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NMR characterization of a 264-residue hyperthermostable endo-β-1,3-glucanase

Johannes H. Ippel [a,1,3], Sotirios Koutsopoulos [b,2,3], Sanne M. Nabuurs [a], Willem J.H. van Berkel [a], John van der Oost [b], Carlo P.M. van Mierlo [a,*]

[a] Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands
[b] Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands

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ABSTRACT

Insight into the hyperthermostable endo-β-1,3-glucanase *pf*LamA from *Pyrococcus furiosus* is obtained by using NMR spectroscopy. *pf*LamA functions optimally at 104 °C and recently the X-ray structure of pfLamA has been obtained at 20 °C, a temperature at which the enzyme is inactive. In this study, near-complete (>99%) NMR assignments are presented of chemical shifts of *pf*LamA in presence and absence of calcium at 62 °C, a temperature at which the enzyme is biologically active. The protein contains calcium and the effects of calcium on the protein are assessed. Calcium binding results in relatively small chemical shift changes in a region distant from the active site of *pf*LamA and thus causes only minor conformational modifications. Removal of calcium does not significantly alter the denaturation temperature of *pf*LamA, implying that calcium does not stabilize the enzyme against global unfolding. The data obtained form the basis for elucidation of the molecular origins involved in conformational stability and biological activity of hyperthermophilic endo-β-1,3-glucanases at extreme temperatures.

Introduction

Hyperthermophilic microorganisms flourish in extreme environments of conditions that were until recently considered as incompatible with life. *Pyrococcus furiosus* is an anaerobic hyperthermophile, which was discovered in geothermally heated marine sediments at temperatures exceeding 100 °C [1]. The organism contains a large collection of hyperthermostable enzymes that have potential for applications in biotechnology and industrial biocatalysis [2]. One of these enzymes is the extracellular *pf*LamA, of which several properties have been characterized [3]. *pf*LamA belongs to the class of laminarases (EC 3.2.1.39) of the glycoside hydrolase family GH-16 [4]. The enzyme hydrolyzes 1,3-β-D-glucosidic linkages in 1,3-beta-D-glucans, e.g. laminarins, curdlans, paramylons, and pachymans. *pf*LamA is optimally active at 104 °C, a condition where proteins from mesophilic organisms rapidly denature, whereas it is inactive at room temperature [5,6].

The crystal structure of *pf*LamA at 2.16 Å resolution has been recently elucidated (PDB entry 2VY0) [7]. In general, protein structures belonging to the GH-16 family have a beta jelly-roll fold with a highly concave side to which a glycan polymer substrate binds and contain at least one metal-binding site [8]. Indeed, X-ray structural analysis shows that *pf*LamA contains one calcium ion, which is located opposite to the active site of the enzyme (Fig. 1). The dissociation constant of the enzyme-calcium complex is 30 nM at 20 °C [9]. Catalytic residues E170, D172, and E175 of *pf*LamA are involved in hydrolysis of the 1,3-β-glycosidic bond and are highly conserved in the GH-16 family [8]. These three amino acids are located in a single β-strand and their side chains point towards the substrate-binding pocket. Mutation of either one of the aforementioned two glutamate residues abolishes enzymatic activity almost completely.

Currently, virtually all three-dimensional structures of hyperthermostable proteins have been obtained using crystallography, as is the case for *pf*LamA. X-ray data are extracted from protein crystals at temperatures far lower than the normal operating temperature of the proteins involved. Although supported by little experimental data, it is generally believed that protein crystals grown at ambient temperatures are imperfect proxies for the conformational properties of hyperthermostable proteins that are active at temperatures above 100 °C [10]. NMR spectroscopy is a suitable technique to solve the three-dimensional structure of a protein in solution at temperatures well above room temperature. Most solution structures of thermo- and hyperthermostable proteins have been derived from data obtained at temperatures

Abbreviations: *pf*LamA, endo-β-1,3-glucanase from *Pyrococcus furiosus*; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; HSQC, heteronuclear single quantum correlation; DSC, differential scanning calorimetry.

* Corresponding author. Fax: +31 317 484801.
E-mail address: carlo.vanmierlo@wur.nl (C.P.M. van Mierlo).
[1] Present address: NMR Spectroscopy Research Group, Bijvoet Center for Biomolecular Research, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.
[2] Present address: Massachusetts Institute of Technology, 77 Massachusetts Av., Center for Biomedical Engineering NE47–307, Cambridge, MA 02139, USA.
[3] These authors contributed equally to this work.

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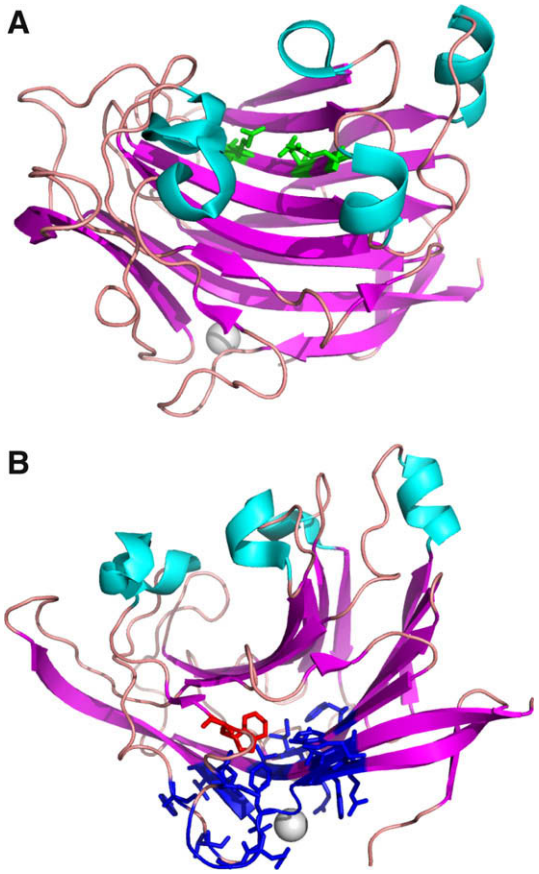

Fig. 1. Cartoon drawings of *pf*LamA, with calcium shown as gray sphere. Beta sheets and alpha-helices are colored magenta and cyan, respectively. (A) Top view of *pf*LamA with E170, D172, and E175, involved in substrate binding, colored green. (B) Side view of *pf*LamA with amino acids that are identified by NMR spectroscopy to be affected by Ca^{2+} binding colored blue; W64, located near the active site of *pf*LamA, is also affected by calcium binding (see Fig. 3) and is highlighted in red.

between room temperature and 40 °C. Only five solution structures have been deduced from NMR data obtained at 50–65 °C, all of small, less than 100 residues-containing proteins [11–15].

With this work we aim to elucidate the molecular origins involved in conformational stability and biologically activity of the 264-residue hyperthermophilic *pf*LamA from *P. furiosus* at extreme temperatures. Currently, for no (hyperthermostable) glucanase NMR assignments are available and thus no solution structures of these proteins are known. To understand the (sub)molecular properties of hyperthermostable endo-β-1,3-glucanases, we aim to solve the solution structure of *pf*LamA in its catalytically active state at elevated temperatures. The latter is considered important, as the X-ray structure of *pf*LamA has been obtained at 20 °C, a temperature at which the protein is biologically inactive. The data presented here of *pf*LamA at 62 °C reveal insight into the role of Ca^{2+} in *pf*LamA's conformational characteristics and form the basis for elucidation of its solution structure.

Materials and methods

Protein expression and purification. The sequence of the *pf*LamA gene has been deposited in the GenBank™ database (Accession No. AF013169). Residues 34–297 of *pf*LamA were expressed in transformed *E. coli* BL21 (DE3) cells using the T7 expression system, as described elsewhere [3]. Cells were grown at 37 °C in M9 minimal medium containing $^{15}NH_4Cl$ and ^{13}C-glucose. The M9 medium was supplemented with 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 10 μM $FeCl_3$, and contained thiamin, biotin, and kanamycin. After expression of *pf*LamA, the cells were centrifuged at 4 °C, resuspended in Tris buffer and lysed by sonication. Purification involved heat incubation of the cell extract at 85 °C for 45 min to denature the non-hyperthermostable proteins of *E. coli*; protein aggregates were removed by centrifugation. Further purification of the *pf*LamA-containing supernatant was performed using a phenyl-Sepharose and a Superdex 200 column (Amersham Pharmacia, Sweden).

NMR samples of 1.5 mM uniformly [^{13}C, ^{15}N]-labeled *pf*LamA were prepared in 10 mM sodium phosphate, pH 7.0, with 10% D_2O (Cambridge Isotope Laboratory, Cambridge, MA), 100 μM of 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS), and 0.01% (w/v) azide. Samples were stored at 4 °C; under these conditions *pf*LamA was stable for at least four years.

Mass spectrometry. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF, Voyager DE-RP, PerSeptive Biosystems, Framingham, MA, USA) and size exclusion chromatography showed that the 264-residue *pf*LamA in solution is a monomer.

Trypsinized *pf*LamA was characterized by electrospray ionization-quadrupole-time-of-flight (ESI-Q-TOF) mass spectrometry. Separation of the *pf*LamA peptide fragments was performed using a Zorbax 300 Extend-C18 column (Agilent Technologies, Palo Alto, CA) and an Agilent 1200 series chromatography system coupled to Agilent 6510 Q-TOF; spray voltage of 3.8 kV, gas temperature of 275 °C, nebulizer gas at 10 psi, and a drying gas of 4 l/min were used. Positive ion data-dependent acquisition range in the scan mode was 100–1799 *m/z* and for the MS/MS mode 100–2000 *m/z*. The charge priority of MS/MS acquisition was +2 (charges), +3, >+3, unknown, +1. Data were collected and analyzed using the Agilent MassHunter software (Agilent, Technologies, Palo Alto, CA). Peptide identification and assignment of possible amino acid chemical modifications was confirmed by inspection of the MS/MS spectra and by using the ProteinProspector software (http://prospector.ucsf.edu/).

NMR spectroscopy. Two sets of spectra were collected for *pf*LamA: one set was obtained using a sample in which half of the *pf*LamA molecules had their calcium-binding site filled with Ca^{2+}. The other set was obtained using a sample in which all *pf*LamA molecules had their calcium-binding site filled with Ca^{2+} (achieved by adding $CaCl_2$ to the sample to a final concentration of approximately 10 mM). Preliminary titration and back-titration NMR experiments were performed with increasing $CaCl_2$ and EDTA concentrations, respectively prior to determining the optimal conditions for the study.

NMR data were acquired on Bruker AMX500, DRX700, and DRX900 spectrometers, equipped with triple-resonance probes with *z*-gradients. ^1H–^{15}N HSQC, ^1H–^{13}C HSQC, 3D-^{15}N-edited NOESY, 3D-^{13}C-edited NOESY and CBCA(CO)NH, HNCACB, HNCO, HN(CA)CO, HBHA(CO)NH, HN(CO)CA, and HCCH-TOCSY triple-resonance experiments were collected to complete ^1H, ^{13}C, and ^{15}N resonance assignments of calcium-containing and no calcium-containing *pf*LamA molecules. To assign side-chain resonances of tryptophans, 2D-TOCSY and 2D-NOESY spectra were collected on unlabeled samples of *pf*LamA.

All NMR data were processed with Xwinnmr (Bruker, Rheinstetten, Germany) and analyzed with CARA v1.5.5/NEASY (Institute of Molecular Biology and Biophysics, ETH Zürich, Switzerland) and Sparky (TD Goddard and DG Kneller, University of California, San Francisco, USA).

Differential scanning calorimetry. Calorimetric studies were carried out in a VP-DSC calorimeter (MicroCal Inc., Northampton, MA). The effect of calcium ions on thermal stability of *pf*LamA was investigated through addition of 1 mM $CaCl_2$ or 1 mM EDTA to the protein solution. Heat exchanges of non-labeled *pf*LamA