SANS investigation of assembly state of proteasome activator 28 and the 20S proteasome

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Abstract. The state of proteasome activator 28 (PA28) and the formation of the proteasomal complex in an aqueous solution are investigated with small-angle neutron scattering (SANS). The most appropriate state of PA28, which well reproduces the observed SANS profile, is the dissociation equilibrium between dimer and monomer with dissociation degree of 0.5. In addition, it is revealed that the packing of PA28 in the dimer is same as that in a crystal. It is also revealed that the proteasomal complex in which two PA28s connects to both basal planes of the 20S proteasome is spontaneously formed in the mixture solution of PA28 and the 20S proteasome.

1. Introduction

Small-angle neutron scattering (SANS) enable us to analyze a nano scale particle structure in an aqueous solution[1]. In addition, since neutron is a very gentle probe for samples, we have little or no worry about protein damage caused by the neutron irradiation. Therefore, SANS is one of the most suitable methods to investigate the formation and disruption of assembly state of proteins, which are deeply related with expression and loss of their functions, in an aqueous solution.

We have been focusing on the proteasomal systems consisting of several proteins, of which combinations depend upon their functions. The main component in the system is the 20S proteasome (PRS) with the molecular mass of ∼700 kDa known as a protein degradation machinery in cells[2, 3]. PRS has a hollow cylindrical shape constructed with four rings (α-β-β-α rings), each of which consists of seven subunits[4, 5]. The function of this huge protease complex is regulated through the attachment of other protein complexes termed proteasome activators (PAs): PAs identify proteins which are degraded by PRS. For example, proteasome activator 28 (PA28) with the molecular mass of ∼200 kDa has a truncated cone shape[6] consisting of highly homologous α- and β-subunits[7]. PA28 connects to both basal planes of PRS[8] and this protease complex (PRS + two PA28; PRS2PA28) contributes to the processing of antigenic...
proteins into peptides for presentation via the MHC class I pathway\cite{9, 10, 11, 12}. Therefore, PA28, PRS and PRS2PA28 are very important in our immune system.

For a better understanding of the assembly mechanisms in this proteasomal system, it is necessary to reveal the state of PA28, PRS and PRS2PA28 in an aqueous solution. Therefore, as the first step in our research, using a SANS method, we characterized the state of PA28 and then examined the formation of PRS2PA28 in a solution of PA28 mixed with PRS.

2. SANS experiment and simulation method

2.1. Sample preparation

The cDNAs of mouse PA28\(\alpha\) and PA28\(\beta\) were kindly gifted by Dr. Keiji Tanaka (Tokyo Metropolitan Institute of Medical Science). The PA28 \(\alpha\)-subunit lacking the loop segment (residue Val69 to Lys97) was generated using standard PCR and genetic-engineering techniques. A mutated \(\alpha\)-subunit and a wild-type \(\beta\)-subunit were subcloned into the expression vectors pET21d and pET23a, respectively. Each subunit was separately expressed using \textit{Escherichia coli} (BL-21) and purified by ammonium sulfate precipitation, hydroxyapatite chromatography, and ion-exchange chromatography. The hetero-oligomer of PA28 was constructed by mixing the two subunits and purified by gel filtration chromatography. Hereafter, this hetero-oligomeric PA28 will be simply referred to as PA28. PA28 was concentrated to 5.0 mg/mL in a buffer solution composed of the following: 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and 99.9% \(\text{D}_2\text{O}\).

The strain of \textit{S. cerevisiae} for expression of FLAG-tagged PRS was also kindly gifted by Dr. Keiji Tanaka. Cells were grown to an absorbance of 600 nm at 2.0 in YPD medium at 27°C. The grown cells were harvested and suspended in 50 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl and 10% glycerol, then lysed by mixing with glass beads. The extract was centrifuged at 26,740 g for 30 min at 4°C, then the supernatant was subjected to the affinity chromatography using anti-FLAG M2-agarose beads (Sigma). Anti-FLAG M2-agarose beads, to which the 26S proteasome had been bound, were incubated with 50mM Tris-HCl buffer (pH 7.5) containing 500mM NaCl, 10% glycerol and 10% Triton X-100 for 30 min at 25°C to dissociate the 19S components from the 26S proteasome. After washing the column, PRS was eluted with 3×FLAG peptide and the concentration was tuned to be 3.8 mg/mL in same buffer.

In order to examine the formation of PRS2PA28 in an aqueous solution, both prepared samples were mixed with the molar ratio of [PA28] : [PRS] = 2.6:1. The concentration of the mixture sample was also tuned to be 4.1 mg/mL.

2.2. SANS experiment

SANS experiments of three samples were performed with a SANS-U spectrometer of the Institute for Solid State Physics, University of Tokyo, installed at JRR-3 of the Japan Atomic Energy Agency, Tokai, Japan\cite{13}. The SANS data were measured at two sample-detector distances (SDLs) of 8 and 2 m. The total \(q\)-range covered was 0.008-0.2 \(\text{Å}^{-1}\) using 7.0 \(\text{Å}\) neutrons. The measurement times for the low (SDL=8 m) and middle (SDL=2 m) \(q\)-ranges were 3 and 1 hr, respectively, and temperature was kept constant at 25°C. The observed SANS intensities were corrected for background, cell, buffer scattering, and transmission factor.

2.3. SANS simulation

The SANS profiles are simulated as follows\cite{14, 15}. Firstly, a appropriate protein structure, monomer, dimer and/or their modulated ones, is fabricated using with atomic coordinates downloaded from Protein Data Bank: the examined structure models are described later. Because the samples were in the aqueous solution with heavy water and SANS cannot ignore hydrogen, we should make two improvements on the downloaded atomic coordinates. One is to add protons to the atomic coordinates at the most probable position, and the other is to
replace all exchangeable protons with virtual hydrogen atoms and remove the dissociatable protons/deuterons under the experimental condition (pH 7.5 and 25°C). The virtual hydrogen atom has an average scattering length depending on the ratio of light water and heavy water in the solution. Both procedures are simultaneously carried out using the computer program CNS[16] with a neutron-dedicated parameter file[17, 18].

Next, to reduce the calculation time, the amino acid residues are replaced with simple beads which have the volumes of the corresponding amino-acid residues[19] and the scattering contrast. With this simplified protein structure, SANS intensity $I(q)$ is calculated as follows,

$$I(q) = \sum_i |f_i(q)|^2 + 2 \sum_{i>j} f_i(q) f_j(q) \frac{\sin(q \cdot |r_i - r_j|)}{q \cdot |r_i - r_j|} + I_{inc},$$ (1)

$$f_i(q) = 3v_i \Delta \rho_i \frac{\sin(q R_i) - (q R_i) \cos(q R_i)}{(q R_i)^3},$$ (2)

$$I_{inc} = \frac{1}{4\pi} \sum_k \sigma_{inc}^k + N_x \left( <b_x^2> - <b_x>^2 \right),$$ (3)

$$<b_x^2> = c_{H_2O} b_H^2 + c_{D_2O} b_D^2,$$ (4)

$$<b_x>^2 = (c_{H_2O} b_H + c_{D_2O} b_D)^2,$$ (5)

where $f_i(q)$, $r_i$, $v_i$, $\Delta \rho_i$ and $R_i (=3v_i^{1/3}/(4\pi))$ are the form factor, the center of mass, the volume, the scattering contrast and average radius of the $i$-th amino acid, respectively, $\sigma_{inc}^k$ is the incoherent scattering cross section of the $k$-th atom, $N_x$ is the total number of replaceable hydrogen atoms, $c_{H_2O}$ and $c_{D_2O}$ are the ratios of light water and heavy water in the solvent, and $b_H$ and $b_D$ are the scattering lengths of proton and deuteron, respectively.

3. Results and analysis

Figure 1 show SANS profiles of PA28 (black circles), PRS (cyan circles) and their mixture (blue circles), respectively.

Figure 2. Guinier plots of SANS profiles of PA28 (black circles), PRS (cyan circles) and their mixture (blue circles), respectively. The straight lines show the results of the least square fittings with Guinier formula.

The results of the least square fitting of Guinier formula: $I(q) = I(0) \exp\left(-\frac{(R_g)^2}{3}q^2\right)$, where $R_g$ is a gyration radius. $R_g$s are found to be 44.2±0.9 Å, 61.9±0.7 Å and 87.2±0.8 Å of PA28, PRS and their mixture, with Guinier
Table 1. Concentrations and the results of Guinier analysis of PA28, PRS and their mixture.

|                | PA28 | PRS | Mixture |
|----------------|------|-----|---------|
| Concentration  | 5.0  | 3.8 | 4.1     |
| $R_g$ [Å]      | 44.2±0.9 | 61.9±0.7 | 87.2±0.8 |
| $I(0)/I_{PR5}(0)$ | 0.58 | 1   | 1.42    |

formula, respectively. The ratios of the SANS intensities of PA28 and the mixture at $q=0$ Å$^{-1}$ to that of PRS, which should be the monodisperse system, are 0.58 and 1.42, respectively.

3.1. PA28 in an aqueous solution

We start from the examination of the state of PA28 in an aqueous solution by simulating the SANS profiles and the gyration radii of several models: the atomic coordinates of PA28[6] was downloaded from PDB. As shown in Fig. 3, the simulated SANS profile of a single PA28 (s-PA28) cannot reproduce the experimental one (see green line and closed circles). In addition, $R_g$ of s-PA28 was calculated to be 33.7 Å with the simulation. This is quite smaller than the experimental value (44.2±0.9 Å). Therefore, it was considered that PA28 makes aggregation in an aqueous solution.

![Figure 3. Simulated and experimental SANS profiles of PA28. Green, blue and red lines indicate the simulated SANS profiles of s-PA28, d-PA28 and state of the dissociation equilibrium between d-PA28 and s-PA28 at 0.5 (see text), respectively. Closed circles denote the experimental SANS profile.](image)

Figure 4. The projection of crystal structure of PA28 to ac-plane[6]. The crystal structure belongs to the space group C2 with the lattice parameters, $a=162.600$, $b=134.300$, $c=116.200$.

The crystal structure of PA28[6] gave us a good starting point for considering the assembly state in an aqueous solution. As shown in Fig. 4, two PA28s closely locate in the crystal, facing the planes each of which connects to PRS. Therefore, we assumed that PA28 could make a
similar dimer in an aqueous solution; a dimer of PA28 will be abbreviated to d-PA28. The SANS profile of d-PA28 is also shown in Fig. 3 (see blue line) and its $R_g$ was calculated to be 47.4 Å. The SANS profile and the gyration radius of d-PA28 became closer to the experimental ones than those of s-PA28. However, there were still discrepancies both in the experimental and simulated ones: The simulated SANS profile of d-PA28 showed the deviation from the experimental one between 0.03 Å$^{-1}$ and 0.08 Å$^{-1}$. In addition, $R_g$ of d-PA28 was slightly larger than the experimental one.

**Figure 5.** Dissociation equilibrium state of PA28 in an aqueous solution: d1-PA28 $\rightleftharpoons$ s-PA28 + s-PA28. Red circles and a red arrow indicate the centers of mass of s-PA28s and the distance between them $l$, respectively.

Here, we noticed that the experimental SANS profile and $R_g$ are almost the average between those of d-PA28 and s-PA28. Therefore, it is rational that the state of PA28 in an aqueous solution is in the dissociation equilibrium between d-PA28 and s-PA28 as shown in Fig. 5. In other words, d-PA28 and s-PA28 could coexist in an aqueous solution. Furthermore, to make two s-PA28s get closer is another possibility to fit $R_g$ of d-PA28 to the experimental one: the outside chains around the connecting plane are random coils and can move freely in aqueous solution. Based on above mentioned consideration, we made the further simulation with two parameters: one was the dissociation degree, $x = ([s-PA28]/(2[d-PA28] + [s-PA28]))$, and the other was the distance of the centers of mass between two PA28s in d-PA28, $l$ (see a red arrow in Fig. 5). $l$ is varied from where two PA28s slightly touch (68 Å) as in the crystal to where two PA28s are embedded by the edge of columns of $\alpha$-helix each other (59 Å).

**Figure 6.** Relation between the error sum of the squares $\chi$ and the dissociation degree $x$ in the distance $l$ of 68-59Å.

**Figure 7.** Relation between the gyration radius $R_g$ and the dissociation degree $x$ in the distance $l$ of 68-59Å. The zone between two broken lines indicates the gyration radii satisfying the experimental one (44.2 ± 0.9 Å).

Figure 6 shows the relation between the error sum of the squares $\chi$ and $x$ in $l$ of 68-59Å: $\chi^2 = \Sigma_i [W_i (I_i^{exp} - I_i^{sim})/I_i^{exp}]^2$, where $I_i^{exp}$ and $I_i^{sim}$ are the experimental and simulated SANS intensities at the $i$-th data point, respectively, and $W_i$ is the weight function, which is the
normalized inverse of experimental error at the \( i \)-th data point. As shown in Fig. 6, \( \chi \) becomes minimum at \( x=0.5 \), \([\text{d-PA28}]:[\text{s-PA28}]=1:2\), for all distances.

Figure 7 shows the relation between \( R_g \) and \( x \) in \( l \) of 68-59 Å. The zone between two broken lines indicates the gyration radii satisfying the experimental one (44.2 \( \pm \) 0.9 Å). Therefore, considering that the error sum of squares becomes minimum at \( x=0.5 \) shown in a black straight line, the most appropriate distance between two s-PA28s is 67 \( \pm \) 1 Å, which is almost same as the distance in the crystal. The simulated SANS profile of the resulted state \( (x=0.5 \text{ and } l=67 \text{ Å}) \) as PA28 in an aqueous solution well reproduces the experimental one as shown in Fig. 3 (see red line).

The obtained dissociation ratio \( ([\text{d-PA28}]:[\text{s-PA28}]=1:2) \) is also supported with \( I_{\text{obs}}(0) \) obtained with Guinier analysis (Table 1). The obtained \( I_{\text{obs}}(0) \) is described with the number of particles in a solution, \( N \), and the intensity of one particle at \( q=0 \text{ Å}^{-1} \), \( I_p(0) \), as follows.

\[
I_{\text{obs}}(0) = NI_p(0) = N \left\langle \int_{V_p} \rho_p(r) - \rho_s \exp(\mathbf{q} \cdot \mathbf{r}) d\mathbf{r} \right\rangle^2,
\]

where \( V_p \), \( \rho_p(r) \) and \( \rho_s \) are the particle volume, the scattering length density of particle and that of solvent, respectively. In the samples with same optical paths, \( N \) is proportional to \( d/M_w \), where \( d \) and \( M_w \) are the density in weight percent and the molecular weight of the particles, respectively. In addition, \( I_p(0) \) is expressed as follows, \( I_p(0) = |(\rho_p - \rho_s)V_p|^2 \): \( \rho_p \) is the average of the scattering length density of particle. Here, since \( \rho_p \) is almost same \( (~0.3 \text{ fm} \text{Å}^{-3}) \) and \( V_p \) is proportional to \( M_w \) in proteins, \( I_p(0) \) is proportional to \( M_w^2 \) in the same solvent. As a result, the obtained \( I_{\text{obs}}(0) \) is also described in the solution with same solvent and optical path as follows.

\[
I_{\text{obs}}(0) \propto dM_w.
\]

Considering that PRS should be monodisperse in the solution, \( I_{\text{PA28}}(0)/I_{\text{PRS}}(0) \) are calculated to be 0.38, 0.75 and 0.56 for the monodisperse solutions of s-PA28 and d-PA28 and the solution with \([\text{d-PA28}]:[\text{s-PA28}]=1:2\), respectively, where \( ds \) are indicated in Table 1 and \( M_ws \) of s-PA28 and PRS are \( 4 \times 10^5 \) and \( 7 \times 10^5 \), respectively. Therefore, it means that the solution of PA28 is not a monodisperse system of s-PA8 nor d-PA28 but in the dissociation equilibrium between d-PA28 and s-PA28. In addition, \( I_{\text{PA28}}(0)/I_{\text{PRS}}(0) \) calculated with the dissociation ratio \( ([\text{d-PA28}]:[\text{s-PA28}]=1:2) \) also shows a good agreement with the observed one \( (0.58) \).

In conclusion, the state of PA28 in an aqueous solution is the dissociation equilibrium between d-PA28 and s-PA28. In addition, the structure of d-PA28 could be almost same as that in the crystal.

### 3.2. Mixture of PA28 and the 20S proteasome

Based on the above shown result, it is considered that the following two steps could be necessary to fabricate the 20S proteasome-PA28 complex (PRS2PA28).

\[
\text{d-PA28} \rightleftharpoons \text{s-PA28} + \text{s-PA28}, \quad (8)
\]

\[
\text{s-PA28} + \text{s-PA28} + \text{PRS} \rightleftharpoons \text{PRS2PA28}. \quad (9)
\]

Here, it is important to clarify whether the second step proceeds spontaneously or with help of any chaperon when PA28 and PRS coexist in the solution. We began to examine it with the SANS results. The simulated \( R_g \)s of PRS and PRS2PA28 were calculated to be 60.6 Å and 85.6 Å, respectively. These values show good agreement with the observed \( R_g \)s of PRS and the mixture of PA28 and PRS as indicated in Table 1, respectively. This result means that PRS2PA28 was fabricated in the mixture of PA28 and PRS.
Figure 8. Simulated and experimental SANS profiles. Cyan and blue circles show PRS and the mixture of PA28 and PRS, respectively. Green and red curves denote the simulated SANS profiles of PRS and PRS2PA28, respectively.

Figure 8 shows the simulated SANS profiles of PRS and PRS2PA28, and the experimental ones of PRS and the mixture of PA28 and PRS, respectively. The simulated SANS profile of PRS2PA28 reproduced the experimental one of the mixture of PA28 and PRS. This result strongly supports that PRS2PA28 was spontaneously fabricated in the mixture of PA28 and PRS.

4. Conclusion and further remarks

The state of PA28 in an aqueous solution is the dissociation equilibrium between d-PA28 and s-PA28. This is a suitable solution to store surplus PA28s for association to PRSs when they are introduced. In addition, it is revealed that PA28s spontaneously connect to PRS and make PRS2PA28.

However, the reaction rate of the association of PA28s to PRS has not been clarified yet. In other words, the association rate of equation (9) is unclear. As shown in Fig. 8, there is little deviation between the simulated SANS of PRS2PA28 and the experimental one of the mixture of PA28 and PRS in the q range of 0.03-0.05 Å\(^{-1}\). This suggested that a small amount of PRS could remain: all PRS could not be introduced to PRS2PA28. This is also supported with analysis of \(I_{obs}(0)\). As indicated in Table 1, \(I_{mixture}(0)/I_{PRS}(0)\) is 1.42 even though \(I_{PRS2PA28}(0)/I_{PRS}(0)\) should be 1.70 with equation (7). This means that not all PRS and PA28 become PRS2PA28. Therefore, it is important to clarify the association rate of equation (9) and the change of dissociation degree of equation (8) when PRS is introduced into a solution of PA28. For the clarification, we have to estimate four partial SANS intensities, SANS intensities of s-PA28, d-PA28, PRS and PRS2PA28, in one SANS profile. However, it is difficult without any other information. Therefore, in order to solve this difficulty, SANS experiments with the deuterated PA28 and employing a contrast variation method are now in progress.

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