Metal–peptide Interaction: Influence of the Aminoacid Sequence on the Binding of Co(II) to Glycyltryptophan and Tryptophylglycine Studied by ^{1}HNMR and Fluorescence

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1 Introduction

Aim of this work is to investigate the nature of the interactions between transition metals and peptides.

Peptides bind metal ions in various manner depending on their aminoacid composition, on the pH of the solution as well as on the metal/peptide ratio. Among the various aminoacids those with a charged side-chain are the most efficient for metal binding, though, ions can also be coordinated by the peptidic nitrogen as well as by the terminal amino group. It is well known that the binding of transition metals to peptides decreases the apparent pK_a of both the terminal amino group and of the peptidic NH. The fluorescence of the aromatic aminoacids (Trp, Tyr and Phe) can be used to monitor those changes. It is well rocognized that fluorescence spectroscopy is suitable for the study of peptides and proteins, and that Trp is the best internal probe due to its high quantum efficiency and molar absorption as compared to Tyr and Phe. Stemming from these considerations, conformational changes of proteins or peptides can be monitored following the modifications of the Trp fluorescence. Similarly, the interaction of Trp with transition metal ions can be easily detected by measuring the fluorescence quenching induced by the metal ions themselves.

Recently [1], [2] we have been studying the interaction of Cu(II) and Ni(II) with Trp and Gly–Trp and we found not only that the quenching of Trp fluorescence is mainly due to a ground state interaction but also that, for the two metals, the formation of the metal complex with the dipeptide and the AA is different, both in terms of binding constants and stoichiometry.

Another technique that can be used for the study of metal-peptide complexes is high resolution ^{1}HNMR. The possibility of a transition metal to act as a line broadening or as a shift reagent is strictly associated to its electronic relaxation time. Paramagnetic ions with a short relaxation time are responsible for changes in chemical shift without line broadening while, if the relaxation time is long enough, the effect is a strong line broadening with no changes of the chemical shift. Therefore the study of the chemical shift variation as a function of the metal concentration can lead to interesting results relevant for a better understanding of the complexe's stoichiometry. At the same time relaxation studies will provide valuable information on their geometry.

2 Materials and Methods

Tryptophylglycine (Trp–Gly) and glycyltryptophan (Gly–Trp) were obtained from Sigma Co. S. Louis, MO, their purity was checked by gas–chromatography. $CoCl_2 \cdot 6H_2O$ was obtained from Merk, Darmstad, Germany and used without further purification.

^{1}HNMR experiments were carried out using a Bruker AMX 400 spectrometer, operating at 9.41 T, static fluorescence experiments were carried out using a Perkin Elmer MPF 44A spectrofluorimeter, time resolved fluorescence experiments were carried out with a time correlated single photon counter equipped with an Edinburgh F199 nanosecond flash lamp operating in a N_2 flux of 1 ℓ/min, a Philips XP2020Q fast photomultiplier and an EG&G Ortec fast NIM electronics. Time resolved fluorescence data analysis was carried out using the *Global Analysis* [3].

The temperature for all the experiments was 25°C.

Peptide's solutions for ^{1}HNMR were freshly prepared in double distilled water, with 10% D_2O, at a final concentration of 10 mM. Solutions for fluorescence experiments were obtained by appropriate dilution of 1 mM stock solution in double distilled water. No buffers were used. $CoCl_2$ solutions were 3 M for ^{1}HNMR and 1 M for fluorescence experiments.

Distinct protocols were used to obtain the pH titration of the two peptides in ^{1}HNMR and fluorescence experiments. In ^{1}HNMR experiments small aliquots (μℓ) of 0.1 M HCl or 0.1 M NaOH were added to 0.5 mℓ of the sample in order to obtain a given pH. As for the fluorescence measurements, additions of 1 M HCl or 1 M NaOH were made on 50 mℓ in order to avoid dilution artifacts, the same pHmeter was used. The data from pH titrations, for both ^{1}HNMR and fluorescence, were fitted using the Henderson–Hasselbach equation in order to obtain the pK_a values .

In the case of fluorescence experiments correction for the inner filter effect was made using Parker's equation [4].

3 Results and Discussion

Fluorescence quenching experiments demonstrate that the complexes formed by Gly–Trp and Trp–Gly with Co(II) have distinct properties. The binding of the metal ion is strictly influenced by the aminoacid sequence and by pH. Moreover Co(II) like other paramagnetic transition metals such as Cu(II) and Ni(II), is known to influence the pK_a of the ionizable groups in aminoacids and peptides [1], [2].

In the case of the binding of metal ions to fluorescent aminoacids a non fluorescent ground state complex is formed, thus, it is possible to calculate the binding constant from the variation of the fluorescence intensity. Co(II) binding to Trp–Gly and Gly–Trp produces biphasic quenching curves [5], indicating that at least two different complexes are formed in each case. In table I the binding constants of Co(II) for the two peptides are reported together with the fraction of the two complexes present at the given pHs.

Unfortunately, fluorescence spectroscopy is not able to give the required informations for the direct determination of the stoichiometry of the complexes and of their geometry. To overcome these limitations ^{1}HNMR has been used.

The pK_a values of the ionizable groups (carboxylate and amino group) in the absence and in the presence of Co(II) have been obtained from fluorescence data. These values turn out to be quite different with respect to those obtained using the same technique and reported in a recent publication [6].

To verify these values, the determination of the two pK_as was carried out by means of ^{1}HNMR. We studied the pH dependence of the proton chemical shifts for the two dipeptides in the absence of the metal ion. Figure 1A reports the plot of the data for the two α protons of glycine and figure 1B for the NH proton and for the indolic one. From these data the pK_as of the carboxylate and of the amino group (Table II) were calculated. The pK_as values of the amino group obtained from pH dependence of the chemical shift of the various protons were in good agreement with those obtained from fluorescence experiments. In the case of the indolic and the NH proton resonances, as they disappear above pH 7.5, probably because of their fast exchange with H_2O, the fitting of the data was obtained imposing, as pK_{a2} values, the average values obtained from all the other protons. As can be seen the fitting is quite satisfactory.

When Co(II) is present, while with fluorescence spectroscopy it is possible to carry out a pH titration of the dipeptide–metal complex up to pH 12, NMR is limited to pH 7. In fact, due to its high sensitivity fluorescence spectroscopy allows to operate at μM concentration for the dipeptides and for Co(II), thus avoiding the precipitation of the complex at high pH NMR, being less sensitive, requires concentrations in the range of 10 mM, therefore, above pH 7.5 we observe the formation of a mixed complex with OH that precipitates, with a consequent disappearing of the signal.

TABLE I: Binding constants (K_1 and K_2) obtained from fluorescence quenching experiments, f_1 and f_2 represent the fraction of complex formed

Dipeptides	f_1	K_1 (M^{-1})	f_2	K_2 (M^{-1})
Trp–Gly pH 3.2	1	0	--	--
Gly–Trp pH 3.2	0.36	2810.6	0.64	33.5
Trp–Gly pH 8.2	0.22	896.6	0.78	131.3
Gly–Trp pH 8.2	0.55	900.6	0.45	12.1

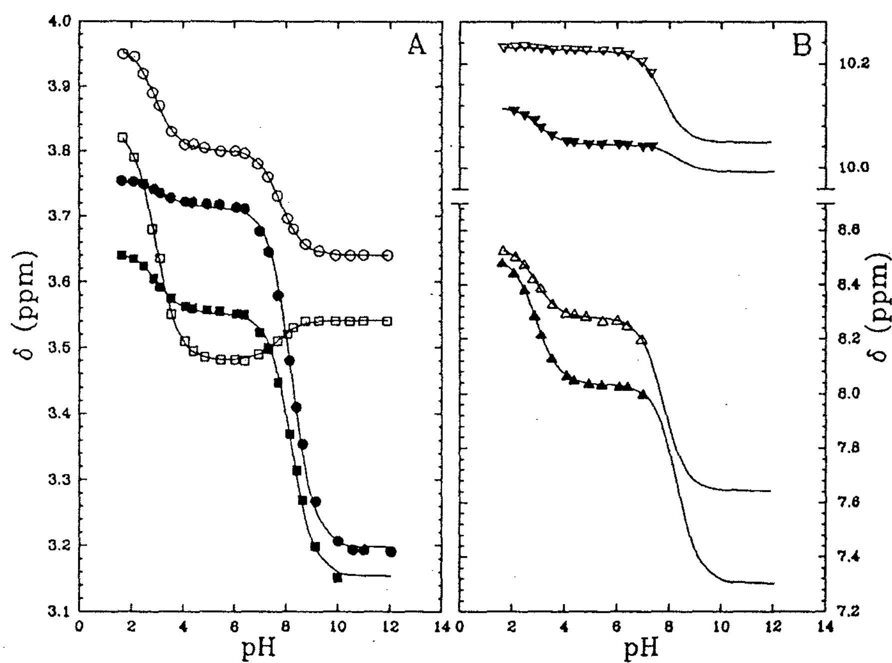

Figure 1 A Glycine α protons chemical shifts of glycyltryptophan (•,■) and tryptophylglycine (○,□).
B Indolic proton (▾,▿) and NH (▴,▵) chemical shifts of glycyltryptophan (▾,▴) and tryptophylglycine (▿,▵).

TABLE II: pK_as obtained from fluorescence and from ^1H NMR experiments

	Gly–Trp	Gly–Trp:Co^{++}	Trp–Gly	Trp–Gly:Co^{++}
Fluorescence intensities				
pK_{a1}	2.74	1.85	2.59	2.51
pK_{a2}	8.27	7.46	7.73	7.05
pK_{a3}	--	9.66	--	9.15
^1H NMR Chemical Shifts				
pK_{a1}	3.13	2.89	2.86	2.88
pK_{a2}	8.30	--	7.74	--

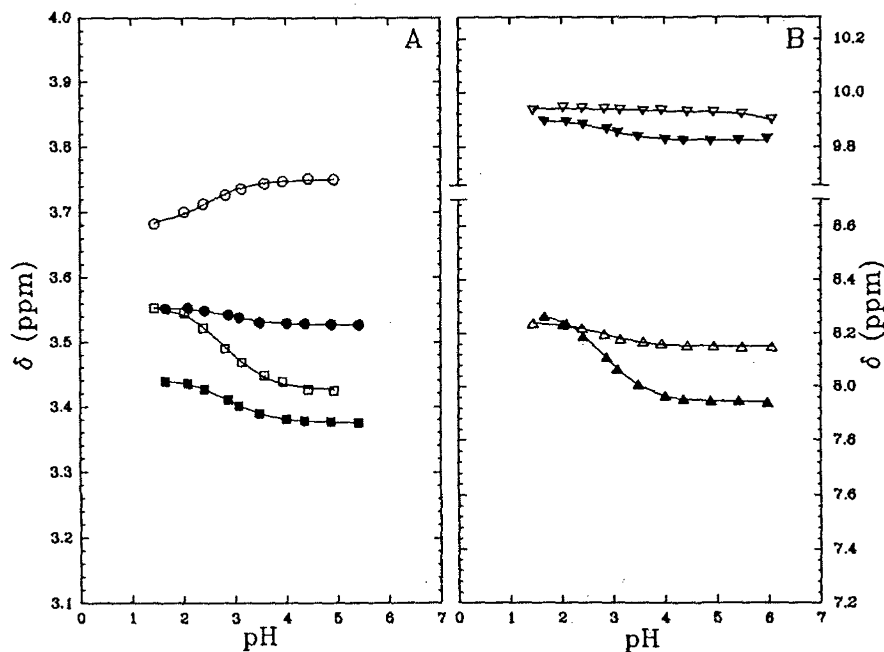

Figure 2 A Glycine α protons chemical shifts of glycyltryptophan (•,■) and tryptophylglycine (○,□) in presence o Co(II) (1:5 peptide metal ratio).

B Indolic proton (▼,▽) and NH (▲,△) chemical shifts of glycyltryptophan (▼,▲) and tryptophylglycine (▽,△ in presence of Co(II) (1:5 peptide metal ratio).

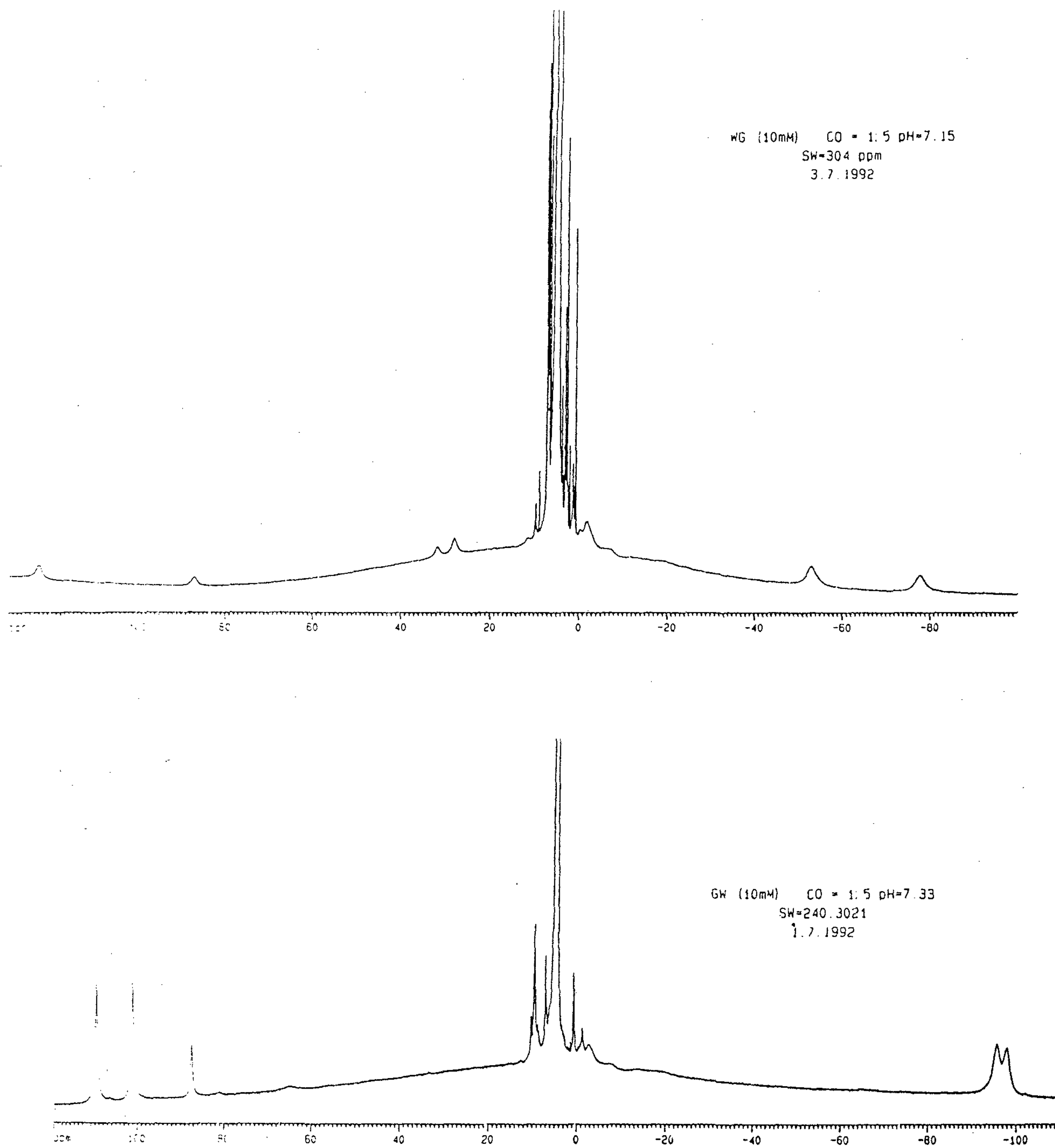

Figure 3 Top ^{1}HNMR spectrum of tryptophilglycine in presence of Co(II) (1:5) at pH 7.15.
Bottom ^{1}HNMR spectrum of glycyltryptophan in presence of Co(II) (1:5) at pH 7.33.

In figure 2A and 2B the ^{1}HNMR titration of the glycine α protons and of the indolic and NH protons in the presence of Co(II) (1:5 ratio) are reported. It can be seen that above pH 7.5 no signal was detectable due to the formation of a bluish precipitate and that, as a consequence only the carboxylate pK_a has been calculated and reported in Table II.

Despite these limitations, we have been able to detect the proton NMR spectrum for the two dipeptides complexes (Figure 3). A group of resonances is shifted between -50 ppm to -100 ppm and two or three peaks appear between 80 ppm and 130 ppm. Interestingly, though the assignment of the peaks is still to be completed, it can be seen that the NMR profile for the two complexes is quite different suggesting that Trp-Gly and Gly-Trp, indeed, are forming two distinct complexes. The half line width is approximatively 200 Hz for Gly-Trp and 1000 Hz for Trp-Gly indicating that Co(II) is either closest or more tightly bound to Trp-Gly as compared to the Gly-Trp. Indeed the Trp-Gly binding constants suggests an average high affinit of this peptide respect to Gly-Trp. The NMR spectra of the two peptide-metal complexes present one single peak with the same chemica shift.A that peak tend to disappear upon D_2O addition we believe it is a proton bound to a nitrogen atom. The NMR spectra of the two complexes posses other peculiar characteristics. The chemical shifts of their resonance lines are insensitive to the pH variation suggesting a high stability of the complexes. Moreover, the NMR spectra disappear below pH 5.2 for Trp-Gly and below pH 4.15 for Gly-Trp. We do believe these evidences are an indication both of the need for the carboxylic group to be ionized as well as of the requirement for a small fraction (1/1000) of NH_2 (the pK_a for the amino group are 8.27 and 7.73 respectively) in order to have metal binding.

In conclusion, these preliminary results indicate that, because of the complementarity of NMR and fluorescence spectroscopy, it is possible to better evaluate the pK_as of the dissociable groups and the metal binding properties of small peptides. We believe that such an integrated approach can be relevant for the study of more complex macromolecules such as polypeptides and proteins.

4. References

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