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Effects of hydrostatic pressure on the quaternary structure and enzymatic activity of a large peptidase complex from *Pyrococcus horikoshii*

Eva Rosenbaum [a,b,c], Frank Gabel [a,b,c], M. Asunción Durá [a,b,c], Stéphanie Finet [d], Cécile Cléry-Barraud [e], Patrick Masson [a,b,c,e], Bruno Franzetti [a,b,c,*]

[a] Group Extremophiles and Large Molecular Assemblies (ELMA), CEA, Institut de Biologie Structurale, 41 rue Jules Horowitz, 38027 Grenoble, France
[b] CNRS, UMR5075, 38027 Grenoble, France
[c] Université Joseph Fourier, 38000 Grenoble, France
[d] Institut de Minéralogie et de Physique des Milieux Condensés (IMPMC), CNRS-UPMC, UMR7590, 4 place Jussieu, 75252 Paris Cedex, France
[e] Département de Toxicologie, Institut de Recherche Biomédicale des Armées, Antenne La Tronche-CRSSA, BP 87, 38702 La Tronche Cedex, France

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ABSTRACT

While molecular adaptation to high temperature has been extensively studied, the effect of hydrostatic pressure on protein structure and enzymatic activity is still poorly understood. We have studied the influence of pressure on both the quaternary structure and enzymatic activity of the dodecameric TET3 peptidase from *Pyrococcus horikoshii*. Small angle X-ray scattering (SAXS) revealed a high robustness of the oligomer under high pressure of up to 300 MPa at 25 °C as well as at 90 °C. The enzymatic activity of TET3 was enhanced by pressure up to 180 MPa. From the pressure behavior of the different rate-constants we have determined the volume changes associated with substrate binding and catalysis. Based on these results we propose that a change in the rate-limiting step occurs around 180 MPa.

Introduction

A large portion of the earth's biosphere is a high pressure environment [1]. Around 79% of the marine biosphere lies at depths below 1000 m, i.e. at pressures of 10 MPa or higher. Recent oceanographic campaigns revealed the existence of a large number of species in the deep-sea [2,3]. In organisms that are adapted to live at atmospheric pressure, exposure to high pressure will lead to growth arrest and eventually to cell death [4]. On the other hand piezophilic organisms require elevated pressure for optimal growth [1,5,6].

According to Le Chatelier's principle hydrostatic pressure shifts chemical equilibria and kinetic processes towards the state with smaller volume. This affects all biochemical or physiological processes in an organism when it is exposed to high pressure. The effect of high pressure resembles that of high temperature as it destabilizes the quaternary and tertiary structure of proteins and it resembles that of low temperatures since it has a similar effect on protein synthesis and membrane structure [7]. However, compared to temperature effects, much less energy is involved in pressure effects, which act exclusively on weak interactions. Regarding the quaternary structure of protein complexes pressure can lead to dissociation or partial unfolding and aggregation [8–11]. Whether dissociation or aggregation occurs depends on the volume change associated with the breaking of the intersubunit interactions within the assembly and on the volume difference between the native and the unfolded/dissociated states. In most cases, changes in the quaternary structure will have an effect on enzyme activity. However, pressure will directly affect enzymatic reactions through volume effects as well. Depending on the sign of the activation volume $\Delta V^{\neq}$, i.e. the volume difference between the transition state and the initial state, the reaction will be slowed down or enhanced by pressure [12]. A hint that the growth arrest of microorganisms might be related to pressure-induced loss of enzyme function is the fact that its kinetics resemble the inactivation kinetics of proteins [13,14].

The archaea *Pyrococcus horikoshii* was isolated from the Okinawa Trough in the Pacific Ocean at a depth of 1395 m [15]. At pressure conditions between 0.1 and 15 MPa growth is optimal at 95 °C, whereas at higher pressure (30 MPa) the optimal growth temperature is 100 °C [16]. We recently solved the structure of the TET3 peptidase from *P. horikoshii* (PhTET3) [17]. TET peptidases are a new class of large selfcompartmentalized proteases found in archaea. In contrast to other known cytosolic bacterial or eukaryotic proteases, these proteins do not form barrel-shaped

* Corresponding author at: Group Extremophiles and Large Molecular Assemblies (ELMA), CEA, Institut de Biologie Structurale, 41 rue Jules Horowitz, 38027 Grenoble, France. Fax: +33 (0)4 38 78 54 94.
E-mail address: franzetti@ibs.fr (B. Franzetti).

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assemblies, but dodecameric tetrahedral complexes of about 470 kDa [18]. The six edges of the tetrahedron are each formed by a dimer, which we have identified as the building-block of the dodecamer [19]. On the faces of the tetrahedron a 9 Å opening is located in the center of the triangle formed by three dimers. On the vertices small pores of 4 Å diameter exist. We proposed that peptides access the active site in the interior of the protein through the openings on the faces and that the reaction products, free amino acids, are expelled through the pores [17].

In the MEROPS classification system TET proteins belongs to peptidase family M42, which is part of the clan MH [20]. All TET proteases for which high resolution structures are available have the same active site architecture as aminopeptidase AAP, the type example of clan MH [17,21–23]. In the proposed catalytic mechanism for AAP, a glutamate residue first acts as a general base, removing a proton from the water molecule that bridges the cocatalytic zinc ions. The attack of the resulting hydroxide on the carbonyl of the scissile peptide bond leads to the tetrahedral intermediate. The protonated glutamate residue is then thought to provide a proton to the amino nitrogen, leading to the breakage of the C–N bond [24]. In a last step the products are released and a water molecule binds to the zinc ions to return the enzyme to its original state. The breaking of the C–N bond is the rate-limiting step of the reaction, independently of the temperature at which the reaction takes place [25]. The reaction can be summarized by the following reaction scheme:

$$E + S \overset{K_m}{\rightleftharpoons} ES \overset{k_2}{\rightarrow} EA \overset{k_3}{\rightarrow} E + P.$$

The behavior of enzymes under pressure has garnered more and more interest in recent years because of the role of pressure as a fundamental thermodynamic variable, the discovery of the large biodiversity in high-pressure environments such as the deep sea and the increasing implementation of high pressure in biotechnology. Nevertheless, few studies exist that combine the investigation of the influence of high pressure on enzyme activity and on structural integrity [11], especially compared to the extensive research that has been done on the effect of temperature. Several studies have been carried out on the pressure-resistance of proteins from hyperthermophiles, mostly on the increase in half-life at high temperature with increasing pressure [26–28]. However, these studies have focused on pressures of up to 75 MPa and report effects on residual activity, which does not allow conclusions on whether oligomer dissociation, unfolding or more subtle structural changes are at the origin of the observed effects.

In order to advance our understanding of the effect of hydrostatic pressure on the quaternary structure and the catalytic activity of high molecular weight complexes *in situ*, we have used the PhTET3 peptidase as a model system. In this study we combine small angle X-ray scattering (SAXS)[1] and enzymatic activity measurements to investigate the limits of the stability of the quaternary structure and of the enzymatic activity of PhTET3 under high pressure.

Theory

From the pressure dependence of the activity of an enzyme and from its catalytic parameters, information on the volume changes associated with different steps in the catalytic mechanism can be obtained. The partial derivative of a rate constant k with respect to pressure can be expressed in terms of the activation volume $\Delta V^{\neq}$ [12,29,30].

$$\left(\frac{\partial(\ln k)}{\partial P}\right)_T = \frac{-\Delta V^{\neq}}{RT}, \tag{1}$$

where R and T are the gas constant and temperature in Kelvin, respectively. $\Delta V^{\neq}$ represents the volume difference between the transition state and the initial state of the system in the reaction with rate constant k. Similarly, a volume change ΔV_{bind} associated with a binding constant K_{bind}, can be defined as:

$$\left(\frac{\partial(\ln K_{bind})}{\partial P}\right)_T = \frac{-\Delta V_{bind}}{RT}. \tag{2}$$

In the most straight forward case, when the volume change is pressure independent, the rate constant will have a linear pressure dependence. If the volume change is pressure dependent, the rate-constant will show a non-linear behavior with pressure. This can be due to a change in the isothermal compressibility β of a protein, which is defined as the change of its molar volume V_i with pressure [13]

$$\beta = \frac{-1}{V_i}\left(\frac{\partial V_i}{\partial P}\right)_T.$$

If the non-linearity of the pressure behavior of the rate constant is due to compressibility changes, then its pressure dependence can be described by the following quadratic equation [30]:

$$\ln(k) = ln(k_0) - \frac{\Delta V^{\neq}}{RT}P + \frac{\Delta \beta^{\neq}}{2RT}P^2. \tag{3}$$

Methods

Production of recombinant PhTET3

The protein was produced and purified as described earlier [17].

Small angle X-ray scattering

All small angle X-ray scattering (SAXS) experiments were carried out at the high brilliance beamline ID02 at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. For the high pressure measurements the cell described in [31] was used allowing access to a pressure range from atmospheric to 300 MPa. The sample to detector distance was set to 2 m. The X-ray energy used was 16.5 keV which corresponds to a wavelength of 0.751 Å. All samples were measured at a concentration of 1 mg mL^{-1} in 50mM HEPES-NaOH buffer at pH 7, except for the sample which was progressively heated, for which HEPES buffer adjusted with NaOH to pH 9 at 90 °C was used. Exposure times were 5 times 0.3 s for the sample and 10 times 0.1 s for the buffer. A test run showed that PhTET3 can be exposed to the beam for at least 130 s in intervals of 1 s or less without suffering measurable radiation damage. During data reduction the dark images were subtracted from the data, which were then corrected for spatial distortion and divided by a flatfield image. Data reduction was automatically done during the data acquisition at ID02. The program Fit2d [32] was used for masking of the beamstop, the detector edges and any parasitic scattering from the diamond windows of the pressure cell. Averaging, subtraction of the mask and azimuthal integration were done using the 'SAXS program package' by Boesecke [33]. In a last step scaled buffer intensities were subtracted from the sample intensities using the program PRIMUS [34]. Parasitic scattering from the diamond windows of the pressure cell led to bad data quality at very small angles in some data sets. Therefore, pair distribution functions and the corresponding radii of gyration (R_g) were calculated using the program GNOM [35]. For all points within a data series calculations were done over the same q-range. The program CRYSOL [36] was used to

[1] *Abbreviations used:* SAXS, small angle X-ray scattering; ESRF, European Synchrotron Radiation Facility; BuChE, mutant butyrylcholinesterase; H-lysine-pNA, [1]H-lysine-para-nitroanilide.