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Influence of pressure on structure and dynamics of bovine pancreatic trypsin inhibitor (BPTI): Small angle and quasi-elastic neutron scattering studies

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Abstract

We have studied the influence of pressure on structure and dynamics of a small protein belonging to the enzymatic catalysis: the bovine pancreatic trypsin inhibitor (BPTI). Using a copper-beryllium high-pressure cell, we have performed small angle neutron scattering (SANS) experiment on NEAT spectrometer at HMI (Berlin, Germany). In the SANS configuration, the evolution of the radius of gyration and of the shape of the protein under pressures up to 6000 bar has been studied. When increasing pressure from atmospheric pressure up to 6000 bar, the pressure effects on the global structure of BPTI result on a reduction of the radius of gyration from 13.4 Å down to 12.0 Å. Between 5000 and 6000 bar, some transition already detected by FTIR [N. Takeda, K. Nakano, M. Kato, Y. Taniguchi, *Biospectroscopy*, 4, 1998, pp. 209–216] is observed. The pressure effect is not reversible because the initial value of the radius of gyration is not recovered after pressure release. By extending the range of wave-vectors to high q, we have observed a change of the form factor (shape) of the BPTI under pressure. At atmospheric pressure BPTI exhibits an ellipsoidal form factor that is characteristic of the native state. When the pressure is increased from atmospheric pressure up to 6000 bar, the protein keeps its ellipsoidal shape. The parameters of the ellipsoid vary and the transition detected between 5000 and 6000 bar in the form factor of BPTI is in agreement with the FTIR results. After pressure release, the form factor of BPTI is characteristic of an ellipsoid of revolution with a semi-axis a, slightly elongated with respect to that of the native one, indicating that the pressure-induced structural changes on the protein are not reversible. The global motions and the internal dynamics of BPTI protein have been investigated in the same pressure range by quasi-elastic neutron scattering experiments on IN5 time-of-flight spectrometer at ILL (Grenoble, France). The diffusion coefficients D and the internal relaxation times $<\tau_2>$ of BPTI deduced from the analysis of the intermediate scattering functions show a slowing down of protein dynamics when increasing pressure.

Keywords: BPTI; Protein Folding; Denatured States; Small Angle Neutron Scattering; Quasi-elastic Neutron Scattering; High Pressure

1. Introduction

Most of recent studies about pressure effects on biological systems have been mentioned in general publications by Silva and Weber [1] in 1993, by Prehoda et al. [2] in 1997, by Heremans and Smeller [3] in 1998, by Mentré and Hui Bon Hoa [4] in 2000, and by Heremans [5] in 2004 for the more recent publication. A collection of reviews edited by Balny, Masson and Heremans [6] in *Biochemica et Biophysica Acta* in 2002 brings together a large panel of studies realised with several techniques and on several proteins.

The study of the influence of temperature and pressure on proteins has a practical interest because these two parameters are employed for sterilization process and bioconservation in food and pharmaceutical industries. From a biological point of view, the influence of pressure on proteins is interesting because in physiological media these macromolecules can be submitted at pressures from a few millibar in blood circulation up to nearly 1000 bar in Deep Ocean. Organisms called barophiles can support pressure values up to 5000 bar like *Bacillus licheniformis* [7].

Concerning the protein folding investigation, pressure can induce unfolding pathways different from thermal unfolding. Instead of temperature that induces changes in the reaction volume and in the thermal energy, pressure affects only the reaction volume. This makes the thermodynamic of unfolding

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more easy to be followed. Moreover, pressure unfolding is accompanied by a reorganisation of hydration water at the surface of proteins.

Structural studies have been done by small angle neutron scattering (SANS) technique that gives information about the global structure of molecules as proteins. In the Guinier regime, one gets the radius of gyration and in the Porod regime, the specific surface of molecule. In order to get the influence of pressure on radius of gyration and on intermolecular interactions of horse heart metmyoglobin, SANS experiments were performed at pressures up to 3000 bar by Loupiac et al. [8]. The evolution of the specific molar volume of metmyoglobin as a function of applied pressure has been determined by this technique. It has been shown that the specific molar volume of this protein decreases by a factor of 5.4% in this range of pressure. Moreover, the authors have shown that the radius of gyration of metmyoglobin remains constant and that, between atmospheric pressure and 3000 bar, the intermolecular interactions are always repulsive [8]. In order to study the influence of pressures up to 3000 bar on the structure of staphylococque nuclease, Paliwal et al. [9] have used small angle neutron scattering in complement of molecular dynamics simulation. They have observed the folding–unfolding transition of the protein. When the pressure is applied, a two-step increase of the radius of gyration and a modification of the shape of the protein are obtained. At the highest pressure, the protein is less compact and more elongated than in the native state.

In protein, several motions occur on a wide time scale, from femtosecond for electronic transitions, to picosecond and nanosecond for vibrational and diffusive motions, and millisecond for conformational changes, and second to minute for kinetics. Incoherent quasi-elastic neutron scattering allows us to explore individual motions from picosecond to nanosecond time scale [10]. Using this technique, the markers of protein motion are the hydrogen atoms (due to the anomalously large incoherent neutron scattering cross-section of the ^{1}H nucleus) that are uniformly distributed throughout the protein. The quantity measured is the incoherent dynamic structure factor $S_{inc}(q, \omega)$ which is the space-time Fourier transform of the self-correlation of the position of an atom at time 0 with the position of the same atom at time t.

The influence of pressure between atmospheric pressure and 900 bar on the internal dynamics of trypsin in solution [11] has been recently studied. The authors have shown a diminution of the volume inside which the internal motions of non-labile protons occur and a narrowing of dynamic structure factor $S_{inc}(q, \omega)$ showing a slowing down of internal dynamics of trypsin. In 2003, Doster and Gebhardt [12] have observed the influence of pressure up to 7000 bar on internal dynamics of myoglobin by quasi-elastic neutron scattering. The authors have observed a slowing down of the dynamics with a transition at 3000 bar. Beyond this pressure, myoglobin does not keep its native structure [8].

Our interest focused on BPTI protein that is a small protein belonging to the enzymatic catalysis. Its function is to inhibit protease-like trypsin by inserting its lysine 15 inside the catalytic site. It is a small protein composed by 58 amino acid residues and with a molecular weight of about 6500 Da. Its crystallographic structure is well known at 1.7 Å resolution [13]. A particularity of this protein is the presence of three disulphide bridges and three salt bridges that induce a very high stability throughout temperatures up to 95 °C and pressures up to 14 kbar. This model system was studied at high temperature by differential scanning calorimetry [14] and Raman spectroscopy [15]. BPTI begins to be unfolded above 95 °C. The influence of pressure on BPTI in solution has been studied by FTIR [16,17] showing that some secondary structures still exist at 14 kbar.

In this paper, we present results from small angle neutron scattering performed on NEAT spectrometer at HMI (Berlin) and from quasi-elastic neutron scattering on IN5 spectrometer at ILL (Grenoble), on a 85 mg/ml concentrated solution of BPTI, as a function of applied pressure between 1 bar and 6000 bar and after pressure release.

2. Material and methods

2.1. Sample preparation

BPTI was purchased from SIGMA-ALDRICH (A-4529) as a lyophilised powder and used without any further purification. Deuterated acetic acid and D_2O were purchased from EURISOTOP. BPTI lyophilised powder is dissolved overnight at room temperature in a deuterated acetic acid buffer at 50 mM concentration in D_2O with 100 mM ammonium sulphate salts. The solution of BPTI is filtered through a 0.22 μm pore diameter filter, then dialysed against the same buffer during 24 or 48 h in order to complete H/D exchange of the labile protons of the protein. The solution of BPTI is concentrated with 3 kDa MWCO CENTRICON in order to have the highest concentration of protein as possible. We have obtained a solution of BPTI of 85 mg/ml.

2.2. Neutron-scattering experiment

A detailed description of the neutron scattering theory can be found in the reference [18]. Small angle neutron-scattering principles can be found in references [19] and [20]. In the following, we recall only the expression of the form factors used in our analysis.

In the case of a monodisperse system containing particles with a spherical symmetry, the scattered intensity $I(q)$ is isotropic and given by the following expression:

$$I(q) = \frac{cM_W}{N_A} K^2 P(q) S(q) \quad (1)$$

where c is the protein concentration, M_w is the molecular weight, N_A is the Avogadro constant, K is the contrast between the protein and the solvent, $P(q)$ is the form factor of protein describing the correlation between the positions of atoms inside a protein, and $S(q)$ is the correlation function of protein positions in the solution or structure factor of the protein. The contrast term can be expressed as follows:

$$K = (\langle\rho\rangle_p - \langle\rho\rangle_s) \quad (2)$$

where $\langle\rho\rangle_p$ and $\langle\rho\rangle_s$ are, respectively, the scattering length densities of the protein and of the solvent.

In the Guinier regime ($qR_g < 1$) the form factor is given by [19,20]:

$$P(q) = e^{-\frac{(qR_g)^2}{3}} \quad (3)$$

so, a Guinier representation of intensity, i.e., a plot of $\mathrm{Ln}(I(q))$ as a function of q^2, gives a linear behaviour with a slope proportional to the radius of gyration R_g of the macromolecule.