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Studies on the parameters controlling the stability of the TET peptidase superstructure from *Pyrococcus horikoshii* revealed a crucial role of pH and catalytic metals in the oligomerization process

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ABSTRACT

The TET proteases from *Pyrococcus horikoshii* are metallopeptidases that form large dodecameric particles with high thermal stability. The influence of various physico-chemical parameters on PhTET3 quaternary structure was investigated. Analytical ultracentrifugation and biochemical analyses showed that the PhTET3 quaternary structure and enzymatic activity are maintained in high salt and that the complex is stable under extreme acidic conditions. Under basic pH conditions the complex disassembled into a low molecular weight species that was identified as folded dimer. Metal analyses showed that the purified enzyme only contains two equivalent of zinc per monomer, corresponding to the metal ions responsible for catalytic activity. When these metals were removed by EDTA treatment, the complex dissociated into the same dimeric species as those observed at high pH. Dodecameric TET particles were obtained from the metal free dimers when 2 mM of divalent ions were added to the protein samples. Most of the dimers remained assembled at high temperature. Thus, we have shown that dimers are the building units in the TET oligomerization pathway and that the active site metals are essential in this process.

1. Introduction

Proteolysis is involved in a number of essential cellular functions. First of all, the degradation of cytosolic proteins and peptides regenerates the pool of amino acids needed to produce new proteins. When peptides serve as an external carbon source, intracellular proteases are responsible for the hydrolysis of these peptides. Additionally, proteolysis is implicated in processes as diverse as the cell cycle and cell division, regulation of transcription factors and antigen presentation [1,2]. In all three domains of life cytosolic proteolysis is carried out by sophisticated protein degradation machines that have little in common with small extracellular proteases. To avoid unspecific degradation, intracellular proteases have either very high substrate specificity or are self-compartmentalized complexes, in which the active sites are shielded away from the cytoplasm inside cavities within the complex [3,4]. A new class of self-compartmentalized proteases was discovered in archaea by Franzetti et al. [5]. In contrast to other known bacterial and eukaryotic protease complexes, these proteins do not form barrel-shaped assemblies, but dodecameric, tetrahedral complexes [5]. This new class was therefore named TET [5]. TET proteases belong to family M42 according to the MEROPS classification [6]. X-ray crystallographic structures and biochemical characterizations have been published for three TET proteases from *Pyrococcus horikoshii* [7–10]. All three proteins form tetrahedral, dodecameric complexes with active sites located inside the particle and accessible through four openings located on the facets of the protein complex [7–10]. Each apex of the tetrahedrons contains a catalytic chamber in which three active sites are located [9]. Within the active sites of the TET proteases two divalent cations, either zinc or cobalt, are bound [7–10]. The TET proteases from *P. horikoshii* are strict aminopeptidases that are activated by cobalt ions [9–11]. They exhibit complementary substrate specificity and are therefore not redundant in the cytosol [9].

When recombinant PhTET3 is purified from *E. coli* lysate, it is found exclusively in its dodecameric form. The oligomerization pathway of TET is unknown and so far no oligomerization intermediates have been detected. Nevertheless it seems unlikely that such a complex assembly could be formed in a one-step process. Schoehn et al. [10] suggested that dimers should be the building block of the tetrahedron as it appears as if all six edges of the complex are formed by a dimer. Assembly and disassembly of the TET proteases could be a means of controlling the enzyme's activity *in vivo*. Therefore understanding the TET oligomerization process is important.

P. horikoshii is a hyperthermophilic archaeon isolated from deep-sea thermal vents. Therefore TET particles from this organism have to

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resist elevated temperatures. Oligomerization is known to increase the thermostability of a number of proteins and has been proposed as a means of temperature adaptation in thermophiles [12,13]. Hence the determination of the physico-chemical limits under which TET particles remain assembled will help to understand the molecular adaptation of TET to an extremophilic environment and to specify the range of conditions in which TET could be used for biotechnological applications. Parameters that could presumably influence the oligomeric state of PhTET3 include temperature, salt concentration and pH, as those attain extreme values in hydrothermal vents, the habitat of *P. horikoshii* [14,15] and it has been shown that *P. horikoshii* can grow in a wide range of these parameters [16]. Furthermore, each TET dodecamer contains 24 cations in its active sites. The very high metal content in the enzyme complex prompted us to investigate whether it could also contribute to the folding of the protein.

We have used analytical ultracentrifugation (AUC) combined with enzymatic assays and metal content analysis to study the influence of the above-mentioned parameters on PhTET3 oligomerization *in vitro*. This approach permitted to determine the mass of the different oligomeric states present in the solution and gave accurate information on the hydrodynamic shape of the different protein subspecies, which is a reliable indicator whether a protein is unfolded or not [17].

2. Materials and methods

2.1. Production of recombinant PhTET3

The protein was purified as described previously [9].

2.2. Analytical ultracentrifugation

All analytical ultracentrifugation (AUC) experiments were carried out at 20 °C using a protein concentration of 0.3 mg/mL. 20 mM Tris at pH 7.5 was used as a standard buffer to which other solutes were added, except when testing the influence of pH, where acetate (pH 4.0 and 4.5), MES (pH 5.5 and 6.5), PIPES (pH 7.5), HEPES (pH 7.5), TAPS (pH 8.5), CHES (pH 9.5) and CAPS (pH 10.5 and 11.0) were used. Sedimentation velocity experiments were performed at 42000 rpm on a XL-I analytical centrifuge (Beckman). Experiments were carried out either with an An-Ti50 8-hole rotor (Beckman) or with an An-Ti60 4-hole rotor (Beckman). Two-channel centerpieces were used with an optical path of 12 mm. All experiments were done with sapphire windows. Scans were recorded at 280 nm with radial spacing of 0.005 cm. The program Sednterp [18] was used to estimate the partial specific volume $\bar{v}$ from amino acid composition as well as the density ρ and viscosity η of the buffer. Data was then analyzed with the program Sedfit [19] using a continuous c(s) distribution model. Theoretical sedimentation coefficients $s_{20,w}$ were calculated according to the following equation:

$$s_{20,w} = \frac{M\left(1-\rho_{20,w}\,\bar{v}\right)}{N_A(f/f_0)6\pi\eta_{20,w}\left(\frac{3}{\pi}\frac{M\bar{v}}{N_A}\right)^{1/3}}$$

with $\rho_{20,w}$ and $\eta_{20,w}$ being the density and viscosity of water at 20 °C, N_A Avogadro's number and M and f/f_0 the protein's mass and frictional ratio, respectively. The values obtained for different hypothetical oligomers of PhTET3 are resumed in Table 1.

2.3. Enzymatic studies

PhTET3 activity was measured at pH 7.4 and 85 °C with Lys-pNA[1] and using the assay procedure previously described [9] using the same buffer composition except for KCl concentration which ranged from 0 to 2 M.

Table 1
Sedimentation coefficients $s_{20,w}$ and hydrodynamic radius $s_{20,w}$ of hypothetical oligomers (with number of subunits SU) of PhTET3 calculated for two different frictional ratios, both typical for folded proteins, with $\bar{v}$ = 0.742 mL/g.

	$f/f_0 = 1.20$		$f/f_0 = 1.33$	
SU	$s_{20,w}$ (S)	R_H (Å)	$s_{20,w}$ (S)	R_H (Å)
1	3.3	27.1	3.0	30.0
2	5.2	34.1	4.7	37.8
3	6.8	39.0	6.2	43.3
4	8.3	43.0	7.5	47.6
6	10.9	49.2	9.8	54.5
8	13.1	54.1	11.9	60.0
10	15.3	58.3	13.8	64.6
12	17.2	62.0	15.5	68.7

2.4. Metal content measurement

Two enzyme samples were prepared, containing about 1.5 mg of protein in a total mass of 9.8 g of buffer. Metal analyses were performed using ICP-AES[2] (Perkin Elmer, Optima 3300 DV) at the Equipe Géochimie de l'environnement, LGIT/CNRS in Grenoble, France. Buffer was used as a blank and duplicate measurements were done on every sample. Seven standard solutions at different concentrations were used for calibration, each containing a mixture of cobalt, zinc, calcium, manganese, iron and magnesium.

2.5. Crystallographic structure representation

The dodecameric assembly of PhTET3 was generated by applying crystallographic symmetry operations on the crystallographic structure of PhTET3 that is available in the Protein Data Bank with accession code 2VPU. All figures were prepared using the program PyMol [20].

3. Results

3.1. PhTET3 oligomerization is salt-independent

Intracellular salt concentration in Pyrococcus species has been found to be 350 mM [21]. Our analysis of the 3D-structure of PhTET3 revealed that only salt bridges and hydrogen bonds are present on the intermolecular interfaces within the dodecameric complex [9]. These electrostatic interactions and therefore the oligomeric state of the protein could depend on the salt concentration in the solvent. In order to elucidate the effect of salt on the oligomeric state of PhTET3, AUC experiments on samples containing 0, 0.5 and 1 M KCl were carried out. In all three samples more than 90% of the protein sediments with a sedimentation coefficient of about 16(Fig. 1). The peak has been assigned as dodecameric PhTET3, in agreement with the theoretical sedimentation coefficient for PhTET3 (see Table 1). This shows that the TET oligomeric structure remains assembled in high as well as in low salt conditions.

Whereas the quaternary structure of an enzyme is often linked to its activity, it does not necessarily mean that an enzyme is active. We have therefore tested PhTET3 activity in different salt concentration (see Fig. 2). The enzyme is optimally active at salt concentration between 0.075 and 0.25 M KCl. Remarkably, more than 70% of the activity is maintained at 2 M KCl, showing that the hyperthermophilic enzyme is stable and functional under hyper saline conditions.

[1] H-Lysine-p-nitroanilide (Bachem).

[2] Inductively coupled plasma atomic emission spectrophotometry.