_3	201–202	59.69	5.51	16.24	59.19	5.42	16.93
Z-His-Gly-Gly-Trp-Gly-OCH_3	193 (dec)	58.17	5.49	16.96	58.03	5.57	16.81

Pauly reagent[13] was used for the specific detection of histidine-containing peptides. The melting points were determined with a Tottoli instrument and are uncorrected. The microanalyses were performed by the microanalytical laboratory of the Technisch-Chemisches Laboratorium of the ETH, Zurich.

The aqueous solutions for the spectrophotometric measurements were prepared with quartz-redistilled water. The pH was adjusted by adding small amounts of 0.1 N $HClO_4$ or 0.1 N NaOH and was measured by inserting a microelectrode directly into the cuvettes. All measurements were made at room temperature. The concentrations of the tetra- and hexapeptide solutions were calculated on the basis of an extinction coefficient of 5530 M^{-1} cm^{-1} at 280 nm. UV-absorption spectra were recorded with an Acta M VI spectrophotometer and fluorescence spectra with an Aminco SPF 1000 spectrofluorimeter. The corrected emission spectra were obtained by excitation at 279 nm. The bandwidths for excitation and emission were 5 and 1 nm, respectively. For measurement of the quantum yield, both bandwidths were set at 2 nm, in order to minimize photodecomposition of tryptophan. The absolute quantum yields were determined by comparison with the quantum yield of tryptophan (0.144).[3] CD spectra were measured on a Jasco J40AS dichrograph with 1 and 10 mm path length cells.

The ^1H NMR spectra were obtained by using 5–10 mM solutions on a Bruker HXS 360 spectrometer. Chemical shifts in D_2O solutions are relative to internal TSP and were corrected for the small pH dependence of the reference compound.[14] The pH meter readings were used without corrections. The low-temperature measurements were carried out by using methanol solution as described in a previous paper.[15] The coupling constants of the ABX spin systems (computed with program LAME) were used to calculate the rotamer distribution around the dihedral angle χ_1, according to the method of Kopple[16] since the method of Feeney,[17] used in our previous papers,[4,15] led to negative fractions of G^+ for the tryptophan residue of H-Gly-His-Trp-Gly-OH. Apparently, the J_T and J_G values used by Feeney for his approach are not valid in this particular case. A detailed comparison of several methods of evaluation of the relative conformer content is given elsewhere.[18]

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TABLE II: UV-Absorption Properties of Cooligopeptides of Glycine, Histidine, and Tryptophan in Water at pH 3.1[a]

compd	λ_1	λ_2	$\lambda_{min,1}$	λ_3	$\lambda_{min,2}$	R_0	R_1	R_2	R_3
H-His-Trp-OH	287.5	279	245	218	204	2.68	1.18	6.79	1.39
H-His-Trp-Gly-OH	287.5	279	245	216.5	205	2.55	1.18	6.87	1.25
H-Gly-His-Trp-Gly-OH	288	279.5	246.6	218.5	206.5	2.60	1.17	7.01	1.24
H-Gly-His-Gly-Trp-Gly-OH	287.5	279	248.5				1.18		
H-Gly-His-Gly-Gly-Trp-Gly-OH	287.5	279	245	217.5	207	2.70	1.17	7.11	1.19

[a] λ_1, λ_2, and λ_3 are the wavelengths of the various absorption maxima and $\lambda_{min,1}$ and $\lambda_{min,2}$ are absorption minima. $R_0 = \epsilon(\lambda_2)/\epsilon(\lambda_{min,1})$, $R_1 = \epsilon(\lambda_2)/\epsilon(\lambda_1)$; $R_2 = \epsilon(\lambda_3)/\epsilon(\lambda_2)$; $R_3 = \epsilon(\lambda_3)/\epsilon(\lambda_{min,2})$. λ in nanometers and $\epsilon(\lambda_2) = 5530$ M^{-1} cm^{-1}.

Synthesis. Fully Protected Penta- (XXI) and Hexapeptides (XXVI). The Z-protected peptides XIX and XXIV were hydrogenolysed in the presence of a slight excess of HCl. The crude products XX and XXV were dissolved in water, and 2 equiv of $NaHCO_3$ were added, followed by addition of 1 equiv of Z-Gly-ONSu in concentrated methanolic solution. The reaction mixture was stirred at room temperature and the reaction was monitored with thin-layer chromatography. After 3 h the resulting suspension was filtered and the residue was washed with methanol. The recovered products were used for the next steps without purification.

Z-Protected Oligopeptide Acids (XII, XV, XXII, and XXVII). The protected peptide was suspended in a small quantity of methanol and hydrolyzed by adding 1–2 equiv of 1 N NaOH. After about 1 h at room temperature the reaction mixture became clear and completion of the reaction was determined by thin-layer chromatography. The solution was acidified with 1 N HCl to pH 5–6 and evaporated under reduced pressure at room temperature.

Free Oligopeptides (XIII, XVI, XXIII, and XXVIII). The free oligopeptide was prepared by catalytic hydrogenolysis of the corresponding Z-protected oligopeptide acid, obtained above. The reaction was carried out at room temperature and at 1-atm pressure in a mixture of methanol and water (9/1, v/v) in the presence of palladium on charcoal (5%, 50 mg per 100 mg of peptide). The reaction was monitored by thin-layer chromatography and was complete after 1–2 h. The catalyst was filtered off (membrane filter, 0.45 μm) and the filtrate was evaporated under reduced pressure at room temperature to dryness. The crude product (including the commercial dipeptides) was purified by chromatography on a Sephadex LH20 column (2.6-cm diameter V_t = 270 mL). The column was loaded with 2–50 mg of peptide in 1–3 mL of solution. The eluent was 10^{-3} M $HClO_4$ or 0.05 M acetic acid. This procedure was very effective in removing the oxidation products of the tryptophan residue,[19] which otherwise markedly affect the spectroscopic data.

For a racemization test, H-Gly-His-Trp-Gly-OH was hydrolyzed in 6 N HCl at 110 °C for 3 h. The reaction mixture was evaporated under reduced pressure to dryness. The hydrolyzed product was chromatographed on a Sephadex LH20 column with 10^{-3} M $HClO_4$ as eluent, which yielded pure fractions of histidine and tryptophan. The UV absorption and ellipticity of these fractions were measured, and, by comparison with standard absorption and ellipticity values, the concentration and optical purity of both histidine and tryptophan were determined. No racemization was detected by this criterion (the sensitivity is ≤2%).

Results and Discussion

Synthesis. The synthesis of histidine- and tryptophan-containing peptides was carried out by the classical stepwise procedure outlined in Schemes I and II. The π-nitrogen (in position 3 of the imidazole ring) is a good nucleophile and can lead to by-products in peptide-coupling reactions. Furthermore, the imidazole ring is a good base and can catalyze racemization of amino acid residues. These problems were bypassed by using the azide method, which does not require protection of the imidazole ring,[12,20] in all the coupling steps involving a histidyl residue as the carboxyl-activated moiety. The azides, prepared in situ with $NaNO_2$, were reacted with the appropriate oligopeptide esters. All other coupling steps were made with the *N*-hydroxysuccinimide ester method. The free peptides were obtained by basic hydrolysis and catalytic hydrogenolysis and were purified as described in the Experimental Section. The colorless products were pure, as established by thin-layer chromatography and UV and ^1H NMR spectroscopy measurements. No racemization of end products was detected by high-field ^1H NMR spectroscopy (searching for diastereomers), or by the spectroscopic method outlined in the Experimental Section.

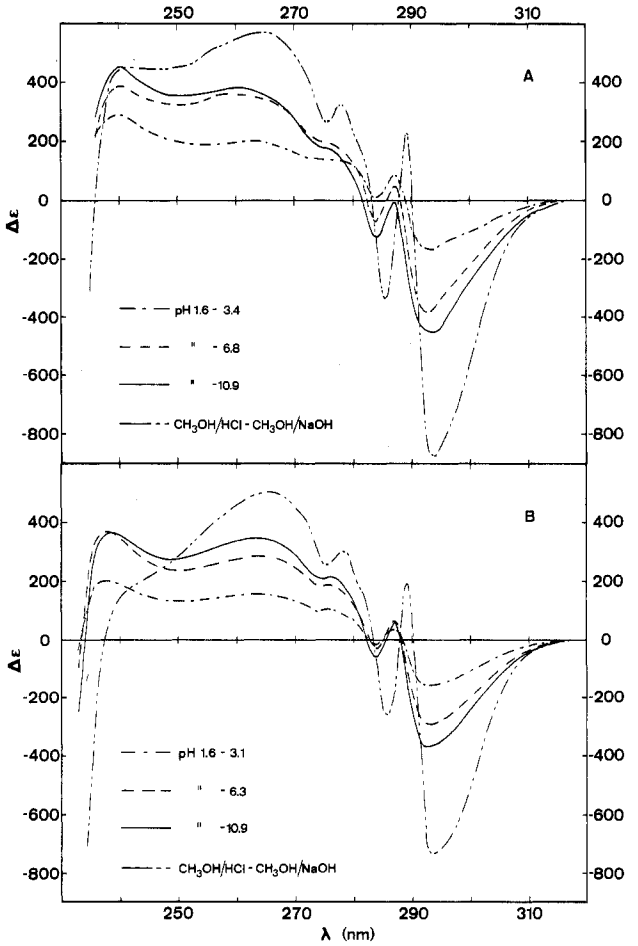

Figure 1. UV pH-difference spectra of H-His-Trp-OH (A) and H-Gly-Trp-OH (B). The p*K* of the histidine residue is 6.4.

UV Absorption and Circular Dichroism. Our group, utilizing simpler peptides,[1,15] has already studied the influence of primary structure, pH, and temperature changes on indole and imidazole. The absorption spectra (Table II) are dominated by the transitions of the indole chromophore, since the extinction coefficient of the imidazole π–π^* transition is about 6 times smaller than that of tryptophan. Moreover, above 235 nm the UV absorption is due only to the indole chromophore and therefore its interpretation is easier.

The intensities of the L_a and L_b transitions are practically the same as those for simple tryptophan peptides.[1] The maximum at lower wavelength is at 218 nm, as found also for the cooligo-

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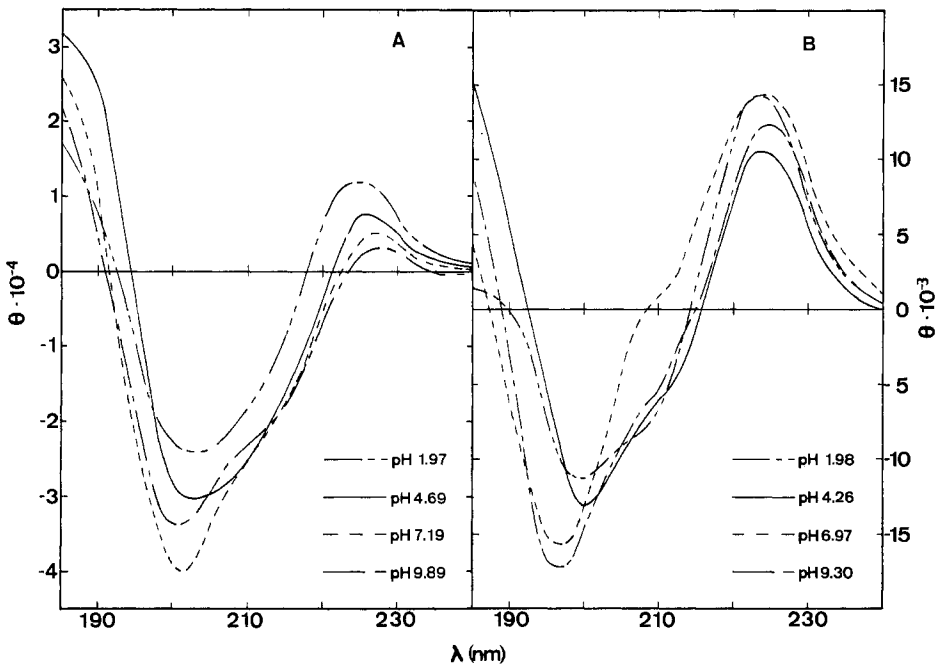

Figure 2. CD spectra of the different ionic forms of H-Gly-His-Trp-Gly-OH (A) and H-Gly-His-Gly-Gly-Trp-Gly-OH (B) in water.

peptides of tryptophan and glycine.[1] However, the extinction coefficient is higher, due to the absorption of the histidine chromophore.

Figure 1 shows the influence of pH on the absorption properties of H-His-Trp-OH (A) and H-Gly-Trp-OH (B). Note, for instance, that the deprotonation of the carboxyl group gives the largest effect at 240 nm, whereas the deprotonations of the imidazole ring and of the amino group have less of an influence. It is interesting that all three deprotonation steps produce shifts of the absorption bands in the same direction, namely, toward the red. This is in agreement with observations of Donovan et al.[21,22]

The difference spectra of the two dipeptides in methanol (acid minus basic solution) are also very similar. However, the spectral perturbations are 50–100% more intense than those for water. This is not unexpected since the charged groups are less solvated in methanol than in water and therefore their influence through the bond should be larger. Moreover, the comparison shows that even in methanol the histidine ring, despite this larger charge effect, has no marked influence on the L_a and L_b transitions of the indole chromophore. For more details about these spectroscopic properties, the reader is referred to ref 18.

The CD spectra of the four ionic species of H-Gly-His-Trp-Gly-OH and H-Gly-His-Gly-Gly-Trp-Gly-OH are shown in Figure 2. The significant maximum at 224 nm can safely be attributed to the B_b transition of the indole chromophore, since at this wavelength the histidine oligopeptides have not more than one-fourth as much ellipticity.[15]

Compare the two compounds and notice that in the hexapeptide, where two glycyl residues are spacing the two aromatic groups, both of the Cotton effects at about 198 and 224 nm have more or less the same intensities. The pH dependence of the CD spectrum of H-Gly-His-Gly-Gly-Trp-Gly-OH is relatively small but comparable to that of the tetrapeptide. The deprotonation of the imidazolium ring is attented by the formation of a positive shoulder at 208 nm, which disappears upon deprotonation of the amino group. The minimum at 200 nm is shifted to 197 nm and becomes more intense upon deprotonation of the imidazolium ring. The CD spectrum of the hexapeptide corresponds to the sum of the two spectra of the tripeptides H-Gly-His-Gly-OH and H-Gly-Trp-Gly-OH. As is the case of the hexapeptides of the phenylalanine and tryptophan families,[2] this additivity can indicate that there is no specific intramolecular interaction between the two aromatic rings.

Let us now consider in more detail the case of the tetrapeptide, where, because the two aromatic residues are next to each other, the spectroscopic and conformational properties are expected to be more interesting. This expectation is borne out also from our earlier studies with the Trp-Trp peptide family where "anomalous" spectroscopic properties derived from a particular intramolecular interaction.[2,23] In fact, in contrast to the "control" hexapeptide, the CD spectra of the tetrapeptide cannot be reconstituted as either the sum of the spectra of the two possible tripeptides H-Gly-His-Gly-OH and H-Gly-Trp-Gly-OH or the sum of the spectra of the dipeptides H-Gly-His-OH and H-Trp-Gly-OH, or a combination of all. The spectra for the solutions at the various pHs are similar in shape, but the negative Cotton effect at 202 nm increases upon deprotonation of the carboxyl group and of the imidazolium ring, whereas the positive band at 224 nm decreases. This must reflect either a change of the conformational equilibrium or a direct influence on the indole transitions (e.g., via electronic effects).

In conclusion, CD spectra are shown to be sensitive to the distance between the two aromatic rings and some spectroscopic differences between the tetra- and hexapeptides can safely be attributed to conformational effects, and in particular to differences in intramolecular interactions.

Circular dichroism does not, however, enable one to specify the nature of such interactions. It furnishes a first level of screening information, upon which a detailed NMR analysis can efficiently be based. This is presented in the next sections.

^1H NMR. pH Dependence of Chemical Shifts. The ^1H NMR spectra of H-His-Trp-OH and H-Gly-His-Trp-Gly-OH were investigated as a function of pH with aqueous solutions. The aim of this study was to show whether and to what extent the charged end groups influence the side-chain conformation, and whether an interaction between the two rings would be pH dependent.

Figure 3A shows the titration curves for the aromatic protons of the dipeptide H-His-Trp-OH. The protons of the histidine ring, as expected, undergo the largest changes. For the dipeptide, the pK's of the amino group and the imidazole ring are very close, so that it is not clearly distinguishable that there are two titration steps. By a comparison with the NMR data of Tanokura et al.[24]

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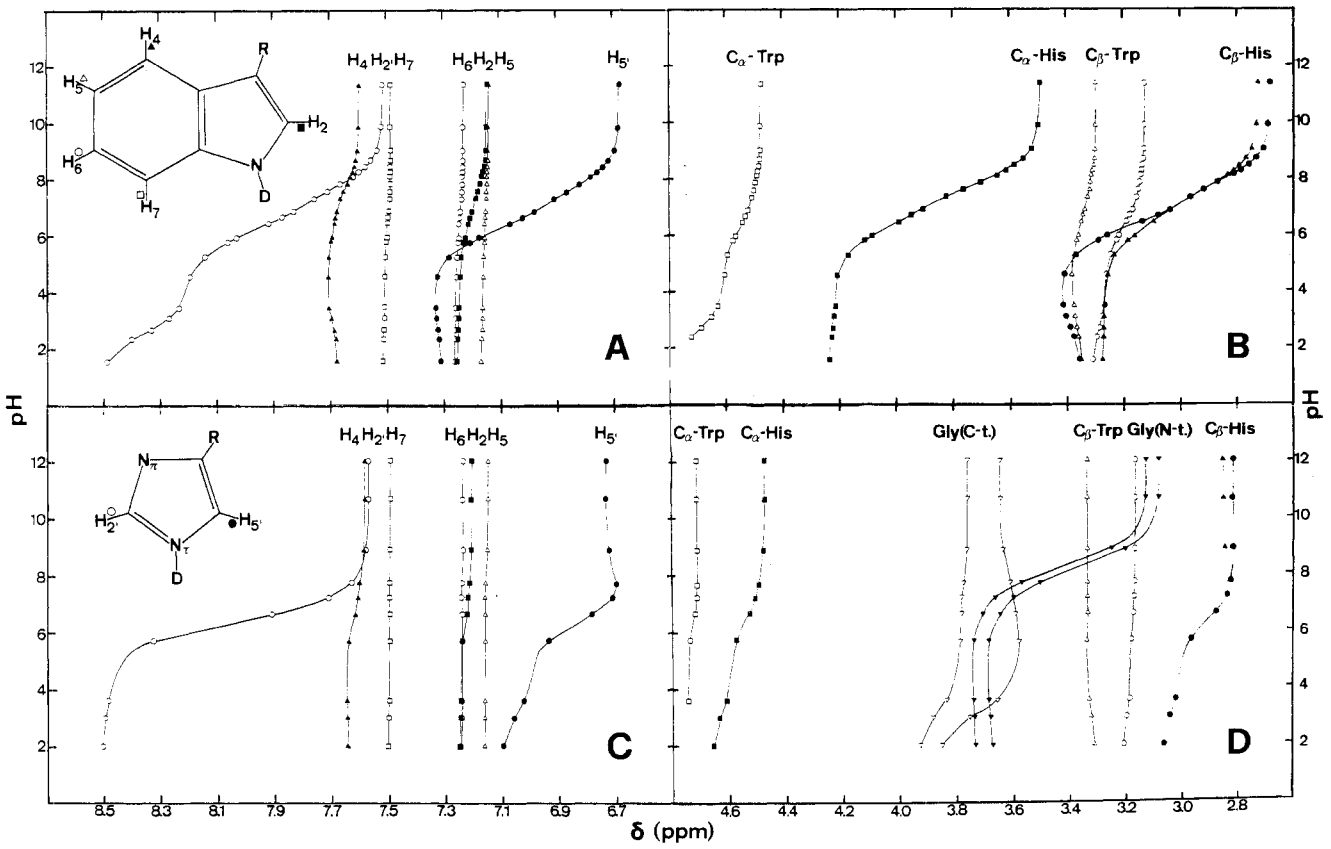

Figure 3. ^{1}H NMR titration curves of H-His-Trp-OH (A, B) and H-Gly-His-Trp-Gly-OH (C, D).

for other dipeptides of the type H-His-X-OH (X = Ala, Leu, Ser, Lys, or Gly), it can be established that the ring current of the tryptophan residue has its largest effect in the cationic state (a shift to the higher field of 0.46 ppm for $H_{2'}$ and 0.15 ppm for $H_{5'}$, with respect to the other dipeptides). The pH dependence of this ring current effect can be interpreted on the basis of a pH-induced conformational change, in particular by a specific interaction between the protonated histidine ring and the carboxylate group as was reported for the C-terminal -His-Gly-O^- peptide sequence.[15,25]

Upon protonation of the carboxyl group, the ring current effect is reduced, and consequently there is an unusually large titration shift for the $H_{2'}$ proton. For the tetrapeptide (Figure 3C) the influence of the ionization of the amino group upon the chemical shift of the histidine ring protons is generally smaller than for the dipeptide, probably due to the larger distance from the amino group. The small anomalous shift in the titration behavior of $H_{5'}$ (i.e., the shift to higher field upon protonation of the amino group) can be attributed to an interaction between the ammonium group and the free nitrogen of the histidine ring. On the average, this brings the $H_{5'}$ proton closer to the indole ring, which gives rise to a small ring current effect. Of course, in the anionic state there is still an interaction (but weaker) between the amino group and the histidine ring (similar to H-Gly-His-O^-[15]). The carboxylate group's titration effect on the resonances of the $H_{5'}$ proton of the histidine ring must be due to a conformational change, since the carboxylate is two residues away from the histidine ring.

After this analysis of the aromatic histidine protons (some NMR properties of the aromatic tryptophan protons will be discussed in the section on charge transfer), let us now consider the aliphatic C_α and C_β protons of the aromatic residues. Figure 3, B and D, shows the NMR titration curves of the aliphatic protons of the di- and tetrapeptides. Surprisingly, in the tetrapeptide the protons of the histidine residue are sensitive to the protonation of the carboxylate group even more than in the dipeptide, despite the larger distance. This could imply a somewhat preferred conformation in the cationic state, where the proton resonances are shifted to higher field by the neighboring tryptophan ring.

Let us now direct our attention to the anisochrony of the methylene protons of the two aromatic residues. This anisochrony is another NMR parameter useful for conformational analysis. As outlined in our previous papers,[4,15] large anisochrony values can be correlated, with some restrictions, to "conformational rigidity".[38] Note the enhanced anisochrony for the histidine C_β protons in the cationic state of the dipeptide. The effect is twice as large as in the model peptide H-His-Gly-OH.[25] This could be ascribed to a lower flexibility of the histidine side chain and/or to an amplification effect brought about by ring current effect of the neighboring tryptophan moiety.

Anisochrony can also be observed in the glycine methylene groups of the tetrapeptide. Figure 3D shows that the two glycine residues can be easily identified from their pH dependences. The resonances of the N-terminal glycine show a larger shift to lower field upon protonation of the adjacent amino group, and a pK of 8.2 has been determined. The C-terminal glycine is influenced by the titration of the carboxyl group with a decrease of the anisochrony and with a shift of its resonances. The anisochrony is largest (0.21 ppm) in the cation. A plausible explanation is the following: Upon protonation of the imidazole ring, the interaction between the ammonium group and the uncharged imiazole ring (see above) disappears, and another conformation stabilized by the interaction of the two end groups may become more populated. Such an interaction is in agreement with the influence of the carboxylate group titration on the chemical shifts of most histidine protons. We will return to this point in the Conclusion.

^{1}H NMR. Temperature Dependence of Chemical Shifts. The ^{1}H NMR spectra of the whole histidine family including H-His-Trp-OH and H-His-Trp-Gly-OH were measured on alkaline methanol solutions in the temperature range 200–320 K. In the

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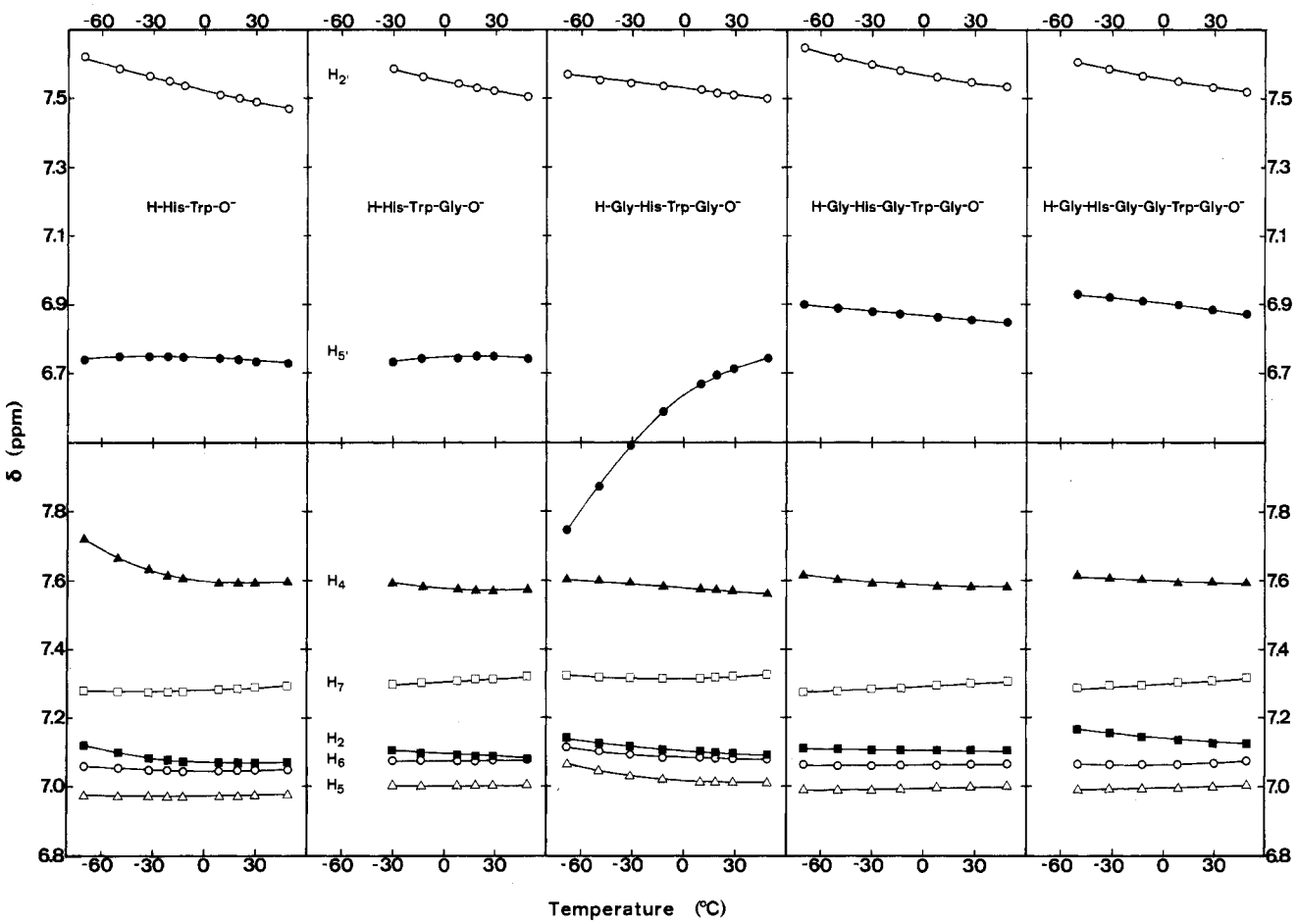

Figure 4. Temperature dependence of the chemical shifts of the aromatic protons.

case of the tryptophan peptides studied earlier it was found that all those peptides with a larger temperature dependence of their NMR properties[4] showed some unusual features also in their CD spectra.[1,23] In turn, this was interpreted as an indication of an anomalous conformational behavior. Therefore, the NMR low-temperature study is used here as a screening for all of the histidine peptides. We present here only the data which are directly relevant to the conformational properties (the detailed NMR analysis is presented elsewhere[18]).

Let us first consider the aromatic protons. Figure 4 shows the temperature dependence of the chemical shifts of the aromatic protons in the five investigated peptides. The only sizable effect is present in the tetrapeptide. In this case, $\delta(H_{5'})$ shows a strong shift to higher field of about 0.5 ppm. Since the aromatic resonances per se generally have only a little structure and temperature dependence, this must be due to a large ring current effect arising from an interaction with the tryptophan ring. By comparing the chemical shifts of the aromatic protons at 50 °C, one observes that actually only the $H_{5'}$ resonance of the histidine ring shows significant structure dependence. Thus, in H-His-Trp-OH, H-His-Trp-Gly-OH, and H-Gly-His-Trp-Gly-OH where the histidine residue is adjacent to the tryptophan residue, the $H_{5'}$ signal is about 0.1 ppm at higher field than in the penta- and hexapeptides or in the shorter histidine oligopeptides.[15]

Let us now examine the temperature behavior of the glycine resonance shown in Figure 5. In H-Gly-His-Trp-Gly-OH the N-terminal glycine resonances are at somewhat higher field, similar to H-Gly-Trp-Trp-OH and H-Gly-Trp-Trp-Gly-OH. Interpreting this shift to be a small ring current effect would lead us to the conclusion that the aromatic residue in position 2 is oriented toward the amino end, this effect being more pronounced than in the case of the peptides with a single aromatic residue.[4,26,27]

The anisochrony of the N-terminal glycine protons of H-Gly-His-Trp-Gly-OH increases asymmetrically from 0 to 0.22 ppm with decreasing temperature: only one of the two resonances is shifted to lower field; the other remains unchanged. The same observation was found for H-Gly-Trp-Trp-Gly-OH.[4] As far as the C-terminal glycine protons are concerned, they all have an average chemical shift of 3.70 ± 0.05 ppm. Their anisochrony is much larger than that for the N-terminal glycines. The increase of the anisochrony with decreasing temperature is the largest for H-Gly-His-Trp-Gly-OH, where at −70 °C $\Delta\delta$ amounts to 0.6 ppm, similar to H-Gly-Trp-Trp-Gly-OH. Such large anisochrony values of the glycine protons could be explained by an increase of the conformational rigidity of the peptide backbone, as discussed in detail in our previous papers.[4,15]

Other interesting similarities to the tryptophan series are found in the temperature behavior of the internal glycine protons of the penta- and hexapeptides. For example, in the pentapeptides the anisochrony increases markedly when the temperature is decreased. Moreover, for both pentapeptides, there is a marked asymmetry; namely, one of the two resonances remains nearly constant with a chemical shift (3.93 ppm) that at room temperature is identical with that of the internal glycine of H-Gly-Gly-Gly-OH. In contrast to that, no large temperature effects are observed for the internal glycine protons in the hexapeptide, which is consistent with a larger conformational flexibility.

Let us now consider the temperature dependence of the aliphatic protons of the histidine and tryptophan residues, as shown in Figure 5 (the assignment of the β-protons to the resonances A and B of the ABX system is discussed in the next section). Note the change of the anisochrony sign of the histidine β-protons in H-His-Trp-OH: $\Delta\delta$ changes from 0.20 to −0.20 ppm at −70 °C. It is striking that only the resonance of the H_S proton shifts to lower field, the

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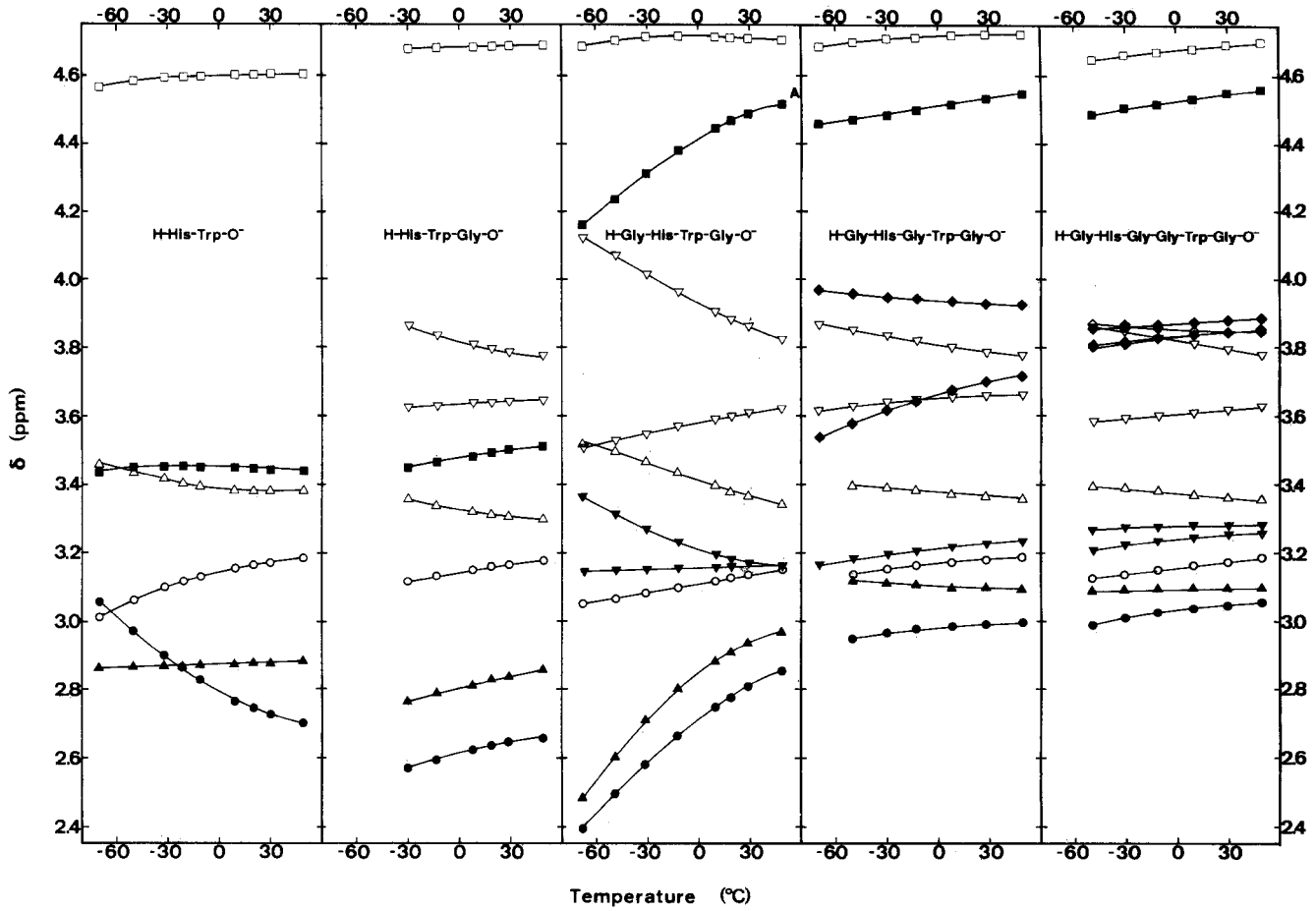
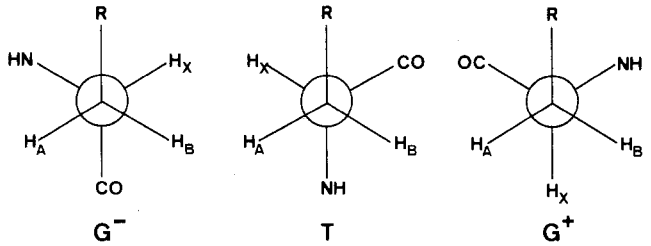


Figure 5. Temperature dependence of the chemical shifts of the aliphatic protons: ABX protons of the histidyl (●, ▲, ■) and tryptophyl (○, △, □) residues and C-terminal (▽), N-terminal (▼), and internal (◇, ◆) glycine protons.

TABLE III: Rotamer Distribution[a] around χ_1 According to Kopple[b]

		histidyl			tryptophyl		
compd	ion	g^-	t	g^+	g^-	t	g^+
H-His-Trp-OH	0++	0.25	0.55	0.20	0.44	0.28	0.28
H-His-Trp-OH	−++	0.08	0.49	0.43	0.47	0.15	0.38
H-His-Trp-OH	−0+			0.38	0.55	0.17	0.28
H-His-Trp-OH	−00	0.39	0.27	0.34	0.51	0.17	0.32
H-Gly-His-Trp-Gly-OH	0++			0.14	0.57	0.36	0.07
H-Gly-His-Trp-Gly-OH	−++			0.16	0.59	0.31	0.10
H-Gly-His-Trp-Gly-OH	−0+			0.19	0.56	0.27	0.17
H-Gly-His-Trp-Gly-OH	−00	0.45	0.36	0.18	0.57	0.24	0.19



[a] In the papers of our group, g^-, t, and g^+ signify the rotamer populations, and G⁻, T, and G⁺ the actual geometrical isomers. [b] $J_T = 12.40$ Hz; $J_G = 3.25$ Hz.

other resonance remaining constant. A similar observation was made for H-Trp-Trp-OH.[4]

Again the largest temperature dependence is found in the tetrapeptide, where the signals of the α- and β-protons of histidine are strongly shifted to higher field. The tryptophan analogue H-Gly-Trp-Trp-Gly-OH shows exactly the same behavior for the corresponding residue in position 2.[4] Therefore, it seems that both tetrapeptides prefer similar conformations as the temperature is decreased. In the case of the tryptophan side chain the largest temperature effect is also found in the di- and tetrapeptides. The anisochrony of their β-protons increases about 0.25 and 0.30 ppm in the investigated temperature range.

^1H NMR. Side-Chain Conformation. In the previous sections we have discussed chemical shifts and anisochrony data. We will now consider coupling constants, mostly with the aim of determining the conformation of the aromatic side chains.

The vicinal coupling constants between the aliphatic protons were used to calculate the rotamer distribution around the dihedral angle χ_1. The first problem to be solved was the assignment in the ABX system for the two β-protons. For each case it was

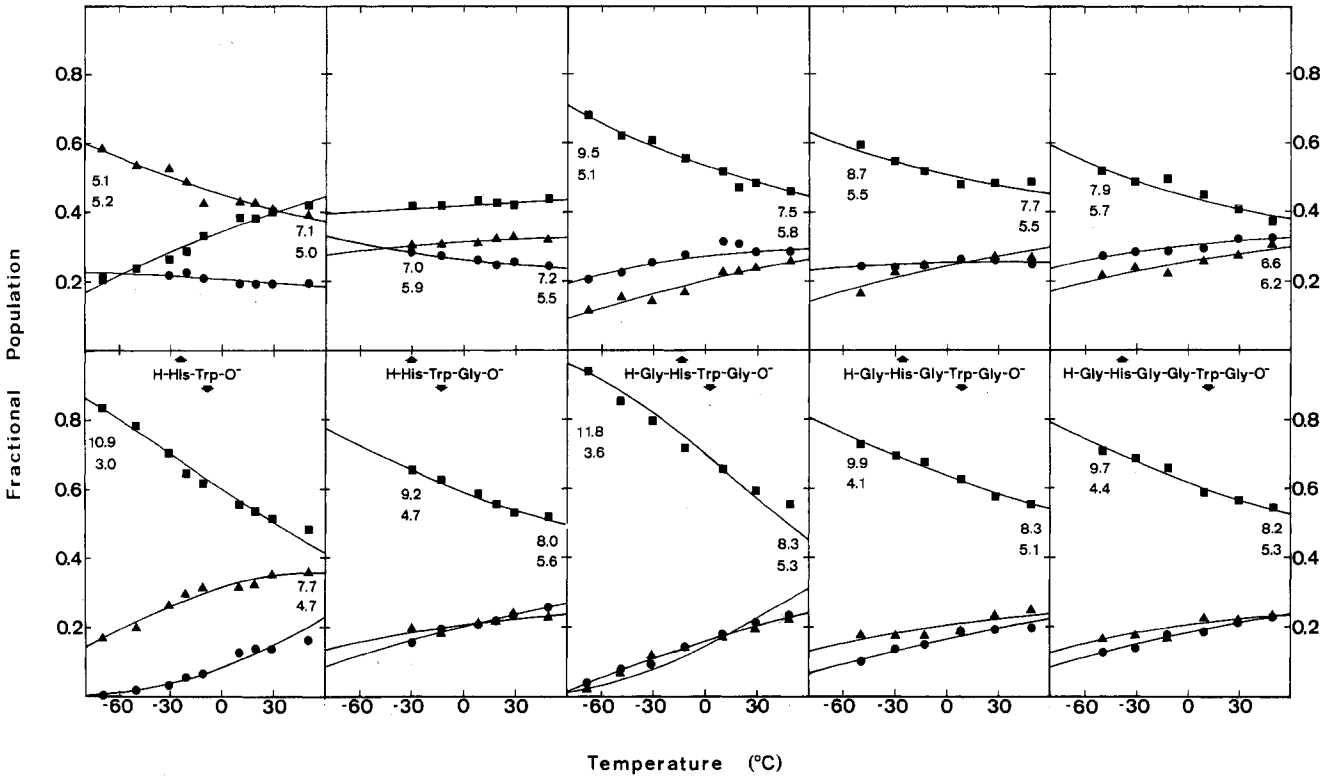


Figure 6. Temperature dependence of the χ_1 rotamer populations. G^- = ■, T = ●, G^+ = ▲. Curves have been calculated by using the thermodynamic parameters reported in Table IV. For each side chain J_{AX} (upper) and J_{BX} are given in hertz and were measured at the temperature extremes.

TABLE IV: Thermodynamic Properties[a,b] Elaborated from the Temperature Coefficients of the Rotamer Distribution[c] in Basic Methanol

compd	ΔH_1	ΔH_2	ΔS_1	ΔS_2
H-*His*-Trp-OH	−1.3 (0.2)	−1.1 (0.3)	−0.4 (0.7)	−4.9 (1.0)
H-His-*Trp*-OH	1.5 (0.2)	4.2 (0.9)	4.3 (0.8)	11.4 (3.5)
H-*His*-Trp-Gly-OH	0.1 (0.2)	−0.4 (0.2)	−0.4 (0.8)	−2.4 (0.5)
H-His-*Trp*-Gly-OH	0.9 (0.5)	1.4 (0.2)	1.3 (1.7)	3.1 (0.5)
H-Gly-*His*-Trp-Gly-OH	1.4 (0.4)	0.8 (0.1)	3.0 (1.5)	1.5 (0.4)
H-Gly-His-*Trp*-Gly-OH	3.6 (1.6)	2.5 (0.4)	10.0 (6.4)	6.3 (1.5)
H-Gly-*His*-Gly-Trp-Gly-OH	1.0 (0.6)	0.4 (0.3)	2.1 (2.2)	0.0 (1.1)
H-Gly-His-Gly-*Trp*-Gly-OH	0.9 (0.5)	1.5 (0.3)	1.1 (2.0)	2.7 (1.3)
H-Gly-*His*-Gly-Gly-Trp-Gly-OH	1.0 (0.3)	0.7 (0.3)	2.3 (2.0)	1.8 (1.3)
H-Gly-His-Gly-Gly-*Trp*-Gly-OH	0.9 (0.5)	1.3 (0.2)	1.3 (1.8)	2.4 (0.8)

[a] $\Delta H_1 = H(G^+) - H(G^-)$, $\Delta H_2 = H(T) - H(G^-)$ in kcal/mol. $\Delta S_1 = S(G^+) - S(G^-)$, $\Delta S_2 = S(T) - S(G^-)$ in eu. [b] In parentheses the 95% confidence limits are given. [c] According to the method of Kopple.

assumed that the same assignment holds for basic solutions as for the free amino acids, i.e., that the H_S proton of the histidine residue and the H_R proton of the tryptophan residue absorb at higher field (see discussions in our prevous papers[4,15]). Results for the pH dependence of the di- and tetrapeptides are shown in Table III.

Because of the isochrony of the β-protons of the histidine residues, in some cases only the population of the G^+ rotamer could be estimated. In H-His-Trp-OH, g^+ is largest for the cation and decreases upon protonation of the carboxyl group. On the other hand, g^- is in the cation very low. A similar behavior was reported for H-His-Gly-OH, where an interaction between the carboxyl group and the protonated histidine ring has been proposed.[25] Because of steric hindrance, such an interaction would only be possible in the T and G^+ rotamers. This would explain the unusually small population of G^- and is a good argument that the assignment of the β-protons is correct, since otherwise g^- and t should be exchanged.

This pH-induced change in the histidine side-chain conformation is attented by an increase of the G^+ rotamer in the tryptophan residue of the cationic form of the dipeptide. This set of experimental observations, coupled with the information contained in the titration curves of Figure 3, suggests a specific conformation for the cationic dipeptide, as will be discussed in the Conclusions.

In the case of the tetrapeptide, the rotamer distribution shows a smaller pH dependence than that for the dipeptide. In both residues the G^+ rotamer is the least populated one. The G^- rotamer is dominant in the tryptophan side chain and this seems to be the case also in the histidine residue (Table III).

Let us now consider the temperature dependence of the side-chain rotamer distribution. This has been carried out by using the anionic form of all the compounds in the temperature range −70 to +50 °C in alkaline methanol. The results are shown in Figure 6, which also shows the vicinal coupling constants J_{AX} and J_{BX} of the ABX systems at the two extreme temperatures. All tryptophan residues have diverging pairs of coupling constants with decreasing temperature. For the histidine side chain, the behavior is not so uniform and the differences between the coupling constants are smaller. In both side chains the largest effects are observed for the di- and the tetrapeptides.

The lines in Figure 6 were calculated with the parameters obtained from a thermodynamic analysis which has been extensively discussed in our previous papers.[4,15] The parameters are reported in Table IV. The agreement between the calculated curves and the experimental points is satisfactory for the tri-, penta-, and hexapeptides. In the case of the di- and tetrapeptides, however, there are significant deviations for the tryptophan residue. Rotamer G^- is generally enthalpically preferred in comparison

to T and G^+. The only exception is the N-terminal histidine residue in the di- or tripeptide. As seen in Figure 6 the G^- rotamer is also the most populated one at room temperature despite an unfavorable entropic term (Table IV). By comparing the rotamer fractions of the histidine family with those of the tryptophan family[4] one observes that in the histidine series the tryptophan residue generally shows a larger G^- fraction than the corresponding residue in the tryptophan series. This could be explained by the smaller steric hindrance (imidazole instead of indole) due to the aromatic residue on the amino side.

Charge-Transfer Complexes. Let us consider more specifically the question of the CT complexes. As is well-known, CT complexes are formed by interaction of an electron donor with an electron acceptor. The term "charge-transfer complex" is a simple generalization, which usually describes a situation in which one electron is transferred from a donor to an acceptor, and in which the energy contribution from the covalent dative structure is usually small, while electrostatic and polarization interactions are generally more significant.[28,29] The appearance of a new electronic absorption band, which is not observed in the separate components, is characteristic for CT complexes.

It has been known for several years that indole forms yellow complexes when mixed with concentrated solutions of NAD^+.[5] A broad absorption band, attributed to CT complexes, was observed in the intramolecular system indolylethylnicotinamide;[6] and interactions between imidazolium and indole of the CT type have also been postulated by Shinitzky and co-workers[7,8] on the basis of fluorescence studies.[9] However, Bushueva et al. have provided an alternative explanation[10] of such fluorescence data.

Considering now the UV-absorption properties, it appears that the main argument presented in the literature to argue in favor of the existence of CT complexes is the presence of a hyperchromic effect in the 240-nm region at pH values where the acceptor (imidazole in our case) is positively charged. Therefore, it was important to study in our case the absorption spectra as a function of pH. In particular, the difference spectra of H-His-Trp-OH (Figure 1A) should be considered vis-à-vis the spectra of H-Gly-Trp-OH (Figure 1B), where no histidine is present. It is immediately clear that the pH-induced spectral perturbations shown in Figure 1, A and B, are very similar, both in form and in magnitude. This evidences that the spectral differences are due to perturbations from the end groups and not to CT complexes. In conclusion, UV-absorption spectroscopy does not support in any way the notion presented by other authors that new electronic bands, and therefore possible CT complexes between histidine and tryptophan, exist in the ground state.

Let us consider now the ^1H NMR properties, in particular the aromatic protons of the indole and imidazole rings. If such a complex were present in the ground state of H-His-Trp-OH and H-Gly-His-Trp-Gly-OH, some of the aromatic protons of the two rings should show changed chemical shifts and a strong pH effect should occur, since above pH 7 the histidine ring is deprotonated and no longer acts as a good electron acceptor. However, as shown above, the pH dependence of these resonances is very modest. In the dipeptide H-His-Trp-OH the H_2 and H_4 protons of the tryptophan ring are somewhat (ca. 0.08 ppm) shifted to lower field with protonation of the imidazole ring, but the other aromatic resonances remain constant. In the tetrapeptide, where the relative distance between the two rings is the same, only a much smaller effect is observed. The aliphatic protons of the tryptophan side chain in the two peptides show a similar behavior as in the case of the aromatic protons, namely, a small influence of the histidine protonation in the dipeptide and none in the tetrapeptide. In conclusion, this analysis shows that there is no significant pH-dependent intrinsic interaction between the two rings. This is in agreement with the finding of Bushueva et al.[10] with a 0.1 M mixture of histidine hydrochloride and tryptophan (only small shifts to higher field for both the imidazole (0.06 ppm) and indole (0.03 ppm) resonances were found).

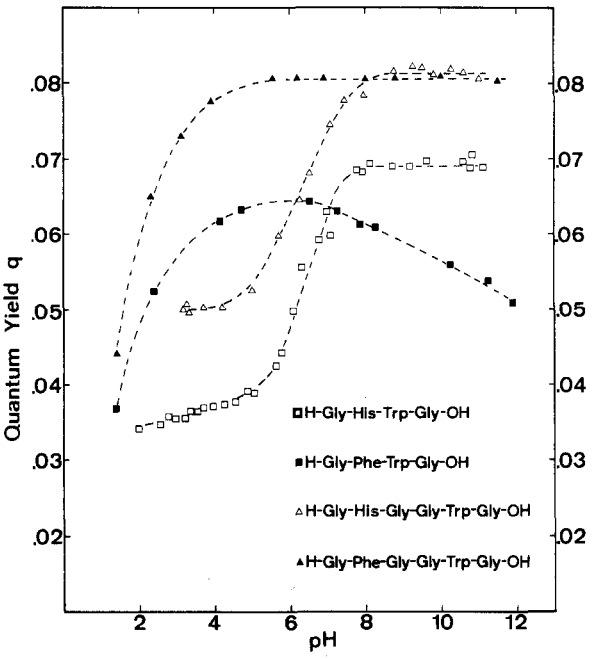


Figure 7. pH dependence of the quantum yield.

Further evidence that there are no CT complexes in the ground state is given by the comparison of the aromatic resonances in H-Gly-His-Trp-Gly-OH with the model peptide H-Gly-Gly-Trp-Ala-OH.[30] Below pH 6 (histidine side chain is protonated) the chemical shifts of the indole protons are identical within 0.01 ppm in the two compounds.

Fluorescence. We have used UV-absorption and NMR data to rule out the presence of CT complexes between tryptophan and histidine in the ground state. Now, we will analyze the fluorescence properties of the His-Trp peptides, asking the question of whether there is an interaction between the two aromatic residues in the excited state. As already mentioned, the fluorescence properties of indole have been studied in conjunction with the question of CT complexes.[7,8,10] The fluorescence properties of the peptides investigated in this work are shown in Figure 7. In the tetrapeptide H-Gly-His-Trp-Gly-OH the two aromatic residues are vicinal to each other and therefore it might be expected that this compound would have a fluorescence behavior similar to that for the His-Trp derivatives investigated by Shinitzky et al.[8] The titration curves in Figure 7 show that this is so: protonation of the imidazole ring brings about a marked quenching of the indole fluorescence both in the tetrapeptide and also in the hexapeptide, where the two aromatic residues are separated by two glycyl residues. A comparison with the analogous peptides of the phenylalanine family can be useful in this regard, since under our conditions (λ_{ex} = 279 nm) the phenylalanine chromophore does not contribute to the observed fluorescence. Such a comparison confirms that the decrease of the quantum yield of the histidine- and tryptophan-containing peptides between pH 9 and 4 is due to an interaction with the protonated imidazole ring. The protonation of the amino group, two residues away, has no effect on the fluorescence quantum yield. It is therefore most striking that in the hexapeptide H-Gly-His-Gly-Gly-Trp-Gly-OH, where the protonated imidazole ring is separated from the fluorophore by two glycyl residues, the fluorescence is quenched nearly as much as in the tetrapeptide. Therefore, the quenching efficiency of the imidazolium ring must be larger than that of the protonated amino group.

This observation leads us to the question of the quenching mechanism. For tackling this point, let us consider first some literature data. For the intermolecular indole/N_α-acetyl-

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TABLE V: Fluorescence Properties of Histidine- and Tryptophan-Containing Peptides

compd	pK	q_0[a]	q[b]	$\Delta q/q_0$	k_Q'/k_f[c]	ref[d]
c-(His-Trp)	6.44	0.085	0.019	0.78	40.8	8
c-(His-D-Trp)	6.20	0.065	0.030	0.54	18.0	8
N_α-Ac-His-Trp-OMe	6.58	0.041	0.024	0.41	17.3	8
N_α-Ac-His-Trp-OH	6.80	0.054	0.026	0.52	20.0	8
H-(His-Trp)$_3$-OH	6.75	0.060	0.017	0.72	42.2	8
H-Gly-His-Trp-Gly-OH	6.4	0.070	0.038	0.46	12.1	this work
H-Gly-His-Gly-Gly-Trp-Gly-OH	6.3	0.082	0.050	0.39	7.7	this work
α-MSH	6.4	0.111	0.094	0.15	1.6	8
ACTH	6.3	0.106	0.094	0.11	1.2	8
glucagon		0.104	0.104	0	0	8

[a] pH 9. [b] pH 4.5. [c] $=\Delta q/(qq_0)$. [d] The quantum yields of the peptides reported by Shinitzky have been corrected for the standard quantum yield of tryptophan of 0.144 (3).

histidinamide hydrochloride system, Shinitzky[31] has determined a Stern–Volmer constant $K_{SV} = 28\ M^{-1}$ according to eq 1, where

$$q_0/q = 1 + k_Q\tau_0[Q] = 1 + K_{SV}[Q] \quad (1)$$

q_0 and q are the quantum yield in the absence and the presence of the quencher, respectively, [Q] is the concentration of the quencher, k_Q is the quenching rate, and τ_0 is the lifetime of the fluorophore. By rearranging this equation we can estimate the quenching amount due to intermolecular interaction in histidine- and tryptophan-containing peptides.

$$\frac{q_0 - q}{q_0} = \frac{\Delta q}{q_0} = \frac{K_{SV}[Q]}{1 + K_{SV}[Q]} \quad (2)$$

On the basis of the above-mentioned K_{SV} value and our peptide concentration (3.6×10^{-5} M) one obtains an intermolecular quenching $\Delta q/q_0$ of about 0.1%, which is negligible compared with the observed total quenching of 46% for the tetrapeptide and 39% for the hexapeptide (Table V). This shows that in our case intermolecular interactions do not play a significant role and the quenching therefore must be intramolecular.

In looking for possible quenching mechanisms one should consider the work by Steiner and Kirby,[32] who studied the fluoresence quenching of acetyltryptophan by various electron scavengers, histidine hydrochloride in particular. They found a parallel decrease of the lifetime with the fluorescence intensity, which was taken to indicate that collisional quenching was the main contribution to the fluorescence quenching. As a consequence, the static quenching part, possibly due to CT-complex formation,[8,9,31] has to be much smaller, since the lifetime is influenced only by dynamic quenching. It seems therefore reasonable to propose that also in the intramolecular tryptophan/histidine system the static quenching contribution is negligible and therefore no CT complex is present. This implies that in eq 3[9] the complex formation constant β is zero.

$$q_0/q = (1 + k_Q'\tau_0)(1 + \beta) \quad (3)$$

From this one obtains directly $k_Q'\tau_0$:

$$k_Q'\tau_0 = q_0/q - 1 = \Delta q/q \quad (4)$$

This quantity can be understood as being the ratio between the quenching rate by collisions and the fluorescence radiation rate. By dividing these values by q_0 one obtains the ratio k_Q'/k_f of the quenching rate k_Q' and the undisturbed fluorescence radiation rate k_f, which is almost constant ($5.5 \times 10^7\ s^{-1}$) in these peptides.[33]

It is now interesting to compare in terms of k_Q'/k_f our compounds with a series of compounds from literature data, in order to have an idea of the relative dynamic flexibility (Table V). It appears that c-(His-Trp), where the two rings are on the same side of the diketopiperazine ring, has the largest quenching rate, twice as much as in c-(His-D-Trp), where the two rings are not on the same side. The collision quenching rate k_Q' of c-(His-Trp) ($2.25 \times 10^9\ s^{-1}$) is in the same range as the maximal transfer frequency ($6.7 \times 10^9\ s^{-1}$) for α-(3-α-naphthylpropyl)naphthalene.[34] If we compare the tetrapeptide H-Gly-His-Trp-Gly-OH with the protected dipeptide Ac-His-Trp-OMe, we can interpret the smaller quenching rate of the tetrapeptide in terms of a smaller probability for the side chains to assume the correct relative geometry for quenching (in other words the tetrapeptide is less flexible than the dipeptide). Such a conclusion cannot be derived from a comparison between the tetra- and hexapeptides because of the different relative positions of the two aromatic residues. However, by comparing the hexapeptide with α-MSH or ACTH, where His and Trp are spaced also by two amino acid residues, a 5-times larger quenching rate is found in the hexapeptide, which agrees quite well with the findings in the CD and NMR section, indicating a flexible conformational state.

Conclusions

The data gathered in this extensive investigation are useful for the general purpose of constructing an accurate data-bank system for the relationship between chemical structures and spectroscopic properties of solutions. The fact that, if one includes previous work, the data refer to a series of parent compounds, having the general structure H-Gly-X-(Gly)$_n$-Trp-Gly-OH (n = 0, 1, 2) with X = Trp, Phe, Tyr, and His, and the fact that this study includes the shorter terms of the series like H-Gly-X-OH and H-X-Gly-OH, adds, in our opinion, to the meaning of this systematic analysis. The compounds have been investigated with different spectroscopic techniques, i.e., UV absorption, circular dichroism, fluorescence, and NMR.

The combined spectroscopic information can be utilized to depict the conformational behavior in solution. Our approach is based on an initial screening of the CD properties. Despite its inherent lack of specificity, this technique is the best in providing rapid qualitative information as to which compound behaves "abnormally". For example, one can readily detect whether pH changes bring about significant overall conformational changes or whether the conformational equlibrium is sensitive to temperature changes.[1]

The step successive to such CD screening was a "zooming" by NMR at the most interesting conformational situations. In two particular cases, it is possible to construct the most preferred conformation by requiring that the model should be consistent with several independent experimental observations. One case is shown in Figure 8A for the cationic dipeptide $^+H_2$-His$^+$-Trp-O$^-$. The conformation represented in the figure is consistent with the following body of information: (i) preferred side-chain conformations, as presented in Table III; (ii) the ring current effect of $H_{2'}$ of the histidine ring, which indicates a proximity to the indole ring, and in a way by which the $H_{5'}$ is less involved then $H_{2'}$; (iii) the pH effect (Figure 3A) which suggests an interaction between the $-COO^-$ and the protonated histidine ring; (iv) the anisochrony of the histidine β-protons (Figure 3B), which suggests a decreased flexibility of the side chain. In this conformation, the backbone is oriented in such a way so as to build a hydrogen bond between the protonated π-nitrogen of the histidine ring and the carboxylate

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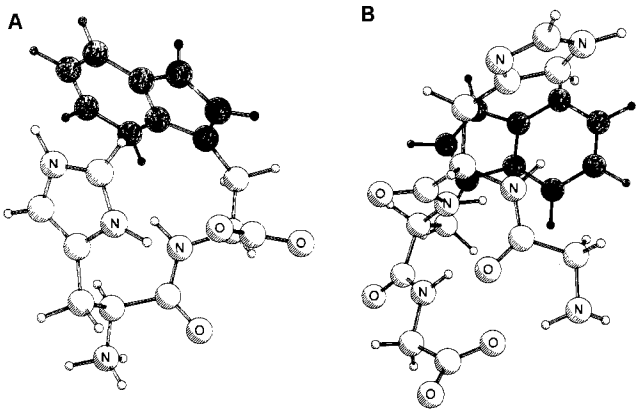

Figure 8. Computer drawings for the most stable conformations of $^{+}H_2$-His^{+}-Trp-O^{-} (A) ($\phi(1) = 60°$, $\psi(1) = 150°$, $\chi_1(1) = -170°$, $\chi_2(1) = -90°$, $\phi(2) = -70°$, $\psi(2) = -30°$, $\chi_1(2) = 60°$, $\chi_2(2) = 80°$) and H-Gly-His-Trp-Gly-O^{-} (B) ($\phi(1) = 30°$, $\psi(1) = -170°$, $\phi(2) = -60°$, $\psi(2) = -30°$, $\chi_1(2) = -50°$, $\chi_2(2) = -90°$, $\phi(3) = -70°$, $\psi(3) = -10°$, $\chi_1(3) = -50°$, $\chi_2(3) = -80°$, $\phi(4) = 170°$, $\psi(4) = -30°$). The tryptophan rings are darkened.

group. The suggested conformation is in agreement with literature data, for example, the NMR data obtained by Tran and coworkers on H-His-Gly-OH.[25] In such a conformation the two aromatic rings tend to get in close proximity—but not in a parallel stacked conformation. The two rings are tilted by about 45° from each other.

Figure 8B gives the case of the anionic tetrapeptide H-Gly-His-Trp-Gly-O^{-} at low temperature. Such a proposal is consistent with the following: (i) the most stable side-chain conformations that result from Figure 6; (ii) the large ring current effect for $H_{5'}$ (Figure 4) which suggests the proximity of the two rings, and again in a way that gives nonequivalent effects for $H_{2'}$ and $H_{5'}$; (iii) the strong upfield shift of the aliphatic histidine protons (Figure 5), indicating the closeness of the tryptophan ring; (iv) the anisochrony of the terminal CH_2-protons, and their temperature effect (Figure 5) which is indicative of a decreased flexibility of the chain terminals; (v) the previous observation is in agreement with the pH effect of Figure 3D for the C-terminal glycine. Although such an effect refers to the cationic form, the very small pH effect observed in the CD spectra when going from the anionic to the cationic form of the tetrapeptide indicates that the chain conformation remains more or less the same in the two ionic forms. Notice that the conformation presented in Figure 8B corresponds to a β-turn-like structure (4 → 1 hydrogen bridge) with an additional hydrogen bond (1 → 4) between the end groups. There are data in the literature, for compounds having similar structural properties, which support the high probability of such a conformation.[35-37]

The relative position of the rings of this last conformation is quite similar to that which we proposed for H-Trp-Trp-OH in the anionic form.[4] Again, the two rings happen to be close to each other and almost perpendicular to each other. In regard to the relative position of the two rings, we would like to point out that none of our data indicate a parallel stacked conformation. We have already discussed this point in previous papers dealing with the Trp-Trp family.[3,4,23] Here, it is important to mention that a stacked interaction between Trp and His was proposed by Bushueva et al.[10] for the intermolecular Trp-His system. These authors ruled out, as we do, the existence of charge-transfer complexes in the ground state, but then they proceeded to argue that a stacked interaction is the best way to explain certain small differences between the NMR properties of the mixture of H-His-OH and H-Trp-OH and the NMR properties of the individual amino acids. We believe that such small differences (of the order of 0.05 ppm) can be explained by simple concentration effects.

The present study has clarified that there is no charge-transfer complex betwen indole and protonated histidine in the ground state. Quenching fluorescence data evidence that the excited state responds readily to the ionization state of histidine; however, this phenomenon can be attributed to a dynamic quenching mechanism. These considerations reflect a more general problem of fluorescence spectroscopy in regard to conformation. First of all, fluorescence is a property of excited states and, in principle, has no bearing on the ground state—a point often overlooked. Furthermore, fluorescence is very dependent on dynamic processes, a shown by the present examples. These considerations show that fluorescence spectroscopy is able to produce useful, but only complementary, information about the conformational behavior of molecules in solution.

In addition to the proposals of Figure 8, the present investigation has been instrumental in elucidating many detailed conformational properties, relative for example, to the response of the overall conformation to pH and temperature changes, and to the relationship between the main-chain and side-chain conformations. On the basis of all these observations, we can reconsider now the question raised in the Introduction of this work, as to whether we have satisfactory knowledge about the conformational behavior of these flexible molecules in solution. The answer to this question depends, of course, one one's definition of "conformation" as well as satisfactory "knowledge". We believe that such definitions should pivot on a compromise between two distinct, somewhat contradictory points: the qualitative understanding of the average conformational properties of the molecule on the one hand, and the detailed understanding of only a few most stable conformers on the other. Within the limits of this definition, we believe that our investigation has been successful. The fact that so many experimental observations are needed for this relatively limited goal is indeed the inherent difficulty of studying the conformational behavior of flexible molecules in solutions.

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