Metal compound-mediated hydrolytic cleavage of oxidized insulin B chain: Regioselectivity and influence of peptide secondary structure

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The interaction of oxidized insulin B chain (B) with *cis*-[Pd(en)Cl_2] (en = ethylenediamine), *cis*-[Pd-(dtco-3-OH)Cl_2] (dtco-3-OH = dithiacyclooctan-3-ol) and $CuCl_2$ was studied by electrospray mass spectrometry. It is discovered that the binding of Pd(II) complexes and the sites of cleavage are highly dependent on the secondary structure and local environment of B. The hydrolytic cleavage of denatured B by Pd(II) complexes was monitored by HPLC. The reaction is regioselective and follows first order kinetics with half-life of 4.8 days at 40℃. Two amide bonds, *i.e.* at Leu6-Cys7 and at Gly8-Ser9, which are close to the two potential Pd(II) binding sites His5 and His10, are selectively cleaved. In the case of Cu(II) ion as promoter, only one cleavage site was observed which is located at Gly8-Ser9 bond. These results provide improved understanding on the design of artificial metallopeptidase.

Keywords Insulin B chain, hydrolytic cleavage, palladium(II) complexes, Cu(II) ion, electrospray mass spectrometry

Introduction

Selectively chemical cleavage 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 designed and their activity towards the directly hydrolytic or oxidative cleavage of amide bonds in peptides and proteins have been investigated.[1-28] The interaction of palladium(II) complexes with methionine, cysteine and histidine-containing di- or tripeptides and the hydrolytic cleavage of the corresponding amide bond by Pd(II) complexes have been extensively studied.[6-23] However, reports concerning the directly selective hydrolysis of proteins or long peptides are very rare due to the difficulty involved in their cleavage. It appears that only five proteins have been really hydrolyzed by transition metal complexes.[24-28] This forms a strong contrast with the fact that in the wide rang of practical applications, such as protein sequencing, structure mapping and functional analysis, analysis of protein-DNA interaction and development of new peptide drugs, involves mainly proteins and long peptides. Therefore, there is a huge scope in the development of metal-based cleaving agents, those could selectively hydrolyze proteins and long peptides.

Insulin is one of the most widely studied peptide hormones, and continues to play a central role in the treatment of diabetes. It is composed of two chains, A and B linked by two intermolecular disulfide bonds.[29,30] Oxidized insulin B chain (designated **B** in short) contains two histidyl residues His5 and His10, which are the potential bonding sites for Pd(II) complexes and Cu(II) ion.

The different behavior in selective cleavage of amide bonds between short peptides and proteins is likely due to their conformational differences. In order to assess the possible effect of the secondary structure and lo-

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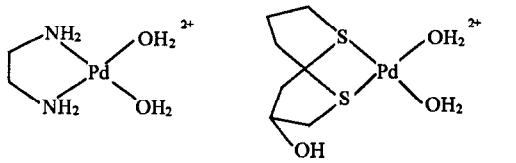
cal environment of bonding sites in the proteins and long peptides on the cleavage, we have chosen to study the selective cleavage of oxidized insulin B chain by *cis*-[Pd(en)Cl_2], *cis*-[Pd(dtco-3-OH)Cl_2] and $CuCl_2$. The sequence of the oxidized insulin B chain and structural formula of the Pd(II) complexes are listed in chart 1.

Chart 1

Palladium(II) complexes



cis-[Pd(en)$(H_2O)_2$]$^{2+}$ *cis*-[Pd(dtco-3-OH)$(H_2O)_2$]$^{2+}$

Sequence of oxidized insulin B chain (**B**)

H_2N·Phe-Val-Asn-Gln-His-Leu-Cys(-SO_3H)-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys(-SO_3H)-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala.

Experimental

Chemicals

Double distilled water was used for preparation of solutions. Hog insulin was obtained from Sigma Co. and acetonitrile for HPLC grade was from Fisher Chemical Co. $PdCl_2$ and $AgBF_4$ were obtained from Aldrich Co. All other chemicals were of reagent grade.

The following Pd(II) complexes were prepared and re-crystallized by the published procedures: *cis*-[Pd(en)Cl_2],[31] *cis*-[Pd(dtco-3-OH)Cl_2].[27,32] The corresponding aqua complexes were obtained by stirring the precursors with 2.0 mol equiv. of $AgBF_4$ in aqueous solution for 4 h at pH 2 and 35℃. AgCl was removed by centrifugation. These procedures were completed in the dark.

The oxidized insulin B chain was prepared according to the published procedures.[33] 1.0 g of insulin was oxidized to yield 0.12 g of oxidized insulin B which was characterized by electrophoresis and electrospray mass spectrometry.

Measurement

Bio-Rad Mini-Protean II instrument was used for electrophoresis of the oxidized insulin B. An 18% polyacrylamide gel for separations was overlaid by a 7% polyacrylamide gel for staking. The electrophoresis proceeded under the constant voltage 100V at room temperature. The gel was stained by a 0.1% solution of Coomassie blue R-250 in an aqueous solution that contained 10% of acetic acid and 40% of methanol.

LCQ electrospray mass spectrometry (ESMS, Finnigan MAT) was applied to determine the m/z of the polypeptide and its complexes with Pd(II) or Cu(II). The sample was dissolved in water and diluted to 100 $\mu mol \cdot L^{-1}$. 1.0 μL of such solution was loaded into the injection valve of the LCQ unit and then injected into the mobile phase solution (50% aqueous methanol containing 1% acetic acid), and carried through the electrospray interface into the mass analyzer at a rate of 200 $\mu L \cdot min^{-1}$. The employed voltage at the electrospray needles was 5 KV and the capillary was heated to 200℃. A maximum ion injection time of 200 msec along with 10 scans was set. Positive ion mass spectra were obtained. Zoom scan was used in these experiments. Predicted isotope distribution patterns for each of complexes were calculated using IsoPro 3.0 program.

The pH was measured with an Orion 901 instrument and a Phoenix Ag-AgCl reference electrode.

The cleaving reaction was monitored by a high performance liquid chromatography (HPLC) with an ALLTIMA C18 column of size 4.6 × 200 mm, filled with 5-μm beads. The flow rate was 1.0 $mL \cdot min^{-1}$ at 35℃. A 0.09% solution of CF_3COOH in acetonitrile was admixed into a 0.1% solution of CF_3COOH in water in a gradient increasing from 30% to 40% within 20 min. Then, the previous solution was run for 5 min. The absorption at 280 nm was recorded.

An Applied Bio-system Procise was used for *N*-terminal amino acid sequencing of the cleaved fragment separated by HPLC.

Cleavage of oxidized insulin B chain with Pd(II) *complexes and* Cu(II) *ion*

Oxidized insulin B chain (**B**) was stored in aqueous solution of H_3PO_4 at pH 2.5, with concentration of 20 $mg \cdot mL^{-1}$. After mixing 10 μL of the solution with 1.5—2.9 μL of Pd(II) complexes or 1.5 μL Cu(II) (both with concentration of 100 $mmol \cdot L^{-1}$) in a certain mole ratio, 11.0 mg urea was then added, and the total

volume of the mixture solution was made to be 30 μL, then it was incubated at 40 ± 0.5℃. Na_2HPO_4 (2 mol·L^{-1}) was added to adjust the pH value of mixture solution if necessary. The hydrolytic products were separated and monitored by HPLC, and their concentrations (C_t and C_∞) were calculated based on their peak areas. Reaction of the cleavage was carried out in first order of kinetics: $-\ln[(C_\infty - C_t)/C_\infty] = k_{obsd} \times t$, over at least three half-lives with correlation coefficients of 0.94—0.99. The reactions were run for six half-lives.

Results and discussion

Characterization of the oxidized insulin B chain

The oxidized insulin B chain (**B**) obtained was analyzed by SDS gel electrophoresis and only one band appeared. The m/z of the **B** was determined by ESMS, and two groups of peaks were attributed to ions of **B** with +2 and +3 charges. The molecular mass determined is 3496.0, precisely equal to 3495.9 calculated by IsoPro3.0 program for $C_{157}H_{232}N_{40}O_{47}S_2$.

Interaction of Pd(II) *complexes with the oxidized insulin B chain*

Pd(II) complex, *cis*-$[Pd(en)(H_2O)_2]^{2+}$ (**1**) or *cis*-$[Pd(dtco\text{-}3\text{-}OH)(H_2O)_2]^{2+}$ (**2**), was added to **B** in acidic solution (pH 2.5) at room temperature in a molar ratio of 5:1. Two ESMS spectra obtained for the mixed solutions, both are associated with the complex ion $[PdL\mathbf{B} + H^+]^{3+}$ in which L is en or dtco-3-OH. Fig. 1 shows the ESMS spectrum for the mixed solution of **1** and **B** with a zoom scan spectrum inserted. It indicates that only one molecule of Pd(II) complex was bound to **B** under this condition, although there are three potential binding sites in the **B**, including terminal NH^{3+}, imidazole groups of His5 and His10. The secondary structure of **B** in solution seems to be similar to that in the crystal structure of the hexamer including an extended region (B1—B8), an α-helix (B9—B19), a β-turn (B20—B23) and another extended strand (B24—B28).[34] It is possible that His5 located at the extended region and His10 at α-helix section are less accessible to Pd(II) than the terminal NH_3^+ due to the steric hindrance. Another potential binding site is the deprotonated amide nitrogen of Val2. Either ligand en or dtco-3-OH remains attached to Pd(II).

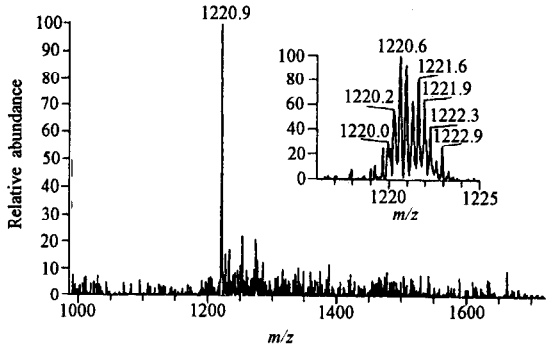


Fig. 1 ESMS spectrum measured 4 h after mixing of **B** with *cis*-$[Pd(en)(H_2O)_2]^{2+}$ at room temperature and pH 2.5 with a zoom scan of the highest abundance inserted which corresponds to calculated isotope distribution pattern of $[\mathbf{B} + Pd(en) + H^+]^{3+}$

Fig. 2 shows an ESMS spectrum which was measured 4 h after adding urea to the mixture solution of complex **1** and **B**. It was found that the complexes $[Pd_2L_2\mathbf{B} - H^+]^{3+}$ and $[Pd_3L_3\mathbf{B} - 3H^+]^{3+}$ were formed besides complex $[PdL\mathbf{B} + H^+]^{3+}$ and $[PdL\mathbf{B}]^{2+}$, with the highest m/z values of 1275.6 and 1330.7. This change accounts for coordination of His5 and His10 through N-3 atom of imidazole to Pd(II) owning to denaturation of the **B**, while the ligand en is still attached to Pd(II) in all species, similar to that observed in the absence of urea. ^1H NMR spectrum also confirms the coordination of His5 and His10 to Pd(II). The chemical shift of free imidazole H-2 is δ 8.41, which shifted to δ 7.92 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 $[Pd_2L_2\mathbf{B} - H^+]^{3+}$ and $[Pd_3L_3\mathbf{B} - 3H^+]^{3+}$, the remaining binding site is likely to be deprotonated amide nitrogens of His5 and His10, forming six-membered chelate ring.

Interaction of the oxidized insulin B chain with Cu^{2+} *ion*

An ESMS spectrum (Fig. 3A) was measured 4 h after mixing $CuCl_2$ with **B** (molar ratio 2:1) in aqueous solution of pH 2.5 at room temperature. Besides free **B**, the species $[Cu\mathbf{B} + H]^{3+}$, $[Cu_2\mathbf{B} - H^+]^{3+}$ and $[Cu_3\mathbf{B} - 3H^+]^{3+}$ were detected which correspond to the high-

est m/z values of 1186.5, 1206.9 and 1227.7, respectively. The same species were observed in the presence of 6 mol·L^{-1} urea. Similar to Pd(II) complexes,

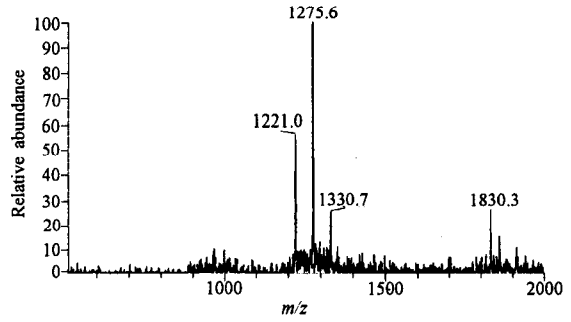


Fig. 2 ESMS spectrum measured 4 h after adding 6 mol·L^{-1} of urea to the mixed solution of **B** and *cis*-[Pd(en)-$(H_2O)_2$]$^{2+}$ at room temperature and pH 2.5. The three groups of peaks, separated by 0.3 m/z each, are attributed to [PdL**B** + H$^+$]$^{3+}$ (1221.0), [Pd_2L_2**B**-H$^+$]$^{3+}$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

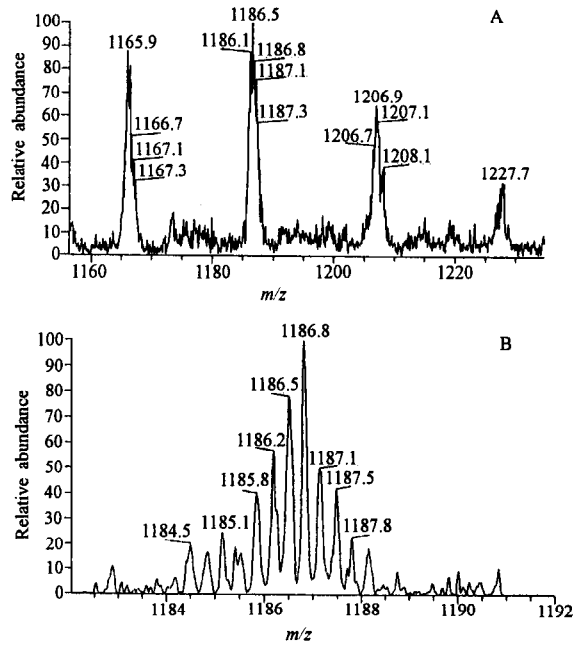


Fig. 3 ESMS spectrum measured 4 h after mixing **B** with $CuCl_2$ at room temperature and pH 2.5 (A), and a zoom scan of the peaks with m/z = 1185.1—1187.8 (B). The three groups of peaks, separated by 0.3 m/z each, are attributed to [Cu**B** + H$^+$]$^{3+}$ (1186.5). [Cu_2**B**-H$^+$]$^{3+}$ (1206.9) and [Cu_3**B**-3H$^+$]$^{3+}$ (1127.7), respectively. Peaks with m/z = 1165.9 belong to free **B**.

the favored binding sites of the **B** for Cu^{2+} in three species are N-terminal NH_3^+, two imidazoles of His5 and His10, and deprotonated amide nitrogens from Val2, His5 and His10.

Cleavage of the oxidized insulin B chain with Pd(II) *complexes*

The control experiments with and without urea proceeded at 40℃ and pH 2.5 for 32 days and were monitored by HPLC and ESMS. There is no any fragment of cleavage observed.

A mixture solution of **B** and Pd(II) complex (molar ratio 5∶1) was incubated at 40℃ and pH 2.5 for 10 days. After digestion, it was investigated by HPLC. There is no fragment of cleavage found and only molecular ion peaks of the **B** were observed by ESMS after removing Pd(II) by N,N-diethyl-dithio-carbomate (ddtc). As discussed above, in this case, only one Pd(II) complex is coordinated to **B** through deprotonated amino nitrogen of Phe1 and deprotonated amide nitrogen of Val2. Therefore, no cleavage reaction occurs.

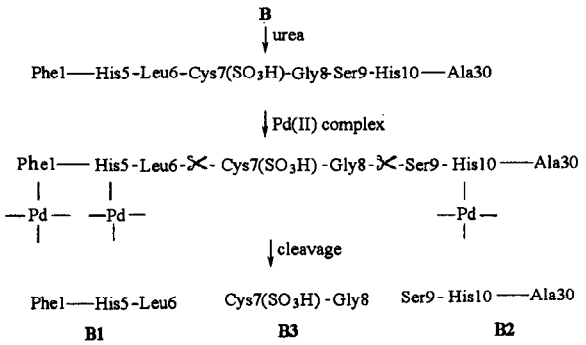
After adding urea, the mixture solution of Pd(II) complex and **B** was incubated at pH 2.5 and 40℃ for 10 days, which was then separated into two portions. One was directly analyzed by HPLC. Two fractions with retention time of 4.05 min and 12.67 min were collected and further analyzed by ESMS, whose spectra are shown in Figs. 4A and 4B, respectively. The peaks with m/z = 454.7—458.1 in Fig. 4A (RT = 4.05 min) are attributed to ion [Pd(II) + **B1** + H_2O + CH_3OH]$^{2+}$, where **B1** stands for a fragment Phe1-Leu6. The ion peaks in Fig. 4B with m/z = 1326.1—1330.1 and 1336.7—1341.1 (RT = 12.67 min) are attributed to [Pd(II) + **B2**]$^{2+}$ and [Pd(II) + **B2** + Na$^+$ − H$^+$]$^{2+}$ respectively, where **B2** stands for the fragment from Ser9 to 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 ddtc to remove Pd(II) and then analyzed by HPLC. Both components with retention time of 4.05 min, and 12.67 min disappeared, while a new component appeared with retention time of 12.73 min, which was analyzed by ESMS (Fig. 5). The molecular masses obtained from Fig.5 are consistent with the ions of [**B2** + 4Na$^+$ − H$^+$]$^{3+}$ (2636.8) and [**B2** + 2Na]$^{2+}$ (2594.8). It is well known that peptides capture Na$^+$ during electrospray ionization process.

This component was further analyzed by *N*-terminal amino acid sequencing, and the first ten amino acid sequence of fragment **B2** is listed in Table 1.

Taken together, the cleavage reaction of **B** by Pd(II) complexes can be summarized in Scheme 1. First, Pd(II) complex, *cis*-[Pd(en)$(H_2O)_2$]$^{2+}$ or *cis*-[Pd(dtco-3-OH)$(H_2O)_2$]$^{2+}$, is anchored to His5 and His10 of the denatured oxidized insulin B chain, 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 **B3** stands for the fragment Cys7-Gly8.

As shown in Scheme 1, the denatured oxidized insulin B chain 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 previous studies.[10,13,21,26,27] In peptides[26] and proteins (*e. g.* myglobin)[27] 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 (*vide infra*). 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 of His5 and directs it closer to Leu6 and leads to cleavage of Leu6-Cys7 bond.[19,21]

Scheme 1



The kinetics of hydrolytic cleavage reaction is also studied by HPLC. The increases in intensity of components **B1** and **B2** with time are monitored by HPLC (Fig. 6), and the data are fitted to first order reaction equation, $-\ln[(C_\infty - C_t)/C_\infty] = k_{obsd} \times t$. The observed rate constants k_{1obsd} and k_{2obsd} of **B1** and **B2** are listed in Table 2.

As can be seen from Table 2 that the rate of cleavage of **B1** is nearly equal to that of **B2**, and the variation of pH values does not have significant impact on the hydrolytic rate of cleavage. This means that the complexes **1** and **2** could cleave **B** at near neutral pH with a comparable rate to that at pH 2.5. 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.

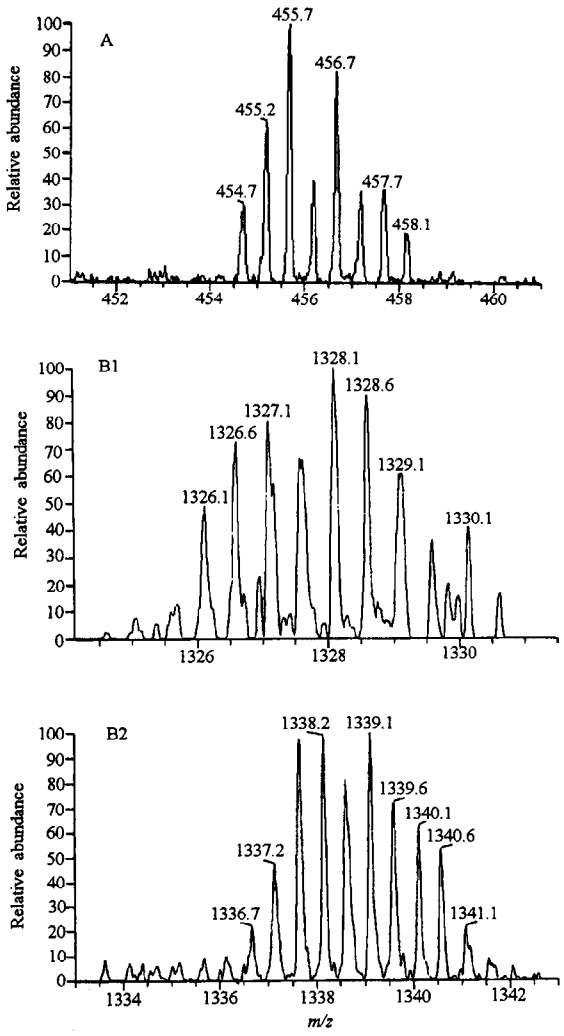


Fig. 4 A zoom scan spectrum of ESMS of **B1** with Pd(II) anchored (A), cooresponding to calculated isotope distribution pattern of [Pd(II) + **B1** + H_2O + CH_3OH]$^{2+}$; two zoom scan spectra of ESMS of **B2** with Pd(II) anchored (B), corresponding to calculated isotope distribution patterns of [**B2** + Pd(II)]$^{2+}$, [**B2** + Pd(II) + $Na^+ - H^+$]$^{2+}$.

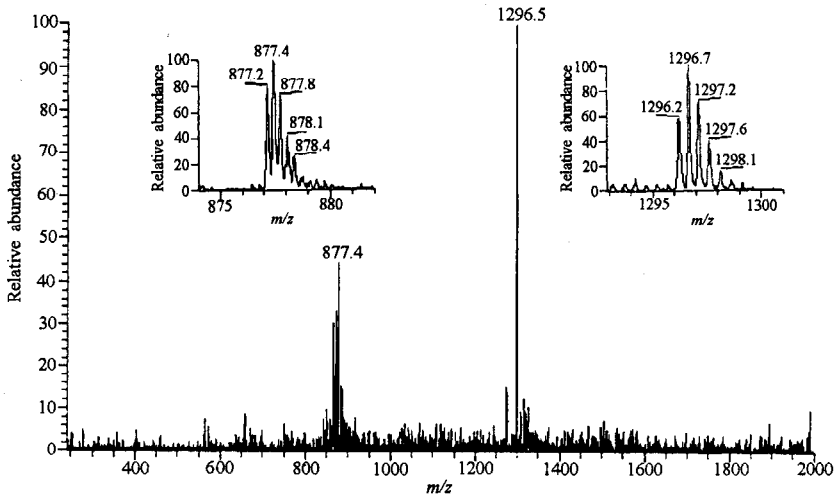


Fig. 5 ESMS spectrum of **B2** with zoom scans of the two groups of peaks inserted which correspond to calculated isotope distribution patterns of $[\mathbf{B2}+2Na^+]^{2+}$ and $[\mathbf{B2}+4Na^+-H^+]^{3+}$.

Table 1 *N*-Terminal amino acid sequencing of **B2** fragment

No.	Running time (min)	Correct time (min)	Amino acid
1	4.55	4.60	Ser
2	6.92	7.10	His
3	16.33	16.28	Leu
4	12.80	12.75	Val
5	5.73	5.72	Glu
6	7.51	7.55	Alu
7	16.36	16.28	Leu
8	9.60	9.62	Tyr
9	16.34	16.28	Leu
10	12.82	12.75	Val

Cleavage of the denatured oxidized insulin B chain with Cu(II) *ion*

It was reported by Allen *et al.*[26] that Cu(II) cleaved short histidyl-containing peptides and a humanised monoclonal IgGI antibody at amide bond near His. Furthermore, cleavage of peptides containing Ser-His or Thr-His sequence at the second amide bond left from His demonstrates a specificity of 10-50-fold higher than that for other histidyl-containing peptides, and over 100-fold higher than that for peptides lacking histidinyl residue. We present here another example of Cu(II) promoted cleavage of peptide containing Ser-His sequence.

After mixing Cu(II) with **B** in a molar ratio 2:1, urea was added (a precipitate was formed during one day of incubation without urea). An ESMS spectrum of the digestion solution, as shown in Fig. 7, was obtained after incubated at 40℃ and pH 2.5 for 10 days. Two groups of peaks in Fig. 7 are produced by double-charged positive ions: one of them with m/z = 1296.1—1297.6 fits to ion $[\mathbf{B2}+2Na^+]^{2+}$, while the other one with m/z = 1304.5—1308.0 corresponds to the ion $[Cu\mathbf{B2}]^{2+}$. Therefore, Cu(II) only selectively cleaves **B** at second amide bond left from His10.

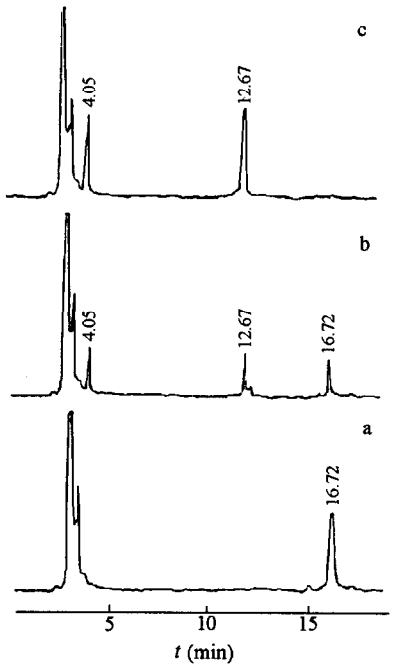


Fig. 6 HPLC spectra measured during the reaction of *cis*-$[Pd(en)(H_2O)_2]^{2+}$ with **B** at 40℃ and pH 2.5. (a): 4 h; (b): 10 days; (c): 30 days.

Table 2 Observed rate constants for formation of **B1** (k_{1obsd}) and **B2** (k_{2obsd}) at 40℃

	Pd(II) complex	$k_{1obsd} \times 10^4$ (min^{-1})	$k_{2obsd} \times 10^4$ (min^{-1})
pH 2.5, **B**:Pd(1:5)	$[Pd(en)(H_2O)_2]^{2+}$	1.10 ± 0.07	0.97 ± 0.03
	$[Pd(dtco\text{-}3\text{-}OH)(H_2O)_2]^{2+}$	0.92 ± 0.02	0.83 ± 0.05
pH 6.0, **B**:Pd(1:5)	$[Pd(en)(H_2O)_2]^{2+}$	0.97 ± 0.05	0.90 ± 0.04
	$[Pd(dtco\text{-}3\text{-}OH)(H_2O)_2]^{2+}$	0.89 ± 0.03	0.81 ± 0.02
pH 2.5, **B**:Pd(1:2)	$[Pd(en)(H_2O)_2]^{2+}$	Very slow	Very slow
	$[Pd(dtco\text{-}3\text{-}OH)(H_2O)_2]^{2+}$	Very slow	Very slow

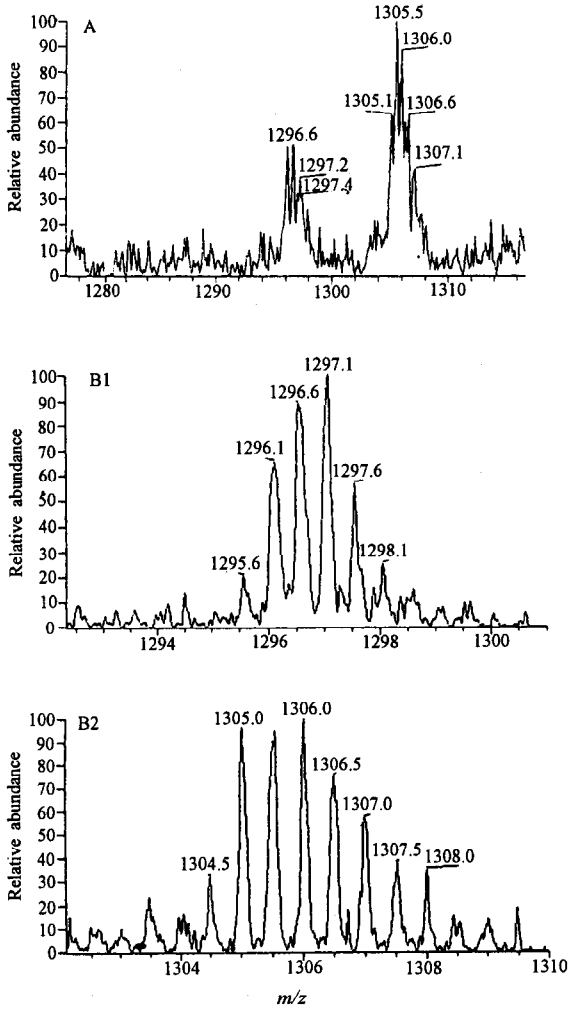


Fig. 7 ESMS spectrum of the fragment of **B** cleaved by $CuCl_2$ (A) and zoom scans of two gruops of peaks in A (B1 and B2) which correspond to calculated isotope distribution patterns of $[\mathbf{B2} + 2Na^+]^{2+}$ and $[\mathbf{B2} + Cu(II)]^{2+}$.

Conclusion

This study indicates that cleavage of the oxidized insulin B chain is affected by its overall conformation. The Pd(II) complexes are bound to *N*-terminal amino group and the imidazoles of His5 and His10 only in presence of urea, and followed by cleavage of amide bond in the vicinity of above two histidyl residues. The cleavage of Gly8-Ser9 bond by both Pd(II) complexes and Cu(II) is associated with the special sequence Ser9-His10, which provides potential application in cleavage of proteins and long peptides containing Ser-His sequence. The cleavage of Leu6-Cys7 bond by Pd(II) complexes also reveals the importance of regioselectivity. The Cys7-(SO_3^-) residue attracts Pd(II) complex toward the backbone of Lue6-Cys7 and accelerates its cleavage. It should be emphasized that Pd(II) complex can cleave the subtract even at near neutral pH, with the same rate of cleavage as at pH 2.5, which is crucial for Pd(II) complex to become a real reagent in selective cleavage of proteins and long peptides.

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