Pd(II) Complexes Mediated Hydrolytic Cleavage of Insulin B Chain: Regioselectivity and Influence of Peptide Secondary Structure

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Electrospray mass spectrometry studies show that the binding of Pd(II) complexes and the site of cleavage are highly dependent on the secondary structure and local environment of oxidized insulin B chain (**B**). The hydrolytic cleavage of denatured **B** by Pd(II) complexes is monitored by HPLC, which follows first order kinetics with half-life of 4.8 days at 40 °C. Two amide bonds at Leu6-Cys7 and Gly8-Ser9 are selectively cleaved.

Over the past decade, there are considerable interests in designing artificial metallopeptidases. A variety of metal complexes have been designed and their activity towards the directly hydrolytic cleavage of amide bonds in peptides have been investigated.[1–6] However, there are only scant information on the directly selective hydrolysis of proteins or long peptides.[7–12] This forms a strong contrast with the fact that in the wide range of practical applications, such as protein sequencing, structure mapping and functional analysis, analysis of protein–DNA interaction and development of new peptide drugs, mainly involves proteins and long peptides.

The different behavior in selective cleavage of amide bonds between peptides and proteins is likely due to their conformational difference. In order to understand the possible effect of the secondary structure and local environment of bonding sites in the proteins and long peptides on the cleavage, oxidized insulin B chain is chosen for this purpose.

The hog oxidized insulin B chain was prepared and purified according to the published procedures,[13] and was stored in aqueous solution of H_3PO_4 at pH 2.5 with concentration of 20 mg·mL^{-1}. Its sequence is as follows:
FVNQHLC(-SO_3H)GSHLVEALYLVC(–SO_3H)GERGFFYTPKA

Electrospray mass spectrometry (ESMS, LCQ, Finnigan MAT) determined the *m/z* of the purified **B** and the molecular mass determined is 3496.0, precisely equal to 3495.9 calculated by IsoPro3.0 program for $C_{157}H_{232}N_{40}O_{47}S_2$.

The interaction between Pd(II) and **B** under acid condition was studied by ESMS. Pd(II) complex, *cis*-[Pd(en)$(H_2O)_2]^{2+}$(**1**) (en: ethylenediamine) or *cis*-[Pd(dtco-3-OH)$(H_2O)_2]^{2+}$(**2**) (dtco: dithiacyclooctane), was added to **B** in acidic solution (pH 2.5) at room temperature in a molar ratio of 5:1. Two ESMS spectra were obtained for the mixed solutions, both are associated with the complex ion [PdL**B** + $H^+]^{3+}$ in which L is en or dtco-3-OH, indicating that only one molecule of Pd(II) complex was bound to **B** under this condition, although there are three potential binding sites in **B**, including terminal NH_3^+, imidazole groups of His5 and His10.[14] The secondary structure of **B** in solution seems to be similar to that in the crystal structure,[15] it is reasonable that the binding site is the terminal amino group which is less steric hindrance than the other two histidines. Either ligand en or dtco-3-OH remains attached to Pd(II).

When 6 mol·L^{-1} urea that was used to denature **B** to a random coil conformation was incubated with the mixture solution of complex **1** and **B** for 4 h at room temperature and pH 2.5, two new peaks were observed in the ESMS spectrum which correspond to the formation of complexes [Pd_2L_2**B** – $H^+]^{3+}$ (m/z: 1275.6) and [Pd_3L_3**B** – $3H^+]^{3+}$ (*m/z*: 1330.7) besides complexes [PdL**B** + $H^+]^{3+}$ and [PdL**B**$]^{2+}$ (Figure 1). This change is likely due to the coordination of Pd(II) to N-3 atom of imidazole of His5 and His10 in **B** upon denaturation. The ligand en remains attached to Pd(II) in all species, similar to that observed in the absence of urea. ^{1}H NMR spectrum also confirms the coordination of His5 and His10 to Pd(II). The chemical shift of free imidazole H-2 is 8.41 ppm, which shifted to 7.92 ppm after addition of Pd(II) complex. The chemical shift of H-5 was not observed because of the overlapping with aromatic ring protons of Phe residue. Based on molecular masses, charges and loss of protons in the species of [Pd_2L_2**B** – $H^+]^{3+}$ and [Pd_3L_3**B** – $3H^+]^{3+}$, the remaining binding site is likely to be deprotonated amide nitrogens of His5 and His10, forming six-membered chelate ring.

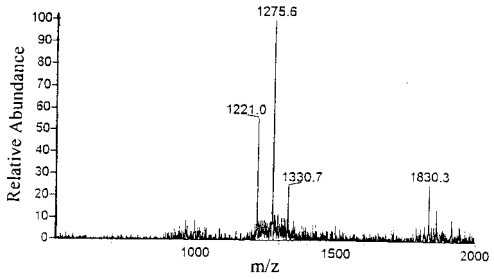


Figure 1. ESMS spectrum recorded 4 hours after adding 6 mol·L^{-1} of urea to the mixed solution of **B** and *cis*-[Pd(en)$(H_2O)_2]^{2+}$ at room temperature and pH 2.5.The three groups of peaks, separated by 0.3 m/z each, are attributed to [PdL**B**+$H^+]^{3+}$ (1221.0), [Pd_2L_2**B**-$H^+]^{3+}$ (1275.6) and [Pd_3L_3**B**-$3H^+]^{3+}$ (1330.7), respectively. A group of peaks, separated by 0.5 m/z is attributed to [PdL**B**$]^{2+}$ (1830.3).

The mixture solution of Pd(II) complex and **B** in a molar ratio of 5 : 1 was incubated with 6 mol·L^{-1} urea at pH 2.5 and 40 °C for 10 days, which was then separated into two portions. One of them was directly analyzed by HPLC (Figure 2). Two fractions with retention time (RT) of 4.05 min and 12.67 min were collected and analyzed by ESMS. For fraction one (RT = 4.05 min), an ionic species with m/z = 454.7–458.1 was observed which can be attributed to [Pd(II) + **B1** + H_2O + $CH_3OH]^{2+}$ (**B1** = fragment of Phe1–Leu6). For fraction two (RT = 12.67 min) two ionic peaks with m/z = 1326.1–1330.1 and 1336.7–1341.1 were observed and attributed to [Pd(II) + **B2**$]^{2+}$ and [Pd(II) + **B2** + Na^+ – $H^+]^{2+}$, respectively (**B2 =** fragment of Ser9–Ala30). In all species, the ligand en is detached from Pd(II) during digestion. Another portion of the digestion solution was treated with 20-fold excess of *N*,*N*-diethyldithiocarbomate to remove Pd(II) and then analyzed by ESMS. Besides Cys(–SO_3H)–Gly, there are also two components present which correspond to **B1** and **B2**, and there is no fragment detected which is associated with

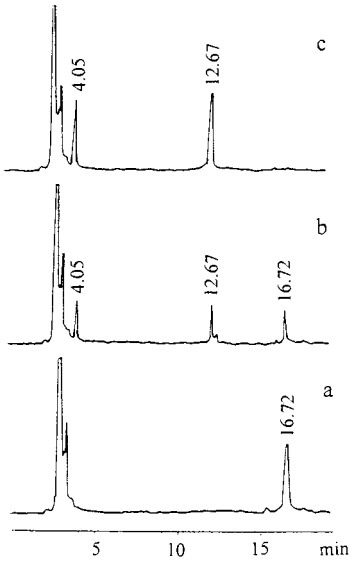
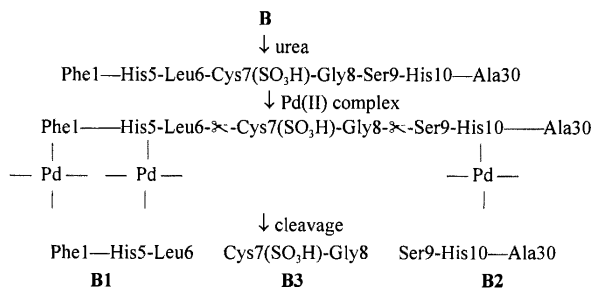


Figure 2. HPLC chromatograms recorded during the reaction of *cis*-$[Pd(en)(H_2O)_2]^{2+}$ with **B** at 40 °C and pH 2.5. (a): 4 h; (b): 10 d; (c): 30 d. The data are fitted to first order reaction equation, $-\ln[(C_x-C_\infty)/C_x] = k_{obsd} \times t$. The observed rate constants k_{1obsd} and k_{2obsd} of **B1** and **B2** are listed below. For $[Pd(en)(H_2O)_2]^{2+}$ with a molar ratio of 5 : 1 (Pd : **B**) , $k_{1obsd} \times 10^4(min^{-1}) = 1.10 \pm 0.07$ (pH 2.5), 0.97 ± 0.05 (pH 6.0); $k_{2obsd} \times 10^4(min^{-1}) = 0.97 \pm 0.03$ (pH 2.5), 0.90 ± 0.04 (pH 6.0). For $[Pd(dtco\text{-}3\text{-}OH)(H_2O)_2]^{3+}$ with a molar ratio of 5 : 1 (Pd : **B**), $k_{1obsd} \times 10^4(min^{-1}) = 0.92 \pm 0.02$ (pH 2.5), 0.89 ± 0.03 (pH 6.0); $k_{2obsd} \times 10^4(min^{-1}) = 0.83 \pm 0.05$ (pH 2.5), 0.81 ± 0.02 (pH 6.0). As shown from the data, the k_{1obsd} and k_{2obsd} are almost same, even though the two additional intermediate peptides, Phe1-Gly8 and Cys7-Ala30, are present, their amounts are undetected, and the variation of pH values does not have significant impact on the hydrolytic rate of cleavage. However, the molar ratio of Pd(II) to **B** does remarkably affect the cleavage. When the molar ratio of Pd(II) to **B** is 2:1, the cleavage reaction is very slow.

the cleavage of Asn3–Gln4 bond. Further separation was proceeded by HPLC. Both components with retention time of 4.05 min and 12.67 min disappeared, while only a new component with retention time of 12.73 min appeared, which was analyzed by ESMS. The molecular masses obtained are consistent with the ionic species $[\mathbf{B2} + 4Na^+ - H^+]^{3+}$ (2636.8) and $[\mathbf{B2} + 2Na^+]^{2+}$ (2594.8). This component was further analyzed by *N*-terminal amino acid sequencing, and the first ten amino acid sequence of fragment **B2** is SHLVEALYLV. The other component (**B1**) is too short to be retained by the chromatograghic column.

Taken together, the cleavage reaction proved that the total denaturation of oxidized insulin **B** by treatment of 6 mol·L^{-1} urea is required for the successful metallation and hydrolysis, and summarized in Scheme 1. First, Pd(II) complex, *cis*-$[Pd(en)(H_2O)_2]^{2+}$ or *cis*-$[Pd(dtco\text{-}3\text{-}OH)(H_2O)_2]^{2+}$, is anchored to His5 and His10 of the denatured **B**, then the vicinal amide bonds of them are cleaved by attack of water molecule which may coordinate to Pd(II) or come from solvent. The denatured **B** is cleaved by Pd(II) complexes at two sites: one is at the second amide bond right from His5, and the other is at the second amide bond left from His10. A similar pattern of cleavage was observed in peptides and proteins.[9–11] In peptides[9] and protein (myglobin)[10] containing Ser–His sequence, the cleavage site is always at the second amide bond left from histidine. The hydroxyl group of side chain in serine may play a special role in the cleavage. The cleavage of second amide bond right from His5 is probably associated with negative charge of Cys(SO_3^-)7. The electrostatic interaction attracts Pd(II) anchored to imidazole

Scheme 1.



of His5 and directs it closer to Leu6 and accelerates the cleavage of Leu6-Cys7 bond.[11]

The control experiments with and without 6 mol·L^{-1} urea proceeded at 40 °C and pH 2.5 for 32 days were monitored by HPLC and ESMS. There was no any fragment of cleavage observed. This clearly shows that the selective cleavage is promoted by Pd(II) complexes which is anchored to histidine residues.

The Pd(II) complexes mediated hydrolytic cleavage of oxidized insulin B chain under denaturing conditions provides an example, indicating that its cleavage is highly affected by the secondary structure of the peptide and is also regioselective. The different behavior in cleavage between short and long peptides is probably associated with their secondary structures. The cleavage of Gly8–Ser9 bond by Pd(II) complexes is due to the special sequence of Ser9–His10, which provides a potential application in cleavage of proteins and long peptides containing Ser–His sequence. It should be emphasized that Pd(II) complex can cleave the substrate even at near neutral pH, with the similar rate of cleavage to that at pH 2.5, which is crucial for Pd(II) complex becoming a real reagent in selective cleavage of proteins and long peptides.

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References and Notes

1 X. Chen, L. Zhu, X. You, and N. M. Kostić, *J. Biol. Inorg. Chem.*, **3**, 1 (1998).
2 G. B. Karet and N. M. Kostić, *Inorg. Chem.*, **37**, 1021 (1998).
3 X. Chen, X. Luo, Y. Song, S. Zhou, and L. Zhu, *Polyhedron*, **17**, 2271 (1998).
4 X. Luo, X. Chen, and L. Zhu, *Chem. Lett.*, **1998**, 1079.
5 X. Luo, W. Huang, Y. Mei, S. Zhou, and L. Zhu, *Inorg. Chem.*, **38**, 1474 (1999).
6 T. N. Parac, G. M. Ullmann, and N. M. Kostić, *J. Am. Chem. Soc.*, **121**, 3127 (1999).
7 L. Zhu, L. Qin, T. N. Parac, and N. M. Kostić, *J. Am. Chem. Soc.*, **116**, 5218 (1994).
8 E. L. Hegg and J. N. Burstyn, *J. Am. Chem. Soc.*, **117**, 7015 (1995).
9 G. Allen and R. O. Campbell, *Int. J. Pept. Protein Res.*, **48**, 265 (1996).
10 L. Zhu, R. Bakhtiar, and N. M. Kostić, *J. Biol. Inorg. Chem.*, **3**, 383 (1998).
11 T. N. Parac and N. M. Kostić, *Inorg. Chem.*, **37**, 2141 (1998).
12 F. Dou, F. Qiao, J. Hu, T. Zhu, X. Xu, D. Zhu, and L. Zhu, *Prep. Biochem. & Biotechnol.*, **30**, 69 (2000).
13 F. Sanger, *Biochem. J.*, **44**, 126 (1949).
14 Considering pK_a of side amino group of lysine29 is 10.8 and Ref. 11, it is not considered a potential binding site of Pd(II) at low pH solution.
15 B. Hawkins, K. Cross, and D. Crail, *Int. J. Pept. Protein Res.*, **46**, 424 (1995).