带羟乙基侧臂三脚架多胺 Cu(Ⅱ)配合物切割肌红蛋白所得片断的质谱指认

蒋 疆[1,2] 赵春梅[2] 洪 瑾[2] 唐惠炜[2] 朱龙根*,[2]
([1]福州大学化学化工学院,福州 350002)
([2]南京大学配位化学国家重点实验室,南京 210093)

摘要:采用电喷雾质谱和串联质谱以及聚丙烯酰胺凝胶电泳技术研究了[CuL(H$_2$O)](BF$_4$)$_2$(L 为 2-[二(2-氨乙酸)氨基]乙醇)与马心肌红蛋白的键合作用和水解切割。聚丙烯酰胺凝胶电泳研究显示在中性及 60 ℃条件下,切割效率与[CuL(H$_2$O)]$^{2+}$的浓度和温育时间密切相关。电喷雾质谱和串联质谱分析显示,[CuL(H$_2$O)]$^{2+}$通过与肌红蛋白的氨基酸 His36,His93,His116 和 Arg139 侧链的结合,并在羟乙基侧臂的促进下,选择性地水解了肽键 Phe33-Thr34,Gln91-Ser92,Ala94-Thr95,His116-Ser117 和 Asn140-Asp141。

关键词:肌红蛋白;质谱;Cu(Ⅱ)配合物;切割
中图分类号:O657.6 **文献标识码**:A **文章编号**:1001-4861(2007)02-0243-10

Mass Spectrometry Assisted Assignments of Fragments of Myoglobin Cleaved by Copper(Ⅱ) Complex with Tripodal Polyamimine Bearing an Hydroxyethyl Pendant

JIANG Jiang[1,2] ZHAO Chun-Mei[2] HONG Jin[2] TANG Hui-Wei[2] ZHU Long-Gen*,[2]
([1]*College of Chemistry and Chemical Engineering, Fuzhou University, Fuzhou* 350002)
([2]*State Key Laboratory of Coordination Chemistry, Nanjing University, Nanjing* 210093)

Abstract: The bonding interaction and hydrolytic cleavage of horse heart myoglobin with [CuL(H$_2$O)](BF$_4$)$_2$, where L is 2-[bis (2-aminoethyl)amino]ethanol, were investigated by electrospray ionization mass spectrometry(ESI-MS), tandem mass spectrometry (MS/MS) and SDS-PAGE electrophoresis. The SDS-PAGE electrophoresis showed that the cleavage yield was dependent on the concentration of [CuL(H$_2$O)]$^{2+}$ and incubation time. The ESI-MS and MS/MS analysis revealed that with the assistance of the pendant hydroxyl group in [CuL(H$_2$O)]$^{2+}$, [CuL(H$_2$O)]$^{2+}$ may selectively hydrolysis the peptide bonds of Phe33-Thr34, Gln91-Ser92, Ala94-Thr95, His116-Ser117 and Asn140-Asp141 of myoglobin by the binding of [CuL(H$_2$O)]$^{2+}$ to the side chains of His36, His93, His116 and Arg139 of myoglobin.

Key words: myoglobin; mass spectrometry; Cu(Ⅱ) complex; cleavage

0 Introduction

Endo-proteolytic cleavage has been of interest for a long time, which is one of the most common and most important procedures in biochemistry and bioanalytic chemistry involved in protein sequencing [1], foot printing[2], folding studies[3], protein semisynthesis[4], proteomics[5] and purification of fusion proteins[6]. Proteases cleave proteins selectively and catalytically under mild conditions, but only a few of them are usable in

收稿日期:2006-09-27。收修改稿日期:2006-10-25。
国家自然科学基金资助项目(No.NSF20271027)。
*通讯联系人。E-mail:Zhulg@public1.ptt.js.cn;fuzhoujiang_1969@yahoo.com.cn
第一作者:蒋 疆,女,36 岁,副教授;研究方向:生物无机化学。

practice because they often produce short fragments that are ill-suited for automatic sequencing[1]. Unlike enzymes, the existing synthetic reagents, such as cyanogens bromide, BNPS-skatole and N-bromosuccinimide, which often require harsh conditions, must be applied in high excess and often give incomplete selectivity and relatively low yields[7]. Therefore, new cleavage reagents for efficient and selective cleavage of peptides or proteins are required. New chemical proteases should be capable of binding to certain residues and promote selective cleavage of a peptide bond near the binding sites. Relatively mild conditions, equimolar amount or small molar excess over the substrate, and easy removal of the reagent after cleavage are desirable features. However, the extreme stability of the peptide bond has placed limits on the number of reagents available[8~10].

In recent years, a variety of transition-metal complexes have emerged as new synthetic reagents for cleavage of peptide bonds in short peptides and proteins[11~27]. These artificial proteases can overcome thermal, chemical, and mechanical instability of natural proteases and hydrolytically cleave short peptides and proteins selectively and quickly. Hydrolyses of short peptides promoted by metal ions of Cu(Ⅱ)[11], Zn(Ⅱ)[12], Ni(Ⅱ)[13], Ce(Ⅳ)[14], Zr(Ⅳ)[15] and by the complexes of Cu(Ⅱ)[16], Ce(Ⅳ)[17], Fe(Ⅱ,Ⅲ)[18], Pd(Ⅱ)[19], Pt(Ⅱ)[19c,19g,19j,19m,19n,20], Co(Ⅱ,Ⅲ)[21] under either near neutral or acidic conditions have been reported. Although many studies proved the ability of transition metals to promote cleavage of small compounds containing amide groups and peptides, reports concerning the directly selective hydrolysis of proteins were only for a very limited number of proteins, such as myoglobin, lysozyme, cytochrome c, bovine serum albumin etc. The site-specific cleavages of proteins in the presence of metal ions and/or complexes of Fe(Ⅲ)[22], Cu(Ⅱ)[23], Co(Ⅱ,Ⅲ)[23f,24], Pd(Ⅱ)[19h,19j,25], and Pt(Ⅱ)[19j,26] are successful examples.

Myoglobin (Mb) is a small single heme protein acting as an oxygen transportation in life, whose structure has been reported[27,28]. Up to now, several kinds of metal complexes including Co(Ⅲ) complexes[23f], copper(Ⅱ) complexes[16b,23d~23f], $CuCl_2$[23c], Pd(Ⅱ) and Pt(Ⅱ) complexes[19h,19j,25b] have been examined for their ability to hydrolyze the peptide bonds in myoglobin, but most of them have low catalytic rates and relative low selectivity especially under physiological conditions. Currently, Pd(Ⅱ) and Pt(Ⅱ) complexes have been investigated in some details. These studies revealed that only the peptide bond downstream from methionine residues of myoglobin was cleaved by the Pt(Ⅱ) reagents, and the Pd(Ⅱ) complexes-mediated cleavage consistently occurred at the second or third peptide bond from the residues of methionine, arginine and histidine. For the Pd(Ⅱ) and Pt(Ⅱ) complexes, the hydrolytic cleavages of myoglobin were achieved in a weak acid condition. In fact, there are few examples of Cu(Ⅱ)-promoted hydrolysis of peptide bonds in myoglobin. For a review, the peptide bonds of Gln91-Ser92 and Ala94-Thr95 in myoglobin were cleaved by Cu(Ⅱ) ion anchored to the side chain of His93 residue at neutral pH [23c]. As for the Cu(Ⅱ) complex of cycen, Cu(Ⅱ)A-PS, Cu(Ⅱ)B-PS and [Cu(Ⅱ)-Cyc]$_2$-PS[23d,23e], four cleavage sites near histidine residues in myoglobin were determined by MALDI-TOF MS. These results prove that the peptide bonds downstream or upstream from the histidinge residues are more susceptible to cleavage by coordination of His to Cu(Ⅱ) complexes.

As known from previous studies, Cu(Ⅱ) ion was one of the transition metal ions that shows significant activities of artificial metallopeptidases for hydrolytic cleavage of peptides and proteins [11a,16b,23a,23d,23e,29]. However, how to design the new Cu(Ⅱ) complexes with a variety of ligands is still a hot topic for improving their selectivity and efficiency of hydrolytic cleavage. It has been reported in some serine-containing peptidases, the hydroxyl group of serine plays a key role in catalytic hydrolysis of peptide bond in proteins[25d,30]. In this report, a new Cu(Ⅱ) complex with a hydroxyethyl pendant, [CuL(Cl)](ClO_4), is designed as a model compound to mimic the function and myoglobin is taken as a target protein. This study reveals that the complex of [CuL(Cl)](ClO_4) is able to selectively hydrolytic cleavage of myoglobin with high efficiency and may extend to cleavage of other proteins in future.

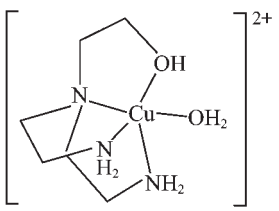
1 Materials and methods

1.1 Chemicals

Horse heart myoglobin was obtained from Sigma. 2-[bis(2-aminoethyl)amino]ethanol(L)[31] and [CuL(Cl)](ClO_4)[32] were synthesized according to the literature procedures. Trifluoroacetic acid (TFA) was purchased from Aldrich and acetonitrile of HPLC grade was obtained from Fisher. All other chemicals were of reagent grade.

1.2 Sample preparation

Double distilled water (dd H_2O) was used for preparation of solutions. An aqueous solution of myoglobin with a concentration of 100 mg·mL^{-1} was centrifuged to remove insoluble residue and its concentration was 2.72 mmol·dm^{-3} determined by UV-Visible spectroscopy at 557 nm (ε_{557}=13.8 mmol^{-1}·dm^3·cm^{-1}) after the addition of sodium dithionite[33]. The molecular mass of myoglobin detected by LC-ESI-MS is 16951.0 Da, that is in excellent agreement with 16 951.5 Da calculated by IsoPro3.0 program for apomyoglobin. 100.0 mmol·dm^{-3} of $[CuL(H_2O)]^{2+}$ was obtained by mixing $[CuL(Cl)](ClO_4)$ with one equivalent of $AgBF_4$ in aqueous solution, stirring at 37 ℃ for 4 h in the dark and removing the white AgCl precipitate by centrifugation. The *m/z* value of the $[CuL(H_2O)]^{2+}$ determined by ESI-MS was 209.0, which is assigned to $[CuLH_{-1}]^+$.



$[CuL(H_2O)]^{2+}$

01 GLSDGEWQQVLNVWGKVEAD 20
21 IAGHGQEVLIRLFTGHPETLEKF 20
44 DKFKHLKTEAEMKASEDLKKH 20
65 GTVVLTALGGILKKKGHHEAEL 86
87 KPLAQSHATKHKIPIKYLEFISD 109
110 AIIHVLHSKHPGDFGADAQGAM 131
132 TKALELFRNDIAAKYKELGFQG 153

Horse heart myoglobin

Scheme 1

1.3 Electrophoresis

Bio-Rad Mini-Protean Ⅱ instrument was used for the SDS-PAGE electrophoresis of the digested myoglobin. An 18% polyacrylamide gel for separation was overlaid by a 7% polyacrylamide gel for stacking. The electrophoresis proceeded under a constant voltage of 100 V at 25 ℃ for about 2.0 h. The peptide bands were visualized by staining with a 0.10% solution of Coomassie Blue R-250 in an aqueous solution that contained 10% of acetic acid and 40% of methanol. The intensities of the blue band corresponding to the intact protein was scanned by a densitometer UVP white/ultraviolet transilluminator and calculated by the ImageQuant TL v2003.03 software. The estimated relative error of the densitometric analysis was 10%. The percentage of cleavage was calculated based on the difference between the relative intensities of the intact bands obtained in the presence and in the absence of the $[CuL(H_2O)]^{2+}$ in solution containing equal concentrations of the protein under the same experimental conditions; the intensities of the band obtained in the absence of the $[CuL(H_2O)]^{2+}$ was taken to be 100%. The reported percentage of cleavage can be semi-quantitatively considered.

In a typical cleavage experiment, an aqueous solution of 60.0 L containing 1.0 mg of myoglobin and various concentrations of $[CuL(H_2O)]^{2+}$ at pH value of 6.8 adjusted by 20.0 mmol·dm^{-3} phosphate buffer (pH 7.27) was incubated at 60 ℃ for 3 days. The cleavage reaction was quenched by addition of EDTA with 10-fold excess over $[CuL(H_2O)]^{2+}$, and then, 360 μL of the standard SDS-reducing buffer was added. The mixture solution was heated at 95 ℃ for 5 min and cooled at room temperature later. 5.0 μL of the reaction solution was loaded on a 7% polyacrylamide gel for stacking and an 18% polyacrylamide gel for separation in a Mini-Protean Unit.

1.4 pH measurements

pH value was measured with a PHS-3C pH instrument and a phoenix Ag-AgCl reference electrode standardized with pH 4.00, 7.00 and 10.00 buffers.

1.5 Mass spectrometry

ESI mass spectra and tandem mass spectra were recorded by a Finnigan MAT LCQ ion-trap mass spectrometer in a positive ionization mode. For LC-ESI-MS, a chromatographic column directly connected

to the injection valve of mass spectrometer was used for analysis of myoglobin and its fragments-containing samples. Typically, 2.0 μL of pre-treated degradation solution was first injected into HPLC with a Hypersil 5 C8 column of size 2.1 mm × 100 mm using a gradient of water/acetonitrile, both containing 0.1% CF_3COOH. The gradient was made by increasing the amount of acetonitrile from 0 to 60% in 70 min, at a flow rate of 0.2 mL·min^{-1}. The isolated solution was then directly transported into ESI-MS instrument. The molecular mass was determined by transformation of ESI-MS raw data into a true molecular mass scale using Bioexplore software. The measured molecular mass of a given fragment was compared with the value calculated by PAWS software, obtained from ProteoMetrics, LLC. For MS/MS analysis, the most intense ion in a spectrum was selected as the precursor ion, and a collision-induced dissociation scan with an isolation width of 3 *m/z* was performed. The optimal relative collision energy of 30% gave satisfactory information about the cleaved fragmentation of the protein. The employed voltage at the electrospray needles was 4.5 kV, N_2 sheath gas flow was 35 units (arbitrary for the LCQ-Deca system) and the heated capillary temperature was 200 ℃. Helium gas was admitted directly into the ion trap, and was used as the damping gas to improve trapping efficiency and as collision gas in the Collision-Induced Dissociation experiments.

1.6 Interaction of $[CuL(H_2O)]^{2+}$ with myoglobin

0.55, 2.75, 5.50, 11.00 and 22.00 μL of 50.0 mmol·dm^{-3} $[CuL(H_2O)]^{2+}$ were respectively added to 10.0 μL of 2.72 mmol·dm^{-3} myoglobin solution, with molar ratios of 1∶1, 5∶1, 10∶1, 20∶1 and 40∶1, and then extra dd H_2O was added to make total volume of the mixture solution up to 120.0 μL and pH value of 6.5. These mixed solutions were incubated at 37 ℃ and then measured by LC-ESI-MS.

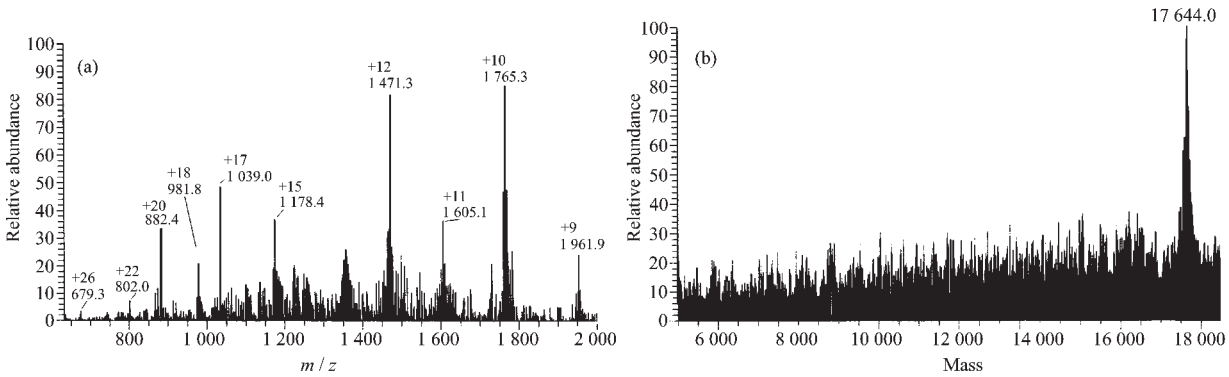
1.7 Cleavage of myoglobin with $[CuL(H_2O)]^{2+}$

15.0 μL of 2.72 mmol·dm^{-3} myoglobin was mixed with 8.25 μL of 100.0 mmol·dm^{-3} $[CuL(H_2O)]^{2+}$ solution in a molar ratio of myoglobin/$[CuL(H_2O)]^{2+}$ to be 1∶20, and dd H_2O was added to make total volume of the mixture solution up to 80.0 μL with pH 6.5. The solution was incubated at 60 ℃ for 3 days. The cleavage reaction was quenched by addition of 16.0 μL of 0.50 mol·dm^{-3} EDTA and was then centrifuged. The obtained clear solution was analyzed by LC-ESI-MS and MS/MS. 20.0 μL of 6.0 mol·dm^{-3} urea was added to the remaining precipitation and the mixture was incubated at 37 ℃ for 12 h, and was then centrifuged. The above clear solute was also tested by LC-ESI-MS.

2 Results and discussion

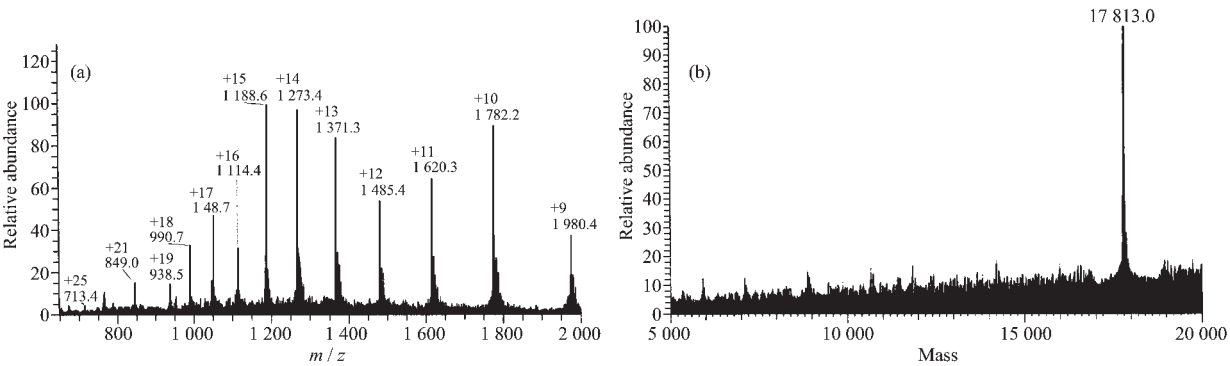
2.1 Interaction of myoglobin with $[CuL(H_2O)]^{2+}$

ESI mass spectra were measured after incubation of mixed solutions of $[CuL(H_2O)]^{2+}$ and myoglobin in molar ratios of 1∶1, 5∶1, 10∶1, 20∶1, 40∶1 at 37 ℃ and pH value of 6.5, labeled as samples **a**, **b**, **c**, **d**, **e**. After 4 h of incubation of samples **a**, **b**, **c**, **d**, **e**, besides free myoglobin, the singly Cu(Ⅱ)-coordinated species $[CuLH_{-1}]$Mb and the doubly Cu(Ⅱ)-coordinated species $[CuLH_{-1}Na]_2$Mb were detected, corresponding to molecular mass of 17 160.0 Da and 17 415.0 Da, respectively, but the intensities were very weak. After 12 h of incubation for the samples **b**, **c**, **d**, **e**, the singly Cu(Ⅱ)-coordinated species $[CuLH_{-1}]$Mb and the doubly Cu(Ⅱ)-coordinated species $[CuLH_{-1}Na]_2$Mb disappeared, and the triply Cu(Ⅱ)-coordinated species $[CuLH_{-1}Na]_3$Mb was strongly detected, as shown in Fig.1, which corresponds to molecular mass of 17 644.0 Da. As the incubation time was prolonged to 24 h, the triply-coordinated species was decreased and a new peak appeared at 17 813.0 Da, shown in Fig.2, which is assigned as quadruply-coordinated species $[(CuL)_3(CuLH_{-1}Na)]$ Mb. These two species were always observed, as the molar ratio of $[CuL(H_2O)]^{2+}$ to myoglo-bin was varied from 5∶1 to 40∶1 under the same experimental conditions. Any adduct of myoglobin anchored by more than four $[CuL(H_2O)]^{2+}$ was not det-ected after extra 24 h of incubation, and the quadruply-coordinated species became the dominant one in the processing of time. It is evident that there are four binding sites in myoglobin that are preferred by $[CuL(H_2O)]^{2+}$ during the incubation time. In the experiment



(a) raw spectrum; (b) transformed spectrum calculated by Bioexplore

Fig.1　ESI-mass spectra of Cu(Ⅱ)-coordinated species of $[CuLH_{-1}Na]_3$Mb measured after purification on HPLC with a Hypersil 5 C8 column



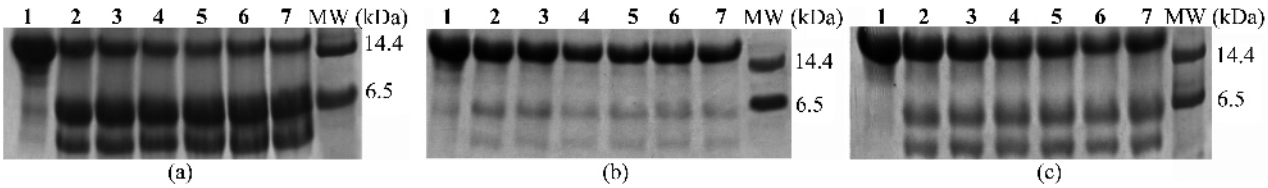
(a) raw spectrum; (b) transformed spectrum calculated by Bioexplore

Fig.2　ESI-mass spectra of Cu(Ⅱ)-coordinated species of $[(CuL)_3(CuLH_{-1}Na)]$Mb measured after purification on HPLC with a Hypersil 5 C8 column

of cleavage, the quadruply-coordinated species was also detected in a clear solution obtained by treating precipitate of the digested solution with urea. It further indicates that the quadruply-coordinated species is the predominant one even the mixture solution is incubated at 60 ℃ for 3 days.

2.2　Degradation of myoglobin with $[CuL(H_2O)]^{2+}$

To examine the influence of the concentration of $[CuL(H_2O)]^{2+}$ on the cleavage, myoglobin was incubated with $[CuL(H_2O)]^{2+}$ in $[CuL(H_2O)]^{2+}$/myoglobin molar ratios of 0, 10, 20, 30, 40, 50, 60 respectively at 60 ℃ for 3 days. As shown in Fig.3(a), control experiment indicats that no cleavage occurs in the absence of $[CuL(H_2O)]^{2+}$ (lane **1**). Therefore, the cleavage of myoglobin is mediated by $[CuL(H_2O)]^{2+}$. In lanes **1**~**7**, a band just below the band of intact myoblobin is observed. This band was also found even when myoglobin was freshly prepared, therefore, this band which was also observed in the cleavage of myoglobin with Pd(Ⅱ) complexes [25b] is due to the minor impurity of myoglobin and not a product of backbone cleavage. The molecular mass of this band in this study was detected by the LC-ESI-MS to be 14 699.0 Da. Besides intact band of myoglobin, two major fragmental bands with molecular weights of ca. 6.5 kDa and 3 kDa in lanes **2**~**7** were observed from the electrophoretogram. From Lanes **2** to **7**, the percentage of cleavage is 36.2%, 43.6%, 45.7%, 61.1%, 70.6% and 71.0%, respectively. It is evident that raising the molar ratio of $[CuL(H_2O)]^{2+}$ to myoglobin significantly increases the extent of cleavage, however, when the molar ratio of $[CuL(H_2O)]^{2+}$/myoglobin is greater than 50, the degree of cleavage does not increase again. In the previous study, when the molar ratio of $CuCl_2$/myoglobin was greater than 15, specificity of the cleavage was reduced [23c]. By contrast to this, [CuL



Lanes in the SDS polyacrylamide gel electrophoretogram: (1) control experiment in the absence of $[CuL(H_2O)]^{2+}$; (2) 10:1; (3) 20:1; (4) 30:1; (5) 40:1; (6) 50:1; (7) 60:1. (a) 60 ℃ for 3 days; (b) 60 ℃ for 1 days; (c) 60 ℃ for 1 day in 6.0mol dm^{-3} urea

Fig.3 Myoglobin was incubated with $[CuL(H_2O)]^{2+}$ in different $[CuL(H_2O)]^{2+}$/myoglobin molar ratios at pH value of 6.8

$(H_2O)]^{2+}$ shows high regioselectivity even the molar ratio of $[CuL(H_2O)]^{2+}$ to myoglobin is raised up to 60. The hydrolytic cleavage was sensitive to incubated time. As shown in Fig.3(b), the efficiency of cleavage incubated at 60 C for 1 day is much lower than that at 60 ℃ for 3 days. As shown in Fig.3(c), the cleavage reaction is also carried out in the presence of 6.0 mol·dm^{-3} urea. The denaturation of the myoglobin by urea increases the rate of cleavage and does not alter the pattern of cleavage. This result implies that the selectivity of cleavage does not depend on the secondary structure of the myoglobin. This situation was also observed in the cleavage of myoglobin with $CuCl_2$[23c].

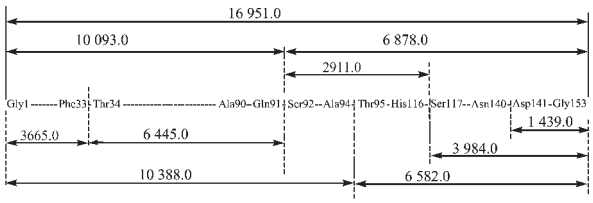
2.3 Cleavage sites of myoglobin promoted by $[CuL(H_2O)](BF_4)_2$

As mentioned above, there are four binding sites in myoglobin towards the $[CuL(H_2O)]^{2+}$ complex. Only knowing the composition of $[CuL(H_2O)]^{2+}$-bound Mb is not enough, it is necessary to further determine the sites to which the $[CuL(H_2O)]^{2+}$ has been anchored. As far as the $[CuL(H_2O)]^{2+}$-bound Mb is concerned, MS/MS analysis is unsuitable for direct sequencing because it is too large. In this study, therefore, the binding sites in $[CuL(H_2O)]^{2+}$-bound Mb were determined through observation of cleaved fragments. As demonstrated by the LC-ESI-MS data and in Table 1, the molecular mass of fragments produced by cleavage is 1 439.0, 2 911.0, 3 665.0, 3 984.0, 6 445.0, 6 582.0, 6 878.0, 10 093.0 and 10 388.0 Da, which correspond to the cleavage sites of Phe33-Thr34 (site **1**), Gln91-Ser92 (site **2**), Ala94-Thr95 (site **3**), His116-Ser117 (site **4**) and Asn140-Asp141 (site **5**) respectively. The four cleavage sites of **1**, **2**, **3** and **4** are exactly the same as that of the Cu(Ⅱ) (cyc)-promoted cleavage of myoglobin[23e]. The sites **2** and **3** were also observed in the cleavage of myoglobin with $CuCl_2$[23c] at neutral pH and with Pd(Ⅱ) complex[25b]. The cleaved site **5** was observed for the first time. Previous studies showed that a peptide bond, which is the second or third peptide bond upstream or downstream from histidine residue in myoglobin can be cleaved by Cu(Ⅱ) and Pd(Ⅱ) complexes[23c~23e,25b]. In this study, it is observed that the four cleavage sites of **1**, **2**, **3**, **4**, which are in proximity to the histidine residues, are also cleaved by $[CuL(H_2O)]^{2+}$. This report provides a new evidence of Cu(Ⅱ) complexes-promoted specific cleavage of the histidine-containing proteins and peptides[23b,34,35]. It was reported that in degradation of myoglobin by Cu(Ⅱ) Cyclen, Gln91-Ser92 and Ala94-Thr95 were the major initial cleavage sites, and the fragments of Gly1-Gln91 and Gly1-Ala94 produced were degraded rapidly afterwards, whereas Ser92-Gly153 and Thr95-Gly153 were more stable during the time of incubation[23e]. Interestingly, this situation was also found in this study. As shown in Fig.3, there is no band observed at ca. 10 kDa and the intensive bands appeared at ca. 6.5 kDa and 3 kDa. These results semi-quantitatively indicates that although fragments of Gly1-Gln91 and Gly1-Ala94 with the molecule mass of 1 0093.0 and 1 0388.0 Da are detected by ESI-MS because of its high sensitivity, they are unstable during the incubation and are cleaved further. As for the cleavage site **5** of Asn140-Asp141, the cleavage seems occur at the sec-ond peptide bond downstream from arginine139 residue, that is, the X-Y bond in the sequence segments Arg-X-Y, in which X and Y are any noncoordinating residues. Latest research shows, a Cu(Ⅱ) complex of cyclen acting as a protein-cleaving catalyst, can selectively cleave lysozome at peptide bond of

Table 1　ESI mass spectra for fragments of myoglobin cleaved by $[CuL(H_2O)]^{2+}$

Fragment	Multiply charged ions (*m/z*)	Averaged molecular mass	
		Observed	Calculated
Asp141-Gly153	480.6(+3), 720.6(+2), 1 439.9(+1)	1 439.0	1 439.6
Ser92-His116	728.9(+4), 971.5(+3), 1 456.7(+2)	2 911.0	2 911.4
Gly1-Phe33	733.7(+5), 917.1(+4), 1 222.1(+3)	3665.0	3665.1
Ser117-Gly153	571.3(+7),798.0(+5),997.2(+4),1328.8(+3),	3 984.0	3 983.5
Thr34-Gln91	645.3(+10), 717.8(+9), 806.4(+8), 1 074.9(+6), 1 611.8(+4)	6 445.0	6 445.4
Thr95-Gly153	598.9(+11), 732.5(+9), 941.4(+7), 1098.3(+6), 1 317.3(+5), 1 646.5(+4)	6 582.0	6 581.6
Ser92-Gly153	625.2(+11), 861.2(+8), 983.6(+7), 1 147.1(+6), 1 376.5(+5), 1 720.3(+4)	6 878.0	6 876.9
Gly1-Gln91	649.5(+16), 800.2(+13), 866.5(+12), 1 040.1(+10), 1 154.1(+11), 1 299.6(+8), 1 732.4(+6)	10 093.0	10 092.6
Gly1-Ala94	673.8(+15), 843.3(+12), 918.7(+11), 1 010.5(+10), 1 122.4(+9), 1 262.8(+8), 1 443.7(+7), 1 683.9(+6)	10 388.0	10 387.9

Arg114-Cys115 and Arg125-Gly126, which is near arginine residue[23e,24c]. Shields[36] proposed that the Cu (I) ion could bind to the guanidine group of arginine residue and fragmentation occurred remote from the Cu(I) ion-attached site. Lavanant[37] also indicated that the sites of cleavage were the same in the $[MCu(I)]^+$ and $[M-H+Cu(II)]^+$ complexes, in which M represents the dipeptides containing arginine or lysine. Therefore, it is reasonable to believe that the cleavage site **5** is relative to arginine139. The $[CuL(H_2O)]^{2+}$ anchors to the side chain of Arg139, resulting in cleavage of the second peptide bond downstream from the binding sites.



Scheme 2

2.4　Sequencing cleaved fragments with MS/MS analysis

There is a current interest in using tandem mass spectrometry as a tool for sequencing biomolecules instead of N-terminal amino-acid sequencing by Edman degradation. In our previous studies, LC-ESI-MS combined with MS/MS analysis has been proven to be a powerful technique for understanding interactions of metal complexes with peptides and proteins, especially for determination of binding sites in metal complexes-bound peptides and proteins[19m,20c,26b,38]. In order to further confirm the sites of cleavage, the MS/MS analysis in this study was employed to sequence the cleaved fragments. From the cleaved fragments with molecular mass of 1 439.0, 2 911.0, 3 665.0, 3 984.0 Da, we chose the most intense peaks of 720.6^{2+}, 971.5^{3+}, $1\,222.1^{3+}$, 997.2^{4+} as precursor ions, respectively. We characterized only the b_n and y_n ions, which are the most common fragments on account of their greater stability towards further dissociation. The successive N-terminal and C-terminal fragment ions that exactly match the sequence of Asp141-Gly153, Ser92-His116, Gly1-Phe33, and Ser117-Gly153 in myoglobin. An example of MS/MS spectrum recorded for $[M + 2H]^{2+}$ (*m/z* 720.6) for the cleaved fragment of Asp141-Gly153 is illustrated in Fig.4. The successive N-terminal fragment ions b_3-b_{12} and C-terminal fragment ions y_3-y_{12} assigned in Fig.4 exactly match the sequence of Asp141-Gly153 in myoglobin. These results further confirm that the sites of cleavage are at Phe33-Thr34, Gln91-Ser92, Ala94-Thr95, His116-Ser117 and Asn140-Asp141.

2.5　Selectivity of binding and cleavage in $[CuL(H_2O)]^{2+}$-bound myoglobin

Horse heart myoglobin consists of 153 amino acid residues and only of α helices spans by short connecting links that have a coil conformation. Because the hydrolytic cleavage of myoglobin promoted by $[CuL(H_2O)]^{2+}$ proceeds at neutral pH, the conformation of myoglobin may be conserved. As mentioned above, the histidine residues and arginie residues are the potential bonding sites preferred by $[CuL(H_2O)]^{2+}$. As shown in Fig.5, among 11 histidines

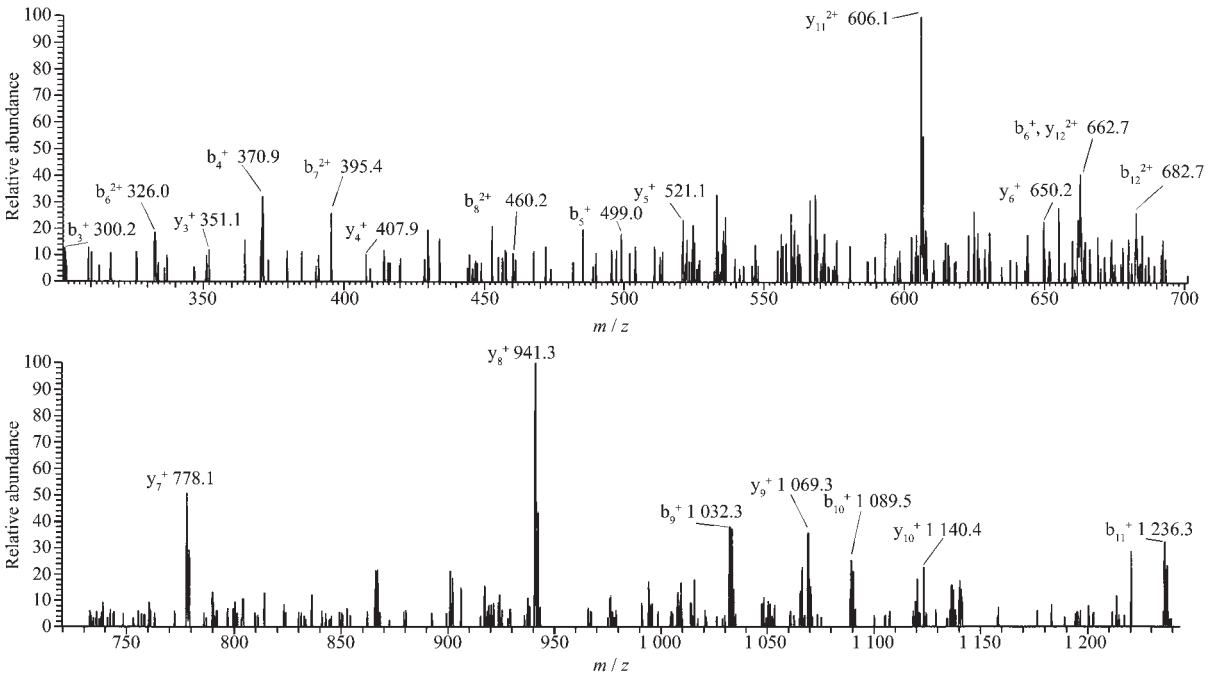


Fig.4 MS/MS spectra recorded for $[M + 2H]^{2+}$ (*m/z* 720.6) for the cleaved fragment of Asp141~Gly153 with molecular mass of 1 439.0 Da

and 2 arginines in myoglobin, the four binding sites confirmed are located at the positions which are easily attached by $[CuL(H_2O)]^{2+}$. His36, His116 and Arg139 are on the surface of the myoglobin, and His93 is located at the passage through the center of heme pocket. Therefore, according to the information of the cleaved fragments and the visualization of the conformation of myoglobin, His36, His93, His116 and Arg139 residues are more susceptible to binding with $[CuL(H_2O)]^{2+}$, giving rise to selective cleavage of peptide bonds in the vicinity of these binding sites.

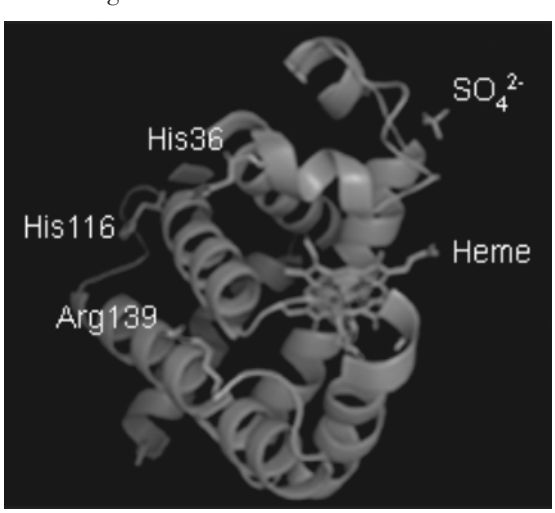


Fig.5 Three-dimentional view of myoglobin with the putative binding sites. The figure was prepared with Pymol. DeLano, W.L. The PyMOL Molecular Graphics System 2002, DeLano Scientific, San Carlos, CA, USA. Citation: protein data bank, pdb ID: 1YM

As well known, to perform the metal complex-mediated cleavage, two conditions are required: firstly, the metal ion must be anchored to a site which is in the vicinity of the aimed peptide bond; secondly, the metal ion must retain at least one aqua ligand after anchoring. If either condition is absent, cleavage does not occur[19h]. In this study, the $[CuL(H_2O)]^{2+}$ complex is designed as a new "inorganic protease", in which the pendant hydroxyl group may assist hydrolysis of peptide bond in myoglobin, which was also observed in hydrolysis of peptide bond in methionine-containing dipeptides and oxidized insulin B lately reported[39,40]. In the $[CuL(H_2O)]^{2+}$ complex, the pK_a value of hydroxyl group was determined to be 8.5[32]. In the experimental condition at pH value of 6.8, $[CuL(H_2O)]^{2+}$ is a dominant species with occupancy of 98%, and the deprotonated one only has occupancy of 2% . Although the deprotonated species is present in small amount, the negative charge exhibits greater nucleophilic ability. It is reasonable to believe that both protonated and

deprotonated species may assist the cleavage of peptide bonds downstream or upstream from anchored residues.

In conclusion, myoglobin is remarkably cleaved by $[CuL(H_2O)]^{2+}$ with explicable specificity, and only five cleaved sites were detected. The cleavage tends to occur at the second or third peptide bond upstream, and the first or second peptide bond downstream from the binding sites. It is worth emphasizing that the degradation of myoglobin with $[CuL(H_2O)]^{2+}$ is accomplished at neutral pH. Compared to Cu(Ⅱ) (cyc)[23e], the Cu(Ⅱ) complex is simple and easily synthesized. In comparison with $CuCl_2$[23c], the new Cu(Ⅱ) complex is more efficient and more specific, especially in high molar ratio. Importantly, the ability of the Cu(Ⅱ) complex exhibited in selective hydrolytic cleavage of myoglobin with high efficiency provides a clue to design new metal complexes bearing the pendant hydroxyl group to mimic the function of serine-containing peptidases.

References:

[1] Croft L R. *Handbook of Protein Sequence Analysis*, 2*nd ed*. Wiley: Chichester, UK, **1980**.

[2] Heyduk T, Baichoo N, Heyduk E. *Met. Ions Biol. Syst.*, **2001**, **38**:255~287

[3] Hubbard S, Beynon R J. *Proteolysis of Native Proteins as a Structural Probe*. New York: Oxford University Press, **2001**.

[4] Wallace C J A. *Protein Engineering by Semisynthesis*. Boca Raton, FL: CRC Press, **2000**.

[5] Thomas J J, Bakhtiar R, Siuzdak G. *Acc. Chem. Res.*, **2000**, **33**:179~187

[6] Thorner J, Emr S D, Abelson J N. *Applications of Chimeric Genes and Hybrid Proteins, Part A*: *Gene Expreeion and Protein Purification*. Methods in Enzymology 326; Eds. New York: Academic Press, **2000**.

[7] Walker J M. *The Protein Protocols Handbook*. Totowa: Humana Press, NJ, **2002**.

[8] Radzicka A, Wolfenden R. *J. Am. Chem. Soc.*, **1996**,**118**: 6105~6109

[9] Smith R M, Hansen D E. *J. Am. Chem. Soc.*, **1998**,**120**:8910~8913

[10]Bryant R A R, Hansen D E. *J. Am. Chem. Soc.*, **1996**,**118**: 5498~5499

[11](a)Allen G, Campbell R O. *Int. J. Pept. Protein Res.*, **1996**,**48**: 265~273

(b)Smith M A, Easton M, Allen G, et al. *Int. J. Pept. Protein Res.*, **1996**,**48**:48~55

(c)Luo X M, He W J, Zhu L G, et al. *Chin. J. Chem.*, **2000**, **18**:855~862

[12](a)Yashiro M, Sonobe Y, Komiyama M, et al. *Org. Biomol. Chem.*, **2003**,**1**:629~632

(b)Bazzicalupi C, Bencini A, Valtancoli B, et al. *Eur. J. Inorg. Chem.*, **2003**,**10**:1974~1983

[13](a)Bal W, Liang R T, Kasprzak K S, et al. *Chem. Res. Toxicol.*, **2000**,**13**:616~624

(b)Bal W, Lukszo J, Kasprzak K S, et al. *Chem. Res. Toxicol.*, **1998**,**11**:1014~1023

[14]Takarada T, Yashiro M, Komiyama M. *Chem. Eur. J.*, **2000**,**6**: 3906~3913

[15]Kassai M, Ravi R G, Grant K B, et al. *Inorg. Chem. Commun.*, **2004**,**43**:6130~6132

[16](a)Fujii Y K, Kiss T, Yashiro M, et al. *J. Biol. Inorg. Chem.*, **2002**,**7**:843~851

(b)Hegg E L, Burstyn J N. *J. Am. Chem. Soc.*, **1995**,**117**:7015~7016

[17]Yashiro M, Takarada T, Komiyama M, et al. *J. Chem. Soc. Chem. Commun.*, **1994**:1757~1758

[18](a)Rana T M, Meares C F. *J. Am. Chem. Soc.*, **1990**,**112**: 2457~2458

(b)Rana T M, Meares C F. *J. Am. Chem. Soc.*, **1991**,**113**:1859~1861

[19](a)Zhu L G, Kosti N M. *Inorg. Chem.*, **1992**,**31**:3994~4001

(b)Zhu L G, Kosti N M. *J. Am. Chem. Soc.*, **1993**,**115**:4566~4570

(c)Djuran M I, Milinkovic S U. *Polyhedron*, **2000**,**19**:959~963

(d)Parac T N, Kostic N M. *J. Am. Chem. Soc.*, **1996**,**118**:51~58

(e)Chen X, Zhu L G, Yan H, et al. *J. Chem. Soc., Dalton Trans.*, **1996**:2653~2658

(f)Chen X, Zhu L G, You X Z, et al. *J. Biol. Inorg. Chem.*, **1998**,**3**:1~8

(g)Milovi N M, Kosti N M. *Met. Ions Biol. Syst.*, **2001**,**38**: 145~186

(h)Milovi N M, Kosti N M. *Inorg. Chem.*, **2002**,**41**:7053~7063

(i)Milovi N M, Kosti N M. *J. Am. Chem. Soc.*, **2002**,**124**: 4759~4769

(j)Milovi N M, Dutc L M, Kosti N M. *Inorg. Chem.*, **2003**,**42**: 4036~4045

(k)Milovi N M, Kosti N M. *J. Am. Chem. Soc.*, **2003**,**125**:781~788

(l)Milovi N M, Badji J D, Kosti N M. *J. Am. Chem. Soc.*, **2004**,**126**:696~697

(m)Sun X J, Jin C, Zhu L G, et al. *Inorg. Chem.*, **2004**,**43**:290~296

(n)Stoffregen S A, Griffin A K K, Kosti N M. *Inorg. Chem.*, **2005**,**44**:8899~8907

(o)Yang G S, Miao R, Zhu L G, et al. *Dalton Trans.*, **2005**:1613~1619

[20](a)Burgeson I E, Kostic N M. *Inorg. Chem.*, **1991**,**30**:4299~4305

(b)Kaminskaia N V, Kosti N M. *Inorg. Chem.*, **2001**,**40**:2368~2377

(c)Miao R, Yang G S, Zhu L G, et al. *Rapid Commun. Mass Spectrom.*, **2005**,**19**:1031~1040

[21](a)Buckingham D A. *Met. Ions Biol. Syst.*, **2001**,**37**:2141

(b)Chae P S, Kim M S, Suh J, et al. *J. Am. Chem. Soc.*, **2005**,**127**:2396~2397

[22](a)Alanna S, Bernard C. *J. Am. Chem. Soc.*, **1990**,**112**:3247~3249

(b)Pan Q H, Jiang W, Liu C L, et al. *Inorg. Chem. Commun.*, **2006**,**45**:490~492

(c)Pan Q H, Jiang W, Liu C L, et al. *Chin. Sci. Bulletin*, **2005**,**50**:1869~1873

[23](a)Buranaprapuk A, Leach S P, Kumar C V, et al. *Biochim. Biophys. Acta*, **1998**,**1387**:309~316

(b)Humphreys D P, King L M, Stephens P E, et al. *Protein Engng.*, **2000**,**13**:201~206

(c)Zhang L, Mei Y H, Zhu L G, et al. *Inorg. Chem.*, **2003**, **42**:492~498

(d)Yoo C E, Chae P S, Suh J, et al. *J. Am. Chem. Soc.*, **2003**, **125**:14580~14589

(e)Yoo S H, Lee B J, Suh J, et al. *J. Am. Chem. Soc.*, **2005**, **127**:9593~9602

(f)Jeon J W, Son S J, Suh J, et al. *Bioorg. Med. Chem.*, **2003**, **11**:2901~2910

(g)De Oliveira M C B, Scarpellini M, Soares M R, et al. *Inorg. Chem.*, **2005**,**44**:921~929

(h)Shrivastava H Y, Kanthimathi M, Nair B U. *Biochem. Biophys. Res. Commun.*, **1999**,**265**:311~314

[24](a)Kumar Challa V, Buranaprapuk A, Nicholas J T, et al. *Proc. Natl. Acad. Sci. USA.*, **1998**,**95**:10361~10366

(b)Kumar Challa V, Buranaprapuk A, Cho A. et al. *Chem. Commun.*, **2000**:597~598

(c)Kumar Challa V, Thota J. *Inorg. Chem.*, **2005**,**44**:825~827

(d)Aoki T, Sato D, Stato H, et al. *J. Biochem.*, **2005**,**137**:95~99

(e)Saha M K, Mukhopadhyay U, Bernal I. *Dalton Trans.*, **2004**:1466~1473

[25](a)Zhu L G, Qin L, Parac T N, et al. *J. Am. Chem. Soc.*, **1994**,**116**:5218~5224

(b)Zhu L G, Bakhtiar R, Kostic N M. *J. Biol. Inorg. Chem.*, **1998**,**3**:383~391

(c)Qiao F, Hu J Z, Zhu L G, et al. *Polyhedron*, **1999**,**18**:1629~1633

(d)Zhu L G, Kosti N M. *Inorg. Chim. Acta*, **2002**,**339**:104~110

(e)Vicente J, Arcas A. *Coordin. Chem. Rev.*, **2005**,**249**:1135~1154

[26](a)Milovi N M, Dutc L M, Kosti N M. *Chem. Eur. J.*, **2003**,**9**:5097~5106

(b)Yang G S, Miao R, Zhu L G, et al. *J. Mass Spectrom.*, **2005**,**40**:1005~1016

(c)Dutca L M, Ko K S, Kosti N M, et al. *Inorg. Chem.*, **2005**, **44**:5141~5146

[27]Brayer G D, Evans S V. *J. Biol. Chem.*, **1988**,**263**:4263~4273

[28]Brayer G D, Evans S V. *J. Mol. Biol.*, **1990**,**213**:885~897

[29]Gallagher J, Zelenko O, Sigma D S, et al. *Biochemistry*, **1998**,**37**:2096~2104

[30]Corey D R, Craik C S. *J. Am. Chem. Soc.*, **1992**,**114**:1784~1790

[31]Bobylev V A, Chechik V D. *Zh. Obshch. Khim.*, **1990**,**60**:2721~2725

[32]Xia J, Li S A, Tang W X, et al. *J. Chem. Soc.-Dalton Trans.*, **2001**:2109~2115

[33]Stryer L. *J. Mol. Biol.*, **1965**,**13**:482~495

[34]Humphreys D P, Smith B J, Slephens P E, et al. *Protein Engng.*, **1999**,**12**:179~186

[35]Luo X M, He W J, Zhu L G, et al. *Chem. Lett.*, **2000**:1030~1031

[36]Shields S J, KBluhm B, Russell D H. *J. Am. Chem. Soc. for Mass Spectrum.*, **2000**,**11**:626~638

[37]Lavanant H, Hoppilliard Y. *DCMR, Ecole Polytechnique, Palaiseau, Fr. Eur. Mass Spectrom*, **1999**,**5**:41~50

[38]Hong J, Miao Y, Zhu L G, et al. **2005**,**40**:91~99

[39]Yang G S, Tang H W, Zhu L G, et al. *Inorg. Chem. Commun.*, **2005**,**8**:862~865

[40]Hong J, Miao R, Zhu L G, et al. *J. Mass Spectrom.*, **2006**,**41**:1061~1072