Nanoreactors Hot Paper

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Quantitative Packaging of Active Enzymes into a Protein Cage

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Abstract: *Genetic fusion of cargo proteins to a positively supercharged variant of green fluorescent protein enables their quantitative encapsulation by engineered lumazine synthase capsids possessing a negatively charged lumenal surface. This simple tagging system provides a robust and versatile means of creating hierarchically ordered protein assemblies for use as nanoreactors. The generality of the encapsulation strategy and its effect on enzyme function were investigated with eight structurally and mechanistically distinct catalysts.*

Compartmentalization of enzymes within restricted spaces is a common strategy for controlling otherwise incompatible biosynthetic processes in complex intracellular environments. In bacteria, for example, protein-bounded microcompartments coordinate the activity of catalysts involved in carbon fixation and small-molecule metabolism.[1] Like the lipid membrane of eukaryotic organelles, the protein shells of such assemblies restrict diffusion into and out of the enclosed chamber, thereby providing a means of regulating the encapsulated enzymes, concentrating volatile reactants, sequestering toxic intermediates, and avoiding undesired side reactions.[1]

Liposomes,[2] synthetic polymers,[3] and protein cages[4] have been extensively used as artificial microcompartments to investigate how confinement and spatial organization affect enzyme activity. Virus-like particles are particularly attractive in this regard because of their biocompatibility, spontaneous self-assembly into highly organized structures of defined size and shape, and ready modification by chemical and genetic methods.[5] Diverse strategies have been exploited to load virus capsids with cargo molecules, including diffusion methods,[4a] coiled-coil[4b] and electrostatic[4c] tagging systems, RNA-mediated loading,[4d] and genetic fusion to scaffolding proteins.[4e–g] Because enzyme stoichiometry can influence pathway output,[6] control over guest loading is highly desirable in such systems. Experiments with cowpea chlorotic mottle virus (CCMV) have shown that both covalent and noncovalent approaches can afford considerable control over packing density.[7]

We recently developed an alternative encapsulation system based on the non-viral capsid-forming enzyme lumazine synthase from *Aquifex aeolicus* (AaLS).[8] Introduction of four glutamate residues on the lumenal surface of the protein yielded AaLS-neg, which forms inclusion complexes with complementarily charged guest molecules bearing a deca-arginine (R10) tag in vivo.[8a] AaLS-neg was further optimized by directed evolution, resulting in an improved variant, AaLS-13, with higher loading capacity under physiological conditions.[8b] In addition to R10-tagged proteins, AaLS-13 efficiently encapsulates the positively supercharged green fluorescent protein GFP(+36) in vitro.[8c] Here we show that GFP(+36) can also be used as a genetically encodable tag to target cargo enzymes to the interior of AaLS-13 capsids (Figure 1A,B).

As a model enzyme, we selected a computationally designed and experimentally optimized retro-aldolase (RA) that catalyzes the cleavage of (±)-4-hydroxy-4-(6-methoxy-2-naphthyl)-2-butanone (methodol, **1**) to 6-methoxy-2-naphthaldehyde (**2**) and acetone (Figure 1C).[9] The K210M

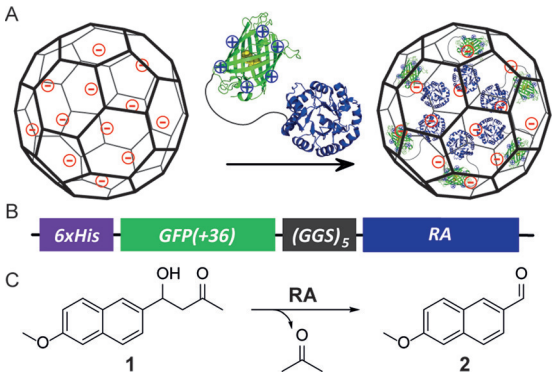

Figure 1. A strategy for encapsulating active enzymes in a protein cage. A) GFP(+36) (green) serves as a directing tag to load appended cargo proteins (blue) into empty AaLS-13 capsids. B) Designed gene for the GFP(+36)-fused retro-aldolase (RA). C) The retro-aldol reaction catalyzed by RA.

variant of RA95.5–8[9] ($k_{cat} = 0.18\ s^{-1}$, $k_{cat}/K_M = 1800\ M^{-1}s^{-1}$) was N-terminally fused to GFP(+36) through a (Gly-Gly-Ser)$_5$ linker (Figure 1B). Despite an approximately 10-fold decrease in catalytic activity ($k_{cat} = 0.018\ s^{-1}$, $k_{cat}/K_M = 170\ M^{-1}s^{-1}$), the unoptimized GFP(+36)–RA fusion construct still efficiently cleaves the chromogenic substrate. The resulting absorbance change provides a facile means of monitoring enzymatic activity upon encapsulation.

Like GFP(+36), GFP(+36)–RA was readily taken up by purified AaLS-13 capsids.[10] Following the simple method described for the encapsulation of GFP(+36),[8c] GFP(+36)–RA was mixed with empty AaLS-13 capsids in aqueous buffer (pH 8.0, ionic strength = 0.34 M) at various ratios. Size-exclusion chromatography of the mixtures, followed by SDS-PAGE analysis of the capsid fraction, showed that the tagged aldolase associated with AaLS-13 (Figure 2A,B and Figure S1 in the Supporting Information), whereas only negli-

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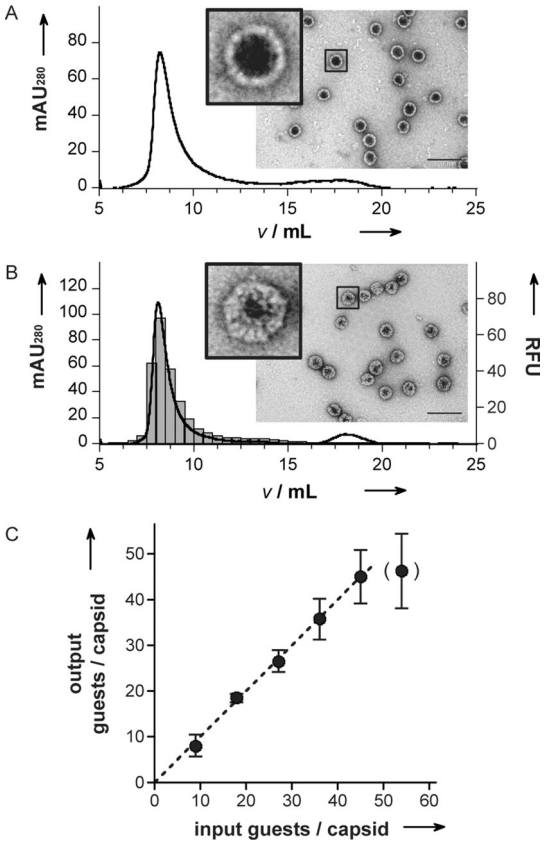

Figure 2. Quantitative loading of GFP(+36)–RA into empty AaLS-13 capsids. Size-exclusion chromatogram of A) empty capsids and B) capsids mixed with 45 equivalents of GFP(+36)–RA. Traces and bars correspond to $\lambda = 280$ nm absorbance and fluorescence readings from individual fractions, respectively. Insets show TEM images of A) empty and B) filled AaLS-13 capsids (scale bar: 100 nm); a representative particle from each sample is enlarged. C) Average number of encapsulated GFP(+36)–RA fusion proteins per AaLS-13 capsid as a function of input mixing ratio. Errors indicate the standard deviation for three independent experiments. The linear fit (excluding the point in parentheses) has a slope of 0.995 (dashed line).

gible association was observed for untagged RA (Figure S2). Negative-staining transmission electron microscopy (TEM) of the capsid particles confirmed that the fusion protein localized to the capsid interior (Figure 2B, inset). Host–guest stoichiometry was estimated from the $\lambda = 280$ nm/488 nm absorbance ratio. A linear correlation between input and output host/guest ratios was observed up to 45 GFP(+36)–RAs per capsid, assuming that AaLS-13 adopts a 180-subunit $T = 3$ structure[8b] (Figure 2C). Increasing the input ratio further did not afford higher loading densities owing to co-precipitation of both proteins. These results show that cargo encapsulation is essentially quantitative up to around 45 guests per $T = 3$ capsid. The observed encapsulation efficiency is comparable to that of GFP(+36) alone.[11] Recent studies on the loading mechanism suggest that GFP(+36) associates tightly with the interior surface of the AaLS-13 capsid shell,[12] thus leaving ample space to accommodate a fusion partner in the central cavity (Figure 2B inset). Approximately 1.5 GFP molecules would fit on the lumenal surface of one pentameric subunit of AaLS (Figure S4), which is in good agreement with the observed ratio of around 45 guests per 180-mer capsid (1.25 guests per pentamer).

Although an R10 tag has been successfully employed to direct GFP and HIV protease to the lumen of AaLS-neg and AaLS-13 in vivo,[8] it is less effective than GFP(+36) for in vitro encapsulation. When 18 equivalents of an R10-tagged retro-aldolase (RA–R10) were mixed with AaLS-13, only 2.7 ± 1.1 enzymes co-purified with each capsid (Figure S5); higher cargo/capsid ratios caused precipitation. The low net charge of RA–R10 (+4.3 at pH 8.0 versus +29.9 for GFP (+36)–RA and +20.0 for the originally studied HIV protease–R10 dimer) may account for its comparatively poor in vitro encapsulation efficiency.

The activity of the encapsulated aldolase was found to be sensitive to packing density. At low loading (e.g., ca. 8 guests per capsid), bound GFP(+36)–RA exhibited nearly the same k_{cat} value ($0.018 \pm 0.001\ s^{-1}$) as free enzyme in solution but a 4.5-fold higher K_M value ($470 \pm 120\ \mu M$; Figure 3A). Increasing the concentration of enzyme within the capsid had little effect on k_{cat}/K_M but led to a gradual decrease in k_{cat} (Figure 3B), likely owing to inactivation of the enzyme by the aldehyde product **2**, which can form a covalent Schiff base adduct with the catalytic lysine.[13] The generation of high concentrations of this reactive metabolite in the vicinity of the densely packed enzymes would be expected to exacerbate this problem. In analogy to the cascade reactions promoted in natural bacterial micro-compartments,[1] however, further processing of the aldehyde by additional co-encapsulated enzymes should be possible.

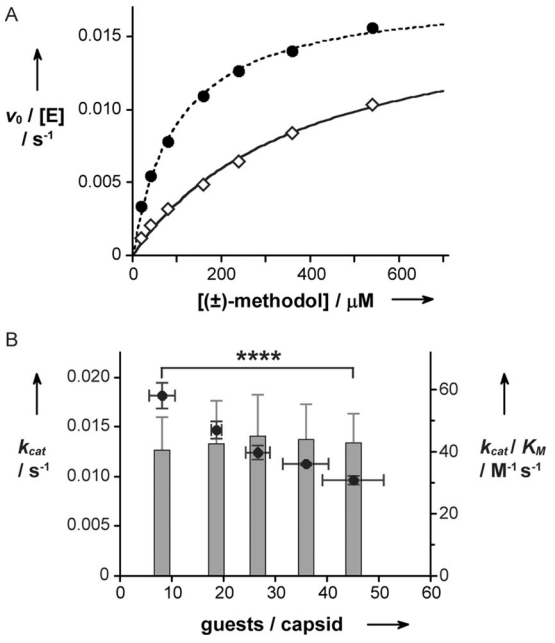

Figure 3. Retro-aldolase activity in AaLS-13. A) Michaelis–Menten plots for GFP(+36)–RA in solution (circles) and in AaLS-13 (diamonds; 8.6 GFP(+36)–RA fusion proteins per capsid). B) k_{cat} (circles) and k_{cat}/K_M (bars) parameters for encapsulated GFP(+36)–RA as a function of catalyst loading density. Errors indicate the standard deviation for three independent experiments. ****: $P < 0.001$.

The generality of this simple targeting system was assessed by fusing GFP(+36) to seven other enzymes: a computationally designed Kemp eliminase (KE),[14] a TEM β- lactamase (βLac),[15] a cyclohexylamine oxidase (CHAO),[16] a catalase-peroxidase (KatG),[17] an NADH oxidase (NOX),[18] an aldehyde dehydrogenase (AldH),[19] and a monoamine oxidase (MAO).[20] These proteins cover a broad range of properties in terms of molecular mass (29 kDa to 78 kDa), quaternary state (monomer, dimer, and tetramer), and net charge (−23.9 to +2.5 at pH 8.0; Table S1). Monomeric cargo enzymes with intermediate charge, like GFP(+36)–KE and GFP(+36)–βLac, were encapsulated by AaLS-13 as efficiently as GFP(+36)–RA (Figure 4A and Figure S6A,B, Table S1). Encapsulation of very negatively charged proteins, like CHAO and KatG (Table S1), was less straightforward, however. Although GFP(+36)–CHAO and GFP(+36)–KatG quantitatively associated with AaLS-13 (Figure 4A), TEM images of isolated particles suggest that the guests partially attached to the exterior of the capsid (Figure S6C,D). Conversely, cargo molecules with too much positive charge led to precipitation and hence lower loading efficiencies. This was observed with the homodimeric enzyme GFP(+36)–NOX, which has a calculated net charge of +78.2 at pH 8.0 (Figure 4A and Table S1). Precipitation was even more severe with the tetrameric enzymes AldH and MAO bearing four copies of the positively supercharged GFP(+36) tag. Further fine-tuning of the electrostatic interaction between guests and host capsids will be necessary for efficient encapsulation of such proteins.[12, 21]

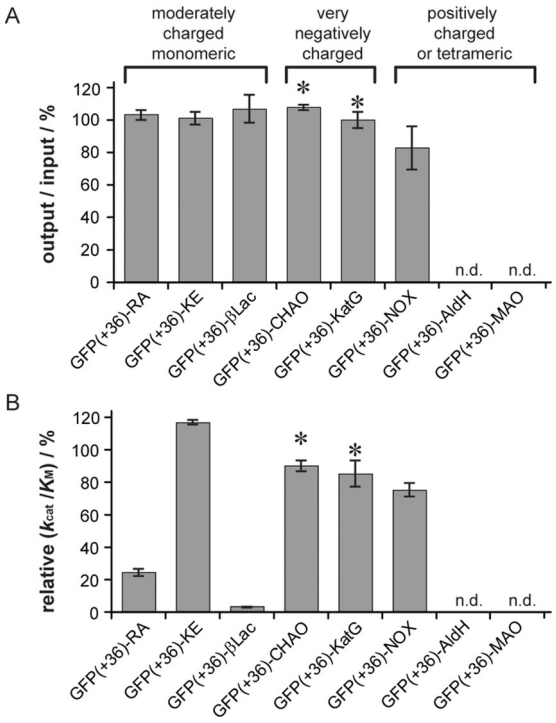

Figure 4. Scope of the targeted encapsulation strategy. A) Fraction of the GFP(+36) fusion enzyme associated with AaLS-13. B) Activity of the GFP(+36)–enzyme/AaLS-13 complexes relative to that of free enzyme. *: includes some guests associated with the capsid exterior (see Figure S6C, D). n.d., not determined due to protein precipitation. Experimental details are summarized in Tables S1 and S2.

The model enzymes in this study act on a range of substrates that vary in both size and charge. Remarkably, and in contrast to encapsulated GFP(+36)–RA, which was around 5-fold less efficient than the corresponding free enzyme, GFP(+36)–KE, GFP(+36)–CHAO, GFP(+36)–KatG, and GFP(+36)–NOX retained nearly full activity with their native substrates upon complexation with AaLS-13 (Figure 4B and Table S2). Although the full scope and mechanism of molecular transport still needs to be elucidated, the high turnover of encapsulated GFP(+36)–KE (k_{cat} = 380 s^{-1}) is especially notable, since it implies fast diffusion of the neutral 5-nitrobenzisoxazole substrate across the capsid wall. Retention of NOX activity additionally shows that the negatively charged capsid shell does not prevent the uptake of negatively charged substrates like NADH. Molecules capable of reacting directly with the host or guest proteins, like aldehyde **2**, are potentially problematic of course. Activity could also be adversely affected by unfavorable interactions between the encapsulated enzyme and the negatively charged capsid lumen that block the active site and/or populate an inactive form of the catalyst. Such effects may be responsible for the more than 10-fold drop in catalytic efficiency observed for encapsulated GFP(+36)–βLac, for example (Figure 4B). These limitations notwithstanding, the facility with which assembled AaLS-13 cages rapidly take up a wide range of cargo molecules irrespective of size and charge promises to be broadly useful for efforts to engineer artificial microcompartments for novel applications.

In summary, this study establishes GFP(+36) as a useful, genetically encodable tag for efficiently packaging active enzymes in AaLS-13 protein cages. The encapsulation procedure is easy and robust. Simply mixing host and cargo under mild aqueous conditions leads to internalization. No pH or temperature changes are required. Loading is nearly quantitative up to around 45 guest enzymes per $T = 3$ capsid, thereby affording precise control over the density of guest enzymes in the lumenal space. Although encapsulation is straightforward for most monomeric enzymes, modulating the surface charge of the enzyme or GFP may be needed to avoid difficulties encountered with very negatively charged or oligomeric enzymes. Nevertheless, the properties of this simple encapsulation system set the stage for creation of more complex nanoreactors by co-encapsulation of sequentially acting enzymes. The competitive advantage such cascades provide to organisms is currently under investigation in our laboratory.

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[10] Although packaging of GFP(+36) could also be achieved starting from unassembled capsid components, mixing GFP(+36)–RA with AaLS-13 fragments only afforded low yields of capsid–cargo complexes (Figure S3). The fusion partner may sterically interfere with capsid assembly.

[11] We previously reported that AaLS-13 encapsulates up to 100 GFP(+36) proteins based on Bradford analysis of total protein concentration and determination of GFP(+36) concentration by fluorescence.[8c] Loading yields calculated independently from $\lambda = 280$ nm/488 nm absorbance ratios suggests that the earlier values were overestimated by a factor of around 2, probably because the staining efficiency of the supercharged GFP(+36) and AaLS-13 proteins differs from that of bovine serum albumin, which was used as a standard in the Bradford assay.

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