Biochemical and Biophysical Research Communications 416 (2011) 106–110

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Preparation and characterization of cobalt-substituted anthrax lethal factor

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ARTICLE INFO

Article history:
Received 15 October 2011
Available online 10 November 2011

Keywords:
Anthrax lethal factor
Electronic spectroscopy
Metal substitution
Thioglycolic acid
Zinc proteases

ABSTRACT

Anthrax lethal factor (LF) is a zinc-dependent endopeptidase involved in the cleavage of mitogen-activated protein kinase kinases near their N-termini. The current report concerns the preparation of cobalt-substituted LF (CoLF) and its characterization by electronic spectroscopy. Two strategies to produce CoLF were explored, including (i) a bio-assimilation approach involving the cultivation of LF-expressing *Bacillus megaterium* cells in the presence of $CoCl_2$, and (ii) direct exchange by treatment of zinc-LF with $CoCl_2$. Independent of the method employed, the protein was found to contain one Co^{2+} per LF molecule, and was shown to be twice as active as its native zinc counterpart. The electronic spectrum of CoLF suggests the Co^{2+} ion to be five-coordinate, an observation similar to that reported for other Co^{2+}-substituted gluzincins, but distinct from that documented for the crystal structure of native LF. Furthermore, spectroscopic studies following the exposure of CoLF to thioglycolic acid (TGA) revealed a sequential mechanism of metal removal from LF, which likely involves the formation of an enzyme:Co^{2+}:TGA ternary complex prior to demetallation of the active site. CoLF reported herein constitutes the first spectroscopic probe of LF's active site, which may be utilized in future studies to gain further insight into the enzyme's mechanism and inhibitor interactions.

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1. Introduction

Anthrax lethal factor (LF) is a 90 kDa Zn^{2+}-dependent endopeptidase responsible for the excision of an N-terminal peptide segment from most members of the mitogen-activated protein kinase kinase family of signaling proteins [1–3]. The Zn^{2+} ion in the active site of LF is coordinated to the side chains of His686, His690 and Glu735, and to a water molecule, which serves as the nucleophile in the cleavage of the substrate [4]. In addition, Glu687, which is part of the thermolysin-like HExxH consensus motif, has been shown to be essential for enzyme function [5], presumably by serving as a general base in the catalytic mechanism [4]. In view of the participation of the aforementioned amino acid residues in forming the active site, LF can be classified as a gluzincin [6].

In the case of a considerable number of Zn^{2+}-dependent enzymes, insights into the nature and geometry of metal coordination at the active site, and the type of intermediates involved in the catalytic mechanism have been gained by replacement of the spectroscopically silent Zn^{2+} with Co^{2+}, which is amenable to spectroscopic investigations [7,8]. Although a recent report has demonstrated the ability of Co^{2+} to reactivate the apoform of LF to yield an enzyme with slightly higher (by 35%) activity than that noted for the parent enzyme (ZnLF) [9], a Co^{2+}-substituted analog of LF (CoLF) has yet to be isolated and studied in detail. The observation that many Co^{2+}-substituted gluzincins display significantly higher catalytic activities than their native zinc counterparts prompted an investigation into alternative methods of metal replacement.

The current report concerns the preparation and characterization of CoLF. The enzyme was prepared in high yields by (i) cultivating LF-expressing cells in the presence of $CoCl_2$ (bio-assimilation approach), and (ii) treatment of ZnLF with excess Co^{2+} (direct exchange approach). Regardless of the method of preparation, CoLF was found to be twice as active as its native Zn^{2+}-containing counterpart, a finding similar to that documented previously for other gluzincins including thermolysin [10]. The spectroscopic features of CoLF (in the absence and presence of a metal-chelating thiol inhibitor) are discussed in the context of those encountered with other Zn^{2+}-dependent proteases.

2. Materials and methods

2.1. General

Chromogenic anthrax lethal factor protease substrate II, *S*-pNA (Ac-GYβARRRRRRRRVLR-pNA, pNA = para-nitroanilide) was obtained from EMD Biosciences (La Jolla, CA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO). All solutions were prepared using MilliQ ultrapure water ($\geqslant$18.2 MΩ cm resistivity). Hepes buffer (50 mM, pH 7.4) was depleted of trace metals with the aid of Chelex-100 resin.

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doi:10.1016/j.bbrc.2011.11.005

2.2. Isolation of LF

Wild-type LF was isolated from *Bacillus megaterium* containing the plasmid pWH1520-LF (MoBiTec, Göttingen, Germany) as documented in the literature [11], with some minor modifications. Cells were grown in Terrific Broth (12 g/L tryptone, 24 g/L yeast extract, 0.4% (v/v) glycerol, 2.31 g/L KH_2PO_4, 12.54 g/L K_2HPO_4) supplemented with tetracycline (10 μg/mL) at 37 °C with moderate agitation (900 rpm) using a BioFlo 110 Fermenter (New Brunswick Scientific, Edison, NJ). Upon growth of the culture to an absorbance value of 0.3 at 600 nm (typically after 3 h), LF production was initiated by introduction of 0.5% (w/v) D-xylose to the medium, and growth was allowed to proceed for an additional 6 h. After removal of the cells by centrifugation (12,000*g* for 30 min), the culture fluid was treated with an equal volume of PEG-8000 (40% (w/v)) to facilitate protein precipitation. The mixture was stirred for 16 h at 4 °C, and the LF-containing protein precipitate was recovered by centrifugation (12,000*g* for 2 h).

Following resolubilization in Tris/HCl buffer (20 mM, pH 8.0), LF was subjected to chromatography on Q-Sepharose equilibrated with the same medium using a discontinuous NaCl gradient. LF was recovered in the 350 mM NaCl fraction, and subsequently concentrated to approximately 1–2 mL using an Amicon Ultra-15 filtration device with a 30 kDa molecular weight cut-off (Millipore, Bedford, MA). The recovered protein preparation was finally subjected to size exclusion chromatography on a HiLoad (16/60) Superdex 200 column using an AKTA FPLC system (Amersham Biosciences, Uppsala, Sweden). Tris/HCl buffer (20 mM, pH 8.0) containing NaCl (200 mM) served both as equilibration and elution media. Fractions harboring LF were collected, pooled and concentrated by Amicon filtration prior to storage at −80 °C. The homogeneity of all LF preparations was assessed by SDS–PAGE according to the method of Laemmli [12]. The concentration of LF was determined spectrophotometrically at 280 nm, using an extinction coefficient of 74,200 M^{-1} cm^{-1} [9].

2.3. Preparation of CoLF

CoLF was prepared by two independent methods. The first procedure involved the isolation and purification of the protein in a fashion analogous to that described for ZnLF, except for the inclusion of $CoCl_2$ (at a final concentration of 1 mM) in the growth medium (bio-assimilation approach). In addition, CoLF was prepared by exposing ZnLF to excess Co^{2+} (direct exchange approach). In brief, ZnLF (3–4 mg), purified as outlined above, was loaded onto a Q-Sepharose column (5 mL bed volume) equilibrated with Hepes buffer. The Zn^{2+} ion in LF was subsequently allowed to exchange with Co^{2+} by passing 50 mL of 5 mM $CoCl_2$ in Hepes buffer through the column. Excess Co^{2+} and any released Zn^{2+} were removed by washing the column with 50 mL of Hepes buffer. Following elution of CoLF with 10 mL of Hepes buffer containing 350 mM NaCl, the protein was concentrated (to approximately 150 μM) by Amicon Ultra-15 filtration.

2.4. Enzymatic assays

The enzymatic activity of LF was assessed spectrophotometrically at 405 nm with the aid of the chromogenic *S*-pNA substrate according to published protocols [9,13]. Steady-state kinetic parameters (K_M, k_{cat}) for the hydrolysis of *S*-pNA were estimated by a non-linear least squares fit of the initial velocity data to the Michaelis–Menten equation using the Grafit 4.0 software package (Erithacus Software Ltd., Staines, UK) and a $\Delta\varepsilon$405 nm value of 9920 M^{-1} cm^{-1} [13].

2.5. Determination of metal content

The Zn^{2+} content of ZnLF was assessed using the chromophoric chelator 4-(2-pyridylazo)resorcinol (PAR) according to published procedures [9]. The metal content (Co^{2+} and Zn^{2+}) of CoLF was determined with PAR using the direct spectrophotometric method outlined in the literature [14].

2.6. Determination of dissociation constant

The dissociation constant of CoLF was determined using metal-buffered media (with dipicolinic acid (DPA) serving as the chelator) in a manner analogous to that employed previously in the estimation of the K_d value for ZnLF [9]. In brief, the K_d value of CoLF was determined by assessing the activity of the enzyme at various concentrations of free Co^{2+} in the medium using the following equation:

$$\text{Relative activity} = \frac{[Co^{2+}]_{free}}{[Co^{2+}]_{free} + K_d} \quad (1)$$

The (total) concentration of DPA ($[DPA]_{tot}$) required to achieve a particular concentration of free Co^{2+} was calculated by first solving Eq. (2) before substituting the obtained concentration of free DPA into Eq. (3),

$$\beta_2[DPA]^2 + \beta_1[DPA] + \left(1 - \frac{[Co^{2+}]_{tot}}{[Co^{2+}]_{free}}\right) = 0 \quad (2)$$

$$[DPA]_{tot} = [DPA] + [Co^{2+}]_{free}(\beta_1[DPA] + 2\beta_2[DPA]^2) \quad (3)$$

where $[Co^{2+}]_{tot}$ is the total concentration of Co^{2+} in the assay, [DPA] is the concentration of the free chelator, and β_1 and β_2 denote the conditional stability constants of the Co^{2+}(DPA) and $Co^{2+}(DPA)_2$ complexes (at pH 7.4: β_1 (Co) = 10^7; β_2 (Co) = 3.2×10^{12} [15,16]), respectively. The catalytic competence of CoLF (50 nM) was assessed in a typical enzyme assay (using 10 μM *S*-pNA) after exposure of the protein to Co^{2+} (50 μM) and DPA for 1 h at 25 °C. The K_d value of CoLF was estimated by fitting the enzyme activities to Eq. (1) by non-linear regression using GraFit 4.0.

2.7. UV–Vis spectroscopy

Electronic spectra of CoLF were recorded at 25 °C with an OLIS RSM-1000 spectrophotometer (Bogart, GA) using a 10-mm pathlength micro quartz cell (Hellma, Concord, ON) and 100 μL samples containing approximately 150 μM (13.5 mg LF/mL) protein in Hepes buffer (50 mM, pH 7.4). Since samples containing LF at such high concentrations were slightly turbid, all spectra were corrected for scattering using the reciprocal relationship between the intensity of scattered light and wavelength ($I \sim 1/\lambda^4$).

3. Results

3.1. Preparation of CoLF

Preliminary studies on the cytotoxicity of Co^{2+} on *B.* megaterium cultures revealed an absence of impairment of cellular growth up to a concentration of 1 mM $CoCl_2$ in the growth medium (data not shown). Thus, $CoCl_2$ at a final concentration of 1 mM was added as a supplementary component to the standard growth medium, and cell cultivation and purification of LF were performed according to the protocols employed for the isolation of ZnLF. Protein yields were identical to those obtained for the Zn^{2+} enzyme (ca. 5 mg of LF/L of cell culture). The metal content (mol of metal per mol of protein) of LF prepared in this manner was found to be 0.98 (±0.10) for

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