

Effect of Zn(II) on the Structure and Biological Activity of Natural β -NGF

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Abstract Only β -NGF, the subunit of the 7S NGF complex, exhibits NGF activity, but the function of the zinc ion in native β -NGF has received little attention. Flameless atomic absorption spectroscopy (FAAS) measurements reveal that native β -NGF contains Zn(II) with a Zn(II)/ β -NGF stoichiometry of 1:14.6. The presence of Zn(II) in the native molecule results in significant changes of the secondary structure and local tertiary structure around Trp(s) with respect to those of apo β -NGF, as suggested by spectra of fluorescence and circular dichroism. Stopped-flow studies show that there are at least two steps during the interaction of Zn(II) with the apo form. In comparison with its apo form, the native β -NGF shows a higher ability to trigger the proliferation of TF1 cells and mediate the survival of PC12. Thus it is most likely that the structural changes caused by the presence of Zn(II) directly lead to the increase in the biological activity of β -NGF. All results indicate that Zn(II) in native β -NGF plays an important role in the structure and the biological activity of the protein.

Key words Zn(II); β -NGF; apo β -NGF; secondary structure; tertiary structure; biological activity

NGF is a well-characterized member of the neurotrophin family involved in a variety of processes of neurons such as signaling, cell differentiation and survival, growth cessation and apoptosis [1–3]. β -NGF is a 2.5S protein, exhibiting the survival and neurite-promoting activities associated with NGF. β -NGF is composed of two identical polypeptides of 118 amino acid residues. In the mouse submaxillary gland, β -NGF forms a 7S complex with a stoichiometry of $\alpha_2\beta\gamma_2$ by independently binding two other subunits, α and γ , which belong to the kallikrein family of serine proteases [4–6].

Zinc is an essential component of more than 300 kinds of proteins and plays structural, catalytic and regulatory roles in these proteins [7,8]. Zinc has also been known to bind to 7S NGF with a Zn(II)/NGF stoichiometry of 1:1 [9]. Upon the loss of zinc, the 7S complex is autocatalytically cleaved to release of β subunit [9,10]. Similarly, X-ray diffraction analysis indicates that 2.5S NGF has a zinc-binding site with His⁸⁴/Asp¹⁰⁵ as ligands [11]. It has been suggested that zinc ion play a role in the structure and the biological activity of 2.5S NGF [11],

although addition of extra Zn(II) to native 2.5S NGF could result in its inactivation [12–14]. However, little attention has been paid to the role of Zn(II) in native 2.5S NGF.

In the present paper, we provide evidence that zinc ion is indeed bound to 2.5S native NGF and has a significant effect on the structure and biological activity of 2.5S NGF.

Materials and Methods

Materials

Mice were obtained from Beijing Animal Center. 2.5S NGF was extracted from mouse submaxillary gland as previously described [15,16]. β -NGF concentration was determined by measuring the absorbance at 280 nm [6]. Apo β -NGF was prepared as previously reported [6]. All chemicals used were of analytical or high purity grade. Circular dichroism (CD) spectra were performed on a Jasco J-715 spectrophotometer. Fluorescence measurements were run on a Shimadzu RF-540 spectrophotometer. Flameless atomic absorption spectroscopy (FAAS) measurements were carried out on a Perkin-Elmer 560 instrument with a graphite furnace. Kinetic studies were performed on a Union Giken RA-401 stopped-flow

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spectrophotometer, equipped with a versatile Union Giken RA-451 data processor and a Union Giken RA-454 refrigerated circulating bath (temperature accuracy is 0.1 K).

Determination of the ratio of Zn^{2+}/β -NGF in native β -NGF

2 ml native β -NGF (48.0 μ M) in 10 mM pH 7.2 Tris-HCl was dialyzed three times against 1 L deionized water for 24 h at 4 °C. NGF concentration was determined by measuring the absorbance at 280 nm [6]. The zinc content in native β -NGF (1.0 mg/ml) was detected by FAAS by suspending the solution in 2% nitric acid and incubating it at 50 °C for 24 h to hydrolyze the protein. The ratio of Zn^{2+}/β -NGF is the molar concentration of zinc versus the molar concentration of the protein.

CD spectroscopy

0.5 ml apo β -NGF (32.0 μ M) or native β -NGF (32.0 μ M) in 10 mM pH 7.2 Tris-HCl buffer was warmed to room temperature (25 \pm 1) °C prior to analysis. Each sample was scanned in the range of 180–250 nm. CD spectrum was obtained as the average of four scans with the buffer background subtracted.

Fluorescence spectroscopy

Excitation-emission spectra were measured at 25 °C and processed to obtain the emission spectra (λ_{em} : 300–500 nm) at the maximum excitation wavelength (295 nm).

Stopped-flow fluorescence studies

Equal volumes of 2.490 μ M apo β -NGF and 250 μ M Zn^{2+} in 10 mM pH 7.2 Tris-HCl buffer were mixed (the dead time for mixing is 0.5 ms). The emission fluorescence intensity at 340 nm excited at 295 nm was monitored for 50 s. The experiment was conducted at 25 °C under pseudo-first-order condition with $Zn(II)/apo$ β -NGF (1.245 μ M) stoichiometry of about 100:1.

Cell lines and bioassays

TF1 (the human erythroleukemia) cells were routinely grown in RPMI 1640 medium containing 10% fetal calf serum and 10 ng/ml GM-CSF. The TF1 cell proliferation assay was measured in microtiter plates by incubating 15,000 cells per well in the presence of the indicated concentration of β -NGF. After 40-h culture, 10 μ l of MTT solution (5 mg/ml) was added for an additional 4-h incubation and the optical density was determined at 570 nm (A_{570}) in a microtiter plate reader according to previously described colorimetric assay [17].

Cells of the rat pheochromocytoma cell line PC12 were grown in RPMI 1640 medium with 5% fetal bovine serum and 10% donor horse serum at 37 °C 5% CO₂ in poly-*L*-lysine hydrobromide (mol. wt. 150–300 kD) coated culture dishes. Prior to measurement, the growth medium was removed; the cells were rinsed, and then cultured with 200 μ l of RPMI 1640 containing β -NGF in different doses. The PC12 cell survival measurement was achieved in poly-*L*-lysine coated 96-well plate by incubating 40,000 cells per well in the presence of the indicated concentration of β -NGF without serum. After 24-h incubation, the PC12 cell survival was measured by crystal violet staining as previously reported [18].

Results

Zn(II)/ β -NGF stoichiometry

Based on earlier findings that 7S NGF contains tightly bound zinc ion [9,10] and recent X-ray diffraction analysis that 2.5S NGF also has a natural affinity for Zn(II) [11], we have measured the zinc content in native β -NGF. Three different preparations yield 0.161, 0.178 and 0.167 μ g Zn(II) per mg native β -NGF, respectively ($\bar{x}\pm s=0.169\pm 0.009$). On the basis of the present result, a Zn(II)/ β -NGF molar ratio of 1:14.6 is obtained.

Effect of Zn(II) on the secondary and tertiary structure of β -NGF

The circular dichroism spectra of apo β -NGF, native β -NGF and native β -NGF plus Zn(II) are shown in Fig. 1. The principal feature of the CD spectra of β -NGF is

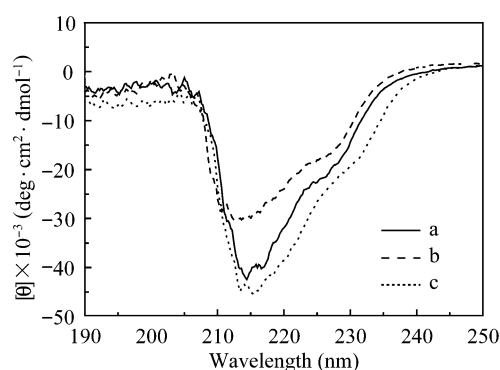


Fig. 1 The far UV CD spectra of the β -NGF

The far UV CD spectra of β -NGF in 10 mM pH 7.2 Tris-HCl buffer were measured at 25 °C. a, 32.0 μ M native β -NGF; b, 32.0 μ M apo β -NGF; c, 32.0 μ M native β -NGF plus 30 μ M $ZnCl_2$.

an intense negative peak at 218 nm, which is the characteristic of β -sheet structure [6,19]. The best fits for spectra between 190 and 250 nm produce 26.5%, 35.4%, and 37.5% β -sheet for apo β -NGF, native β -NGF, and native β -NGF plus 30 μ M Zn(II), respectively, a result consistent with previous X-ray crystallography that a secondary structure of the β -NGF is rich in β -sheet but contains no α -helix [20].

In order to probe the effects of Zn^{2+} on the tertiary structure of β -NGF, the intrinsic fluorescence emission of the protein was investigated (Fig. 2). On excitation at 295 nm, the emission maximum was observed at around 340 nm. A major increase in total fluorescence was seen in the native NGF with respect to its apo NGF and the enhanced fluorescence was not accompanied by a shift in the λ_{max} , suggesting that Zn(II) can also cause a change of the local tertiary structure around Trp residues in the β -NGF. The fluorescence intensity of the native β -NGF further increases upon adding 30 μ M Zn(II), being consistent with the above observation (Fig. 2c).

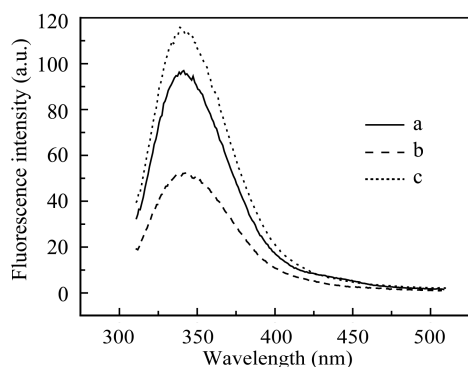


Fig. 2 Fluorescence emission spectra of β -NGF

Fluorescence emission spectra of 32.0 μ M β -NGF in 10 mM pH 7.2 Tris-HCl buffer were measured at 25 $^{\circ}$ C. Excitation wavelength is 295 nm. a, the native protein; b, the apo β -NGF; c, the native β -NGF plus 30 μ M ZnCl_2 .

Stopped-flow studies

Stopped-flow measurement of Zn(II) binding to apo β -NGF was conducted to shed some insight into the binding process using monitoring intrinsic fluorescence intensity of the protein. The kinetic curve is shown in Fig. 3. After 48 s, no further change was observed, indicating that the fluorescence change in the first 48 s covers the whole process of conformation change induced by Zn(II). It was observed that the quenching of fluorescence began at 1 s and reached a maximum at 6 s. The

observed pseudo-first-order rate constant for the quenching reaction was obtained as $(0.780 \pm 0.014) \text{ s}^{-1}$. After 15 s, the fluorescence resumed to a higher level than its initial one, which accords with fluorescence spectra that the native β -NGF shows a stronger intrinsic fluorescence than its apo form. The stopped-flow result shows obviously that there are at least two steps during the interaction of Zn(II) with the apo molecule.

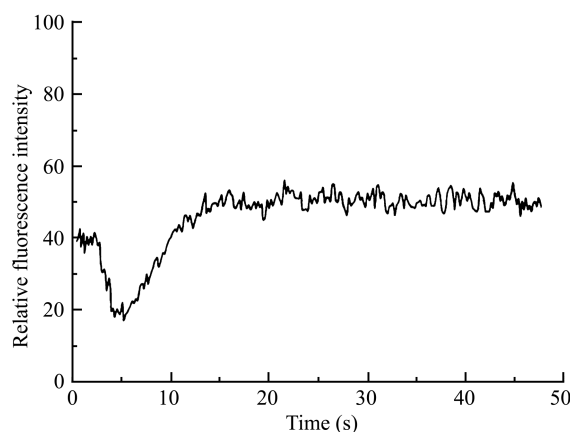


Fig. 3 Conformation change of the apo β -NGF during the Zn(II) binding

The conformation change was monitored by the tryptophan fluorescence as a function of time. Excitation wavelength is 295 nm; Emission wavelength is 340 nm. 1.245 μ M apo β -NGF was in 20 mM pH 7.2 Tris-HCl buffer; $[\text{Zn(II)}]/[\text{apo } \beta\text{-NGF}]=100$.

Cell bioassay

In order to illustrate the biological effect of Zn(II) on the β -NGF, the proliferation of TF1 cell triggered by both the native β -NGF and the apo β -NGF was determined by MTT method (Fig. 4) [17]. Fig. 4(A) shows that the proliferation effect increases with the increasing of the native β -NGF concentration. Although the native β -NGF at the low concentration (less than 2.5 ng/ml) has no significant proliferation effect on TF1 cells, the dose-response curve increases sharply at the concentration higher than 2.5 ng/ml, and reaches a maximum at 25.0 ng/ml. It is also observed that the native β -NGF triggered TF1 cells proliferation with a half-maximal effect at approximately 5 ng/ml β -NGF. Therefore, 5.0 ng/ml β -NGF is chosen as an example to compare native form with its apo form for the proliferation effect on TF1 cells [Fig. 4(B,C)]. Both native form and apo form show higher ability to trigger the proliferation of the TF1 cells than the control. Given that the proliferation effect of the growth medium on the TF1 cells was deducted, at the same concentration the

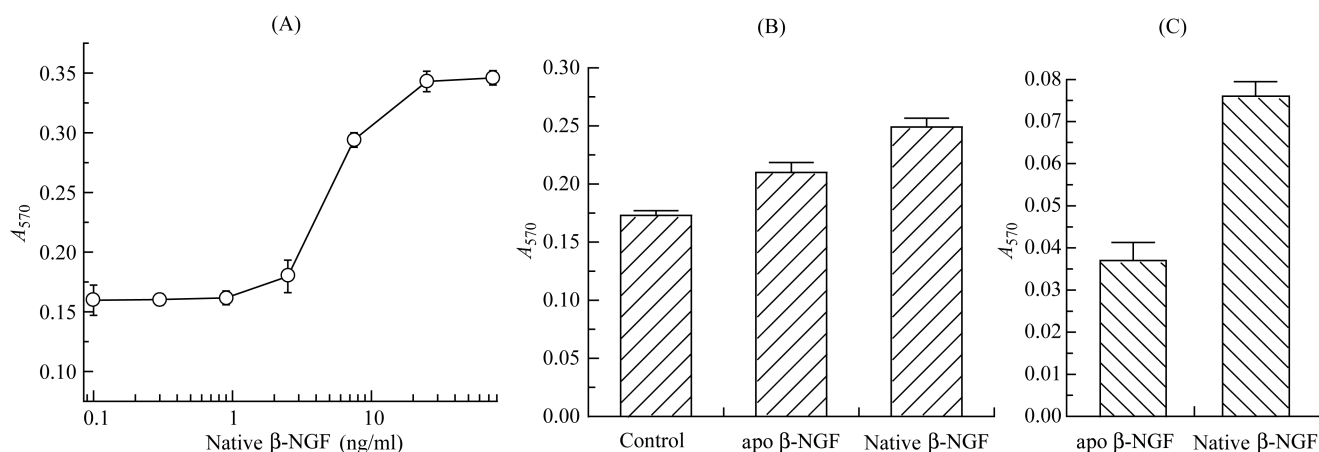


Fig. 4 The effect of apo β -NGF and native β -NGF on TF1 cells

(A) The proliferation of TF1 cells at different concentration of native β -NGF. (B) The proliferation effect of control, apo β -NGF (5 ng/ml) and native β -NGF (5 ng/ml) on TF1 cells. (C) The resulted proliferation effect of apo β -NGF and native β -NGF on TF1 cells after being subtracted by control, respectively. TF1 cell proliferation assay was performed in triplicate in microtiter plate by MTT method as described in methods.

native form is as almost 2-fold potent as the apo form for the proliferation effect.

The biological assessment of the apo form and the native form was also carried out using the PC12 cells. Fig. 5 shows that cell survival increases with increasing the concentration of both native and apo β -NGF. When β -NGF is at low concentration, there is no big difference between the native β -NGF and the apo β -NGF in increasing cell survival. However, the native protein shows a much higher ability to improve the cell survival than its apo β -NGF upon increasing the concentration to 3–100 ng/ml. However, at the concentration of

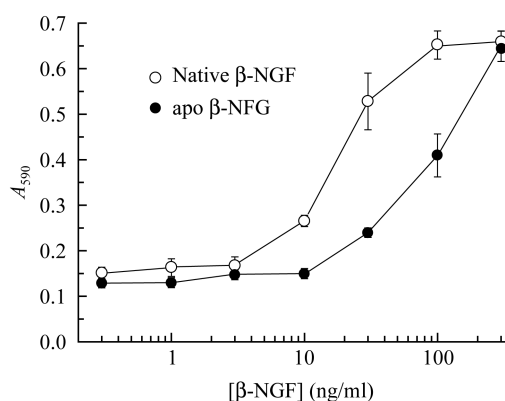


Fig. 5 The survival of PC12 cells at different concentration of both the native β -NGF and the apo β -NGF

The survival of PC12 cells 24 h after being seeded at 40,000 cells per well in a 96-well tissue culture plate coated with poly-*L*-lysine, the survival of PC12 cells at different concentration of both the native β -NGF and the apo β -NGF was measured by crystal violet staining of fixed cells as described in methods. The data point represents $\bar{x} \pm s$ ($n=4$).

300 ng/ml there is no difference between two β -NGFs in sustaining the survival of PC12 cells, possibly because the binding-sites of the β -NGF to the receptor p75 on the cell surface have been saturated with such a high concentration of the β -NGF (see below).

Discussion

In this paper we report that β -NGF contains Zn(II) with a Zn(II)/ β -NGF stoichiometry of 1:14.6, a finding consistent with X-ray diffraction analysis that the protein has a naturally binding site for zinc ion [11]. As compared with a Zn(II)/NGF stoichiometry of 1:1 in the 7S NGF [9], the Zn(II) content in the 2.5S NGF is much lower. However, it is possible that this low content reflects the loss of zinc during purification, as suggested by the low zinc content determined by FAAC. The similar observation that transition metal cation was lost in the process of the protein purification has been reported [21].

The fluorescence emission spectra of apo β -NGF, native β -NGF and native β -NGF plus 30 μ M Zn²⁺ consist of a single peak at 340 nm upon excitation at 295 nm, but the fluorescence intensity of the apo protein was greatly decreased and was weakest among three samples (Fig. 2), indicating that the fluorescent Trp(s) must be in a different environment, reflecting difference in conformation, resulting in substantial quenching compared with that in the β -NGF containing Zn(II). Thus the presence of bound Zn(II) in the protein must be responsible for the significant increase in the overall fluorescence

intensity. However, it is noted from the stopped-flow result that the binding of zinc ion to the β -NGF firstly results in the fluorescence quenching of the intrinsic fluorescence (Fig. 3) and the pseudo-first-order rate is estimated to be 0.780 s^{-1} . 5 s later, a conformational change stage (corresponding to the second step of the interaction of zinc ion with the apo β -NGF) following the Zn(II) binding increases the fluorescence intensity of the apo protein to a higher level than the initial fluorescence (Fig. 3), a result agreeing with the fluorescence spectra that the native protein exhibits an increase in the fluorescence emission intensity as compared with its apo form (Fig. 2). Naturally bound Zn(II) in the native NGF causes an evident increase in the content of β -sheet by about 9% as compared to apo β -NGF, indicating that Zn(II) is closely associated with the stability of the secondary structure of β -NGF. The proposal is further proven by the observation that the content of β -sheet in β -NGF keeps increasing upon the addition of $30 \mu\text{M Zn}^{2+}$ to the native β -NGF (Fig. 1). We believe that the tertiary structural change in the apo protein induced by Zn(II) is possibly accompanied by an increase in β -sheet as reflected in circular dichroism.

The measurements of the biological activity show a concerted result that, as compared with apo β -NGF, the native β -NGF has a stronger ability to trigger the proliferation of TF1 cells and mediate the survival of PC12 cells [Fig. 4(B,C), Fig. 5], a finding suggesting that the presence of Zn(II) in the native molecule effectively promotes the biological activity of this protein. The increased activity may stem from the change of the secondary and tertiary structure of the apo β -NGF induced by Zn(II) (Fig. 1–3). Such a structural change is most likely due to the direct binding of Zn(II) to ligands such as His⁸⁴ and Asp¹⁰⁵ at the natural binding-sites in the protein as suggested by X-ray diffraction analysis [11]. On the other hand, NGF is believed to act via an NGF receptor on the surface of responsive neurons [2,22,23]. Two types of NGF receptor, high-affinity TrkA and low-affinity p75, have been identified based on the affinity for the NGF molecule. It is reported that receptor TrkA but not p75 is responsible for the proliferation triggered by NGF on TF1 cells [17]. On the contrary, the survival of PC12 cells is enhanced by NGF as a result of the binding of NGF to the low-affinity receptor p75, mainly because the binding blocks the apoptotic effect of the receptor p75 on PC12 cells after serum withdrawal [24–26]. Therefore, the conformational change of the β -NGF due to the presence of Zn(II) may facilitate the binding of the β -NGF to the two receptors, resulting

in an increase in the biological activity of the β -NGF. But the mechanism is out of the scope of the present study.

The paper demonstrates that, Zn(II) is associated with 2.5S NGF, as with 7S NGF, to be a part of the overall complex. The presence of Zn(II) in the native NGF can significantly change the secondary and tertiary structure around Trp(s) of the β -NGF as suggested by the fluorescence and circular dichroism spectra, which likely leads to an increase in the ability of the native β -NGF to trigger the proliferation of TF1 cells and improve the survival of PC12 cells. All the results suggest that Zn(II) in the native β -NGF plays an important role in both the structure and the biological activity of this protein.

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