Chemical Physics 468 (2016) 53–62

Contents lists available at ScienceDirect

Chemical Physics

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

Structural investigation of ribonuclease A conformational preferences using high pressure protein crystallography

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ARTICLE INFO

Article history:
Received 31 July 2015
In final form 28 January 2016
Available online 12 February 2016

Keywords:
Ribonuclease A
High pressure macromolecular crystallography
Diamond anvil cell

ABSTRACT

Hydrostatic pressure in range 0.1–1.5 GPa is used to modify biological system behaviour mostly in biophysical studies of proteins in solution. Due to specific influence on the system equilibrium high pressure can act as a filter that enables to identify and investigate higher energy protein conformers. The idea of the presented experiments is to examine the behaviour of RNase A molecule under high pressure before and after introduction of destabilizing mutation. For the first time crystal structures of wild-type bovine pancreatic ribonuclease A and its markedly less stable variant modified at position Ile106 were determined at different pressures. X-ray diffraction experiments at high pressure showed that the secondary structure of RNase A is well preserved even beyond 0.67 GPa at room temperature. Detailed structural analysis of ribonuclease A conformation observed under high pressure revealed that pressure influences hydrogen bonds pattern, cavity size and packing of molecule.

1. Introduction

Hydrostatic pressure that induces conformational changes in protein, at moderate pressure causes compression of the molecule at highest values leads to denaturation and lost of the native structure. Therefore, pressure is used as a tool to study kinetics, dynamics and lately as a perturbant that can reveal conformational changes in protein structure [1]. In most cases those kinds of studies are performed for protein in solution. Since 1986 when Kundrot and Richards proved that lysozyme crystals diffract after pressurization to up 100 MPa [2], high pressure technique was introduced into protein crystallography field. During high pressure X-ray diffraction experiments number of technical difficulties have to be overcome. Therefore, experiments performed so far were mostly done for the simple model proteins using synchrotron radiation and ultra-short wavelengths [3]. Nevertheless, the field of protein crystallography under high pressure is now a developing technique that can provide structural information of the same quality as results obtained at ambient pressure conditions [4]. Recent progress in macromolecular crystallography adapted to high pressure measurements has permitted refined analysis of the effect of pressure on biomacromolecules at submolecular level. Since the number of protein structures solved at high hydrostatic pressure is limited, all potential features of this new method are still uncovered. However, some scientific application of high pressure macromolecular crystallography (HPMX) could be drawn. Different aspects of pressure influence on proteins were studied including protein solvation [5,6], protein compressibility [2,7–10] and protein flexibility [11]. Furthermore, as referred in latest papers combining high pressure technique with standard crystallography led to improving of crystal quality due to ordering effect [4,12,13]. Results presented here indicate that high pressure macromolecular crystallography experiments can be conducted without using high brilliance X-ray source as well. All described high pressure measurements were performed at home using NoniusKappaCCD diffractometer with sealed Mo tube.

Over a period of about 50 years the structure and function of bovine pancreatic ribonuclease A (RNase A; EC 3.1.27.5) have been extensively studied [14]. RNase A is a small, 124 residue protein that is still widely used as a model system to develop new methodologies, as well as to investigate various protein features including mechanism of kinetic reactions, unfolding process and structure–stability relationships [15,16]. RNase A is an enzyme which

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http://dx.doi.org/10.1016/j.chemphys.2016.01.010
0301-0104/

catalyses RNA degradation by cleaving the 3'-end of pyrimidine nucleosides. The catalytic site is composed of His12, His119 and Lys41. Several other amino acid residues serve as substrate binding subsites (Thr45, Asp83 and Phe120 in B1 subsite; Gln11 in P1 subsite; and Asn71 and Glu111 in B2 subsite). Beside numerous studies of RNase A enzymatic kinetics, this stable enzyme was also investigated in order to understand mechanism of its folding/unfolding processes. Results of those studies led to the identification of residues comprising regions that play initiative role in RNase A folding, so-called chain folding initiation site. This site compraises of residues from Lys62 to Ala64, Cys72 to Ser75, Ile106 to Cys110, Val111 to His119, Val54 and Val57 [17]. As a protein that can be nowadays easily expressed, in bacterial system, purified and crystallized, RNase A provides one of the best-studied example used in crystallographic experiments [18–20] RNase A crystals exhibits different forms that diffract even to an atomic resolution [21]. Consequently, number of three dimensional structures determined with different space group symmetries are already known, including modified variants [22–25], oligomers [26], complexes with ligands [27,28] and its cellular inhibitor [29].

Two variants of bovine pancreatic ribonuclease A, the wild-type RNase A and protein mutated at position 106 (Ile → Ala), were studied. Conformational stability of I106A variant, with amino acid substitution at the cavity within the main hydrophobic core, is noticeable lower than that of the wild-type enzyme. Previous investigation of thermodynamical parameter $p_{1/2}$ (half-denaturation pressure) of unfolding process showed that the variant I106A ($p_{1/2}$ = 0.039 GPa) is dramatically less stable than wild-type enzyme ($p_{1/2}$ = 0.525 GPa) [30]. To the best of our knowledge this is the first study in which two variants of the same protein with dramatically different pressure stability determined in solution were examined at high pressure. In the present report, high pressure diffraction data sets were collected for wild-type and I106A variant of RNase A crystals at 0.67 GPa and 0.48 GPa, respectively.

2. Experimental

Proteins were obtained using the T7 expression system in *Escherichia coli* strain BL21 (DE3) from Novagen (Madison, WI). Oligonucleotides used for site-directed mutagenesis and molecular biology enzymes were from Roche (Switzerland). Other chemicals were purchased from Sigma (ST. Louis, MO). Mutant plasmid used to express I106A variant was constructed on pBXR vector by site-directed mutagenesis [31]. The recombinant proteins were purified essentially as previously described [31]. Protein purity and homogeneity was confirmed by SDS–PAGE and mass spectrometry.

Crystals were grown at 293 K using the hanging drop vapour diffusion method according to Kadonosono et al. [32]. Lyophilized protein was dissolved in MiliQ water to the final concentration of 24 mg/ml. A 4 µl drop containing an equal volume of the protein and the reservoir solution were equilibrated against 0.5 ml of reservoir solution containing: 1.5 M NaCl, 3.0 M ammonium sulphate and 0.16 M sodium acetate pH 6.0 (298 K, atmospheric pressure).

For data collection crystals were mounted in the diamond anvil cell (DAC) inside steel gasket with thickness of 0.3 mm and with a whole diameter 0.45 mm using procedure described in Kurpiewska and Lewiński [3]. The crystals were transferred into the chamber previously filled with crystallization mother liquor. Pressure measurements using ruby fluorescence method [33] were performed at ambient temperature, on a BETSA PRL spectrometer, with an accuracy of 0.05 GPa (for experiments up to 1 GPa).

X-ray diffraction data were collected with the KappaCCD detector on Nonius diffractometer (Bruker Nonius) at 298 K using Mo radiation (wavelength λ = 0.71069 Å) and generator running at 55 kV, 30 mA. The crystal to detector distance was 110–120 mm. Because of limited opening of the DAC (60°), data collection was carried using four ω scan in range ±30° at κ = 0°, 30°, 60°, 90°. Data were processed and scaled with *DENZO*/*HKL2000* and *SCALEPACK* [34].

The structures were solved by molecular-replacement method implemented in *CNS* [35] using the coordinates of wt RNase A PDBid: 1FS3 [36] as a starting model. The mutated amino acid (Ile106) was replaced manually by Ala residue in search model. Cross-rotation and translation functions were calculated using data from 15 and 4 Å resolution range without any σ-cut off.

Crystallographic refinement was carried out with cycles of energy minimisation, torsion-angle simulated annealing and temperature-factor optimisation using maximum-likelihood target functions implemented in *CNS*. Each round of refinement alternated with a round of model rebuilding using *COOT* [37]. 5–10% of the data were randomly excluded from the refinement and used as a test data set to monitor R_{free} [38]. As phases improved, ordered solvent molecules were added to the model using peak-searching algorithm in *CNS* program. Chloride anions and number of the water molecules were added manually. Finally, the structures were validated using *PROCHECK* [39].

Superposition of all structures and calculation of the root-mean-square deviation for atomic coordinates (r.m.s. deviation) were performed using *LSQKAB* [40] program from *CCP4* suite (Collaborative Computational Project, 1994). Difference distance matrices (DDM) were calculated using *ESCET* program [41]. Buried surface area was calculated with server *PISA* [42]. Molecular volume and accessible surface area were calculated with program *GRASP* using radius equal 1.4 Å [43]. Protein compressibility was calculated from molecular volume changes.

VOIDOO program was used to detect volume changes of the main cavity volume [44]. The calculations were carried out using a probe radius 0.8 Å. Probes of smaller or larger radius resulted in detecting cavities opened to the surface or no detection of cavities at all. The value 0.8 Å gave us most consistent results and was used for all compared structures. For each structure calculations were repeated ten times (with "randomly oriented" molecules) and results were averaged. The estimated standard deviation of cavity volume V_{cavity} was 0.7 Å^3. The hydrogen bond pattern was calculated using program *DSSP* [45]. Calculation of number of contacts was done using *CONTACT* program from *CCP4* suite (Collaborative Computational Project, 1994). Two residues were considered as in contact if distance between two atoms was found below 4 Å. The energy computation was done with *AMBER* force field implementation in *HyperChem* (Hypercube, Inc., 1115 NW 4th Street, Gainesville, Florida 32601, USA). Figures were prepared using *PyMOL* (The PyMOL Molecular Graphics System, Version 0.99 Schrödinger, LLC.).

3. Results

3.1. Visual inspection of the sample

The mother liquor, which was used as a pressure medium, contained high concentration of ammonium sulphate. During pressurization the constant growing of salt crystals inside the DAC was observed. Salt precipitation was used as an early indicator of successful process of pressurization (Fig. 1). Salt crystals had no influence on protein crystals behaviour inside the gasket and no displacement of RNase crystal inside the DAC was observed. In fact, salt precipitation can be assumed as an alternative way of protein crystal immobilization inside the DAC without applying any alternative method of holding crystals in place [46]. One important