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Serum Amyloid A 2.2 Refolds into a Octameric Oligomer that Slowly Converts to a More Stable Hexamer

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Abstract

¹Serum Amyloid A (SAA) is an inflammatory protein predominantly bound to high-density lipoprotein in plasma and presumed to play various biological and pathological roles. We previously found that the murine isoform SAA2.2 exists in aqueous solution as a marginally stable hexamer at 4–20°C, but becomes an intrinsically disordered protein at 37°C. Here we show that when urea-denatured SAA2.2 is dialyzed into buffer (pH 8.0, 4°C), it refolds mostly into an octameric species. The octamer transitions to the hexameric structure upon incubation from days to weeks at 4°C, depending on the SAA2.2 concentration. Thermal denaturation of the octamer and hexamer monitored by circular dichroism showed that the octamer is ~10°C less stable, with a denaturation mid point of ~22°C. Thus, SAA2.2 becomes kinetically trapped by refolding into a less stable, but more kinetically accessible octameric species. The ability of SAA2.2 to form different oligomeric species *in vitro* along with its marginal stability, suggest that the structure of SAA might be modulated *in vivo* to form different biologically relevant species.

Keywords

kinetic control; oligomerization; intrinsically disordered; inflammation; folding; SAA

Introduction

Serum Amyloid A (SAA) proteins are highly conserved throughout vertebrates and play a major role in the acute phase inflammatory response [1]. Although the functions of SAA remains poorly understood, SAA has been linked to a number of functions related to cholesterol metabolism and transport and host survival during an inflammatory response [2–7]. The homeostatic concentration of SAA in serum is 1–3 µg/mL [8]. However, during inflammation resulting from infection, injury, or trauma, the concentration of SAA can

¹Abbreviations: AUC, analytical ultracentrifugation; CD, circular dichroism; MW, molecular weight; PB, phosphate buffer; RT, room temperature; SAA, serum amyloid A; SE, sedimentation equilibrium; SEC, size exclusion chromatography; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

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increase up to 1000 fold to a final concentration of over 1.0 mg/ mL [8,9]. The biological reasons for this dramatic increase in SAA expression is not yet clear, but the prolonged high levels of SAA during chronic inflammation sometimes lead to amyloid A (AA) amyloidosis, a disease characterized by the deposition of SAA amyloid fibrils in the liver, spleen and kidney [10,11]. Furthermore, SAA has been linked to other diseases, including, heart disease, cancer, and Alzheimer's disease [6,12]. Thus, it is intriguing to understand the molecular basis by which a protein of only 103 amino acids might be involved in many physiological and pathological processes.

In prior *in vitro* studies, we found that murine SAA2.2 forms a hexameric structure with a putative central channel [13]. Further studies revealed that the hexamer loses its quaternary, tertiary, and secondary structures upon incubation in either ~ 2M urea or 37°C [14,15]. Thus, hexameric SAA2.2 is not only marginally stable, but it is an intrinsically disordered protein *in vitro* at physiological temperature. The ability of many proteins to form intrinsically disordered structures appears to confer them the ability for regulation, signaling and control either by being able to interact directly with multiple targets or by adopting various structures with multifaceted functions [16]. Consistent with this quality, we have observed that SAA has a tendency to form various self-assembled structures under mild conditions, including amyloid fibrils [15]. In this study we report that after denaturation in urea, the refolding of SAA2.2 results in the formation of an octameric species that slowly converts to the hexameric oligomer upon incubation at 4°C. Thermal denaturation experiments showed that the SAA2.2 octamer is less stable than the hexamer. These results show that the oligomerization of SAA2.2 during refolding is kinetically controlled leading to the formation of a less stable, but more kinetically accessible octameric oligomer. The ability of SAA2.2 to form different oligomeric species has implications for the various putative functions of SAA proteins.

Materials and methods

All chemicals and supplies were purchased from Fisher Scientific unless specified. Size exclusion columns and chromatofocusing columns were purchased from GE Healthcare. Disposable microfilters were purchased from Whatman.

SAA2.2 expression and purification

The murine SAA2.2 cDNA was cloned into a pET21-a(+) vector between the NdeI and BamHI restriction sites and transformed into Escherichia coli strain BL21 (DE3) pLysS competent cells, as previously described [17]. SAA2.2 expression and purification procedures were modified from the methods previously described [13] with the following modifications. Six tubes, each containing 15 mL of LB broth (2.5% LB, 100 µg/mL ampicillin and 34 µg/mL chloramphenicol) were inoculated with a single colony, and cells were allowed to grow at 37°C until an OD₆₀₀ of ~ 0.7. These starter cultures were poured into six-100 mL LB broth in 500 mL flasks and grown until an OD₆₀₀ of ~ 0.7. Medium growth cultures were poured into six-700 mL LB broth in 2000 mL flasks and grown until an OD₆₀₀ of ~ 0.7. IPTG was then added to a final concentration of 1 mM. SAA2.2 was expressed at 30°C while shaking at 220 rpm for 3 h. Cultures were cooled in an ice bath for 20 min and then spun at 4,000 rpm and 4°C for 20 min. Cell pellets were stored at -20°C.

To purify SAA2.2, frozen cells were thawed and suspended in ~ 50 mL TSB buffer (20 mM Tris/150 mM NaCl, pH 8.2). Sonication was applied 7 times in 30-sec bursts to break the cell. The cell lysate was transferred to 250 mL of Buffer A (6 M urea / 20 mM Tris, pH 8.2 at 4°C), and concentrated to 50mL by ultrafiltration using a Millipore 3,500 Da cut-off membrane. 200 mL of Buffer A was added again followed by ultra-filtration. This was repeated again, after which the ~ 50 mL lysate was centrifuged at 15,000 rpm and 4°C for 30

min. The supernatant was then loaded onto a DE52 anion exchange column with Buffer A on an AKTA™ purifier UPC 10 (GE healthcare) and eluted with buffer B (Buffer A + 0.4 M NaCl) using a 10 to 65% gradient. The relevant fractions were pooled and desalted via several rounds of ultrafiltration and then loaded onto a PBE 94 chromatofocusing column (Amersham Pharmacia Biotech) equilibrated with Buffer A. SAA2.2 was eluted with buffer A containing 7% polybuffer 96 (GE healthcare), 3% polybuffer 74 (GE healthcare), pH 5.5. SAA2.2 fractions were pooled, concentrated and precipitated with 70% ammonium sulfate to remove polybuffer resins. The precipitate was subjected to additional washes with 70% ammonium sulfate to remove traces of polybuffer. The precipitate containing pure SAA2.2 was solubilized by resuspending in buffer A. Minimal volume of buffer A was used to achieve high concentrations of SAA, after which the solution was filtered through a 20 nm microfilter and stored at -80°C.

Refolding of SAA2.2

SAA2.2 stored at -80°C in urea was thawed overnight at 4°C. It was then loaded in a 1 mL capacity red-cap dialyzer fitted with a 3K MWCO membrane and dialyzed against 500 – 1000X Tris buffer (20 mM Tris, pH 8.2 at 4°C), for 45 min, followed by buffer changes at 2 h and overnight to remove traces of ammonium sulfate and urea. The dialyzed SAA was carefully aliquoted and its absorbance measured at 280nm (Molecular weight: 11670, Extinction coefficient: 25440 M⁻¹ cm⁻¹). Following dialysis, ~20µg of SAA2.2 (20µl from a 1mg/ml stock) was loaded in an analytical gel filtration column to determine the oligomeric state of refolded SAA.

Size exclusion chromatography (SEC)

SEC of SAA2.2 was carried out on a Superdex 200 HR 10/300 column GL (GE Healthcare) using an AKTA™ purifier UPC 10 (GE healthcare). The column was equilibrated with TBS buffer (20mM Tris, 150mM NaCl, pH 8.2 at 4°C), after which 5 – 50 µL of SAA2.2 was loaded, and the protein was eluted using a flow rate of 0.5 mL/min.

Circular dichroism (CD) and thermal denaturation

Far-UV CD spectra were recorded on an OLIS RSM 1000 CD instrument (Bogart, GA). SAA2.2 samples (0.1 mg/mL) were prepared by diluting the stock in PB buffer (20 mM, pH 7.4) and loaded in a circular quartz cuvette (20 mm path length). Three spectra were collected from 190 - 260 nm at 4°C and averaged. SAA2.2 thermal denaturation experiments were carried out by programming the software to increase the temperature automatically. Each temperature point was stabilized for 15 min before recording the signal for 1 min at 222 nm. The signal at 255 nm was used for baseline subtraction.

Analytical ultracentrifugation (AUC)

Equilibrium sedimentation experiments were performed using a Beckman XL-I analytical ultracentrifuge with an AN-50Ti rotor and operating at 4°C. Different protein concentrations (6 to 20 µM) in 20 mM Tris pH 8.2, 150 mM NaCl buffer were loaded into six-sector cells and spun at 10,000, 12,000 and 19,000 rpm. The absorbance at 280 nm was recorded as a function of the radial position. Equilibrium was considered attained when replicate scans six hours apart were indistinguishable. The data were globally fitted to a model of single species in solution, according to equation 1, using the nonlinear regression software (NLREG) and in-house written scripts.

$$A(r)=I+A_o(r_o) \cdot \exp \left[\frac{\omega^2}{2RT} M(1 - \nu\rho)(r^2 - r_o^2) \right] \quad (1)$$

where $A(r)$ is the absorbance of the protein at a radius r , $A_o(r_o)$ is the absorbance at a radius r_o , I is the baseline offset constant, ω is the angular velocity, R is the gas constant ($8.134 \times 10^7 \text{ erg mol}^{-1} \text{ K}^{-1}$), T is the temperature in Kelvin, M is the molecular mass, v is the partial specific volume of the protein ($0.71423 \text{ cm}^3/\text{g}$ calculated from the amino acid composition according to Makhatadze et. al. [18]) and ρ is the density of the solution (considered 1 g/cm^3). The goodness of the fit was assessed by the quality of the residuals.

Results and discussion

Because of the inherent tendency of SAA to aggregate during refolding, the protein is purified under denaturing solvent conditions (i.e. 6M urea). The last step in our purification protocol involves a refolding step in which the buffer is exchanged via dialysis to 20 mM Tris, pH 8.1 at 4°C (see Materials and methods for details). After this dialysis step, analysis of refolded SAA2.2 by size-exclusion chromatography (SEC) resulted in a major peak whose retention time was consistent with the molecular mass (93 kDa) of an octameric species. A smaller overlapping peak (about 30% area) from hexameric SAA2.2 was also observed, as well as a much smaller (<10%) monomer peak (Fig. 1A). Interestingly, when refolded SAA2.2 was incubated in the refrigerator and monitored at different times by SEC at 4°C, the size of the octamer peak decreased compensated by an increase in hexamer peak (Fig. 1A). This octamer to hexamer conversion was slow, requiring 3-4 weeks for most of the SAA2.2 to acquire the hexameric structure.

To confirm the oligomeric state of fresh SAA2.2 (soon after dialysis) and mature SAA2.2 (after 30 days of incubation at 4°C), analytical ultracentrifugation (AUC) sedimentation equilibrium (SE) experiments were performed using different protein loading concentrations and three centrifugation speeds. The sedimentation traces were globally fitted according to the single ideal species model (eq 1 in Materials and methods). Fig. 1B shows the AUC profiles for the “mature” protein. The molecular weight (MW) obtained from the global fit was $70.5 \pm 0.6 \text{ kDa}$, in excellent agreement with the theoretical MW for the hexameric protein (70.0 kDa). Analysis of the AUC traces for the “fresh” protein according to equation 1 resulted in a MW of $85.1 \pm 0.5 \text{ kDa}$. This is significantly larger (22%) than the MW for the hexameric protein and notably lower (9%) than that of the octameric protein, but it is consistent with the MW expected for the 2:1 octamer:hexamer distribution observed by SEC (Fig. 1A), suggesting that these species are in equilibrium.

The conversion from octameric to hexameric SAA2.2 suggests that the latter is more stable than the former. Both oligomers showed similar CD spectra with high alpha helical content (Fig. 2A). Therefore, we monitored the temperature-induced denaturation of octameric and hexameric SAA2.2 at 222 nm by far-UV circular dichroism (CD). In previous studies we showed that the thermal denaturation of SAA2.2 hexamer is irreversible and exhibits a denaturation midpoint (T_m) of $\sim 32^\circ\text{C}$ [15]. It should be noted that because the thermal denaturation of SAA2.2 is irreversible, the T_m values obtained are not equilibrium parameters. Interestingly, the thermal denaturation of freshly refolded SAA2.2 at low concentration (0.1 mg/ml) for which the octamer represents more than 95% (see Fig. 4A) revealed that indeed the SAA2.2 octamer is less stable than the hexamer, exhibiting a T_m of $\sim 22^\circ\text{C}$ (Fig. 2B). The T_m of octameric SAA2.2 suggests that if refolding were performed at RT ($\sim 22^\circ\text{C}$) instead of 4°C, the octameric species would be significantly reduced, perhaps allowing more SAA2.2 monomers to refold into the hexamer. Hence, SAA2.2 was refolded via dialysis at RT, and as a control, SAA2.2 from the same stock was also refolded at 4°C using the same buffer and dialysis procedure. SEC analysis after dialysis at RT for $\sim 12 \text{ h}$ showed that the fraction of octameric SAA2.2 formed was reduced to about 50-55%, consistent with the population of octamer expected at its T_m (Fig. 3).

The mechanisms of SAA2.2 octamer and hexamer formation, and the subsequent octamer to hexamer conversion are not clear. One scenario is that SAA2.2 may fold into a dimeric conformation that favors either the octamer or the hexamer. The lack of populated SAA2.2 intermediates in SEC experiments suggests that formation of the putative oligomeric intermediate(s) is the rate-limiting step, after which the oligomers quickly assemble to form either octamer or hexamer. If that were the case, then the conversion of SAA2.2 octamer to the more stable hexamer should be concentration-dependent because hexamer formation must occur through the rate-limiting formation of a smaller (e.g. dimeric or trimeric) intermediate. Therefore, we refolded SAA2.2 at several concentrations (0.25, 0.8, and 1.8 mg/mL), and monitored at 4°C the octamer to hexamer conversion. Fig. 4 shows that the octamer to hexamer transition was accelerated at higher SAA2.2 concentrations, in support of a mechanism where the self-assembly of a dimeric or trimeric intermediate is the rate-limiting step in hexamer formation.

The ability of SAA2.2 to form multiple oligomeric structures is not unique, as other proteins have shown this feature, including interleukin-6 (IL-6), [19] UvrD helicase [20], geranylgeranyl diphosphate synthase [21], protective antigen protein subunit of the anthrax toxin [22,23], N-terminal domain of leucine-rich repeat kinase 2 [24], LIM domain binding proteins [25], and Apolipoprotein A-I [26]. This ability to switch structures appears to be a biological mechanism for a protein to regulate or carry out multiple functions. A most interesting and well-studied example is human porphobilinogen synthase, which largely exists in an equilibrium between high-activity octamers and low-activity hexamers that interconvert via different dimer conformations [27,28]. The existence of multifunctional proteins being regulated by changes in oligomeric structure might be more common than previously anticipated, and have been categorized by the term “morphoein”, which refer to proteins with the ability to fold into monomers of different conformation that self-assemble into various functionally distinct quaternary structures [29]. Thus, the ability of SAA2.2 to form different oligomers appears to be consistent with the concept of morphoein.

Because the structure of the SAA2.2 hexamer and octamer are unknown, one cannot rule out the possibility that they might have significant differences in their overall fold. This would not be surprising, as there is an increasing number of proteins that possess low stability and are able to fold into more than one three dimensional structures [30]. Although the SAA2.2 oligomers described here are marginally stable *in vitro* and SAA might be intrinsically disordered *in vivo*, different SAA structures might be stabilized by crowding effects and ligand binding, as well as by the nearly 1000-fold increase in SAA levels during inflammation. Given the many examples of proteins that can form multiple structures it seems implausible that the formation of these SAA2.2 quaternary structures is coincidental. Therefore, we suggest that SAA's seemingly multifaceted biological roles might be allowed by possessing an intrinsically disordered conformation that is modulated *in vivo* to form different functionally distinct quaternary structures.

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Research Highlights

- SAA2.2 folds into a kinetically favored octameric species that slowly converts to a hexamer at 4°C.
- The octamer is ~10°C less stable than the hexamer, explaining the octamer to hexamer conversion.
- Data suggest that a dimeric/trimeric intermediate is the rate-limiting step in hexamer formation.
- SAA might be modulated *in vivo* to form different biologically distinct oligomeric species.

Fig. 1.

Refolding of SAA2.2 monitored by size-exclusion chromatography (SEC) (A), and analytical ultracentrifugation (AUC) sedimentation equilibrium (SE) analysis of “mature” (B) and “fresh” (C) SAA2.2. Denatured SAA2.2 (0.8 mg/mL, 20 mM Tris, 150 mM NaCl, 6M Urea, pH 8.2) was dialyzed against Tris buffer (20mM Tris, pH 8.2) at 4°C and its oligomeric structure was followed by SEC for 1 month. SAA2.2 refolds initially to an octameric species that later rearranges to a hexamer (A). Representative AUC-SE traces at 10,000 rpm (○), 12,000 rpm (□) and 19,000 rpm (△) for SAA2.2 (20 mM Tris pH 8.2, 150 mM NaCl, 4°C) are shown, where the solid lines represent the global fit of mature (B) and fresh (C) SAA2.2 according to equation 1. The lower panel shows the residuals for each fit. The molecular weight (MW) obtained from the fit for the “mature” and “fresh” protein was 70.5 ± 0.6 kDa and 85.1 ± 0.5 kDa, respectively.

Fig. 2. CD secondary structure characterization (A) and thermal denaturation (B) of SAA2.2 hexamer and octamer. Far-UV CD spectrums of SAA2.2 hexamer (■) and octamer (◆) at 4 $^{\circ}\text{C}$ show that they have similar secondary structure. Thermal denaturation curves of SAA2.2 hexamer (■) and octamer (◆) monitored by far-UV CD indicate that the octamer is ~10 $^{\circ}\text{C}$ less stable than the hexamer. Samples contained 0.1 mg/mL SAA2.2 in 20 mM phosphate buffer.

Fig. 3.

Refolding of SAA2.2 (0.8mg/ml in 20mM Tris, 6M Urea, 150mM NaCl, pH 8.2) was performed at room temperature (RT) against Tris buffer (20 mM Tris, pH 8.2). After ~12h ~25 μ l of the sample was loaded in an analytical gel filtration column to determine the oligomeric state of SAA2.2. A sample from the same SAA2.2 stock was refolded at 4°C as a control, and formed mostly an octamer species.

Fig. 4.

Concentration dependence conversion of SAA2.2 octamer to hexamer. SAA2.2 was purified and dissolved in buffer A (20mM Tris, 6M Urea, 400mM NaCl, pH 8.) and aliquoted at three different concentrations (A: 0.25mg/ml, B: 0.8mg/ml and C: 1.8mg/ml) and allowed to refold at 4°C in for 14hrs in 500 – 1000X refolding buffer (20mM Tris, pH 8). SAA was aliquoted in sterile tubes and stored under refrigerated conditions. At regular intervals (as specified in the figure) samples were analyzed on a Superdex 200® analytical gel filtration column.