Protein Dimerization

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Protein Dimerization Induced by Supramolecular Interactions with Cucurbit[8]uril**

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The application of supramolecular chemistry to proteins provides a means of reversibly controlling protein properties. For example, proteins have been decorated with synthetic guest ligands for immobilization on host-molecule-modified surfaces,[1] and vesicles decorated with β-cyclodextrin have been used as a platform to immobilize adamantane-modified enzymes.[2] The supramolecular dimerization of two peptides enables sequence-specific recognition of DNA,[3] and a β-cyclodextrin-based host–guest system can be used to control the functional reassembly of two fragments of green fluorescent protein (GFP).[4] The supramolecular interaction of β-cyclodextrin with lithocholic acid, both of which were attached to protein C termini, was exploited to induce selective protein heterodimerization in solution and in cells.[5]

Control over protein dimerization with synthetic molecules is highly desirable, and approaches in which small molecules bind to specific, additionally incorporated protein domains have been very successful.[6] Control over protein dimerization by means of a very small, genetically encoded peptide motif is an attractive goal in this respect as it would require only very minor protein modifications. Synthetic supramolecular host molecules such as the cyclodextrins[7] and cucurbituril[8] have been shown to selectively recognize amino acids, and synthetic receptors have been developed to recognize protein elements,[9] some of which with a high degree of recognition affinity and selectivity.[10] However, synthetic supramolecular host molecules have not been applied thus far as inducers of protein dimerization. Herein we show that the supramolecular host molecule cucurbit[8]uril can be used to induce and reversibly control the dimerization of proteins having a genetically incorporated N-terminal phenyalanine–glycine–glycine (FGG) peptide motif (Figure 1). The FGG peptide tag is easily incorporated through molecular biology techniques. The induction of protein dimerization is stable and can be reversed by addition of a synthetic supramolecular guest molecule.

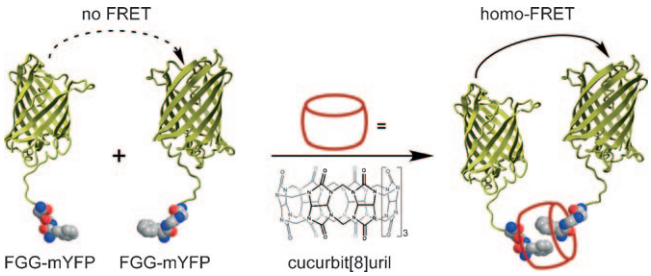

Figure 1. Schematic representation of two monomeric yellow fluorescent proteins having an N-terminal FGG peptide motif and their dimerization which is induced by supramolecular interactions with cucurbit[8]uril.

The FGG tripeptide has been shown to bind as a dimer in the cavity of a cucurbit[8]uril molecule; a key interaction is that between the N-terminal amine functionality of the peptide and the carbonyl rim of cucurbit[8]uril.[10] We therefore incorporated this motif at the N terminus of the proteins tested as a recognition element to induce protein dimerization mediated by cucurbit[8]uril. Proteins are typically expressed with an N-terminal methionine. Therefore intein-based protein expression was used to generate proteins with an N-terminal phenylalanine. Two monomeric fluorescent proteins[11] incorporating the FGG motif, FGG-mCFP (monomeric cyan fluorescent protein) and FGG-mYFP (monomeric yellow fluorescent protein), were designed and generated. The monomeric character of the proteins ensures the absence of unspecific protein aggregation.[5,11] Apart from an N-terminal intein domain, for postexpression autocleavage, the proteins featured an N-terminal chitin-binding domain (CBD) for purification. The FGG proteins were expressed at 15 °C, and subsequently the N-terminal intein tag was released from the target proteins by overnight incubation at room temperature and pH 7.0. Two normal fluorescent reference proteins featuring an N-terminal methionine were also generated (Met-CFP and Met-YFP). All proteins were isolated in pure form as evidenced by SDS-PAGE and LC-ESI-MS (see the Supporting Information).

We first studied the cucurbit[8]uril-induced homodimerization of FGG-mYFP (Figure 1) with Förster resonance energy transfer[12] (homo-FRET) studies (Figure 2a). The homodimerization of two fluorescent proteins typically results in a decrease of the fluorescence anisotropy as a result of intermolecular energy transfer. Indeed, upon the addition of cucurbit[8]uril to a solution of FGG-mYFP, the anisotropy of the YFP fluorescence decreased. The aniso-

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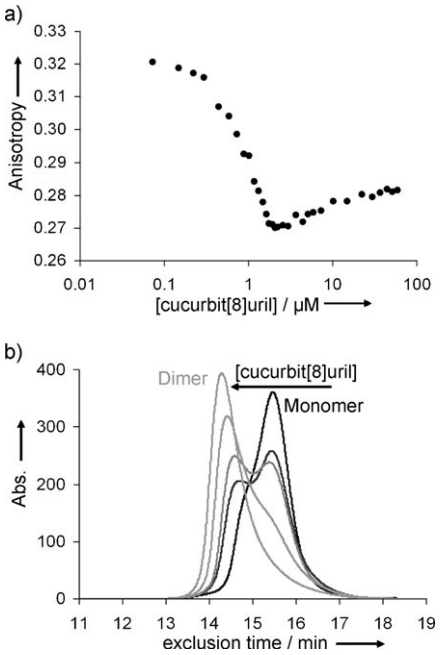

Figure 2. a) Fluorescence anisotropy titration of cucurbit[8]uril to 2 μM FGG-mYFP in 10 mM phosphate buffer at pH 7. b) Size-exclusion chromatograms (superdex 200 column) of 20 μM FGG-mYFP with increasing concentrations of cucurbit[8]uril (0, 2, 5, 10, and 20 μM).

tropy decrease is dependent on the cucurbit[8]uril concentration, as expected for a supramolecular recognition process. The cucurbit[8]uril thus binds to the proteins and induces their dimerization. Addition of a large excess of cucurbit[8]uril results in a small increase of the fluorescence anisotropy, possibly resulting from a less optimal protein orientation for FRET as a result of low-affinity cucurbit[8]uril binding to the amino acid side chains. The induced protein dimerization could also be proven by size exclusion chromatography (Figure 2b). Addition of increasing amounts of cucurbit[8]uril to a solution of FGG-mYFP resulted in the appearance of a second signal at higher molecular weight, which increased with increasing cucurbit[8]uril concentration. The molecular weight of this second species is approximately twice that of the individual FGG-mYFP, and this species could also be observed by mass spectrometry (see the Supporting Information). The ternary complex coexists next to the monomeric form of the protein at intermediate concentrations of curbit[8]uril. At cucurbit[8]uril/protein ratios above 1:2 only the protein dimer can be observed. These results show not only that the cucurbit[8]uril binds two FGG motifs and induces protein dimerization, but also that the induced protein dimer is stable and can be separated on a purification column.

Isothermal calorimetry (ITC) measurements (see the Supporting Information) confirmed the high affinity of the proteins for cucurbit[8]uril and also showed that the process of complex formation is more multifaceted for proteins than for peptides.[10] This is apparent because the ITC data could not be fitted correctly with the model derived for the peptides[10] and, for example, because the fluorescence anisotropy was observed to increase at higher cucurbit[8]uril concentrations (Figure 2a). Most probably additional interactions such as those between the two proteins and between the peptide sequence following the FGG motif and the cucurbituril account for this.

To further evaluate and investigate the scope of the cucurbit[8]uril-induced FGG-protein dimerization, we performed protein heterodimerization studies with CFP and YFP variants (Figure 3a). Addition of cucurbit[8]uril to an equimolar mixture of FGG-mCFP and FGG-mYFP resulted in the occurrence of a strong hetero-FRET (Figure 3b). The strong energy transfer is even more notable, considering that the induced protein heterodimerization in this case is most probably accompanied by 50% homodimerization, which does not contribute to the hetero-FRET. The hetero-FRET system was used to evaluate the selectivity of the cucurbit[8]uril-induced FGG-protein dimerization over proteins with an N-terminal methionine. The addition of cucurbit[8]uril to a solution of FGG-mCFP and FGG-mYFP

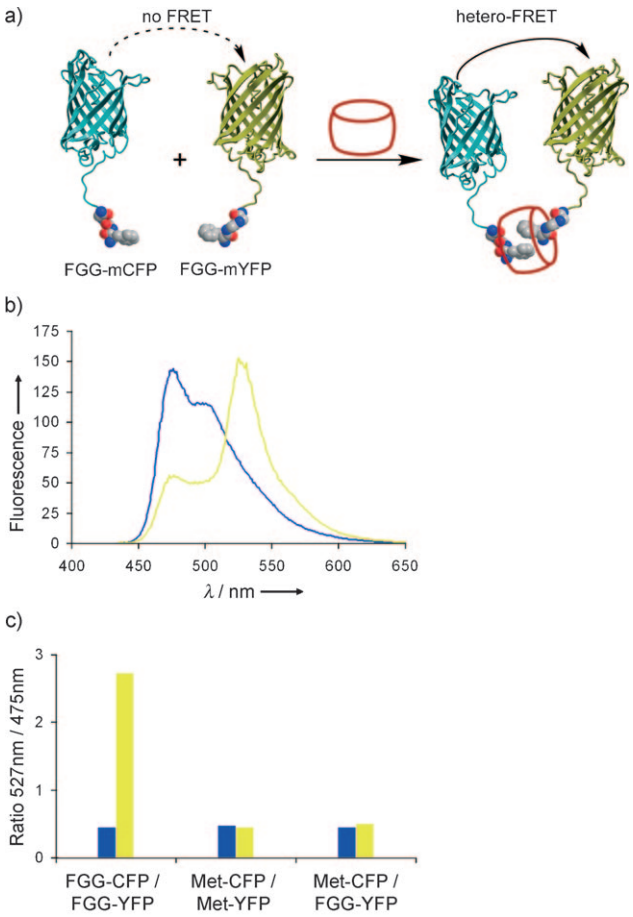

Figure 3. FRET studies with CFP–YFP protein pairs under the control of cucurbit[8]uril. a) Schematic model of the induced protein dimerization induced by supramolecular interactions. b) Representative spectra of a mixture of FGG-mCFP and FGG-mYFP (both at 1 μM) in the absence (blue) and presence (yellow) of cucurbit[8]uril (1.5 μM). c) Comparison of the 527 nm/475 nm FRET ratios observed without (blue bars) and with (yellow bars) cucurbit[8]uril (1.5 μM) for different protein mixtures (both at 1 μM).

resulted in an increase of the peak ratio (527 nm/475 nm) from 0.46 to 2.73 (Figure 3c). Addition of the same amount of cucurbit[8]uril to a mixture of two reference proteins with N-terminal methionine residues did not result in an increase of the 527 nm/475 nm ratio (from 0.48 to 0.46). Neither was an increase in the FRET ratio observed in a mixture of a CFP protein with a methionine and a YFP protein with a FGG motif (from 0.45 to 0.50). The cucurbit[8]uril-induced protein dimerization is thus selective for proteins featuring the FGG motif over proteins with an N-terminal methionine. These results additionally show that the protein dimerization does not result from unselective interactions of the cucurbit[8]uril with the amino acid side chains at the periphery of the protein.

As this protein dimerization is induced by supramolecular interactions, it should be possible to noncovalently reverse the induced protein dimerization by addition of a small synthetic molecule (Figure 4a). Therefore a solution of cucurbit[8]uril-induced fluorescent protein heterodimers was titrated with a solution of methyl viologen or paraquat.[13] Upon addition of the competitive methyl viologen ligand, a rapid decrease of the hetero-FRET effect was observed (Figure 4b,c), resulting in the restoration of the original CFP fluorescence (Figure 4b, blue line), devoid of energy transfer to YFP. These results show that the FGG motif can be displaced by methyl viologen, thus accomplishing a complete reversal of the induced protein dimerization with a bio-orthogonal supramolecular ligand.

These results show that a very short, genetically encoded, N-terminal FGG peptide motif can be used as a handle to control protein dimerization when the N termini interact with the supramolecular host molecule cucurbit[8]uril in buffered solution. The induced protein dimers are significantly stable, they can be separated by size-exclusion chromatography, and the recognition is selective for the FGG motif over the classical N-terminal methionine residue. Finally, the induced protein dimerization can be reversed efficiently with a small synthetic ligand that competes with the peptide motif for cucurbit[8]uril binding. The combination of the very short peptide motif with the easy accessibility of both the genetically encoded proteins and the synthetic supramolecular molecules holds great promise for applications as supramolecular inducers of protein dimerization, both for protein homodimerization, such as that observed in dimerizing enzymes and membrane proteins, and for the stabilization of weakly associating protein heterodimers.

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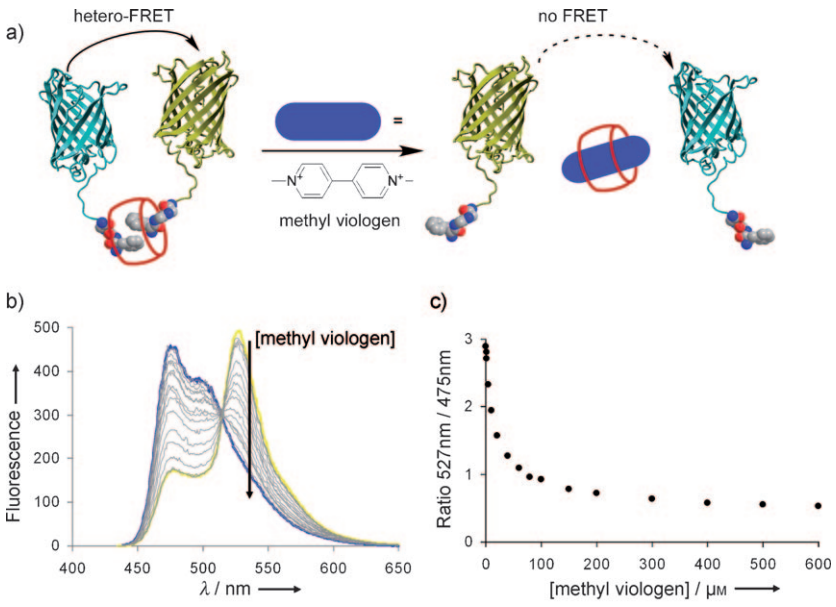

Figure 4. FRET studies on the supramolecular reversal of protein dimerization. a) Schematic model of the supramolecular inhibition of protein heterodimerization. b) Spectra of the FGG-mCFP and FGG-mYFP heterodimer (both at 1 μM) induced by interaction with cucurbit[8]uril (1.77 μM) recorded with increasing concentrations of methyl viologen; the blue curve represents the fluorescence of FGG-mCFP (1 μM) without added FGG-mYFP. c) FRET peak ratios (527 nm/475 nm) from (b) upon the addition of increasing concentrations of methyl viologen to the protein dimer.

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