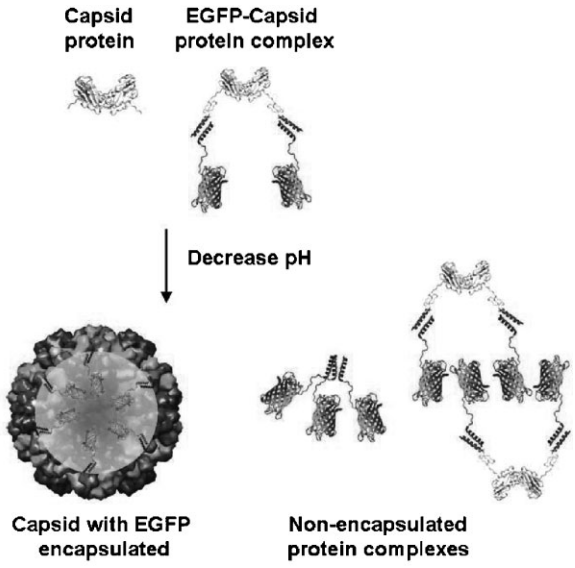
Complex Assembly Behavior During the Encapsulation of Green Fluorescent Protein Analogs in Virus Derived Protein Capsules[a]

Inge J. Minten, Roeland J. M. Nolte, Jeroen J. L. M. Cornelissen*

Enzymes encapsulated in nanocontainers are a better model of the conditions inside a living cell than free enzymes in solution. In a first step toward the encapsulation of multiple enzymes inside the cowpea chlorotic mottle virus (CCMV) capsid, enhanced green fluorescent protein (EGFP) was attached to CCMV capsid proteins. The capsid protein–EGFP complex was then co-assembled with wild-type capsid protein (wt CP) in various ratios. At higher complex to wt CP ratios, the number of EGFP per capsid decreased instead of leveling off. We propose that this unexpected behavior is caused by pH-induced disassembly of the capsid protein–EGFP complex as well as by concentration and ratio dependent dimerization of the complex, making it partially unavailable for incorporation into the capsid.



Introduction

The coat proteins of the cowpea chlorotic mottle virus (CCMV) have the remarkable ability to self-assemble in absence of their indigenous RNA. This self-assembly is triggered by lowering the pH, i.e., at pH 7.5 the capsid proteins are in a dimeric state, while at pH 5.0 exactly 90 dimers self-assemble into the native capsid conformation of 28 nm in diameter.[1] Due to the ability to self-assemble the CCMV capsid can be used to encapsulate guest molecules, by adding them to the capsid protein dimers, and subsequently lowering the pH. This method works especially well when the guest molecules are negatively charged, since in that case they presumably interact with the positively charged capsid interior, thereby providing a driving force for the selective encapsulation of the guest molecules. This principle has been shown to work for inorganic salts,[2] as well as negatively charged polymers.[3] When the guest molecules are neutral, or even positively charged, this method is considerably less efficient. Nonetheless, the encapsulation of neutral or positively charged guest molecules such as enzymes would be of great interest. The encapsulation of single enzymes by statistical

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encapsulation has already been shown to provide new insights in the way single enzymes function.[4] Following up on these studies, it would be very interesting to see how multiple different enzymes inside a single capsid would affect, for example, their kinetics. The confined space of a capsid could bring these enzymes in close proximity, possibly altering reaction rates. Such a system would resemble the natural organization of enzymes inside a cell organelle more closely than enzymes that are free in solution.[5] Since enzymes are usually not negatively charged at acidic pH, a different method is needed to encapsulate multiple enzymes inside one capsid.

We have recently demonstrated a method for the efficient and controlled encapsulation of enhanced green fluorescent protein (EGFP) into the CCMV virus capsid.[6] In short, this was accomplished via the non-covalent attachment of EGFP to the capsid proteins via a short heterodimeric coiled-coil sequence. Coiled-coils are oligopeptides able to form stable dimers. The particular pair chosen for this study, called E-coil and K-coil, have a very high binding constant ($K_d = 7 \times 10^{-8}$),[6] while being one of the shortest coiled-coil sequences known to date. EGFP was provided with the *E*-coil (GE) via molecular biology techniques, and the capsid protein was likewise provided with the *K*-coil (CK). Mixing of the two proteins resulted in an EGFP–capsid protein complex (GE–CK complex). In order to encapsulate the EGFP, this complex was mixed with wild-type capsid protein (wt CP) in various ratios, to allow control over the amount of encapsulated EGFP and to prevent overcrowding of the capsid. The pH was subsequently lowered to assemble the capsid (Figure 1).

The formed capsids contained different amounts of EGFP depending of the initial [GE–CK complex]/[total protein] ratio (Figure 2, closed triangles and diamonds). When this ratio increases, however, the number of encapsulated EGFP proteins per capsid first increases and then decreases again. Clearly, there must be a maximum to the number of proteins that can be encapsulated, due to limited space inside the capsid. But there does not seem to be an obvious reason why the number of encapsulated EGFP proteins per capsid should decrease with increasing amounts of EGFP

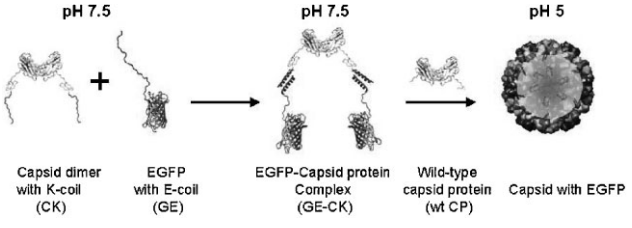

Figure 1. Schematic representation of EGFP encapsulation. The capsid protein with K-coil is mixed with EGFP with E-coil, to form the GE–CK complex. This complex is mixed with wt CP, and dialyzed to pH 5.0 to induce capsid formation.

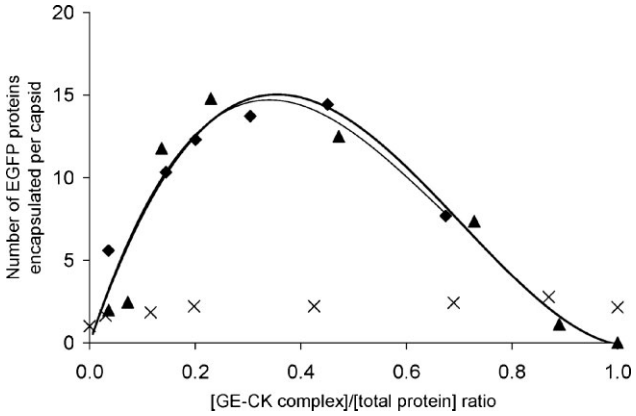

Figure 2. Number of EGFP proteins encapsulated per capsid as function of the GE–CK complex/total protein ratio. Filled triangles depict data points of experiment #1, filled diamonds depict data points of experiment #2. Crosses depict data points of negative controls, in which wt CP was mixed with GE instead of the GE–CK complex.

present. In this paper we provide an explanation of this seeming discrepancy between theory and practice.

Experimental Part

Expression and Purification of wt CP, GE, and CK

Expression and purification of wt CP, GE, and CK, as well as the assembly of the GE–CK complex were carried out according to procedures previously described.[6]

EGFP Encapsulation Experiments

CK–GE complex or GE (negative control) in buffer pH 7.5 (50 mM Tris–HCl, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.5) were added in different ratios to wt CP in the same buffer (Table S1, Supporting Information). The proteins were allowed to mix for 5 min, before dialyzing the mixture overnight to buffer pH 5.0. The mixtures were then analyzed on a fast performance liquid chromatography (FPLC) system equipped with a Superose 6 column.

Encapsulation Experiment With Labeled wt CP

DyLight 649 NHS ester (Thermo Scientific) was added to wt CP in a 50 mM phosphate buffer pH 7.5 with 0.5 M NaCl. The unreacted dye was removed by extensive dialysis against buffer pH 7.5 and the labeling efficiency was determined according to the procedure described in the DyLight manual, which resulted in a labeling efficiency of 0.14 mol of the dye per mol wt CP. The labeled wt CP was mixed with GE–CK complex in a [GE–CK complex]/[total protein] ratio of 0.7. The concentration of wt CP in the sample was 2.01×10^{-5} M and that of GE–CK complex was 4.69×10^{-5} M. The mixture was then dialyzed to buffer pH 5.0 and analyzed on FPLC equipped with a Superose 6 column.

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Investigation of the [GE–CK Complex]/[Total Protein] Ratio on the Aggregation Behavior of GE–CK

GE–CK (8.28×10^{-5} M) was mixed with wt CP (9.55×10^{-5} M) in a GE–CK/total protein ratio of 0.7. The mixture was divided in two equal portions which were each dialyzed to buffer pH 5.0. After 2 h, a concentrated solution of wt CP (6.92×10^{-4} M, pH 7.5) was added to one of the mixtures resulting in a GE–CK/total protein ratio of 0.4. The two mixtures were further dialyzed to buffer pH 5.0 for approximately 10 h before analysis on an FPLC equipped with a Superose 6 column.

Calibration Curves of FPLC Columns

Calibration curves were made for the Superdex 200 and Superose 6 column using a protein-based low molecular weight and high molecular weight gel filtration calibration kit from Amersham Biosciences.

Results and Discussion

Capsids containing multiple encapsulated EGFP molecules per capsid were analyzed on FPLC. A typical FPLC spectrum of these encapsulation experiments is shown in Figure 3. EGFP has a specific absorption at $\lambda = 395$ nm at pH 5.0. By monitoring both this EGFP specific absorption and the protein absorption at $\lambda = 280$ nm during the run, EGFP can be distinguished from the capsid protein. Formed capsids typically elute at $V = 1.1$ mL and since the EGFP specific absorption is present at $V = 1.1$ mL, this indicates that EGFP is encapsulated. Free proteins like EGFP, and the capsid dimers elute between $V = 1.6$ and 1.9 mL. A careful look at the FPLC graphs of the encapsulation experiments, shows that the peak around $V = 1.6$–1.9 shifts to lower elution volumes with increasing [GE–CK complex]/[total protein] ratios, which is indicative of the formation of a bigger complex (Figure 4A).

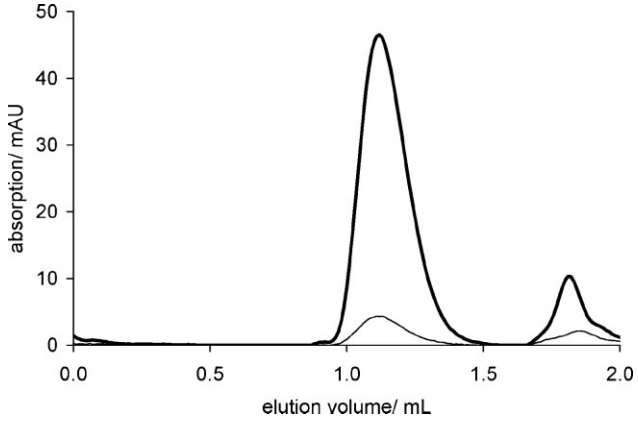

Figure 3. FPLC trace of a mixture of GE–CK complex with wt CP at pH 5.0. The FPLC system is equipped with a Superose 6 column. The thick line represents the protein absorption at $\lambda = 280$ nm, and the thin line the EGFP specific absorption at $\lambda = 395$ nm. The capsid with encapsulated EGFP elutes at $V = 1.1$ mL, and the unassembled capsid dimers and GE–CK complex elute at ≈1.8 mL.

It is hypothesized that this unknown complex (UC) consists of several GE–CK complexes. If this is true, the GE–CK contained in this UC would presumably be unavailable for incorporation into the capsid structure. This might explain the decrease in EGFP encapsulation efficiency with increasing [GE–CK complex]/[total protein] ratios.

To investigate this hypothesis, we first examined whether the UC was solely composed of the GE–CK complex, or of the GE–CK complex and the wt CP. This was done by labeling wt CP with a fluorescent dye (DyLight 649) and repeating an encapsulation experiment with a [GE–CK complex]/[total protein] ratio that was known to cause the formation of the UC. The sample was analyzed by FPLC equipped with a Superose 6 column, and the resulting graph again showed a peak associated with the UC at $V = 1.65$ mL, but the labeled wt CP eluted at $V = 1.73$ mL (Figure 5). Hence, the UC seems to be composed of the GE–CK complex.

In the encapsulation experiments, the concentration of GE–CK complex is increased, while keeping the total concentration of capsid proteins (wt CP and CK) approximately at an equal level, to obtain increasing [GE–CK complex]/[total protein] ratios. Therefore the effect of the concentration of GE–CK complex on the UC formation is studied. During the GE–CK complex purification on FPLC it was already observed that at pH 7.5 the UC was formed, so the GE–CK complex was studied in more detail at this pH using a Superdex 200 FPLC column. This column is better suited for the mass range of the complex and the UC than the Superose 6 column used for the encapsulation experiments. The complexes were studied at several concentrations spanning the range of the concentrations used for the encapsulation experiments. The elution volumes increased with increasing GE–CK complex concentrations, this is indicative of the formation of a bigger complex, presumably the UC. In order to estimate the molecular weights corresponding to the elution volumes of these complexes, a calibration curve was made for this column (Figure S1).

This calibration curve was then used to calculate the molecular weights for the complexes formed. The resulting graph of GE–CK complex concentration versus molecular weight (Figure 6), shows a gradual increase in molecular weight with increasing GE–CK concentrations. Since the proteins of which the complex consists, have a discrete mass, a gradual increase can best be explained by a shifting equilibrium between two, or more distinct forms of the GE–CK complex. According to the calibration curve, the molecular weights corresponding to the smallest elution volume, $V = 1.3$ mL and the largest elution volume, $V = 1.4$ mL are 239 and 127 kDa, respectively. The theore-

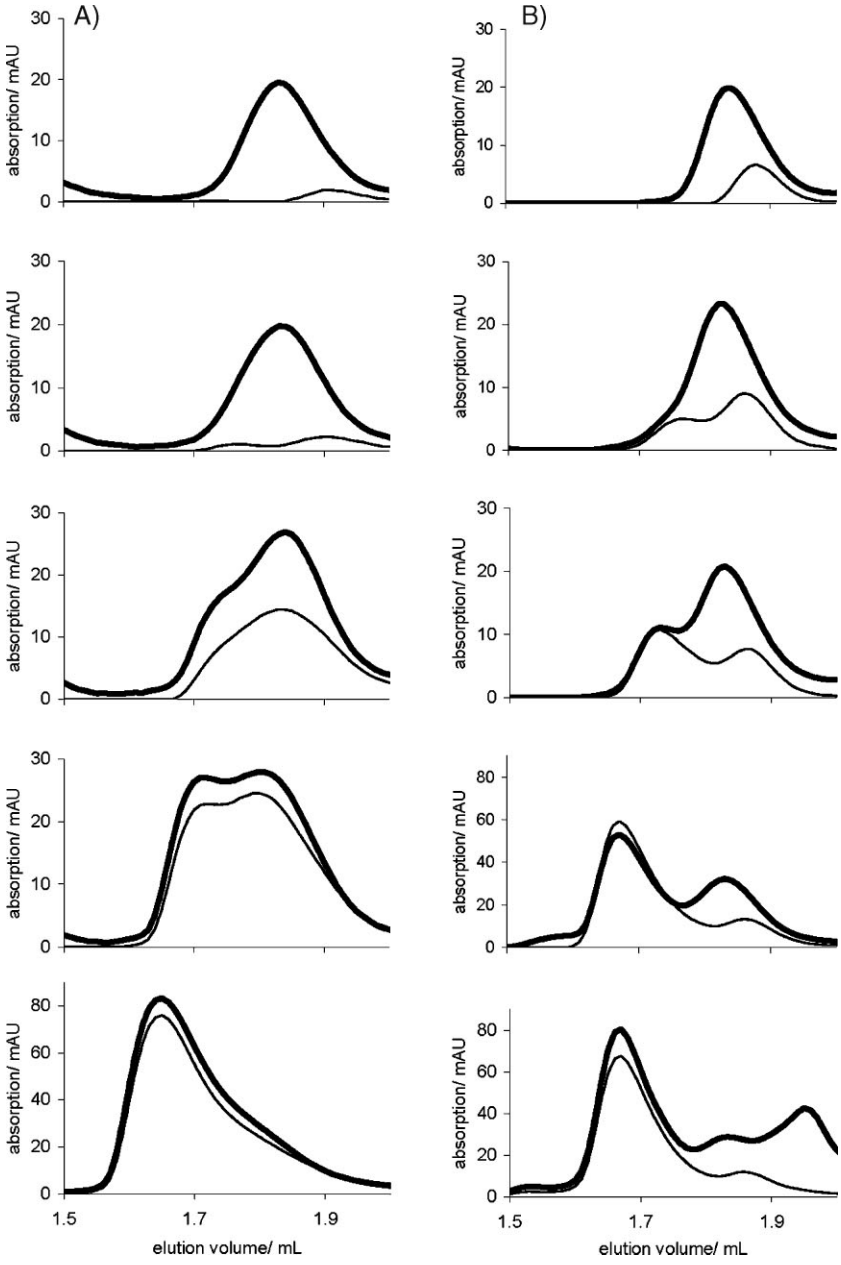

Figure 4. Part of the FPLC graphs of the various encapsulation experiments. FPLC is equipped with a Superose 6 column. Thick lines represent the protein absorption at $\lambda = 280$ nm and thin lines the EGFP absorption at $\lambda = 395$ nm. (A) Graphs from the encapsulation experiment with increasing amounts of GE–CK complex. [GE–CK complex]/[total protein] ratios increase in the following order from top to bottom: 0.03, 0.14, 0.29, 0.43, and 0.66. (B) Graphs from encapsulation experiment in which uncomplexed GE was used. [GE]/[total protein] ratios increase in the following order from top to bottom: 0.03, 0.12, 0.20, 0.43, and 0.69.

tical molecular weight for the GE–CK complex is 113 kDa, which corresponds to the $V = 1.4$ mL peak. The molecular weight corresponding to the 1.3 mL peak (239 kDa) could correspond to two times the molecular weight of the GE–CK complex, i.e., 226 kDa. Thus, the two distinct forms that are in equilibrium could be the GE–CK complex and a complex of twice that molecular weight. Since the graph of GE–CK complex concentration versus $\overline{M}_w$ has not leveled off completely at the highest concentration used for EGFP encapsulation, it is possible that at higher concentrations even bigger complexes are formed (not studied in further detail).

Since the capsid formation is induced by lowering the pH from 7.5 to 5.0, it was also investigated whether the same effect would be seen at pH 5.0. This indeed seemed to be the case. At low concentrations of the GE–CK complex (6.3 μM), the complex has an elution volume of $V = 1.4$ mL, which corresponds to the molecular weight of the GE–CK complex. At high concentrations (37.9 μM), the elution volume has shifted to $V = 1.3$ mL, corresponding to the molecular weight of two times the GE–CK complex.

To determine whether the elution volume of the UC on a Superose 6 FPLC column ($V \approx 1.6$ mL) also corresponded to twice the molecular weight of the GE–CK complex, a calibration curve was also made for the Superose 6 column (Figure S1b). This indicated that the elution volumes of the GE–CK complex and that of a complex of twice that weight should have an elution volume of $V = 1.73$ and 1.64 mL, respectively. Which in turn suggests that the peak of the UC at $V = 1.6$ mL on the Superose 6 column indeed originates from a complex that has twice the weight of the GE–CK complex.

Since it seems that the UC is twice the molecular weight of the GE–CK complex, the UC might be a dimeric form of the GE–CK complex. A possible explanation for the formation of a dimeric complex might be that the EGFP proteins from two GE–CK complexes dimerize. The crystal structure of GFP shows GFP dimerized in an antiparallel fashion,[7,8] and EGFP also has a weak tendency to dimerize.[9] GE also seems to have a tendency to dimerize at high concentrations, as is suggested by FPLC data (measured on an FPLC equipped with a Superdex 200 column). At low concentra-

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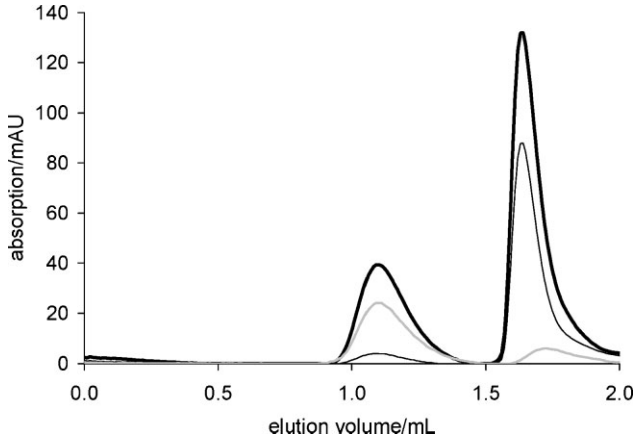

Figure 5. FPLC graph of GE–CK complex mixed with labeled wt CP in a [GE–CK complex]/[total protein] ratio of 0.7 and dialyzed to pH 5.0. FPLC is equipped with a Superose 6 column. The thick black line represents protein absorption at $\lambda = 280$ nm, the thin black line represents EGFP absorption at $\lambda = 395$ nm, and the gray line represents labeled wt CP absorption at $\lambda = 647$ nm.

tions (16 μM) GE has an elution volume of $V = 1.64$ mL, which corresponds to a molecular weight of 32 kDa. The theoretical molecular weight for monomeric GE is 33 kDa. High concentrations of GE (390 μM), however, have an elution volume of $V = 1.51$ mL, which corresponds to a molecular weight of 68 kDa. This is about twice the mass of the monomeric GE, thus indicating dimerization of GE proteins and making it conceivable that this is the cause of the apparent dimerization of the GE–CK complex. The GE proteins bound to the capsid protein are positioned parallel with respect to each other (Figure 7), whereas the GFP dimers in the crystal structure are dimerized in an antiparallel fashion. It is possible that this prevents the

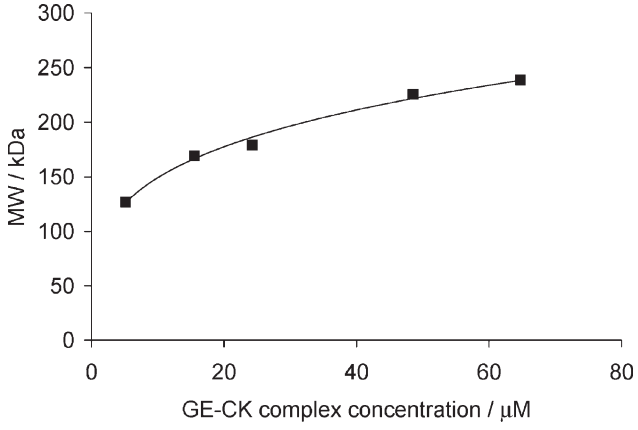

Figure 6. Graph of GE–CK complex concentration versus the molecular weight of the resulting complex. Closed squares represent data points. The elution volumes corresponding to the calculated molecular weights of the data points are 1.40 mL for 127 kDa, 1.35 mL for 169 kDa, 1.34 mL for 179 kDa, 1.30 mL for 225 kDa, and 1.29 mL for 239 kDa.

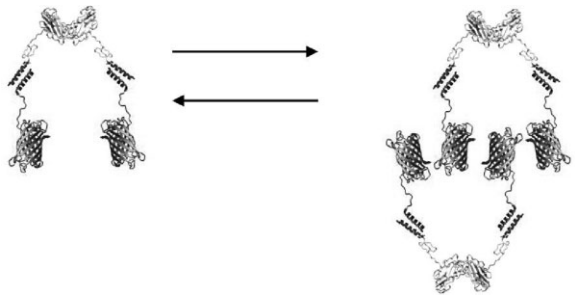

Figure 7. Schematic representation of the possible structure of the CK–GE complex and the CK–GE complex dimer.

internal dimerization of the GE proteins in the GE–CK complex. GE proteins of two different GE–CK complexes, however, might be able to align in an antiparallel fashion in that way enabling the formation of a dimeric complex. Hence, it is possible that the UC is a dimeric form of the GE–CK complex.

Besides the concentration of the GE–CK complex, other factors might also have an influence on the formation of the dimeric GE–CK complex. Since in the encapsulation experiments not only the concentration of the GE–CK complex is changed, but also the [GE–CK complex]/[total protein] ratio, the effect of this ratio was also investigated. The proteins were mixed in a ratio that was known to cause the formation of the dimeric GE–CK complex, i.e., a ratio of 0.7 (Figure 4), and dialyzed to buffer pH 5.0. This mixture was measured on an FPLC equipped with a Superose 6 column, and as expected, the majority of the GE–CK complex was in the dimerized form (Figure 8A). A very concentrated solution of the wt CP at pH 7.5 was then added to the mixture, thereby shifting the ratio from 0.7 to 0.4. Due to the small volume of the wt CP solution this did not significantly affect the pH, or the total concentration of GE–CK complex in the sample. The mixture was equilibrated for one night and was then again measured using FPLC. The resulting FPLC graph (Figure 8B) showed a decrease in the amount of the dimeric GE–CK complex formed, and also an increase in the number of encapsulated EGFP proteins per capsid. This experiment shows that the [GE–CK complex]/[total protein] ratio also affects the formation of the dimeric GE–CK complex, and that it is a dynamic equilibrium.

Since it appears that both the concentration of the GE–CK complex and the [GE–CK complex]/[total protein] ratio have an effect on the formation of the dimeric GE–CK complex, and both these variables were changed in the encapsulation experiments, a new encapsulation experiment was performed, in which the concentration of the GE–CK complex was kept constant. That is, only the [GE–CK complex]/[total protein] ratio was varied. This resulted in a more or less constant encapsulation of about 9 EGFP proteins per capsid (Figure 9).

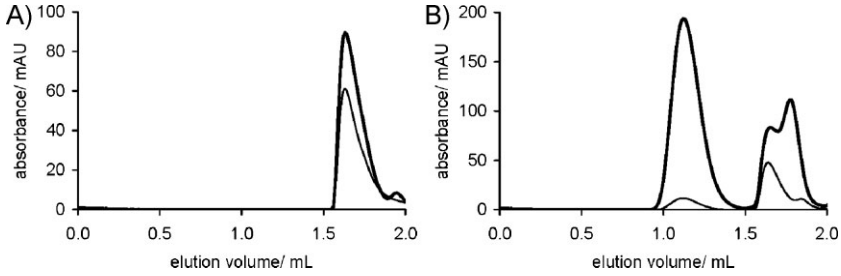

Figure 8. FPLC graphs of experiments to investigate the effect of the [GE–CK complex]/[total protein] ratio on the ability of the complex to form capsids. The FPLC system was equipped with a Superose 6 column and the samples were measured at pH 5.0. Thick lines represent protein absorption at $\lambda = 280$ nm and thin lines represent EGFP absorption at $\lambda = 395$ nm. (A) [GE–CK complex]/[total protein] ratio of the sample is 0.7. (B) [GE–CK complex]/[total protein] ratio of the sample was changed from 0.7 to 0.4.

The form of this graph is significantly different from that of the previous encapsulation experiments, in which the concentration of capsid proteins (wt CP and CK) instead of the concentration of the GE–CK complex was kept constant. This indicates that not only [GE–CK complex]/[total protein] ratio is of importance for the encapsulation efficiency of EGFP into the capsids, but also that the absolute concentrations of the individual proteins are also critical.

It is likely that the dimeric GE–CK complex cannot be incorporated into the capsid. This would explain the decrease in encapsulation efficiency with increasing [GE–CK complex]/[total protein] ratios. However, this does not appear to be the only cause, since the FPLC peaks around 1.7 mL that are probably caused by the dimeric GE–CK complex are also observed when negative control encapsulation experiments are performed in which only GE is added to the wt CP instead of the GE–CK complex. The peaks around $V = 1.6$–1.9 mL of the resulting FPLC graphs (Figure 4B) show a remarkable similarity to the peaks around $V = 1.6$–1.9 mL of the normal encapsulation experiments (Figure 4A). So these peaks could also be caused by the GE proteins without CK. To check if this was also the case for the normal encapsulation experiments with the GE–CK complex, FPLC fractions of both the capsid peak at $V = 1.1$ mL and the peaks around $V = 1.6$–1.9 mL were analyzed by SDS–PAGE and visualized by silver staining (Figure S2). This showed that the GE and CK proteins were not present in a 1:1 ratio in either the capsid peak at $V = 1.1$ mL or the peaks between $V = 1.6$ and 1.9 mL. This indicates that the GE–CK complex dissociates to certain extends, since otherwise the amount of GE and CK in the FPLC fractions would be equal. It is known from literature[10] that the E/K coil interaction is pH sensitive. At pH 7.5 the coiled-coil complex is stable, but at pH 5.0 the coiled-coils partially disassemble. Three disassembled E-coils can then form a trimeric coiled-coil structure. This would indicate that at pH 5.0 the (dimeric) GE–CK complex could partially disassemble and reassemble into free CK proteins and trimeric GE proteins. If this disassembly would take place faster than the capsid formation, this could influence the efficiency with which EGFP is encapsulated as it would result in a decrease of the amount of GE–CK complex in solution.

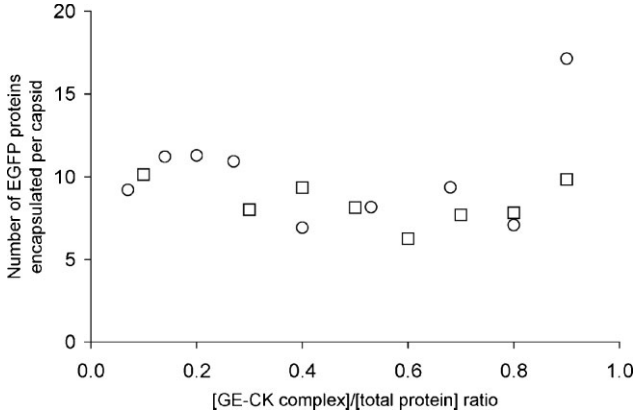

Figure 9. Number of EGFP proteins encapsulated per capsid as function of the GE–CK complex/total protein ratio. To obtain different ratios only the concentration of the wt CP was varied, the concentration of GE–CK was kept constant. Open squares depict data points of experiment #3 and open circles depict data points of experiment #4. Experiments #3 and #4 are duplicate experiments.

Conclusion

Inclusion of EGFP in the capsid of the CCMV can be controlled to a certain extent by introducing non-covalent interactions between the guest and the capsid proteins, resulting in the formation of a stable GE–CK complex at pH 7.5. The subsequent encapsulation process in the presence of wild-type capsid has an unexpected optimum when the [GE–CK complex]/[total protein] ratio is increased. It was expected that the GE–CK complex concentration in solution would be equal to the amount of GE–CK complex added. This does not seem to be the case, however. We have shown that at pH 7.5, part of the GE–CK complex tends to dimerize as a result of both increasing concentration of the complex as well as the increasing [GE–CK complex]/[total protein] ratio. This dimeric complex also seems to be present at pH 5.0. Presumably the dimeric complex cannot be incorporated into the capsid, so the actual GE–CK complex concentration in the mixture is lower than expected. Another factor contributing to the decrease in GE–CK complex concentration in the mixture, is the pH dependent

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dissociation of the GE–CK complex. At pH 5.0, the (dimeric) GE–CK complex partially dissociates into CK proteins and GE proteins. The latter will then probably re-associate to form a trimeric GE complex as is reported in detail for these coiled-coil systems by Apostolovic and Klok.[10]

So the mechanism of EGFP encapsulation as proposed by Minten et al.[6] and outlined in Figure 1 is more complex. Presumably part of the GE–CK complex dimerizes at pH 7.5 with increasing concentration and [GE–CK complex]/[total protein] ratio. At pH 5.0 this dimerized complex is not incorporated into the capsid. Due to the dissociation of the coiled-coils at this pH, both the dimerized GE–CK complex and the non-dimerized GE–CK complex probably partially disassemble, forming trimerized GE complexes and free CK. This leads to a mixture of five different species: i.e., the wt CP, free CK, and GE–CK complex, which will assemble to an EGFP filled capsid, and the trimerized GE and dimerized GE–CK complex, which will not be incorporated into the capsid and elute from the FPLC column around $V = 1.7$ mL (Figure 10).

In summary, both the concentration and ratio dependent dimerization of the GE–CK complex as the pH dependent dissociation of the GE–CK complex result in a decrease of the available amount of GE–CK complex in solution, which in turn leads to a decrease in encapsulation efficiency.

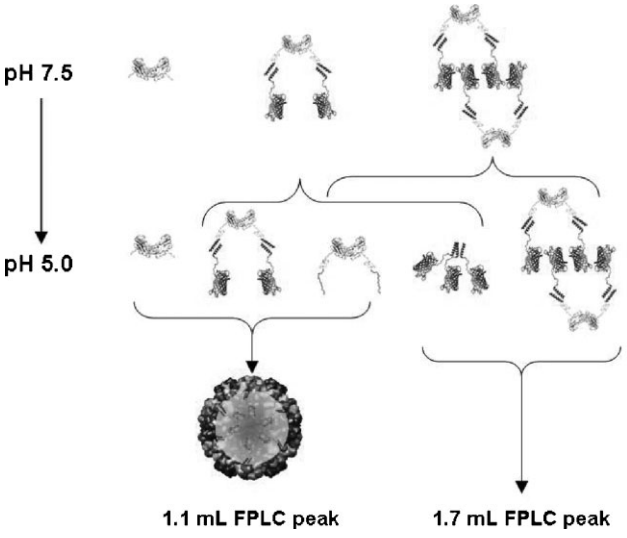

Figure 10. Proposed EGFP encapsulation mechanism. The top row represents (from left to right): the wt CP, GE–CK complex, and dimeric GE–CK complex at pH 7.5. Upon lowering the pH to 5.0 the GE–CK complex is thought to partially dissociate, resulting in a mixture of CK, trimeric GE and intact GE–CK complex, the dimeric GE–CK complex probably also partially dissociates, resulting in a mixture of CK, trimeric GE, and dimeric GE–CK complex. The wt CP, together with GE–CK complex and dissociated CK can then presumably form a capsid with EGFP encapsulated, giving rise to a $V = 1.1$ mL peak on the FPLC, while the trimeric GE and dimeric GE–CK complex probably give rise to the peaks between $V = 1.6$ and 1.9 mL.

Since formation of the dimeric GE–CK complex might be due to specific properties of the EGFP, it is possible that this effect will not occur with other proteins. This might mean that the current maximum of 15 EGFP proteins encapsulated per capsid is not necessarily the maximum for all proteins. Further studies are needed to answer this question.

Abbreviations

CK	capsid protein with K-coil
DLS	dynamic light scattering
E-coil	negatively charged coiled-coil, amino acid sequence: $(EIAALEK)_3$
EGFP	enhanced green fluorescent protein
FPLC	fast performance liquid chromatography
GE	EGFP with E-coil
K-coil	positively charged coiled-coil, amino acid sequence: $(KIAALKE)_3$
TEM	transmission electron microscopy
Wt CP	wild-type capsid protein

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