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Thermophilic Ferritin 24mer Assembly and Nanoparticle Encapsulation Modulated by Interdimer Electrostatic Repulsion

Katherine W. Pulsipher, Jose A. Villegas, Benjamin W. Roose, Tacey L. Hicks, Jennifer Yoon, Jeffery G. Saven, and Ivan J. Dmochowski*

University of Pennsylvania, Department of Chemistry, 231 South 34th Street Philadelphia, PA 19104, Phone: 215-898-6459

Abstract

Protein cage self-assembly enables encapsulation and sequestration of small molecules, macromolecules, and nanomaterials for many applications in bionanotechnology. Notably, wild-type thermophilic ferritin from *Archaeoglobus fulgidus* (AfFtn) exists as a stable dimer of four-helix bundle proteins at low ionic strength, and the protein forms a hollow assembly of 24 protomers at high ionic strength (~800 mM NaCl). This assembly process can also be initiated by highly charged gold nanoparticles (AuNPs) in solution, leading to encapsulation. These data suggest that salt solutions or charged AuNPs can shield unfavorable electrostatic interactions at AfFtn dimer-dimer interfaces, but specific “hot-spot” residues controlling assembly have not been identified. To investigate this further, we computationally designed three AfFtn mutants (E65R, D138K, A127R) that introduce a single positive charge at sites along the dimer-dimer interface. These proteins exhibited different assembly kinetics and thermodynamics, which were ranked in order of increasing 24mer propensity: A127R < WT < D138K << E65R. E65R assembled to the 24mer across a wide range of ionic strengths (0 – 800 mM NaCl), and the dissociation temperature for the 24mer was 98 °C. X-ray crystal structure analysis of the E65R mutant identified a more compact, closed-pore cage geometry. A127R and D138K mutants exhibited wild-type ability to encapsulate and stabilize 5-nm AuNPs, whereas E65R gained ability to remain assembled in apo-form. This work illustrates designed protein cages with distinct assembly and encapsulation properties.

Graphical abstract

*Corresponding Author: Department of Chemistry, University of Pennsylvania, 231 S. 34th St. Philadelphia, PA 19104. Phone: (215) 898-6459. ivandmo@sas.upenn.edu.

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Protein cages are large, multi-subunit structures with hollow interiors. Because of their size and unique structural properties, these cages are useful in many applications including bio-targeting,^{1,2} drug delivery,^{3,4} catalysis,^{5,6} and nanoelectronics.⁷ Ferritins make up a ubiquitous family of protein cages, important for iron storage in most organisms. Maxi-ferritins share a similar overall structure: 24 tetrahelical subunits, assembled to form a hollow, roughly spherical shape. Eukaryotic ferritins remain stably assembled as 24mers over a wide pH range.⁸ Some prokaryotic ferritins exist as mixture of fully assembled 24mer and disassembled dimer.⁹ The dimer is a common intermediate in ferritin self-assembly, and the 24mer/dimer distribution can be altered by single point mutations. Orner et al. identified several “hot spot” residues in *E. coli* bacterioferritin and ferritin-like DNA binding protein from starved cells (DPS) whose mutation either shut down assembly entirely^{10,11} or stabilized it.^{12,13} They accomplished this by modulating hydrophobic interactions, either by plugging water pockets with aromatic amino acids,^{12,13} by disrupting electrostatics along the dimer interface,¹¹ or by replacing amino acids with alanine.^{10,14} In *E. coli* ferritin A, single point mutations to alanine at the 3-fold axes decreased 24mer cage stability.¹⁵ Similarly, Theil et al. prevented assembly of bullfrog ferritin cages by inserting positively charged amino acids in the loop region of each subunit, leading to repulsion of like charges.¹⁶ More extensive modification involving cleavage of the last 23 amino acids in the monomer polypeptide chain enabled changes in the pH-dependent self-assembly of human H ferritin.¹⁷ Human H ferritin was also redesigned to self-assemble only in the presence of copper ions.¹⁸

In this paper, we investigate the self-assembly of thermophilic ferritin from the archaeon *Archaeoglobus fulgidus* (AfFtn), which has unique salt-dependent assembly, not-yet-found in other ferritins. Shown in Figure 1, at high ionic strength (800 mM NaCl), the 24mer is the dominant assembly state. At lower ionic strength ([NaCl] <200 mM), the protein disassembles into stable dimers, which we refer to herein as the protein subunits.^{19,20} We have used the ionic strength-dependent self-assembly of AfFtn to encapsulate citrate- or bis(*p*-sulfonatophenyl)phenylphosphine (BSPP)-functionalized gold nanoparticles (AuNPs), rendering them more biocompatible and stable to salt-induced precipitation.^{20–22} The encapsulation process happens under mild conditions, at room temperature with gentle agitation. The resulting AfFtn-AuNP assembly maintains native protein secondary structure, subunit stoichiometry, melting temperature, and ferroxidase activity, thus highlighting unusual protein-AuNP complementarity.

The crystal structure of AfFtn (PDB ID 1SQ3)¹⁹ contains a trimeric interface rich in negatively charged residues. We hypothesized that electrostatic repulsion at this interface prevents subunit assembly at low ionic strength and neutral pH, given that the estimated pI

of AfFtn is 4.7. Subunit-subunit electrostatic repulsion was found to be important in governing the rate of self-assembly of *E. coli* ferritin A, which typically exists only as 24mer except in acidic conditions.²³ As other ferritins do not feature salt-mediated self-assembly, AfFtn provides a unique opportunity to investigate the role of subunit interface electrostatics in protein cage formation.

We hypothesized that decreasing electrostatic repulsion between anionic subunits by designed amino acid substitution should promote 24mer formation at low-salt concentrations. We tested this hypothesis by introducing positively charged groups at various positions along the dimer-dimer interface. As we have employed previously in the redesign of ferritin proteins,^{24,25} a statistical computational design strategy was used to calculate theoretical amino acid probabilities at selected sites. The resulting probabilities were used to guide the selection of point mutations likely to be compatible with the overall protein structure as well as the supramolecular assembly. Three single-point mutants (shown in Figures 1 and 2) were experimentally characterized, where each mutation substituted a single negatively charged or neutral residue for a positively charged one. We investigated the thermal stability of the mutants and explored changes in the self-assembly equilibrium, kinetics, and reversibility at different salt concentrations. Finally, we tested the capability of each mutant to encapsulate and stabilize AuNPs. We conclude that altering electrostatic interactions between ferritin subunits provides a versatile approach to modulating protein cage assembly.

Experimental Procedures

Computational Design of AfFtn Mutants

The template structure consisted of a trimer of subunits comprising chains G, H and J from the crystallographic structure of *Archaeoglobus fulgidus* thermophilic ferritin 24mer (PDB ID 1SQ3¹⁹). Amino acid probabilities were calculated independently for sites 34, 65, 127, 131, and 138. Sites 127, 131, and 138 are situated along a helical interfacial region. Sites 34 and 65 were chosen as they form, along with site 138, the center of a carboxylate-rich pore. Eighteen natural amino acids were considered at each site selected; cysteine and proline were precluded. For each mutation calculation, residues other than the site of interest were constrained to the crystal structure conformations. Side-chain conformational states of mutated sites were taken from a rotamer library.^{26,27} An entropy-based, probabilistic formalism was used to calculate the probabilities of the amino acids and their rotamer conformations.^{28–31} Using CHARMM19,²⁶ hydrogen atoms were added, and energies were calculated using the dihedral, van der Waals, and electrostatic terms, with a non-bonded cut-off of 8 Å. The probabilistic approach has an effective parameter β that is conjugate to the average energy over sequences and side chain conformations; $\beta = 0.5$ mol/kcal for these calculations.^{28–30} The probability of each amino acid at a mutated site was the sum of the calculated probabilities of its side-chain conformations.

Mutagenesis

Site-directed mutagenesis was used to generate the mutant AfFtn plasmids using the Quik Change kit (Stratagene). Primers used to generate the mutations were purchased from

Integrated DNA Technologies and are listed in the SI. Mutations were confirmed using Sanger Sequencing on ABI 3730, provided by the DNA Sequencing Facility at the University of Pennsylvania.

Protein Expression and Purification

The plasmids were transformed in *E. coli* BL21-CodonPlus(DE3)-RP cells and cultured overnight in LB medium at 30 °C, supplemented with 100 µg/mL ampicillin and 35 µg/mL chloramphenicol. The cultures were then transferred to 1 L terrific broth (TB) supplemented with the same antibiotics and grown at 37 °C until OD₆₀₀ reached ~0.6-0.8. The cells were then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h. Cells were lysed by treating with lysozyme (~1 mg/mL) at rt for 30 min, followed by sonication (amplitude of 30, 1 s on, 1 s off, 15 min total processing time). The cell-free extract was obtained by centrifugation (30 min, 6 krpm, 4 °C), and after adding MgCl₂ to a final concentration of 2 mM, the supernatant was treated with DNase for 15 min at rt. The solution was then heat shocked at 80 °C for 10 min and soluble protein was obtained following centrifugation (1 h, 9 krpm, 4 °C). The supernatant was filtered and buffer exchanged to assembly buffer (2 M NaCl, 1 mM EDTA, 20 mM phos, pH 7.6). The protein was concentrated and injected onto a HiLoad 16/60 column equilibrated with high-salt buffer (800 mM NaCl, 20 mM phos, pH 7.6) on an AKTA FPLC (GE Healthcare). Purity was confirmed using SDS-PAGE (4–15% tris-HCl, Mini-Protean TGX gel), shown in Figure S1. By UV-vis spectroscopy no significant DNA contamination was observed (Figure S2). Identity was confirmed using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) as shown in Figure S3, using sinapinic acid as a matrix and linear positive mode ion generation. Protein concentration was determined using the Bradford Assay, with bovine gamma globulin as a standard. Average protein yield was 12 mg/L TB.

Transmission Electron Microscopy (TEM)

TEM samples were prepared using glow-discharged carbon-coated copper grids (Electron Microscopy Sciences). The grids were floated on 5 µL of sample for 5 min, washed with 2 drops of distilled water, and stained with 2% ammonium molybdate or uranyl acetate. Micrographs were obtained using a Tecnai T12 electron microscope (FEI) operating at 120 keV. Images were analyzed using ImageJ.³²

Circular Dichroism Spectroscopy (CD)

CD data were obtained using an Aviv 410 CD spectrometer (Aviv Biomedical; Lakewood, NJ) with 1 mm quartz cuvette (Hellma USA). Protein samples were 0.3 mg/mL in high-salt buffer. For melting point determination, the signal at 222 nm was monitored as temperature increased 1-96 °C, with a 1 min equilibration at each 1 °C step. Signal was averaged over 10 s at each temperature.

Differential Scanning Calorimetry (DSC)

A VP-DSC microcalorimeter (Malvern) was used to measure the T_m of E65R and wt proteins. Buffer scans were run overnight prior to sample measurement. Samples of 0.5

mg/mL protein in high-salt buffer were degassed with stirring at 10 °C before loading into the sample chamber. The temperature range was 10-125 °C with a scan rate of 60 °C/h.

Ferroxidase Activity Assay

Ferritin samples were buffer exchanged to 25 mM tris, pH 7.0 and diluted to 0.1 mg/mL, 800 µL total volume. A fresh solution of 10 mM FeSO₄ was prepared in 5 mM HCl to prevent premature oxidation. Ferroxidase activity was monitored at 315 nm and 37 °C with stirring using an Agilent 8453 UV-visible spectrometer. The absorbance was measured at 10 s intervals over a total time of 600-800 s, adding 480 eq iron per AfFtn 24mer after an initial ~20 s.

Tryptophan Fluorescence

Steady-state fluorescence spectra were collected on a Varian Cary Eclipse fluorescence spectrophotometer (Cary Eclipse software, 2003) using 100 µL quartz cuvette (Starna Cells). Protein samples were prepared at 5 mg/mL and incubated overnight at 4 °C in the appropriate high-salt or no-salt buffer (20 mM phos, pH 7.6). Samples were excited at 295 nm, scanned 300-400 nm, with a PMT voltage of 700-750 V, at 25 °C.

Dynamic Light Scattering (DLS)

DLS data were collected on a Zetasizer Nano ZS (Malvern). Protein samples (5 mg/mL) were prepared in 0.22 µm filtered high-salt or no-salt buffer and incubated overnight at 4 °C. The measurements were done in plastic micro cuvettes at 25 °C, with backscatter detection at an angle of 173°. For kinetics experiments, %24mer was calculated based on the area under the peaks in the intensity distribution.

Size Exclusion Chromatography (SEC)

Samples of 5 mg/mL protein were prepared in 20 mM phos, pH 7.6, varying concentrations of NaCl, and incubated overnight at 4 °C. Samples of 200 µL were injected onto a Superdex200 Increase 10/300 GL column equilibrated with the same buffer, using an ATKA FPLC (GE Healthcare). The samples were run at 4 °C with a flow rate of 0.9 mL/min.

Native Gel Electrophoresis

For PAGE analysis, 2.4 µg protein was loaded onto 4–20% tris– HCl gels (Mini-Protean TGX) and run at 150 V for 90 min, on ice. The running buffer was tris-glycine (25 mM tris, 192 mM glycine, pH 8.3). Agarose gels (0.7%) were run at 100 V for 20 min at room temperature, with 5 mM NaCl, 20 mM phos, pH 7.6 as the running buffer.

X-ray Crystallography

Crystals of E65R were prepared by mixing 2 µL of protein solution (10 mg/mL E65R in high-salt buffer) with 2 µL of precipitant solution (0.45 M potassium phosphate monobasic, 0.3 M sodium phosphate monobasic, 5% (v/v) glycerol) in a sitting drop suspended over a 500 µL reservoir of precipitant solution. The sitting drop was streak seeded with E65R crystals previously grown in the same conditions. The crystallization tray was incubated at 21 °C, and large octahedral crystals appeared within 24 h. Single crystals of E65R were

briefly immersed in cryoprotectant solution (0.8 M potassium phosphate monobasic, 0.8 M sodium phosphate monobasic, 20% (v/v) glycerol) before being flash-cooled in liquid nitrogen. X-ray diffraction data from single crystals of E65R were collected at beamline 9-2 at the Stanford Synchrotron Radiation Lightsource (SSRL). Diffraction data were integrated using iMosflm³³ and processed using AIMLESS³⁴ from the CCP4 suite of programs.³⁵ The structure of E65R was phased in PHENIX^{36–38} by molecular replacement using a monomer of wild-type AfFtn (PDB ID 1SQ3).¹⁹ Structure refinement was performed in PHENIX and manual model adjustments were made in COOT.³⁹ Initial rounds of refinement showed ambiguous density in the 2Fo-Fc map for protein sidechains, so a polyALA model for E65R was used in which all protein sidechains were replaced with alanine. The lack of density in the Fo-Fc difference map after refinement with polyALA E65R affirmed that sidechain conformations could not be confidently assigned based on experimental data. Refinement proceeded until R_{free} converged at its lower limit. The quality of the final model was assessed using MolProbity⁴⁰ and the wwPDB validation server. Data collection and refinement statistics are recorded in Table S1. Figures were generated using PyMOL.⁴¹

Salt Stability Assay

Citrate-capped AuNPs (5 nm) were purchased from TedPella. Ligand exchange to BSPP was performed as previously published.²¹ Protein-AuNP samples were prepared by first disassembling AfFtn overnight in no-salt buffer at 4 °C. Disassembled protein was mixed with AuNPs to a final concentration of 0.6 μM AfFtn, 0.6 μM AuNP in 0 mM NaCl buffer. The sample was incubated at rt with gentle agitation (gel rocker) for 48 h. After incubation, 50 μL sample was mixed with 50 μL increased salt buffer (0, 400, 800, 1200, 1600 mM NaCl, 20 mM phos, pH 7.6) for final salt concentrations of 0, 200, 400, 600, 800 mM NaCl. UV-vis spectra were collected using an Infinite M1000 microplate reader (Tecan).

Results

Computational Design of AfFtn Mutants

In the design calculations, we sought to identify mutations that both introduce a positively charged residue and span the interface between adjacent protomers. We focused on the probabilities of amino acid type (a) at each site i , $P_i(a)$, or the ratio relative to the wild-type residue $P_i(a)/P_i(a_{\text{wt}})$. At site 34, the wild-type Asp was the most probable residue, and as a result, this site was not selected for mutation. At site 65, Arg was the most probable amino acid ($P_{65}(\text{R}) = 0.83$), yielding the suggested mutation E65R. At site 127, Arg was the most probable amino acid ($P_{127}(\text{R}) = 0.63$), and the mutation A127R was selected. At site 131, Lys was the most probable amino acid $P_{131}(\text{K}) = 0.93$. Upon examining the resulting model structures, the conformations of this side chain directed the ammonium group within the protomer, and the side-chain interactions did not span an interface with other protomers; this site was not selected for mutation. At site 138, the four most probable amino acids were Arg, Asn, Asp, and Lys, and $P_{138}(\text{K})/P_{138}(\text{D}) = 1.49$. The mutation D138K was selected at this site.

General Protein Characterization

We prepared E65R, A127R, and D138K single point mutants using standard site-directed mutagenesis. After expression and purification, protein purity was verified by SDS-PAGE and UV-vis spectroscopy (Figures S1-S2) and identity was verified by MALDI-TOF MS (Figure S3). We then verified cage formation in 800 mM NaCl by transmission electron microscopy (TEM). Shown in Figure 3 and summarized in Table 1, cages with approximately the same diameter as wild type (wt) were observed in TEM micrographs, showing the mutations did not inhibit self-assembly in high ionic strength solution. Dynamic light scattering (DLS) also showed similar results, with all mutants having average particle diameters within 2 nm of wt ($D_{\text{avg}}(\text{wt}) = 13.5 \text{ nm}$). Circular dichroism (CD) spectra for all three mutants showed almost no change compared to wt, demonstrating a lack of perturbation in secondary structure (see Figure S4). This is unsurprising, as ferritins and ferritin-like proteins have been shown to be stable to extensive mutagenesis.²⁵

Next, we investigated the thermal stability of the mutants using CD spectroscopy and calorimetry (Figure S5). At 0.3 mg/mL, the temperature-dependent molar ellipticities of A127R and D138K yielded nearly identical thermal stability to that of wt AfFtn ($T_m = 84 \text{ }^{\circ}\text{C}$, Table 1). However, E65R did not unfold for $T < 96 \text{ }^{\circ}\text{C}$, and we were unable to measure its T_m by CD. Instead, we turned to differential scanning calorimetry (DSC), which uses a pressurized, sealed sample chamber, enabling us to measure higher melting temperatures in aqueous conditions. By DSC at 0.5 mg/mL, we measured a $T_m = 98 \text{ }^{\circ}\text{C}$ for E65R; using the same technique, we measured $T_m = 84 \text{ }^{\circ}\text{C}$ for wt, which confirmed the CD-determined values (Figure S5b). Remarkably, E65R has thermal stability enhanced by $14 \text{ }^{\circ}\text{C}$ compared to the hyperthermophilic wt protein and other two mutants. Although CD monitors changes in protein secondary structure and does not necessarily yield information about the assembly state, the single sharp transitions observed in the CD and DSC suggest that disassembly of the 24mer may be concomitant with unfolding of the subunits under these conditions.

Shown in Figure S6, we also investigated the enzymatic activity of the mutants using an absorbance-based ferroxidase assay.⁴² An aliquot with 480 eq of Fe^{2+} was added to each sample and oxidation to Fe^{3+} was monitored by the increase in iron mineral absorbance at 315 nm. E65R had slightly enhanced activity compared to wt, while D138K had slightly diminished activity and A127R had significantly diminished activity. A127R is closest to the ferroxidase site of the protein and likely interacts with glutamates in positions 128 and 131, potentially perturbing their activity. In light of minimally affected structural features and thermal stability mentioned above, this represents a localized disruption of the ferroxidase site compared to wt AfFtn.

Self-assembly Equilibria

After general characterization of all mutants, we were interested in how mutagenesis affected the dimer-24mer equilibrium. To investigate the assembly state of the proteins at varying salt concentrations, we used size exclusion chromatography (SEC), tryptophan fluorescence, DLS, and native gel electrophoresis.

SEC was performed after incubating each protein overnight at 5 mg/mL in solutions of different salt concentrations, as seen in Figure 4. The peak in the SEC trace at ~10 mL was attributed to 24mer, while the peak at ~14 mL was attributed to dimer (see Figure S8), based on column MW calibration. The area under the peaks was used to quantify the percentage 24mer for each protein at each salt concentration. As expected, at high-salt conditions (800 mM NaCl), all proteins showed nearly 100% 24mer. At lower salt conditions (< 200 mM NaCl), different behaviors were observed. A127R is slightly less likely to self-assemble in low-salt conditions compared to wt, while D138K has slightly higher propensity to assemble, with higher 24mer populations than wt at 0, 20, and 100 mM NaCl. E65R shows >90% 24mer under all salt conditions tested, demonstrating a dramatic change in self-assembly equilibrium.

To corroborate SEC results, we used Trp fluorescence, which is reflective of the solvation environment of Trp residues in the protein (Figure 5a). AfFtn contains four Trp residues per single-chain subunit, two of which are predicted to see some change in solvation upon disassembly (Trp44 and Trp124). For wt, in 800 mM NaCl buffer where AfFtn is completely assembled, the Trp emission maximum is at 332 nm. In 0 mM NaCl buffer, where AfFtn is disassembled into dimers, the emission red shifts to 337 nm, consistent with the Trp residues becoming more solvent exposed. We measured the Trp fluorescence spectrum for each protein in 0 and 800 mM NaCl buffer. All of the mutants exhibited similar fluorescence spectra to wt at 800 mM NaCl, with similar peak shapes and maximum emission wavelengths. E65R showed a slight blue shift compared to wt, with an emission maximum of 331 nm compared to 332 nm for wt. At 0 mM NaCl, significant changes were observed. A127R and wt had the largest red shifts, moving to 337 and 338 nm, respectively, indicating disassembly for both proteins. D138K had a red shift of only 2 nm, while E65R only shifted by 1 nm. These trends for D138K and E65R match those observed by SEC, with E65R showing minimal structural changes with changing salt concentration, and D138K showing smaller changes than wt.

DLS corroborated Trp fluorescence results showing A127R with similar self-assembly behavior to wt, with decreased 24mer population at low-salt conditions (Figure 5b). Like wt, A127R 24mer was present at 800 mM NaCl and not at 0 mM NaCl. DLS also indicated that at 5 mg/mL protein and 0 mM NaCl, A127R, wt, and D138K (to a smaller extent) form a broad range of higher molecular weight aggregates. These aggregates are too weakly associated to withstand FPLC treatment as no aggregates were observed on the sizing column under any conditions. Aggregate formation is concentration-dependent, as wt samples at 1 mg/mL had individual dimer visible in DLS data, but at 5 mg/mL only aggregate was present (Figure S9). D138K was predominantly 24mer at 800 mM NaCl and mostly dimer (with some 24mer) at 0 mM NaCl, whereas E65R remained fully assembled at both salt concentrations. Native gel results also support the uniqueness of the E65R mutant, as shown in Figure S10, where E65R was the only protein to run similarly to horse spleen apoferritin (HSAF, used as a control 24mer due to its lack of salt-mediated disassembly). WT, D138K, and A127R all ran as smaller species.

Self-assembly Kinetics

By monitoring particle size using DLS, we were also able to investigate differences in the kinetics of 24mer assembly for wt, A127R, and D138K. Because E65R does not disassemble with decreasing ionic strength, no changes were observed by DLS or SEC upon changing the buffer conditions. We started with disassembled protein in 0 mM NaCl buffer, which was transferred to 800 mM NaCl buffer to induce self-assembly. The rate of assembly was concentration-dependent, particularly for A127R and wt (Figure 6). For wt protein at 5 mg/mL, assembly appeared to be complete within 4 h. For A127R at 5 mg/mL, assembled 24mer population increased up to 75% over 4 h, and then stalled with no further assembly. At 2 mg/mL, both wt and A127R took approximately 24 h to reassemble. At 1 mg/mL, wt assembly occurred within 10 min (the time it took to prepare samples and take the DLS measurement), and A127R was >90% 24mer within 2 h. Assembly was significantly faster for D138K compared to wt and A127R at 2 and 5 mg/mL. At these concentrations the D138K samples were >90% 24mer within 10 min. At 1 mg/mL, assembly was still quite fast, with a population of >80% 24mer within 10 min. Rapid assembly kinetics with D138K at all concentrations tested is consistent with lack of aggregate formation, and an orderly process of subunit assembly. This is in contrast to wt and A127R, both of which started from large aggregates, particularly at 5 mg/mL (see Figure S11 for representative size distributions at varying time points). This feature should lead to more reversible and higher-yielding ferritin disassembly and assembly processes with D138K, e.g., as required for cargo loading.

We observed a less dramatic difference among the protein disassembly kinetics, moving samples from 800 mM NaCl to 0 mM NaCl. By DLS, A127R and wt appeared to disassemble immediately, producing large aggregates. The disassembly of D138K was more difficult to monitor by DLS due to the relative similarity in diameter of 24mer and dimer (and lack of aggregates). By SEC, as shown in Figure S12, we could observe disassembly for all three proteins within 25 min (the measurement time). Some 24mer remained for D138K within this time frame, which matched the results for the 0 mM NaCl equilibrium measurement (Figure 4).

X-ray Crystallography

To better understand the significantly enhanced stability of E65R, we obtained a 3.08 Å resolution X-ray crystal structure (PDB ID 5V5K). Although the quality of the electron density maps does not allow for sidechain conformations to be confidently modeled, the crystallography data do offer insights pertaining to the global structure of the E65R mutant. As shown in Figure 7, the structure of 24mer E65R shows a shift in the symmetry of the assembled cage to octahedral (as opposed to the tetrahedral structure of wt), resulting in a lack of the large triangular pores. The 544,000 Å³ volume calculated from the structure of the E65R cage using the Voss Volume Voxelator program⁴³ is roughly 10% smaller than the 600,000 Å³ calculated for a polyALA version of wt (PDB ID 1SQ3)¹⁹ – a finding also reflected in our TEM and DLS results (Table 1). These volumes correspond to outer diameters of 10.1 nm and 10.5 nm for E65R and wt, respectively.

Gold Nanoparticle Encapsulation

Having established differences in self-assembly patterns for the various proteins, we next investigated their interaction with 5 nm AuNPs coated in BSPP. We showed previously that BSPP-coated, 5-nm AuNPs are more stably encapsulated within wt-AfFtn compared to citrate-coated AuNPs.²¹ To encapsulate the NPs, we first incubated the proteins at 0 mM NaCl overnight at 4 °C to allow for maximum disassembly. We then added AuNPs and incubated for 48 h at room temperature with gentle agitation. After 48 h the presence of AuNP did not disrupt the 2° structure of any of the proteins when incubated at a ratio of 1:1 AfFtn 24mer:AuNP (Figure S13). However, by native agarose gel electrophoresis some differences were observed among the samples. By 48 h, wt, A127R, and D138K appeared to successfully encapsulate AuNPs as judged by cleanly overlapping blue protein and red AuNP bands, while the AuNP bands in the E65R-containing sample remained diffuse (Figure S14). Shown in Figure 8, successful encapsulation was observed for wt, A127R, and D138K at a ratio of 1:1 AfFtn 24mer:AuNP. In contrast, the AuNP bands for the E65R-containing samples were significantly more diffuse, indicating greater variety in particle charge:mass ratio. Two bands are visible in the Coomassie stained image, with the less intense band overlapping with the darkest part of the AuNP bands. This suggests that although some AuNPs may be encapsulated within the E65R cavity, many are not, likely due to less disassembly of the protein cage.

We investigated the ability of the protein to stabilize the AuNPs against salt-induced aggregation. With increasing ionic strength, electrostatically-stabilized AuNPs begin to aggregate, causing a red shift in the surface plasmon resonance (SPR) peak.⁴⁴ By monitoring the SPR peak with increasing concentration of NaCl, we could observe how well the proteins passivated the AuNP surface (Figure 9). Bare AuNPs had the largest SPR red-shift of over 40 nm, from 0 to 800 mM NaCl. E65R-AuNP had the next largest shift of approximately 20 nm, while wt-AuNP, A127R-AuNP, and D138K-AuNP all had similarly small red shifts of less than 10 nm. This suggests that E65R does not passivate the surface of the AuNP, in agreement with the native gel results. As a 24mer, E65R likely interacts with the AuNP surface, but provides a smaller stabilizing effect compared to wt, A127R, and D138K. By TEM (Figure S15), several 24mer cages can be seen in contact with the AuNP surface, supporting this hypothesis.

Discussion

We have expressed and characterized three novel AfFtn mutants, each replacing a negative or neutral residue with one that is positively charged. None of the mutations decreased the thermal stability nor hampered the ability of the protein to self-assemble into a nanocage at high ionic strengths. However, the results presented here demonstrate the profound effect a single point mutation can have on the self-assembly of AfFtn. This is in keeping with recent literature, where *E. coli* bacterioferritin,¹¹ ferritin-like DNA binding protein from starved cells (Dps),¹⁰ and bullfrog ferritin¹⁶ were also shown to be susceptible to changes in self-assembly through minor mutagenesis. While the AfFtn mutations changing a negative residue to a positive one (D138K and E65R) showed increased 24mer population in low ionic strength solutions, changing a neutral residue to a positive one (A127R) showed slight

destabilization of the 24mer. Even between D138K and E65R there were significant differences in the favorability of 24mer assembly, with E65R remaining >90% 24mer in all salt concentrations tested and D138K disassembling at low-salt conditions. The specific interdimer location of the point mutation greatly affects self-assembly.

It is notable that E65R shows enhanced thermal stability in addition to the formation of stable 24mer assemblies at low ionic strengths. Increased thermal stability can often go hand-in-hand with enhanced cage stability. For example, when the self-assembly equilibrium of *E. coli* bacterioferritin¹² was shifted from a mixture of dimer and 24mer to 100% 24mer, the T_m increased by over 20 °C. Similarly, destabilization in favor of dimers has led to decreased thermal stability in mycobacteria ferritin,⁴⁵ *E. coli* ferritin A,¹⁵ Dps,¹⁰ and *E. coli* bacterioferritin.¹¹ For wt AfFtn, the T_m in low-salt conditions was found to be essentially the same as that in high-salt, indicating that stability of the dimer is very similar to that of the assembled cage and that 24mer assembly does not increase the stability of the dimer.²¹ However, in *E. coli* bacterioferritin, mutations designed to plug an interdimer water pocket with hydrophobic residues led to significantly enhanced thermal stability ($T_m > 20$ °C) but greater dimer population compared to the wt, as the geometry of the more stable dimers prevented cage formation.¹³ Although A127R appears to favor dimer at low-salt conditions, its subunit thermal stability is identical to wt. For D138K, its enhanced 24mer stability at low-salt conditions also does not appear to be linked to thermal stability, as it too exhibits the wt T_m . The stabilities of the individual protein subunits, their oligomeric assemblies, and the 24mer assembly are coupled and can be difficult to resolve.

At low ionic strengths, all the results support enhanced cage stability for E65R and D138K. The symmetry shift seen in the crystallographic structure of E65R is striking (Figure 10a), but such closed-form (octahedral) structures need not exhibit enhanced cage stabilities. A double mutant of AfFtn, K150A/R151A, was previously shown to yield octahedral cage symmetry; however, this mutant maintained the salt-dependent disassembly and reassembly behavior of the wt protein.⁴⁶ The crystallographic structures of E65R and K150A/R151A are highly similar, with an alpha-carbon coordinate root-mean-square-deviation (RMSD, as calculated using VMD⁴⁷) of RMSD = 2.19 Å for the assembled 24mer cage (see alignment of structures in Figure S16). A trimer of subunits from the tetrahedral wild-type structure was used in the computational design of the mutants. The structure of this trimer is retained in both the open (tetrahedral, wild-type) structures and in the closed (octahedral) structures of the mutants E65R and K150A/R151A. The crystallographic structures these trimers in the wild-type, E65R and K150A/R151A are similar, with an alpha-carbon RMSD = 1.40 Å between E65R and wild-type and RMSD = 1.32 Å between E65R and K150A/R151A (see alignment of structures in Figure S17). It is remarkable that in our study a single point mutation led to such a dramatic change in assembly behavior. This symmetry shift can be rationalized using the structures of the 24mer and the computationally predicted side chain conformation of R65. In the wildtype tetrahedral assembly, the amino acid exists in two distinct environments due to the symmetry of the cage. In one environment, R65 on one subunit is positioned directly across from R65 of a neighboring subunit (Figure 10b), resulting in electrostatic repulsion. In the octahedral assembly, however, R65 is in only one environment. The residue is not in proximity to an R65 residue on a separate protomer, which is consistent with octahedral assembly being preferred (Figure 10a). Within the model

of E65R, R65 and D138 form a salt-bridge at the interface present in both the tetrahedral and octahedral assemblies (Figure 10c), which could lead to the enhanced cage stability observed. A127R was also predicted to form complementary electrostatic interactions (Figure 10d). However, Arg127 is conformationally constrained at an interface that is more sterically crowded than the pore environment where the mutations E65R and D138K were introduced. With A127R, this introduction of a large residue at a subunit interface may reduce the stability of the 24mer at low-salt concentrations relative to the wild type. Within the model of D138K, a K138-D34 salt bridge is observed between neighboring subunits, which may increase the population of 24mer relative to wild type (Figure 10e).

It is striking that we see substantial differences in self-assembly kinetics for the proteins alone, and yet in the presence of AuNPs, protein assembly encapsulating the AuNP seems to be similarly fast for wt, A127R, and D138K. High ionic strength solution perhaps does not model the charged AuNP surface and thus differences seen in protein-only assembly are not observed in the presence of AuNPs. We hypothesize that the AuNP nucleates protein assembly at its surface and may thereby increase the effective concentration of protein in solution, while favoring assembly over possible aggregation pathways. Our DLS results suggest that protein concentration has a large effect on the rate of protein cage assembly, with decreasing protein concentration in some cases leading to faster assembly. This is likely due to minimal aggregation of dimers at low concentrations, allowing assembly to occur. We were unable to monitor reassembly by DLS at 0.3 mg/mL due to low signal-to-noise ratio, but based on our results from 1, 2, and 5 mg/mL samples, we would expect assembly to occur rapidly at 0.3 mg/mL for all three proteins that disassemble.

When NPs are introduced into a biological medium, protein adsorption is rapid and evolves with time. This shell of protein on the NP surface is termed the protein corona.⁴⁸ Protein adsorption has been shown to sterically stabilize NPs against aggregation with increasing salt concentration,^{49,50} similar to our results. A127R, D138K, and wt protein all successfully encapsulated AuNPs as seen by native gel, and prevented aggregation of AuNPs with increasing ionic strength compared to particles without protein present. Although E65R does not encapsulate AuNPs at the same high yields as the disassembling proteins, there is still some level of AuNP stabilization, as the SPR red shift for the E65R-AuNP sample was smaller compared to bare particles. The fully-assembled E65R cage may be adsorbed to the AuNP surface. It is possible that the cage dynamics of E65R are such that some AuNPs are encapsulated, as indicated by faint bands overlapping by native gel (Figure 8). Such is the case for lumazine synthase from *Aquifex aeolicus*, a protein cage capable of encapsulating large protein cargo without first disassembling.⁵¹ The greater 24mer stability and cargo selectivity exhibited by E65R opens up the possibility of designing more specific ferritin-cargo interactions in the future. We are exploring new types of cargo for ferritin encapsulation.

Conclusions

We have shown that single point mutations of AfFtn can have varying effects on thermal stability, assembly symmetry, and self-assembly equilibrium, kinetics, and reversibility. More dramatic charge changes such as changing negatively charged residues to positive ones

increased the stability of the 24mer in decreasing ionic strength, while a less dramatic change, changing a neutral residue to a positive one, had a slightly destabilizing effect. The E65R mutant shows enhanced cage formation as well as thermal stability, formation of the 24mer at low-salt conditions, and self-assembly in octahedral symmetry rather than tetrahedral. The kinetics of self-assembly were also affected by mutation, with A127R showing slower nanocage assembly compared to wt, and D138K assembling faster. These results corroborate earlier studies in other ferritin species, demonstrating the generality of single point mutations along subunit interfaces dramatically affecting cage self-assembly. All mutants showed some salt stabilization of AuNPs compared to bare particles, but only mutants that retained their ability to disassemble showed full AuNP encapsulation. Enhanced control over protein cage assembly could have applications in delivery, nanomaterials separations, and controlled inorganic NP synthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Salt-dependent assembly for wild type AffFn. At high ionic strength (800 mM NaCl), the 24mer cage predominates. At low ionic strength (<200 mM NaCl), the protein disassembles into twelve dimers. The dimer is highlighted in blue in the 24mer cage on the left. Inset shows close-up of mutation positions at the trimeric interface. The crystallographic structure of AffFn (PDB ID 1SQ3) was used to generate the figure.¹⁹

Figure 2. Computationally designed mutations along the trimeric interface. Wild-type residues (dark blue border): (a) E65, (b) A127, and (c) D138. Single-point mutations: (d) A127R (violet border), (e) D138K (teal border), and (f) E65R (orange border). Different protomers (chains) are rendered distinct colors: cyan, yellow and pink.

Figure 3. TEM micrographs and size distributions for wt and mutant AfFtn. Similar cage structures were observed for all samples, indicating mutations did not prevent self-assembly. Grids were stained with either 2% uranyl acetate or 2% ammonium molybdate negative stain. Particle size was measured manually using ImageJ.³² Scale bars are 100 nm.

Figure 4.

Size exclusion chromatography was used to quantify the amount of 24mer present at various salt concentrations for all proteins. Compared to wt (dark blue) at low-salt concentrations, D138K has a slightly larger percentage of the fully formed assembly (orange), and A127R has a slightly larger percentage of the dimer (teal). E65R has greater than 90% assembly at all salt concentrations tested, [NaCl] = 0-800 mM (violet).

Figure 5.

(a) Tryptophan fluorescence results for wt and mutants. (b) Dynamic light scattering results. All proteins in 800 mM NaCl show complete 24mer assembly. At 0 mM NaCl, only E65R remains assembled, while D138K forms discrete dimers, 24mer, and some aggregate, and wt and A127R predominantly form aggregates of dimers. Volume and number size distributions can be found in Figure S7.

Figure 6.

DLS was used to monitor assembly of wt and mutants, starting from dimers. Assembly rate was concentration-dependent for A127R, with faster assembly at lower protein concentrations. WT showed fastest assembly at 1 mg/mL, followed by 5 mg/mL and 2 mg/mL. D138K assembled within the time it took to take the measurement for all protein concentrations tested.

Figure 7.

E65R exists exclusively in its 24-mer state in a closed-pore assembly. (a) Cartoon of E65R crystal structure (PDB 5V5K) with residue 65 highlighted in purple. (b) Open-pore wt AffFn (PDB 1SQ3) with residue 65 highlighted in purple.

Figure 8.

Native gel electrophoresis showed AuNP association for wt, A127R, and D138K at ratios of 1:1 AfFtn 24mer:AuNP, but not E65R, which shows lower propensity for disassembly.

Figure 9.

Changes in SPR peak maximum with respect to salt concentration show higher stability for AuNPs that appear associated with proteins by gel.

Figure 10.

Inter-protomer interactions in mutants of AfFtn. Crystallographic structures of AfFtn with site 65 highlighted as purple sphere (a, b). (a) Crystallographic structure for E65R reported herein (closed pore, octahedral structure, PDB 5V5K). (b) Structure of wt AfFtn (open pore, tetrahedral structure, PDB 1SQ3). Two R65 are in close proximity at one interface. (c-e) Computationally modeled structures of mutants with most probable conformations of mutated side chains. Distinct protomers (chains) have different colors: cyan, yellow, and pink. (c) Within E65R, potential R65-D138 salt bridge. (d) Within A127R, a potential R127-E65 salt bridge within a sterically crowded local environment. (e) Within D138K, a potential K139-D34 salt bridge.

Table 1

Assembled Cage Characterization in 800 mM NaCl.

Sample	D _{avg} TEM (nm) ^a	N ^b	D _{avg} DLS (nm) [PDI] ^c	T _m (°C) ^d
wt	13.2 ± 1.1	101	13.5 [0.035]	84
A127R	13.3 ± 0.9	117	13.6 [0.052]	85
D138K	12.3 ± 1.3	110	14.9 [0.103]	84
E65R	11.1 ± 0.9	173	12.9 [0.059]	98

^aD_{avg} is average diameter.^bN is number of particles that were measured manually using ImageJ³² to calculate average diameter.^cBased on intensity distributions, though volume and number distributions gave essentially the same results. All distributions can be found in Figure S7.^dT_m was measured by CD for wt, A127R, D138K and by DSC for E65R.