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Low Resolution Structure of RAR1-GST-Tag Fusion Protein in Solution

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RAR1 is a protein required for resistance mediated by many R genes and function upstream of signaling pathways leading to H_2O_2 accumulation. The structure and conformation of RAR1-GST-Tag fusion protein from barley (*Hordeum vulgare*) in solution was studied by the small angle scattering of synchrotron radiation. It was found that the dimer of RAR1-GST-Tag protein is characterized in solution by radius of gyration $R_G = 6.19$ nm and maximal intramolecular vector $D_{max} = 23$ nm. On the basis of the small angle scattering of synchrotron radiation SAXS data two bead models obtained by *ab initio* modeling are proposed. Both models show elongated conformations. We also concluded that molecules of fusion protein form dimers in solution via interaction of GST domains.

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1. Introduction

Plants are continuously attacked by numerous microorganisms but unlike higher eukaryotes they do not possess specialized types of cells to fight them. They are able to recognize and respond to the pathogen infection via two innate immunity systems: the pathogen associated molecular pattern (PAMP)-triggered immunity system (PTI) and the effector triggered immunity system (ETI) [1]. PAMP triggers the immunity systems use of transmembrane receptors to recognize evolutionary conserved pathogen molecules such as protein flagellin from bacteria flagellum. In effector-triggered immunity the pathogen effectors, i.e. molecules that are injected by the bacteria through secretion system, are specifically identified by the group of intracellular resistance proteins (R proteins). ETI system usually induces reactive oxygen species release, production of antimicrobial compounds and programmed cell death. R proteins share structural similarities: nucleotide binding domain (NBD) and leucine rich repeat (LRR) suggesting existence of common signaling pathways during infections.

RAR1 (Required for Mla12 Resistance) is a protein required for resistance mediated by many R genes [2] and function of upstream of signaling pathways leading to H_2O_2 accumulation [3]. In many plant species RAR1 forms complex with Heat Shock Protein 90 (HSP90) and a Suppressor of the G_2 allele of skp1 (SGT1) [4], which suggest the RAR1 role as a co-chaperone. In *Nicotiana benthamiana* RAR1 together with SGT1 associate with the COP9 signalosome into a complex involved in protein degradation, and SCF-Ubiquitin E3 ligase complex which binds to substrate protein and transfer ubiquitin molecule onto it, marking it for degradation [5]. The RAR1 protein together with SGT1 protein may pass on signals triggered by the R genes into a SCF complex for ubiquitin-mediated protein degradation.

RAR1 protein consists of two 60 amino acid domains containing six cysteine and three histidine residues, called N-terminal CHORD-I and C-terminal CHORD-II domains. In addition, in plant these two domains are separated by cysteine and histidine rich motif CCCH [3]. The biochemical analysis has shown that each CHORD domain coordinates two zinc ions and the CCCH motif coordinates one zinc ion, and they are essential for structural stability of the protein-protein interaction. According to native-state electron ionization spray mass spectrometry, RAR1 protein is a monomer in solution and its molecular mass was found as 24.9 kDa [6].

Yeast two-hybrid screening assay experiments have shown that CHORD-I domain interacts with the N-terminal ATPase domain of HSP90 [4] and CHORD-II domain interacts with the CS domain of SGT1 [7] as shown schematically in Fig. 1.

This study was aimed at determining the shape (low resolution structure) of the molecule of RAR1-GST-Tag fusion protein in solution on the basis of small angle X-ray scattering (SAXS). GST-Tag protein purification system used in this study is based on high affinity of glutathione S-transferase (GST) to its substrate — glutathione. The expressed protein fused with glutathione binding domain can be easily separated from cell proteins fraction using affinity chromatography. Further cleavages

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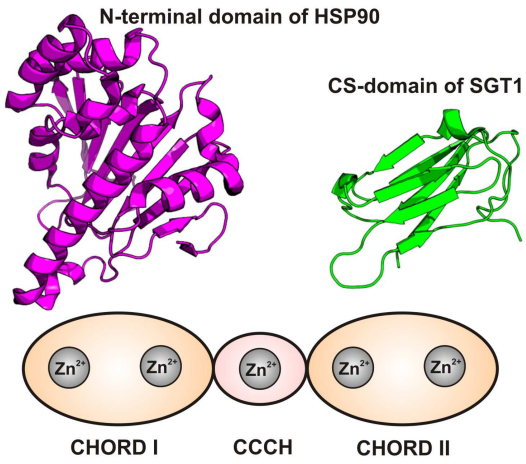

Fig. 1. Schematic representation of RAR1 interaction with SGT1 and HSP90.

of such fusion systems with dedicated proteases allow obtaining the protein without GST-Tag.

2. Material and methods

2.1. Protein expression and purification

The full length cDNA encoding barley (*Hordeum vulgare*) RAR1 was cloned into the expression vector pGEX 4T-2 (GE Healthcare Life Science). The RAR1-GST-Tag fusion protein was purified using Glutatione Sepharose 4 Fast Flow (GE Healthcare Life Science) according to the standard manufacturer's protocol. The purity of recombinant protein was checked by SDS/PAGE, and protein concentration was estimated using the Bradford method (BioRad).

2.2. Data collection and processing

SAXS measurements were preformed on the X-33 EMBL beamline at DESY, Hamburg (Germany) [8, 9] using the Pilatus photon counting detector. Protein samples (4.7, 7.5, 15.3 mg/ml) in 50 mM Tris/HCl pH 7.6 were measured using synchrotron radiation (wavelength $\lambda = 0.15$ nm) at 283 K. The sample-to-detector distance was 1.7 m, corresponding to the scattering vector range from 0.057 to 5.178 nm^{-1} ($s = 4\pi \sin\theta/\lambda$, where 2θ is the scattering angle). The detector s-axis was calibrated using the diffraction patterns of silver behenate [10]. The experimental data were normalized to the incident beam intensity, corrected for nonhomogeneous detector response and the scattering of the buffer was subtracted. A solution of xylose/glucose isomerase from *Streptomyces rubiginosus* of known concentration ($\approx$ 3 mg/mL) was used as the reference for molecular weight calibration [11]. The final scattering curve was obtained by merging the scattering data collected at a lower concentration and higher concentration using the program PRIMUS [12]. The radius of gyration was calculated from the low s-region of solution scattering curve, using the Guinier equation (1) [13]:

$$\lg\left(I(s)\right) = \lg\left(I(0)\right) - \frac{s^2 R_{\mathrm{G}}^2}{3}\,. \tag{1}$$

The pair distance distribution $p(r)$ function (2) was calculated from the experimental scattering data by the program GNOM [14, 15]:

$$p(r) = \frac{r}{2\pi^2}\int_0^\infty sI(s)\sin(sr)\,\mathrm{d}s\,. \tag{2}$$

The radius of gyration R_{G} was also calculated from the $p(r)$ function (3):

$$R_{\mathrm{G}}^2 = \int_0^{\mathrm{D_{max}}} r^2 p(r)\,\mathrm{d}r \Big/ \int_0^{\mathrm{D_{max}}} p(r)\,\mathrm{d}r\,. \tag{3}$$

The low-resolution structure of the RAR1-GST-Tag molecule in solution was determined by *ab initio* program DAMMIN v5.3. Detailed description of the shape determination method used is given by Svergun [16].

3. Results and discussion

The experimental solution scattering curve for RAR1--GST-Tag is presented in Fig. 2. The overall shape of the scattering curve is characteristic of the elongated molecule. The radius of gyration ($R_{\mathrm{G}} = 6.23 \pm 0.03$ nm) was obtained by a linear fit of data to the Guinier equation (1) (see Fig. 2).

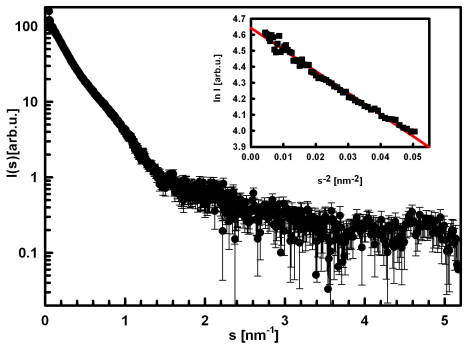

Fig. 2. SAXS data collected for RAR1-GST-Tag fusion protein and Guinier plot (inset).

The distance distribution $p(r)$ function was calculated on the basis of the full scattering data by indirect Fourier transformation. The $p(r)$ function represents the frequency of the distances r within a protein molecule obtained by combining any volume element with any other volume element.

This function, presented in Fig. 3, also offers an alternative method of calculation of radius of gyration based on the full scattering curve. RAR1-GST-Tag protein forms dimer in solution via interaction of GST domains. The dimer of RAR1-GST-Tag protein is characterized in solution by $R_{\mathrm{G}} = 6.19$ nm, which is in good agreement with the R_{G} obtained from the fit to Guinier equation, and maximal intramolecular vector $D_{\mathrm{max}} = 23$ nm. Low resolution solution models were obtained using DAMMIN v53 [16]. The packing radius of the dummy atoms was

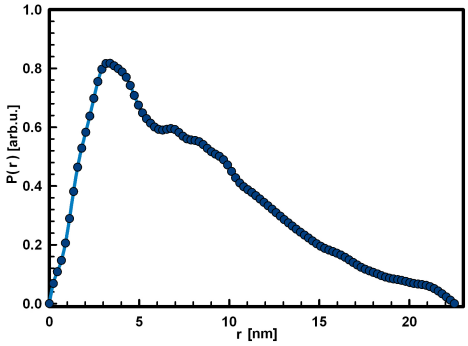

Fig. 3. Intramolecular distances distribution function $p(r)$ calculated for the RAR1-GST-Tag fusion protein in solution.

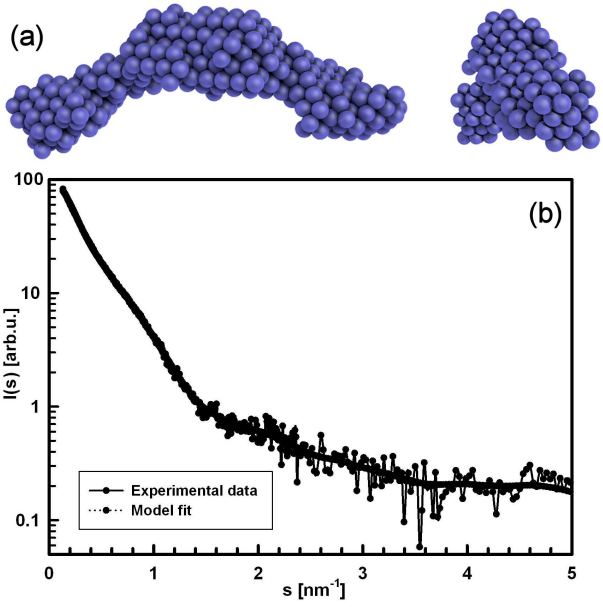

Fig. 4. Low resolution model of RAR1-GST-Tag fusion protein in solution (curved form) (a) and the fit of the model to the experimental data (b). The models are rotated clockwise by 90° around the y-axis.

0.49 nm and the total number of non-solvent dummy atoms used was about 8900. First calculations with $P1$ symmetry indicated that the structure was characterized by 2-fold symmetry axis and next runs were performed for the assumed $P2$ symmetry. After 30 independent runs we noticed two distinct shapes, and the best models within those groups were selected for further averaging. The final model was obtained by DAMAVER [17]. The exemplary models and their fits to the experimental SAXS data are presented in Figs. 4 and 5.

Both models of RAR1-GST-Tag fusion protein have elongated shapes which is in agreement with analytical centrifugation analysis data of RAR1 protein [6]. Additionally, $p(r)$ function shows a shape characteristic of the model built on two ellipsoids [18].

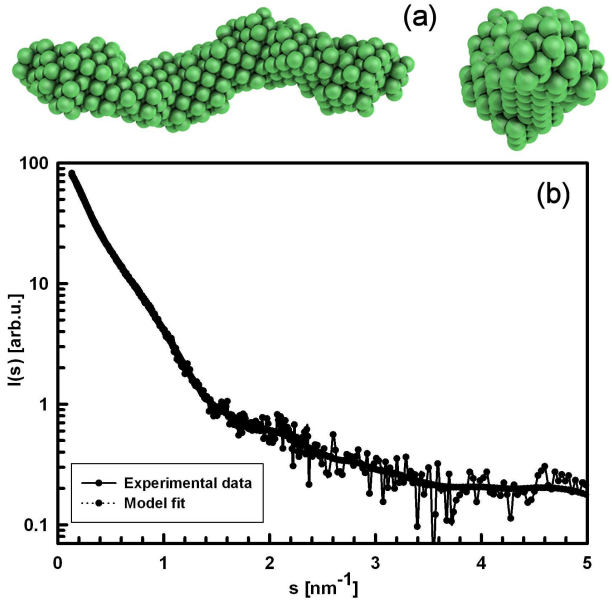

Fig. 5. Low resolution model of RAR1-GST-Tag fusion protein in solution (elongated form) (a) and the fit of the model to the experimental data (b). The models are rotated clockwise by 90° around the y-axis.

4. Conclusions

In conclusion, our study has demonstrated that it is possible to use SAXS measurements to determine the low resolution of fusion proteins. Both models of RAR-GST--Tag fusion protein obtained by the shape determination method show elongated conformations. We also concluded that the molecules of fusion protein form dimers in solution via interaction of GST domains. It was also proved possible to obtain structural information on the protein of interest using the GST-Tag construct, and due to dimerization it was possible to get stronger scattering signal and then better data. It may be useful when the protein studied has low molecular mass.

Acknowledgments

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