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Proton nuclear magnetic resonance and potentiometric studies on the palladium(II)–prolylglycylalanylhistidine binary system †

Panayotis Tsiveriotis,[a] Nick Hadjiliadis *,[a] and Imre Sovago [b]

[a] *Department of Chemistry, University of Ioannina, 451 10 Ioannina, Greece*
[b] *Department of Inorganic and Analytical Chemistry, Lajos Kossuth University, 4010 Debrecen, Hungary*

Potentiometry measurements were used to investigate equilibria of the palladium(II)–prolylglycylalanylhistidine binary system. The results obtained by this method were confirmed by ^{1}H NMR techniques in one and two dimensions. In strongly acidic solutions, pH < 1.0, Pd^{II} most probably reacts simultaneously with both proline amine and one of the imidazole nitrogens (N^1 and N^3) to form a macrochelate structure. At higher pH deprotonated histidine amide nitrogen is co-ordinated, while further increase of the pH results in terminal carboxylate deprotonation.

The histidine residue (His) is found in the active sites of several metalloenzymes,[1–4] *e.g.* haemoglobin, hemerythrin, *etc.* or metal transport proteins,[5] *e.g.* human serum albumin. The sequence NH_2–X–Y–His (X, Y are amino acids) is encountered in human serum albumin, involved in the transport of Cu^{II} [6,7] and in human fetoprotein.[8] Moreover, the growth-stimulating factor glycylhistidyllysine (Gly-His-Lys) is known to bind Cu^{II} and other metal ions *in vivo*.[9,10] For these reasons, extensive studies on interactions of various metal ions with histidine peptides have been undertaken.[11–18]

On the other hand, metal–peptide systems with histidine at the fourth position of the peptide chain or even further may resemble biological systems even more closely since in proteins His is distant from the N-terminus. Thus, Pettit *et. al.*[19] compared the donor properties of terminal amino and imidazole nitrogen, in peptides like Ala-Gly-Gly-His and derivatives towards Cu^{II}. They found that the primary anchoring site for the metal was the N^3 of the imidazole ring of histidine, which may lead to the formation of a specific macrochelate ring involving the terminal NH_2 group of alanine (Ala).

Bal *et al.*[20] also concluded that the N^3 of the imidazole ring is the primary anchoring site for Ni^{II} in Ala-Gly-Gly-His and related peptides. A 2N complex involving either the imidazole and peptide nitrogens of histidine or the terminal NH_2 group and the peptide nitrogen of Gly is the second species formed in this case. This is followed by the final formation at pH 8 of a 4N species with the deprotonated imino protons involved also in bonding. Similar behavior was also found in the copper(II) angiotensin system.[21]

The situation is different when the peptide chain does not contain a histidine residue and the N-terminal amino group is the first anchoring site for metals like Cu^{II}, Ni^{II}, Pd^{II} and Pt^{II}.[11,12] Here easy deprotonation and subsequent co-ordination of successive peptide nitrogens forms, depending on the length of the peptide, up to three five-membered chelate rings.

Thus, the presence of a histidine residue in the peptide sequence alters dramatically the co-ordination behavior of the named peptides and in general more stable complexes are formed.[19–21]

In the present paper we report on the results of the interaction of Pd^{II} with the tetrapeptide prolylglycylalanylhistidine (Pro-Gly-Ala-His), which contains histidine at position 4 of the peptide chain, combining potentiometric and ^{1}H NMR spectroscopic techniques. The application of potentiometric methods to elucidate the mechanism of the interaction of histidine-containing peptides with Pd^{II} seemed to be extremely difficult in the past, due to ammonium or amide nitrogen deprotonation of peptides promoted by the metal in strongly acidic solutions.[11,12] For example, Pd^{II} co-ordinates with His-Ala,[22] His-Gly-Ala,[22] Gly-His,[23] Gly-His-Gly[23] and Gly-His-Lys [14] is found to ionize these groups at pH ⩽ 2, while other metals such as Cu^{II} [11] do the same at pH 4–6. The complexes formed were characterized mainly by ^{1}H NMR spectroscopic techniques but no reliable stability constants were obtained since accurate pH measurement in the range below 2 is not possible. On the other hand, palladium(II) complex-formation reactions are characterized by slow kinetics compared to those of Cu^{II}, making difficult equilibrium investigations. Despite these difficulties, we were able completely to characterize the above binary system by means of potentiometric and ^{1}H NMR techniques (SUP 57288). Two-dimensional ^{1}H NMR techniques in combination with potentiometry proved to be useful in understanding the mechanism of the interaction of Pd^{II} with the named peptide, since they allowed the full identification of the species formed as a function of pH. These results show that the previous belief of the amide nitrogen deprotonation of peptides by Pd^{II} in strongly acidic solutions does not in fact prevent such studies.

Experimental

All solvents and chemicals from commercial sources were of the highest available purity and used without further purification. The protected L-amino acids Fmoc-His(Trt)-OH, Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Pro-OH (Fmoc = fluoren-9-ylmethoxycarbonyl, Trt = trityl) and the resin 2-chlorotrityl chloride were from CBL Chemicals Ltd. Patras-Greece.

Peptide synthesis

The peptide Pro-Gly-Ala-His was synthesized in the solid state, using the 2-chlorotrityl chloride resin (substitution 1.1–1.6 mequivalents g^{-1}) as the solid support. The Fmoc group was used for temporal protection of the α-amine group and dicyclohexylcarbodiimide (dcc)–Bu^{t}OH were used as coupling reagents. The procedure followed is described in detail in the literature.[22] Peptide cleavage from the resin was performed in $MeCO_2H$–CF_3CO_2H–CH_2Cl_2 (1 : 2 : 7 v/v). The imidazole pro-

† *Supplementary data available* (No. SUP 57288, 14 pp.): full assignment of NMR spectra, stability constant calculations. See *J. Chem. Soc.*, *Dalton Trans.*, Issue 1, 1997.

Table 1 Stability constants of complexes of H^+ and Pd^{II} with Pro-Gly-Ala-His

log β	HA	8.94(2)	Pd(HA)	17.58(10)
	H_2A	15.93(3)	PdA	15.72(4)
	H_3A	18.69(3)	$PdAH_{-1}$	11.95(5)
	log $K_{im\ NH}$	6.99	log $K_{Pd(HA)}$	1.86
	log K_{CO_2H}	2.76	log K_{PdA}	3.77

tecting group (Trt) was removed using 20% trifluoroacetic acid in CH_2Cl_2–CF_3CO_2H (6:1 v/v). Peptide purity was controlled by thin-layer chromatography using the solvent mixtures BuOH–$MeCO_2H$–water (4:1:1 v/v) and BuOH–$MeCO_2H$–pyridine–water (30:6:20:24 v/v).

Potentiometric studies

Potentiometric studies were carried out at 25 °C using a Russell glass–calomel combined electrode, connected to a Radiometer pHmeter. Base solution volumes (0.208 M, CO_2-free KOH) were measured using a Radiometer automatic microburette. All parts of this system were calibrated by literature methods.[24] Sample volumes were 25 cm^3 and the ionic strength 0.2 M was adjusted by KNO_3. The concentration of the peptide was 1.205×10^{-3} M while that of the metal (K_2PdCl_4) was 1.205×10^{-3} M. Argon bubbling was used to stir the solution and also to eliminate CO_2. All calculations were performed using the PSEQUAD program[25] (SUP 57288).

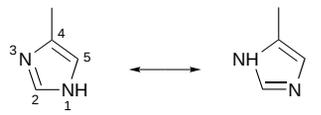
One- and two-dimensional ^{1}H NMR spectra

One-dimensional ^{1}H NMR spectra at 200 or at 400 MHz were recorded at room temperature (25 ± 1 °C) on a Bruker AC 200 or on an AMX 400 spectrometer respectively; D_2O was used as solvent and sodium 3-(trimethylsilyl)tetradeuteriopropionate as external reference. The pD values were calculated by adding 0.4 logarithmic units to pHmetric readings. The concentration of the samples for the ^{1}H NMR measurements was of the same order as that used for the potentiometric measurements. Field stabilization was provided by an internal deuterium lock-signal. The spectrometer conditions consisted of 4000 and 8000 Hz sweep width at 200 and 400 MHz respectively; 64 scans were used and 16 K data points.

Two-dimensional ^{1}H NMR spectra were recorded at room temperature (25 ± 1 °C) on the Bruker AMX 400 spectrometer. Correlation (COSY) spectra were acquired in the magnitude mode. The MLEV-17 mixing pulse sequence (50 ms) was used in total correlation spectroscopy (TOCSY) experiments.[26] For both experiments, the sweep width was typically 5050 Hz. 256 Free induction decays were recorded in 1024 data points; each consisted of 32 scans. Prior to Fourier transformation, the initial 1024 × 256 data matrix was zero filled in the t_1 dimension and multiplied by a sine-bell function in both t_1 and t_2 dimensions. Full assignment of NMR spectra are available (SUP 57288).

Results and discussion

The tetrapeptide Pro-Gly-Ala-His contains three groups that can be deprotonated over the pH range. These are ammonium and carboxyl terminals and also the imidazole ring nitrogens. Calculated protonation constants and stepwise protonation constants for the tetrapeptide are given in Table 1. These values compare well with literature values calculated for similar peptides.[19–21] Although both imidazole nitrogens are protonated in strongly acidic solution yielding a positive net charge on the ring, only one of them is deprotonated in the range pH 0–14. The resulting monoprotonated imidazole ring is neutral and can exist in two tautomeric forms in which one proton shifts from N^3 to N^1 (Scheme 1). Proton NMR and potentiometric



Scheme 1

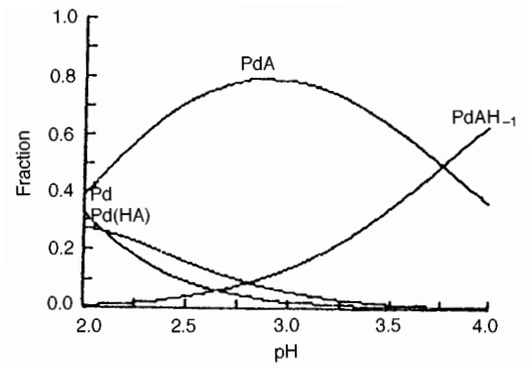


Fig. 1 Species distribution diagram for a 1:1 mixture of K_2PdCl_4 and Pro-Gly-Ala-His

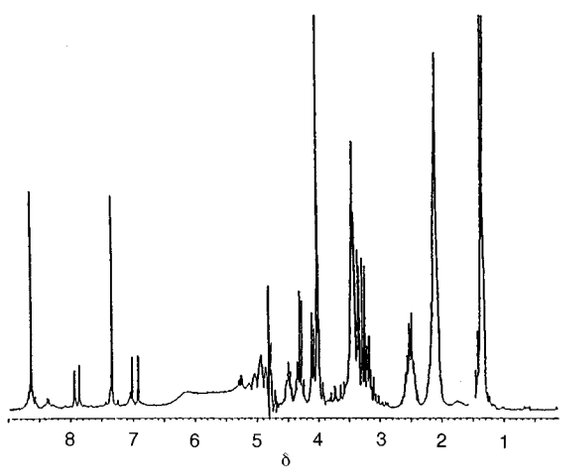


Fig. 2 Proton NMR spectrum of a 1:1 mixture of K_2PdCl_4 and Pro-Gly-Ala-His at pD 1.2

studies suggest that the N^1-protonated tautomer is favored over the N^3-protonated tautomer.[27]

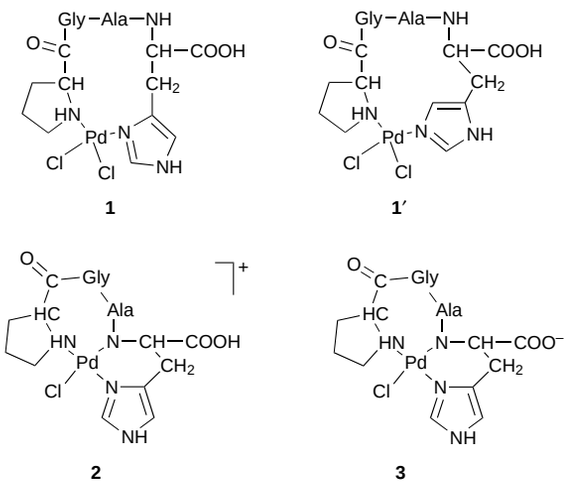
Peak assignment of the ^{1}H NMR spectrum of the free peptide at pD 2 was made using COSY and TOCSY spectra. Selected ^{1}H NMR data are given in Table 2.

Equilibrium investigation by potentiometry of the binary system Pd^{II}–Pro-Gly-Ala-His suggests a model in which only species of the type Pd(HA), PdA and $PdAH_{-1}$ may exist in aqueous solution. Computer treatment of the potentiometric data rules out formation of any bis species. Calculated log β and log K values for the species proposed by the model are also given in Table 1, while the species distribution diagram for a 1:1 mixture of K_2PdCl_4 and Pro-Gly-Ala-His is depicted in Fig. 1. The fact that the results reported here are the first of this kind do not allow any comparison. However we can confirm these results by ^{1}H NMR spectroscopy since by this technique we can determine the structure of the various species formed, as they are dictated by the species distribution diagram (Fig. 1). Based on these results we have tried to identify the species PdHA, PdA and $PdAH_{-1}$ at pD 1.2, 2.8 and 6.2.

At pD 1.2 we observe two pairs of new peaks in the aromatic region of the spectrum in addition to peaks at δ 8.60 and 7.30 of the free peptide. These new peaks are at δ 7.93, 7.85 and 7.02, 6.91 and are of the same intensity. On the other hand, the intensity of the Pro C_α–H peak at δ 4.42 decreased and a new peak appeared at δ 4.02 (Fig. 2). These results can be explained by

Table 2 Selected ^{1}H NMR data for the free peptide and its palladium(II) complexes

		Imidazole protons				
Compound	pD	C^2–H	C^5–H	His C_α–H	His C_β–H_2	Pro C_α–H_2
Pro-Gly-Ala-His	2.4	8.6	7.3		3.32	4.42
Pd(HA)	1.2	7.93/7.86	7.02/6.91		3.41	4.02
PdA	2.8	7.85	6.86	3.42	2.07/2.47	4.02
$PdAH_{-1}$	6.2	8.14	7.13	3.40	2.51/2.05	3.97



Scheme 2

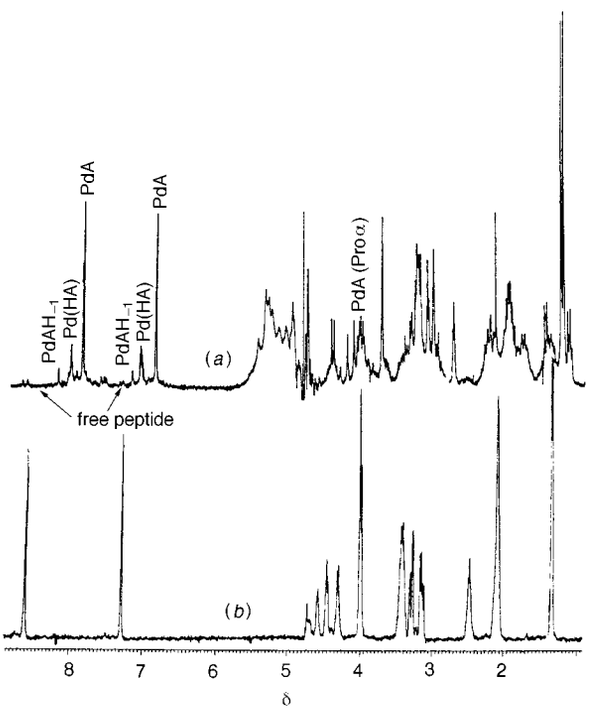


Fig. 3 Proton NMR spectrum of a 1:1 mixture of K_2PdCl_4 and Pro-Gly-Ala-His (*a*) and of Pro-Gly-Ala-His (*b*) at pD 2.8

the formation of two 2N complexes, with the peptide co-ordinated to the metal by either the N^3 or N^1 imidazole nitrogen and the proline amine nitrogen. Viscosity measurements rule out the formation of poly- or oligo-meric species.

The formation however of two dimeric complexes, one involving two proline residues bound to one Pd^{II} and two imidazoles to the other Pd^{II}, and the second with one imidazole and one proline bound to each Pd^{II}, cannot be ruled out, but seems less likely, since a selectivity of N^3 over N^1 was found only in cases where a six-membered chelate ring involving N^3 and the N_α of histidine with Pd^{II} and Pt^{II} was formed. In addition palladium(II) salts like $[Pd(dien)(H_2O)]^{2+}$ (dien = diethylenetriamine) and $[(CH_3NH_2)_2Pt(mcyt)_2PdCl]^+$ (mcyt = methylcytosine) containing bulky ligands imposing geometrical constraints do not show any selectivity of N^3 over N^1 and *vice versa*. At this pD therefore a mixture of two 2N complexes may form, with the peptide co-ordinated by either the N^3 or the N^1 imidazole nitrogen and the proline amine nitrogen (**1**, **1′** in Scheme 2). Peak integration shows that about 30% of the peptide co-ordinates at this pD value. Assuming formation of the above described macrochelate monomeric complexes, these can be formulated as Pd(HA), since the carboxylate group is protonated.

At pD 2.8 the species distribution diagram suggests the existence of three different species, namely Pd(HA), PdA and $PdAH_{-1}$, the PdA being in large excess over the other two, together with a small amount (≈ 10%) of free peptide. All these observations are in agreement with ^{1}H NMR investigations. For example in the aromatic region of the spectrum at pD 2.8 (Fig. 3) three different products are observed. Each gives rise to a pair of peaks at this region for C^2–H and C^5–H respectively. These pairs were identified by COSY analysis. The pair at δ 8.00 and 7.06 is easily assigned to one of the linkage isomers formed at pD 1.2. The other linkage isomer observed at pD 1.2 might also be present but its C^2–H and C^5–H resonances are overlapping with the peaks of the main product at this pD, PdA.

Peaks in the aromatic region attributed to PdA species appear at δ 7.85 and 6.86. Comparing these values to corresponding literature ones[22,23] we may conclude that histidine imidazole is co-ordinated preferentially through N^3. On the other hand, the peak at δ 4.42 due to C_α–H of the Pro residue in the free peptide almost disappears, confirming imine co-ordination of Pro as described above. The resonance of co-ordinated Pro C_α–H now appears at δ 4.02, as the COSY and TOCSY spectra indicate. Peaks due to other proline protons are also shifted almost to the same extent. Moreover, a new peak appeared at δ 3.42 which was attributed to His C_α–H on the basis of COSY and TOCSY spectral analysis. This shift by 1.38 ppm of His C_α–H in the spectrum of the mixture compared to that of the free peptide indicates co-ordination of histidine amide nitrogen.[22,23] Histidine β protons appear at δ 2.47 and 2.07 and are shifted upfield by ≈ 0.80 ppm compared to the free peptide. Thus PdA is a 3N species, in which the peptide is co-ordinated through proline amine, deprotonated histidine amide and imidazole N^3 nitrogen. The carboxylate group remains protonated (see **2** in Scheme 2). Since peaks from Pd(HA) species disappear as PdA forms, it is obvious that on passing from Pd(HA) to PdA a rearrangement of the metal-ion co-ordination sphere takes place. Thus the Pd–N^1 co-ordination breaks and a Pd–N (amide) co-ordination of histidine takes place instead. The formation of a six-membered chelate ring involving the N^3 and the deprotonated amide of histidine is favored over the possible N^1 co-ordination and formation of a seven-membered chelate ring involving the N^1 and the deprotonated amide nitrogen of histidine.[12] Minor peaks appearing at δ 8.14 and 7.13 are assigned to the $PdAH_{-1}$ species. Peaks of the free peptide (≈ 10%) are very difficult to identify and can hardly be distinguished from noise.

These peaks are the main peaks in the ^{1}H NMR spectrum at pD 5.8 (Fig. 4) and account for more than 95% of the total

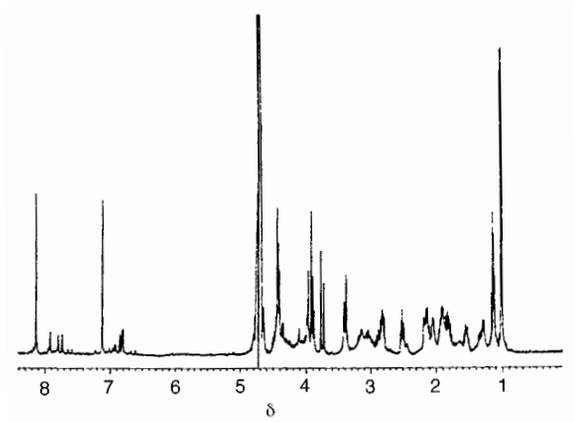


Fig. 4 Proton NMR spectrum of a 1:1 mixture of K_2PdCl_4 and Pro-Gly-Ala-His at pD 5.8

peptide. At this pH we were not able to apply potentiometric techniques because pH readings were unstable perhaps due to slow rearrangement of the co-ordination sphere, *e.g.* formation of $PdAH_{-2}$ species or its polymeric form. Such oligo- or polymeric structures were also detected in Pd–His-Ala and His-Gly-Ala systems. It is worthwhile mentioning that although ^1H NMR spectra of the samples at lower pD values do not change after the samples are left to stand, those of the sample at this pD decrease with time and new minor peaks appear in the aromatic region. However, two-dimensional COSY and TOCSY spectra allowed us to determine the structure of $PdAH_{-1}$. Thus, it appears also to be a 3N species of the same co-ordination mode as PdA. Taking into account that $PdAH_{-1}$ starts forming after the addition of 3 equivalents of base to the reaction mixture, it is obvious that on passing from PdA to $PdAH_{-1}$ only carboxylate deprotonation takes place. This product is compound **3** in Scheme 2. Further support is taken from the calculated log *K* value of 3.76 for the carboxylate deprotonation ($PdA \rightleftharpoons PdAH_{-1} + H$) found in the present study which compares well with the value calculated by Rabenstein *et al.*[23] for the palladium(II) complex of Gly-His.[23]

Conclusion

We have shown that at least in the Pd^{II}–peptide system studied here it was possible to calculate formation constants of the complexes formed at low pH values using potentiometric equilibrium techniques. This is a very important tool in the understanding of such systems. Knowledge of the formation constants helps not only to identify the various species formed, but also to understand better the chemical behavior of the system under investigation. Comparing now the stability of the palladium(II) complexes to those of the corresponding complexes of Cu^{II} [19] and Ni^{II} [20] with similar peptide ligands, it is obvious that the former are 5–7 orders of magnitude more stable than the corresponding ones of Cu^{II} and Ni^{II}. However the stability differences between the various species of Cu^{II} [19] and Ni^{II} [20] formed are much higher than those between the various palladium(II) species. Thus, formation of the 1N species observed in the case of Cu^{II} and Ni^{II} by co-ordination of histidine imidazole N^3 cannot be observed in the case of Pd^{II} where 2N species are formed directly. On the other hand, Pd^{II} in these 2N species co-ordinates to the same extent to N^1 and N^3 and there is no selective co-ordination to N^1 as is the case with Cu^{II} [19] and Ni^{II}.[20] It seems likely that there is no significant stability difference between N^1 and N^3 co-ordination in the palladium(II) system in contrast to the case of Cu^{II} and Ni^{II}. Furthermore, formation of the 3N complex PdA takes place with rearrangement of the co-ordination sphere of the 2N complex but not in the same way as in the case of Cu^{II} [19] and Ni^{II}.[20] In the 3N species, Pd^{II} selectively co-ordinates the N^3 of imidazole while the proline imine nitrogen remains co-ordinated. The third N-donor atom is provided by the deprotonated histidine amide nitrogen.

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