Effects of metal ions and an inhibitor on the fluorescence and activity of acutolysin A from Agkistrodon acutus venom

Xianghu Liu¹, Xiaolong Xu, Jiexia Chen¹, Wenqi Liu² and Qingliang Liu¹* ¹Department of Chemistry, University of Science and Technology of China, Hefei, Anhui, 230026, P. R. China ²Center for Physical Sciences, University of Science and Technology of China, Hefei, 230026, P. R. China

Received 24 May 2004; revised 28 February 2005

Acutolysin A, a protein isolated from the venom of Chinese Five-pace snake (Agkistrodon acutus) has shown marked hemorrhagic and proteolytic activities. In the present study, the effects of metal ions and an inhibitor EDTA on the fluorescence and function of acutolysin A have been studied, by following fluorescence and activity measurements. Acutolysin A contains a Ca²⁺-binding site, which provides it with important structural stability, and a Zn²⁺-binding site, which is essential for its enzymatic activities. The removal of metal ions in acutolysin A by incubation with EDTA results in irreversible inhibition and complete denaturation, and a marked decrease in its fluorescence intensity. The fluorescence intensity of acutolysin A is also decreased in the presence of Cu²⁺, Co²⁺, Mn²⁺ or Mg²⁺, but does not change in the presence of Ca²⁺, Cd²⁺ or Tb³⁺. Caseinolytic activity of acutolysin A is enhanced by Co²⁺, Ca²⁺ and Mg²⁺, but is partly inhibited by Cu²⁺, Mn²⁺ and Tb³⁺, and completely inhibited by Cd²⁺. Both Zn²⁺ and Co²⁺ recover the loss of activity of the protein caused by Cd²⁺.

Keywords: Acutolysin A; fluorescence; metal ion; Agkistrodon acutus, zinc-metalloproteinases, EDTA, snake venom

IPC Code: A61K35/58

Hemorrhagins, the biologically active proteins are found widely distributed in snake venoms and can cause the hemorrhage and damage of animal tissuesⁱ². Most of these proteins have metal ions and some of them are zinc-metalloproteinases³. Snake venom zinc-metalloproteinases have similar active site and biological function with matrix metalloproteinases (MMP), and are responsible for degrading the proteins of extracellular matrix. Many of them are able to act on collagens in connective tissues. They belong to the MMP super-family and therefore can become the key targets in the drug design for anti-tumor and anti-arthritis drugs⁴. In addition, they are useful as convenient tool in studying the mechanisms of the ligand-receptor and cell-cell interactions⁵.

The venom of Chinese Five-pace snake (Agkistrodon acutus) contains several biologically active proteins that interfere with the coagulation process⁶⁷. Four hemorrhagins, namely acutolysins A-D showing hemorrhagic and proteolytic activities have been purified from the venom⁸¹¹. Recently, the structures of acutolysins A and C have been reported¹²,¹³. Both acutolysins A and C have a molecular mass of 22 kDa and contain a Zn²⁺ binding in the active site and a Ca²⁺ binding on the molecular surface. It has been demonstrated that Zn²⁺ is essential for the activity of acutolysin A¹³, but as to the function of Ca²⁺, it is not clear. Chelators, such as Chelex-100 and EDTA³ can remove the metal ions in native acutolysin A, like acutolysin D. The Ca²⁺- and Zn²⁺- containing acutolysin A is termed as holo-acutolysin A and the Ca²⁺- and Zn²⁺-free form as apo-acutolysin A.

Fluorescence spectroscopy is an important analytical technique suitable for multicomponent analysis, due to its inherent sensitivity, selectivity and versatility¹⁴,¹⁵. Acutolysin A contains both Trp and Tyr residues and the change of its conformation or microenvironment caused by the metal ions or inhibitor can be detected by fluorescence spectroscopy¹⁵. In this paper, we report the effect of metal ions and an inhibitor EDTA on the conformation and function of acutolysin A using fluorescence and activity measurements.

Materials and Methods

Materials

Lyophilized venom powder was provided by the TUN-XI Snakebite Institute (Anhui, P. R. China).
DEAE-Sephadex A-50 and Sephadex G-75 were obtained from Pharmacia (Uppsala, Sweden) and casein from the Shanghai Institute of Biochemistry (Shanghai, P. R. China). Chelex-100 was purchased from Bio-Rad Laboratories. All other reagents were of analytical reagent grade. Milli-Q purified water was used throughout.

**Acotolysin preparation**

Holo-acotolysin A was purified using a modified procedure of Gong *et al* \(^\text{13}\). Briefly, crude venom (4 g) was dissolved in 15 ml of starting buffer (0.02 M Tris-HCl, pH 8.0) and a small amount of precipitate was removed by centrifugation. The clear supernatant was loaded onto a DEAE-Sephadex A-50 column (3.6 × 100 cm), preequilibrated with starting buffer, and then eluted with a linear gradient from 0-0.5 M NaCl in the same buffer. The eluted fractions were tested for their hemorrhagic and caseinolytic activities by the following assays. The fraction containing acotolysin A was pooled and further purified by gel filtration on a Sephadex G-75 column (2.6 × 100 cm) equilibrated and eluted with 0.15 M NaCl. All the purification steps were done at about 4 °C. The apo-acotolysin A was prepared by dialysis of purified holo-acotolysin A against a suspension of Chelex-100 (1 g/L; Bio-Rad) in 0.02 M Tris-HCl (pH 8.0).

**Caseinolytic activity of acotolysin A**

Caseinolytic activity (CA) was measured by the method described earlier \(^\text{16}\). The reaction mixture containing 0.1 ml protease solution (1 mg/ml) and 2.0 ml casein solution (1%, w/v, 0.1 M Tris-HCl, pH 9.0) was incubated at 37°C for 30 min. Then 3.0 ml of trichloroacetic acid (5%) was added and the mixture further incubated at 40°C for 30 min, centrifuged at 4500 g for 15 min and the absorbance of the supernatant was recorded at 280 nm. One unit of caseinolytic activity was defined as the amount of enzyme, which induced a 0.001 absorbance unit (AU) increasing in absorbance per min.

**Solutions of metal ions**

The solutions of Ca\(^{2+}\), Zn\(^{2+}\), Cu\(^{2+}\), Cd\(^{2+}\), Co\(^{2+}\), Mn\(^{2+}\) and Mg\(^{2+}\) ions were prepared from their respective chlorides. Tb\(_2\)O\(_3\) was dissolved in conc. HCl by gentle heating to dryness, and then dissolved in Milli-Q super-pure water, respectively. The pH values of solutions were adjusted to 6.0 with HCl or NaOH. All metal ion solutions were standardized by titration with standard EDTA solution.

**Fluorescence measurements**

All fluorescence measurements were performed on a Shimadzu RF-5000 spectrophuorometer using a 1 cm quartz cuvette at an excitation wavelength of 280 nm at 25°C. The bandwidths for excitation and emission were both set to 5 nm. Each spectrum is the average of 3 consecutively acquired spectra. All spectra were corrected for buffer base-line fluorescence.

**Results**

**Effect of ionic strength on CA of holo-acotolysin A**

Holo-acotolysin A (Ca\(^{2+}\)- and Zn\(^{2+}\)-containing acotolysin A) was purified by a two-step chromatography procedure of anion-exchange chromatography and gel permeation and its homogeneity was judged by PAGE and SDS-PAGE \(^\text{13}\). Before analyzing the effect of metal ions on the CA of holo-acotolysin A, the effect of ionic strength on CA was determined. Since holo-acotolysin A has an optimum of pH 9.0 for CA (data not shown), pH 9.0 was used for analyzing the effects of ionic strength. As shown in Fig. 1, upon addition of NaCl, very little change of CA of holo-acotolysin A was observed.

**Effect of metal ions and EDTA on CA of holo-acotolysin A**

Holo-acotolysin A contains both Zn\(^{2+}\) and Ca\(^{2+}\), which can be replaced by other metal ions. The effect of metal ions on its CA of holo-acotolysin A was determined and the results are shown in Fig. 2. Addition of Co\(^{2+}\), Ca\(^{2+}\), or Mg\(^{2+}\) ions increased the CA of holo-acotolysin A. However, while addition of Cu\(^{2+}\), Mn\(^{2+}\), Zn\(^{2+}\) or Tb\(^{3+}\) decreased the CA to different extents, addition of Cd\(^{2+}\) resulted in the loss of the CA. In the presence of 0.1 mM Cd\(^{2+}\), CA was restored by 80% upon the addition of Zn\(^{2+}\) ion, and completely restored upon the addition of both Zn\(^{2+}\) and Ca\(^{2+}\).
and Ca\(^{2+}\) (Fig. 3), however, the addition of Ca\(^{2+}\) did not restore the CA. Interestingly, CA was also recovered after addition of Co\(^{2+}\) (Fig. 4).

EDTA, as an inhibitor of zinc metalloproteinase completely inhibited the CA (Fig. 5A). CA was not recovered after addition of Ca\(^{2+}\), Zn\(^{2+}\) or both Ca\(^{2+}\) and Zn\(^{2+}\) to apo-acutolysin A (Ca\(^{2+}\)- and Zn\(^{2+}\)-free acutolysin A). The fluorescence intensity of holo-acutolysin A decreased after removal of Ca\(^{2+}\) and Zn\(^{2+}\) by addition of EDTA (Fig. 5B), and was not restored after addition of Ca\(^{2+}\), Zn\(^{2+}\) or both Ca\(^{2+}\) and Zn\(^{2+}\).

**Effect of metal ions on fluorescence of holo-acutolysin A**

As acutolysin A contains both Trp and Tyr residues and belongs to type B proteins, its fluorescence emission by excitation at 280 nm should be dominated by Trp residues fluorescence, due to the intramolecular energy transfer from Tyr to Trp residues\(^{17}\). To examine the metal ions-induced conformational change around Trp residues, fluorescence measurements were performed at an excitation wavelength of 280 nm. The wavelength \(\lambda_{\text{max}}\) for Trp depends on its microenvironment. Specifically, a low polarity, hydrophobic micro-environment is characterized by \(\lambda_{\text{max}} \approx 331\) nm, while for Trp in an aqueous phase \(\lambda_{\text{max}}\) is 350-353 nm\(^{18}\). There are three Trp residues in acutolysin A\(^{13}\), thus the average microenvironment of Trp residues can be assessed. The maximum excitation and emission of holo-acutolysin A are at 280 nm and 342 nm, respectively. The about 10 nm blue shift of \(\lambda_{\text{max}}\) of holo-acutolysin A relative to that of Trp in an aqueous phase suggests that some Trp residues are located in hydrophobic environment.

The fluorescence spectra of holo-acutolysin A in the presence of different metal ions are shown in Fig. 6A. No obvious changes for the maximum emission wavelength were observed after addition of different metal ions, suggesting that metal ions have no significant influence on the polarity of microenvironment of Trp residues. Addition of Cu\(^{2+}\), Co\(^{2+}\) and Mn\(^{2+}\) markedly decreased the fluorescence intensity of the protein, while Mg\(^{2+}\) slightly decreased and Ca\(^{2+}\), Tb\(^{3+}\) and Cd\(^{2+}\) showed no effect on fluorescence intensity (Fig. 6B).
Discussion

The present study was aimed to investigate the effects of metal ions on the activity and fluorescence of acutolysin A. Holo-acutolysin A loses all CA after removal of Ca$^{2+}$ and Zn$^{2+}$ or addition of Cd$^{2+}$. The loss of activity caused by Cd$^{2+}$ is restored, after addition of Zn$^{2+}$, but not with Ca$^{2+}$, suggesting that Zn$^{2+}$ is essential for its enzymatic activity. Ca$^{2+}$ is important for the structural integrity of the snake venom zinc-endopeptidase adamalysin II$^{19}$. In holo-acutolysin A, Ca$^{2+}$ probably plays a role similar to that in adamalysin II. This is based on the fact that the presence of Ca$^{2+}$ markedly enhances its CA. Holo-acutolysin A loses its CA in the presence of 0.1 mM Cd$^{2+}$, and the activity is restored by 80% upon the addition of Zn$^{2+}$ ion, and completely restored upon the addition of both Zn$^{2+}$ and Ca$^{2+}$ suggesting that Ca$^{2+}$ provides the protein with important structural stability.

To explore the metal ions-induced fluorescence spectroscopy change of acutolysin A, Trp residue fluorescence measurement was performed. Protein fluorescence intensity depends upon the degree of exposure of Trp side chain to the polar, aqueous solvent and upon its proximity to specific quenching groups, such as protonated carboxyl and imidazole groups and deprotonated amino groups$^{20}$. Metal ions have different effects on the fluorescence intensity of the protein. Earlier, we reported that Co$^{2+}$, Ca$^{2+}$, Mg$^{2+}$, Cd$^{2+}$, Mn$^{2+}$, Zn$^{2+}$ and Tb$^{3+}$ have no direct collisional quenching effect on the fluorescence of tryptophan$^{21}$. The complex formation between protein and metal ions, which in turn perturbs the microenvironment around the relevant tryptophan residue(s) may be
of metallo-enzymes are known to contain zinc, a diamagnetic metal ion at the active site of such enzymes. In contrast, cobalt is a paramagnetic and gives rise to the visible spectra and therefore can be a very good environmental probe of Zn$^{2+}$ ion$^{24}$. Thus, the replacement of Zn$^{2+}$ by Co$^{2+}$ is very important and useful.

In conclusion, Zn$^{2+}$ is essential for the enzymatic activities of holo-acutolysin A, while Ca$^{2+}$ is important to stabilize its conformation. EDTA completely inhibits its activity and markedly decreases its fluorescence intensity irreversibly. Caseinolytic activity of holo-acutolysin A is enhanced by Co$^{2+}$, Ca$^{2+}$ or Mg$^{2+}$, but is partly inhibited by Cu$^{2+}$, Mn$^{2+}$ or Tb$^{3+}$, and completely inhibited by Cd$^{2+}$. Both Zn$^{2+}$ and Co$^{2+}$ recover the loss of activity caused by Cd$^{2+}$, and Ca$^{2+}$ can help Zn$^{2+}$ to recover the activity. The fluorescence intensity of holo-acutolysin A decreases in the presence of Cu$^{2+}$, Co$^{2+}$, Mn$^{2+}$ or Mg$^{2+}$, and does not change in the presence of Ca$^{2+}$, Cd$^{2+}$ or Tb$^{3+}$.

MMP super-family is involved in various connective-tissue diseases, such as arthritis and breast cancer$^3$. The present study may prove useful for understanding the biochemical basis of acutolysin A, as a MMP, and also for designing the anti-tumour and anti-arthritic drugs. Zn$^{2+}$, Ca$^{2+}$, Co$^{2+}$, Mg$^{2+}$, Cu$^{2+}$, Mn$^{2+}$, and EDTA may be used as the regulators for the activity of acutolysin A in its application in drug design. Also, Co$^{2+}$ and Tb$^{3+}$ may be useful as the paramagnetic probes of Zn$^{2+}$ and Ca$^{2+}$, respectively for studying the structures of the metal ion sites in holo-acutolysin A by electron paramagnetic resonance spectroscopy.

Acknowledgement
The work was supported by grants from the National Natural Science Foundation of China (No. 20171041, X. L. Xu) and the Anhui Provincial Natural Science Foundation (No. 00044428, X. L. Xu).

References
LIU et al.: EFFECT OF METAL IONS ON FLUORESCENCE AND ACTIVITY OF ACUTOLYSIN A

16 Van der Walt S J & Joubert F J (1971) Toxicon 9, 153-161
17 Weber G & Rosenheck K (1964) Biopolymers Symp 1, 333-344