Mechanisms of ferritin assembly studied by time-resolved small-angle X-ray scattering

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Abstract

The assembly reaction of *Escherichia coli* ferritin A (EcFtnA) was studied using time-resolved small-angle X-ray scattering (SAXS). EcFtnA forms a cage-like structure that consists of 24 identical subunits and dissociates into dimers at acidic pH. The dimer maintains native-like secondary and tertiary structures and can reassemble into a 24-mer when the pH is increased. The time-dependent changes in the SAXS profiles of ferritin during its assembly were roughly explained by a simple model in which only tetramers, hexamers, and dodecamers were considered intermediates. The rate of assembly increased with increasing ionic strength and decreased with increasing pH (from pH 6 to pH 8). These tendencies might originate from repulsion between assembly units (dimers) with the same net charge sign. To test this hypothesis, ferritin mutants with different net charges (net-charge mutants) were prepared. In buffers with low ionic strength, the rate of assembly increased with decreasing net charge. Thus, repulsion between the assembly unit net charges was an important factor influencing the assembly rate. Although the differences in the assembly rate among net-charge mutants were not significant in buffers with an ionic strength higher than 0.1, the assembly rates increased with increasing ionic strength, suggesting that local electrostatic interactions are also responsible for the ionic-strength dependence of the assembly rate and are, on average, repulsive.

Keywords Assembly · Electrostatic interaction · Ferritin · Net charge · Small-angle X-ray scattering (SAXS)

Introduction

Time-resolved small-angle X-ray scattering (TR-SAXS) is a useful technique to follow assembly reactions of large, complex supramolecular structures, such as microtubules (Kutter et al. 2016; Shemesh et al. 2018), F-actin filaments (Oda et al. 2016), and viral capsids (Kler et al. 2012; Tresset et al. 2013; Tuma et al. 2008). Recently, this technique has been used to study the assembly of ferritin (Sato et al. 2016a, b). However, there were some difficulties when TR-SAXS was applied to monitor the assembly reactions of ferritin. Firstly, the assembly rate is a function of the protein concentration and is too fast to measure at the protein concentration that is needed for SAXS

detection. This difficulty has been overcome by the recent development of a photon-counting PILATUS detector (Kraft et al. 2009), which makes it possible to observe two-dimensional scattering images every few milliseconds. Secondly, the dissociation of mammalian ferritins is frequently irreversible at least partly. Most previous studies to address the assembly mechanism of ferritin were done with commercially available horse spleen ferritin (HSF) (Gerl and Jaenicke 1987; Gerl et al. 1988; Kim et al. 2011; Stefanini et al. 1987). Mammalian ferritins consist of structurally similar subunits H-chain and L-chain. The H-chain has an iron-oxidizing (ferroxidase) activity, and the L-chain is involved in iron core nucleation and mineralization. The ratio of the two types of subunits depends on tissues. HSF is known to contain approximately 90% L-chain and 10% H-chain (Stefanini et al. 1982). Although HSF dissociates into the subunits at acidic pH and reassociates at neutral pH, the process is not fully reversible (Crichton and Bryce 1973; Kim et al. 2011). Although HSF can refold and reassemble from the dissociated and unfolded state in concentrated guanidinium chloride, amorphous aggregate is formed during the folding and reassembly reaction (Gerl et al. 1988). Such aggregates scatter X-rays strongly and interfere with the interpretation of SAXS data. Recombinant human ferritin H-chain and L-chain

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were reported to show similar irreversible denaturation (Kuwata et al. 2019; Santambrogio et al. 1993). In contrast to mammalian ferritins, bacterial ferritins are composed of the same kind of subunits resembling H-chain of mammalian ferritins. Recently, Ohtomo et al. have found that *Escherichia coli* ferritin A (EcFtnA) dissociates into dimers at acidic pH and maintains its secondary and tertiary structures and that the dissociation is completely reversible without the formation of larger aggregates (Ohtomo et al. 2015). This property of EcFtnA was suitable for studying the assembly mechanism using TR-SAXS.

Assembly reaction monitored by SAXS

EcFtnA was dissociated into dimers at pH 2.5, and then, reassembly reactions were induced by a pH jump from 2.5 to 8.0. The time course of the changes in the scattering profiles was monitored using a high-speed photon-counting detector. A typical example is shown in Fig. 1. As the reaction proceeded, the scattering intensity at zero angle $I(0)$ increased and fringes that are characteristic of large particles emerged. From each scattering profile, $I(0)$ and R_{app} values were estimated using the Guinier approximation:

$$I(Q) = I(0) \exp\left(-\frac{R_{\text{app}}^2 Q^2}{3}\right), \quad (1)$$

where the scattering vector $Q = (4\pi/\lambda) \sin\theta$ (λ is the wavelength, and 2θ is the scattering angle), and $I(Q)$ is the scattering intensity at a given Q value. In a system in which a single particle is present in a solution, R_{app} is the radius of gyration of that particle, and $I(0)$ is proportional to the mass concentration, the molecular weight, the beam intensity, and the square of the difference in the electron density between the particle and the solvent (Glatter and Kratky 1982). In a system in

which two or more particles are present in a solution, $I(0)$ and R_{app} are complex. $I(0)$ is an arithmetic average of the values obtained for the scattering bodies present in the solution. R_{app} is not a simple population average of the scattering bodies' R_g s; rather, it is biased toward the values of the larger scattering bodies. Assuming that all intermediate oligomers are spherical, the observed $I(0)$ and R_{app} are expressed by the following equations:

$$I(0) = \sum_i C_i \cdot I(0)_i, \quad (2)$$

$$R_{\text{app}} = \sqrt{\frac{\sum_i C_i \cdot I(0)_i R_{g,i}^2}{\sum_i C_i \cdot I(0)_i}}, \quad (3)$$

where C_i , $I(0)_i$, and $R_{g,i}$ denote the mass concentration, the forward scattering intensity (per unit mass concentration), and the radius of gyration of the i -th species, respectively.

Kinetic model

To determine the reaction order, the initial velocity of assembly reaction was determined at various protein concentrations (Sato et al. 2016a). The initial velocity was found to depend on the 2.44th power of the protein concentration, which suggests that both second- (formation of 4-mers) and third-order reactions (formation of 6-mers) contribute to the initial increment of $I(0)$.

Based on this observation and the model proposed by Gerl et al. (1988), the authors suggested a model illustrated in Fig. 2. Rate equations were built for this model, and the approximate solution of the rate equations was obtained using the fourth-order Runge–Kutta method (Griffiths and Higham 2010). If $I(0)_i$ and $R_{g,i}$ values can be obtained for species M_2 , M_4 , M_6 , and M_{12} , it is possible to construct the time courses of $I(0)$ and R_{app} using Eqs. 2 and 3. $I(0)_i$ and $R_{g,i}$ values were calculated as follows. First, the theoretical scattering intensity was calculated from the atomic coordinates of oligomers extracted from the crystal structure of the 24-mer (Stillman et al. 2001) using CRY SOL software (Svergun et al. 1995). Although four and 24 different isomers are possible for 6-mers (M_6) and 12-mers (M_{12}), respectively, the authors assumed that only one isomer for 6-mers and 12-mers was present, as shown in Fig. 2. Using the oligomer-specific $I(0)_i$ and $R_{g,i}$ values and a differential equation solver (Berkeley Madonna; <http://www.berkeleymadonna.com/>), the authors fitted the calculated $I(0)$ and R_{app} curves to all experimentally observed data at various protein concentrations and optimized rate constants. The fitting was fair, and the model was able to reproduce assembly kinetics at various protein concentrations with a set of rate constants. To confirm the validity of the model, the authors calculated the scattering profiles at various time points and compared them with the experimental data. At various reaction times, the

Fig. 1 An example of changes in the SAXS profiles during the assembly of EcFtnA (5 mg/mL) at pH 8.0 and 25 °C

Fig. 2 A simplified model of the ferritin assembly mechanism. Each dimer unit is shown in a different color

calculated scattering profiles showed a fair agreement with the experimental ones. Therefore, the model represented in Fig. 2 is valid, at least as a first approximation.

To assess the accumulation of intermediate oligomers during the assembly reaction, the authors calculated the population changes of the intermediate using the rate constants obtained (Fig. 3). At 1 mg/mL, the maximum populations of 6-mers and 12-mers were 18% and 32%, respectively. At 4 mg/mL, the populations of 6-mers and 12-mers reached 27% and 41%, respectively. The accumulation of 12-mers is consistent with the results of a cross-linking study of horse spleen ferritin (Gerl and Jaenicke 1987), in which 12-mers accumulated up to 14% at 5.6 $\mu\text{g/mL}$. However, 6-mers were not detected in that study. Because the populations of both 6-mers and 12-

mers decreased with decreasing protein concentrations, the fact that a larger population was detected compared with that detected in the cross-linking study may be ascribed to the differences in the protein concentrations used in the two studies. The authors described that if they simulated the kinetics at 5.6 $\mu\text{g/mL}$ using the rate constants obtained in our TR-SAXS study, the population of 12-mers was a few percent, and that of 6-mers was less than 0.1%.

Ionic strength and pH dependence of assembly rates

In homooligomers, all subunits have net charges of the same sign. Therefore, electrostatic interactions between the net charges of subunits should be repulsive when they come close

Fig. 3 Population changes during the assembly of EcFtnA. The populations of dimers, 4-mers, 6-mers, 12-mers, and 24-mers were calculated using a set of optimized rate constants at protein concentrations of 1 mg/mL (a) and 4 mg/mL (b). The curves were calculated with a set of optimized parameters; $k_1 = 1.06 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $k_2 = 6.22 \times 10^8 \text{ M}^{-2} \text{ s}^{-1}$, $k_3 = 2.51 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $k_4 = 7.39 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $k_5 = 1.54 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $k_{-1} = 1.50 \times 10^2 \text{ s}^{-1}$, $k_{-2} = 4.82 \times 10^{-2} \text{ s}^{-1}$, $k_{-3} = 1.87 \times 10^{-6} \text{ s}^{-1}$, $k_{-4} = 1.36 \times 10^{-7} \text{ s}^{-1}$, $k_{-5} = 6.75 \times 10^{-6} \text{ s}^{-1}$ (Sato et al. 2016a)

to each other during the assembly process. To understand how electrostatic interactions influence the assembly reaction, the assembly reaction was measured either at various ionic strengths at constant pH 8.0 or at various pH values and constant ionic strength $\mu = 0.08$ (Sato et al. 2016b). The assembly rate increased with increasing ionic strength at pH 8.0. Given that the electrostatic interactions between the assembly unit (AU, dimer in this case) net charges are repulsive, the ions may screen the electrostatic repulsion and accelerate the assembly. The assembly rate also increased with decreasing pH. Since the isoelectric point (pI) of EcFtnA is 5.43, the net charge of the AU is expected to be negative and to become more negative at higher pH values. Therefore, the pH dependence of the assembly rate may be explained by increased electrostatic repulsion due to increased AU net charges at

higher pH. To verify this hypothesis, the authors designed several mutant proteins that exhibit a range of net charges.

Assembly reactions of net-charge mutants

To construct mutants with different net charges (net-charge mutants), Sato et al. selected charged residues that (1) held side chains that were exposed to the solvent, (2) did not participate in the formation of salt bridges, and (3) were located far from the subunit interfaces (Sato et al. 2016b). Selected residues were Glu5, Glu8, Glu12, Glu85, and Glu89 of the outer surface of the shell (see Figure 2 of Sato et al. 2016b). They replaced one to five Glu residues with Gln. The mutants were named EEEEEQ, EQQEE, EQQEQ, EQQQQ, and QQQQQ, according to the position of the Glu to Gln mutation(s). For example, EQQEE denotes the mutant in which Glu8 and Glu12 are glutamine, and the others are glutamate. Although QQQQQ could be purified, its solubility was poor, and it was not used for TR-SAXS experiments. Far- and near-UV CD spectra of EEEEEQ, EQQEE, EQQEQ, and EQQQQ were similar to those of the wild type (WT), indicating that the secondary structure and the packing around the aromatic residues are similar to those of WT both in the native and acid-dissociated states. Gel filtration experiments indicated that the four mutants formed 24-mers and 2-mers in buffers at neutral and acidic pH, respectively. The SAXS profiles of the four mutants were also indistinguishable from those of the WT. The pIs of the mutants were determined by isoelectric focusing. As expected, the pI values became larger with increasing numbers of Glu to Gln mutations.

The assembly reactions of net-charge mutants were investigated at pH 8.0 and at three different ionic strengths ($\mu = 0.08, 0.11$, and 0.17). It was expected that WT and mutants would be negatively charged at pH 8.0 and that the absolute value of the AU net charge would decrease as the number of mutations from Glu to Gln increases. The time courses of changes in $I(0)$ and R_{app} during the assembly reactions under three different ionic strengths are shown in Fig. 4. It can be seen clearly that at $\mu = 0.08$, the assembly rate increases with increasing numbers of mutations (Fig. 4a). This can be explained by reduced electrostatic repulsion between AU net charges, which decreases with increasing number of mutations. However, the difference in the assembly rate between WT and mutants was not remarkable at $\mu = 0.11$ and $\mu = 0.17$ (Fig. 4b, c). Therefore, the electrostatic repulsion between AU net charges is not significant under the conditions in which μ is > 0.1 and is not a serious problem for the assembly under physiological conditions (intracellular solutions contain 140 mM potassium ions) (Alberts et al. 2002). Rather, local electrostatic repulsions between charges located near the AU interface seem to be important. The assembly rates at $\mu = 0.17$ were much faster than those at $\mu = 0.11$, although the difference between WT and net-charge mutants was not significant

Fig. 4 Changes in $I(0)$ were monitored during the assembly reactions of WT and net-charge mutants at a protein concentration of 2.5 mg/mL, in 25 mM Tris, 25 mM phosphate, 1 mM EDTA buffer (pH 8.0) containing appropriate concentrations of NaCl to adjust $\mu = 0.08$ (a), $\mu = 0.11$ (b), and $\mu = 0.17$ (c). Adapted with permission from Sato et al. (2016b). Copyright 2016 by the American Chemical Society

under either condition (Fig. 4b, c). This can be interpreted as follows. If two AUs are far apart, their net charges can be regarded as point charges. In such cases, the probability that two AUs are separated by a certain distance is lower at a shorter distance because of the repulsion between net charges. This tendency is important when the ionic strength is low. At high ionic strength, however, the distribution becomes relatively uniform except for very close distances; therefore, the

assembly rate becomes independent of the net charges of AUs. In a situation in which two oligomers are in close proximity, electrostatic interactions between specific charges at the interface must be considered. In this case, the electrostatic interaction may be either attractive or repulsive depending on the charge pair considered. The authors call this “local electrostatic interaction.” The fact that the assembly rates at $\mu = 0.17$ were much faster than those at $\mu = 0.11$ suggests that the local electrostatic interactions are, on average, repulsive and that they are screened by ions.

Other SAXS studies on ferritins

The SAXS has been used for structural characterization of apoferritins from more than half a century ago (Bielig et al. 1966; Fischbach and Anderegg 1965). The pH-dependent dissociation and reassembly were also studied by SAXS (Kim et al. 2011). SAXS is also useful to study the morphology of iron mineral core, its formation, and iron release process (Balejckova et al. 2017; Ciasca et al. 2012; Fischbach and Anderegg 1965; Gálvez et al. 2008; Kim et al. 2011; Melnikova et al. 2014). All of these studies were performed using HSF and encountered some experimental difficulties such as irreversibility of dissociation, aggregate formation, or destruction of the self structure upon iron loading.

Sana et al. characterized the structures of *Archaeoglobus fulgidus* apoferritin (AfFtn) and its mutant AfFtn-AA by SAXS (Sana et al. 2013). AfFtn has a unique tetrahedral (2-3) assembly of 24 subunits with large triangular pores in the AfFtn shell (Johnson et al. 2005). Sana et al. made a mutant in which amino acid residues K150 and R151 were replaced by alanine (AfFtn-AA). Interestingly, AfFtn-AA showed the conventional octahedral (4-3-2) assembly (Sana et al. 2013). AfFtn and AfFtn-AA dissociate to dimers at low salt concentrations and reassemble to 24-mers at high salt concentrations (Johnson et al. 2005; Sana et al. 2013). SAXS showed that the solution structures of both proteins at a high salt concentration are consistent with the crystal structures described above (Sana et al. 2013).

Conclusion

TR-SAXS is a quite useful technique to study the assembly reaction mechanisms of macromolecules. Information about the stoichiometry of assembled particles is available from the forward scattering intensity, and the SAXS profile includes information about the shape of the particles. Using synchrotron light sources and PILATUS detectors, the time resolution of TR-SAXS becomes a few milliseconds. For ferritin, the required sample concentration is comparable to that needed to obtain circular dichroism spectra (~ 0.5 mg/mL). Previously, the assembly reaction was followed by chemical

cross-linking coupled with sodium dodecyl sulfate–polyacrylamide gel electrophoresis or high-pressure liquid chromatography analysis (Gerl and Jaenicke 1987). Although this method can separate and quantify intermediate species, its time resolution is limited by the speed of the cross-linking reaction. Therefore, it is not easy to investigate protein concentration dependence or pH dependence of an assembly reaction by the cross-linking method, whereas it is easy to investigate it using TR-SAXS. Another promising method is fluorescence resonance energy transfer (FRET) (Carmona et al. 2017). By coassembling subunits labeled with donor and acceptor fluorophores, the assembly reaction can be monitored by FRET. Although the time resolution of this method is also high, it provides less structural information. These methods are complementary with each other, and we can elucidate the ferritin assembly mechanisms in much more detail by combining them with site-directed mutagenesis techniques.

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Compliance with ethical standards

Conflict of interest Daisuke Sato declares that he has no conflict of interest. Masamichi Ikeguchi declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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