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Cucurbit[8]uril Reactivation of an Inactivated Caspase-8 Mutant Reveals Differentiated Enzymatic Substrate Processing

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Caspase-8 constructs featuring an N-terminal FGG sequence allow for selective twofold recognition by cucurbit[8]uril, which leads to an increase of the enzymatic activity in a cucurbit[8]uril dose-dependent manner. This supramolecular switching has enabled for the first time the study of the same caspase-8 in its two extreme states; as full monomer and as cucurbit[8]uril induced dimer. A mutated, fully monomeric caspase-8 (D384A), which is enzymatically inactive towards its natural substrate caspase-3, could be fully reactivated upon addition of cucurbit[8]uril. In its monomeric state caspase-8 (D384A) still processes a small synthetic substrate, but not the natural caspase-3 substrate, highlighting the close interplay between protein dimerization and active site rearrangement for substrate selectivity. The ability to switch the caspase-8 activity by a supramolecular system thus provides a flexible approach to studying the activity of a protein at different oligomerization states.

Introduction

Control over protein dimerization events is crucial for the study of protein function and the interplay between protein oligomerization state and activity.[1,2] A variety of protein engineering and small-molecule-based approaches have been developed to induce or inhibit the dimerization of proteins in a controllable manner.[3,4] Keeping perturbations of the natural protein oligomerization mechanism to a minimum and the use of the same protein construct for studying different oligomerization states are key to obtaining reliable insights in the effects of the protein oligomerization state on protein activity; requirements typically not provided when the dimerization state is tuned using interface mutations.[5] Caspases are prototypical examples of enzymes the activity of which is strictly under control of regulated oligomerization mechanisms.[6,7] Kept inactive in a monomeric state, their activity is switched-on upon signaling through dimerization, resulting in induction of apoptotic pathways.[8] Studies of caspase dimerization mechanisms and subsequent enzymatic activation have been recurrent research questions and require well-defined protein constructs allowing the dissection of the molecular effects of the individual oligomerization states.[7,9–11] Specifically, caspase-8 (casp-8) has received significant attention in this respect, but studies are complicated by its potentially mixed oligomerization state in dilute solution.[12–14]

Casp-8 is a so-called initiator caspase which features a catalytic domain consisting of a large and a small subunit. Fas ligand, binding to the Fas death receptor, activates a signaling event, which activates casp-8 via the death-inducing signaling complex (DISC).[15,16] The procaspase-8 is autocleaved at proteolytic sites Asp216, Asp374, and Asp384, thereby generating the active casp-8.[14] A key step in casp-8 activation is the dimerization of the monomers after proteolytic cleavage, which results in a number of conformational changes coupled to active site activation.[17] Studies using designed casp-8 constructs have investigated this molecular mechanism in more detail.[9] Fusion of casp-8 to a FKBP-rapamycin associated protein domain allowed a dimeric FK506 small molecule analogue to induce dimerization of this fusion construct, resulting in increased casp-8 activation.[18,19] Redesigned casp-8 dimer interfaces enable improved dimerization, while not enhancing apoptosis.[20] Structural and biochemical studies on casp-8 have provided new insights through a fully monomeric engineered casp-8, which features abolished or, interestingly, only reduced enzymatic activity.[13,21] In these studies different casp-8 variants were used to study either the monomer or the dimer state, thus limiting the possibility to uncouple point-mutation-induced allosteric effects from the effects of the dimerization process on the active site.

Supramolecular chemistry has recently emerged as a powerful tool for reversible control over protein function, dimerization, and assembly.[22–28] Synthetic host–guest systems have shown relevant orthogonality to proteins. Especially the synthetic donut-shaped cucurbituril class of host molecules has

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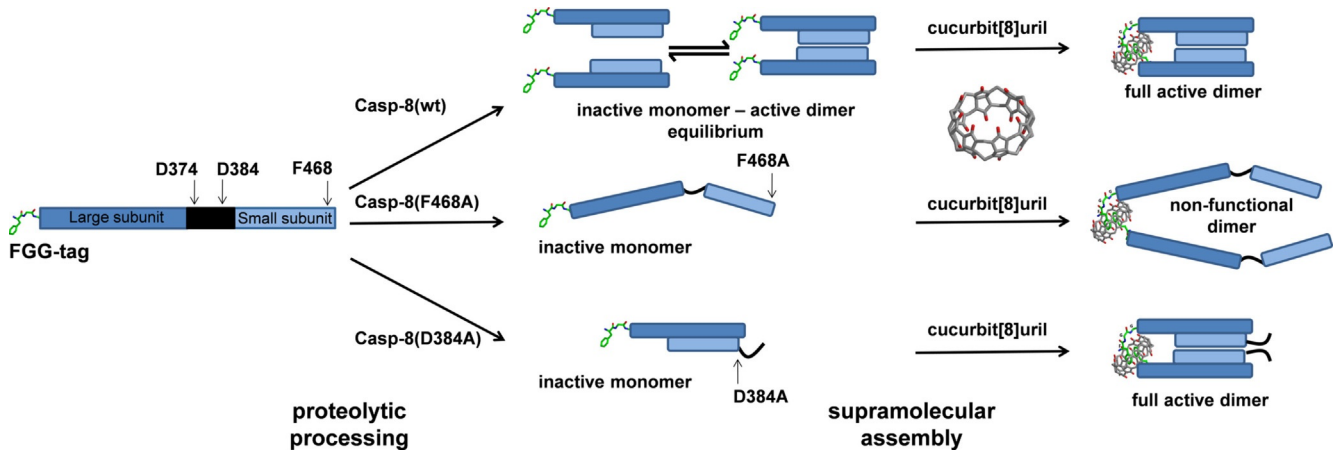

Scheme 1. Library of engineered caspase-8 (casp-8) proteins and concept of casp-8 activity regulation by monomer–dimer equilibrium and supramolecular rescue of dimerization and activity through twofold binding of cucurbit[8]uril to the N-terminal FGG-tags. Casp-8(wt) is fully processed into the two subunits and the intrinsic monomer–dimer equilibrium is shifted to the active dimer upon cucurbit[8]uril binding; Casp-8(F468A) is not proteolytically processed and cucurbit[8]uril binding does not induce a functional reorganization. Casp-8(D384A) features only a single cleavage site resulting in the linker sequence remaining attached to the small subunit and inhibition of background dimerization and activity, which can be rescued by cucurbit[8]uril, leading to the formation of catalytically active dimers.

proven to be a strong and selective protein binding element, capable of inducing the assembly of proteins into well-defined dimers and larger oligomers.[29–35] The cucurbit[8]uril variant can simultaneously bind two N-terminal phenylalanine-glycine-glycine (FGG) peptide elements.[36] This very small genetically encodable tag is easily incorporated into proteins and is selective for cucurbit[8]uril binding,[37] allowing for example to induce the dimerization of FGG-tagged caspase-9 constructs in a more efficient manner than by dimerization interface point mutations.[29,38] Here, we describe the design and generation of wild-type casp-8 and monomeric mutants of casp-8 featuring an N-terminal FGG motif and their cucurbit[8]uril-induced activation (Scheme 1). The thus generated potential to address different activity states of the same casp-8 protein revealed substrate-specific processing, depending on the assembly state of the protein.

Results

Engineering casp-8

Wild-type casp-8 exists in equilibrium between a monomeric and a dimeric state in buffered solutions.[39] The casp-8 variants, casp-8(D384A) and casp-8(F468A) (Scheme 1) are fully monomeric in buffered solution, even at high protein concentrations.[13] The aspartic acid to alanine mutation prevents the proteolytic cleavage at amino acid site 384 of casp-8(D384A), resulting in the small subunit still featuring the central ten amino acids of the intersubunit linker, which may sterically interfere with the dimer formation of casp-8(D384A).[13,21] Casp-8(F468A) is not processed at all, and the phenylalanine to alanine mutation removes cross-strand intermolecular interactions, thus making casp-8(F468A) incompetent to dimerize.[21] The catalytic domains (residues 217–479) of wild-type casp-8(wt) and the monomeric mutants casp-8(D384A) and casp-8(F468A) were therefore selected to provide insights in the effects of protein oligomerization state on enzyme activity modulated with cucurbit[8]uril (Scheme 1).

The crystal structure of the catalytic domain of casp-8 (PDB ID: 1F9E) reveals the two N termini of a casp-8 dimer to be in close proximity of around 18 Å.[40] Therefore, casp-8(wt), casp-8(D384A) and casp-8(F468A) were genetically provided with a four amino acid spacer incorporating an FGG motif or an MGG motif, as non-cucurbit[8]uril binding reference, at their N termini, not unlike our previous approach for caspase-9.[29] For efficient expression and purification, these constructs were genetically fused with an intein domain, as autocleavable N-terminal tag. After expression, the casp-8 constructs were autocleaved by intein splicing at room temperature and pH 7. After chromatographic isolation, the proteins were analyzed by SDS-PAGE and LC-ESI-MS, confirming their purity and integrity (Supporting Information).

Casp-8 activity on synthetic substrate

The enzymatic activity of the casp-8 constructs, alone and in the presence of cucurbit[8]uril, was first determined for the synthetic substrate *N*-acetyl-Ile-Glu-Thr-Asp-7-amino-4-(trifluoromethyl)coumarin (Ac-IETD-AFC).[9] Active casp-8 cleaves this substrate at the site C terminus to the aspartic acid, thereby releasing fluorescent AFC. In the absence of cucurbit[8]uril, FGGcasp-8(wt) and FGGcasp-8(D384A) featured specific activities of around 600 and 240 units mg^{-1}, respectively (Table 1). The activity of the two reference proteins, MGGcasp-8(wt) and MGGcasp-8(D384A), was the same as for the FGG-tagged constructs, showing that the type of amino acid at the N terminus does not modulate activity. The casp-8(D384A) constructs featured an around 2.5-fold lower activity than the casp-8(wt) and thus, though demonstrated to be monomeric,[21] still featured catalytic activity on the synthetic peptide substrate, in line

Table 1. Catalytic efficiencies[a] of casp-8 activity for synthetic substrate.

	−Cucurbit[8]uril	+Cucurbit[8]uril[b]	FE
MGGcasp-8(wt)	713±20	645±14	0.9
FGGcasp-8(wt)	606±17	1024±9	1.7
MGGcasp-8(D384A)	223±3	210±7	0.9
FGGcasp-8(D384A)	238±4	1000±18	4.2
FGGcasp-8(F468A)	n.a.	n.a.	–

[a] U mg^{-1}: one unit cleaves 1.0 nmole of Ac-IETD-AFC substrate per hour at pH 6.5 at 37 °C; FE: fold enhancement.; n.a.: not active. [b] Cucurbit[8]-uril at 1 μM.

with previous literature reports.[13] The monomeric and not proteolytically processed FGGcasp-8(F468A) did, as expected, not feature any enzymatic activity on the synthetic substrate.

Addition of cucurbit[8]uril to FGGcasp-8(wt) and FGGcasp-8(D384A), resulted in significant cucurbit[8]uril concentration-dependent increase of the enzymatic activity (Table 1, Figure 1). Both FGGcasp-8(wt) and FGGcasp-8(D384A) increased their enzymatic activity to the same end-level of around 1000 units mg^{-1}. The activity of FGGcasp-8(wt) was thus enhanced 1.7-fold by cucurbit[8]uril (Table 1). In contrast, the intrinsically less active FGGcasp-8(D384A) featured an over fourfold activity increase upon cucurbit[8]uril addition. The addition of excess cucurbit[8]uril to both FGGcasp-8(wt) and FGGcasp-8(D384A) did not further affect the activity of the enzymes (Figure 1). Next to showing the absence of nonspecific cucurbit[8]uril effects, the constant enzymatic activity at large cucurbit[8]uril excess supports the notion that this supramolecularly induced protein dimerization is cooperative.[41] The bivalent interaction of the supramolecular motif and the intrinsic protein dimerization act in concert to form a stable protein assembly.[29,32] The two reference proteins, MGGcasp-8(wt) and MGGcasp-8(D384A), and the inactive FGGcasp-8(F468A) did not show responsiveness to cucurbit[8]uril, further supporting the selective mode of action of cucurbit[8]uril.

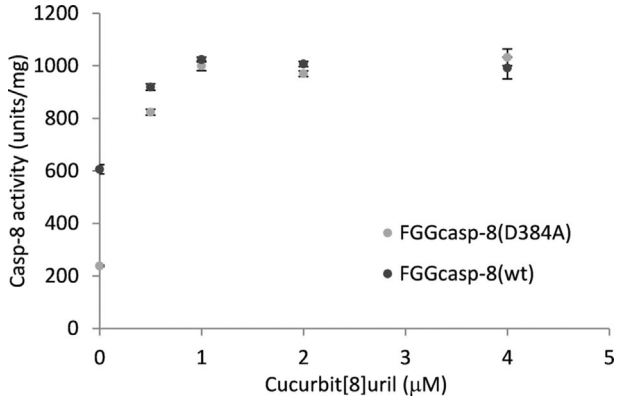

Figure 1. Dose-dependent effect of cucurbit[8]uril on the enzymatic activity of FGGcasp-8(wt) (0.15 μM, black) and FGGcasp-8(D384A) (0.15 μM, light grey) for the Ac-IETD-AFC substrate. The error bars represent the standard deviation based on three measurements.

Casp-8 activity on natural substrate casp-3

In the apoptotic pathway, the initiator casp-8 is responsible for cleavage and activation of caspase-3 (casp-3), which subsequently causes damage of DNA and apoptosis.[6,42] The engineered casp-8 constructs were therefore incubated with the natural substrate casp-3 in the absence and presence of cucurbit[8]uril, and their activity was followed over time and analyzed by SDS-PAGE for casp-3 cleavage (Figure 2 and Table 2). The FGGcasp-8(wt) required approximately 25 minutes to cleave half of the casp-3 substrate. Upon addition of cucurbit-[8]uril, this FGGcasp-8(wt) showed a strong, three- to fourfold,

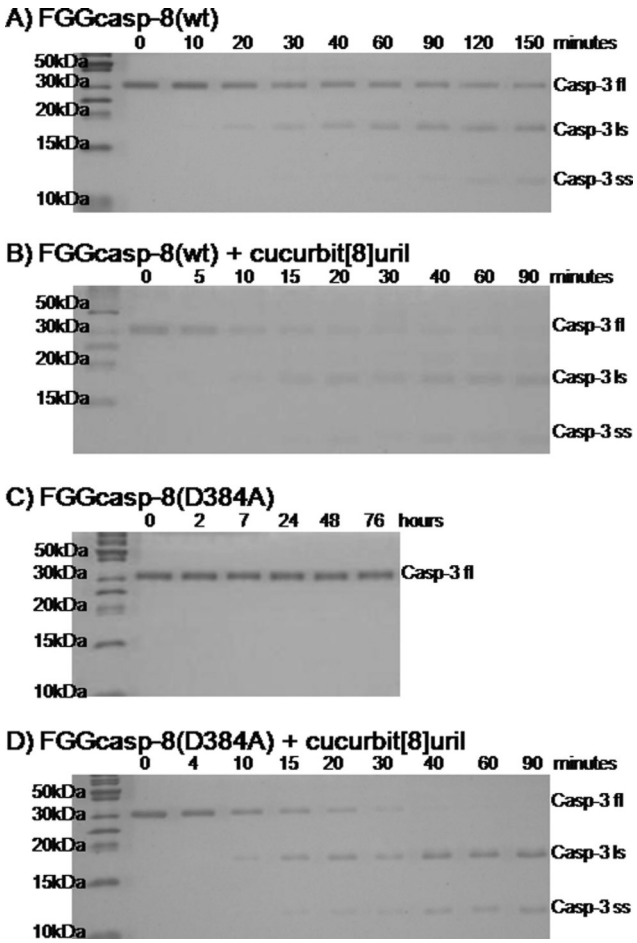

Figure 2. Cleavage activity of FGGcasp-8 (wt) and FGGcasp-8(D384A) (both at 0.15 μM) for casp-3 (4 μM) in the: A, C) absence, and B, D) presence of cucurbit[8]uril (1 μM). Casp-3 fl (full length); ls (large subunit); ss (small subunit).

Table 2. Catalytic efficiencies[a] of casp-8 for natural substrate casp-3.

	−Cucurbit[8]uril	+Cucurbit[8]uril[b]	FE
MGGcasp-8(wt)	≈25	≈25	1
FGGcasp-8(wt)	≈25	≈7	3.5
FGGcasp-8(D384A)	>4560	≈10	>456

[a] Time required for 50% cleavage of casp-3 by casp-8 [min]. [b] Cucurbit[8]uril at 1 μM. FE: fold enhancement.

increase in enzymatic activity; this supramolecular complex required only about 7 minutes to cleave half of the casp-3 substrate (Figure 2B). The activity enhancement effect of cucurbit[8]uril for FGG-casp-8(wt) is thus stronger for the natural substrate than for the small synthetic substrate (Table 1). The monomeric FGGcasp-8(D384A) alone was completely incompetent to cleave casp-3 (Figure 2C); even after 76 hours, no casp-3 cleavage could be detected. This is in contrast to the significant activity of this monomeric construct for the synthetic substrate Ac-IETD-AFC. Interestingly, addition of cucurbit[8]uril to the inactive FGGcasp-8(D384A) fully reinstated the enzymatic activity for the natural casp-3 substrate. The cucurbit[8]uril-induced FGGcasp-8(D384A) dimer required only about 10 min to cleave half of the casp-3 substrate (Figure 2D). The resulting FGGcasp-8(D384A) is, within the error of this assay, just as active as the wild-type FGGcasp-8(wt) in the presence of cucurbit[8]uril. The MGGcasp-8(wt) was similarly active as FGGcasp-8(wt), but in contrast did not show any response in activity upon addition of cucurbit[8]uril (Table 2 and Figure S4 in the Supporting Information).

Discussion

Dimerization has been demonstrated as a crucial prerequisite for casp-8 activation.[6,12,39] The excision of the ten amino acids (374–384) of the intersubunit linker of casp-8(wt) provides an advantage for the arrangement of two subunits in close proximity and stabilization of the active site loop, resulting in enzymatic activity of casp-8(wt). The mutant casp-8(F468A) exists completely as a monomer even at high concentration[13] and does not show catalytic activity. The incompetence for dimerization of casp-8(F468A) is explained by the absence of a cross-strand intermolecular interaction, resulting in the absence of self-proteolytic processing.[21] The lack of enzymatic activity of casp-8(F468A) is thus connected to its impaired dimerization capacities, supporting the notion of enzyme active-site rearrangement upon dimerization. In surprising contrast, the mutated casp-8(D384A) is still enzymatically active for the synthetic substrate, even though casp-8(D384A) has been demonstrated to exist in monomeric form in buffered solution. These conflicting results are difficult to reason or further interrogate using the existing point-mutated casp-8 analogues; point mutations in the catalytic protein domain could lead to a plethora of molecular effects, including loss of dimerization affinity and impaired active site rearrangement. The complexity of the mechanism of casp-8 activation is further illustrated by the processing of the synthetic Ac-IETD-AFC substrate by casp-8(D384A), but inactivity of the same enzyme towards the natural casp-3 substrate. An orthogonal means to modulate casp-8 dimerization, uncoupled from active site rearrangement, would help to shed light on the molecular processes involved.

Cucurbit[8]uril-induced protein dimerization[26,29,32,34,37] provides an important biochemical tool to study the correlation between dimerization and catalytic activity of casp-8. Addition of cucurbit[8]uril to FGGcasp-8(wt) and FGGcasp-8(D384A) increases the enzymatic activity of both proteins for the synthetic substrate to the same end-value (Figure 1). The cucurbit[8]uril probably stabilizes an intrinsic protein interaction by an increase of the local concentration due to the supramolecular binding of the appended FGG motifs. This facilitated dimerization is significantly different from engineering of the dimerization interface of proteins, which typically leads to a permanent change in dimerization affinity.[2]

The selectivity of cucurbit[8]uril binding only to the FGG N terminus is exemplified by the absence of an effect of cucurbit[8]uril on either MGGcasp-8(wt) or MGGcasp-8(D384A). Interestingly, the maximum activity of the cucurbit[8]uril-induced FGGcasp-8(D384A) dimer reaches the same level as that of cucurbit[8]uril-induced FGGcasp-8(wt) dimer. This demonstrates the ability of cucurbit[8]uril to enable the rearrangement of the active site of casp-8 to full enzymatic activity. Addition of cucurbit[8]uril to FGGcasp-8(F468A) did not induce any enzymatic activity, showing that cucurbit[8]uril binding alone is not sufficient for casp-8 activation. For the FGGcasp-8(F468A), the point mutation not only affects the capacity for dimerization, but also hinders effective active-site rearrangement. These results support the hypothesis that the self-proteolysis process and the cross-strand intermolecular interaction are both critically important for activation of casp-8.[21]

Notable differences for the different casp-8 variants were observed regarding the cleavage of the synthetic and the natural substrates. The cucurbit[8]uril-dependent enhancement of the FGGcasp-8(wt) construct was stronger for the natural substrate casp-3 than for the small synthetic substrate, indicating a different affinity for the substrates between casp-8 monomer and dimer. This effect was most pronounced for the FGGcasp-8(D384A) construct. While this protein is still substantially active towards the synthetic substrate in its monomeric state, it does not show any enzymatic activity towards the natural substrate casp-3. Binding of cucurbit[8]uril however reinstates full activity towards the casp-3 substrate. These results can be reasoned considering the size and structure of the two different substrates. The small synthetic peptide substrate (Ac-IETD-AFC) might easily access the active site pocket of monomeric casp-8, wild type or D384A mutant. In contrast, the large, folded structure of casp-3 does not bind to the active site pocket of the monomeric form of casp-8. These results suggest differences in substrate processing by caspases and provide an explanation for the previously observed, unexpected, catalytic activity of monomeric casp-8(D384A).[13] Apparently, the cucurbit[8]uril binding and resulting dimerization of FGGcasp-8(D384A) rearranges the loop of the active site which allows casp-3 to access the active site pocket. Correct casp-8 dimerization is thus closely coupled to activity and plays a key role in reorganization of the active site for enzymatic activation.

Conclusion

Cucurbit[8]uril selectively induces enzymatic activity of casp-8 variants having an N-terminal FGG tag, probably by facilitating protein dimerization. Differences were observed for a number of mutant casp-8 variants with respect to activity in monomer versus dimer state and regarding activity towards synthetic and natural substrate. Casp-8(D384A) in its monomeric form

cleaves the synthetic substrate, but is incompetent to cleave the natural casp-3 substrate. Cucurbit[8]uril binding to the N-terminal FGG motifs of casp-8(D384A) fully reactivates the enzymatic activity to the level of the wild-type casp-8. These results imply that casp-8 dimerization is tightly coupled with active site rearrangement. Classical protein engineering approaches by introduction of point mutations lead to caspase proteins that can only be studied in a single state, that is, monomeric[13] or dimeric.[43] The application of cucurbit[8]uril now allows the study of these caspases in different states. Cucurbit[8]uril and the very small genetically encoded FGG peptide tag thus provide a powerful approach to studying the molecular mechanism of enzyme dimerization and activation. Further research into the cucurbit[8]uril-FGG approach will hopefully address entries to control homo- versus hetero-dimerization and cellular applicability. It can be envisioned that this concept can then also be applied to other relevant protein activation events in biologically relevant processes.[2,44]

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: caspases · cucurbit[8]uril · protein assembly · protein engineering · supramolecular chemistry

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