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Dependence of hydrolytic cleavage of histidine-containing peptides by palladium(II) aqua complexes on the co-ordination modes of the peptides

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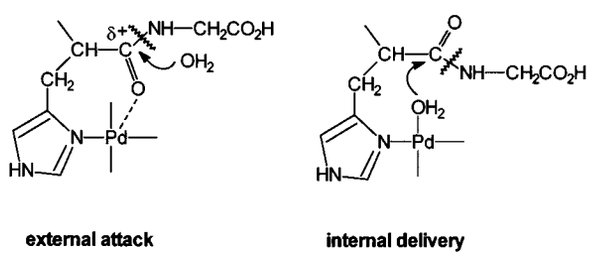
Reactions of palladium(II) complexes *cis*-[$PdCl_2$(en)] and *cis*-[$PdCl_2$(L-HMet-*S*,*N*)], in which en is ethane-1,2-diamine and methionine is an *S*,*N*-bidentate ligand, and their aqua analogs with dipeptides glycyl-L-histidine (Gly-His), L-histidylglycine (His-Gly), and the *N*-acetylated dipeptides MeCO-Gly-His and MeCO-His-Gly have been studied by ^{1}H NMR spectroscopy. In the reactions of *cis*-[$PdCl_2$(L-HMet-*S*,*N*)] and *cis*-[$PdCl_2$(en)] with Gly-His the formation of [Pd(Gly-His)(L-HMet-*S*)]$^+$ and [PdCl(Gly-His)] occurs at 1.5 < pH < 3.5. Tridentate co-ordination of Gly-His causes release of the en from *cis*-[$PdCl_2$(en)] and ring opening of the L-HMet chelate in *cis*-[$PdCl_2$(L-HMet-*S*,*N*)]. The crystal structure of [PdCl(Gly-His)] shows that the peptide is bound to palladium(II) through imidazole N-3, amide, and amino nitrogen atoms. Tridentate chelation of Gly-His to palladium(II) is unfavorable for the hydrolysis of the peptide. The dipeptide His-Gly co-ordinates to palladium(II) as a bidentate ligand, *via* the imidazole N-3 and amino nitrogen atoms. This co-ordination mode also is unproductive for the hydrolysis of the peptide. However, at lower pH this complex converts into a hydrolytically active one, in which the dipeptide is bound to palladium(II) *via* the imidazole N-3 atom only. The dipeptides MeCO-His-Gly and MeCO-Gly-His, in which the terminal amino group is acetylated, exhibit more versatile co-ordination chemistry in the reactions with *cis*-[$PdCl_2$(en)] and *cis*-[$PdCl_2$(L-HMet-*S*,*N*)] and form complexes in which the imidazole N-1 atom co-ordinates to palladium(II). These studies with model complexes contribute to the understanding of selective cleavage of peptides and proteins by palladium(II) aqua complexes.

Various biological processes involve hydrolytic cleavage of peptides and proteins. The half-life for hydrolysis of the unactivated amide bond in neutral aqueous solution is around 500 years.[1] The extreme inertness of the amide bond makes this reaction interesting from the chemical point of view. Several proteolytic enzymes are used for cleavage,[2] but application of enzymes is limited by their rather narrow requirements for temperature and pH. Therefore, new methods for selective hydrolysis of peptides and proteins are required. Recent studies in one of our laboratories showed palladium(II) aqua complexes to be promising reagents for hydrolytic cleavage of peptides and proteins. These complexes bind to the heteroatom in the side chain of methionine[3–7] or histidine[8,9] and promote cleavage of the amide bond involving the carboxylic group of this anchoring amino acid. Palladium(II) aqua complexes selectively cleave proteins upon simple incubation in solution. Successes with the proteins cytochrome c[10a] and myoglobin[10b] bode well for general applicability of these new reagents in biochemistry.

Hydrolysis of the amide bond can occur by two limiting mechanisms (Scheme 1), assuming a histidine anchor; analogous interactions are possible also with the methionine anchor. In the first mechanism palladium(II) acts as a Lewis acid and forms a chelate involving a heteroatom in the side chain of the anchoring amino acid and the oxygen atom in the amide group. The interaction with the oxygen atom polarizes the carbonyl group and activates the carbon atom toward external attack by water. Since water for the hydrolysis of the amide bond comes from the solvent, this mechanism is called external attack. For the reaction to occur by this mechanism the palladium(II) and carbonyl oxygen atoms should be proximate.

In the second mechanism an aqua ligand on palladium(II) is delivered to the carbon atom in the amide bond. Since the water for the hydrolysis comes from the palladium(II) complex



external attack **internal delivery**

Scheme 1

attached to the side chain of the anchoring amino acid this mechanism is called internal attack. For the cleavage of the amide bond to occur by this mechanism an aqua ligand at palladium(II) should be proximate to the carbonyl carbon of the scissile amide bond.

Also possible is a combined mechanism, in which the palladium(II) atom activates the amide bond by binding to the carbonyl oxygen atom and also delivers an aqua ligand to that same bond.

The consistent regioselectivity in the cleavage of histidine-containing peptides by *cis*-[$Pd(H_2O)_2$(en)]$^{2+}$ (en = ethane-1,2-diamine) is not understood yet. Our previous studies have shown that *cis*-[$Pd(H_2O)_2$(en)]$^{2+}$ cleaves the amide bond involving the carboxylic group of histidine in a series of MeCO-His-X peptides, in which X is Gly, Ala, Leu, Ser, Thr, Tyr or Phe.[8,9] Since the amino group in them was acetylated, binding of the palladium(II) atom to this nitrogen atom was suppressed. However, many proteins contain a free terminal amino group, which may co-ordinate to the cleaving agent. In this study, which involves unprotected as well as protected dipeptides, we examine the effect of the various modes of co-ordination on the

occurrence and non-occurrence of that cleavage reaction. A fuller understanding of the co-ordination chemistry of histidine-containing peptides with palladium(II) complexes is necessary for understanding the regioselectivity of protein cleavage promoted by such complexes.

Experimental

Reagents

Distilled water was demineralized and purified to a resistance greater than 10 MΩ cm. The compounds D_2O, DNO_3, NaOD and $K_2[PdCl_4]$ were obtained from Aldrich Chemical Co. All common chemicals were of reagent grade. L-Methionine and dipeptides glycyl-L-histidine and L-histidylglycine were obtained from Sigma Chemical Co. The terminal amino group in the dipeptides was acetylated by standard methods.[4] The complexes *cis*-[$PdCl_2$(en)], *cis*-[$PdCl_2$(L-HMet-*S*,*N*)], and their diaqua analogs were synthesized by the published procedures.[11–13]

Reactions of peptides with palladium(II) complexes

Reactions of *cis*-[$PdCl_2$(en)] and *cis*-[$PdCl_2$(L-HMet-*S*,*N*)], in which L-HMet is L-methionine co-ordinated through sulfur and nitrogen, with Gly-His, MeCO-Gly-His, His-Gly and MeCO-His-Gly were followed by ^{1}H NMR spectroscopy. Equimolar amounts of the palladium(II) complex and the peptide were mixed in an NMR tube. The final solution was 10 mM in each reactant. The pH was varied in the range of 1.5–3.5 for Gly-His and 1.5–5.0 for His-Gly and the *N*-acetylated peptides. All reactions were carried out at room temperature.

The reactions of the hydrolytically active diaqua complexes *cis*-$[Pd(H_2O)_2(en)]^{2+}$ and *cis*-$[Pd(H_2O)_2$(L-HMet-*S*,*N*)$]^{2+}$ with Gly-His, MeCO-Gly-His and His-Gly were also followed by ^{1}H NMR spectroscopy. Equimolar amounts of the complex and the substrate were mixed in an NMR tube, and the final concentration was 10 mM in each. The reaction mixtures were kept at 60 °C for up to 7 d, to allow for the hydrolytic reaction.

Preparation of [PdCl(Gly-His-*N*,*N*,*N*)]·0.5H_2O

The dipeptide Gly-His (0.020 g, 9.5×10^{-5} mol) was dissolved in water (4 cm^3), and to this solution solid *cis*-[$PdCl_2$(en)] (0.022 g, 9.5×10^{-5} mol) was added. The mixture was stirred at room temperature in the dark, until the palladium(II) complex had dissolved; then the pH of the solution was adjusted to *ca.* 2.0 by the addition of 0.10 M HCl. The yellow solution was stirred overnight at room temperature and evaporated to 2 cm^3 at room temperature. The precipitate of the unspent *cis*-[$PdCl_2$(en)] was removed, and the filtrate was left in a refrigerator for several days. The pale yellow crystals of the required compound were filtered off, washed with ethanol, and dried in air. Yield 0.024 g or 70% (Found: C, 26.81; H, 3.20; N, 15.09. Calc. for $C_8H_{13}ClN_4O_{3.5}Pd$: C, 26.46; H, 3.41; N, 15.43%). Elemental microanalyses were performed by the Microanalytical Laboratory, Faculty of Chemistry, University of Belgrade. Single crystals of [PdCl(Gly-His-*N*,*N*,*N*)]·0.5$(CH_3)_2CO$ for X-ray diffraction, designated **5**, were obtained from a mixture of acetone and water. Whereas the microcrystals described above contain water, the single crystals contained one-half molecule of acetone per molecule of the complex.

X-Ray crystallography of [PdCl(Gly-His-*N*,*N*,*N*)]·0.5$(CH_3)_2CO$ 5

Data collection. A crystal of the compound was mounted on a glass fiber on a Siemens P4RA diffractometer for data collection at 293(2) ± 1 K. The cell constants were determined from reflections found from a 360° rotation photograph. Twentyfive reflections in the range θ 21.104–33.754° were used to determine precise cell constants. Pertinent data collection and reduction information are given in Table 1.

Lorentz-polarization corrections were applied and a non-linear correction based on the decay in three standard reflections measured every 97. A series of azimuthal reflections was collected for this specimen; a semiempirical absorption correction was applied.

Structure solution and refinement. The space group $P3_221$ was chosen based on systematic absences and intensity statistics. This assumption proved to be correct as determined by a successful direct-methods solution[14] and subsequent refinement. All non-hydrogen atoms were placed directly from the E-map. All non-hydrogen atoms were treated as riding atoms with individual isotropic displacement parameters. Final refinements were done with SHELXL 93.[15]

The atoms O(10), C(40) and C(41) belong to a disordered solvent molecule. Its structure resembles acetone, but it could not be determined with certainty. Standard deviations are not given for the *y* and *z* coordinates of O(10) and C(41) because these atoms lie at special positions. The coordinates are fixed because of the symmetry of the space group.

The crystal was somewhat disordered, as can be seen in the uncertainty of the solvent molecule and in the high value of R_{int}. This problem could be caused by a minor twin component that does not affect the overall refinement, or by a minor contribution from a satellite on the main crystal.

Crystallographic analysis was done at the Iowa State Molecular Structure Laboratory. Refinement calculations were performed on a Digital Equipment Micro VAX 3100 computer.

CCDC reference number 186/574.

Measurements

All pH measurements were made at 298 K. The pH meter (Iskra MA 5704) was calibrated with Fischer certified buffer solutions of pH 4.00 and 7.00. The results were not corrected for the deuterium isotope effect. Proton NMR spectra of D_2O solutions containing tsp (3-trimethylsilylpropane-1-sulfonate) as internal reference were recorded with Varian Gemini 200 and Bruker DRX 400 spectrometers.

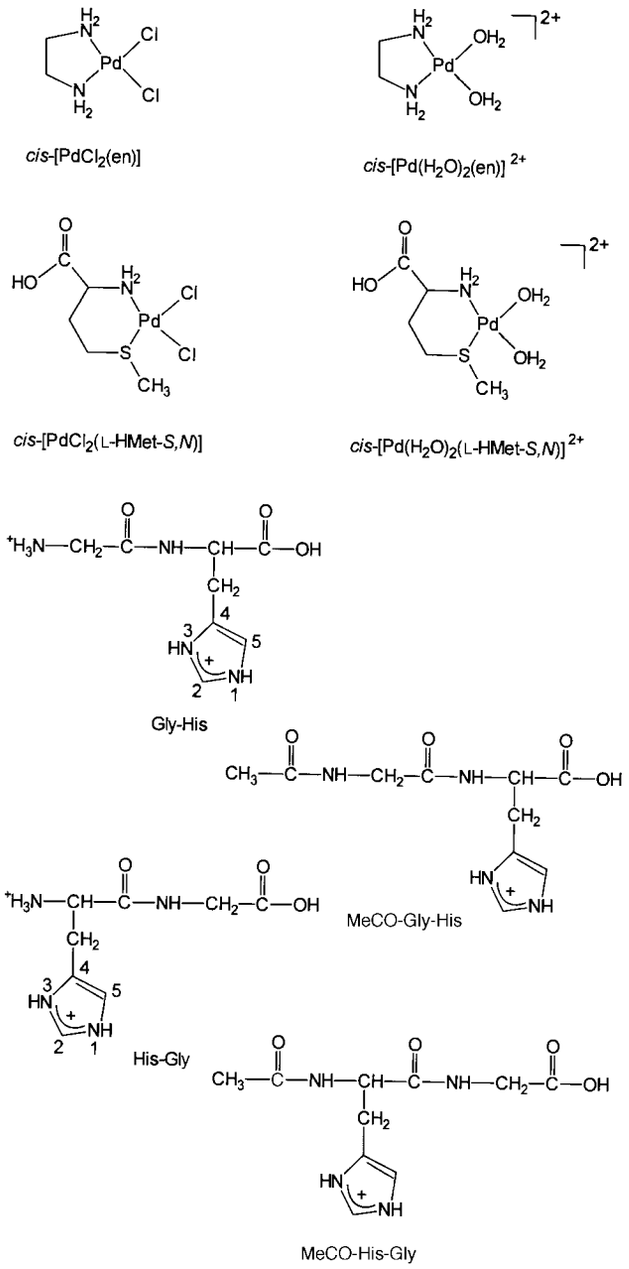
Results and Discussion

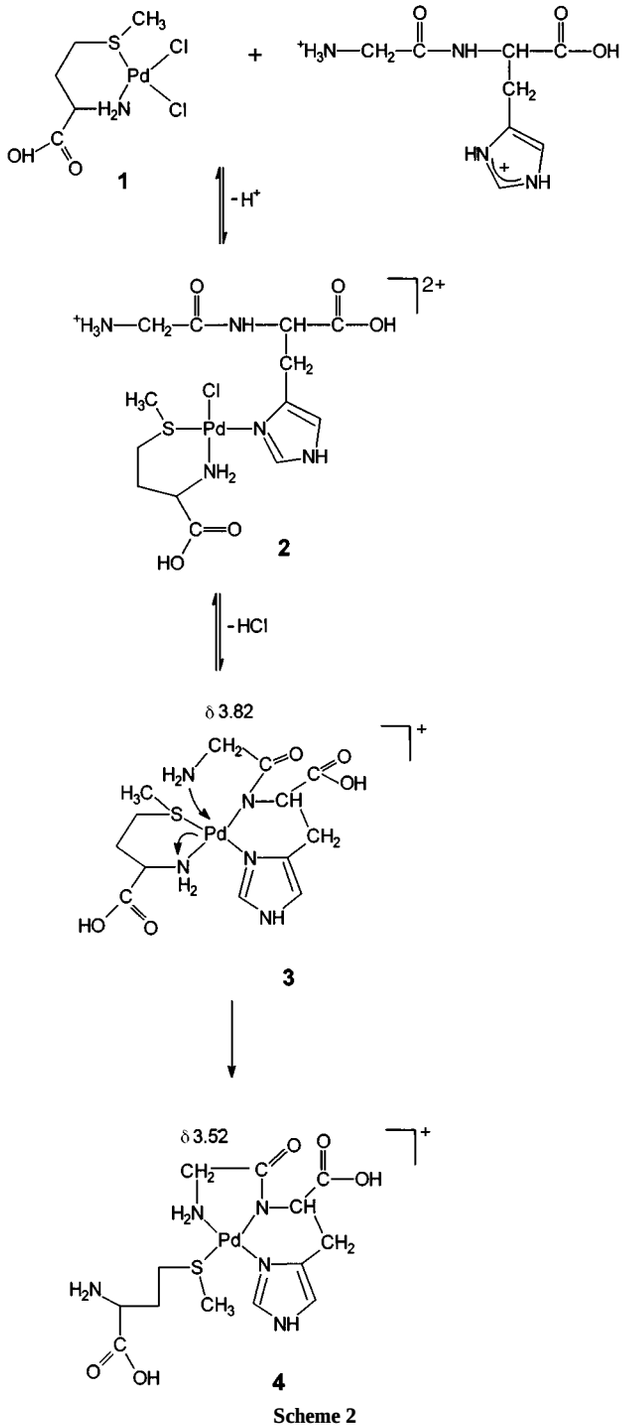
Reactions of palladium(II) complexes with Gly-His

The palladium(II) dichloro complexes and their aqua derivatives are as shown. The chelate ligands are inert to substitution and expected to remain bound to the palladium(II) atom during the reactions with the amino acids and peptides.

The reactions of $[Pd(H_2O)_2(en)]^{2+}$ and *cis*-$[Pd(H_2O)_2$(L-HMet-*S*,*N*)$]^{2+}$ with Gly-His did not cause cleavage of the peptide bond. The absence of hydrolysis was confirmed by ^{1}H NMR spectroscopy. The singlet peak for free glycine at δ 3.88 did not occur after 1 week at 60 °C and pH 2.0.

The chloro analogs of these two aqua complexes were used to explore the co-ordination chemistry of Gly-His in order to find out why this peptide did not hydrolyse. The course of the reaction between *cis*-[$PdCl_2$(L-HMet-*S*,*N*)] and Gly-His at pH 3.0 is shown in Scheme 2. The resonances of the H-2 and H-5 protons are good indicators of imidazole co-ordination to palladium(II). Immediately upon mixing the appearance of new resonances in the imidazole region indicated that complexes **2** and **3** were formed.[8] It is well known that palladium(II) anchored to a side chain, as in compound **2**, is highly effective in displacing the amide proton.[16] The estimated pK_a for this reaction is *ca.* 2.0, and the displacement is observed even in solutions with pH < 2.0.[16–22] The complexes **2** and **3** are in the acid–base equilibrium, but the potentiometric studies confirmed that the imidazole N-3 atom is the first donor atom to





Scheme 2

co-ordinate in the histidyl residue.[23] The deprotonated amide nitrogen ligand in **3** weakens the Pd–N bond in the *trans* position[24] and the amino group of methionine is displaced by the amino group of glycine. This conversion of **3** into **4** is evident in the simultaneous decline of the resonance at δ 3.82 and growth of that at δ 3.52; clearly, **3** is converted into **4**.

The chemical shifts of the imidazole H-2 and H-5 protons of compound **4** are consistent with the tridentate co-ordination of Gly-His.[25,26] The signal at δ 2.14 for the unco-ordinated CH_3S group of methionine did not appear even after the reaction mixture stayed for 10 d at room temperature. The methionine ligand does not seem to be released from the complex **4**. The reaction sequence in Scheme 2 is favorable because in it the number of chelate rings increases and a bidentate ligand is replaced by a tridentate ligand.

Upon mixing Gly-His with $[PdCl_2(en)]$ at pH < 3 the complex **5** formed in 95% yield. A sharp ^1H NMR singlet for free H_2en^{2+}, at δ 3.37, indicates complete detachment of the ethylenediamine ligand from the palladium(II), a process assisted by protonation of the amino groups. The molecular structure of the complex **5** is shown in Fig. 1. The geometry around palladium(II) is square planar with the peptide co-ordinated through imidazole N-3, amide, and amino nitrogen atoms. The fourth ligand is a chloride anion. The crystal structure of [PdCl(Gly-His-*N*,*N*,*N*)] was recently reported,[25] but that of the complex **5** slightly differs. The reported crystal was a monoclinic system and belonged to the space group $P2_1$, while the complex **5** crystallized in the trigonal system and belongs to the space group $P3_221$. Unlike the precedent, **5** does not contain hydrogen bonds between the carboxylic groups of the neighboring molecules. The hydrogen bonds in the crystal of **5** and their lengths are as follows: between N(1)–H and O(2) at $x, y, -z$ (2.45 Å); between N(2g)–H and O(2) at $x, y, -z$ (2.98 Å); and between N(2g)–H and O(10) at $-y, x-y, -\frac{2}{3}+z$ (2.03 Å). There is also a close contact between O(3)–H and Cl at $x, y, -z$ (2.27 Å).

The tridentate co-ordination of Gly-His is unfavorable for the hydrolysis of the peptide because co-ordination of the amide nitrogen atom to the palladium(II) strengthens the amide bond.[27,28] External attack is unlikely because the amide oxygen

Table 1 Structure determination summary

Empirical formula	$C_{9.50}H_{15}ClN_4O_{3.50}Pd$
M	383.11
Color, habit	Yellow, triangular
Crystal size/mm	0.3 × 0.2 × 0.1
Crystal system	Trigonal
Space group	$P3_221$
a/Å	9.8880(10)
b/Å	9.8880(10)
c/Å	23.211(5)
U/Å^3	1965.4(5)
Z	6
D_c/Mg m^{-3}	1.942
μ/mm^{-1}	13.451
F(000)	1146
Radiation (λ/Å)	Cu-Kα (1.541 78)
Monochromator	Graphite
θ Range/°	5.16–56.69
Scan type	ω
hkl ranges	−1 to 10, −10 to 1, −1 to 25
Reflections collected	2529
Independent reflections	1752 (R_{int} = 0.1552)
Observed reflections [$I \geqslant 2\sigma(I)$]	1647
Maximum, minimum transmission	1.0000, 0.2978
Solution	Direct
Refinement method	Full-matrix least squares on F^2
Weighting scheme, *w*	$1/[\sigma^2(F_o^2) + (0.0755P)^2 + 0.9642P]$ where $P = (F_o^2 + 2F_o^2)/3$
Parameters refined	174
*R*1, *wR*2 indices [$I \geqslant 2\sigma(I)$]	0.0396, 0.1031
(all data)	0.0571, 0.1083
Goodness of fit, observed and all data	1.014, 1.029
Largest and mean Δ/σ	−0.001, 0.000
Largest difference peak, hole/e Å^{-3}	0.707, −1.592

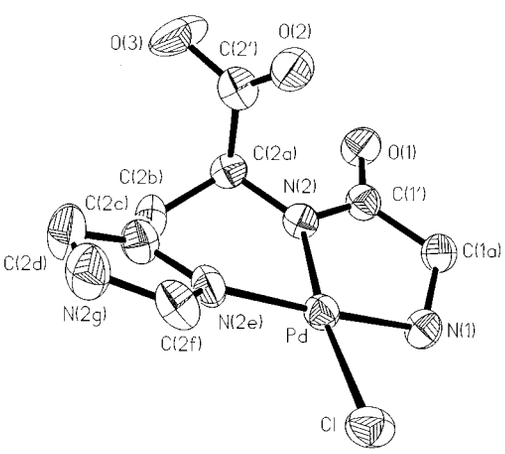
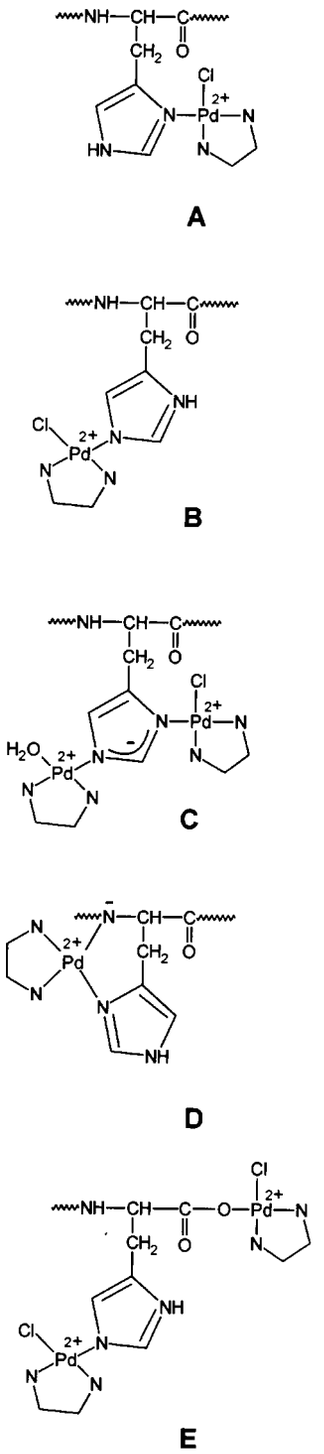


Fig. 1 Molecular structure of the compound **5** showing 50% probability displacement ellipsoids. Hydrogen atoms are omitted for clarity. Selected distances (Å) and angles (°): Pd–N(2e) 1.993, Pd–N(2) 2.003, Pd–N(1) 2.022 and Pd–Cl 2.32; N(2e)–Pd–N(2) 93.0, N(2)–Pd–N(1) 81.4, N(2e)–Pd–Cl 93.3 and N(2)–Pd–Cl 92.3

atom (O-1) does not interact with the palladium(II) atom. Internal attack is also unlikely because in the complex **4** water cannot displace the thioether ligand. In the complex **5** aquation of the chloride ligand is possible, but the aqua ligand and the amide bond would remain coplanar and too distant for the reaction.

Reactions of palladium(II) complexes with His-Gly

Hydrolytic reactions between the dipeptide His-Gly and complexes $[Pd(H_2O)_2(en)]^{2+}$ or *cis*-$[Pd(H_2O)_2$(L-HMet-*S*,*N*)$]^{2+}$ were studied at pH 2.5. In 1 week at 60 °C approximately 15% of the His-Gly bond hydrolysed in the presence of the former complex, and 20% in the presence of the latter. During the reaction, the ^{1}H NMR resonance at δ 3.96 for glycine in the peptide decreased, while that at δ 3.88 for free glycine increased.



In the reaction between $[PdCl_2(en)]$ and His-Gly no release of ethylenediamine was observed. The major product of this reaction at 1.5 < pH < 5.0 is a chelate complex of type **D**. As shown in this complex the peptide co-ordinates to the palladium(II) *via* imidazole N-3 and amino nitrogen atoms.[29,30]

In the reaction between *cis*-$[PdCl_2$(L-HMet-*S*,*N*)$]$ and His-Gly at 1.5 < pH < 5.0 the complexes of types **A** and **D** spontaneously formed. We did not observe opening of the L-HMet chelate even at pH < 1.5.

The previous study with MeCO-His-Gly showed that the complex of type **A** can effect hydrolysis, while the complex of type **D** cannot.[9] The latter complex lacks aqua ligands and the palladium(II) atom is held on the opposite side of the peptide

Table 2 Identification of reaction products according to the chemical shifts of imidazole protons

Reactants	Product type	δ (^{1}H) H-2	H-5
cis-[$PdCl_2$(en)] + MeCO-Gly-His	**A**	8.11	7.08
	B	7.80	6.80
	C	7.59	6.60
	D	7.91	7.04
	E	7.67	6.74
cis-[$PdCl_2$(L-HMet-*S*,*N*)] + MeCO-Gly-His	**A**	8.06	7.14
	B	7.97	6.98
	D	8.05	7.12
cis-[$PdCl_2$(en)] + His-Gly	**D**	7.70	7.08
cis-[$PdCl_2$(L-HMet-*S*,*N*)] + His-Gly	**A**	7.98	7.05
	D	7.94	7.04
cis-[$PdCl_2$(en)] + MeCO-His-Gly	**A**	8.13	7.11
	B	7.85	6.86
	C	7.66	6.60
	D	7.94	7.08
	E	7.73	6.77
cis-[$PdCl_2$(L-HMet-*S*,*N*)] + MeCO-His-Gly	**A**	8.03	7.13
	B	7.95	6.98
	D	8.03	7.11

bond. Neither the external attack nor the internal delivery of a water molecule is possible in this configuration. The co-ordination mode in the complex of type **A** permits the close approach of the metal atom and of its aqua ligands to the peptide bond. These two complexes exist in an acid–base equilibrium. Protonation of the amide nitrogen atom converts the inactive complex **D** into active complex **A**. Since, however, at pH >1.5 the latter is the minor species, the hydrolysis is slow and incomplete.

Reaction of palladium(II) complexes with MeCO-Gly-His and MeCO-His-Gly

When an equimolar amount of *cis*-[$Pd(H_2O)_2$(en)]$^{2+}$ or *cis*-[$Pd(H_2O)_2$(L-HMet-*S*,*N*)]$^{2+}$ was incubated with MeCO-His-Gly at 60 °C and pH 2 complete hydrolysis of the amide bond involving the carboxylic group of the histidine occurred in 2 d. No cleavage of the amide bond involving the amino group of the histidine was detected. When the same complexes were incubated with MeCO-Gly-His no cleavage of the amide bonds was observed after 1 week at 60 °C.

When *cis*-[$PdCl_2$(en)] was mixed with an equimolar amount of MeCO-Gly-His or MeCO-His-Gly at 1.5 < pH < 5.0 five NMR-detectable complexes formed. They are designated from **A** through **E**. The complexes were distinguished on the basis of the chemical shifts of imidazole protons; see Table 2.

Mixing of *cis*-[$PdCl_2$(L-HMet-*S*,*N*)] with an equimolar amount of MeCO-Gly-His or MeCO-His-Gly at 1.5 < pH < 5.0 resulted in a spontaneous formation of three NMR-detectable complexes, designated **A**, **B**, and **D**. The complexes **C** and **E** were not detected in solution even after 5 d. In the interval 0.5 < pH < 1.5 only complexes of the types **A** and **B** were detected in the ^{1}H NMR spectra, because unidentate co-ordination of imidazole *via* the N-1 or N-3 atom is favored at lower pH values.

Acetylation of the amino group in Gly-His and His-Gly lowers the nucleophilicity of the terminal nitrogen atom and its ability to co-ordinate to the palladium(II) atom. Consequently, the other modes of co-ordination become more favorable. In the reactions with *cis*-[$PdCl_2$(en)] the unprotected peptides formed just one complex, while the acetylated peptides formed five complexes. With the unacetylated peptides binding *via* the imidazole N-3 atom is favorable since it can be followed by co-ordination of the amino nitrogen atom resulting in a stable six-membered chelate ring. With the acetylated peptides, however, this chelation is less favorable, and therefore the other co-ordination modes, involving the imidazole N-1 atom, occur.

The lack of cleavage of the amide bond involving the amino group of histidine in both MeCO-Gly-His and MeCO-His-Gly implies that this bond does not interact with the palladium(II) moiety in any of the complexes designated from **A** through **E**. Since MeCO-Gly-His and MeCO-His-Gly behave essentially the same as ligands, this amide bond remains intact in either peptide. However, the co-ordination mode of the type **A** is favorable for cleavage of the amide bond involving the carboxylic group of histidine.[9] Since this amide bond is present in MeCO-His-Gly but not in MeCO-Gly-His, the former peptide is cleaved while the latter peptide remains intact.

Conclusion

This study brings us a step closer to our ultimate goal, namely artificial metaloproteases. The experimental findings suggest that efficient hydrolysis will occur at the His-Y amide bond in the sequence X-His-Y, in which X is an internal amino acid or acetylated N-terminal amino acid. If the X is an unprotected N-terminal amino acid, the tridentate co-ordination involving it and the imidazole N-3 and amide nitrogen atoms will result in the inhibition of hydrolysis. Likewise, the hydrolysis will not occur if histidine is a C-terminal amino acid in the sequence X-Y-His. If histidine is the N-terminal amino acid in the sequence His-X-Y the His-X bond will hydrolyse if co-ordination of the terminal amino group of histidine to palladium(II) is suppressed by sufficiently low pH values. We will continue to investigate the regioselectivity of these new reactions and will apply our findings to selective cleavage of longer peptides and proteins.

Acknowledgements

This work was funded in part by the National Science Foundation, through grant CHE-9404971, and by the Ministry of Science and Technology of the Republic of Serbia. We thank Mrs. B. Mojsilović for assistance with recording ^{1}H NMR spectra.

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Received 28*th February* 1997; *Paper* 7/01491K