

Kinetic Asymmetry of Subunit Exchange of Homo-Oligomeric Protein as Revealed by Deuteration-assisted Small-Angle Neutron Scattering

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Abstract

We developed a novel technique for real-time monitoring of subunit exchange in homo-oligomeric proteins, using deuteration-assisted small-angle neutron scattering (SANS), and applied it to the tetradecamer of the proteasome $\alpha 7$ subunit. Isotopically normal and deuterated tetradecamers exhibited identical SANS profiles in 81% D₂O solution. After mixing these solutions, the isotope sensitive SANS intensity in the low- q region gradually decreased, indicating subunit exchange, while the small-angle X-ray scattering profile remained unchanged confirming the structural integrity of the tetradecamer particles during the exchange. Kinetic analysis of zero-angle scattering intensity indicated that 1) only two of the fourteen subunits were exchanged in each tetradecamer, and 2) the exchange process involves at least two steps. The present study underscores the usefulness of deuteration-assisted SANS, which can provide quantitative information not only on the molecular sizes and shapes of homo-oligomeric proteins, but also on their kinetic properties.

Key words: small-angle neutron scattering, deuteration, homo-oligomeric proteins, subunit exchange kinetics, proteasome

Introduction

Elucidation of the mechanisms underlying the structural kinetics of proteins is one of the fundamental issues to be addressed in biophysics. Kinetic analyses of hydrogen-deuterium exchange observed by spectroscopic and mass spectrometric methods have provided detailed information on secondary structure formation during folding processes and local and global conformational fluctuations of tertiary structures(1, 2). However, because of the lack of appropriate methodology, the detailed formation mechanisms and kinetics of quaternary structures remain largely unknown, particularly in the case of homo-oligomeric proteins, which are the major forms of proteins in living systems(3, 4). Here, we addressed this issue by complementary use of small-angle X-ray scattering (SAXS) and deuteration-assisted small-angle neutron scattering (SANS) focusing on the *subunit exchange kinetics* of a homo-oligomeric protein.

SANS is a powerful method to describe protein quaternary structures in solution(5–7). A fascinating property of this method is its ability to distinguish deuterium from hydrogen owing to the difference in their neutron scattering lengths ($b_D = 6.671$ fm for deuterium and $b_H = -3.7406$ fm for hydrogen). This offers unique opportunities for contrast variation by H₂O/D₂O exchange (8, 9) as well as for subunit labeling in complex or oligomeric particles. Labeling of subunits in a homo-oligomer by selective deuteration for a SANS study is non-invasive, and presents significant advantages in comparison to other subunit-marking methods such as chemical modifications, site-directed mutations, and peptide/protein tagging(10–13). A quantitative analysis of SANS profiles assisted by H₂O/D₂O contrast variation has been applied to unravel the formation and conformational changes of different protein-protein and protein-tRNA complexes between amino-acyl tRNA synthetases and tRNA(14), and, by using selective deuteration, to examine subunit exchange(15). Owing to the recent progress in amplification of neutron beam intensity and computer-assisted simulation technique, the SANS method can now be applied to monitor subunit exchange processes in a homo-oligomer, as the time-dependent changes in the scattering profiles from a mixed solution of deuterated and non-deuterated proteins can be measured in real time. Herein, we developed the quantitative and experimental aspects of this approach and apply it to a model system consisting of the homo-tetradecamer of the proteasome $\alpha 7$ subunits.

The proteasome is a huge oligomeric protein operating as proteolytic machine in the ubiquitin-dependent protein degradation pathway in eukaryotic cells(16, 17). This large machine consists of a proteolytically active 20S core particle (CP) and one or two regulatory particles. The 20S CP is composed of 28 subunits, i.e., two sets of $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, and $\alpha 7$ and two sets of $\beta 1$, $\beta 2$, $\beta 3$, $\beta 4$, $\beta 5$, $\beta 6$, and $\beta 7$, which are arranged in a cylindrical shape of four hetero-heptameric rings, $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ (18, 19). It has recently been revealed that assembly of the proteasome subunit is a chaperone-assisted and ordered process and not a spontaneous self-organization(20, 21). However, among the proteasomal subunits, the $\alpha 7$ subunits spontaneously forms a homo-tetradecamer in the absence of the other subunits(22).

In a previous study, we have characterized the quaternary structure of the $\alpha 7$ homo-tetradecamer in aqueous solution by SANS(23). The analysis indicated a structure made up of two homo-heptameric rings stacked back-to-back to form a double ring structure (Fig. 1A). This suggested that proteasome assembly involves some scrap-and-build processes from homo-heptameric $\alpha 7$ rings to the hetero-heptameric ring composed of seven different subunits. To provide insights into the mechanisms underlying these processes, it is essential to understand the dynamics and stabilities of the quaternary structure of this homo-oligomer. Therefore, herein we assess the dynamic properties of the homo-tetradecamer of proteasome $\alpha 7$ subunits with an attempt to observe subunit exchange by using deuteration-assisted SANS in conjunction with SAXS.

To observe a possible subunit exchange, we prepared two isotopically distinct $\alpha 7$ tetradecamers: one consisted only of deuterated $\alpha 7$ (d- $\alpha 7$) subunits and the other was composed only of non-deuterated (natural abundance) $\alpha 7$ (h- $\alpha 7$) subunits. These tetradecamers show large difference in their neutron scattering length densities: $\rho_d = 0.76 \text{ fm}\cdot\text{\AA}^{-3}$ for the d- $\alpha 7$ tetradecamer and $\rho_h = 0.308 \text{ fm}\cdot\text{\AA}^{-3}$ for the h- $\alpha 7$ tetradecamer in D₂O solution. Given that the mixture of d- $\alpha 7$ and h- $\alpha 7$ tetradecamers results in subunit exchange giving rise to isotopically mixed tetradecamers, they are expected to exhibit scattering length densities between ρ_h and ρ_d depending upon the ratio of d- $\alpha 7$ and h- $\alpha 7$ subunits (bars in Fig. 1B). Accordingly, in 81% D₂O solution, of which scattering length density is intermediate value between those of d- $\alpha 7$ and h- $\alpha 7$ tetradecamers, the scattering contrasts of the h- $\alpha 7$ and d- $\alpha 7$ tetradecamers (defined as the difference in scattering length density between solute and solvent) are equal in absolute

value but are opposite in sign, and any subunit exchange causes a reduction in this absolute value (arrows in Fig. 1B). Therefore, when subunit exchange proceeds in the mixture of both isotopic forms, the SANS intensity (proportional to the square of the scattering contrast) decreases in the low q -region by producing the tetradecamers with a lower scattering contrast, while SAXS (which is not isotope sensitive) is unchanged if the tetradecamer quaternary structure is maintained.

Materials and Methods

Protein samples

The h- α 7 and d- α 7 subunits were separately produced as recombinant proteins in *Escherichia coli* grown in H₂O- and D₂O-based minimal media and assembled into tetradecamers. A detailed protocol for preparation of the tetradecamer solutions used in SANS and SAXS experiments are provided in Supporting Material.

SANS Experiments

SANS experiments were performed using the D22 instrument installed at the Institut Laue-Langevin (ILL), Grenoble, France(24) and the SANS-U instrument of the Institute for Solid State Physics (ISSP), University of Tokyo, installed at the JRR-3M, Japan Atomic Energy Agency, Tokai, Japan (25). The SANS intensities measured in the q -range 0.0085-0.13 \AA^{-1} were accumulated at 15 min intervals for 12 h at a constant temperature of 25 °C. The observed SANS intensity was corrected for background, empty cell and buffer scatterings, and transmission factors, and then converted to the absolute scale by dividing by the SANS intensity of H₂O (26).

SAXS Experiments

SAXS experiments were performed on the small- and wide-angle scattering (SWAXS) instrument installed at the BL40B2 beam line of SPring-8, Hyogo, Japan (27). The SAXS intensities in the q -range 0.005-2.2 \AA^{-1} were measured for 1 s at eight time points in 12 h at a constant temperature of 25 °C. The observed SAXS intensity was

corrected for background, cell, buffer scattering, and transmission factors. The data correction details are described elsewhere (28, 29).

Results and Analysis

Detection of Subunit Exchange

Prior to time-resolved SANS experiments, we checked the structural stability of the $\alpha 7$ tetradecamers in 81% D₂O solution. The SANS profiles of h- $\alpha 7$ and d- $\alpha 7$ tetradecamers before and after 12 h of incubation at 25 °C were in excellent agreement (Fig. 2A), confirming that 1) the h- $\alpha 7$ and the d- $\alpha 7$ tetradecamers have the same absolute value of scattering contrast in 81% D₂O solution and 2) they are structurally stable in this solution at least for 12 h. The structural stability of the $\alpha 7$ tetradecamers in the mixture of both isotopic forms was also confirmed by the time evolution of the SAXS profile, which was unchanged for 12 h after mixing under identical conditions (Fig. 2B).

The SANS intensity in the low q -region gradually decreased after mixing the h- $\alpha 7$ tetradecamer with the d- $\alpha 7$ tetradecamer in 81% D₂O solution (Fig. 2C). As demonstrated by the results of the SAXS experiment, the observed change in SANS profile cannot be attributed to a structural change of the tetradecamers but is ascribed to the subunit exchange between the h- $\alpha 7$ and the d- $\alpha 7$ tetradecamers.

We further analyzed the subunit exchange kinetics, focusing on the time evolution of the zero-angle SANS intensity $I(0, t)$, which is directly proportional to sum of the square of scattering contrasts of tetradecamers in the solution. The time evolution of the normalized zero-angle SANS intensity, ${}^N I(0, t)$, which is defined as $I(0, t)$ against that at the starting point of the time-course, ${}^N I(0, t) \equiv I(0, t)/I(0, 0)$, could not be expressed with a monoexponential decay function, but was well-reproduced with the biexponential decay function.

$${}^N I(0, t) = {}^N I(0, \infty) + k_a \exp(-t/t_a) + k_b \exp(-t/t_b). \quad (1)$$

The best result of the least-square fitting (cyan line in Fig. 2D) was obtained with the parameters, ${}^N I(0, \infty) = 0.76 \pm 0.02$, $k_a = 0.09 \pm 0.02$, $k_b (= 1 - k_a - {}^N I(0, \infty)) = 0.15 \pm 0.03$, $t_a = 1.5 \pm 0.3$ h, and $t_b = 9.1 \pm 3.3$ h, and the normalized zero-angle SANS intensity reaches a non-zero value of 0.76 ± 0.02 .

Number of Exchangeable Subunits

For a quantitative interpretation of the above mentioned results, we first assumed that *all* subunits in the $\alpha 7$ tetradecamer had *equal* probability to exchange *randomly* with *any* subunit in another tetradecamer. Under this assumption, the mixture of h- $\alpha 7$ and d- $\alpha 7$ tetradecamers eventually reaches equilibrium between the isotopically mixed tetradecamers with different numbers of the deuterated subunits. Considering the incidence of the individual isotopic forms of tetradecamer, $^N I(0, \infty)$ was calculated to be 0.0714 (Table), which is considerably smaller than the experimentally estimated value of 0.76 ± 0.02 . This raises the idea that the number of exchangeable subunits in one $\alpha 7$ tetradecamer is limited. Hence, under the constrain of the number of the swappable subunits n , we calculated the number ratio with x deuterated subunits $r_n(x, \infty)$ and the corresponding normalized zero-angle scattering intensities in the equilibrium states ($t = \infty$) as follows.

$$r_n(x, \infty) = \left(\frac{1}{2}\right)^{(n+1)} ({}_n C_x + {}_n C_{x-(14-n)}), \quad (2)$$

$${}_a C_b = 0 \text{ (} a < b \text{ or } b < 0\text{)}, \quad (3)$$

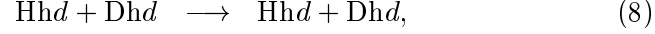
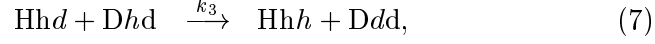
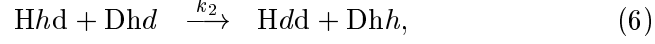
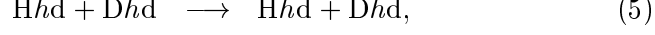
$$^N I_n(0, \infty) = \sum_{x=0}^{14} r_n(x, \infty) \times \left(\frac{7-x}{7}\right)^2. \quad (4)$$

By inspection of the calculated $^N I_n(0, \infty)$ values (Table 1), we concluded that the number of the swappable subunits is two in one $\alpha 7$ tetradecamer; $^N I_2(0, \infty) = 0.745$, which is in good agreement with the experimental value of 0.76 ± 0.02 . This strongly suggests that one subunit could be swappable in one heptameric ring. No more extensive subunit-swapping was observed at least for 48 h (data not shown).

Kinetics of Subunit Exchange

Given that the subunit exchange process can be described as a simple bimolecular exchange model (Fig. 3A), every two swappable subunits in every tetradecamer have an equal exchange probability meaning that every reaction has four subreactions with an equal reaction probability. For example, an exchange reaction between Hhd and Dhd (at the center of the middle line in Fig. 3A) includes the following four

subreactions with equal reaction probabilities.



where H and D represent twelve unswappable subunits, and h, (*h*) and d, (*d*) represent the exchangeable (exchanging) subunits. Here, subreaction 6 yields Hdd and Dhh tetradecamers with probability k_2 , while subreaction 7 yields Hhh and Ddd tetradecamers with probability $k_3 (= k_2)$. The remaining two subreactions 5 and 8 can be ignored because they do not affect number ratio of the tetradecamers. On the other hand, the reaction of (Hhh + Ddd) with k_1 or (Hdd + Dhh) with k_4 (at the top and bottom of the middle line in Fig. 3A, respectively) makes four sets of (Hhd + Dhd) without any branches. It means that the probabilities of the subreactions 6 and 7 are one quarter of that of the reaction of (Hhh + Ddd) or (Hdd + Dhh). Therefore, the exchange probabilities for the reactions in Fig. 3A are $k_1 = k_4 = 4k_2 = 4k_3$. In the same manner, we obtain $k_1 = k_4 = k_9 = k_{11} = 2k_5 = 2k_6 = 2k_7 = 2k_8 = 4k_2 = 4k_3 = 4k_{10} = 4k_{12}$.

Here, the time evolution of the number ratio of each tetradecamer is given as the solutions of a system of differential equations: in the following, as an example, the differential equation of [Hhd(*t*)] is shown,

$$\begin{aligned} \frac{d[\text{Hhd}(t)]}{dt} = & k_1[\text{Hhh}(t)][\text{Ddd}(t)] \\ & - (k_2 + k_3)[\text{Hhd}(t)][\text{Dhd}(t)] \\ & + k_4[\text{Hdd}(t)][\text{Dhh}(t)] - k_5[\text{Hhd}(t)][\text{Ddd}(t)] \\ & + k_6[\text{Dhd}(t)][\text{Hdd}(t)] + k_7[\text{Dhd}(t)][\text{Hhh}(t)] \\ & - k_8[\text{Dhh}(t)][\text{Hhd}(t)] + k_{11}[\text{Hhh}(t)][\text{Hdd}(t)] \\ & - k_{12}[\text{Hhd}(t)]^2. \end{aligned} \quad (9)$$

The differential equations of the number ratio of the other tetradecamers are given in the same manner. The equations were numerically solved starting from the initial state, $[\text{Hhh}(0)] = [\text{Ddd}(0)] = 0.5$ and $[\text{Hhd}(0)] = [\text{Hdd}(0)] = [\text{Dhd}(0)] = [\text{Dhh}(0)] = 0$. In addition, ${}^N I_2(0, t)$

is also given as follows (Eq. 4).

$$\begin{aligned}
{}^N I_2(0, t) = & ([Hhh(t)] + [Ddd(t)]) (7/7)^2 \\
& + ([Hhd(t)] + [Dhd(t)]) (6/7)^2 \\
& + ([Hdd(t)] + [Dhh(t)]) (5/7)^2. \quad (10)
\end{aligned}$$

By substituting the numerical solutions of the six tetradecamers in Eq. 10, we obtained the simulated ${}^N I_2(0, t)$. This time evolution is expressed with a monoexponential decay function (green line in Fig. 2D), which does not reproduce the experimental results, suggesting that the exchange process involves at least two steps.

In general, proteins undergo conformational change to express biological functions such as enzymatic reactions and molecular recognition events. A typical example is the induced-fit mechanism in enzymatic reactions, in which enzymes change their conformations upon binding to substrates to facilitate catalysis (30). In addition, growing evidence indicates that proteins exhibit multiple conformations including those involved in the ligand-binding states even in the absence of the ligands(31). In these cases, ligand-binding processes cannot be expressed as a single exponential process (31).

Such concepts may be applicable to the subunit exchange process of homo-oligomeric proteins: in the two-step exchange system, the swappable subunits are in equilibrium between the active (or exchangeable) and inactive (or resting) states as follows,



where * indicates an inactive state. The exchange occurs when two homo-oligomers each containing the active subunit(s) meet. Therefore, the interconversion given in Eq. 11 is introduced to every exchange reaction with the equal rate constant k_{13} as shown in Fig. 3B. In order to obtain the time evolution of the number ratios of tetradecamers in this model, we improved our numerical calculation approach in the iteration. That is, in every iteration, we re-calculated the number ratios of active tetradecamer for every tetradecamer and then calculated the system of differential equations. For example, the active tetradecamer ratio of Hhh is calculated using the following differential equation,

$$\frac{d[\text{Hhh}(t)]}{dt} = -k_{13} ([\text{Hhh}(t)] - [\text{Hhh}^*(t)]). \quad (12)$$

Subsequently, we performed the calculation as indicated in the simple bimolecular exchange model.

Thus ${}^N I_2(0, t)$ is expressed with a biexponential decay function, where the ratio of time constants depends upon the ratio of k_1 and k_{13} . The best biexponential function for ${}^N I_2(0, t)$ with $k_1/k_{13} = 5.5$ well reproduces the experimental data (blue line in Fig. 2D). According to this model, the conformational transition can be a rate-limiting step in the subunit exchange process.

In perspectives for future work to validate this model, the subunit exchange rates should be measured at various protein concentrations. The exchange reaction rate with a bimolecular step must depend on the concentration of the $\alpha 7$ tetradecamer while the conformational transition rates do not. These experiments can be performed by using a SANS spectrometer with a higher flux neutron beam.

In conclusion, the study demonstrated that only one subunit is exchangeable among the seven $\alpha 7$ subunits constituting one heptameric ring. This means that the $\alpha 7$ heptameric ring is probably not seven-fold symmetric, although the previous structural studies could not predict such an asymmetric property because of low spatial resolution(22, 23). The kinetic asymmetry of subunit exchange of the $\alpha 7$ tetradecamer not only provides important clues to the underlying mechanisms of proteasome subunit assembly but also offers general insights into the formation and dynamics of quaternary structures of homo-oligomeric proteins. Deuteration-assisted, time-resolved SANS has opened up new opportunities for this unexplored field of research.

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|-----------------------|-------|-------|--------|--------|--------|
| n | 0 | 1 | 2 | 3 | 4 |
| ${}^N I_n(0, \infty)$ | 1 | 0.867 | 0.745 | 0.633 | 0.531 |
| n | 5 | 6 | 7 | 8 | 9 |
| ${}^N I_n(0, \infty)$ | 0.439 | 0.357 | 0.286 | 0.224 | 0.173 |
| n | 10 | 11 | 12 | 13 | 14 |
| ${}^N I_n(0, \infty)$ | 0.133 | 0.102 | 0.0816 | 0.0714 | 0.0714 |

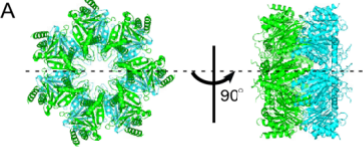
Table 1: Normalized zero-angle scattering intensity in the equilibrium state ${}^N I_n(0, \infty)$ calculated for varying numbers of swappable subunits, n .

Figure Legends

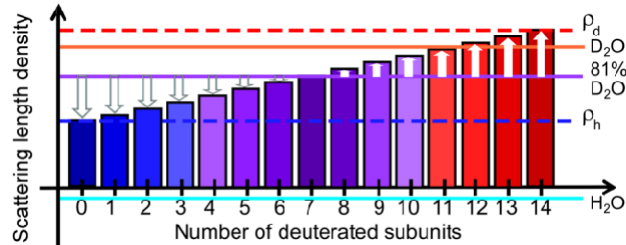
Figure 1: Three-dimensional structural model and neutron scattering length densities of the tetradecamer of proteasomal $\alpha 7$ subunit computed from the 3D structural model. (A) Top and side views of the double-ring structure of the $\alpha 7$ tetradecamer derived from the SANS data in conjunction with the crystal structure of the 20S core particle(19, 23). Each $\alpha 7$ heptameric ring with seven-fold symmetry is drawn in green or cyan. The diameter and thickness are ~ 120 Å and 50 Å, respectively. (B) Neutron scattering length densities of tetradecamers of proteasomal $\alpha 7$ composed of different numbers of h- $\alpha 7$ and d- $\alpha 7$ subunits computed from its 3D structural model, shown with bars having color gradient. Blue and red broken lines express the scattering length densities of h- $\alpha 7$ (ρ_h) and d- $\alpha 7$ (ρ_d) tetradecamers, respectively, while cyan, orange and purple solid lines express the scattering length densities of H₂O, D₂O, and 81% D₂O, respectively. The arrows indicate the scattering contrasts of tetradecamers in 81% D₂O solution.

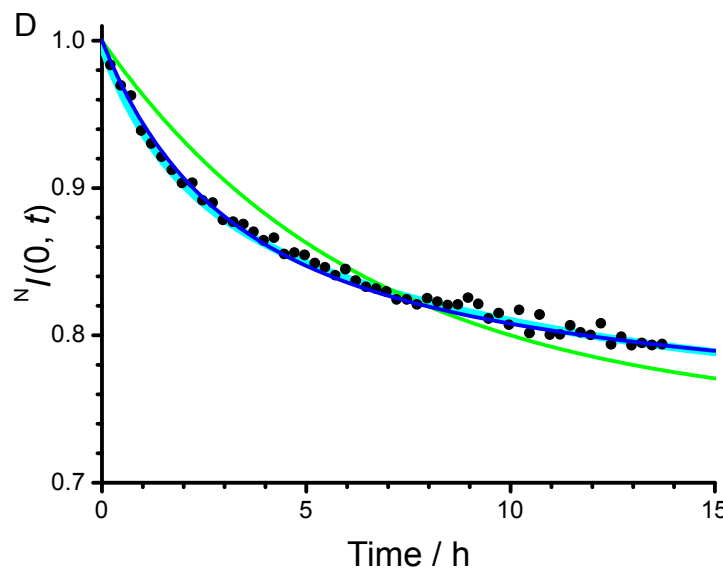
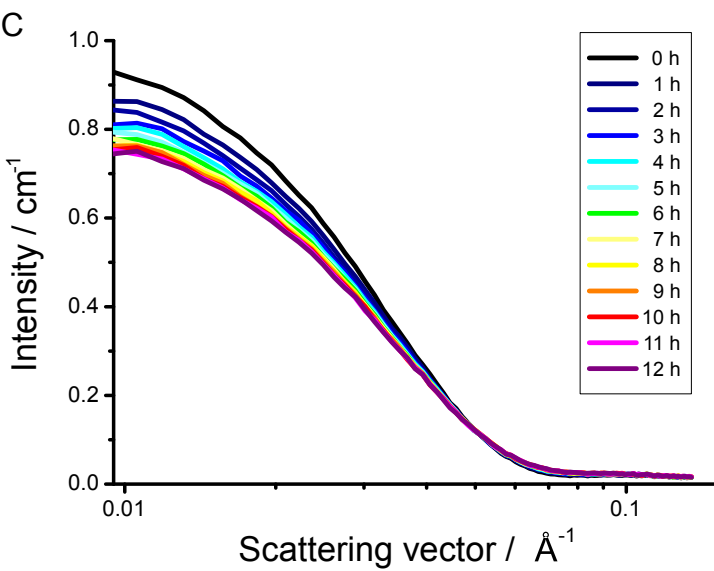
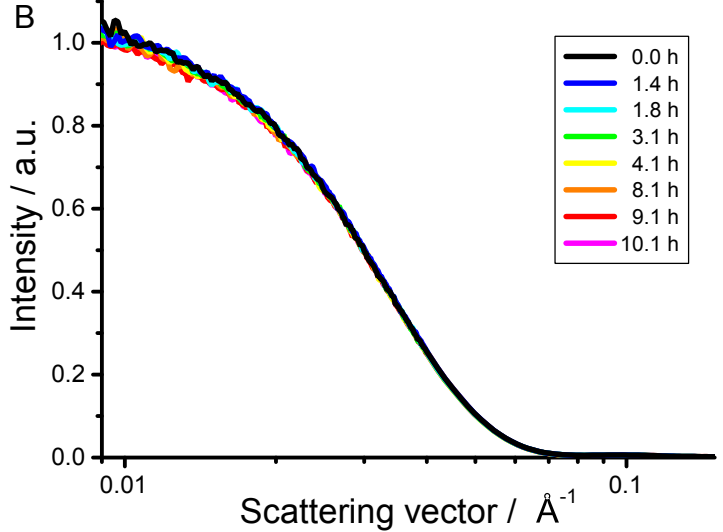
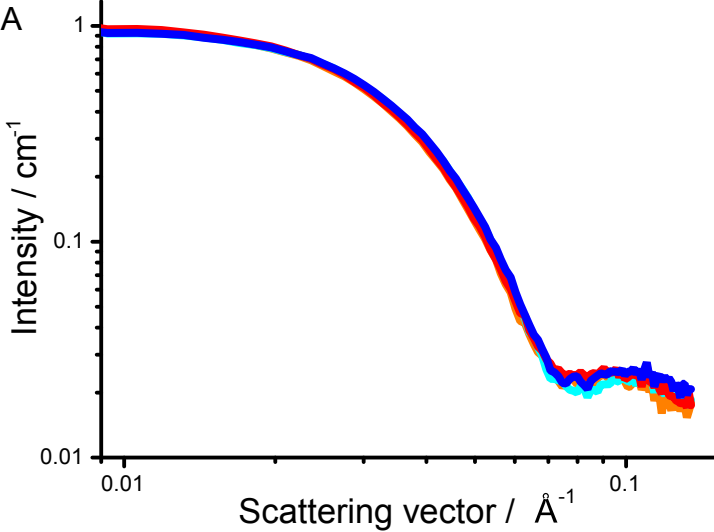
Figure 2: Time evolution of scattering profiles. (A) SANS profiles just after dissolving into 81% D₂O solutions (blue and red for h- α 7 and d- α 7 tetradecamers, respectively) and after 12 h (cyan and orange for h- α 7 and d- α 7 tetradecamers, respectively). (B) Time evolution of SAXS profile after mixing the h- α 7 tetradecamers with the d- α 7 tetradecamers in 81% D₂O solution. (C) Time evolution of SANS profile after mixing the h- α 7 tetradecamers with the d- α 7 tetradecamers in 81% D₂O solution. (D) Time evolution of normalized zero-angle scattering intensity, $^NI(0, t)$. Filled circles show the experimental result calculated with the Guinier formula. Cyan line represents the result of the least-square fitting of the biexponential decay function, Eq. 1 (see text), while green and blue lines show the best simulated results using the two-subunit-swapping model with (blue) and without (green) the assumption that the swappable subunits are in equilibrium between active and inactive states (Fig. 3B and see text).

Figure 3: Subunit exchange schemes of two kinetics models. (A) Scheme of simple bi-molecular exchange model. The tetradecamer constitution is expressed by H and D for twelve unswappable subunits, and h and d for two swappable subunits. For simplicity, the exchange reactions which do not cause intensity change, such as $Hhh+Dhh \rightleftharpoons Hhh+Dhh$ (an italic letter means an exchanging subunit), are not shown. Top, middle, and bottom rows correspond to the α 7 tetradecamers with zero, one, and two differently labeled subunit(s), respectively. Typical α 7 tetradecamers are drawn on the left and right sides, where the blue and red spheres show h- α 7 and d- α 7 subunits, respectively. $k_1 - k_{12}$ denote the exchange probabilities in the reactions, where $k_1 = k_4 = k_9 = k_{11} = 2k_5 = 2k_6 = 2k_7 = 2k_8 = 4k_2 = 4k_3 = 4k_{10} = 4k_{12}$ (see text). (B) Extended exchange scheme by the two-step exchange model assuming the transition between active and inactive(*) states of the swappable α 7 subunit. It is a modification of the reaction scheme $Hhh+Ddd \rightleftharpoons Hhd+Dhd$ (boxed with dashed line in panel A) and exemplifies the extension of the model. k_{13} is the rate constant of the transition.

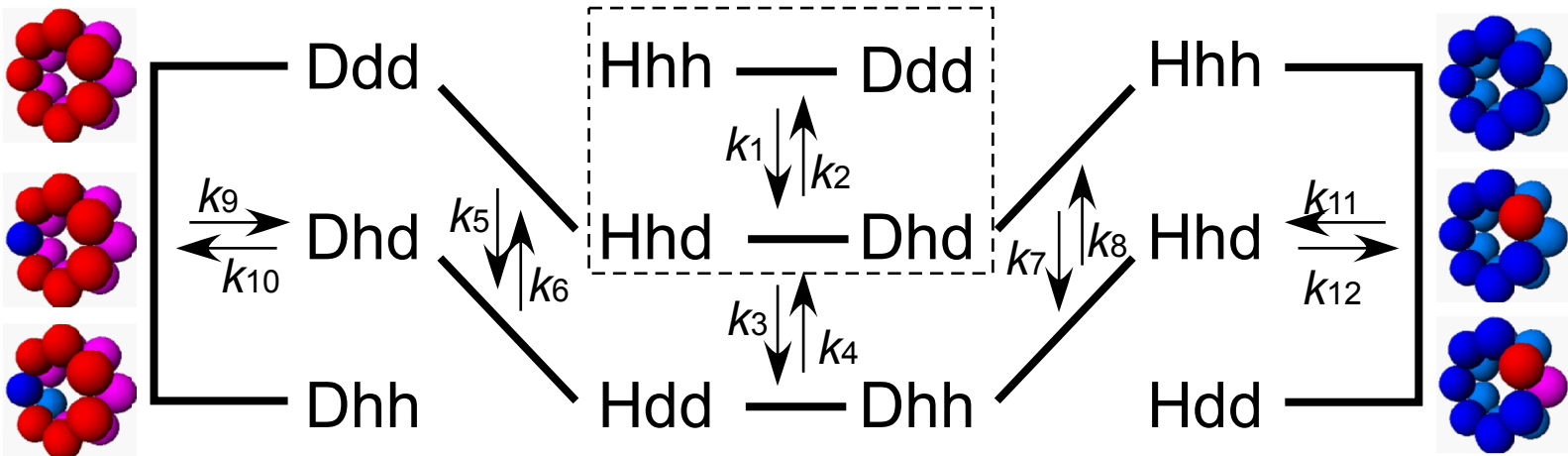


B

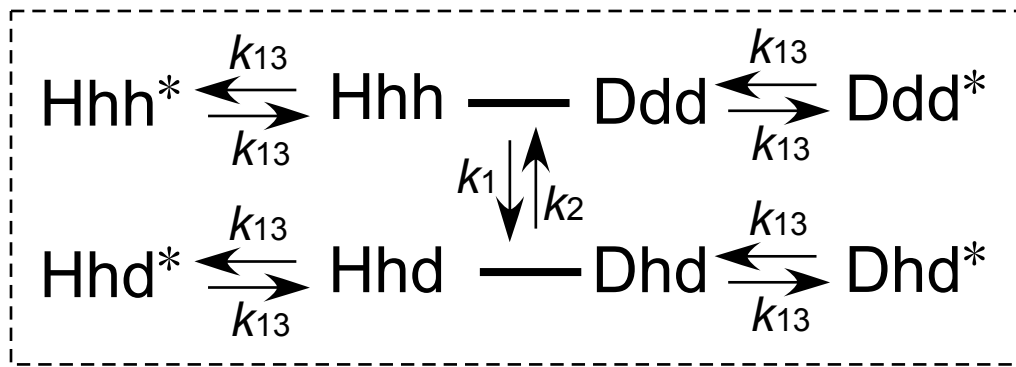




A



B



Kinetic Asymmetry of Subunit Exchange of
Homo-Oligomeric Protein as Revealed by
Deuteration-assisted Small-Angle Neutron
Scattering

July 8, 2011

Supporting Materials

Sample preparation

Human proteasome $\alpha 7$ subunit was expressed using *Escherichia coli* [BL21(DE3)]. For the preparation of deuterated subunits, the cells were grown in M9 minimal media containing deuterated glucose (2 g/L) and 99.8% D₂O. After sonication and centrifugation, cell lysates were subjected to anion exchange chromatography (DEAE Sepharose Fast Flow, GE Healthcare). Roughly purified samples were dialyzed and further purified by HPLC system sequentially using an anion exchange column (RESOURCE Q, GE Healthcare) and gel-filtration column (HiLoad 26/60 Superdex 200 pg, GE Healthcare). The purified proteins were concentrated to 5 mg/ml in buffer solutions composed of 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and 100% H₂O or 98.2% D₂O. In the aqueous solution, the $\alpha 7$ subunits simultaneously form the double heptameric ring, which was confirmed by the SANS experiment.

The protocol for scattering experiments is schematically illustrated in fig. S1. In step 1, four isotopically different $\alpha 7$ solutions were prepared: h- $\alpha 7$ in H₂O (solution #1), h- $\alpha 7$ in D₂O (solution #2), d- $\alpha 7$ in H₂O (solution #3), and d- $\alpha 7$ in D₂O (solution #4). In step 2, the 81% D₂O solution of the h- $\alpha 7$ tetradecamer (solution #5) was prepared by mixing solution #1 with solution #2, whereas the 81% D₂O solution of the d- $\alpha 7$ tetradecamer (solution #6) was prepared by mixing solution #3 with solution #4. After

mixing, time evolution of SANS of solutions #5 and #6 were measured, the results of which are shown in Fig. 2A. Finally, in step 3, the 81% D₂O solution of the 1:1 mixture of the h- α 7 and the d- α 7 tetradecamers (solution #7) was prepared by mixing equal amounts of solutions #5 and #6. Again, after the mixing, time evolutions of SAXS and SANS of this solution were measured for 12 h, the results of which are shown in Fig. 2B and 2C, respectively.

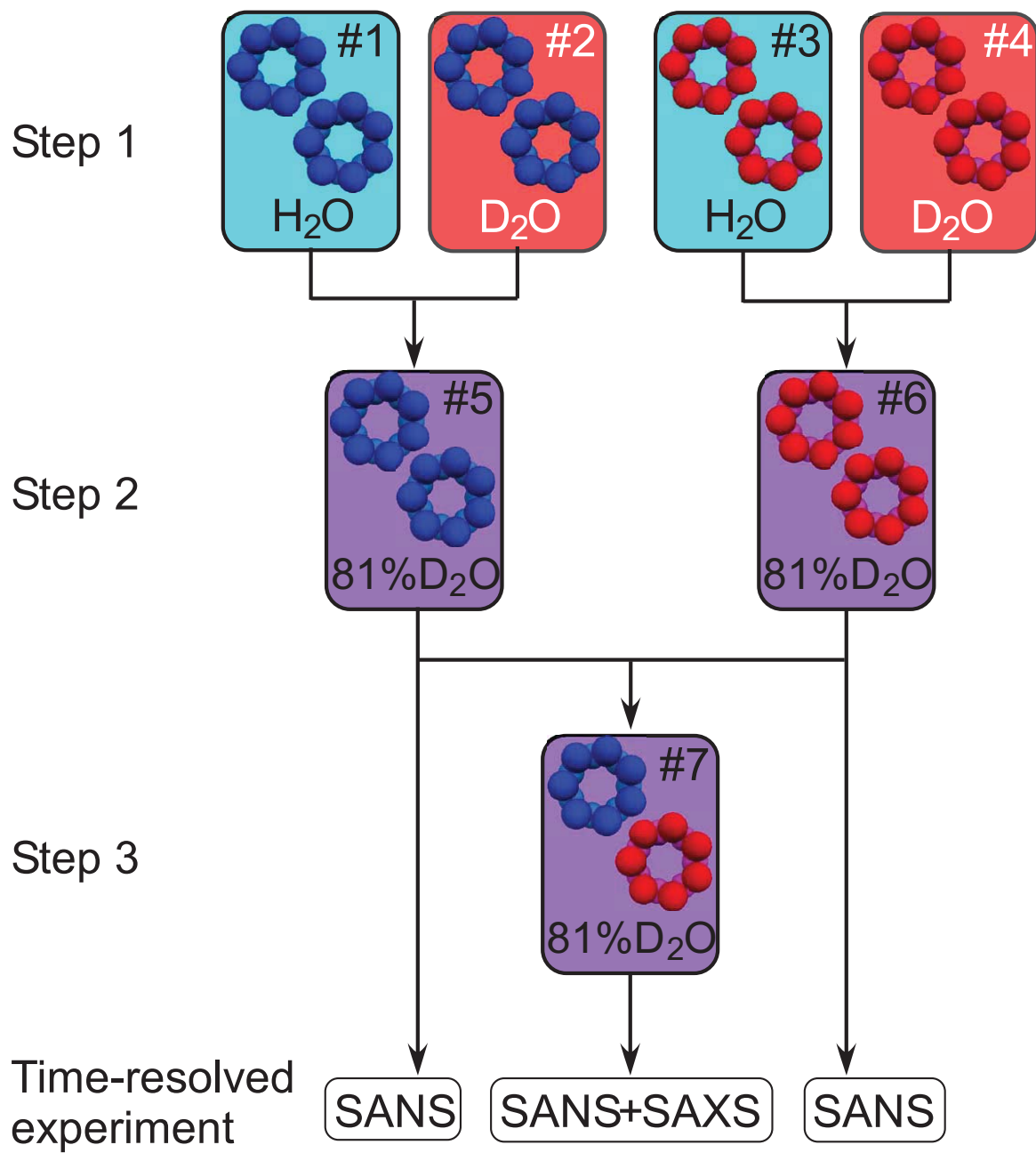


Figure 1: Sample preparation for scattering experiments.