New insights into the palladium-mediated selective hydrolysis of the His18–Thr19 peptide bond in cytochrome *c*: ^{1}H NMR and density functional theory investigation for model compounds

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The peptides, CH_3CO-Met-His-GlyH, CH_3CO-CysMe-His-GlyH, CH_3CO-CysMe-His-Gly-OEt and its imidazole derivatives, N^{τ}-benzyl, N^{τ}-tosyl, N^{τ}-benzyl-N^{π}-phenacyl, have been synthesized and used as model compounds for the mechanistic study of the selective cleavage of cytochrome *c* promoted by Pd(II) complexes. The peptide bond cleavage of these substrates by *cis*-$[Pd(en)(solvent)_2]^{2+}$ (solvent: D_2O or CD_3OD) was monitored by ^{1}H NMR spectroscopy. The results showed that the methionine-containing tripeptide differs from the *S*-methylcysteine-containing tripeptides in the mode of coordination to Pd(II). The former coordinates to Pd(II) through a sulfur atom, an amide nitrogen of methionine and an N^{π} atom of imidazole, forming a polycyclic chelate, and is resistant to hydrolysis. The latter, as a model compound for cleavage of the His18–Thr19 bond in cytochrome *c* with Pd(II) complexes, coordinates to Pd(II) *via* a sulfur atom, an amide nitrogen and a carbonyl oxygen of histidine to form a polycyclic chelate in which the His–Gly peptide bond is cleaved. Kinetic studies showed that protonation of the N^{π} atom of imidazole in the *S*-methylcysteine-containing tripeptides is one of the key factors in controlling the cleavage of the His–Gly bond. In order to obtain theoretical guidance on the cleavage reaction, the geometries of a representative N^{π} protonated tripeptide cation of CH_3CO-CysMe-His-GlyNMe and its Pd(II) complex with and without ancillary water molecules are optimized at the B3LYP density functional theory level using 3-21G, 6-31G(d) and LanL2DZ basis sets. Based on the experimental and theoretical results obtained from the model compounds, a mechanism is proposed for the first time to explain the nature of selective cleavage of the His18–Thr19 bond in cytochrome *c* promoted by Pd(II) complexes. Coordination of Pd(II) to the carbonyl oxygen of histidine and hydrogen bond formed between the C=O and ancillary dimer water weaken and polarize the C=O double bond of histidine, giving rise to cleavage of the peptide bond.

Selective hydrolysis of peptides and proteins is one of the most important reactions in both chemical and biochemical processes. Over the past decade, a variety of metal complexes have been developed for directly hydrolytic cleavage of peptide bonds in peptides[1–22] and proteins.[23–34] Simple palladium(II) and platinum(II) complexes were found to promote effectively the hydrolytic cleavage of peptides containing methionine,[6,7,11,12,20–22] cysteine,[5,8,15] histidine[9,10,14,20–22] or tryptophan residues[19] through coordination of the side chains. The use of Pd(II) complexes was also extended to site-specific cleavage of proteins such as horse heart cytochrome *c*,[27,31] horse myoglobin,[30] oxidized insulin B chains[16,17] and albumin from cow, pig and chicken eggs.[33] However, up to now, little is known about the factors governing the site specificity of the cleavage reactions.

In cytochrome *c*, there are two cysteine residues (Cys14 and Cys17) and two methionine residues (Met65 and Met80) as well as three histidine residues (His18, His26 and His33), which are the potential anchoring sites for Pd(II) complexes. However, only the His18–Thr19 peptide bond was hydrolyzed under the experimental condition described in the literature,[27,31] though additional cleavage sites were more recently observed and identified using a 10-fold excess of Pd(II) over the protein and a higher incubation temperature.[21] These results indicated that the His18–Thr19 peptide bond is more susceptible to cleavage, compared to other sites of cleavage. Previous studies on hydrolysis of CH_3CO-Cys-His-AlaH and CH_3CO-CysMe-His-GlyH with Pd(II) complexes showed that only the His–Ala and His–Gly bonds were cleaved and cleavage of Cys–His and CysMe–His was not observed. These findings further corroborate findings with cytochrome *c* by showing the special reactivity of the peptide bond in the -Cys-His-X- sequence. Alkylation of the sulfur atom does not affect the regioselectivity of cleavage.[27] In order to explain the selectivity of cytochrome *c* cleavage, therefore, the short tripeptide CH_3CO-CysMe-His-GlyH and its derivatives, in which CysMe is used to mimic the vinylCys in cytochrome *c*, are chosen as models for mechanistic studies of cytochrome *c* cleavage.

To prove the necessity of the -Cys-His- sequence for cleavage of the peptide bond at the C-terminal of histidine, in this report, we also prepared CH_3CO-Met-His-GlyH. The interaction of the tripeptide with the palladium(II) complex explained the importance of the cysteine residue in the cytochrome *c* cleavage. On the other hand, three imidazole derivatives of CH_3CO-CysMe-His-GlyOEt were synthesized and their kinetics of cleavage with the palladium(II) complex were measured in order to test the hypothesis that protonation of imidazole facilitates the hydrolytic cleavage. The geometries of a N^{π}-protonated tripeptide cation of CH_3CO-CysMe-His-GlyNMe, and its Pd(II) complex in the presence and the absence of ancillary water molecules are optimized by B3LYP density functional methods with 3-21G, 6-31G(d) and LanL2DZ basis

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sets. The interesting results obtained are very helpful for exploiting the nature of site-specific cleavage of the His18–Thr19 peptide bond in the cytochrome *c* with Pd(II) complexes.

Experimental

Materials

The deuterium solvents were obtained from Aldrich. The *N*-terminal-protected amino acids and EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) were obtained from Sigma. The terminal amino group in each amino acid or peptide was acetylated. The complex *cis*-[Pd(en)Cl_2] was prepared by the published procedure[35] and converted to *cis*-[Pd(en)$(CD_3OD)_2]^{2+}$ or *cis*-[Pd(en)$(D_2O)_2]^{2+}$ by treatment with 2 equiv. of anhydrous $AgBF_4$ in CD_3OD or in D_2O.[6,7] Other common chemicals were of reagent grade.

Measurements

Proton NMR spectra at 500 MHz of D_2O or CD_3OD solutions, using sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DDS) as an internal reference, were recorded with a Bruker AM-500 spectrometer. The sample temperature was kept at 40 ± 0.5 °C. The pH was measured with an Orion 901 instrument and a phoenix Ag–AgCl reference electrode. The uncorrected values in D_2O solution and nominal values in CD_3OD solution are designated pH*. The molecular masses of the synthesized peptides were measured by VG-ZAB-HS mass spectrometer.

Model compounds

CH_3CO-Met-His-GlyH. This was prepared in solution following the synthetic route: N^{α}-Cbz-N^{τ}-Cbz-histidine → N^{α}-Cbz-N^{τ}-Cbz-histidylglycine ethyl ester → N^{α}-H-N^{τ}-Cbz-histidylglycine ethyl ester → acetyl-methionyl-histidyl-glycine.[36,37] The molecular mass observed was m/z 386; calculated value for $C_{15}H_{23}N_5O_5S$ is 385.4.

CH_3CO-CysMe-His-Gly-OEt. This was prepared in solution as follows. N^{α}-Boc-N^{τ}-tosyl-histidine → N^{α}-Boc-N^{τ}-tosyl-histidylglycine ethyl ester → N^{α}-H-N^{τ}-tosyl-histidylglycine ethyl ester → acetyl-*S*-methylcysteinyl-histidylglycine. The molecular mass calculated for $C_{16}H_{25}N_5O_5S$ is 399.5; the observed value was 400.

CH_3CO-CysMe-His(N^{τ}-tosyl)-Gly-OEt. This was also synthesized following the same procedure as that of CH_3CO-CysMe-His-GlyH. Molecular mass calculated for $C_{23}H_{31}N_5O_7S_2$ is 553.6,: the observed value was 554.

CH_3CO-CysMe-His(N^{τ}-benzyl)-Gly-OEt. This was prepared following the same procedure as CH_3CO-CysMe-His(N^{τ}-tosyl)-Gly-OEt except that N^{α}-Boc-N^{τ}-tosyl-histidine was replaced by N^{α}-Boc-N^{τ}-benzyl-histidine. The molecular mass calculated for $C_{23}H_{31}N_5O_5S$ is 489.6; the observed value was 490.

CH_3CO-CysMe-His(N^{τ}-benzyl-N^{π}-phenacyl)-Gly-OEt imidazolium nitrate. *N*-CH_3CO-*S*-methylcysteine-*N*-hydroxysuccinimide ester was prepared by the standard ethyl chloroformate method in anhydrous tetrahydrofuran (THF).[38] After filtering triethylamine hydrochloride, the THF solution was directly used for further coupling reaction without purification. N^{α}-Boc-N^{τ}-benzyl-histidylglycine ethyl ester was synthesized by the EDC procedure.[36] Phenacylation of N^{π} of imidazole was followed by the method of Fletcher *et al.*[39] The preceding dipeptide ester (0.86 g, 2 mmol) and phenacyl bromide (0.4 g, 2 mmol) were dissolved in 20 mL of THF and stirred at room temperature for 4 days. The solvent was removed under reduced pressure. The residue was dissolved in 5 mL of trifluoroacetic acid (TFA) for 45 min. The TFA was then evaporated under vacuum. The residue was triturated with ether. The resulting pale solid was dissolved in 10 mL of THF and 1 equiv of triethylamine was added. A THF solution containing *N*-CH_3CO-*S*-methylcysteine-*N*-hydroxysuccinimide ester, which was prepared from 0.44 g (3.0 mmoL) of CH_3CO-CysMe and 0.35 g (3.0 mmoL) of *N*-hydroxysuccinimide, was added to the above solution. The mixture was stirred overnight at room temperature. After removing the solvent under vacuum, the residue was dissolved in 50 mL of chloroform and sequentially washed with saturated NaCl solution, 5% acetic acid, 5% $NaHCO_3$, and saturated NaCl solution. The chloroform was dried over sodium sulfate and evaporated to dryness. A slight excess of solid $AgNO_3$ (0.22 g, 1.3 mmol) was added to 20 mL aqueous solution of the residue. After vigorous stirring for 15 min, the white AgCl precipitate was removed by filtration. The filtrate was saturated with KNO_3 and extracted three times with a total of 25 mL chloroform. The chloroform solution was evaporated to dryness. The residue was recrystallized from chloroform and ether. The total yield was 52%. The molecular mass observed was 609; the calculated value for $C_{31}H_{38}N_5O_6S$ is 608.7.

Cleavage study of peptide bond

cis-[Pd(en)$(solvent)_2]^{2+}$ solution (solvent = CD_3OD or D_2O) was freshly prepared to minimize formation of polymeric complexes. Equimolar amounts of substrate and of the Pd(II) complex, both dissolved in CD_3OD or D_2O, were mixed rapidly in an NMR tube. The solution was 20 mM in each and the total volume was 600 μL. Because the pH difference was always less than 0.10, pH* was measured only at the end of the experiments. Acquisition of the ^1H NMR spectra started immediately after mixing, and 16 scans were collected for each spectrum. The cleaved product, free glycine or free glycine ethyl ester, was monitored by enhancement of a singlet at 3.87 or 3.82 ppm, with decrease of the multiplet at 4.06 or 4.01 ppm of the glycyl residue in the substrate. At the end of the experiments, no new resonance appeared upon addition of the authentic glycine or glycine ethyl ester to the reaction mixture. The error in integration of these resonances was estimated at ±5%. First-order logarithmic plots of substrate concentration or product concentration *versus* time were linear for a period of 3 half-lives. Typical plots consisted of 10–20 points and correlation coefficients were 0.990–0.999.

Optimization of conformations

The structures of the N^{π}-protonated tripeptide cation of CH_3CO-CysMe-His-GlyNHCH$_3$ and its Pd(II) chelates involving a thioether, an amide nitrogen and a carbonyl oxygen of histidine, and one aqua ligand, in the presence and the absence of ancillary water molecules, were optimized in two steps—first optimized by the HyperChem v. 6.0 software employing the MM^+ method of molecular mechanics with a constraint of a hydrogen bond formed between the protonated N^{π} atom of histidine and the carbonyl oxygen of glycine, then optimized by the density functional B3LYP theory in the Gaussian 98 program package[40] without any constraint using the 6-31G(d) basis set for the tripeptide cation, the LanL2DZ basis set for its Pd(II) chelates with and without the ancillary water molecules, and the 3-21G basis set for all compounds above-mentioned. The RMS gradient was less than 0.01 kcal $Å^{-1}$ mol^{-1} for the MM^+ method and 10^{-8} atomic units of convergence for B3LYP method.

Results and discussion

Interaction of CH_3CO-Met-His-Glyh with *cis*-[Pd(en)$(D_2O)_2]^{2+}$ in D_2O

When mixing CH_3CO-Met-His-GlyH with *cis*-[Pd(en)-$(D_2O)_2]^{2+}$ in a weak acid solution of pH* ~1.5, the thioether

group at the side chain of methionine rapidly reacts with the Pd(II) complex, accompanied by a prominent migration of the CH_3S ^1H resonance downfield to 2.48 ppm. At the same time, ethylenediamine in the complex is detached from Pd(II) by acidic action and by the strong *trans* effect of the thioether group. Free enH_2^{2+} was readily detected by the growth of a sharp ^1H NMR singlet at 3.37 ppm. During the reaction, a new singlet resonance at 2.06 ppm was enhanced with the concomitant decrease of the acetyl group CH_3 ^1H NMR resonance of the free peptide at 2.02 ppm. It implied that the new resonance was associated with the acetyl group. After the reaction was complete, a small amount of acetic acid was added to the reaction mixture, whereupon a new resonance at 2.08 ppm appeared. It was evident that the resonance at 2.06 ppm did not belong to free acetic acid. When NaI was added to the solution, PdI_2 precipitated immediately.[6] After removing the precipitate, no new resonance that was different from the original tripeptide was observed. All these results indicate that the Pd(II) anchors to the side chain of methionine, followed by the coordination of the deprotonated amide nitrogen of methionine, with migration of the acetyl group CH_3 resonance to 2.06 ppm. This coordination reaction was capable of proceeding to completion, as judged by ^1H NMR spectra. The observed rate constant, k_{obsd}, for the reaction was $21.6 \pm 3 \times 10^{-3}$ min^{-1} at 40 °C and pH* = 1.53. In addition, it was found that the imidazole of His also coordinated to Pd(II) *via* the N^{π} atom, though it is protonated in the free tripeptide ($pK_a = 6.5$). The H2 and H5 resonances of imidazole moved from 8.65 and 7.33 to 8.10 and 7.10 ppm upon coordination. The coordination of Pd(II) to the N^{τ} atom of imidazole was not observed in the NMR spectra. Although the detailed structure is unclear as yet, the ^1H NMR spectra confirmed that the Pd(II) can be coordinated by the sulfur atom and the amide nitrogen of methionine, and the N^{π} atom of histidine. It should be emphasized that hydrolytic cleavage of the peptide bond does not occur in this case.

Hydrolysis of CH_3CO-CysMe-His-GlyH in D_2O

CH_3CO-CysMe-His-GlyH differs apparently from CH_3CO-Met-His-GlyH in coordination to Pd(II) complexes. When the *S*-methylcysteine containing peptide was mixed with *cis*-$[Pd(en)(D_2O)_2]^{2+}$ in a molar ratio of 1:1, the ^1H NMR signal of SCH_3 moved downfield from 2.14 to 2.45 ppm, and ethylenediamine was rapidly released, however, the ^1H NMR resonances of CH_3 of the acetyl group, and of H2 and H5 of imidazole were unaffected. The hydrolysis of the His–Gly peptide bond was observed by ^1H NMR monitoring, with observed rate constant $k_{obsd} = 4.4 \times 10^{-3}$ min^{-1} at 40 °C. The rate of hydrolysis is slightly faster than that of alcoholysis determined in methanol, as Table 1 shows. Electrospray mass spectrometry (ESMS) also detected the complex [Pd(*S*,*N*,*N*,*O*-CH_3CO-CysMe-His-GlyH)] in which Pd(II) was coordinated by the *S* atom of CysMe, the amide nitrogen and the carbonyl oxygen of histidine, and the N^{π} atom of imidazole.[41] The coordination of Pd(II) to the carbonyl oxygen of histidine in the complex may be associated with cleavage of the His–Gly bond. In acidic solution (pH ~ 1.0) used for the hydrolysis studies of peptides and cytochrome *c*,[27,31] the imidazole nitrogen in the complex was detached from Pd(II) and was protonated ($pK_a = 5.6$), which was replaced by an aqua ligand.

Table 1 Kinetic data of *cis*-$[Pd(en)(CD_3OD)_2]^{2+}$-promoted cleavage of His–Gly bond in CH_3CO-CysMe-His-Gly-OEt and its imidazole derivatives at 40 °C and pH* 1.1 ± 0.1

Substrate	k_{obsd}/min^{-1}
CH_3CO-CysMe-His-Gly-OEt	2.3×10^{-3}
CH_3CO-CysMe-His(Bzl)-Gly-OEt	4.0×10^{-3}
CH_3CO-CysMe-His(Tos)-Gly-OEt	very slow
CH_3CO-CysMe-His(Bzl, phenacyl)-Gly-OEt	not detected

Alcoholysis of CH_3CO-CysMe-His-Gly-OEt and its imidazole derivatives in methanol

Because the imidazole derivatives of CH_3CO-CysMe-His-Gly-OEt are soluble in methanol, rather than in water, the kinetic studies of Pd(II) complex-mediated cleavage of peptide bond for these substrates were carried out in methanol solution, instead of water, for comparison. As shown in Table 1, the groups linked to the imidazole remarkably affected the alcoholysis of these substrates. CH_3CO-CysMe-His-Gly-OEt was alcoholized to give glycine ethyl ester. When a benzyl group (an electron-donating group) is linked to the N^{τ} atom of imidazole, it would be expected to enhance the basicity of the imidazole. As a result, the resonances of H2 and H5 of imidazole moved toward high field in CD_3OD solution of pH* 1.1, from 8.96 and 7.51 ppm for CH_3CO-CysMe-His-Gly-OEt to 8.88 and 7.35 ppm for the benzyl group-substituted derivative. Corresponding to this, CH_3CO-CysMe-His(N^{τ}-benzyl)-Gly-OEt was alcoholized to give free glycine ethyl ester with $k_{obsd} = 4.0 \times 10^{-3}$ min^{-1}, which was slightly faster than that of CH_3CO-CysMe-His-Gly-OEt. When tosyl (tolylsulfonyl) group, a well known strong electron-withdrawing group, was connected to the N^{τ} atom of imidazole, it was anticipated to greatly reduce the basicity of the imidazole. Its ^1H NMR resonances of H2 and H5 of imidazole shifted downfield and appeared at 9.41 and 7.79 ppm at pH* 1.1. Only trace amounts of glycine ethyl ester were detected after 24 h of incubation at 40 °C. No glycine ethyl ester was detected for the substrate of CH_3CO-CysMe-His(N^{τ}-benzyl-N^{π}-phenacyl)-Gly-OEt imidazolium nitrate, in which both N^{τ} and N^{π} atoms of imidazole were blocked. Therefore, it is concluded that the ability to protonate the N^{π} atom of imidazole is one of the key factors for controlling the Pd(II) promoted alcoholysis reaction of these substrates. It is interested to note that the site of cleavage in alcoholysis of these substrates and hydrolysis of CH_3CO-CysMe-His-GlyH is the same as that in hydrolysis of cytochrome *c*, promoted by Pd(II) complexes. Therefore, the short tripeptide CH_3CO-CysMe-His-GlyH and its imidazole derivatives can be taken as model compounds for mechanistic studies of cytochrome *c* cleavage. The protonation of the N^{π} atom of histidine is required to stabilize the conformation of the tripeptide upon hydrogen bond formation between the protonated N^{π} atom of histidine and the carbonyl oxygen of glycine (*vide infra*).

Conformational optimization of N^{π} atom-protonated CH_3CO-CysMe-His-GlyNCH$_3$ and its Pd(II) complexes

N^{π} atom-protonated CH_3CO-CysMe-His-GlyNCH$_3$. In our previous study,[42] the conformation of the tripeptide cation was optimized by combination of the three-dimensional energy surfaces of AcCysMe and N^{π} protonated AcHisGly cation using the AM1 semiempirical method. The most stable conformer of the tripeptide cation in that calculation exhibits a typical hydrogen bond formed between the protonated N^{π} atom of imidazole and the carbonyl oxygen of glycine, and reveals that the sulfur, amide nitrogen and carbonyl oxygen atoms of histidine are located on the same side which makes their coordination to Pd(II) more favorable with minor perturbation of the conformation. In order to gain further insights into the two important results, the geometry of the N^{π} protonated tripeptide was optimized at the B3LYP/3-21G and B3LYP/6-31G(d) levels. The selected parameters of geometry and charges on atoms are listed in Table 2. As shown in Fig. 1 and Table 2, all these features obtained by the AM1 method are kept in the optimized conformation, though the optimized

Table 2 Select parameters of geometries and charges on atoms in the optimized conformers[a b]

	1	2	3
Pd–S(Cys)		2.426 (2.393)	2.422 (2.398)
Pd–O(H_2O)		2.134 (2.134)	2.079 (2.081)
Pd–N^{α}(His)		1.978 (1.997)	1.992 (1.999)
Pd–O(His)		2.110 (2.084)	2.158 (2.145)
C=O(His)	1.243 (1.226)	1.284 (1.292)	1.290 (1.296)
C–N(Gly)	1.368 (1.364)	1.337 (1.346)	1.333 (1.345)
S–Pd–O(H_2O)		92.20 (88.46)	90.84 (89.66)
S–Pd–O(His)		91.26 (96.59)	90.94 (94.50)
N^{α}(His)–Pd–O(His)		80.54 (81.96)	78.10 (79.92)
O(H_2O)–Pd–O(His)		96.71 (93.00)	100.29 (95.88)
N^{π}(His)–H···O(Gly)	2.626 (2.722)	2.612 (2.628)	2.615 (2.630)
N^{π}(His)–H···O(Gly)	171.6 (174.6)	172.0 (175.8)	176.0 (177.9)
O(H_2O)–H···O(His)			2.618 (2.736)
O(H_2O)–H···O(His)			158.0 (157.7)
Charge on atom:			
C(C=O of His)	+0.693	+0.764	+0.778
O(C=O of His)	−0.492	−0.489	−0.533
N(C–N of Gly)	−0.695	−0.689	−0.679

[a] The conformers from **1** to **3** represent the conformations given in Figs. 1 to 3. [b] The numbers without parentheses are calculated at the B3LYP/3-21G level and the numbers in parentheses are calculated at the B3LYP/6-31G(d) level for conformer **1** and at the B3LYP/LanL2DZ level for conformers **2** and **3**.

geometry may be a local minimum, and the bond lengths and hydrogen bonds calculated at the B3LYP/3-21G and B3LYP/6-31G(d) levels are similar.

Pd(II) complex of the tripeptide cation with one aqua ligand. Based on the above-mentioned experimental and theoretical studies, the initial structure was constructed as follows for geometry optimization by MM^+ molecular mechanics. The Pd(II) was coordinated by the sulfur atom of *S*-methylcysteine, the amide nitrogen and the carbonyl oxygen of histidine, and an aqua ligand, with a hydrogen bond formed between the protonated N^{π} atom of imidazole and the carbonyl oxygen of glycine, or the carbonyl oxygen of histidine, or both. The different initial structures were then optimized at the B3LYP/LanL2DZ level without any constraint. A unique conformation was finally obtained as shown in Fig. 2. For comparison of parameters of geometries and charges on atoms between the free tripeptide cation and its Pd(II) complex, the conformer **2** was also optimized at the B3LYP/3-21G level. The bond lengths calculated by using these two basis sets are very similar, but some deviations of bond angles are evident. The parameters of geometry and charges on atoms for conformer **2** are given in Table 2. As Fig. 2 shows, Pd(II) is coordinated in a square-planar configuration and a typical hydrogen bond between the protonated N^{π} atom of imidazole and the carbonyl oxygen of glycine is kept, similar to the free tripeptide cation. Compared with X-ray crystallographic analysis results,[43] the calculated Pd–S distance is 0.16 Å longer and

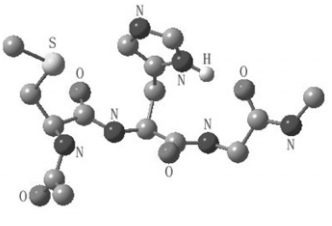

Fig. 1 The conformation of N^{π}-protonated CH_3CO-CysMe-His-GlyNCH_3, optimized at the B3LYP/6-31G(d) level.

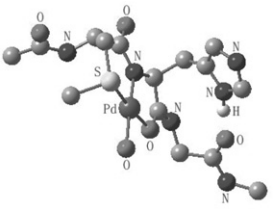

Fig. 2 The conformation of the Pd(II) complex, which contains N^{π}-protonated CH_3CO-CysMe-His-GlyNCH_3 cation and one aqua ligand, is optimized at the B3LYP/LanL2DZ level.

the Pd–N distance is similar. As shown in Table 2, the coordination of Pd(II) to the carbonyl oxygen of histidine lengthens the C=O bond but shortens the C–N bond distance due to π electron donation from nitrogen to carbon. In other words, the C=O bond, rather than the C–N bond, is weakened upon coordination. The positive charge on the carbonyl carbon is also increased by means of the coordination, from +0.6934 e in conformer **1** to +0.7640 e in conformer **2**. The increased positive charge of this carbon atom and the weakened C=O double bond are favorable for nucleophilic attack by solvent water to form a tetrahedral carbon intermediate, followed by breaking of the C–N bond.

Water-assisted cleavage of the His–Gly bond. Two mechanisms[2,21,22] have been proposed for interpretation of the hydrolytic cleavage of the peptide bond by transition metal complexes. A transition-metal ion either binds to amide oxygen of the scissile peptide bond, resulting in breaking of the peptide bond by external attack of solvent water molecules, or delivers an aqua ligand to the scissile amide carbon, then cleaving it. Although the two mechanisms are kinetically distinguishable, our experimental and theoretical results reported here corroborate the external attack mechanism. Because methanol is more nucleophilic than water for external attack on carbonyl carbon,[11] it would be anticipated that the alcoholysis of the CysMe-containing tripeptide would be faster than the hydrolysis. However, as indicated above, in fact, the hydrolysis is slightly faster than the alcoholysis. This implies that water molecules may assist the cleavage reaction besides external attack by solvent water molecules. On the other hand, since almost all Pd(II)-mediated hydrolysis of peptides and proteins is carried out in bulk water, the effect of the water on the cleavage should be properly considered. In our case, one and two water molecules are involved in geometry optimization. As far as only one molecule of water is concerned, the water molecule is close to the aqua ligand upon hydrogen bonding. As Fig. 3 shows, only the dimer water molecules involved will assist the cleavage of the His–Gly bond. The hydrogen bond formed between the dimer water and the carbonyl oxygen of His causes the C=O and C–N distances to be further lengthened and shortened respectively, from 1.284 Å to 1.290 Å and from 1.337 Å to 1.333 Å,. The charges on the carbonyl

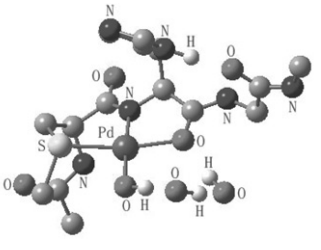

Fig. 3 The conformation of Pd(II) complex containing the tripeptide cation, one aqua ligand and dimer water molecules is optimized at the B3LYP/LanL2DZ level. This conformation is associated with water-assisted cleavage of the His–Gly bond.

carbon and oxygen are further increased respectively from +0.7640 e to +0.7780 e and from −0.4889 e to −0.5325 e. Although the variation of these values is small, it is indicated again that the C=O double bond is further weakened and polarized. Therefore, the dimer water assists the cleavage of the His–Gly bond. These results are consistent with the fact that the hydrolysis of the His–Gly bond is slightly faster than the alcoholysis.

Conclusion

The results obtained satisfactorily explain the necessity of both the -Cys-His- sequence and the hydrogen bond formed between the N^{π} atom of histidine and C=O of glycine for cleavage of cytochrome *c* and its model compounds. The hydrogen bond stabilizes the conformation of the tripeptide cation in which the sulfur atom of the cysteine residue, the amide nitrogen (upon deprotonation) and the oxygen of histidine can readily coordinate to Pd(II). This type of coordination does not occur in the methionine-containing peptide of CH_3CO-Met-His-GlyH, because of formation of a labile seven-membered chelate ring. Although the carbonyl oxygen of histidine is a weak ligand, its coordination to Pd(II) is facilitated by the chelate effect. This type of coordination increases the positive chage on the carbonyl carbon and weakens the C=O double bond, rather than the C–N bond. The cleavage of the C–N bond is probably caused by nucleophilic attact of solvent water, resulting in a tetrahedral carbon intermediate. The assistance of the ancillary dimer water futher weakens and polarizes the C=O double bond to make the cleavage of the His–Gly bond more feasible.

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