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Engineered protein cages with phosphate-dependent reversible opening

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Abstract

Engineered protein cages are promising nanocarriers for molecular delivery. Reversible and non-destructive control over their structural opening and closing for cargo manipulation remains a key challenge. Here, we present protein cages formed by an engineered variant of *Aquifex aeolicus* lumazine synthase, called AaLS-POF, that exhibits a rapid and reversible transition between closed dodecahedral and open tetrahedral states in response to phosphate or analogous oxyanions. This dynamic opening-closing behavior, induced by a non-invasive stimulus, enables controlled retrieval and packaging of various cargoes through enzymatic methods. Cryo-EM and mutagenesis identified a key phosphate-binding site and revealed how local recognition of a simple oxyanion is transmitted across subunit interfaces to reshape the geometry of a megadalton-scale assembly. The elucidated molecular mechanism and design principles make the AaLS-POF system a promising platform for engineering customized nanocontainers with desired opening characteristics.

21 Main text

22 Protein cages are naturally occurring multimeric protein architectures with defined nanoscale
23 dimensions and internal cavities¹. These structures provide confined spaces for cargo molecules,
24 enabling their protected transport and localized biochemical processes, as primarily exemplified by
25 viral capsids and bacterial microcompartments^{2, 3, 4}. Beyond these natural roles, their inherent
26 monodispersity, biocompatibility, and amenability to genetic and chemical modification have inspired
27 active efforts to develop protein cages with customized structural and functional characteristics for
28 applications in nanomedicine and synthetic biology^{1, 5}.

29 Engineering of protein cages that can open and close in response to stimuli offers a means for
30 packaging cargo molecules and their controlled release at desired times and locations. Naturally
31 occurring protein cages are often highly stable, and their disassembly requires harsh conditions such as
32 extreme pH or high concentration of chaotropic reagents that potentially damage sensitive cargo and
33 are incompatible with biological systems^{6, 7, 8}. Hence, substantial efforts have been directed toward
34 control over protein cage assembly by a mild trigger, such as small changes in pH⁹ or ionic strength¹⁰,
35 redox potentials¹¹, metal ions^{12, 13}, protein ligand¹⁴, and protease-mediated cleavage^{15, 16}. However,
36 existing systems have considerable limitations, including irreversibility or inefficiency in their
37 structural switch, limited cargo types that can be packaged, and poor blocking ability against
38 damaging macromolecule entry. None of the systems has solved them all simultaneously, impeding
39 the full potential of protein cages for versatile cargo delivery.

40 Lumazine synthase from *Aquifex aeolicus* (AaLS) exemplifies the challenges in protein cage
41 engineering. This naturally occurring enzyme, forming a dodecahedral assembly composed of 12
42 pentameric subunits¹⁷, has been extensively engineered to provide tailorable bionanotechnological
43 platforms¹⁸. A circularly permuted variant of AaLS (cpAaLS), for instance, exhibits a reversible and
44 controllable assembly switch in response to ionic strength, and possesses interior-facing termini that
45 enable modifiable cargo loading through genetic fusion or binding partners^{10, 19, 20, 21, 22}. However, the
46 structures formed by cpAaLS are porous and do not limit the entry of macromolecules¹⁰. While

47 wildtype AaLS (AaLS-wt) cages lack such large pores^{17, 23}, none of the previously engineered AaLS
48 variants exhibited a triggered conformation switch involving the wildtype-like assembly.

49 Here, we report an engineered variant of AaLS that undergoes rapid and reversible
50 conformational change between the closed dodecahedron and a porous tetrahedral structure in
51 response to phosphate or analogous oxyanions. We refer to this variant as AaLS-POF, reflecting its
52 PUFferfish-like expansion when PO₄ comes OFF. This assembly switch, triggered by a mild, non-
53 invasive stimulus under physiological conditions, enables the controlled retrieval and packaging of
54 cargo molecules through enzymatic methods while preserving cargo integrity. Cryo-electron
55 microscopy, together with mutagenesis, reveals the molecular basis underlying this phosphate-
56 dependent transition, providing design principles for engineering protein cages with customizable
57 opening responses.

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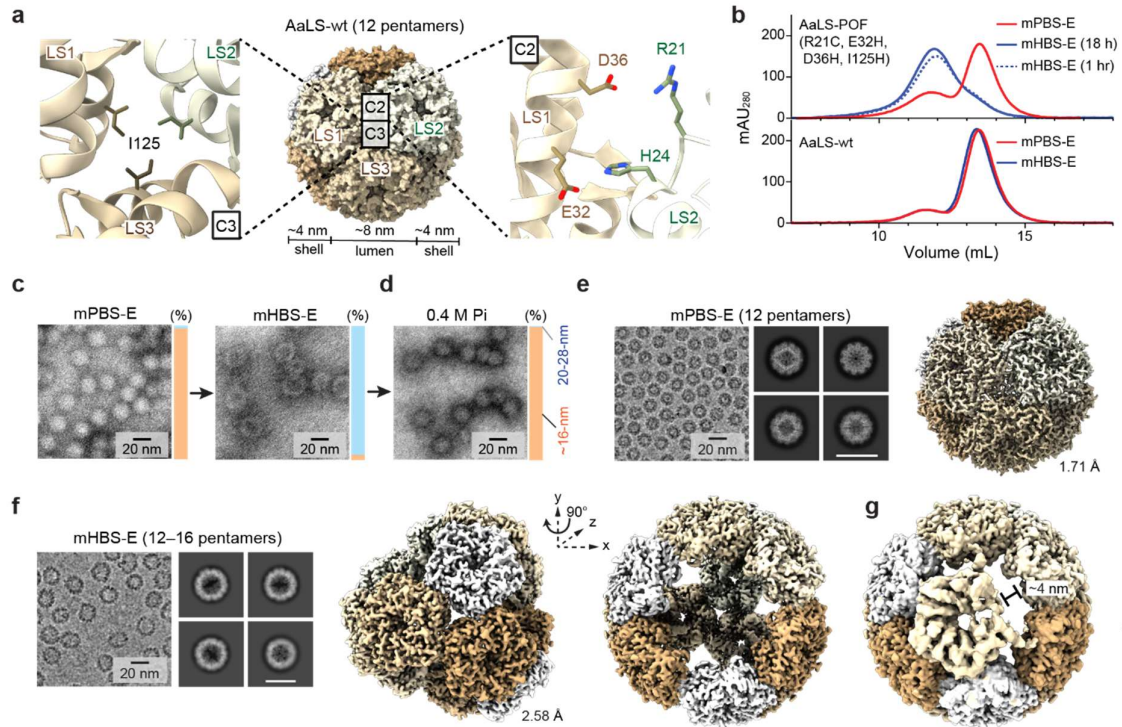
59 **Results and discussion**

60 **Phosphate-dependent AaLS variant, AaLS-POF**

61 The AaLS variant with phosphate-dependent morphology change was initially designed with
62 the aim of imparting the ability to assemble in response to metal ions. For this purpose, we employed
63 the metal-templated interface redesign (MeTIR) strategy that comprises the introduction of metal-
64 coordination motifs at protein assembly interfaces to disrupt native interactions while enabling cage
65 formation only in the presence of metal ions^{13, 24}. The pentameric subunits of the wildtype AaLS
66 dodecahedral assembly are held together by interaction networks at the 2- and 3-fold symmetrical, C2
67 and C3, interfaces¹⁷, and both must be sufficiently destabilized to induce cage disassembly by
68 mutagenesis²⁵. Therefore, we substituted one isoleucine at the C3 hydrophobic core with a histidine
69 (I125H) to generate a three-His coordination site and three charged amino acid residues at the C2
70 interface with one cysteine and two histidines (R21C, E32H, and D36H) to form an H3C1 motif with
71 the native His24 (Fig. 1a). However, contrary to expectations, size-exclusion chromatography (SEC)
72 and negative-stain transmission electron microscopy (TEM) revealed that the resulting AaLS variant

73 formed wildtype-like cage assemblies even in the absence of metal ions (Fig. 1b,c and Extended Data
 74 Fig. 1a).

75



76

77 **Fig. 1 | Phosphate-responsive cage morphology of AaLS-POF.** **a**, Amino acid residues selected for
 78 mutagenesis to yield AaLS-POF, presented in the structure of AaLS-wt dodecahedral assembly (PDB
 79 1HQK [<https://doi.org/10.2210/pdb1hqk/pdb>])¹⁷, where regions surrounding the C2 and C3 symmetry
 80 axes are enlarged. Individual pentamers are shown in distinct white-brown shades, and three
 81 symmetry-related pentamers are labeled LS1-LS3 for correspondence between the views. The
 82 substitutions were originally designed for metal binding, with I125H generating a three-His
 83 coordination site at C3 and R21C, E32H, and D36H forming an H3C1 motif with native His24 at C2.
 84 **b,c**, Size-exclusion chromatograms (SEC) (**b**) and negative-stain transmission electron microscopy
 85 (TEM) images (**c**) of AaLS-POF in modified phosphate- or HEPES-buffered saline containing EDTA
 86 (mPBS-E, red; mHBS-E, blue). For AaLS-POF in mHBS-E, dotted and solid blue lines show SEC
 87 profiles after 1 and 18 h of incubation, respectively. The SEC profile of AaLS-wt is shown for
 88 comparison. mAU₂₈₀, milli absorbance units at 280 nm. The AaLS-wt dodecahedral assembly elutes at

~13.5 mL with a shoulder around 11.5 mL corresponding to cage oligomers⁸. The expanded assemblies and these cage oligomers are indistinguishable by SEC due to similar elution volumes. The bars next to the TEM images indicate the relative populations of ~16-nm (orange) and 20-28-nm (light blue) assemblies based on particle counting (Extended Data Fig. 1a). **d**, Recompressing of the expanded assemblies. The expanded assemblies isolated by SEC in mHBS-E were transferred to 0.4 M sodium phosphate buffer (Pi, pH 7.5) and imaged by TEM. **e,f**, Cryo-EM analysis of AaLS-POF assemblies in mPBS-E (**e**; PDB 34II [<https://doi.org/10.2210/pdb34ii/pdb>]) and mHBS-E (**f**; PDB 34IJ [<https://doi.org/10.2210/pdb34ij/pdb>]), shown as representative micrographs, 2D class averages and 3D density maps. Scale bars, 20 nm. Map resolutions are indicated at the bottom right. The expanded-assembly map in mHBS-E is a composite, generated by positioning four copies of the locally refined three pentamers in the consensus map with tetrahedral symmetry. **g**, Additional pentamer within a hexagonal opening of the expanded assembly, identified by masked 3D classification, leaving a residual gap of ~4 nm (EMD-59870 [<https://www.ebi.ac.uk/emdb/EMD-59870>]).

The AaLS variant possessing two metal-coordination motifs displayed a change in its assembled structures, not in response to metal ions but rather to the removal of phosphate ions. This was observed when the sample buffer was exchanged from a modified phosphate-buffered saline [20 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl, 6 mM KCl, and 1 mM ethylenediaminetetraacetate (EDTA)] to a HEPES-buffered saline [50 mM HEPES-NaOH buffer (pH 7.5) containing 150 mM NaCl and 1 mM EDTA], referred to hereinafter as mPBS-E and mHBS-E, respectively. Upon the buffer replacement and following incubation for 18 hours, SEC and TEM showed an expansion in cage morphology from ~16-nm regular to 20-28 nm polydisperse assemblies (Fig. 1b,c and Extended Data Fig. 1a). Analogous experiments using a Tris-HCl buffer confirmed that this conformation change is caused by removal of phosphate ions rather than addition of HEPES (Extended Data Fig. 1b). In contrast, no notable morphology change was observed for AaLS-wt, which forms only a ~16 nm assembly irrespective of these buffers (Fig. 1b and Extended Data Fig. 1c). We

therefore refer to this variant as “AaLS-POF”, reflecting its pufferfish-like cage expansion when PO_4 comes OFF.

AaLS-POF cage expansion occurs rapidly and efficiently upon phosphate removal. To monitor this morphological transition, samples in mPBS-E were buffer-exchanged into mHBS-E by ultrafiltration and immediately analyzed by SEC, with the entire process taking approximately one hour. The resulting chromatogram showed a prominent peak corresponding to expanded assemblies, alongside a minor shoulder likely representing remaining wildtype-like structures (Fig. 1b, blue dashed line). This pattern was similar to that observed after 18 hours in mHBS-E (Fig. 1b, blue solid line), where ~96% of particles adopted expanded morphologies, as judged by TEM (Fig. 1c and Extended Data Fig. 1a). Dynamic light scattering (DLS) showed progressive cage expansion upon phosphate depletion, with a half-maximal effective phosphate concentration of 1.2 ± 0.1 mM (Extended Data Fig. 1d).

The phosphate-responsive assembly switch of AaLS-POF is reversible. When the expanded cages, isolated in mHBS-E, were placed in mPBS-E, the wildtype-like 16-nm assemblies reappeared (Extended Data Fig. 1e). However, this recompacting process was notably less efficient than the expansion, with approximately 80% of the particles remaining in the expanded state after an 18-hour incubation in mPBS-E. A high phosphate concentration can promote this recompacting process. Treatment of the same sample in 0.4 M sodium phosphate buffer (pH 7.5) yielded >99% reformation of the wildtype-like assemblies (Fig. 1d and Extended Data Fig. 1e).

Cryo-EM single-particle reconstruction of the AaLS-POF structures revealed that the phosphate-dependent morphology change is attributed to reorganization of pentameric subunits (Fig. 1e-g, Extended Data Figs. 2 and 3, and Extended Data Table 1). In the presence of phosphate, AaLS-POF formed a dodecahedral cage composed of 12 pentameric subunits, resembling the wildtype assembly (Fig. 1e). Upon phosphate removal, AaLS-POF adopts heterogeneous assemblies, with a prominent structural class characterized by a tetrahedral arrangement of 12 pentamers with four large hexagonal openings (Fig. 1f and Extended Data Fig. 3). Local analysis at the openings identified an additional pentameric subunit (Fig. 1g) that appears to adopt variable positions, potentially contacting

three protomers from the framework while distorting the overall tetrahedral geometry. Such plasticity likely limits local definition of the consensus map and may also underlie the heterogeneous assemblies (Extended Data Fig. 3). Moreover, the shape mismatch between the pentamer and hexagonal opening leaves structural gaps of 3-4 nm (Fig. 1g), which may widen further through dynamic rearrangement of pentamers and surrounding framework. These structural features render the expanded AaLS-POF assembly highly porous, in marked contrast to the sealed dodecahedral structure formed in the presence of phosphate.

Cargo retrieval and packaging

The reversible opening of AaLS-POF cages offers a compelling platform for controlled encapsulation and release of guest cargo, potentially useful in two application scenarios. One usage centers on the retrieval of cargoes that are encapsulated within the AaLS-POF cage during their synthesis in cells, where cage opening facilitates extraction of the cargo outside the cellular context. The other scenario is cargo packaging outside cells, a procedure requiring a controlled cage opening for loading, followed by cage closure. The mild, non-invasive phosphate trigger is particularly advantageous for preserving the functionality of biomolecular cargoes and enzymes that can be used in these retrieval and packaging processes.

We first examined retrieval of cargo encapsulated in AaLS cages during its synthesis in cells using a guest packaging system we previously established. A model green fluorescent protein was genetically fused to a circularly permuted variant of AaLS via a tobacco etch virus protease (TEVp) recognition peptide, cpAaLS(119)-tev*-GFP, and coproduced with AaLS-POF in *E. coli* cells (Fig. 2a)^{19, 21}. The patchwork assemblies were isolated and treated with TEVp in mPBS-E or mHBS-E. Subsequent SDS-PAGE analysis showed that efficient cleavage occurred only in the absence of phosphate (Fig. 2b). This likely reflects the expanded, porous cage morphology, as indicated by native agarose electrophoresis (AGE) and negative-stain TEM (Fig. 2c,d). In contrast, only trace cleavage was observed in either buffer for the AaLS-wt control (Fig. 2b,d). These results demonstrate the utility

of AaLS-POF cages as a biomolecular reservoir that protects encapsulated cargo from degradative enzymes while allowing their ready retrieval upon phosphate removal.

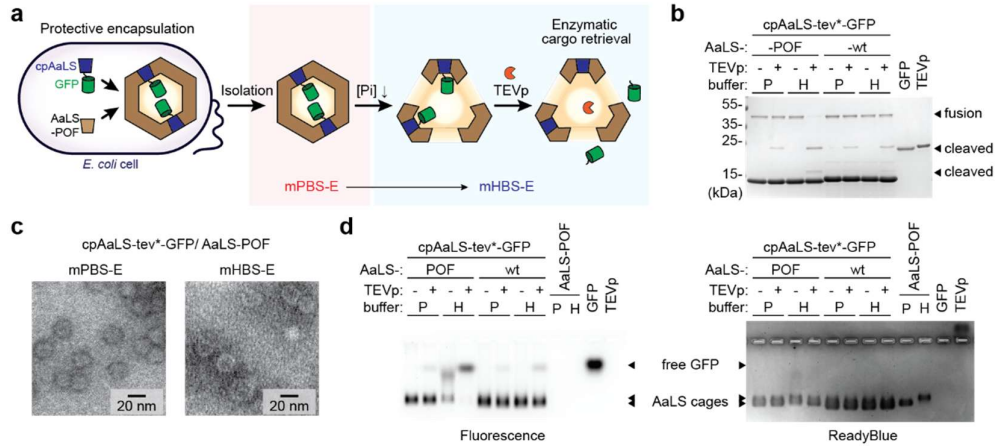


Fig. 2 | Cargo retrieval. **a**, Schematic representation of the cargo retrieval procedure. A model protein GFP (green cylinder) was fused to a circularly permuted variant of AaLS (cpAaLS, blue trapezoid) via a TEV protease recognition peptide (cpAaLS-tev*-GFP) and coproduced with AaLS-POF (beige trapezoid) in *E. coli* cells. After isolation, morphology change was induced by exchanging the solvent from mPBS-E (pink) to mHBS-E (light blue), followed by treatment with TEV protease (TEVp, orange Pacman). Analogous experiments were performed with AaLS-wt instead of AaLS-POF for comparison. **b-d**, TEV protease cleavage in mPBS-E (P) or mHBS-E (H), analyzed by SDS-PAGE (**b**), TEM (**c**), and native-AGE (**d**). Calculated molecular masses are: AaLS-POF, 18.1 kDa; cpAaLS-tev*-GFP, 45.7 kDa; AaLS-wt, 18.1 kDa; TEVp, 28.8 kDa; and cleaved cpAaLS and GFP fragments, 18.7 and 27.0 kDa, respectively. The bands for TEVp and GFP are indistinguishable due to their similar molecular masses. For panel **d**, the same gel was visualized by fluorescence (left) and ReadyBlue staining (right). The upper band of the cpAaLS-tev*-GFP/AaLS-POF complex in mHBS-E is tentatively assigned to assemblies exposing GFP on the exterior.

For controlled cargo packaging outside cells, we employed sortase A-mediated conjugation²⁶. A peptide containing the sortase A recognition sequence (LPSTG) was fused to the C-terminus of

cpAaLS (cpAaLS-srt*) and co-produced with AaLS-POF to form patchwork assemblies displaying the modification unit in the lumen (Fig. 3a). A model small compound, fluorescein isothiocyanate labeled with a tetraglycine unit (G4-FITC), was loaded through an open-load-close procedure, where the cage was opened by phosphate removal, G4-FITC was enzymatically conjugated within the lumen, and the cage was reclosed upon phosphate addition (Fig. 3a). Negative-stain TEM confirmed the expected conformational changes throughout the procedure (Fig. 3b and Extended Data Fig. 4a). SDS-PAGE and native-AGE analysis confirmed that conjugation occurred predominantly in the open state, while the reaction remained residual for the control samples, cpAaLS-srt*/AaLS-POF patchwork assembly in mPBS-E and cpAaLS-srt*/AaLS-wt cages in both buffers (Fig. 3c,d). Quantification by absorbance at 280/494 nm estimated 1.5 ± 0.5 FITC molecules loaded per cage for the reaction with cpAaLS-srt*/AaLS-POF patchwork assembly in mHBS-E (Extended Data Fig. 4b).

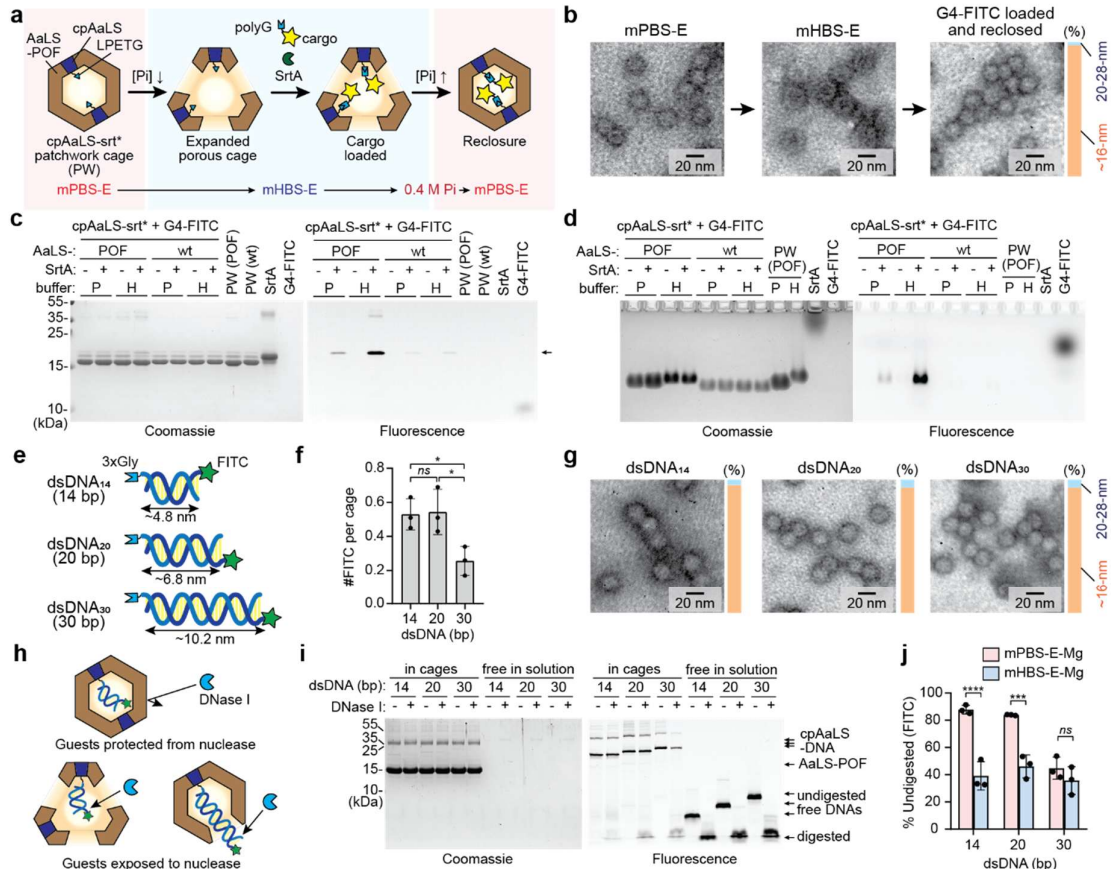


Fig. 3 | Ex-cellulo cargo packaging. **a**, Schematic of the cargo packaging procedure. cpAaLS-srt* was coproduced with AaLS-POF in *E. coli*. After isolation, morphology change was induced by buffer exchange from mPBS-E (pink) to mHBS-E (light blue), followed by treatment with polyglycine-labeled cargo and sortase A (SrtA). Cage reclosure was induced with 0.4 M sodium phosphate buffer (Pi, pH 7.5), followed by buffer exchange to mPBS-E (pink). **b-d**, TEM (b), SDS-PAGE (c), and native-AGE (d) analysis of sortase-mediated conjugation of cpAaLS-srt* with tetraglycine-labeled fluorescein isothiocyanate (G4-FITC) in mPBS-E (P) or mHBS-E (H). Calculated molecular masses are: AaLS-POF, 18.1 kDa; cpAaLS-srt*, 18.4 kDa; AaLS-wt, 18.1 kDa; SrtA, 18.5 kDa; cpAaLS-G4-FITC, 18.9 kDa. Gels were visualized by colloidal Coomassie staining (left) and fluorescence (right). The arrow in panel c indicates cpAaLS-G4-FITC. PW denotes patchwork assemblies of cpAaLS-srt* with AaLS-POF (POF) or AaLS-wt (WT). The bands at ~35 kDa probably correspond to cpAaLS-srt* dimers. **e**, Model double-stranded DNA (dsDNA) constructs (14, 20, and 30 base pairs, bp), in which one strand is labeled with triglycine (3×Gly) and the other with FITC at their respective 5'-termini. **f**, Average number of FITC-labeled dsDNA molecules per cage, calculated from absorbance at 280/494 nm. **g**, TEM images of dsDNA-loaded AaLS-POF after reclosure. The bars next to TEM images in panels b and g indicate the relative populations of ~16-nm (orange) and 20-28-nm (blue) assemblies based on particle counting. **h**, Schematic of possible AaLS-POF conformations and DNase accessibility. **i**, SDS-PAGE analysis of encapsulated and free dsDNA challenged with DNase I. **j**, Protection efficiency calculated from fluorescent SDS-PAGE band intensity. Digestion was performed in mPBS-E or mHBS-E supplemented with 5 mM MgCl₂ (mPBS-E-Mg, pink; mHBS-E-Mg, blue). For panels f and j, data represent means ± standard deviations from triplicate experiments with individual data points overlaid. Statistical significance was assessed by one-way ANOVA with Tukey's test (f) and two-way ANOVA with Sidak's test (j); ns, $p \geq 0.05$; *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

To explore the generality of the sortase-mediated cargo loading, we employed three double-stranded DNA constructs (dsDNA) with 14, 20, and 30 base pairs (dsDNA₁₄, dsDNA₂₀, and dsDNA₃₀),

corresponding to ~4.8-, ~6.8-, and ~10.2-nm lengths, respectively (Fig. 3e and Extended Data Table 2). One strand was labeled with triglycine for sortase-mediated conjugation and the other with FITC for quantification. Given the ~8-nm luminal diameter of the closed AaLS-POF cage, dsDNA₁₄ and dsDNA₂₀ would fit within the cavity, while dsDNA₃₀ was expected to exceed the space limitation. These dsDNAs were loaded into cpAaLS-srt*/AaLS-POF cages using sortase A in mHBS-E, and conjugation was confirmed by SDS-PAGE and native-AGE (Extended Data Fig. 5a,b). Comparable loading efficiencies were observed for dsDNA₁₄ and dsDNA₂₀, while dsDNA₃₀ reached approximately half of that level, likely reflecting limited accessibility due to steric hindrance (Fig. 3f). Despite the reduced loading, all three samples converted to the ~16-nm form upon treatment with 0.4 M sodium phosphate buffer (Fig. 3g and Extended Data Fig. 5c).

To assess cargo protection after reclosure, the dsDNA-loaded cages were challenged with DNase I (Fig. 3h). Approximately 88% and 84% of dsDNA₁₄ and dsDNA₂₀ remained undigested in mPBS-E supplemented with MgCl₂ (mPBS-E-Mg), confirming effective protection upon cage closure (Fig. 3i,j). In contrast, ~55% of dsDNA₃₀ was digested under the same conditions, likely due to partial protrusion from the interior (Fig. 3h-j). The partial protection of dsDNA₃₀ is probably attributed to its configuration rather than wall permeability, as comparable nucleolysis was observed across guest sizes in the open state in mHBS-E (Fig. 3j and Extended Data Fig. 5d). These results demonstrate that AaLS-POF with sortase-mediated conjugation affords a means for effective packaging and protection of oligoglycine-labeled cargo, unless their dimensions exceed those of the cage interior.

Molecular mechanism of phosphate-dependent morphology change

The utility of AaLS-POF for controlled cargo manipulation prompted us to investigate the molecular basis underlying its conformational switch. We first examined the temperature-dependent behavior of AaLS-POF in the presence and absence of phosphate by differential scanning fluorimetry coupled with DLS. In mPBS-E, AaLS-POF exhibited a shift in hydrodynamic radius from ~9 nm, corresponding to the wildtype-like assembly, to ~11 nm, the size comparable to that observed in

mHBS-E, accompanied by a change in the tryptophan fluorescence ratio (350/330 nm) with a midpoint temperature of $\sim 67^\circ\text{C}$ (Fig. 4a and Extended Data Fig. 6a). AaLS-wt showed no such thermal conversion in either buffer (Extended Data Fig. 6b), indicating that the mutations introduced to yield AaLS-POF confer an intrinsic conformational preference for the expanded state, while phosphate stabilizes the wildtype-like dodecahedral assembly.

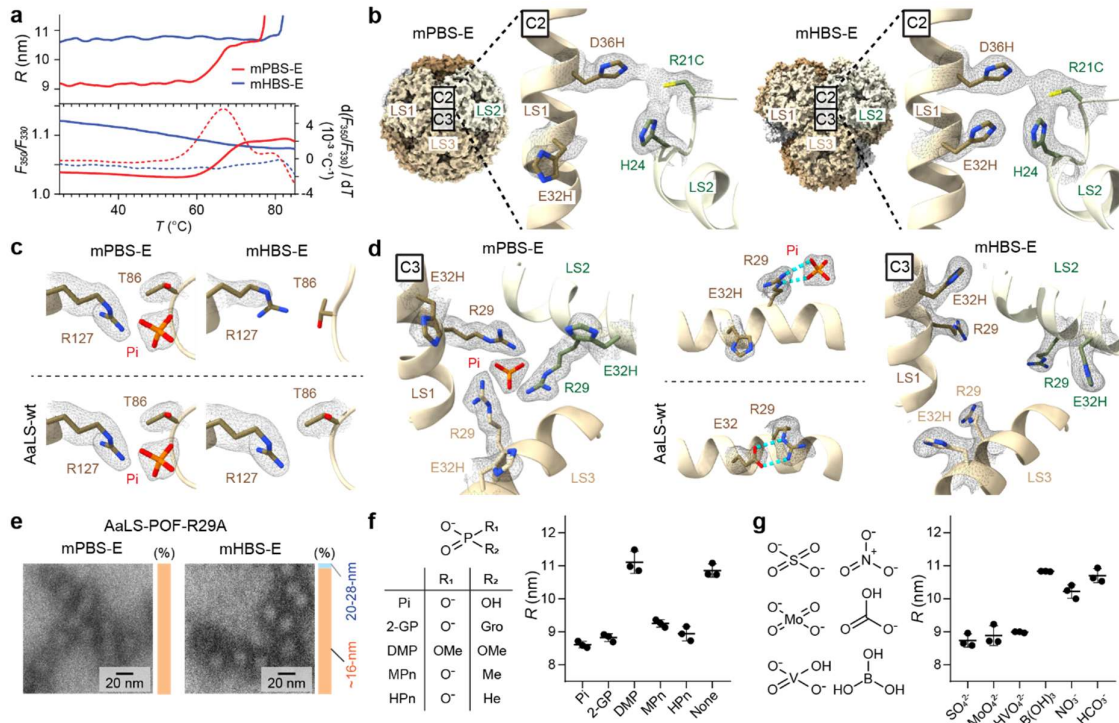


Fig. 4 | Molecular basis of phosphate-dependent morphology change. **a**, Differential scanning fluorometry (DSF), coupled with dynamic light scattering (DLS), of AaLS-POF in mPBS-E (red) and mHBS-E (blue). Data are presented as hydrodynamic radius (R) and fluorescence ratio at 350/330 nm (F_{350}/F_{330}) over temperature (T) from 20 to 90°C . Dashed lines indicate the first derivatives of the fluorescence ratio ($d(F_{350}/F_{330})/dT$). **b**, Cryo-EM structures of AaLS-POF assemblies in mPBS-E (left, PDB 34II [https://doi.org/10.2210/pdb34ii/pdb]) and mHBS-E (right, PDB 34IJ [https://doi.org/10.2210/pdb34ij/pdb]), with enlarged views of the C2 symmetry regions. Cryo-EM densities around the indicated residues are shown as mesh. **c**, The catalytic pocket in mPBS-E and mHBS-E, showing phosphate coordinated by Thr86 and Arg127 in mPBS-E. The corresponding

AaLS-wt structures are shown below dashed line for comparison. **d**, Enlarged views of the C3 symmetry region, showing phosphate (Pi) coordinated by Arg29 from three neighboring protomers in mPBS-E and its absence in mHBS-E. An alternative view focused on Arg29 is shown above the dashed line, with the corresponding Arg29-Glu32 interaction in AaLS-wt below. Cyan dashed lines in the structures indicate electrostatic interactions. **e**, TEM images of AaLS-POF-R29A variant in mPBS-E and HBS-E. The bars indicate the relative populations of ~16-nm (orange) and 20-28-nm (blue) assemblies based on particle counting. **f,g**, Hydrodynamic radius (*R*) of AaLS-POF in the presence of phosphate derivatives (f) or analogs (g) in mHBS-E. 2-GP, glycerol-2-phosphate; Gro, glycerol; DMP, dimethyl phosphate; MPn, methyl phosphonate; HPn, hexyl phosphonate; Me, methyl; He, hexyl. Vanadate was measured without pH adjustment at pH 10.7 to minimize formation of condensed species (e.g., $V_4O_{12}^{4-}$)²⁷. Data are presented as means ± standard deviations from triplicate experiments.

Disruption of native interactions at positions 32 and 125 constitutes a molecular basis driving the preference of AaLS-POF for the expanded conformation. Serine substitutions at these positions, as well as position 36, each retained the phosphate-dependent morphology change, as judged by SEC (Table 1 and Extended Data Fig. 7a). Mutation at position 36 proved dispensable, as the native aspartate retains the morphological switching capability (Table 1 and Extended Data Fig. 7a,b). In contrast, reversion of E32H or I125H to their native residues resulted in variants forming only wildtype-like assemblies in both mPBS-E and mHBS-E (Table 1 and Extended Data Fig. 7b).

Position 21 specifically requires cysteine for the phosphate-dependent conformational change of AaLS-POF. Both reversion to the native arginine and substitution with serine resulted in the variants forming only wildtype-like assemblies in both mPBS-E and mHBS-E (Table 1 and Extended Data Fig. 7a,b). Cryo-EM structure shows that Cys21 potentially forms interactions with His24 from the same protomer and His36 from an adjacent protomer (Fig. 4b and Extended Data Fig. 8a). Whereas substitutions at position 36 retained the switching capability as described above, the H24S mutation

resulted in only wildtype-like assembly (Table 1 and Extended Data Fig. 7c), confirming that the Cys21-His24 interaction is a key determinant for the expanded conformation of AaLS-POF.

Table 1. AaLS-POF mutagenesis and assembly behavior.

AaLS variant	Amino acid position / mutation ^{*1}								Assembly ^{*2}	
	21	32	36	125	24	86	127	29	mPBS-E	mHBS-E
wt	R	E	D	I	H	T	R	R	S ^{*3}	S
POF (#1)	C	H	H	H	H	T	R	R	S	L ^{*4}
#2	-	S	-	-	-	-	-	-	S	L
#3	-	-	S	-	-	-	-	-	S	L
#4	-	-	-	S	-	-	-	-	S	L
#5	-	S	D	-	-	-	-	-	S	L
#6	-	E	S	-	-	-	-	-	S	S
#7	-	-	-	I	-	-	-	-	S	S
#8	S	-	-	-	-	-	-	-	S	S
#9	R	-	-	-	-	-	-	-	S	S
#10	-	-	-	-	S	-	-	-	S	S
#11	-	-	-	-	-	A	-	-	L	ND ^{*5}
#12	-	-	-	-	-	-	A	-	L	ND
#13	-	-	-	-	-	-	-	A	S	S

*1, -, identical to AaLS-POF

*2, Assembly formation estimated by SEC elution profile using a Superose 6 Increase column in mPBS-E and mHBS-E. Chromatograms are provided in Extended Data Fig. 7.

*3, S = small, wildtype-like assembly, characterized by a major peak eluting at ~14 mL, often with a shoulder at a slightly earlier elution volume which corresponds to cage oligomers⁸

*4, L = large, expanded cages, characterized by a broad peak eluting at 11-13 mL

*5, ND, not determined.

The catalytic site of lumazine synthase is known to interact with phosphate, a product of its enzymatic activity. This binding rigidifies the local structure and stabilizes the dodecahedral assembly of bacillus lumazine synthases^{28, 29}, which is likely also the case for AaLS-POF. Cryo-EM structure of AaLS-POF, as well as AaLS-wt, in mPBS-E revealed a tetrahedral-shaped density at the catalytic pocket, likely bound phosphate (Fig. 4c). Alanine substitution of either Thr86 or Arg127, both native

phosphate-recognizing residues, resulted in mutants exhibiting expanded cages in mPBS-E (Table 1 and Extended Data Fig. 7d), indicating that the local interaction network at the catalytic site influences AaLS-POF morphology.

The phosphate-dependent switch of AaLS-POF is not primarily controlled by the catalytic pocket, but by another phosphate-binding site at the C3 pore. Cryo-EM revealed a tetrahedral-shaped density, likely phosphate, at this region, coordinated by Arg29 from three neighboring protomers (Fig. 4d). This arginine residue appears to stabilize the wildtype-like assembly with phosphate bound, while driving cage expansion, likely through electrostatic strain at the C3 interface, when unliganded. The latter role was evident from alanine substitution at position 29, with the resulting variant mostly retaining the wildtype-like morphology in mHBS-E, as judged by SEC and TEM (Fig. 4e and Extended Data Fig. 7e,f). Notably, this secondary phosphate binding is absent in the AaLS-wt assembly where Arg29 is held away from the C3 center by interaction with Glu32 (Fig. 4d and Extended Data Fig. 8). Removal of the native Arg29-Glu32 interaction by the E32H substitution redirects the guanidinium groups toward the C3 symmetry axis, leading to electrostatic repulsion that is in turn counteracted by phosphate coordination.

The phosphate-binding sites of AaLS-POF exhibit a broad yet specific ligand recognition profile. When the wildtype-like form, isolated in mPBS-E, was transferred to mHBS-E supplemented with glycerol-2-phosphate (2-GP), methylphosphonate (MPn), or hexylphosphonate (HPn), a ~9 nm hydrodynamic radius was retained, as judged by DLS (Fig. 4f). Dimethyl phosphate (DMP) induced cage expansion, suggesting the inability of this phosphodiester to maintain the wildtype-like assembly. Furthermore, AaLS-POF recognizes analogous oxyanions beyond phosphorus compounds. Sulfate, molybdate, and vanadate also maintained the ~9 nm radius, whereas borate, nitrate, and bicarbonate triggered cage expansion (Fig. 4g). These results demonstrate that an oxyanion ligand must carry a net -2 charge distributed over at least three equivalent oxygen atoms to preserve the wildtype-like assembly of AaLS-POF, a chemical feature likely required for coordinating the three converging Arg29 residues at the C3 interface and compensating their positive electrostatics.

Structural analysis of AaLS-POF provides a unifying principle based on inter-pentamer geometry for engineering stimulus-responsive AaLS cages (Fig. 5). In the wildtype AaLS assembly, the binding angle between adjacent pentamers is primarily governed by a triangular network comprising the central C2 interface and two flanking C3 hydrophobic cores (Fig. 5a). Removal of the C3 hydrophobic cores potentially provides rotational flexibility around the C2 contact (Fig. 5b), the feature required for non-canonical, pentamer-based AaLS assemblies^{19, 25, 30}. Additional moderate destabilization of the C2 interface is also required for such structural rearrangement, as demonstrated by a cpAaLS variant lacking the domain forming the C3 hydrophobic cluster, cpAaLS(119Δ120-131), assembling into wildtype-like cages exclusively¹⁰. A stimulus-responsive interfacial element can reform the triangular interaction network upon ligand binding (Fig. 5a), thereby restoring the wildtype-like geometry (Fig. 5b). The Arg29-based phosphate response in AaLS-POF exemplifies this principle, with phosphate coordination establishing a new C3 contact, while the unliganded residue additionally contributes to the moderate destabilization required for structural rearrangement (Fig. 5c). This design couples structural plasticity with stimulus-dependent restoration, providing a general route to reversible control of AaLS geometry.

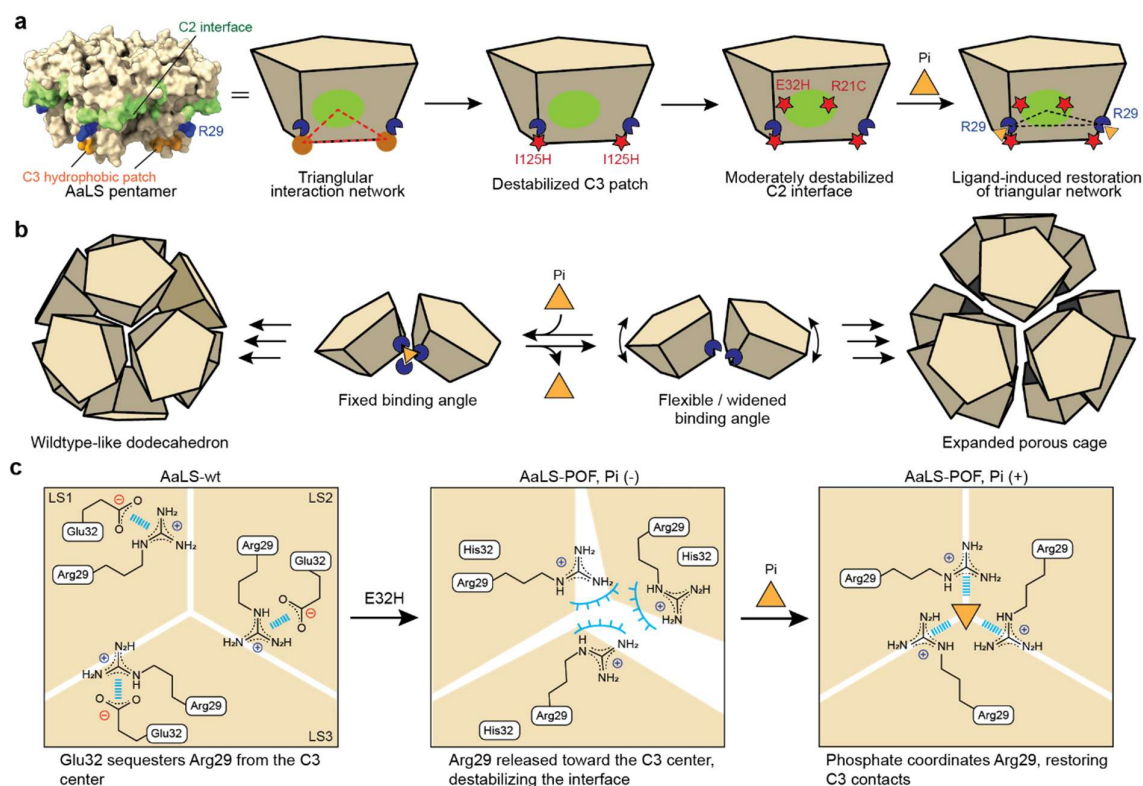


Fig. 5 | Design principle for stimulus-responsive AaLS cages. **a**, Schematic illustration of the stepwise design strategy. (i) The AaLS pentamer (left) features a triangular interaction network (red dashed line) comprising the C2 interface (green) and two flanking C3 hydrophobic patches (dark orange). (ii, iii) Introduction of a mutation destabilizing the C3 patch, e.g., I125H (red stars), followed by substitutions moderately destabilizing the C2 interface, e.g., E32H and R21C (red stars), provides morphological plasticity. (iv) Phosphate (Pi, orange triangle) binding to R29 residues (blue Pacmans) from three neighboring pentamers restores the triangular interaction network. **b**, Schematic illustration of the consequences for cage formation. Phosphate binding to three R29 residues (one pentamer omitted for clarity) fixes the binding angle between adjacent pentamers, driving formation of the wildtype-like dodecahedral assembly (left). Removal of phosphate increases inter-pentamer flexibility and permits widening of the binding angle, leading to the expanded porous cage (right). **c**, Schematic chemical representation of the Arg29-based phosphate switch. In AaLS-wt, each Arg29 guanidinium group interacts with Glu32 from a neighboring protomer (cyan dashed lines), positioning Arg29 away from the C3 center (left, AaLS-wt). The E32H substitution removes these native interactions and

redirects the three guanidinium groups toward the symmetry axis, where electrostatic repulsion (cyan spiked arcs) destabilizes the C3 interface in the absence of phosphate (middle, AaLS-POF, $\text{Pi}(-)$). Phosphate coordination (cyan dashed lines) counteracts this repulsion and establishes a new three-fold contact between neighboring pentamers (right, AaLS-POF, $\text{Pi}(+)$).

Conclusion

In summary, we have converted a rigid protein shell into stimulus-responsive compartments by introducing subtle mutations. AaLS-POF cages undergo a rapid, efficient, and reversible conformational change between a closed dodecahedron and a tetrahedral assembly with a large wall opening, controlled by phosphate or similar tetrahedral oxyanions. This opening/closing capability, combined with our previously established patchwork assembly using a circularly permuted AaLS variant, allowed us to demonstrate enzymatic cargo retrieval and packaging, while the non-invasive phosphate trigger should permit extension to diverse guests and loading/release mechanisms. The protein cage morphology switch arises from mutations that weaken native inter-pentamer contacts, while phosphate coordination at the C3 interface counteracts electrostatic repulsion among converging arginine residues and restores the geometry required for the wildtype-like assembly. This design principle, combining engineered structural plasticity with ligand-dependent stabilization, provides a blueprint for developing customized nanocontainers with tunable opening characteristics for nanodelivery and other bionanotechnological applications.

Methods

Buffers. A modified version of phosphate- or HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-buffered saline corresponds to 20 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl, 6 mM KCl, and 1 mM ethylenediaminetetraacetate (EDTA), or 50 mM HEPES-NaOH buffer (pH 7.5) containing 150 mM NaCl and 1 mM EDTA, and is referred to as mPBS-E or mHBS-E, respectively.

Plasmids. All protein sequences, plasmids, and oligonucleotides used in this study are listed in Supplementary Tables 1, 2, and 3, respectively. The gene encoding AaLS-POF and the gene fragment encoding msfGFP were synthesized by BioCat GmbH (Heidelberg, Germany). The AaLS-POF gene was cloned into pET21, and mutant variants were constructed by site-directed mutagenesis using complementary primer pairs. The msfGFP gene fragment was subcloned downstream of cpAaLS(119)-tev* in pACYC_Ptet_cpAaLS(119)-tev*-LIX²¹, yielding pACYC_Ptet_cpAaLS(119)-tev*-GFP. The sortase A recognition sequence was introduced by replacing the GFP-encoding region by PCR, yielding pACYC_Ptet_cpAaLS(119)-srt*. Oligonucleotides were synthesized by Sigma-Aldrich (Darmstadt, Germany) or Thermo Fisher Scientific (Vilnius, Lithuania). *Escherichia coli* strain DH5 α (Thermo Fisher Scientific) was used for all molecular cloning procedures. The fidelity of all plasmids was confirmed by DNA Sanger sequencing performed by Eurofins Genomics GmbH (Ebersberg, Germany).

Protein production. *E. coli* strain BL21-Gold(DE3) (Agilent Technologies, Santa Clara, CA, U.S.A.), transformed with a pET plasmid encoding the corresponding AaLS variant, was cultured at 37 °C in 2 $\times$ yeast extract – tryptone broth (2 $\times$ YT) medium supplemented with 100 μ g/mL ampicillin until the OD₆₀₀ reached ~0.7–0.9, at which point protein production was induced by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) (0.2 mM). After culturing at 25 °C for ~20 h, cells were harvested by centrifugation at 5,000 $\times$ g, 4 °C for 10 min, and then stored at –20 °C until purification. AaLS patchwork cages were analogously produced using *E. coli* cells that were cotransformed with two plasmids, pET21_AaLS_R21C_E32H_D36H_I125H-StreptII and pACYC-Ptet_cpAaLS-tev-GFP or pACYC-Ptet_cpAaLS-srt vectors, and protein production was induced with IPTG (0.2 mM) and tetracycline (0.1 μ g/mL).

Protein purification. The cell pellet from a 250-mL culture was suspended in 40 mL mPBS-E, supplemented with ~0.1 mg/mL lysozyme, ~0.01 mg/mL DNase I, and ~0.1 mg/mL RNase A. After incubation at 37 °C for 1 h with gentle shaking, the sample was lysed by sonication and cleared by centrifugation at 15,000 $\times$ g and 25 °C. Supernatant was filtered using a 0.45- μ m cutoff membrane, and the filtrate was loaded onto 2-mL StrepTactin Sepharose HP or XT resin (Cytiva, Freiburg im

Breisgau, Germany, or Iba Lifesciences, Göttingen, Germany, respectively) in a gravity column. After washing with 16 mL mPBS-E, the protein was eluted with 12 mL mPBS-E containing 2.5 mM desthiobiotin (Sigma Aldrich) or 50 mM D-biotin (BioShop Canada, Burlington, ON, Canada). Protein concentration was determined using absorbance at 280 nm using the extinction coefficient of AaLS proteins, $\epsilon_{280} = 19,630 \text{ M}^{-1} \text{ cm}^{-1}$, calculated using ProtParam (<https://web.expasy.org/protparam/>).

Size-exclusion chromatography. The proteins isolated using affinity chromatography were placed in a target buffer using an Amicon-15 ultracentrifugal unit (50 k MWCO, Merck-Millipore, Darmstadt, Germany). In addition to mPBS-E and mHBS-E, 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 1 mM EDTA was tested. The protein was kept in a corresponding buffer at room temperature for >18 hours. The samples were then subjected to size-exclusion chromatography using a Superose 6 Increase 10/300 column (Cytiva), running with the corresponding buffer at a flow rate of 0.75 mL/min.

Negative-stain transmission electron microscopy (TEM). The protein solution (0.05 mg/mL, 4 μL) was placed on a glow-discharged carbon-coated copper grid (EM resolution, Newcastle, UK). The excess solution was removed using filter paper, followed by staining with 1.5% (w/v) phosphotungstic acid (Polysciences, Warrington, PA, USA) aqueous solution. Samples were visualized on a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV. Particle counting was performed on CryoSPARC v4.5.3³¹ (Structura Biotechnology, Toronto, Canada), using more than 470 particles from 2-4 images, and the percent population, with respect to the number of particles that fall into individual size classes, is provided as bars next to the TEM images.

Phosphate titration. Protein solution (0.5 mg/mL) was prepared in buffers containing varying phosphate concentrations, prepared by mixing mPBS-E and mHBS-E at appropriate ratios, using ultrafiltration and transferred to a Prometheus high-sensitivity glass capillary (NanoTemper Technologies, Munich, Germany). Dynamic light scattering was performed on a Prometheus PANTA instrument (NanoTemper Technologies) at 25°C, and datasets were analyzed using the PR.PantaAnalysis software. The half-maximal effective phosphate concentration (EC_{50}) was

determined by fitting the mean intensity-based hydrodynamic radius as a function of phosphate concentration to the following equation using GraphPad Prism:

$$R = R_{\max} + (R_{\min} - R_{\max}) \times [\text{Pi}]^n / ([\text{Pi}]^n + \text{EC}_{50}^n)$$

where R is the hydrodynamic radius, $R_{\max}$ and $R_{\min}$ are the radii in the absence and at saturating concentrations of phosphate, respectively, $[\text{Pi}]$ is the phosphate concentration, and n is the Hill coefficient. Experiments and curve fitting were performed in triplicate, and means $\pm$ standard deviations are provided.

Cryogenic transmission electron microscopy (cryo-EM) measurement. Protein solution (1.0 mg/mL, 3.5 μ L) was applied to glow-discharged TEM grids (Quantifoil R2/1, Cu 200 mesh) and plunge-frozen in liquid ethane by a Vitrobot Mark IV (Thermo Fisher Scientific) using the following parameters: humidity, 95%; temperature, 4°C; wait time, 30 s; blot total, 2; blot force, 3; blot time, 3 s. Cryo-EM data were acquired on a Titan Krios G3i microscope (Thermo Fisher Scientific) at the National Synchrotron Radiation Centre in Poland (SOLARIS), operated at 300 kV accelerating voltage, a $\times 105,000$ magnification, and a corresponding pixel size of 0.8456 Å. A K3 direct electron detector used for data collection was fitted with BioQuantum Imaging Filter (Gatan, Pleasanton, CA, USA) using a 20-eV slit and operated in counting mode. Imaged areas were exposed to 40 e⁻/Å² total dose (corresponding to ~ 16 e⁻/px/s dose rate measured in vacuum). Forty-frame movie stacks were obtained using under-focus optical conditions with a defocus range of -2.1 to -0.6 μ m and 0.3 μ m steps. A total of 10,858, 14,378 and 9,478 movies were collected for AaLS-POF in mPBS-E, AaLS-POF in mHBS-E and AaLS-wt in mHBS-E, respectively.

Cryo-EM single particle reconstruction. All three datasets (AaLS-POF in mPBS-E, AaLS-POF in mHBS-E, and AaLS-wt in mHBS-E) were processed using cryoSPARC v4.5.3.³¹ Following patch motion correction and CTF estimation, particles were picked using 3D templates and extracted with 2 $\times$ binning. The binned particles were subjected to two rounds of 2D classification, after which the selected particles were used for ab initio reconstruction. For AaLS-POF in mPBS-E and AaLS-wt in mHBS-E, the selected particles were subjected to ab initio reconstruction followed by homogeneous refinement, both with icosahedral (I) symmetry. The particles were subsequently re-extracted without

binning, subjected to reference-based motion correction and CTF refinement, and further refined with I symmetry. Both final reconstructions reached a global resolution of 1.71 Å, corresponding to the Nyquist limit. For AaLS-POF in mHBS-E, ab initio reconstruction and subsequent heterogeneous refinement were performed with tetrahedral (T) symmetry. One well-resolved class was selected, and the corresponding particles were re-extracted without binning, subjected to reference-based motion correction and CTF refinement, and further refined with T symmetry to generate the “Consensus map”. The AaLS-wt structure in mPBS-E used for comparison was obtained from our previous study²³.

To improve the C3-associated density, particles contributing to the “Consensus map” were T-symmetry expanded and subjected to local refinement. Focused classification was then used to select particles with well-resolved local density of the C3 region, which were reconstructed using local refinement to generate the “Focused-refinement map”. A subsequent classification using a pore-centred mask selected particles with resolved pore-plugging pentamer density. Reconstruction of this subset yielded the “Filled-pore-focused map”. Global and local resolution of the final volumes was assessed using 0.143 FSC cut-off. The three maps reached global resolutions of 3.37, 2.58 and 2.81 Å, respectively. Particle numbers retained at each processing step are indicated in Extended Data Figs. 2 and 3. The “Focused-refinement” and “Filled-pore-focused” maps were independently post-processed using EMReady²³². Four copies of the post-processed maps were then independently combined in ChimeraX v1.10.1³³ with consensus map to generate corresponding composite maps.

Molecular modeling. The initial atomic model of AaLS was derived from the 1.6 Å X-ray crystal structure of wildtype AaLS (PDB: 1HQB [<http://doi.org/10.2210/pdb1HQB/pdb>])¹⁷. Three atomic models were generated using the corresponding cryo-EM maps: AaLS-wt in mHBS-E, AaLS-POF in mPBS-E, and AaLS-POF in mHBS-E using the composite map generated from the “Focused-refinement”. The initial coordinates were rigid-body fitted into the respective maps using ChimeraX, followed by flexible fitting in ISOLDE v1.9³⁴, manual adjustment in WinCoot v0.9.8.96³⁵, and real-space refinement in Phenix v1.20.1-4487³⁶. Model quality was assessed using MolProbity³⁷, and

structural visualizations were prepared in ChimeraX. Model-refinement and validation statistics are presented in Extended Data Table 1.

TEV protease cleavage assay. AaLS cages encapsulating GFP via a peptide containing TEV protease recognition sequence were prepared in mPBS-E or mHBS-E. The reactions were initiated upon the addition of TEV protease^{38, 39, 40} (0.2 equiv. to GFP) to the GFP-containing AaLS cage samples and incubated at room temperature for 18 hours. The samples were then analyzed by SDS-PAGE and native-AGE.

SDS-PAGE. Samples were prepared in SDS-PAGE loading buffer [62.5 mM Tris-HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate, 10% glycerol, 5% (v/v) β -mercaptoethanol, 0.02% (w/v) bromophenol blue], followed by heat denaturation at 95 °C for 10 min. Electrophoresis was performed using 15% polyacrylamide gels, and a Tris-glycine buffer system at 80 V for 10 minutes, followed by 150 V for 60 min. As a protein standard, PageRuler Prestained Protein Ladder (Thermo Fisher Scientific) was used. Protein bands were visualized using ReadyBlue Protein Gel Stain (Sigma-Aldrich).

Native agarose gel electrophoresis (AGE). Samples were prepared in corresponding buffers and supplemented with a 4 $\times$ native-PAGE loading buffer [40% (v/v) glycerol, 0.016% (w/v) bromophenol blue in mPBS-E or mHBS-E]. Electrophoresis was performed using 0.5 μ g of AaLS proteins, 1.2% (w/v) agarose gels, and a running buffer [0.1 M Tris base, pH adjusted with phosphoric acid to ~7.6] at 4 °C and 70 V for 40 min. Fluorescent bands were visualized using a ChemiDoc MP (Bio-Rad, Hercules, CA, USA) in the fluorescein detection mode (460–490 nm excitation and 532/28 nm emission filters). The same gels were also stained with ReadyBlue Protein Gel Stain.

Sortase A-mediated guest loading. Tetraglycine-modified fluorescein isothiocyanate (GGGGK(FITC)-NH₂) was purchased from GenScript Biotech (Rijswijk, The Netherlands). Triglycine-labeled oligonucleotides were purchased from biomers.net GmbH (Ulm, Germany), and FITC-labeled complementary strands from Thermo Fisher Scientific. Three double-stranded DNA constructs with 14, 20, and 30 base pairs (dsDNA₁₄, dsDNA₂₀, and dsDNA₃₀) were prepared by annealing a triglycine-labeled strand (5' to 3': dsDNA₁₄, (Gly)₃-GGACG TTGAC GTTC;

dsDNA₂₀, (Gly)₃-TCCAT GACGT TCCTG ACGTT; dsDNA₃₀, (Gly)₃-AAATC GTCGT TTTGT
CGTTT TGTCG TTTTT) with its FITC-labeled complementary strand (5' to 3': dsDNA₁₄, FITC-
GAACG TCAAC GTCC; dsDNA₂₀, FITC-AACGT CAGGA ACGTC ATGGA; dsDNA₃₀, FITC-
AAAAA CGACA AAACG AAAAA ACGAC GATTT). Equimolar concentrations of complementary
strands (100 μ M each) were mixed in TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA], heated at
95°C, and allowed to cool to room temperature by switching off the heat block.

Sortase A-mediated conjugation was performed using a calcium-independent heptamutant
variant of *Staphylococcus aureus* sortase A (SrtA 7M)²⁶ at room temperature for 3 hours with the
following concentrations: AaLS-POF (60 μ M as monomer), cargo (24 μ M), and SrtA 7M (2 μ M) in
mPBS-E or mHBS-E supplemented with 2 mM dithiothreitol (DTT). Excess cargo and enzyme were
removed by ultrafiltration using Amicon Ultra-0.5 mL centrifugal filters (100 kDa MWCO). For G4-
FITC, the sample was diluted approximately 10-fold and concentrated 4 times. For dsDNA constructs,
the sample was diluted approximately 10-fold and concentrated 8 times. Cage reclosure was
performed by adding 5 volumes of 0.5 M sodium phosphate buffer (pH 7.5) to a final concentration of
0.4 M and incubating at room temperature for > 24 hours. For dsDNA constructs, residual unreacted
cargo was further removed after recompaction by buffer exchange to mPBS-E containing 2 mM
MgCl₂ through three additional 10-fold dilution and concentration cycles.

Quantification of loaded cargo⁴¹. The number of FITC-labeled guest molecules loaded per cage (N)
was calculated as:

$$N = [\text{FITC-guest}] / [\text{cage}]$$

where [cage] represents the molar concentration of AaLS cage assemblies, calculated from the AaLS
monomer concentration divided by 60, assuming a 60-mer assembly:

$$[\text{cage}] = [\text{AaLS monomer}] / 60$$

The AaLS monomer concentration was determined from the absorbance at 280 nm (A_{280}) after
subtracting the contribution of FITC-labeled guest:

$$[\text{AaLS monomer}] = (A_{280} - \epsilon_{280(\text{FITC-guest})} \times [\text{FITC-guest}]) / \epsilon_{280(\text{AaLS})}$$

where A_{280} is the observed absorbance at 280 nm, $\epsilon_{280(\text{FITC-guest})}$ is the molar extinction coefficient of the FITC-labeled guest at 280 nm, and $\epsilon_{280(\text{AaLS})}$ is the molar extinction coefficient of AaLS monomer at 280 nm ($19,630 \text{ M}^{-1}\text{cm}^{-1}$).

The FITC-guest concentration was determined from the absorbance at 494 nm (A_{494}):

$$[\text{FITC-guest}] = A_{494} / \epsilon_{494(\text{FITC})}$$

where A_{494} is the observed absorbance at 494 nm and $\epsilon_{494(\text{FITC})}$ is the molar extinction coefficient of FITC at 494 nm ($70,000 \text{ M}^{-1}\text{cm}^{-1}$). The $\epsilon_{280(\text{FITC-guest})}$ was determined experimentally from the absorbance spectrum of each FITC-labeled cargo prior to loading. Absorbance spectra were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Light scattering contributions were subtracted using software a|c - UV-Vis-IR Spectral Software 1.2 (FluorTools, www.fluortools.com) prior to concentration determination⁴².

DNase challenge. A salt-tolerant variant, DNase I-XT (New England Biolabs GmbH, Frankfurt am Main, Germany), was used to avoid inhibition by 150 mM NaCl present in the buffer. Cleavage reactions were performed in 20 μL volumes with AaLS-POF (20 μM as monomer), 1 unit of DNase I-XT, in mPBS-E containing 5 mM MgCl_2 (mPBS-E-Mg) at 25°C for 90 min. Free dsDNA (0.2 μM) was treated under the same conditions to confirm complete degradation. For comparison, AaLS-POF cages prior to high phosphate recompaction were treated with DNase I-XT in mHBS-E containing 5 mM MgCl_2 (mHBS-E-Mg) to assess cleavage in the open cage state. Samples were analyzed by SDS-PAGE using a Tris-tricine buffer system, with other conditions as described above but with electrophoresis terminated when the bromophenol blue dye front reached ~1 cm above the gel end. FITC-labeled DNA was detected by fluorescence imaging (460–490 nm excitation and 532/28 nm emission), and the same gels were subsequently stained with ReadyBlue Protein Gel Stain. Protection efficiency (percent undigested) was estimated from FITC fluorescence intensities of SDS-PAGE bands quantified using ImageJ software, according to the following equation.

$$\text{Undigested (\%)} = (I_{\text{DNase}(+)} / I_{\text{DNase}(-)}) \times 100$$

where $I_{\text{DNase}(+)}$ and $I_{\text{DNase}(-)}$ are the summed FITC fluorescence intensities of the cpAaLS-srt-DNA conjugate and its dimer bands with and without DNase treatment, respectively.

Thermal denaturation. Protein solution (0.5 mg/mL) was prepared in either mPBS-E or mHBS-E using ultrafiltration and transferred to a Prometheus high-sensitivity glass capillary (NanoTemper Technologies, Munich, Germany) sealed with a dedicated sealing paste. Differential scanning fluorometry, coupled with dynamic light scattering, was performed on a Prometheus PANTA instrument (NanoTemper Technologies) over a temperature range of 20 to 120 °C. The midpoint temperature was determined from the first derivative peak of the tryptophan fluorescence ratio (350/330 nm). Experiments were performed in triplicate. The datasets were merged and analyzed using the PR.PantaAnalysis software. For DLS data, the intensity-based hydrodynamic radii are provided.

Phosphate derivatives and analogues. Sodium beta-glycerophosphate pentahydrate and sodium orthovanadate were purchased from Thermo Fisher Scientific; sodium molybdate dihydrate from ChemPur (Piekary Śląskie, Poland); dimethyl hydrogen phosphate from Apollo Scientific (Manchester, UK); methylphosphonic acid, hexylphosphonic acid, and sodium borate from Sigma-Aldrich (Darmstadt, Germany); sodium sulfate decahydrate from Carl Roth (Karlsruhe, Germany); sodium bicarbonate and sodium nitrate from VWR (Radnor, PA, USA). Their 20 mM solutions were prepared in mHBS-E while pH was adjusted to 7.5 with HCl(aq.) or NaOH(aq.) when needed. Vanadate was used without pH adjustment at pH 10.7 to minimize formation of condensed species.²⁷ Protein solution (0.5 mg/mL) in mPBS-E was transferred to mHBS-E containing 20 mM of each derivative or analogue using ultrafiltration and analyzed by dynamic light scattering on a Prometheus PANTA instrument at 25°C. Experiments were performed in triplicate, and means ± standard deviations of the intensity-based hydrodynamic radii are provided.

Data availability

Cryo-EM micrographs, reconstructions, and atomic models have been deposited in the Electron Microscopy Public Image Archive (EMPIAR), the Electron Microscopy Data Bank (EMDB), and the Protein Data Bank (PDB) under the following accession codes: EMPIAR-13974 (AaLS-wt in mHBS-E), EMD-59863 (<https://www.ebi.ac.uk/emdb/EMD-59863>) and PDB 34IH

(<https://doi.org/10.2210/pdb34ih/pdb>); EMPIAR-13975 (AaLS-POF in mPBS-E), EMD-59864 (<https://www.ebi.ac.uk/emdb/EMD-59864>) and PDB 34II (<https://doi.org/10.2210/pdb34ii/pdb>); EMPIAR-13976 (AaLS-POF in mHBS-E), EMD-59865 (<https://www.ebi.ac.uk/emdb/EMD-59865>) (consensus map), EMD-59867 (<https://www.ebi.ac.uk/emdb/EMD-59867>) (focused refinement map of three pentamers), EMD-59868 (<https://www.ebi.ac.uk/emdb/EMD-59868>) and PDB 34IJ (<https://doi.org/10.2210/pdb34ij/pdb>) (composite map and atomic model of the phosphate-free expanded cage), EMD-59869 (<https://www.ebi.ac.uk/emdb/EMD-59869>) (focused map of four pentamers), and EMD-59870 (<https://www.ebi.ac.uk/emdb/EMD-59870>) (composite map of the phosphate-free expanded cage with filled pores). Source data are provided with this paper.

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Author contributions

V.V.M., L.K., and Y.A. performed experiments and analyzed data. Y.A. conceptualized the project, oversaw the research, acquired the funding, and wrote the original draft. L.K. and Y.A. prepared the figures and edited the manuscript. All the authors contributed to the experimental design, read and approved the final version of the manuscript.

Competing interests

V.V.M., L.K., and Y.A. are inventors on a pending patent application (PCT/EP2025/071715; published as WO 2026/027498 A1) filed by Jagiellonian University and related to the phosphate-responsive protein cage technology described in this manuscript. The other authors declare no competing interests.

Additional information

Extended Data

Extended Data Figs. 1-8

Extended Data Tables 1-2

Supplementary information.

Supplementary Tables 1-3

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Extended Data for

Engineered protein cages with phosphate-dependent reversible opening

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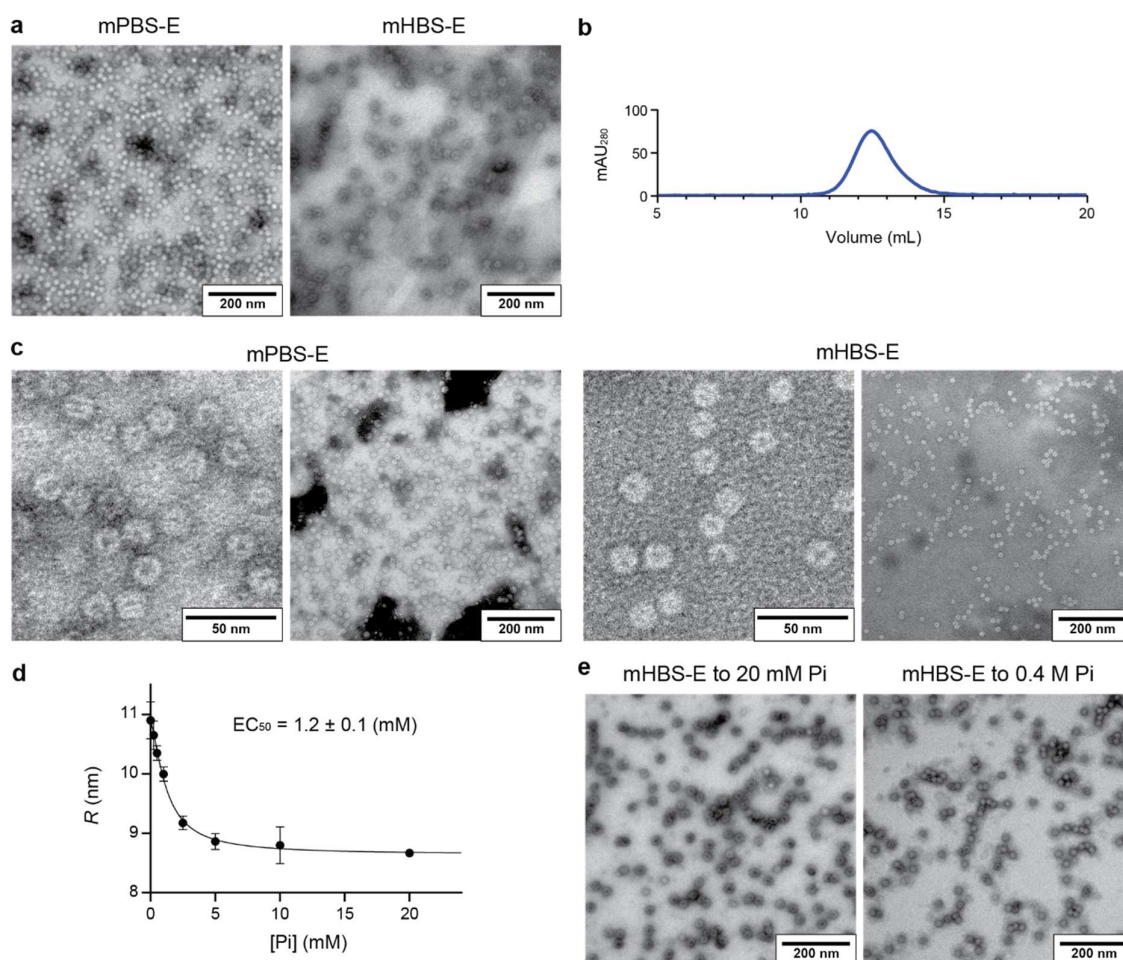
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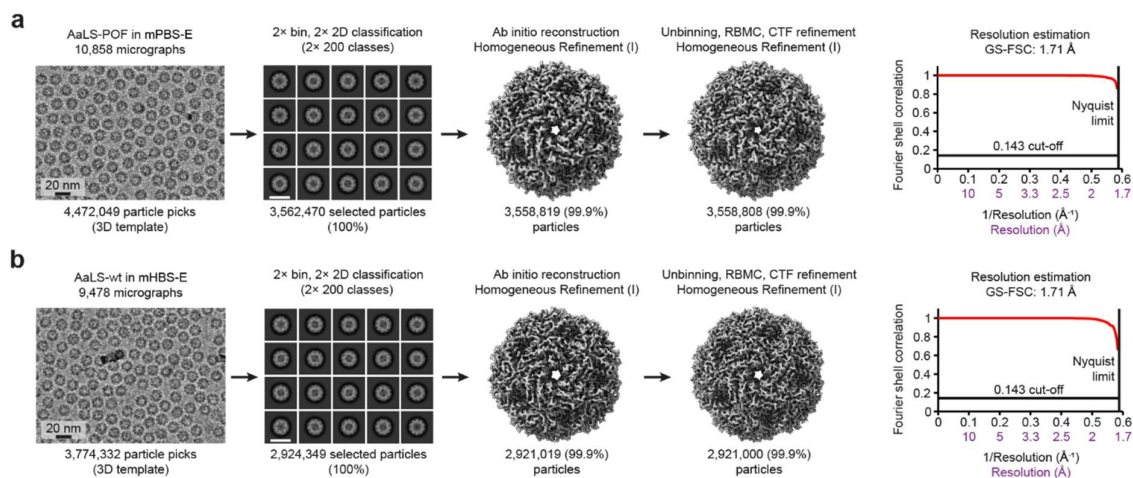
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Extended Data Figs. 1-8

Extended Data Tables 1 and 2



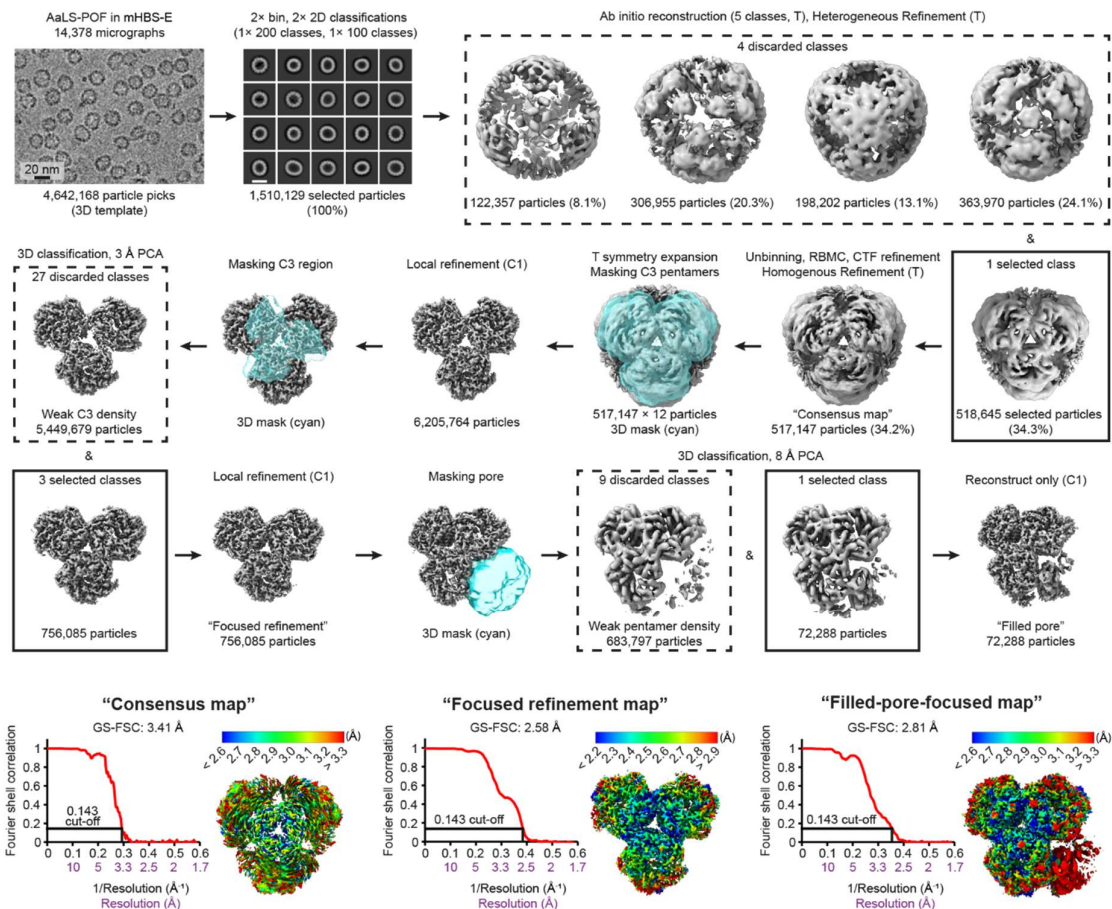
Extended Data Fig. 1 | Characterization of phosphate-dependent morphology change of AaLS-POF. **a**, Negative-stain TEM images of AaLS-POF in mPBS-E (left) and mHBS-E (right), wider field of view corresponding to Fig. 1c. **b**, Size-exclusion chromatography (SEC) of AaLS-POF in 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 1 mM EDTA. mAU₂₈₀, milli absorbance units at 280 nm. **c**, Negative-stain TEM images of AaLS-wt in mPBS-E (left) and mHBS-E (right) at enlarged and wider field of view, showing no detectable morphological change in either buffer. **d**, Hydrodynamic radius (R) of AaLS-POF at varying phosphate concentrations ($[Pi]$), measured by dynamic light scattering (DLS). Data are presented as means $\pm$ standard deviation of triplicate experiments with the curve fitted to the average values. The half-maximal effective phosphate concentration (EC_{50}) was determined from individual titration analysis to be 1.2 ± 0.1 mM. **e**, Phosphate-dependent recompaction of AaLS-POF. Expanded cages isolated by SEC in mHBS-E were transferred to mPBS-E (20 mM Pi, left) or 0.4 M sodium phosphate buffer (0.4 M Pi, right) and imaged by negative-stain TEM. The right image shows a wider field of view corresponding to Fig. 1d.



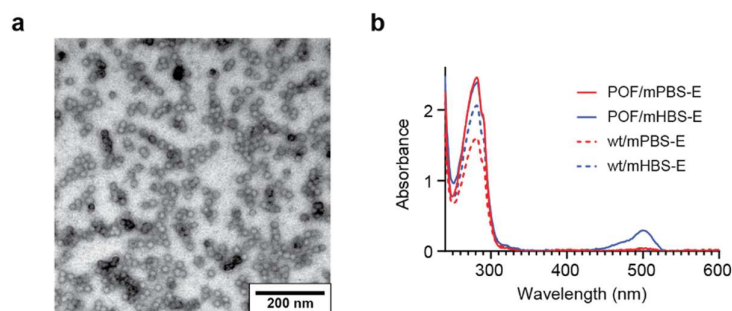
Extended Data Fig. 2 | Cryo-EM single-particle reconstruction of dodecahedral AaLS

assemblies. a,b, Processing pipelines for AaLS-POF in mPBS-E (a) and AaLS-wt in mHBS-E (b).

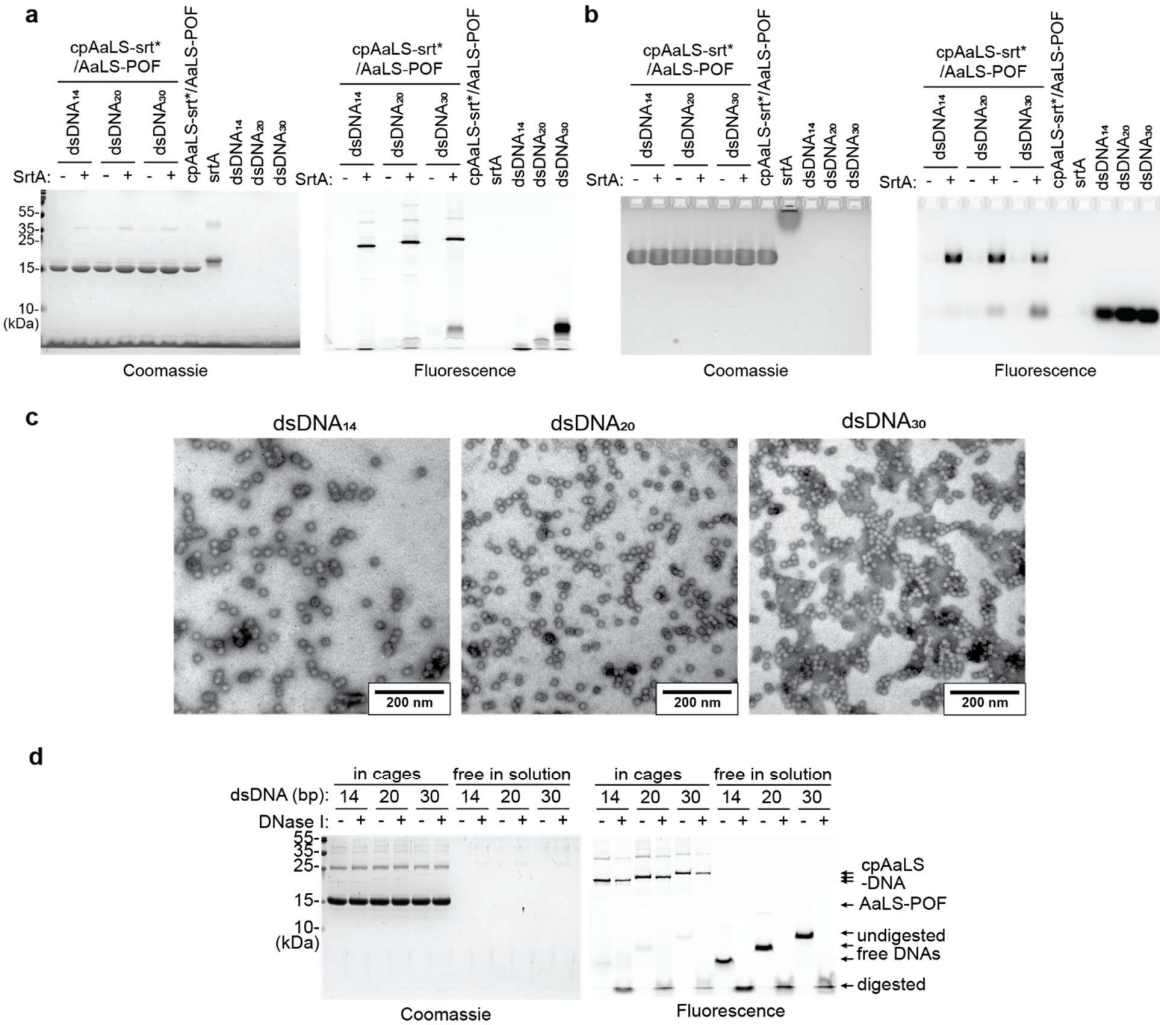
Global resolutions were estimated from gold-standard Fourier shell correlation (GS-FSC) curves using the 0.143 criterion. Particle numbers, percentages, imposed symmetries and relevant processing parameters are indicated at the corresponding steps. Maps are not shown to scale.



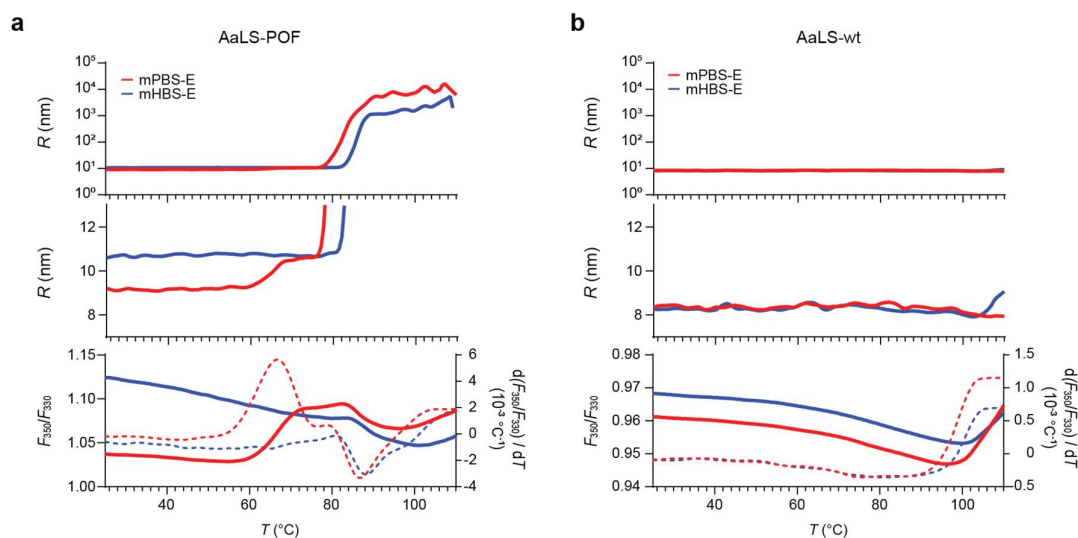
Extended Data Fig. 3 | Cryo-EM single-particle reconstruction of the expanded AaLS-POF assembly. Processing pipeline for AaLS-POF in mHBS-E, yielding three map types: the "Consensus map" after heterogeneous refinement; the "Focused refinement map" after T-symmetry expansion, C3-focused classification and local refinement; and the "Filled-pore-focused map" after additional pore-focused classification. Global resolutions were estimated from gold-standard Fourier shell correlation (GS-FSC) curves using the 0.143 criterion, and local-resolution maps are shown for all three reconstructions. Particle numbers, percentages, imposed symmetries, and relevant processing parameters are indicated at the corresponding steps. Maps are not shown to scale.



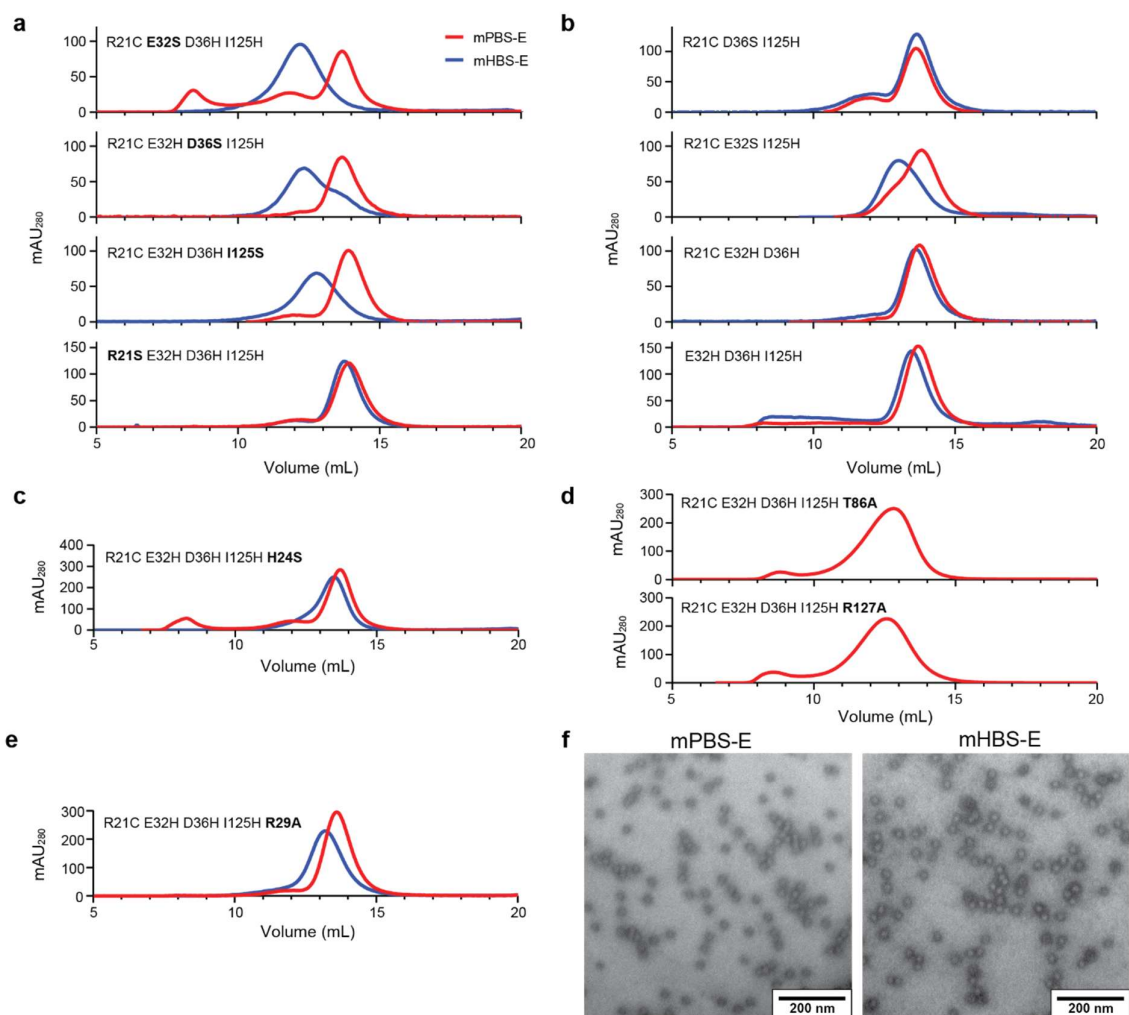
Extended Data Fig. 4 | G4-FITC loading into AaLS-POF cages. **a**, TEM images of G4-FITC-loaded cpAaLS-srt*/AaLS-POF patchwork cages after reclosure by 0.4 M phosphate, a wider view of the image shown in Fig. 3b. **b**, Absorbance spectra of G4-FITC-loaded cpAaLS-srt*/AaLS-POF (solid lines) and cpAaLS-srt*/AaLS-wt (dashed lines) patchwork cages, prepared in mPBS-E (red) or mHBS-E (blue). Data were used to quantify FITC loading efficiency.



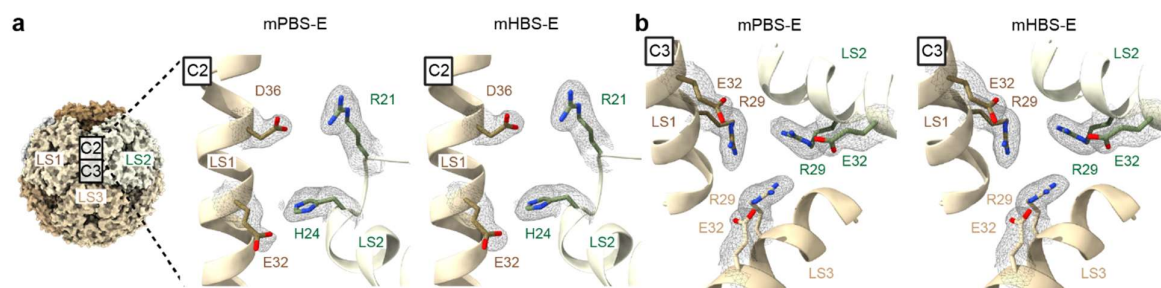
Extended Data Fig. 5 | dsDNA loading into AaLS-POF cages. **a,b**, SDS-PAGE (a) and native-AGE (b) analysis of sortase-mediated conjugation of cpAaLS-srt* with dsDNA₁₄, dsDNA₂₀, and dsDNA₃₀. Gels were visualized by colloidal Coomassie staining (left) and fluorescence (right). Native-AGE confirms association of the fluorescent dsDNA conjugates with AaLS-POF cages. **c**, TEM images of dsDNA-loaded AaLS-POF after reclosure. Wider views of the images shown in Fig. 3g. **d**, SDS-PAGE analysis of encapsulated and free dsDNA challenged with DNase I in mHBS-E-Mg, counterpart to the mPBS-E-Mg experiment shown in Fig. 3i. Fluorescent band intensities from both conditions were used for the quantification shown in Fig. 3j.



Extended Data Fig. 6 | Differential scanning fluorometry and dynamic light scattering assay. a,b, Measurements were performed with AaLS-POF (a) and AaLS-wt (b) in mPBS-E (red) and mHBS-E (blue). Data are presented as the hydrodynamic radius (R) and fluorescence ratio at 350/330 nm (F_{350}/F_{330}) over temperature (T) from 20 to 120 °C. Dashed lines indicate the first derivatives of the fluorescence ratio ($d(F_{350}/F_{330})/dT$). Middle graphs present a Y-axis-amplified view of the top graph. A cropped view of panel a is shown in Fig. 4a.



Extended Data Fig. 7 | SEC analysis of AaLS-POF mutants. a, Serine substitution of individual histidine or cysteine residues introduced in AaLS-POF. **b**, Reversion of individual mutations in AaLS-POF to the original wildtype residue at positions 21, 32, 36, or 125. **c**, Additional H24S mutation in AaLS-POF to evaluate the contribution of the Cys21-His24 interaction. **d**, Additional T86A or R127A mutations in AaLS-POF, to assess the contribution of the native phosphate-binding site to the conformational change. **e**, Additional R29A mutation in AaLS-POF, to evaluate the role of the secondary phosphate-binding site. Chromatography was performed using a Superose 6 Increase column, running in mPBS-E (red) or mHBS-E (blue). **f**, Negative-stain TEM images of AaLS-POF-R29A in mPBS-E or mHBS-E, a wider view of the image shown in Fig. 4e.



Extended Data Fig. 8 | Cryo-EM structures of AaLS-wt in mPBS-E and mHBS-E. a,b, Enlarged views of the C2 (a) and C3 (b) symmetry regions in mPBS-E (PDB 9RYN [<https://doi.org/10.2210/pdb9ryn/pdb>]) and mHBS-E (PDB 34IH [<https://doi.org/10.2210/pdb34ih/pdb>]). The overall dodecahedral assembly is shown at left to indicate the regions examined. Panel b shows the absence of phosphate binding at C3. Cryo-EM densities around the indicated residues are shown as mesh. Panels a and b show the corresponding AaLS-wt views for the AaLS-POF structures presented in Fig. 4b and 4d.

Extended Data Table 1 | Cryo-EM data collection and structure refinement statistics

Variant	AaLS-wt	AaLS-POF	AaLS-POF				
Buffer	mHBS-E	mPBS-E	mHBS-E				
EMPIAR	13974	13975	13976				
Assembly (pentamers)	12	12	12-16	3	12	4	16
Map type	Homogeneous	Homogeneous	Consensus	Focused	Composite	Focused	Composite
EMDB	59863	59864	59865	59867	59868	59869	59870
PDB	34IH	34II	N/A	N/A	34IJ	N/A	N/A
Data collection and processing							
Magnification	105,000 ×	105,000 ×	105,000 ×				
Voltage (keV)	300	300	300				
Electron exposure (e ⁻ /Å ²)	40	40	40				
Defocus range (μm)	-0.6 to -2.1	-0.6 to -2.1	-0.6 to -2.1				
Pixel size (Å)	0.8456	0.8456	0.8456				
Micrographs (no.)	9,478	10,858	14,378				
Initial particle images (no.)	3,319,495	3,847,874	3,550,837				
Final particle images (no.)	2,921,000	3,558,808	517,147	756,085	756,085	72,288	72,288
Symmetry imposed	I	I	T	C1	T	C1	T
Map resolution (Å)	1.71 ¹	1.71 ¹	3.41	2.58	2.58	2.81	2.81
FSC threshold	0.143	0.143	0.143	0.143	0.143	0.143	0.143
Map resolution range 25 th –75 th percentile (Å)	1.71 ¹	1.71 ¹	3.19–4.69	2.45–3.07	2.45–3.07	2.85–4.51	2.85–4.51
Map sharpening B-Factor (Å ²)	55.0	51.0	168.2	60.9	60.9	45.3	45.3
Model building							
Initial model	AaLS PDB ID: 1HQK						
Model composition							
Protein chains	60	60	60				
Protein residues	9,240	9,240	9,240				
Non-hydrogen atoms	73,110	72,970	70,620				
Waters	2,190	1,950	0				
Ligands (PO ₄)	0	80	0				
Protein <i>B</i> factors (mean Å ²)	40.03	36.52	63.08				
Ligand (PO ₄) <i>B</i> factors (mean Å ²)	N/A	66.57	N/A				
Water <i>B</i> factors (mean Å ²)	51.00	47.04	N/A				
R.M.S. deviations							
Bond lengths (Å)	0.005	0.007	0.004				
Bond angles (°)	0.957	0.961	0.923				
Validation							
MolProbity score	0.87	1.02	1.12				
Clash score	0.47	1.10	2.05				
Poor rotamers (%)	0.00	0.00	0.00				
Ramachandran							
Favored (%)	96.71	96.71	97.20				
Allowed (%)	3.29	3.29	2.80				
Outliers (%)	0.00	0.00	0.00				
CC (volume)	0.96	0.95	0.89				
CC (ligands)	0.87	0.88	N/A				

¹ Nyquist limit

869 **Extended Data Table 2 | Model cargo double-stranded DNAs**

name		sequence (5' → 3')
dsDNA ₁₄	strand 1	(Gly) ₃ -GGACGTTGACGTT
	strand 2	FITC-GAACGTCAACGTCC
dsDNA ₂₀	strand 1	(Gly) ₃ -TCCATGACGTTCTGACGTT
	strand 2	FITC-AACGTCAGGAACGTCATGGA
dsDNA ₃₀	strand 1	(Gly) ₃ -AAATCGTCGTTTTGTCGTTTTGTCGTTTTT
	strand 2	FITC-AAAAACGACAAAACGACAAAACGACGATTT

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