ChemComm

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View Article Online
View Journal

# Complete shift of ferritin oligomerization toward nanocage assembly *via* engineered protein–protein interactions†

Cite this: DOI: 10.1039/c3cc40886h

Received 1st February 2013,
Accepted 11th March 2013

DOI: 10.1039/c3cc40886h

www.rsc.org/chemcomm

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**Computational redesign of a dimorphic protein nano-cage at the *C*3-symmetrical interfaces forces it to assemble into the monomorphic cage. These monodisperse assemblies are at least 20 °C more stable than the parent. This approach adds to the toolkit of bottom-up molecular design with applications in protein engineering and hybrid nano-materials.**

The inability to systematically manipulate self-assembling protein nanostructure through molecular design currently impedes the advancement of nanobiotechnology.[1,2] Many of the attempts to rationally design three-dimensionally defined protein quaternary structure have often led to the formation of polydisperse assemblies.[3,4] Only recently have proteins capable of self-assembling into monodisperse nanostructures been successfully designed either through minimal refinement of previously generated fusion proteins[5] or through extensive computational modeling.[6]

Protein cages are nanostructured, hollow and globe-shaped protein complexes whose unique structural properties have been of interest to a wide range of scientific communities from biophysical,[7] medicinal,[8] catalysis,[9] and supramolecular chemistries[10] to materials science.[11] The ferritins are a ubiquitous family of protein cages that are remarkable in that different members of this family can self-assemble into cages of two different sizes and symmetries (12- and 24-mers with either tetra- or octahedral arrangements respectively) in spite of the fact that their monomers have highly similar sequences and structures.[12] Notwithstanding its similarities to other ferritins, bacterioferritin from *E. coli* (*Ec*Bfr) demonstrates a unique property *in vitro*; it exists stably as a mixture of two major oligomerization states—a 24-meric cage and a dimer that, in most ferritins, is believed to be a major self-assembly intermediate.[12–14] The crippled assembly of *Ec*Bfr has been proposed to be due to poorer packing at its twofold and four-fold interfaces compared to those of homologous proteins.[14] We have previously shown that the distribution of these two populations can be altered by subtle mutations.[14,15] Therefore we have exploited this property of *Ec*Bfr to apply it as a model system for the investigation of protein–protein interactions governing the self-assembly of protein cages.

Previously, as part of an alanine scanning study[16] to identify protein–protein interfacial ''hot spots'' that drive self-assembly in the ferritins, we discovered single point mutations at the two-fold dimeric interface of *Ec*Bfr that, while preventing cage formation, resulted in a dimer that was more thermally stable than wildtype (WT).[15] However, this finding was surprising and merely serendipitous, thus leading us to enquire if it would be possible to rationally stabilize oligomers of ferritins through single point mutations at essential protein–protein interactions.

Therefore, in a prequel to this current work, we employed a computational strategy of scanning all possible mutations at the two-fold interface for possible stabilization. This interface was selected because analytical comparisons revealed that, in *Ec*Bfr, its degree of buried surface area is distinctly low among its homologs.[14] Many of the stabilizing mutations predicted by this analysis involved hydrophobic replacement of the residues associated with structural pockets. Based on this observation, we propose a computational design algorithm that is applied for first time in the work presented here (Fig. 1A) (see below). Furthermore, after expression and purification of mutants predicted by this first analysis, we found that three exhibit enhanced thermal stability. However, only one of these demonstrated a shift in the oligomerization state toward cage formation. This lack of correlation between thermal stability and cage assembly, in conjugation with our previous alanine shaving result, suggested the presence of a second, non-assembling and thus non-productive dimer which, while more thermally stable through enhanced ''interfacial packing'', projects the distal protein surfaces with an altered relative geometry, thus arresting further assembly. This notion emphasizes that the partially assembled state must, along with

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3cc40886h

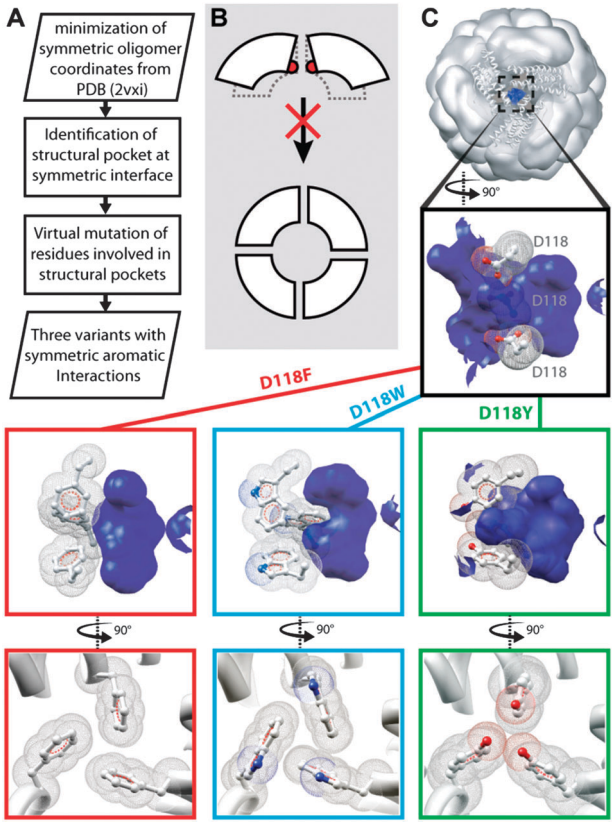


**Fig. 1** (A) Computational workflow to design stabilized protein cages, (B) ''Arch and Keystone'' hypothesis where the red protrusion indicates a mutation that thermally stabilizes a dimer but negatively affects relative monomer orientations and distal protein surfaces, thus disfavoring cage assembly. (C) The mutations D118F, D118W and D118Y bridge the interfacial pocket (the blue surface) at the three-fold axis in *Ec*Bfr (top). Aromatic side chains in the predicted structures of the designed proteins can form edge-to-face interactions (bottom).

presenting the appropriate chemical complementarity, possess the proper geometry to template the docking of additional subunits into the correct nanostructure. This is especially consequential when the final structure is ''closed'' (like cages) as opposed to ''open'' (like fibers, rods, and tapes).[17] Evoking Fisher's ''lock and key'', we styled this concept ''arch and keystone'' from an appreciation that altering the relative surface angles of an architectural keystone can induce geometric frustration of arch assembly (Fig. 1B).

At the initiation of the research documented in this report, we reasoned that targeting the protein–protein interface at the three-fold axes of symmetry would have a greater likelihood of generating a design that achieves both thermal and cage oligomerization enhancement. This expectation is based on the rationale that filling cavities at the three-fold interface would have a lower chance of distorting the vital two-fold symmetric dimer intermediate. This strategy would avoid the disruption of the angular presentation of the distal protein surfaces, thus, the unaltered dimer can easily ''snap'' into the assembling cage. In addition, based on the previous observation that the most stabilizing mutations were associated with interfacial pockets, we reasoned that a more efficient computational search would be to limit our focus to pocket residues rather than the entire interface (Fig. 1A).

Following this minimal computational approach (Fig. 1A), we used CASTp[18] to quantitatively detect the structural pocket at the trimeric three-fold symmetry axis of *Ec*Bfr (PDB ID: 2vxi). The resulting three-fold pocket involves six residues from each protein chain—Y114, R117, D118, I121, E122 and R125 (Fig. S1, ESI†). Virtual mutation of these pocket-forming residues to all other amino acids using the fixed backbone approximation of FoldX[19] revealed a number of stabilizing mutations, many of which converged on hydrophobic replacements of aspartate 118, with D118F, D118W, and D118Y being among the most stabilizing as confirmed by flexible backbone modeling performed using Rosetta Backrub[20] (Table S1, ESI†). The mutations caused the three-fold pocket to contract, with D118W having the strongest effect (Fig. 1C top and Table S3, ESI†). Moreover, analysis of the residue contact map of the mutant three-fold interface indicates that these mutant aromatic interactions unify the three clusters into one extended interaction network across the threefold interface (Fig. S2, ESI†). Based on this analysis, our previous partial success with using aromatic residues to bridge similar pockets, the predominance of aromatic residues in thermodynamically significant protein–protein ''hots spots''[21] and because of the evident edge to face ''pinwheel'' aromatic[22] interactions formed by these residues in the calculated structure, (Fig. 1C, bottom) we selected D118F, D118W, and D118Y for further study.

The three proteins were cloned, expressed, and purified to homogeneity (Fig. S3–S8 and Table S2, ESI†). Full characterization of these proteins demonstrates that they exhibit native-like structure, unaffected by the mutations (Fig. S9 and S10, ESI†). Their ability to form nanoparticulate assemblies was verified through dynamic light scattering (DLS), and transmission electron microscopy (TEM) (Fig. 2A and Fig. S9, ESI†).

To determine whether the redesigned protein–protein interfaces actually result in more thermally stable proteins, the CD signal at 222 nm with respect to temperature was monitored (Fig. 2B and Fig. S11, ESI†). All of the mutants that were engineered to be more stable than the WT protein were indeed more stable as evidenced by an increase in thermal unfolding transition temperature (Tm) by at least 20 °C (Table S3, ESI†) suggesting that our designs are, if nothing else, as successful as those targeting the two-fold axis.[14]

Because we previously found that engineering interfacial interactions at the two-fold symmetry axis of *Ec*Bfr resulted in proteins whose enhanced thermal stability did not correlate with enhancement of cage oligomerization (above), it was essential to rigorously determine the oligomerization state of these new designs focused on the three-fold interface. It was gratifying to observe that all three of the mutants strongly favored the cage state. Using analytical SEC (Fig. 2C), it was determined that WT *Ec*Bfr forms a mixture of dimer and 24-mer, which is consistent with the literature and our previous studies.[12–15] The chromatograms of all the mutants, however, exhibited a single peak with a retention volume similar to the 24-mer of WT *Ec*Bfr with no observable dimer peak. These results were confirmed by native PAGE electrophoresis (Fig. 2D). Consistent with the SEC data, WT *Ec*Bfr separates into two bands corresponding to 24-mer and dimer. All three

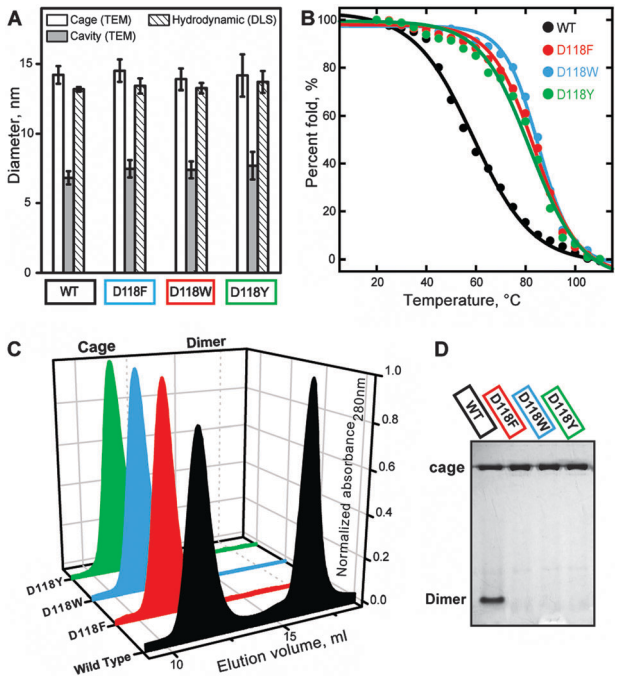


**Fig. 2** Redesigning the three-fold protein–protein interfaces of *Ec*Bfr results in proteins with increased thermostability and a strong preference toward cage formation in solution. (A) The designed mutations do not alter the nanostructure. Cage and cavity sizes of the WT and the designed variants as quantified by the analysis of TEM micrographs (Fig. S9, ESI†) and the hydrodynamic diameter of the protein nanoparticles as calculated from DLS. (B) CD of the wild type (WT) and mutants at 222 nm during thermal denaturation (Full spectra of the denaturation experiments: Fig. S11, ESI†), (C) Distribution of dimer *versus* 24-meric cage as determined by analytical size exclusion chromatography (SEC); curves were normalized so that the height of the highest peak on all curves is identical. (D) Electrophoretic analysis by native PAGE demonstrating the distribution of higher- and lower-order oligomerization states for the WT and each of the mutant proteins. The color-convention follows that of Fig. 1.

mutants displayed a single band with the same motility as the 24-mer band in WT *Ec*Bfr and no dimer was detectable. Thus, it is unambiguous that the designed protein–protein interfaces not only result in proteins that are more thermally stable, they also strongly favor the cage state, conclusively confirming the success of our design strategy.

In conclusion, we have developed and implemented a direct and highly robust strategy utilizing low-level and efficient computation (Fig. 1A) to engineer protein–protein interfaces for the purpose of enhancing self-assembly and thermal stability in a nanostructured protein. By enhancing the self-assembly of this protein nanocage through the bridging of structural pockets at the three-fold symmetric interfaces with aromatic edge to face interactions through single point mutation, we were able to demonstrate the utility of this strategy. Further reinforcing the success of the design and the utility of these proteins, we have recently used them as controls in the development of a novel technique to directly analyze the formation of specific oligomerization states.[23] In addition, this current approach, in comparison to our work at the two-fold axis, suggests that manipulation of interfaces with lower order symmetry has an increased likelihood of negatively affecting the optimum geometry of distal protein surfaces especially when these interfaces control key intermediates in the assembly process. Therefore, our data suggests that the quaternary structure design community could benefit by not only optimizing direct interactions, but also the geometry of assembly intermediates and especially how they project distal surfaces relative to another. More broadly, the approach implemented here represents a new tool for constructing complex protein-based assemblies and biomaterials through minimal sequence variation, and it could be easily ported to other multimeric self-assembling proteins to aid in the formation of highly ordered materials with superior stability and monodispersity. Moreover, similar application of our approach could aid in heightening a fundamental understanding into the nature and evolution of function of the nanocage proteins. The materials eventually produced as a result of these lessons could find utility in engineering nanodevices with wide-ranging applications.

M. S. A. X. L. C. and C. J. F. were supported by an NTU. BPO thanks CBC start-up funds and AcRF Tier 1 Grant (RG 53/06), and start-up funds at King's. We thank Rongli Fan and Yu Zhang and Ai Hua Seow for help. We also thank Mark Peczuh (U. Conn.) for helpful discussions.

## Notes and references

1 A. L. Boyle, E. H. Bromley, G. J. Bartlett, R. B. Sessions, T. H. Sharp, C. L. Williams, P. M. Curmi, N. R. Forde, H. Linke and D. N. Woolfson, *J. Am. Chem. Soc.*, 2012, **134**, 15457–15467.
2 T. O. Yeates, *Nat. Nanotechnol.*, 2011, **6**, 541–542.
3 D. Grueninger, N. Treiber, M. O. P. Ziegler, J. W. A. Koetter, M.-S. Schulze and G. E. Schulz, *Science*, 2008, **319**, 206–209.
4 J. E. Padilla, C. Colovos and T. O. Yeates, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 2217–2221.
5 Y.-T. Lai, D. Cascio and T. O. Yeates, *Science*, 2012, **336**, 1129.
6 N. P. King, W. Sheffler, M. R. Sawaya, B. S. Vollmar, J. P. Sumida, I. André, T. Gonen, T. O. Yeates and D. Baker, *Science*, 2012, **336**, 1171–1174.
7 C. Uetrecht, C. Versluis, N. R. Watts, P. T. Wingfield, A. C. Steven and A. J. R. Heck, *Angew. Chem., Int. Ed.*, 2008, **47**, 6247–6251.
8 Z. Su and Q. Wang, *Angew. Chem., Int. Ed.*, 2010, **49**, 10048–10050.
9 T. Ueno, M. Suzuki, T. Goto, T. Matsumoto, K. Nagayama and Y. Watanabe, *Angew. Chem., Int. Ed.*, 2004, **43**, 2527–2530.
10 F. Hof, S. L. Craig, C. Nuckolls and J. J. Rebek, *Angew. Chem., Int. Ed.*, 2002, **41**, 1488–1508.
11 R. Fan, S. W. Chew, V. V. Cheong and B. P. Orner, *Small*, 2010, **6**, 1483–1487.
12 R. Fan, A. L. Boyle, V. V. Cheong, S. L. Ng and B. P. Orner, *Biochemistry*, 2009, **48**, 5623–5630.
13 S. C. Andrews, J. M. A. Smith, C. Hawkins, J. M. Williams, P. M. Harrison and J. R. Guest, *Eur. J. Biochem.*, 1993, **213**, 329–338.
14 M. S. Ardejani, N. X. Li and B. P. Orner, *Biochemistry*, 2011, **50**, 4029–4037.
15 Y. Zhang, S. Raudah, H. Teo, G. W. S. Teo, R. Fan, X. Sun and B. P. Orner, *J. Biol. Chem.*, 2010, **285**, 12078–12086.
16 L. Jin and J. A. Wells, *Protein Sci.*, 1994, **3**, 2351–2357.
17 G. Grigoryan, Y. H. Kim, R. Acharya, K. Axelrod, R. M. Jain, L. Willis, M. Drndic, J. M. Kikkawa and W. F. DeGrado, *Science*, 2011, **332**, 1071–1076.
18 J. Dundas, Z. Ouyang, J. Tseng, A. Binkowski, Y. Turpaz and J. Liang, *Nucleic Acids Res.*, 2006, **34**, W116–W118.
19 R. Guerois, J. E. Nielsen and L. Serrano, *J. Mol. Biol.*, 2002, **320**, 369–387.
20 C. A. Smith and T. Kortemme, *J. Mol. Biol.*, 2008, **380**, 742–756.
21 L. M. Salonen, M. Ellermann and F. Diederich, *Angew. Chem., Int. Ed.*, 2011, **50**, 4808–4842.
22 G. B. McGaughey, M. Gagné and A. K. Rappé, *J. Biol. Chem.*, 1998, **273**, 15458–15463.
23 T. A. Cornell, J. Fu, S. H. Newland and B. P. Orner, *in preparation*, 2013.