Host–Guest Chemistry

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Supramolecular Control of Enzyme Activity through Cucurbit[8]uril-Mediated Dimerization**

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Protein dimerization is a ubiquitous mechanism to regulate protein activity in a broad range of biological processes including receptor clustering, signal transduction, and apoptosis. Molecular control over these processes is critical to elucidate and perturb the molecular mechanisms of the proteins involved.[1] To be amenable to small-molecule regulation, proteins of interest typically require substantial protein engineering and introduction of additional protein domains that bind the small molecule.[2–4] Control over protein dimerization by using a small, genetically encoded, peptide motif is a highly attractive target in this respect, as a short peptide motif would impose the smallest possible influence on the protein of study. Small molecules do not typically bind to short peptide fragments, however. Synthetic supramolecular molecules, such as the donut-shaped cyclodextrins and cucurbiturils[5] and other more complex synthetic receptors,[6,7] have been shown to selectively recognize amino acids and protein elements.[8] Cucurbiturils are small concave host molecules of diverse ring sizes and feature highly promising biomedical applications.[9–11] Cucurbit[7]uril can selectively recognize insulin[12] or ferrocene-modified proteins.[13,14] Cucurbit[8]uril strongly and specifically binds two short peptide motifs simultaneously.[15–17] The molecular binding mechanism involves the selective recognition of two N-terminal phenylalanines through hydrophobic interactions of the phenyl groups inside the cucurbit[8]uril cavity and interactions of the protonated N-terminal amine functionality with the carbonyl rim of the cucurbit[8]uril.[15] The introduction of an N-terminal phenylalanine-glycine-glycine (FGG) motif in fluorescent proteins allowed cucurbit[8]uril to selectively induce the homodimerization of these proteins in dilute solution.[17] Cucurbit[8]uril thus has great potential as a functional dimerizer and activator of proteins. Herein, the concept of supramolecular protein dimerization to control enzyme activity is described, using cucurbit[8]uril as a supramolecular inducer for caspase-9 (casp-9) dimerization and activation (Figure 1 a). Such supramolecular control of protein dimerization provides a unique approach to reversibly control caspase dimerization and activation, not accessible by only using classical protein engineering techniques.

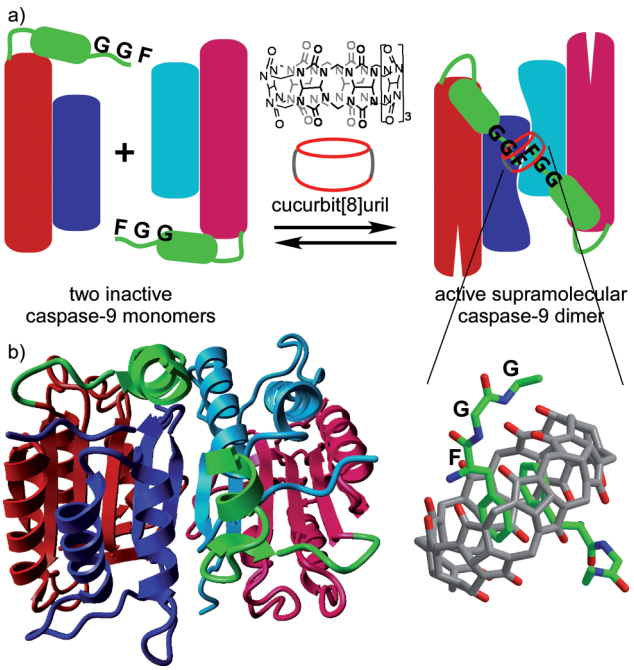

Figure 1. Cucurbit[8]uril-induced caspase-9 dimerization. a) Schematic representation of N-terminal FGG-bearing (green) monomeric caspase-9 (red: large subunit, blue: small subunit) and its dimerization into an enzymatically active homodimer by supramolecular-induced host–guest complexation with cucurbit[8]uril. Zoom in shows the molecular details of the twofold biding of the FGG sequence to cucurbit[8]uril, according to Ref. [15]. b) Crystal structure of a casp-9 dimer (2AR9),[27] portraying the close localization of the two N termini (green) of the two large subunits (red).

Caspases are critical cysteine proteases in the apoptosis pathway and are responsible for cleaving proteins at specific aspartate residues.[18–21] In the cell, casp-9 exists primarily in its inactive monomeric form, becoming activated only upon induced dimerization by auxiliary factors.[22–25] For example, apoptosomes, which include the apoptotic protease activating factor 1, and cytochrome c are responsible for the recruitment

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of monomeric casp-9 to increase the local concentration levels of the protein in favor of dimer-driven activation. In contrast to other caspases, such as casp-8,[26] casp-9 also exists primarily in its inactive monomeric state in dilute solution. Simple biochemical methods to control casp-9 dimerization, independent from the apoptosome, are absent, thus limiting the molecular control achievable over reversible casp-9 activation. Only through engineering of the casp-9 dimerization interface, by using specific point mutations, a constitutively dimeric casp-9 variant could be generated up to now.[27] Principle limitations of such engineered constructs are their lack of controllable reversibility and the unknown effects of the point mutations on the conformation of the active site and activity of the enzyme.

The crystal structure of casp-9 reveals a close colocalization of the two N termini of the large subunits in the protein dimer (Figure 1b).[27] It was envisioned that incorporation of the FGG recognition motif at both these N termini would therefore bode well for an induced dimerization process, which would stimulate the formation of the native active dimeric species. Casp-9 (residues 140–416) was therefore provided with an FGG motif at its N terminus, and this construct was genetically fused to an intein domain, as autocleavable N-terminal tag, and expressed in *E. coli* cells. The fusion protein was captured on a chitin bead column, and the FGG-casp-9 protein was eluted after pH-induced intein auto-cleavage. As reference an MGG-casp-9 construct was generated, featuring an N-terminal methionine in place of the phenylalanine. Additionally, a constitutive dimeric casp-9 construct (FGG-dcasp-9) was generated, bearing the reported constitutively dimeric interface, in which the Gly^{402}-Cys-Phe-Asn-Phe^{406} sequence in the β6 strand was replaced with Cys-Ile-Val-Ser-Met.[27] All proteins were isolated in pure form, featuring their classical large and small subunit after proteolytic auto-cleavage, as evidenced by SDS-PAGE and LC-ESI-MS (Figure 2a,b and the Supporting Information).

Cucurbit[8]uril-mediated casp-9 dimerization was first studied by dynamic light scattering (DLS). The size of the individual FGG-casp-9 was determined to be 2.8 nm. This corresponds, when assuming a spherical particle, to a molecular weight of approximately 37 kDa, which is nicely in line with the calculated mass of 32 kDa. Upon addition of cucurbit[8]uril an increase in the particle size to 3.4 nm was observed (Figure 2c). This hydrodynamic radius correlates with a protein mass of 59 kDa, which is close to the expected molecular weight of the dimerized FGG-casp-9 (64 kDa). The scattering function and size of the particles of the reference construct MGG-casp-9 did not change upon addition of cucurbit[8]uril. Size exclusion chromatography and isothermal calorimetry experiments (see the Supporting Information) additionally confirmed the cucurbit[8]uril-mediated protein dimer formation, analogous to previous observations on fluorescent proteins.[17] These data thus show that cucurbit[8]uril can effectively induce the supramolecular homodimerization of casp-9.

The correlation between supramolecular casp-9 dimerization and enzyme activation was studied by using enzyme activity tests. The catalytic activity of the different casp-9 constructs in cleaving the synthetic fluorescent casp-9 substrate Ac-LEHD-AFC (*N*-acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin) was studied in the presence and absence of cucurbit[8]uril (Figure 3a,b). The FGG-casp-9 alone featured a characteristic, weak background activity. However, addition of cucurbit[8]uril to the solution led to a very strong, ≈50-fold increase in catalytic activity (Figure 3a). The binding of cucurbit[8]uril to the FGG-casp-9 N termini and subsequent casp-9 dimerization allows formation of a highly active casp-9 dimer. The enhancement of the FGG-casp-9 activity by cucurbit[8]uril is concentration-dependent (Figure 3b) as expected for a reversible recognition and binding event. The enzyme activity increases upon increased addition of the supramolecular inducer of dimerization until a maximal activity is reached when all protein is dimerized by cucurbit[8]uril. Interestingly, the ternary formation constant of the cucurbit[8]uril–(casp-9)$_2$ complex as obtained from these data is $(2.7 \pm 0.3) \times 10^{12}\,\text{M}^{-2}$, which is approximately 20-fold higher than that reported for the complex of cucurbit[8]uril with two small FGG peptides (cucurbit[8]uril–(FGG)$_2$, ternary $K = 1.5 \times 10^{11}\,\text{M}^{-2}$).[15] The increased ternary formation constant of the cucurbit[8]uril–(casp-9)$_2$ complex shows that in addition to the interaction between cucurbit[8]uril and the FGG tags, formation of the native casp-9 dimer interface further stabilizes the formation of the ternary complex. The supramolecular host–guest interaction thus strongly enhances the intrinsic, but weak,

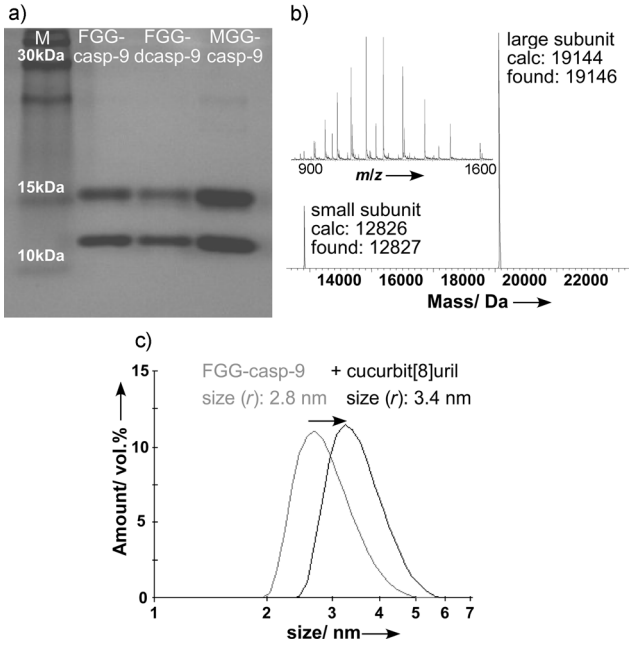

Figure 2. Molecular characterization and cucurbit[8]uril-induced dimerization of casp-9 constructs. a) SDS-PAGE analysis of proteins FGG-casp-9, FGG-dcasp-9, and MGG-casp-9. b) Deconvoluted ESI-MS analysis of FGG-casp-9; inset shows raw spectrum. Caspases undergo proteolytic auto-cleavage into a small and large subunit, as reflected in the SDS-PAGE and MS analyses. c) Dynamic light scattering analysis of FGG-casp-9 (50 μM) in absence (gray) and presence (black) of cucurbit[8]uril (50 μM). Measurements were performed in 10 mM sodium phosphate buffer, pH 7 at 20°C and sizes (radius) reported in nm.

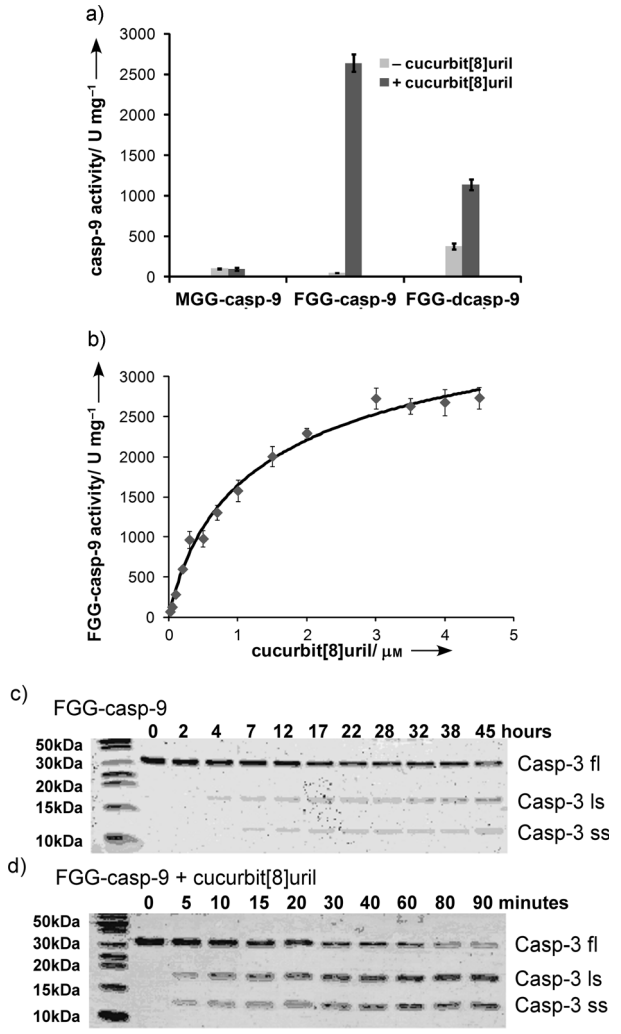

Figure 3. Cucurbit[8]uril-induced casp-9 enzymatic activity. a) Activity for the cleavage of casp-9 substrate Ac-LEHD-AFC (100 μM) of different casp-9 variants (150 nM) in the absence (light gray) and presence (dark gray) of cucurbit[8]uril (4 μM). b) Enzymatic FGG-casp-9 (150 nM) activity as a function of cucurbit[8]uril concentration and Ac-LEHD-AFC as substrate. The solid line represents the best fit using Equation (1), which describes the formation of a ternary cucurbit[8]uril–(FGG-casp-9)$_2$ complex; $K = (2.7 \pm 0.3) \times 10^{12}\,M^{-2}$ ($R^2 = 0.984$). The error bars represent the standard deviation based on three independent measurements. c, d) Cleavage activity of FGG-casp-9 (150 nM) for casp-3 (4 μM) in the absence (c) and presence (d) of cucurbit[8]uril (4 μM). Casp-3 fl (full length); ls (large subunit); ss (small subunit).

dimerization of casp-9. Control experiments using MGG-casp-9 showed a similar activity as observed for FGG-casp-9 in the absence of cucurbit[8]uril, but did not elicit a change in enzyme activity upon addition of cucurbit[8]uril (Figure 3a, Table 1). These results show that the cucurbit[8]uril-induced dimerization and activation is selective for the FGG peptide motif and that cucurbit[8]uril does not affect the active site or the periphery of the enzyme in an unspecific manner. In the absence of cucurbit[8]uril the intrinsically dimerizing FGG-dcasp-9 protein, with the engineered dimerization interface, featured a constitutive activity 7-fold higher than that of FGG-casp-9 in the absence of cucurbit[8]uril. The observed

Table 1: Catalytic efficiencies of caspase-9 activity.

	Ac-LEDH-AFC [U mg^{-1}][a]		
	−Cucurbit[8]uril	+Cucurbit[8]uril[b]	FE
MGG-casp-9	$(1.0 \pm 0.1) \times 10^2$	$(9.9 \pm 1.7) \times 10^1$	1
FGG-casp-9	$(5.0 \pm 0.3) \times 10^1$	$(2.6 \pm 0.1) \times 10^3$	50
FGG-dcasp-9	$(3.7 \pm 0.4) \times 10^2$	$(1.1 \pm 0.1) \times 10^3$	3

[a] U mg^{-1}: One unit cleaves 1.0 nmole of Ac-LEHD-AFC substrate per hour at pH 6.5 at 37 °C; FE: Fold enhancement. [b] A cucurbit[8]uril concentration of 4 μM was used.

basal activity is fully in line with the reported constructs of this protein.[27] Addition of cucurbit[8]uril to the FGG-dcasp-9 construct also induced an increase in catalytic activity. However, this additional induced catalytic activity was only 3-fold, leading to a lower overall activity of the FGG-dcasp-9 dimer than observed for the FGG-casp-9. In fact, the enzymatic activity of the cucurbit[8]uril-induced FGG-casp-9 dimer with the wild-type dimerization interface is 2–3 fold higher than that of the cucurbit[8]uril-induced FGG-dcasp-9 dimer with the engineered dimerization interface.

In the apoptotic pathway, casp-9 catalyzes, amongst others, the activation of caspase-3 through cleavage into a small and a large subunit. The catalytic efficiency of the cucurbit[8]uril-induced casp-9 dimerization was therefore determined using caspase-3 as natural substrate (Figure 3c, d).[28] In the absence of cucurbit[8]uril the FGG-casp-9 monomer requires approximately 22 h to cleave half of the caspase-3 substrate (Figure 3c). Simple addition of cucurbit[8]uril results in a strong >50-fold enhancement of catalytic activity towards the natural substrate, resulting in a half-time of cleavage of approximately only 20 min (Figure 3d). The catalytic efficiency of the reference construct MGG-casp-9 is similar to that of the FGG-casp-9 without cucurbit[8]uril, but in contrast does not increase upon addition of the host molecule (see the Supporting Information), thus again showing the high specificity of the supramolecular interaction. The FGG-dcasp-9 again shows an enhanced basal activity owing to the intrinsic dimerization behavior (see the Supporting Information). Although addition of cucurbit[8]uril to this solution decreased the half-time of cleavage to 70 min, also with this substrate the enzymatic activity of the cucurbit[8]uril–(FGG-dcasp-9)$_2$ complex did not reach the same level as for FGG-casp-9, with the native dimerization interface, in the presence of cucurbit[8]uril.

The induction of FGG-casp-9 dimerization and activation critically depends on the supramolecular complexation with cucurbit[8]uril. Such a supramolecular assembly process provides the opportunity to reverse the protein dimerization with a competitor molecule. Concomitantly, the reversibility of the cucurbit[8]uril-induced casp-9 activation was evaluated through inhibition of complex formation upon addition of a competitor peptide. For this, the enzymatic active complex of cucurbit[8]uril and FGG-casp-9 was treated with increasing amounts of a short FGG peptide (Figure 4). This peptide modulator consisting of 3 amino acids is substantially shorter than other peptide-based regulators that are typically used to compete with protein–protein interactions.[29] Upon addition of this competitor peptide the enzymatic activity of the casp-9

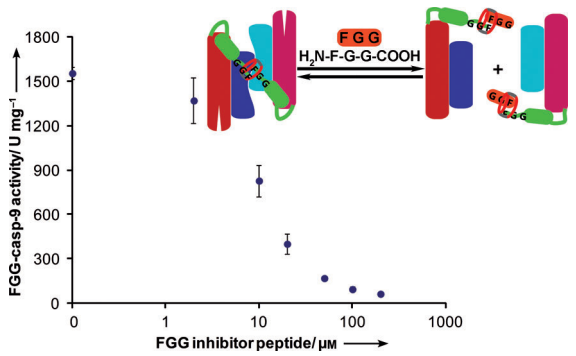

Figure 4. Reversible blockage of casp-9 activation. A preformed supramolecular casp-9 dimer (at 150 nM FGG-casp-9; 1 μM cucurbit[8]uril) was titrated with increasing amounts of FGG peptide. The error bars represent the standard deviation based on 3 independent measurements. Inset shows a representation of the supramolecular inhibition of the cucurbit[8]uril-induced casp-9 dimer by the competitive FGG peptide.

was decreased in a dose-dependent fashion. Inhibition of activation required addition of a relatively large excess of inhibitory FGG peptide; this result is consistent with the enhanced stability of the cucurbit[8]uril–$(FGG\text{-}casp\text{-}9)_2$ complex. The reversibility of the cucurbit[8]uril–FGG system thus illustrates the full control achievable over casp-9 dimerization and activation with the supramolecular approach and the potential to either induce or block protein dimerization with specific small host–guest molecules.

The results show that the supramolecular-induced protein dimerization activates two casp-9 monomers. The supramolecular elements do not affect enzyme activity themselves, as evidenced in the control experiments. Rather the cucurbit[8]uril facilitates the proximity of two casp-9 proteins, thus supporting the enzyme activation.[22,23] The activity of the cucurbit[8]uril-induced casp-9 dimers is not only significantly greater than that of the isolated protein, but is also superior to proteins mutated to have a hydrophobic dimerization interface (Figure 3). This is demonstrated both by the additional increase of the enzymatic activity of FGG-dcasp-9 upon cucurbit[8]uril addition and by the overall higher activity of the supramolecular-dimerized FGG-casp-9. These results show that the molecular pathway by which caspase dimerization is induced can significantly influence the activity of the resulting enzyme assembly.[30,31] The mutations introduced to enhance dimerization[27] in the dcasp-9 constructs at the same time can compromise activity. The mutated dimerization interface probably leads to a molecular packing of the FGG-dcasp-9 dimer diverged from the native state with the active site less optimal arranged. In contrast, the supramolecular, cucurbit[8]uril-facilitated, dimerization of casp-9 makes use of the natural dimerization interface, thereby leading to optimal activity.

In conclusion, the results show that cucurbit[8]uril can act as a supramolecular inducer of caspase dimerization, thereby leading to optimal protein reorganization and enzymatic activity. The facilitated protein dimerization induced by cucurbit[8]uril enables full and reversible control over caspase activity; such control is not achievable by using classical protein engineering approaches, and allows the study of such caspases both in their monomeric and dimerized state. Control over enzyme dimerization by cucurbit[8]uril and a very small genetically encoded FGG peptide motif thus provides a powerful approach to study the molecular mechanisms of enzyme dimerization and activation. It allows for an efficient formation of dimers, prevents perturbation of native active sites, and allows reversible switching. We believe that cucurbit[8]uril-induced protein dimerization holds great promises for studying not only caspases, but also many other protein homodimerization events in a reversible manner, such as dimerizing enzymes and membrane receptor proteins.[32–35]

Experimental Section

Protein expression and purification: The plasmids pMGG-casp-9, pFGG-casp-9, and pFGG-dcasp-9, were transformed into *E. coli* strain BL21 (DE3). The bacteria were cultured in LB medium containing ampicillin (100 $\mu g\,mL^{-1}$), and the cells were grown at 37 °C (shaking at 250 rpm) to an A_{600} value of 0.7, then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.3 mM. The cells were continuously incubated overnight at 15 °C (shaking at 250 rpm) before being harvested. The pellet was resuspended into the BugBuster protein extraction reagent plus benzonase nuclease, and the insoluble material was removed by centrifugation at 20000 rpm for 40 min at 4 °C. The soluble fraction was applied to a column filled with chitin beads (New England Biolabs) through gravity flow, and the column was washed with 40 volumes of sodium phosphate buffer (20 mM, 100 mM of sodium chloride, pH 7) and subsequently incubated for about 6 h at room temperature. The cleaved proteins were then collected in the flow-through by using the phosphate buffer. The purity and identity of the target proteins were confirmed by SDS-PAGE and LC-ESI-MS.

Casp-9 activity screening on a model substrate: The casp-9 activity assays were carried out at 150 nM casp-9 (FGG-casp-9, MGG-casp-9, or FGG-dcasp-9) in the absence or presence of cucurbit[8]uril or the FGG tripeptide in 100 μL of assay buffer (25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 100 mM NaCl, 4 mM $MgCl_2$ and 2 mM triscarboxyethylphosphine (TCEP), pH 6.5) with 100 μM of the synthetic Ac-LEHD-AFC substrate (Enzo Life Sciences) in 96-well plates. AFC cleavage was detected at an excitation wavelength of 400 nm and an emission wavelength of 505 nm using a Tecan Safire II at 37 °C. The specific activity of the specific casp-9 construct was determined by using an AFC calibration curve (see the Supporting Information) and is the average of 3 independent measurements.

Curve fitting of cucurbit[8]uril-dependent casp-9 activity: The casp-9 enzymatic activity at different cucurbit[8]uril concentrations was fitted by using the equation shown below. Assumption in the fit is that casp-9 and cucurbit[8]uril exclusively form a cucurbit[8]uril/$(casp\text{-}9)_2$ complex in this concentration regime, which is a reasonable assumption based on the earlier finding that twofold FGG binding to cucurbit[8]uril is cooperative.[15]

$$Y = P1(((4(P2) + 1/(\beta X)) - \sqrt{(-4(P2) - 1/(\beta X))(-4(P2) - 1/(\beta X)) - 16(P2)(P2)})/8) \quad (1)$$

In which β: the ternary formation constant; X: cucurbit[8]uril concentration (M); $P2$: casp-9 concentration (M); Y: enzymatic activity; $P1$: conversion factor to correlate complex concentration to enzyme activity.

Casp-9 activity screening on casp-3 substrate**:** The activity of the casp-9 variants for casp-3 cleavage was determined in a solution-based assay with casp-9 (0.15 μM) and substrate, casp-3 (4 μM), in the

absence or presence of cucurbit[8]uril (4 μM) diluted in assay buffer (25 mM HEPES, 100 mM NaCl, 4 mM $MgCl_2$, 2 mM TCEP, pH 6.5). Reactions were incubated at 37 °C and samples were collected in time intervals and reactions were stopped by addition of SDS-loading buffer. All samples were separated on 8 to 18% SDS-PAGE and proteins detected with blue staining. The extent of casp-3 cleavage was determined by densitometry using the AlphaEaseFC software (Alpha Innotech).

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