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# Crystal structure of the death effector domains of caspase-8

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ABSTRACT

Caspase-8 is a key mediator in various biological processes such as apoptosis, necroptosis, inflammation, T/B cells activation, and cell motility. Caspase-8 is characterized by the N-terminal tandem death effector domains (DEDs) and the C-terminal catalytic protease domain. The DEDs mediate diverse functions of caspase-8 through homotypic interactions of the DEDs between caspase-8 and its partner proteins. Here, we report the first crystal structure of the DEDs of caspase-8. The overall structure of the DEDs of caspase-8 is similar to that of the DEDs of vFLIP MC159, which is composed of two tandem death effector domains that closely associate with each other in a head-to-tail manner. Structural analysis reveals distinct differences in the region connecting helices α2b and α4b in the second DED of the DEDs between caspase-8 and MC159, in which the helix α3b in MC159 is replaced by a loop in caspase-8. Moreover, the different amino acids in this region might confer the distinct features of solubility and aggregation for the DEDs of caspase-8 and MC159.



## 1. Introduction

Caspase-8 is the major apical caspase that initiates apoptosis through the extrinsic pathway, and has also been shown to play key roles in several non-apoptotic processes such as necroptosis, inflammation, innate immunity, and cell migration [1–7]. Caspase-8 is characterized by the N-terminal prodomain and the C-terminal catalytic protease domain [8]. The C-terminal catalytic protease domain has been extensively studied and characterized by the determined structures of the apo protease domain or the complex of the protease domain in complex with peptide inhibitors or cellular FLICE-inhibitory protein (c-FLIP$_L$) [9,10]. The active protease domain is composed of a heterotetramer with two large and two small subunits [11]. However, the structural study of the N-terminal prodomain is hampered by its strong aggregating tendency and low solubility [12].

The N-terminal prodomain of caspase-8 is composed of two tandem death effector domains (DEDs) that belong to the death domain (DD) superfamily [13–15], which mediates the homotypic interaction with its partner proteins such as Fas associated death domain protein (FADD) in the death-inducing signaling complex (DISC) [16,17], or mediates the self-assembly in the death effector filaments [18,19]. No determined structure is currently available for the DEDs of caspase-8. The crystal structure of the soluble DEDs of vFLIP MC159 is generally used as a model to understand the homotypic interactions mediated by the DEDs of caspase-8 [20,21]. However, considering the low sequence homology (about 22%) and the distinct difference in solubility and aggregation between the DEDs of caspase-8 and MC159, the homology modeling might not be enough to characterize the full structural features of the DEDs of caspase-8. To address this problem, we determined the crystal structure of the tandem DEDs of caspase-8 at 2.2 Å resolution, which provides new insight into understanding the structure and functions of the DEDs of caspase-8.

## 2. Material and methods

### 2.1. Protein expression and purification

All clones were generated with a standard PCR-based cloning strategy, and the individual clones were verified by DNA sequencing. For biochemical assays and crystallization, the DEDs of human caspase-8 (residues 1–188) were subcloned into a pET-28a vector with N-terminal His6 or His-SUMO tags. The relevant mutants of Caspase-8 DEDs (residues1-188) were introduced by Fast

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Mutagenesis System (*TransGen Biotech*). All the Caspase-8 DEDs constructs mentioned above were transformed into Rosetta (DE3) pLysS cells (Novagen). All recombinant protein expression was induced with 0.1 mM IPTG at 20 °C overnight. His-tagged proteins were purified with Qiagen Ni-NTA agarose according to the manufacturer's instructions. For crystallization, the His-SUMO tag was digested with Ulp1 overnight. The digestion was reloaded onto Ni-NTA resin. The flow-through was collected and concentrated to load on a Hiload 16/60 superdex 200 column (GE) to fractionate homogeneous DED proteins. The final purified DED protein was concentrated to 15 mg/ml in buffer A, composed of 20 mM Tris, pH 8.0, 1 mM DTT and 150 mM sodium chloride, and used for crystallization.

### 2.2. Crystallization and structure determination

The final purified caspase-8 DEDs (residues1-188, F122A/I128D) protein was concentrated to 15 mg/ml in buffer A, crystals were initially obtained as small needle clusters using the sitting-drop vapor diffusion method in reservoir buffer that contained 200 mM sodium chloride, 100 mM Tris, pH 8.5, 25% PEG3350. Crystals were optimized using hanging drop vapor diffusion method in reservoir buffer containing 160 mM sodium chloride, 80 mM Tris, pH 8.5, 20% PEG3350. After crystal screening, the diffraction data was collected in homesource Rigaku micromax-002+. Structure was solved by molecular replacement using the phaser-PHENIX software [22,23], using the crystal structure of the tandem DEDs of vFLIP MC159 (PDB code 2BBR) [20] as the initial search model. The final models were manually built in Coot [24] and refined by Refmac in CCP4 software [25,26]. The refinement statistics are shown in Table 1. The protein in the figures was rendered with PyMol [27].

**Table 1**
Data collection and refinement statistics (molecular replacement).

| | |
|---|---|
| PBD ID | 4ZBW |
| *Data collection* | |
| Wavelength (Å) | 1.5418 |
| Space group | P1 |
| Cell dimensions | |
| *a*, *b*, *c* (Å) | 51.36, 52.11, 56.60 |
| *α*, *β*, *γ* (°) | 113.34, 116.89, 90.12 |
| Resolution (Å) | 2.20 (2.28–2.20)[a] |
| Total reflections | 41,944 |
| $R_{merge}$ | 0.052 (0.238)[a] |
| $I/\sigma I$ | 8.76 (2.59)[a] |
| Completeness (%) | 96.6 (93.8)[a] |
| Redundancy | 1.83 (1.59)[a] |
| *Refinement* | |
| Resolution (Å) | 29.22–2.20 |
| Unique reflections | 22,971 |
| $R_{work}/R_{free}$ | 0.204/0.251 |
| No. atoms | 3298 |
| Protein | 3050 |
| Ligand/Ion | |
| Water | 248 |
| Average B factors | 43.1 |
| r.m.s. deviations | |
| Bond lengths (Å) | 0.004 |
| Bond angles (°) | 0.810 |
| Ramachandran statistics | |
| Most Favored (%) | 98.0 |
| Allowed (%) | 2.0 |
| Outlier (%) | 0 |

Equations defining various *R* values are standard and hence are no longer defined in the footnotes.
[a] Values in parentheses are for highest-resolution shell.

### 2.3. Analytical size-exclusion chromatography

Analytical size-exclusion chromatography was performed on an AKTA FPLC system (GE Healthcare). Caspase-8 DED mutant proteins were loaded on to superdex 200 10/300 GL column (GE Healthcare) equilibrated with a buffer containing 20 mM Tris (pH 8.0), 500 mM NaCl, 1 mM DTT. The eluent was monitored by ultraviolet absorbance at 280 nm.

## 3. Results and discussion

### 3.1. Soluble mutants of the death effector domains of caspase-8

Over-expression of the wild-type tandem DEDs of caspase-8 in *Escherichia coli* remains a challenge due to the high aggregation and low solubility. On the other hand, the tandem DEDs of MC159 is soluble and retains the monomeric state in solution, which renders it easier to be characterized by biochemical and biophysical methods. To obtain soluble mutants of the DEDs of caspase-8, we performed a sequence alignment between the DEDs of caspase-8 and MC159, and also performed detailed structural analysis of the crystal structures of MC159 [20,21] to identify the potential residues that might affect the solubility of the DEDs of caspase-8. We found that several solvent-exposed and hydrophobic residues including Leu27, Met43 and Ile128 in caspase-8 are markedly distinct from the corresponding hydrophilic residues in MC159 including His33, Gln44 and Asn123, respectively (Fig. 1A).

To evaluate the influence of these residues on the solubility of caspase-8, three mutants L27H, M43Q, and I128N the DEDs of caspase-8 (residues 1–188) were constructed and overexpressed in *E. coli*. Fortunately, the expression showed that the mutant I128N was efficiently expressed and soluble in aqueous solution (Fig. 1B), but the purified protein precipitated while kept at 4 °C overnight after elution. We then mutated Ile128 to negatively charged aspartic acid, and the generated mutant I128D became more stable after elution. We could obtain root-like microcrystals at the concentration of 2 mg/ml after several rounds of crystallization screening (Fig. 1C), but the protein also precipitated when further concentrated. Inspired by the previous report that the F25Y mutation significantly improved the solubility of FADD [28,29], we thus mutated the corresponding solvent-exposed hydrophobic F122 on DED2 to alanine on the basis of the I128D mutant. The F122A/I128D mutant is highly homogenous and stable in aqueous solution under the concentration up to 15 mg/ml. Finally, we obtained rod-like crystals qualified for data collection after crystallization screening and optimization (Fig. 1D).

### 3.2. Overall structure of the death effector domains$_{1\text{-}188}$

The crystal structure of the DEDs of the F122A/I128D mutant of caspase-8 (hereafter referred to as Casp8$^{F122A/I128D}$ unless otherwise specified) was determined by molecular replacement using the crystal structure of MC159 (PDB code 2BBR) [20] as the template, which was solved at 2.2 Å in the P1 space group with a dimer in the asymmetric unit (Fig. 2A and Table 1). The structure of DEDs contains residues 2–184, residues at the two extreme terminuses were not observed in the solved crystal structure.

The DEDs of Casp8$^{F122A/I128D}$ are composed of two tandem DEDs (DED1 and DED2) that have all α helical fold, which are closely associated with each other to form a dumbbell-shaped structure (Fig. 2B). The intramolecular interface between DED1 and DED2 is mainly formed by the hydrophobic residues from the helices α2a

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