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Contributions of Zn(II)-binding to the structural stability of endostatin

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Abstract Endostatin has a compact structure with a Zn(II)-binding site (His1, His3, His11, and Asp76) at the N-terminus. In this study, the effects of Zn(II)-binding on the folding and stability of recombinant human endostatin were studied. The results show that Zn(II)-binding largely stabilizes the structure of endostatin at physiological pH. Under some proteolytic conditions, Zn(II)-binding also contributes to the integrity of the N-terminus of endostatin, which is critical for endostatin to maintain a stable structure. Moreover, engineering an extra Zn(II)-binding peptide to the N-terminus of human endostatin makes this molecule more stable and cooperative in the presence of Zn(II).
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1. Introduction

Endostatin (ES), a 20 kDa C-terminal fragment of collagen XVIII, was discovered for its potent inhibitory function on angiogenesis and tumor growth without toxicity and acquired drug resistance [1–3]. X-ray crystallography of ES shows a compact structure composed of predominant β-sheets and loops, two pairs of disulfide bonds, and a Zn(II)-binding site at the N-terminus [4,5]. His1, His3, His11, and Asp76 coordinate with Zn(II) in human ES, and these four ligands are also conserved in murine and dog ES [4–6].

Among the four Zn(II)-binding ligands, His1, His3, and Asp76 are critical for Zn(II)-binding in ES [4,6]. The double mutant H1/3A, which cannot bind Zn(II), abolishes ES antitumor activity completely in the Lewis lung carcinoma model [7]. Site mutation in His11 or Asp76 also significantly reduces the antitumor activity of full length ES [7]. Recently, Javaherian and his colleagues reported that Zn(II)-binding is required for antitumor and antimigration activities of an N-terminal 27-amino-acid peptide of ES [8]. However, controversial reports on the role of Zn(II) in the biological activity of ES show that N-terminal deleted mutant of human ES act as efficiently as the wild-type ES [9–11]. In order to further explain these inconsistent results, more studies on the effects of Zn(II)-binding on ES are needed.

On the other hand, it was proposed that Zn(II) is likely to play a structural role in ES [5]. Zn(II)-dependent dimer was observed in the crystal instead of in the solution of human ES [4]. Surface plasmon resonance analysis shows that the binding of ES to heparin and heparan sulfate requires the presence of Zn(II) [12]. As an important constituent of ES, the contribution of Zn(II) to the folding behavior and the overall stability of ES has not been reported yet, nor the binding affinity between ES and Zn(II).

In this study, the effects of Zn(II)-binding on the folding and stability of recombinant human ES were studied using thermal- and guanidine hydrochloride (GdmC1)-induced unfolding monitored by differential scanning calorimetry (DSC) and tryptophan emission fluorescence, respectively. Our results show that upon Zn(II)-binding, ES is more stable against heat, denaturant, and certain proteolytic conditions. Moreover, engineering a Zn(II)-binding peptide (ZBP) to the N-terminus of ES enhances the Zn(II)-binding capacity, which endows holo ZBP-ES a more stable and cooperative structure than holo ES. These observations provide a structural basis for further understanding the molecular behavior involved in the biophysical and biological functions of ES and Zn(II).

2. Materials and methods

2.1. Preparation of ES and its variants

Both ES and ZBP-ES expressed by *Escherichia coli* were provided by Protgen Ltd. Fragment-179 was purified directly from the trypsin digested products of apo ES. Apo ES (0.5 mg/ml) was digested by 25 U/ml trypsin at 37 °C in 10 mM Tris, pH 7.4, for 20 min, then the products were applied on a SP Sepharose High Performance (Amersham) column equilibrated in 10 mM Tris–HCl, pH 8.0, and eluted by linear gradient of NaCl concentration. Protein concentrations were determined by BCA protein assay reagent kit (Pierce).

2.2. Apo and holo sample preparation and Zn(II) content analysis

To prepare the apo and holo samples, 100-fold molar excess EDTA or $ZnCl_2$ was incubated with protein sample in 5 mM Tris–HCl, pH 7.4, at room temperature overnight and then extensively dialyzed to 5 mM Tris–HCl, pH 7.4. The Zn(II) content was measured by atomic emission spectroscopy with an OPTIMA 3300RL instrument.

2.3. Determination of Zn(II)-binding affinity

The dissociation constants for Zn(II)-binding in ES and ZBP-ES were obtained from competition titration as described in Refs.

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Abbreviations: ES, endostatin; FZ, FluoZin-1, tripotassium salt; CD, circular dichroism; FPLC, fast protein liquid chromatography; GdmC1, guanidine hydrochloride; DSC, differential scanning calorimetry; ZBP, Zn(II)-binding peptide; ZBP-ES, Zn(II)-binding peptide modified endostatin

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[13,14], except that Zn(II)-specific fluorescence indicator FluoZin-1 (FZ) (tripotassium salt) was used. FZ can form a 1:1 complex with Zn(II) specifically, with dramatic fluorescence intensity increase at 516 nm when excited at 495 nm [15]. FZ (0.125 μM) alone was titrated with Zn(II) to obtain the dissociation constant (K_0) of the FZ–Zn(II) complex. For determining the Zn(II)-binding affinity to ES or ZBP-ES, the mixture of FZ (0.125 μM) and ES or ZBP-ES (0.5 μM) was titrated with Zn(II). All of the experiments were carried out in 10 mM Tris–HCl, pH 7.4, at 20 °C. The following equilibria were used for calculation:

$$\frac{[\mathrm{FZ-Zn(II)}]}{[\mathrm{FZ}]_{\mathrm{total}}} = \frac{F - F_0}{F_{\max} - F_0} \quad (1)$$

$$\mathrm{FZ-Zn(II)} \overset{K_0}{\leftrightarrow} \mathrm{FZ} + \mathrm{Zn(II)} \quad K_0 = \frac{[\mathrm{FZ}][\mathrm{Zn(II)}]}{[\mathrm{FZ-Zn(II)}]} \quad (2)$$

$$\mathrm{ES-Zn(II)} \overset{K_1}{\leftrightarrow} \mathrm{ES} + \mathrm{Zn(II)} \quad K_1 = \frac{[\mathrm{ES}][\mathrm{Zn(II)}]}{[\mathrm{ES-Zn(II)}]} \quad (3)$$

$$\mathrm{ES-Zn(II)_2} \overset{K_2}{\leftrightarrow} \mathrm{ES-Zn(II)} + \mathrm{Zn(II)} \quad K_2 = \frac{[\mathrm{ES-Zn(II)}][\mathrm{Zn(II)}]}{[\mathrm{ES-Zn(II)_2}]} \quad (4)$$

where F denotes fluorescence intensity measured at the wavelength of 516 nm, F_0 is the fluorescence background of FZ, $F_{\max}$ is the maximal fluorescence intensity, K_1 and K_2 are the dissociation constants for the first and the second Zn(II) ion binding in protein, respectively. For protein which has only one Zn(II)-binding site, Eqs. (1)–(3) are used, while for protein which has two binding sites, Eqs. (1)–(4) are used. In this study K_0 = 7.87 μM was obtained, slightly deviating from the K_0 (8 μM) provided by manufacturer. Based on Zn(II) content and binding affinities of holo ES and ZBP-ES, extra Zn(II) (10-fold molar excess of protein) was added into the holo samples in the following experiments to make sure that holo ES and ZBP-ES were predominantly in the bound forms at neutral pH (>90%, according to the dissociation constants). Higher Zn(II) concentration was avoided taking account of the probability of cation or anion effect brought by $ZnCl_2$ in the unfolding studies.

2.4. Fluorescence measurements

Fluorescence measurements were carried out using an F-4500 fluorescence spectrophotometer (Hitachi) with a cuvette of 1 cm light path. The slit widths for excitation and emission were 5 and 10 nm, respectively. The scan speed was 240 nm/min [16–19].

2.5. GdmCl-induced unfolding

The concentration of ES or its variants was 0.8 μM in 5 mM Tris–HCl, pH 7.4, which contains different GdmCl concentrations ranging from 0 to 6 M. After incubation for over 120 min, fluorescence measurements were carried out. All of the measurements were repeated for three times and data were analyzed according to the procedure of Santoro and Bolen [20].

2.6. Circular dichroism (CD) spectroscopy

The far- and near-UV CD spectra were obtained using a Jasco-715 spectropolarimeter. The concentration of protein was 10 μM, in 5 mM Tris–HCl, pH 7.4. The spectra were measured in 0.1 (far-UV) and 1 cm (near-UV) quartz cuvette. An average of three scans was recorded and corrected by subtracting the baseline spectrum of the buffer. The CD signal was converted to molar ellipticity per residue [θ] (deg cm^2 dmol^{-1}). All measurements were carried out at 20 °C.

2.7. Differential scanning calorimetry (DSC)

DSC thermograms were recorded in a micro DSC III (Setaram, France). Each sample was in 10 mM HEPES buffer at pH 7.0 with a final ES concentration of 2.86 mg/ml. The heating rate is 0.5 °C/min. A reference blank containing all reagents except ES was measured for each sample at identical settings. Data were analyzed as described by Brandts and his colleagues [21].

2.8. Proteolysis assay

EDTA or $ZnCl_2$ (1 mM) was pre-incubated with ES (0.2 mg/ml) for 10 min at 37 °C before trypsin (62.5 U/ml) was added. At each time point, reaction solution was quickly taken out and mixed with reduced SDS–PAGE loading buffer to stop the proteolysis reactions. All of the experiments were carried out in PBS buffer, pH 7.4, at 37 °C.

2.9. Mass spectrometry

The largest fragment of trypsin-digested apo ZBP-ES were separated by SDS-PAGE, and then analyzed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry using a Bruker Biflex linear time-of-flight spectrometer (Bruker Franzen, Bremen, Germany). The MALDI-TOF data were searched in the Swiss-Prot protein data base for protein identification.

3. Results

3.1. Structure analyses and Zn(II)-binding affinity measurement

As described in our previous studies, tryptophan emission fluorescence is a good probe for detecting the tertiary structure changes of ES [16–19]. Fig. 1A shows a 3D structure model of ES, in which the Zn(II)-binding site and the four tryptophan residues are indicated [4]. Apo and holo ES show very similar tryptophan fluorescence spectra at neutral pH (Fig. 1B), which may imply that Zn(II)-binding has little effect on the environment in which the four tryptophan residues are located. The tertiary and secondary structure of apo and holo ES do not change much, as evidenced by the near- and far-UV CD spectra, respectively (Fig. 1C and D). These results show that Zn(II)-binding does not bring evident changes to the overall structure of ES. In our experiment, the dimer formation of holo ES was not detected in solution at physiological pH by Fast protein liquid chromatography (FPLC) size-exclusion chromatography (data not shown).

Atomic emission spectroscopy results show that holo ES contains about one Zn(II) ion per molecule (Zn(II)/ES is 0.9), which is consistent with previous reports [4,6]. The affinity of ES for Zn(II) was determined by competition titration with a Zn(II) fluorescence indicator, FluoZin-1 (FZ). Fig. 2 shows the plots of fluorescence intensity at 516 nm as a function of total added Zn^{2+}. Data of ES were best fitted to Eq. (1)–(3), which describes a single binding site for Zn^{2+}, and the dissociation constant of ES–Zn(II) complex is 6.7 nM.

3.2. Calorimetric study of ES by DSC

The thermograms showing the excess heat capacity (C_p) as a function of temperature are shown in Fig. 3. The transition temperatures (T_m) of apo ES is 52.1 °C. Zn(II)-binding shifts the transition peak towards higher temperature, with a 9.1 °C increase in ΔT_m. These results demonstrate that Zn(II)-binding stabilizes holo ES against heat.

3.3. Limited protease digestion

Trypsin is a commonly used protease, which cleaves C-terminal peptide bonds of the Arg and Lys residues. Since all of the 15 Arg and 5 Lys residues in ES are on the surface of the tertiary structure of the molecule [4], trypsin was used as a tool to probe possible conformational change of ES upon Zn(II)-binding. When digested under the same condition, apo ES was quickly digested within 10 min, while intact holo ES could not be totally digested even after 6 h (Fig. 4).

In addition, different digestion manners were observed with apo and holo ES. Apo ES experienced a two-stage digestion process, during which a dominant fragment was obtained within 3 min and was further degraded. In contrast, intact holo ES follows a continuous digestion process (Fig. 4). These results indicate that Zn(II)-binding may protect certain susceptible cleavage sites of ES thus stabilizing ES against trypsin. Peptide mass fingerprinting shows that only the first four ami-

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