Catalytic capsids: the art of confinement

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Supplementary information

Design of PalB with E-coil and Capsid protein with K-coil.

The PalB variant used contains an N-terminal periplasmic localization signal and a C-terminal histidine tag (His-tag). The His-tag is located at the C-terminus, to prevent cleavage in the periplasm. To couple PalB to the capsid protein, PalB was modified with a coiled-coil sequence, located at the C-terminus between the PalB sequence and the His-tag sequence.
The heterodimeric coiled-coil consists of a negatively charged coiled-coil (E-coil, sequence $EIAALEK_3$) and a positively charged coiled coil (K-coil, sequence $KIAALKE_3$). The capsid protein can only be modified at the N-terminus, since the C-terminus is heavily involved in dimeric contacts. To prevent sticking of the coiled-coil to the highly positively charged capsid interior, the capsid protein is modified with the positively charged K-coil, located between the N-terminal His-tag and the capsid protein. To ensure binding of the PalB to the capsid protein in the proper orientation, the PalB consequently has to be modified with the E-coil at the C-terminus.
After addition of the PalB-capsid protein complex to the wild type capsid protein, the pH is lowered to induce capsid assembly, thus encapsulating PalB. The N-termini of the capsid proteins are located on the capsid interior, and it is therefore expected that PalB will also be located on the capsid interior. In our previous paper[1] we have shown that EGFP, which is encapsulated using the same procedure as used in this contribution to encapsulate PalB, is located inside the capsid by, among others, size analysis and immuno labeling. As also in this case both FPLC and TEM results show no difference in size between empty capsid and capsids with PalB, it is highly unlikely that PalB resides on the capsid exterior.

Diffusion constant CCMV capsid derived from FCCS data.

From the FCCS data a typical dwell time of 2 ms was calculated. For a 250 nm wide focal volume, that gives the CCMV capsid a diffusion constant of 7.4 μm2/s. This corresponds with a Stokes-Einstein radius of 29 nm, which is in reasonable agreement with the size known from TEM images.

Additional methods

Capsid protein with K-coil and His-tag construction in vector pET-15b

The insert containing the K-coil DNA sequence was designed to have a 5' NdeI and a 3' NdeI restriction site overhang after annealing of the strands (table S1). The pET-15b vector containing the capsid protein sequence was digested sequentially with NdeI, dephosphorylated with Antartic Phosphatase, and purified by agarose gel elecrophoresis. The vector was mixed with the annealed insert and ligated with T4 DNA ligase. The resulting plasmids were redigested with NdeI. The insert was designed such that after ligation the plasmid would not contain NdeI restriction sites anymore. Thus ensuring that only plasmids with the insert would not be liniarized by the digestion. The plasmids were then transformed into *E. coli* XL1-Blue cells, the DNA was extracted and the sequence of the capsid protein with K-coil and his-tag was confirmed by DNA sequencing. The plasmids were then transformed into *E. coli* BL21(DE3)pLysS cells, which were used for production of the capsid protein with the K-coil and His-tag.

PalB with E-coil construction in vector pET-22b

The insert containing the E-coil DNA sequence was designed to have a 5' XhoI and a 3' XhoI restriction site overhang after annealing of the strands (table S1).

The pET-22b vector containing the PalB protein sequence was digested with XhoI, dephosphorylated with Antartic Phosphatase, and purified by agarose gel elecrophoresis. The vector was mixed with the annealed insert and ligated with T4 DNA ligase. The resulting plasmids were redigested with XhoII. The insert was designed such that after ligation the plasmid would not contain XhoI restriction sites anymore. Thus ensuring that only plasmids with the insert would not be liniarized by the digestion.

The resulting plasmids were transformed into *E. coli* XL1-Blue cells, the DNA was extracted and the sequence of the PalB with E-coil was confirmed by DNA sequencing. The plasmids were then transformed into *E. coli* BL21(DE3)pLysS cells, which were used for production of the PalB enzyme with the E-coil.

Table S1. DNA and amino acid sequences. DNA sequences for the inserts used for the creation of the His-CK and PalBE proteins. Overhangs are complementary with the overhangs in the vector sequences, created with restriction enzymes. Amino acid sequences for wt CP, His-CK and PalBE protein. K-coil amino acid sequence is depicted in blue; E-coil amino acid sequence is depicted in red

K-coil forward 5'-->3' K-coil reverse 3'-->5'	TAGCAAAATTGCCGCGCTGAAAGAAAAAATTGCGGCCCTGAAAGAAAAAATCGCGGCGCTGAAAGAAGG CGTTTTAACGGCGCGACTTTCTTTTTTAACGCCGGGACTTTCTTTTTTAGCGCCGCGACTTTCTTCCAT
E-coil forward 5'-->3' E-coil reverse 3'-->5'	TCGACGAAATTGCGGCGCTGGAAAAAGAAATTGCGGCGCTGGAAAAAGAAATTGCGGCGCTGGAAAAACTGGTGCCGCGCGGCAGCG GCTTTAACGCCGCGACCTTTTTCTTTAACGCCGCGACCTTTTTCTTTAACGCCGCGACCTTTTTGACCACGGCGCGCCGTCGCAGCT
wt CP amino acid sequence	MSTVGTGKLTRAQRRAAARKNKRNTRVVQPVIVEPIASGQGKAIKAWTGYSVSKWTASCAAAEAKVTSAITISLPNELSSERNKQLKVGRV LLWLGLLPSVSGTVKSCVTETQTTAAASFQVALAVADNSKDVVAAMYPEAFKGITLEQLAADLTIYLYSSAALTEGDVIVHLEVEHVRPTF DDSFTPVY
Capsid with His-tag and K-coil	MGSSHHHHHHSSGLVPRGSHSKIAALKEKIAALKEKIAALKEGMMSTVGTGKLTRAQRRAAARKNKRNTRVVQPVIVEPIASGQGKAIKAW TGYSVSKWTASCAAAEAKVTSAITISLPNELSSERNKQLKVGRVLLWLGLLPSVSGTVKSCVTETQTTAAASFQVALAVADNSKDVVAAMY PEAFKGITLEQLAADLTIYLYSSAALAEGDVIVHLEVEHVRPTFDDSFTPVY
PalB with E-coil amino acid sequence	MKYLLPTAAAGLLLLAAQPAMAMGLPSGSDPAFSQPKSVLDAGLTCQGASPSSVSKPILLVPGTGTTGPQSFDSNWIPLSAQLGYTPCWIS PPPFMLNDTQVNTEYMVNAITTLYAGSGNNKLPVLTWSQGGLVAQWGLTFFPSIRSKVDRLMAFAPDYKGTVLAGPLDALAVSAPSVWQQT TGSALTTALRNAGGLTQIVPTTNLYSATDEIVQPQVSNSPLDSSYLFNGKNVQAQAVCGPLFVIDHAGSLTSQFSYVVGRSALRSTTGQAR SADYGITDCNPLPANDLTPEQKVAAAALLAPAAAAIVAGPKQNCEPDLMPYARPFAVGKRTCSGIVTPLDEIAALEKEIAALEKEIAALEK LVPRGSVEHHHHHH

Expression of capsid protein with K-coil and His-tag

One colony of BL21(DE3)pLysS cells expressing His-CK was used to inoculate 100 mL of LB medium containing ampicilin (0.050 g/L) and chloramphenicol (0.025 g/L). After growth overnight at 30°C this culture was used to inoculate 900 mL of LB medium containing ampicilin (0.05 g/L) and chloramphenicol (0.025 g/L) and grown at 30°C. Protein expression was induced during logarithmic growth (OD_{600} =0.4-0.6) by addition of IPTG to a final concentration of 1 mmol/L. After induction the culture was grown at 30°C for 5 hours.

Expression of PalB with E-coil

One colony of BLR (DE3) pLysS cells expressing PalB with E-coil was used to inoculate 100 mL of 2xTY medium containing ampicilin (0.05 g/L) and tetracycline (0.0125 g/L). After growth overnight at 30°C this culture was used to inoculate 100 mL of 2xTY medium containing ampicilin (0.05 g/L) and tetracycline (0.0125 g/L) and grown at 30°C. Protein expression was induced during logarithmic growth (OD_{600} =0.4-0.6) by addition of IPTG to a final concentration of 1 mmol/L. After induction the culture was grown at 25°C for 20 hours.

Purification of capsid protein with K-coil and His-tag

E. coli cells containing the capsid protein with K-coil and His-tag were harvested by centrifugation at 4000 g for 15 min. at 4°C. The supernatant was discarded and the pelletted cells were stored at -20°C. After thawing, the pelletted cells were resuspended in approximately 10 mL lysisbuffer (50 mM NaH_2PO_4, 10 mM imidazole and 2000 mM NaCl pH 8.0). Approximately 10 mg of lysosyme (EC 3.2.1.17 from Fluka) was added and the solution was incubated at 4°C for 30 min. The solution was sonicated for 5 times 10 s with duty cycle 40 and output control 6 (Branson Sonifier 250, marius instruments Nieuwegein, the Netherlands). RNase (10 µg/mL) and DNase (5 µg/mL) was added and the mixture was incubated at 4°C for 15 min. The solution was centrifuged at 10,000 rpm for 20-30 min. to pellet the cellular debris. The supernatant of was incubated with 0.5 mL of Ni-NTA agarose beads for 1 h at 4°C. The flow-through was collected and the column was washed with 20 mL of wash buffer (50 mM NaH_2PO_4, 25 mM imidazole and 2000 mM NaCl pH 8.0). The capsid protein was eluted from the column using approximately 10 mL of elution buffer (50 mM NaH_2PO_4, 250 mM imidazole and 2000 mM NaCl pH 8.0) and the protein was collected. The capsid protein was dialyzed overnight to buffer pH 7.5, to remove the excess of imidazole. The protein purity was checked with SDS-Page gel electrophoresis (fig S1).

Purification of PalB with E-coil

E. coli cells containing the PalB with E-coil enzymes were harvested by centrifugation at 4000 g for 15 min. at 4°C. The supernatant was discarded and the pelletted cells were stored at -20°C. After thawing, the pelletted cells were resuspended in approximately 10 mL lysisbuffer (50 mM NaH_2PO_4, 10 mM imidazole and 300 mM NaCl pH 8.). Approximately 10 mg of lysosyme (EC 3.2.1.17 from Fluka) was added and the solution was incubated at 4°C for 30 min. The solution was sonicated for 5 times 10 s with duty cycle 40 and output control 6 (Branson Sonifier 250, marius instruments Nieuwegein, the Netherlands). The solution was centrifuged at 10,000 rpm for 20-30 min. to pellet the cellular debris. The supernatant of was incubated with 0.5 mL of Ni-NTA agarose beads for 1 h at 4°C. The flow-through was collected and the column was washed with 20 mL of wash buffer (50 mM NaH_2PO_4, 20 mM imidazole and 300 mM NaCl pH 8.0). PalB was eluted from the column using approximately 10 mL of elution buffer (50 mM NaH_2PO_4, 250 mM imidazole and 300 mM NaCl pH 8.0) and the protein was collected. PalB was dialyzed overnight to phosphate buffer pH

8.5 (100 mM phosphate, 150 mM NaCl), to remove the excess of imidazole. The protein purity was checked with SDS-Page gel electrophoresis (fig S1).

Production and purification of wt CP

The purification of the CCMV virus and the removal of its RNA were carried out according to literature procedures[2].

Formation and purification of PalB-capsid protein complex

PalB with E-coil was labeled with Alexa Fluor® 568. Excess dye was removed by dialyses to buffer pH 7.5. Approximately 1/3 of the obtained labeled PalB with E-coil was further purified by FPLC equipped with a Superdex 200 column. The rest of the labeled PalB with E-coil was added to an two times excess of His-CK and stirred for at least 16 h at 4°C to form the PalB-capsid protein complex. The PalB-capsid protein complex was further purified by FPLC equipped with a Superdex 200 column to remove any non-complexed PalB, capsid protein or other proteins. Pure PalB with E-coil eluted at V=1.6 mL as detected at λ=280 and 586 nm or, whereas the PalB-capsid protein complex eluted between V=1.3-1.5 mL.

PalB with E-coil encapsulation

PalB-capsid protein complex in buffer pH 7.5 (50 mM Tris-HCl, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA pH 7.5) was added in different ratios to wild type capsid protein in the same buffer. For the co-encapsulation of EGFP and PalB, GE-CK complex[1] was also added to the mixture. The proteins were allowed to mix for 5 min., before dialysis of the mixture overnight to buffer pH 5.0. The mixtures and non-encapsulated PalBE were then purified and analyzed on an FPLC system equipped with a Superose 6 column.

Calculations of PalB encapsulation

The number of PalB enzymes in a capsid can be calculated similarly to the number of EGFP molecules in a capsid[1], by using the absorption ratios of the protein absorption (λ=280 nm) and the dye absorption (λ=586 nm) at the capsid peak (V=1.1 mL) from the FPLC data.

1 Inge J. Minten, Linda J. A. Hendriks, Roeland J. M. Nolte et al., *J. Am. Chem. Soc.* **131** (49), 17771 (2009).
2 B. J. M. Verduin, *J. Gen. Virol.* **39** (APR), 131 (1978); B. J. M. Verduin, *FEBS Lett.* **45** (1), 50 (1974).

Additional figures

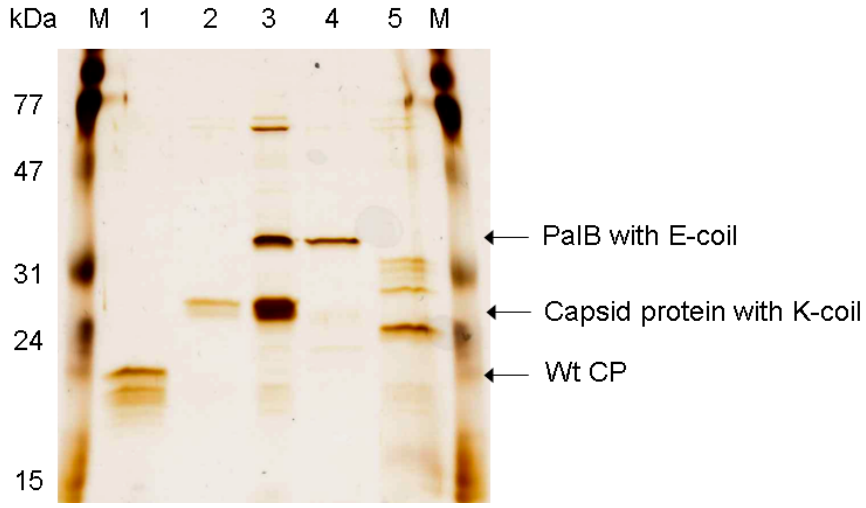

Figure S1 Silverstained SDS-Page gel of proteins and protein complexes used in the experiments. M indicates the marker, molecular weights are given in kDa. Lane 1: wt CP. Lane 2: Capsid protein with K-coil. Lane 3: PalB-capsid protein complex after purification on FPLC. Lane 4: PalB with E-coil. Lane 5: GE-CK complex.

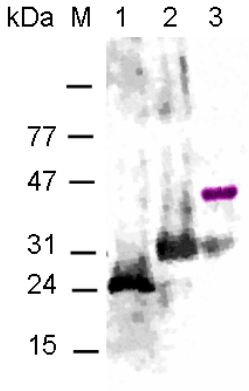

Figure S2 Overlay of immuno blotting data, using PalB (purple) and CCMV capsid protein (black) antibodies. Lane 1: wt CP. Lane 2: Capsid protein with K-coil. Lane 3: Capsid protein with K-coil and PalB with E-coil.

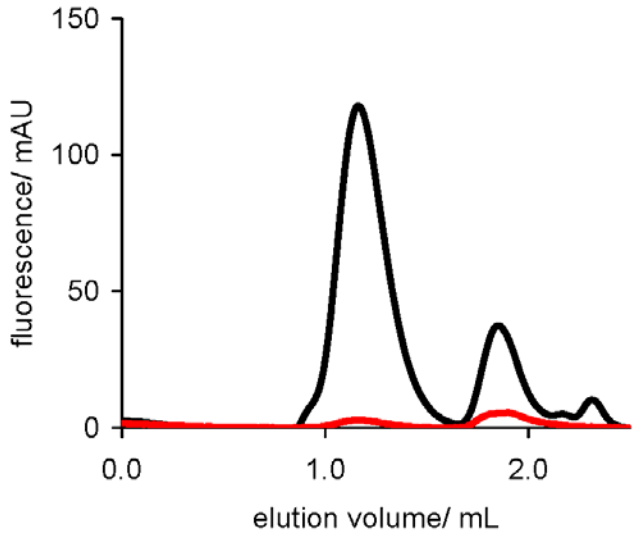

Figure S3 FPLC trace of a mixture of PalB-capsid protein complex with wt CP at pH 5.0. The FPLC system is equipped with a Superose 6 column. The black line represents the protein absorption at λ=280 nm, and the red line the absorption of the dye with which PalB is labeled at λ=586 nm. The capsid with encapsulated PalB elutes at V=1.1 mL, and the unassembled capsid dimers and of PalB-capsid protein complex elute at V ~1.8 mL.

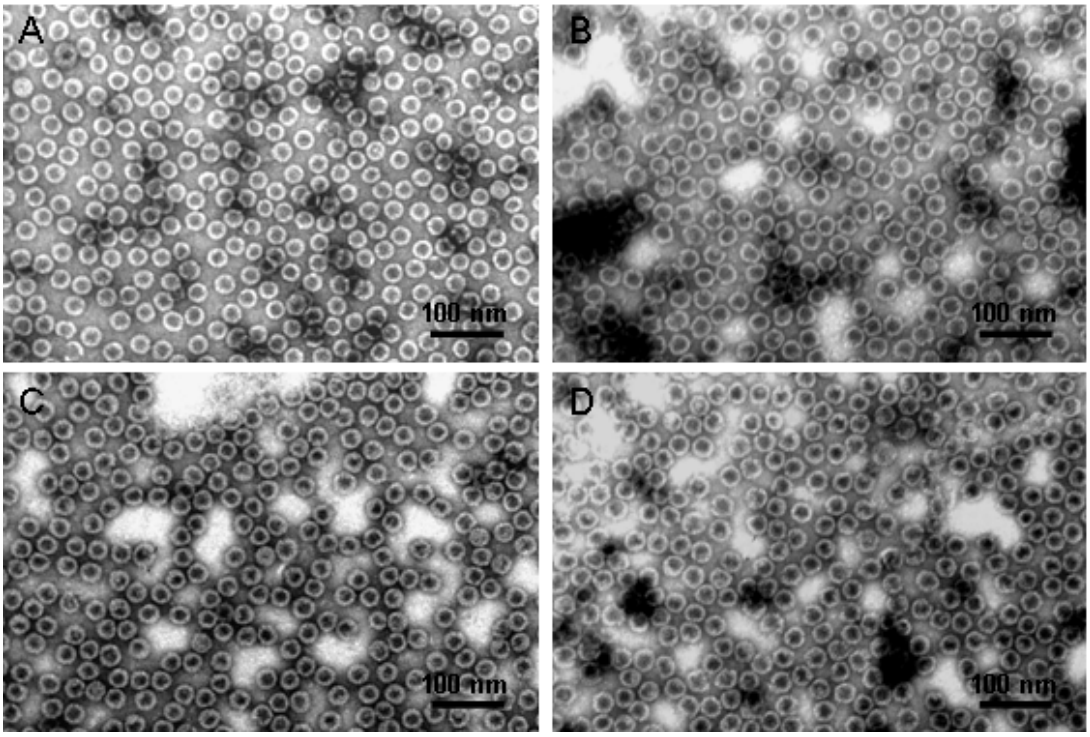

Figure S4 TEM micrographs of uranyl acetate stained samples. A) Empty wild-type capsids. B) Capsids filled with 1 PalB per capsid C) Capsids filled with 4 PalB per capsid. D) Capsids filled with 1 PalB and 7 EGFP per capsid.

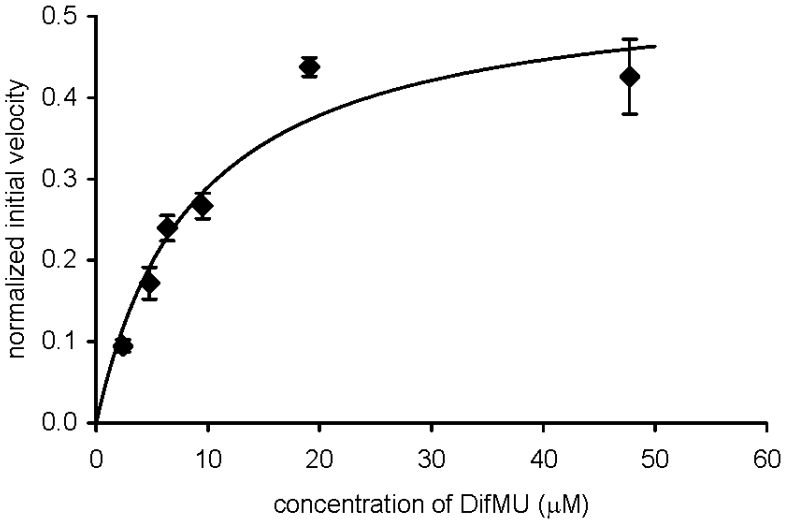

Figure S5 Michaelis-Menten plot of DiFMU concentration versus the normalized initial velocity.

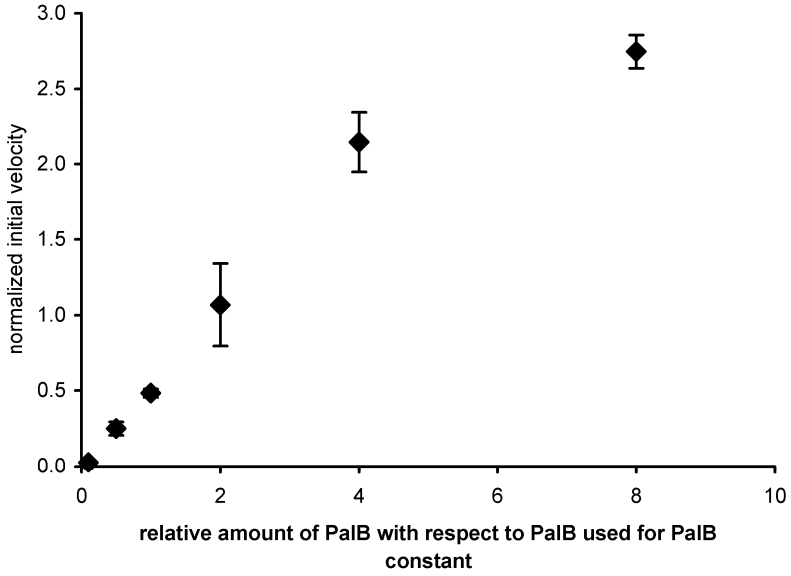

Figure S6 Plot of the normalized initial velocity versus PalB concentration. DiMFU concentration used was 40 μM.

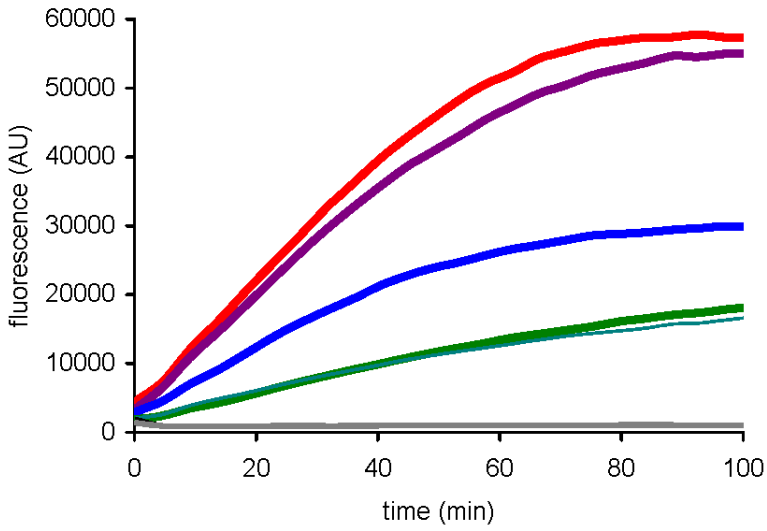

Figure S7 DiFMU substrate conversion curves. The concentration of PalB was the same in all samples. Grey line depicts buffer with DiFMU, thin green line depicts the control sample with non-encapsulated PalB, green line depicts sample with 4 PalB/capsid, blue line depicts sample with 3.5 PalB/capsid, purple line depicts sample with 2 PalB/capsid and red line depicts sample with 1.3 PalB/capsid.