Inorg. Chem. **2003**, *42*, 492–498

Regioselective Cleavage of Myoglobin with Copper(II) Compounds at Neutral pH

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Received March 27, 2002

Selective hydrolytic cleavage of myoglobin was studied with $CuCl_2$, $Cu(ClO_4)_2$, $Cu(AC)_2$, and binuclear Cu(II) complexes of 3,6,9,16,19,22-hexaaza-6,19-bis (2-hydroxyethyl)-tricyclo-[22,2,2,$2^{11,14}$]-triaconta-1,11,13,24,27,29-hexaene (**1**) and 3,6,9,16,19,22-hexaaza-tricyclo-[22,2,2,$2^{11,14}$]-triaconta-1,11,13,24,27,29-hexaene (**2**). The sites of cleavage were precisely determined by LC-ESIMS and further confirmed by an MS/MS method through fragmentation from both the N-terminal and C-terminal. The peptide bonds of Gln91-Ser92 and Ala94-Thr95 were remarkably cleaved by Cu(II) anchored to the side chain of the His93 residue. The data presented in this study show that Cu(II)-mediated cleavage of myoglobin is able to proceed at neutral pH, more selectively than Pd(II)-mediated cleavage, and buffer solution of phosphate and NH_4HCO_3 accelerates the cleavage reaction.

Introduction

Endo-proteolytic cleavage is a subject of ongoing interests and is one of the most common and most important procedures in biochemistry. The reagents that effect degradation of proteins usually are proteolytic enzymes. However, only a few of these proteolytic enzymes are routinely used;[1] the application of them is limited by their narrow requirements for temperature and pH. Therefore, new cleavage agents are required, and transition metal compounds hold promise for cleavage of proteins. Metal ions may bind to the heteroatom in the side chain of methionine, histidine, or tryptophen,[2−13] promoting cleavage of a peptide bond near these anchoring amino acids. Although many studies proved the ability of transition metals to promote cleavage of small compounds containing amide groups and peptides, the hydrolytic cleavage of proteins has been achieved only for several proteins.[14−21]

Myoglobin is a medium sized heme protein having biological functions of dioxygen storage and transport, whose structure has been known in detail.[22−23] It is eminently suitable for studies of cleavage. In addition, copper(II) ion was one of the transition metal ions that showed significant activities of artificial metallopeptidases for oxidative and hydrolytic cleavage of peptides and proteins,[24−31] even

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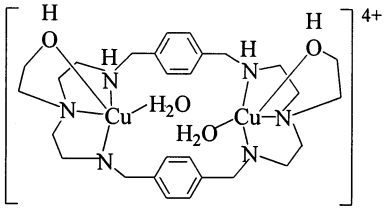
10.1021/ic025619b CCC: $25.00
Published on Web 12/13/2002

extended to cleavage of fusion proteins with some special sequences of ^{N}DKTH^{C} or a related type.[32−33] However, before it becomes an accepted reagent in analytical biochemistry and protein engineering, systematic experiments with various proteins are needed to determine their selectivity, efficiency, and other properties. Up to now, the scission of myoglobin has only been studied with palladium(II) aqua complexes,[19] and the hydrolytic cleavage was achieved in a weak acid condition with rather poor selectivity of up to 13 cleavage sites. We report here the selective hydrolytic cleavage of horse heart myoglobin with copper(II) compounds, which took place at neutral pH and was much more selective than that with Pd(II) complexes.

Electrospray mass spectrometry (ESIMS) has been widely employed because of its ability to measure the exact molecular mass. The convenience of interfacing the ion source to the high performance separation techniques such as liquid chromatography (LC) has made great progress in its biological application.[34−37] In ESIMS, protonated molecules may be further analyzed by fragmentation using collision-induced dissociation (MS/MS) to obtain structural information.[38−40] The spectra of fragment ions produced by collision can be acquired to characterize the peptides with the known sequence. The reported MS/MS spectra, which gave out enough consecutive fragment ions, were limited to peptides with less than 20 amino acid residues.[41−42] So far, for bigger or medium sized peptides, MS/MS analysis mainly focuses on abundant multicharged fragments.[43−47]

The fragments obtained from hydrolytic cleavage of protein are usually determined by the N-terminal amino acid sequence combined with MALDI-TOF and ESIMS mass spectrometries. The ability of MS/MS to sequence peptides encouraged us to apply this method to characterize the scissile fragments. In the study presented here, the sites of cleavage were exactly determined by LC-ESIMS and confirmed by MS/MS analysis from both the N-terminal and C-terminal for a fragment with more than 60 amino acid residues.

Chart 1



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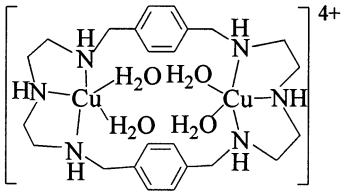

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Materials and Methods

Chemicals. Horse heart myoglobin was obtained from Sigma. Binuclear chloro copper complex of 3,6,9,16,19,22-hexaaza-6,19-bis(2-hydroxyethyl)-tricyclo-[22,2,2,2^{11,14}]-triaconta-1,11,13,24,27,29-hexaene was synthesized according to the literature method.[48] 3,6,9,16,19,22-Hexaaza-tricyclo-[22,2,2,2^{11,14}]-triaconta-1,11,13,24,27,29-hexaene (L) was also synthesized according to the literature method.[49] Reaction of L with $CuCl_2$ in a 1:2 molar ratio in methanol formed a deep blue solid Cu_2LCl_4. The MS of $Cu_2LCl_3^+$ and $Cu_2LCl_2^{2+}$ observed by ESIMS is 644.9 and 304.1; the calculated value is 644.1 and 304.3. The two chloro copper complexes were treated with 2 equiv and 4 equiv of $AgBF_4$, respectively, in water to get corresponding aqua complexes **1** and **2** (Chart 1). All other chemicals were of reagent grade.

Degradation of Myoglobin. Double distilled water was used for the preparation of solutions. An aqueous solution of myoglobin was centrifuged, and its concentration was determined by UV−vis spectroscopy at 557 nm ($\epsilon_{557} = 13.8$ mM^{-1} cm^{-1}).[50] The pH was measured with a pHS-3C pH meter equipped with a phoenix Ag−AgCl reference electrode. In a typical cleavage experiment, an aqueous solution of 60 μL containing 1.0 mg of myoglobin and various concentrations of copper(II) compounds was incubated at 50 °C for 3 days. The cleavage reaction was quenched by addition of EDTA with 10-fold excess Cu(II), and then, 360 μL of the standard SDS-reducing buffer was added. After this mixture solution was heated at 95 °C for 5 min and cooled at room temperature, 5.0 μL of the solution was loaded on a 7% polyacrylamide gel for stacking and an 18% polyacrylamide gel for separation in a Mini-Protean unit (Bio-Rad). The peptide bands were visualized by staining with a 0.10% solution of Coomassie Blue R-250. The

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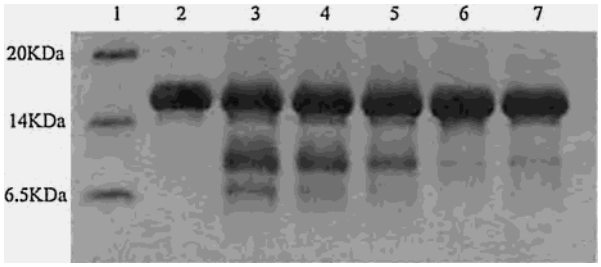


Figure 1. Myoglobin was incubated in aqueous solution with different Cu(II) compounds in a 5:1 molar ratio of Cu(II) to Mb at pH 6.8 and 50 °C for 3 days. Lanes in the SDS polyacrylamide gel electrophoretogram: (1) molecular mass markers; (2) control experiment in the absence of Cu(II) compound; (3) $CuCl_2$; (4) $Cu(AC)_2$; (5) $Cu(ClO_4)_2$; (6) complex **1**; and (7) complex **2**.

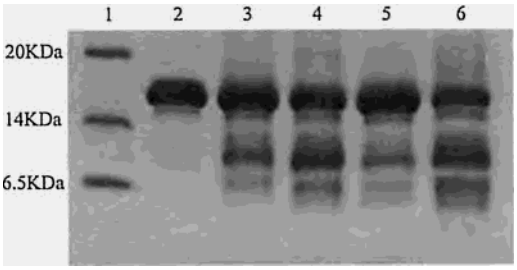


Figure 2. Myoglobin was incubated with $CuCl_2$ in a 5:1 molar ratio of Cu(II) to Mb in different buffer solutions at 50 °C for 3 days. Lanes in the SDS polyacrylamide gel electrophoretogram: (1) molecular mass markers; (2) control experiment in water in the absence of Cu(II) compound; (3) in water, pH 6.8; (4) phosphate buffer (20 mM), pH 7.0; (5) Tris-HCl buffer (20 mM), pH 7.0; (6) NH_4HCO_3 buffer (100 mM), pH 8.3.

intensity of the blue band corresponding to the intact protein was determined by a scanning densitometer UVP white/ultraviolet transilluminator, and the estimated relative error of the densitometric analysis was 10%. The percentage of cleavage was calculated on the basis of the difference between the relative intensities of the intact bands obtained in the presence and in the absence of the Cu(II) ion in solution containing equal concentrations of the protein under the same experimental conditions; the intensity of the band obtained in the absence of the Cu(II) ion was taken to be 100%. The reported percentage of cleavage can be considered semiquantitative.

LC-ESIMS and MS/MS Analysis. Mass spectra were acquired by a model LCQ ion trap mass spectrometer (Finnigan) equipped with a Finnigan MAT electrospray ion source. For LC-ESIMS, the degradation solutions were 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 60 min, at a flow rate of 0.20 mL/min. The isolated solution was then directly entered to ESIMS analysis. The molecular mass was determined by transformation of ESIMS raw data into a true molecular mass scale using Bioexplore software.

For MS/MS analysis, the most intense ion in the spectrum was selected as precursor ion, and a collision-induced dissociation scan with an isolation width of 3u was performed with the relative collision energy 30% to optimize the fragmentation of the peptide. The employed voltage at the electrospray needles was 4.5 kV, and the heated capillary temperature was 200 °C.

Results and Discussion

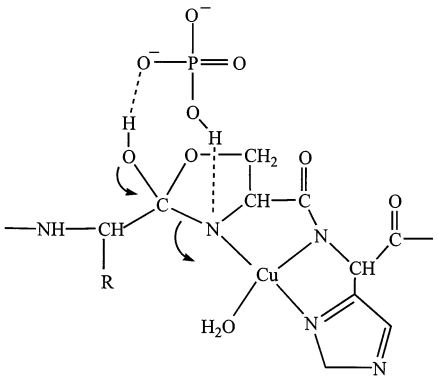
Cleavage of Myoglobin with Various Copper(II) Compounds. Myoglobin was incubated with $CuCl_2$, $Cu(ClO_4)_2$, $Cu(AC)_2$, and complexes **1** and **2** in aqueous solution at 50 °C for 3 days and significantly cleaved as demonstrated in Figure 1. Control experiment indicated that no cleavage was observed in the absence of Cu(II). Therefore, the cleavage of myoglobin is mediated by Cu(II) compounds. All these compounds can selectively scissor the myoglobin backbone to different extents. From lanes 3−7, the percentage of cleavage is 17%, 15%, 11%, 6%, and 7%, respectively. Among them, simple Cu(II) compounds are favorable. As will be discussed later in the text, the different percentages of cleavage induced by $CuCl_2$, $Cu(AC)_2$, and $Cu(ClO_4)_2$ may be related to their configuration existing in water. When myoglobin was incubated with these copper compounds in a given buffer, the difference in cleavage disappeared; nearly the same extent of cleavage was obtained for the three Cu(II) compounds. As shown in Figure 1, a band just below the band of intact myoblobin was observed. This band was also found even when myoglobin was freshly prepared; therefore, this band is due to the minor impurity of myoglobin and is not a product of backbone cleavage. This band corresponding to the minor impurity was also observed in the cleavage study of myoglobin with Pd(II) complexes.[19] Besides the intact band of myoglobin, two major fragmental bands in lanes 3−5 were observed from the electrophoretogram. In lanes 6 and 7, only one fragment band was observed, and because of low efficiency of cleavage with **1** and **2**, a corresponding complementary band with low molecular mass is difficult to visualize. In addition, two other weaker fragment bands, one of which was close to one of the two major bands, were also observed in lanes 3, 4, and 5.

As expected, the rate of cleavage is dependent on the Cu(II) compounds used as well as their concentration, incubation temperature and time, and chemical environment (buffer) sometimes. The cleavage reaction is thermally activated, and susceptible to temperature. Incubation of myoglobin with $CuCl_2$ (Cu/Mb = 5:1) at 60 °C for 1 day may reach the same results as that at 50 °C for 3 days. Raising both temperature and the molar ratio of Cu(II) to myoglobin may dramatically increase the extent of cleavage; however, higher temperature and higher concentration of metal ion result in less regioselectivity. When Cu/Mb > 15 and T > 60 °C, the electrophoretogram obtained by digestion of myoglobin with $CuCl_2$ showed a slight smear, representing that the specificity of the cleavage was reduced.

The cleavage reaction was also carried out in the presence of 6.0 M urea. The denaturation of the myoglobin by urea just increased the rate of cleavage and did not alter the pattern of cleavage. This result implies that the selectivity of cleavage does not depend on the secondary structure of the myoglobin. Increase of the concentration of chloride ion inhibits the cleavage of the protein. NaCl (1 M) evidently reduces the cleavage by 30−50%, and 2 M NaCl almost completely inhibits the cleavage reaction.

To investigate the influence of chemical environment on the cleavage of myoglobin, the cleavage reaction was conducted in different buffers. Figure 2 shows the results of cleavage of myoglobin with $CuCl_2$ in water, phosphate, Tris-HCl, and NH_4HCO_3 buffers. The percentage of cleavage was

Scheme 1



14%, 27%, 11%, and 32%. The hydrolysis of myoglobin exhibits a large buffer effect in the presence of phosphate or NH_4HCO_3: the rate of cleavage increased significantly. There was no evident change in the extent of cleavage when Tris-HCl buffer was used, instead of water. The effect of phosphate and NH_4HCO_3 was interpreted as their bifunctional nature.[51,18] In our case, as shown in Scheme 1, the phosphate buffer (as well as NH_4HCO_3) may have the ability to aid the proton transfer and facilitate the breakdown of the intermediate, which, as will be seen later, is considered as a resemblance to serine protease.

To examine the influence of pH on the cleavage, the various pH values were adjusted with different buffers. The optimal pH range was 6.5−8.5, and the cleavage of myoglobin was slow when pH < 6 or pH > 9, which is consistent with the pH range reported in the previous studies of Cu(II)-mediated cleavage of Ser-His or Thr-His sequence-containing proteins and peptides.[30−31]

Identification of Cleavage Sites of Myoglobin. The digested solution after the addition of EDTA was isolated by HPLC on a C8 column and then directly analyzed by ESIMS. Because heme is weakly attached to the polypeptide backbone, it is easily lost during degradation of myoglobin or at the high interface potential of the ionization source.[52] The molecular mass of the intact myoglobin detected by ESIMS is 16951.0 Da; it is in excellent agreement with 16951.5 Da calculated for apo-myoglobin. The average mass of fragments produced by cleavage was determined to be 10093.0 and 10388.0 Da in Figure 3a, as well as 6876.0 and 6581.0 Da in Figure 3b. Figure 3b also shows a peak of 8477.0 Da that is a double charged ion of apo-myoglobin and was also observed in ESIMS of fresh myoglobin. The emergence of this peak indicates the incomplete separation of the fragments from the intact protein.

The amazing accuracy of ESIMS in measuring the molecular masses of the intact protein and its cleaved fragments provided a powerful tool for determination of the cleavage sites. The cleaved sites determined are at Gln91-Ser92 and Ala94-Thr95, respectively (Scheme 2). The two sites of cleavage were also observed for the study of myoglobin cleavage with Pd(II) complexes in the presence of 6 M urea.[19]

Sequencing Cleaved Fragments with MS/MS Analysis. There is a current interest in using tandem mass spectrometry (MS/MS) as a tool for sequencing biomolecules. To further confirm the sites of cleavage, the MS/MS method in this study was employed to sequence the cleaved fragments instead of N-terminal amino acid sequencing by Edman degradation. So far, the reported MS/MS spectra of polypeptides for sequencing only show some multicharged fragment ions with higher abundance and do not have enough singly charged successive fragment ions. As the first attempt, a cleaved fragment was chosen and successfully analyzed.

From a cleaved fragment with molecular mass of 6876.0 Da, we chose a most intense peak of 983.40^{7+} as a precursor ion. The relative collision energy was increased from 10% to 30%, and the optimal relative energy of 30% gave satisfactory information about the amino acid sequence (Figure 4). The nomenclature used for the fragmentation pattern was based on the conventional notation.[53−54] The amino acid sequence of this fragment is confirmed by interpreting the MS/MS spectrum. The fragment ions are a_n, b_n, c_n when the charge is retained by N-terminal fragment and x_n, y_n, z_n when the charge is retained by C-terminal fragment. We characterized only the b_n and y_n ions, which are the most common fragments on account of their greater stability toward further dissociation.[55] Figure 4 shows the successive N-terminal fragment ions b_3-b_{16} and C-terminal fragment ions y_3-y_{17} that exactly match the sequence of S[92-153]G in myoglobin. These results further confirm that the site of cleavage at Gln91-Ser92 creates the cleaved fragment. The lines, out of b_n and y_n ions, included ions with plus or minus H_2O. We did not mark these lines in the spectrum for the sake of clarity. The further complication in the spectrum is due to the presence of a large number of fragment ions with variable charge states, and the presence of several ions with similar *m*/*z* values, especially between *m*/*z* 800 and 1400. The MS/MS spectrum becomes far more complex because of the derivative fragmentation under ionization conditions, and the process of side chain fragmentation and in-chain or midchain fragmentation. Hence, only the successive b_n, y_n, and some multicharged fragment ions with higher abundance were assigned in Figure 4. A superscript in Figure 4 represents the charge of the fragment ion, and the lack of a superscript denotes a fragment with single charge.

Figure 4 also shows the fragment ions y_{34}^{6+}(*m*/*z* 603.5), y_{34}^{4+}(*m*/*z* 908.7), y_{54}^{7+}(*m*/*z* 854.9), and y_{54}^{6+}(*m*/*z* 996.2), which are created by ion fragmentation at His119/Pro120 and Ile99/Pro100, both relative to proline. Alquicira and Tatlor[43] also observed the two fragment ions y_{34}^{4+}(*m*/*z* 908.6) and y_{54}^{6+}(*m*/*z* 996.5) in their study of myoglobin. Proline is a unique amino acid in which the side chain is covalently bound to the preceding α-amide nitrogen, forming a five-number ring and preventing the rotation of N−C bond. Therefore, it may play a significant role in the conformation

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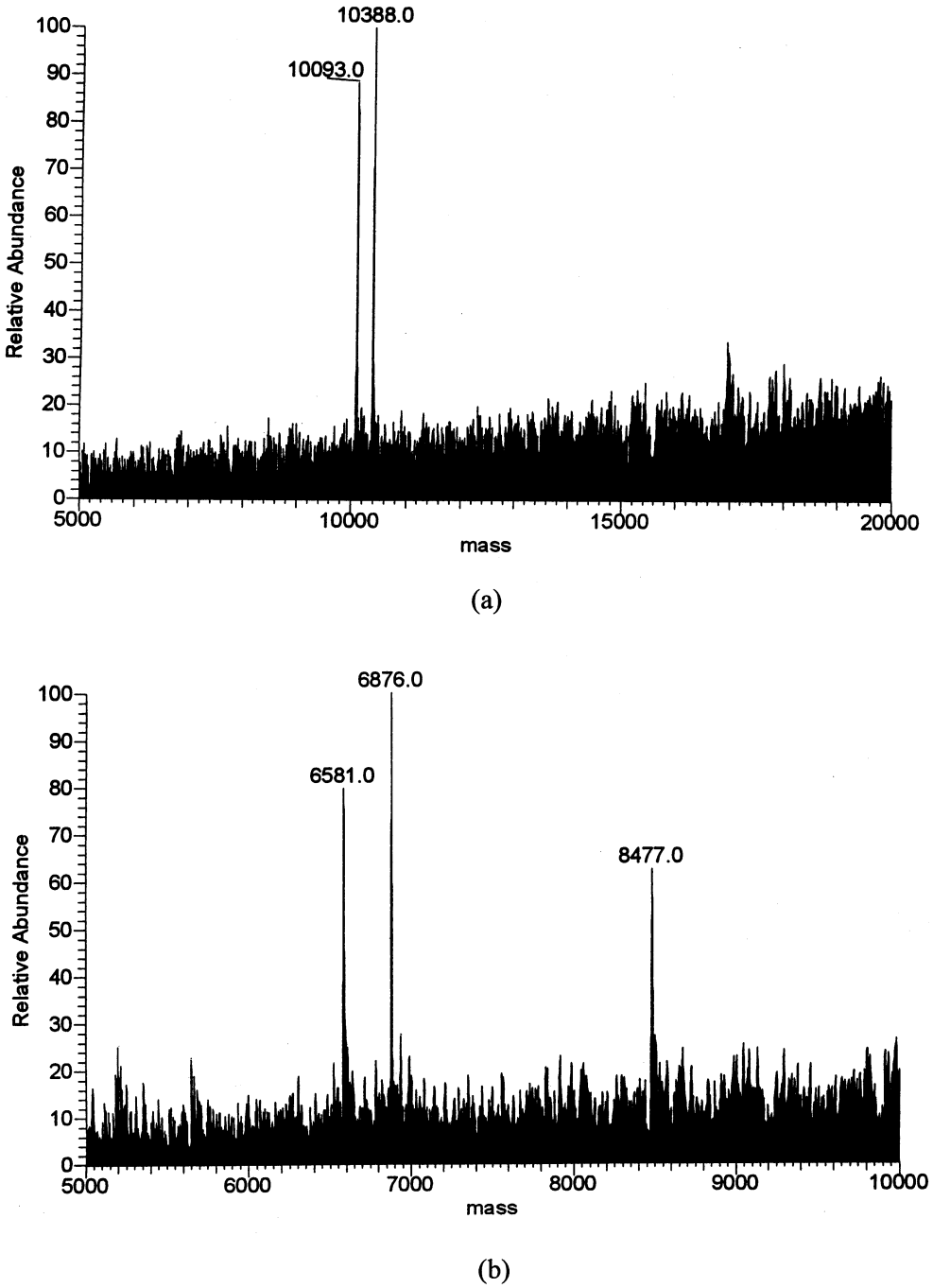
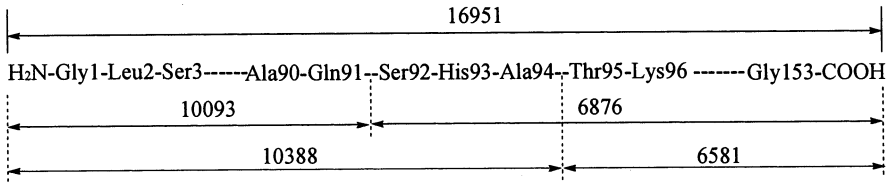


Figure 3. LC-ESIMS spectra recorded for 60 μL of aqueous digestion solution containing 1.0 mg of myoglobin and $CuCl_2$ in a 5:1 molar ratio of Cu(II) to Mb at pH 6.8, incubated at 50 °C for 3 days.

Scheme 2



of the polypeptide backbone and in the MS/MS fragmentation process.[44−47]

Selectivity of the Hydrolytic Cleavage. One of the key questions in hydrolysis of peptides or proteins promoted by artificial metallopeptidase concerns specificity of the cleavage. As a cleavage agent, the coordination of simple metal ions or complexes to a specific site of a protein is required. Previous studies showed that the simple Cu(II) ion is a promising cleavage agent. There is a common feature for all peptides and proteins suffered from cleavage by Cu(II) ion. They contain the Thr-His or Ser-His sequence and coordinate to Cu(II) through the side chain of a histidine

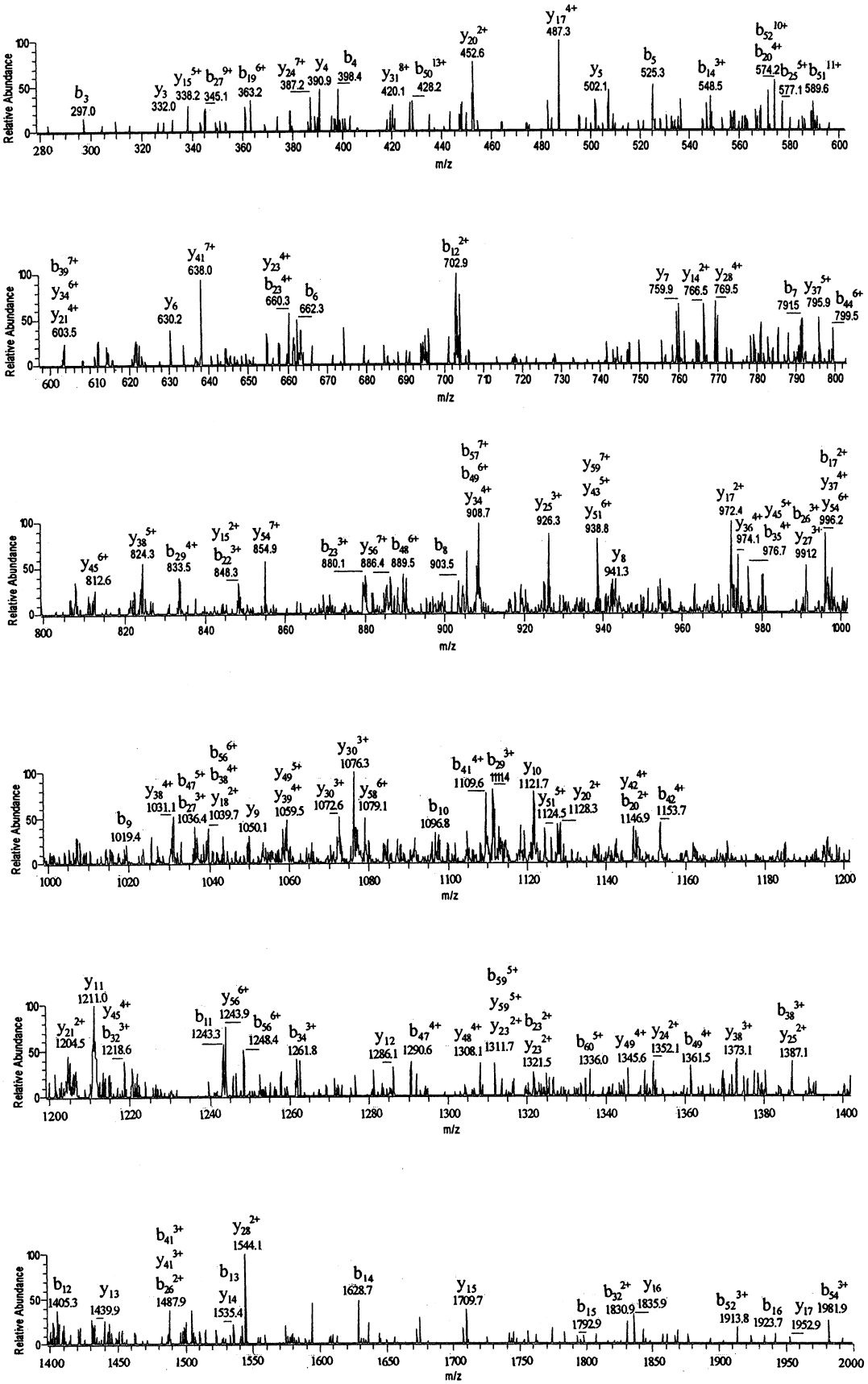


Figure 4. MS/MS spectrum recorded for $[M + 7H]^{7+}$(*m/z* 983.40) from the cleaved fragment of myoglobin with molecular mass of 6876 Da.

residue. Previous studies showed that the cleavage reaction is hydrolytic.[30−31,56] Our ESIMS and MS/MS analyses reveal that both N-terminals of Ser92 and Thr95 are free amino group and both C-terminals of Gln91 and Ala94 are carboxy

group. Therefore, our results further confirm that the Cu(II)-mediated cleavage is a really a hydrolytic reaction. This report provides new evidence for Cu(II) ion promoted specific cleavage of protein containing a Ser-His sequence and a new finding that the peptide bond at the N-terminal of Thr in a His-Ala-Thr sequence can also be cleaved by Cu(II) ion. As already mentioned, this site of cleavage was also observed in myoglobin cleavage by a Pd(II) complex. In the experimental condition used in this study, myoglobin is cleaved by a simple Cu(II) ion with high selectivity; only the two peptide bonds, the second peptide bond upstream and downstream of His93 residue, are cleaved.

Horse heart myoglobin consists of 153 amino acid residues and only of α helices spanned by short connecting links that have a coil conformation. There are 11 histidine residues that are the potential bonding sites toward Cu(II) ion. However, previous study proved that the peptide bond immediately preceding the Ser-His or Thr-His sequence is more susceptible to cleavage upon coordination of His to Cu(II).[32−33,61] Reasonably, in our study, only His93, that is adjoining to Ser and Thr, is detached from iron and returns to coordinate to Cu(II), resulting in the cleavage of the two peptide bonds of Gln91-Ser92 and Ala94-Thr95, though the direct evidence for this displacement is lacking. Because the hydrolytic cleavage of myoglobin by Cu(II) ion proceeds at neutral pH, the conformation of myoglobin may be conserved. Early studies showed that the ligands enter and exit the heme pocket through a pathway between Val68 and His64.[57−58] This channel requires passage through the solvent-exposed side of the heme pocket, which includes residue Lys45 and the heme-6-propionate. The electrostatic and polar interactions involving these groups and surface water molecules have been postulated to be part of the barrier to ligand entry into the heme pocket.[59] Therefore, it is reasonable to propose that both barriers are required for the entrance of Cu(II) ion into heme pocket and for the cleavage of peptide bond. As proved by our experiments, this hydrolytic reaction is sensitive to temperature. At 37 °C, the myoglobin was weakly cleaved by Cu(II) ion in a 10:1 molar ratio of Cu(II) to Mb and 1 day of incubation, but the cleaved band was still visible. Because of the size of complexes **1** and **2**, they enter the heme pocket with more steric blockage; therefore, low extent of cleavage was observed for these two complexes. As far as the three simple Cu(II) compounds are concerned, $CuCl_2$ as trans-square-planar $Cu(H_2O)_2Cl_2$, $Cu(ClO_4)_2$ as an octahedral $Cu(H_2O)_6^{2+}$, and $Cu(Ac)_2$ as dimer $[Cu(Ac)_2]_2$ are present in aqueous solution.[60] Their different geometries may also affect the efficiency of cleavage.

In a previous study, an aa-Ser peptide bond, which is a second peptide bond upstream from a histidine preceded by a serine in oxidized insulin B chain, was cleaved by both Pd(II) and Cu(II).[56,61] Lately, it was found that bovine serum albumin, that contains rich histidine residues (16 histidins), was cleaved by Pd(II) complexes only at the sites upstream from a histidine preceded by serine residue.[62] All these results reveal that Pd(II) and Cu(II) have the same behavior in hydrolytic cleavage of peptides and proteins containing the Ser-His sequence, and the alcoholic side chain of serine residue seems to guide the cleavage of the peptide bond immediately preceding it. In ref 62, a mechanism was proposed for Pd(II)-mediated cleavage of Ser-His(Met) or Thr-His(Met) containing albumins. The mechanism seems to be suitable for the Cu(II)-mediated cleavage of myoglobin, just Pd(II) and Met in Scheme 1 in ref 62 are replaced by Cu(II) and His. As shown in Scheme 1, an intermediate with tetrahedral carbon is formed by nucleophilic attack of alcoholic side chain. This mechanism bears some resemblance to the well-known tetrahedral intermediate in the catalytic mechanism of serine protease.

Myoglobin cleavage with Pd(II) complexes was reported previously.[19] This cleavage reaction proceeded under a weak acidic condition with rather poor selectivity, and 13 sites of cleavage were identified. Corresponding to this, the advantage of myoglobin cleavage with Cu(II) is that the hydrolytic reaction is carried out under neutral pH conditions with high selectivity. In addition, the Cu(II) ion is a common and essential metal for the human body. This study reveals that uptake of excess of Cu(II) ion into the body may bring about a damage to some extent toward the myoglobin.

Conclusion

Horse heart myoglobin is remarkably cleaved by various copper(II) compounds with high specificity for which simple Cu(II) compounds give a greater outcome of cleavage. It is worth emphasizing that the degradation of myoglobin with Cu(II) ion is accomplished at the pH range 6.5−8.5. Raising the incubation temperature and the concentration of Cu(II) ion enables the yield of cleavage to dramatically increase, whereas the specificity of the cleavage decreases. The Cu(II)-mediated hydrolysis of myoglobin exhibits a larger buffer effect, and it is more efficient in a bifunctional buffer of phosphate and NH_4HCO_3. LC-ESIMS and MS/MS accurately determined the sites of cleavage. This is the first attempt for application of the MS/MS method to analyze an amino acid sequence of a bigger cleaved fragment, and it proves that ESIMS and MS/MS are powerful techniques for the cleavage chemistry of proteins.

Acknowledgment. This work was supported by the National Natural Science Foundation of China (29871017, 29823001, and 20271027).

IC025619B

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