The crystal structure of caspase-6, a selective effector of axonal degeneration

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Neurodegenerative diseases pose one of the most pressing unmet medical needs today. It has long been recognized that caspase-6 may play a role in several neurodegenerative diseases for which there are currently no disease-modifying therapies. Thus it is a potential target for neurodegenerative drug development. In the present study we report on the biochemistry and structure of caspase-6. As an effector caspase, caspase-6 is a constitutive dimer independent of the maturation state of the enzyme. The ligand-free structure shows caspase-6 in a partially mature but latent conformation. The cleaved inter-domain linker remains partially inserted in the central groove of the dimer, as observed in other caspases. However, in contrast with the structures of other caspases, not only is the catalytic machinery misaligned, but several structural elements required for substrate recognition are missing. Most importantly, residues forming a short anti-parallel β-sheet abutting the substrate in other caspase structures are part of an elongation of the central α-helix. Despite the dramatic structural changes that are required to adopt a canonical catalytically competent conformation, the pre-steady-state kinetics exhibit no lag phase in substrate turnover. This suggests that the observed conformation does not play a regulatory role in caspase-6 activity. However, targeting the latent conformation in search for specific and bio-available caspase-6 inhibitors might offer an alternative to active-site-directed approaches.

Key words: caspase-6, caspase activation, Huntington’s disease, multi-angle light scattering (MALS), pre-steady-state kinetics, sedimentation velocity.

INTRODUCTION

Apoptosis plays a critical role in the development of the CNS (central nervous system) [1]. Caspases are cysteine proteases that play a significant role in this tightly regulated process of programmed cell death. Initiator caspases, such as caspase-8 and -9, are recruited to and activated by either so-called death receptors or the apoptosome in response to external stimuli during development. The intrinsically monomeric initiator caspases dimerize to produce an active protease, competent to process themselves as well as downstream effectors. This activation triggers a signalling cascade that kills the cell by limited proteolysis of the cellular organelles. Caspase-3 has traditionally been identified as the primary effector caspase in CNS developmental events, based upon the phenotype of the caspase-3 knockout mouse [2]. However, it has recently been reported that developmental axonal pruning is regulated by caspase-6 [3,4]. Furthermore, these results also indicated that the process by which caspase-6 triggers axonal degeneration may involve selective microtubule cytoskeletal destabilization [4].

This same programme, required for the proper development of the CNS, may later become its enemy as previous research has suggested that caspase-6 plays a role in neurodegenerative disease [5,6]. However, the precise role of caspase-6 remains elusive. Caspase-6 activation is observed in primary neuronal cultures after serum deprivation and withdrawal of trophic support [7,8]. Moreover, caspase-6 activation is observed in post-mortem brain tissue from: (i) aged non-cognitively impaired individuals, (ii) aged mild cognitively impaired individuals; and (iii) aged mild, moderate, severe and very severe Alzheimer’s disease individuals [8–10]. In addition, caspase-6 activation is a feature of other neurodegenerative diseases including HD (Huntington’s disease) and caspase-6-dependent proteolytic cleavage of mutant Htt (huntingtin) is proposed to cause HD [11]. However, it is still unclear whether the cell death observed in each of these diseases is apoptotic. In particular, it remains controversial whether the selective ablation of the striatum observed in HD results from an apoptotic process, as opposed to being necrotic (‘cytoplasmic’) or autophagic [10,12]. The recent work identifying caspase-6 as an effector in neuronal pruning, activated by a cascade initiated by APP (β-amyloid precursor protein) signalling via DR6 [death receptor-6, also known as TNFRSF21 (tumour necrosis factor receptor superfamily-21)], also suggests that a similar signalling cascade, involving caspase-6, may contribute to the Alzheimer’s disease pathophysiology [3].

To aid in the discovery of specific caspase-6-directed inhibitors, we solved the structure of active ligand-free caspase-6. Despite activation by limited proteolysis the enzyme rests in an immature, latent conformation, which does not support substrate binding and turnover. However, the latent conformation does not lead to latency in substrate turnover, as shown by pre-steady-state kinetic analysis.

Abbreviations used: Ac-, N-acetyl-; AFC, 7-amino-4-trifluoromethylcoumarin; AMC, 7-amino-4-methylcoumarin; APP, β-amyloid precursor protein; CMK, chloromethylketone; CNS, central nervous system; DR6, death receptor-6; DTT, dithiothreitol; HD, Huntington’s disease; Htt, huntingtin; IMAC, immobilized metal-ion-affinity chromatography; MALS, multi-angle light scattering; RMSD, root mean square deviation; TCEP, tris-(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid.

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EXPERIMENTAL

Materials
All chemicals used were purchased from Sigma–Aldrich unless stated otherwise. Crystallization solutions were all from Hampton Research. The substrates Ac (N-acetyl)-DEVD- AFC (7-amino-4-trifluoromethylcoumarin), Ac-DEVD-AMC (7-amino-4-methylcoumarin) and Ac-VEID-AMC, as well as the inhibitor Ac-LEHD-CMK (chloromethylketone) were obtained from Bachem, Switzerland. All other substrates were purchased from Biosynthan, Germany.

Cloning and protein preparation
The cDNA encoding wild-type human caspase-6 (residues 24–293) was amplified from a human cDNA library using the sense primer 5′-TGAATTCCTGCGACCATGGCAGGATC-CCATATGGGCTTTGATATAAAAGAGAAATG-3′ and the anti-sense primer 5′-TACTCGAGTATTAAGCGGCGGCCGATTAGATTCCGAAAGAATGTC-3′. The cDNA was then cloned using the Topo TA Cloning® Kit into a pCR2.1 vector (Invitrogen) and finally subcloned into a modified pET24a plasmid (Novagen) by using the NdeI and NotI restriction sides. The resulting plasmid encodes caspase-6-(24–293)-Ala 3-His6. The corresponding active sitezymogen mutant (C163A) was generated by single site-directed mutagenesis using the pET24a construct as template using the sense primer 5′-GATAATTATATC-ATTCAGCACGCGGCGGAAACCAGCACGATG-3′ and the anti-sense primer 5′-CATCCTGTGCTGTTTCCCCGGGCTGCCGTTAGCG-GATATTTATC-3′. The DNA sequences of all constructs (both sense and antisense strands) were verified using standard sequencing protocols. Caspase-6-(24–293) as well as the caspase-6-(24–293)-C163A mutant were expressed as soluble proteins from Escherichia coli using standard protocols [BL21-CodonPlus®(DE3)-RIPL cells, LB (Luria–Bertani) medium and induction at 25 °C for 20 h with 0.2 mM IPTG (isopropyl β-D-thiogalactoside)]. The cells were harvested by centrifugation at 5000g for 15 min at 4 °C, cell pellets were washed with 50 mM Tris/HCl, pH 8.0, containing 100 mM NaCl, and were subsequently resuspended in the same buffer and frozen at −20 °C. After thawing, the cells were ruptured using a high-pressure cell disruption system twice at 2.4 kbar (Constant Systems). Insoluble debris was removed by centrifugation at 30 000g for 30 min at 4 °C. The clear supernatant was filtered using a 0.45 μm Stericup® (Millipore).

Next, recombinant caspase-6-(24–293) was captured from the supernatant by IMAC (immobilized metal-ion-affinity chromatography) using a GE Healthcare XK 16/20 column packed with 7 ml of Ni-NTA (Ni2+–nitrotriacetate) superflow from Qiagen. After loading, the column was washed with five column-volumes of 50 mM Tris/HCl, pH 8.0, containing 100 mM NaCl, 20 mM imidazole and five column-volumes of 50 mM Tris/HCl, pH 8.0, containing 100 mM NaCl and 70 mM imidazole. The protein was eluted by increasing the imidazole concentration to 300 mM. To avoid protein precipitation, the pH of the eluate was immediately shifted to pH 5 by adding 1 M acetic acid. Next, solid ammonium sulfate was added up to a final concentration of 1 M. The sample was loaded on to a hydrophobic interaction column (XK 16/20 column packed with 30 ml of phenyl-Sepharose high-performance resin; GE Healthcare) and eluted by decreasing the ammonium sulfate concentration using 50 M sodium acetate, pH 5.5, containing 50 mM NaCl. The pooled and concentrated (final volume 1–5 ml) sample was polished on a size-exclusion column (HiLoad 16/60 Superdex-75 pg with 50 M sodium acetate buffer, pH 5.5, containing 50 mM NaCl). The buffer of the concentrated protein solution (10 mg/ml) was changed with a spin column (Zeba) to 25 mM phosphate buffer, pH 6, containing 100 mM NaCl and 1 mM TCEP [tris-(2-carboxyethyl)phosphine]. Caspase-6-(24–293)-C163A zymogen was expressed and purified as described above, with the difference that protease inhibitor cocktail (Complete EDTA-free; Roche) was added before disrupting the cells.

Analytical size-exclusion chromatography was performed in 50 mM sodium acetate, pH 5.5, containing 50 mM NaCl by loading 100 μl of protein at a concentration of 1 mg/ml on a Superdex 200 HR 10/30 column (GE Healthcare). A MALS (multi-angle light scattering) detector (MiniDAWN, Wyatt; RI-71, Shodex) was coupled to the chromatographic system (Aepta purifier 10, GE Healthcare). Data were evaluated using ASTRA software (Wyatt Technology).

The identity of the caspase-6 preparations was verified by LC-MS using a Poros R1/1 mm × 150 mm column at 80 °C, a flow-rate of 0.06 ml/min with eluent A [0.05 % TFA (trifluoroacetic acid)] and eluent B (acetonitrile/water at a ratio 9:1 and 0.045 % TFA). In all samples analysed the initiator methionine residue was removed. In the case of caspase-6-C163A zymogen one single mass peak at 32183.5 Da was detected. In the case of caspase-6-(24–293), after a three-step purification (see below), two peaks were observed, one at 18067.0 Da (p18 large subunit residues 24–179) and one at 12564.0 Da (p11 small subunit 194–293), as shown in Supplementary Figure S1(A) (available at http://www.BiochemJ.org/bj/423/bj4230429add.htm). Samples that were purified using a two-step procedure [IMAC and size-exclusion chromatography] contained three peaks at 18066.6 Da (large subunit residues 24–179), 19668.1 Da (large subunit 24–193) and 12563.5 Da (p11 small subunit 194–293), as shown in Supplementary Figures S1(B) and S1(C).

Enzymatic characterization
The assay buffer in all experiments was 200 mM Hepes, pH 7.4, containing 1 mM EDTA, 20 mM DTT (dithiothreitol) and 0.05 % CHAPS unless stated otherwise. The enzyme concentration was determined from the absorption measured at 280 nm on a Nanodrop ND-1000 (Witec AG) using the molar absorption coefficient of 25900 M−1 cm−1, calculated with an increment method from the ExPASy Proteomics Server (www.expasy.ch). In addition, the enzyme preparation used in the enzymatic studies was titrated using the irreversible inhibitor Ac-LEHD-CMK and was found to be fully active (results not shown).

The catalytic efficiency of caspase-6 was tested using eleven tetrapeptidic substrates [see Table 1, Supplementary Table S1 and Figure S1 (available at http://www.BiochemJ.org/bj/423/bj4230429add.htm)]. Substrates were selected to include putative optimal and sub-optimal substrates as well as peptides matching natural cleavage sites [13,14]. Substrate hydrolysis under initial velocity conditions was monitored at 23 °C on a Tescan Infinite M-1000 using excitation and emission wavelengths of 370 nm and 505 nm with spectral bandwidths of 10 nm on both sides (for substrate 1) or on a Tescan Safire 2 using the wavelengths 380 nm and 400 nm with the same spectral bandwidths (for substrates 2–11). The Michaelis constants, Ks, were obtained from measurements for at least two different enzyme concentrations (Supplementary Table S1) and various substrate concentrations up to 200 μM. Under conditions where the substrate concentration, [S], is significantly higher than the enzyme concentration and the substrate consumption is below 20 %, the initial enzymatic velocity, V0, can be approximated by the Henri–Michaels–Menten equation (eqn 1):

\[ V_0 = \frac{(V_{\text{max}} \times [S])}{([S] + K_m)} \] (1)
Table 1  Substrate cleavage by caspase-6 under initial velocity conditions

<table>
<thead>
<tr>
<th>Number</th>
<th>Substrate</th>
<th>Description</th>
<th>(K_m) (μM) [according to eqns (1) and (2)]</th>
<th>(k_{\text{cat}}) (s(^{-1})) [according to eqns (1) and (2)]</th>
<th>(k_{\text{cat}}/K_m) (M(^{-1}).s(^{-1})) [according to eqns (1) and (2)]</th>
<th>(k_{\text{cat}}/K_m) (M(^{-1}).s(^{-1})) [according to eqns (3) and (4)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ac-DEVD-AFC</td>
<td>Caspase-3 substrate</td>
<td>87 ± 14</td>
<td>3.2 ± 0.1</td>
<td>3.70 × 10(^4)</td>
<td>nd</td>
</tr>
<tr>
<td>2</td>
<td>Ac-DEVD-AMC</td>
<td>Caspase-3 substrate</td>
<td>126 ± 9</td>
<td>5.5 ± 0.1</td>
<td>4.40 × 10(^4)</td>
<td>2.49 × 10(^4)</td>
</tr>
<tr>
<td>3</td>
<td>Ac-VEDD-AMC</td>
<td>Caspase-6 substrate according to supplier</td>
<td>13.4 ± 1</td>
<td>3.6 ± 0.1</td>
<td>2.70 × 10(^4)</td>
<td>nd</td>
</tr>
<tr>
<td>4</td>
<td>Ac-VEDD-AMC</td>
<td>Optimal caspase-6 substrate according to [13]</td>
<td>246 ± 3</td>
<td>43 ± 0.0</td>
<td>1.75 × 10(^4)</td>
<td>2.66 × 10(^5)</td>
</tr>
<tr>
<td>5</td>
<td>Ac-TEVD-AMC</td>
<td>Caspase-6 cleavage site 103</td>
<td>20.7 ± 4.5</td>
<td>6.0 ± 0.3</td>
<td>2.95 × 10(^4)</td>
<td>nd</td>
</tr>
<tr>
<td>6</td>
<td>Ac-DVDV-AMC</td>
<td>Caspase-6 cleavage site 176</td>
<td>1200 ± 300</td>
<td>1.4 ± 0.4</td>
<td>1.20 × 10(^4)</td>
<td>1.20 × 10(^3)</td>
</tr>
<tr>
<td>7</td>
<td>Ac-IVLD-AMC</td>
<td>Caspase-6 site 586 in Htt [14]</td>
<td>472 ± 124</td>
<td>1.0 ± 0.2</td>
<td>2.53 × 10(^3)</td>
<td>1.51 × 10(^3)</td>
</tr>
<tr>
<td>8</td>
<td>Ac-CLND-AMC</td>
<td>Caspase-3 site 552 in Htt [14]</td>
<td>3600 ± 704</td>
<td>0.14 ± 0.03</td>
<td>3.9 × 10(^3)</td>
<td>nd</td>
</tr>
</tbody>
</table>

\(^{a}\)The experimental conditions \([S]_0 < 0.1 \times K_m\) required for the use of eqn 3 cannot be satisfied.  
\(^{b}\)Under experimental conditions substrate turnover is too low to allow use of eqn 3.

where \(V_{\text{max}}\) is the maximal velocity at saturating substrate concentrations.

Equation (1) can be applied to the curve obtained by plotting the measured enzyme velocities against the corresponding substrate concentrations providing the values for \(K_m\) and \(V_{\text{max}}\) by a non-linear regression fit.

The \(k_{\text{cat}}\) value is then defined as (eqn 2):

\[
k_{\text{cat}} = \frac{V_{\text{max}}}{[E]_0}
\]

where \([E]_0\) is the employed enzyme concentration.

The kinetic values for several substrates could not be determined under initial velocity conditions as the \(K_m\) values were found to be significantly higher than 200 μM and therefore above the limit of our plate-based assay format. Therefore the \(k_{\text{cat}}/K_m\) values have, in addition, been determined by a method described previously in [15]. Briefly, under conditions where the initial substrate concentration, \([S]_0\), used in an enzymatic reaction is significantly lower than one tenth of the \(K_m\) value (i.e. \([S]_0 \ll 0.1 \times K_m\)), the progress of the substrate turnover follows a single exponential curve that can be fitted to the function (eqn 3):

\[
[P(t)] = [S]_0 \times \left[1 - \exp \left( -\frac{t}{\tau} \right) \right]
\]

where \([P(t)]\) is the product concentration at the time \(t\) and \([S]_0\) is the initial substrate concentration. \(\tau\) is defined as (eqn 4):

\[
\tau = \frac{K_m}{k_{\text{cat}} \times [E]}
\]

where \([E]\) is the enzyme concentration used in the experiment.

For each of the substrates 2–11 we monitored the hydrolysis of 1 and 5 μM substrate for 20–120 min, depending on the rate of turnover, and using the assay conditions described above. Enzyme was used at 100 nM for all substrates, with the exception of the efficiently cleaved substrates 3–5 where the enzyme concentration was reduced to 10 nM. Progress curves were fitted to eqn (3) and the catalytic efficiencies \(k_{\text{cat}}/K_m\) were calculated using eqn (4). Although the individual parameters \(k_{\text{cat}}\) and \(K_m\) cannot be determined by this method, the obtained apparent second-order rate constants allow the ranking of substrates. These values are included in Table 1, as far as applicable.

To determine the dependence of the initial rate on the caspase-6 concentration, the caspase-6 concentration was varied from 1 pM to 20 nM. The initial velocities of the product formation were measured with substrate 1 at a concentration of 200 μM. The measurements were performed on a TECAN Infinite M-1000 microtitre plate reader, with excitation and emission wavelengths of 370 and 505 nm respectively, and spectral bandwidths of 10 nm. The total assay volume was 10 μl and the substrate and the enzyme were diluted in assay buffer.

For the determination of the initial rate constants, the experimental data were fitted using the non-linear regression program Origin 7.5SR6 (OriginLab Corporation).

Pre-steady-state kinetics

The hydrolysis of Ac-DEVD-AMC by caspase-6 in the pre-steady-state was analysed with an Applied Photophysics SX 18MV stopped-flow spectrometer (Applied Photophysics). The dead-time of the instrument was 1.8 ms, determined as described previously [16]. The wavelength for the excitation of the AMC dye was 350 nm with a spectral bandwidth of 4.65 nm. The emission was monitored using a 450 nm bandpass filter with a 65 nm bandwidth (450DF65; Laser Components).

The enzyme and substrate solutions were mixed in a 1:1 ratio with volumes of 50 μl each. The concentrations of enzyme were varied from 1 to 15 μM and the substrate concentration was kept fixed at 10 μM. The stopped-flow experiments were performed in 200 mM Hepes, pH 7.5, containing 1 mM EDTA, 20 mM DTT and 0.05% CHAPS. In each run 400 scans were performed.

Each progress curve was averaged over ten repetitions after correction for background signal. The individual traces consisted of 400 data points which were collected over 0.2 s. The first derivative of the resulting averaged traces was calculated and fitted with the non-linear regression program Origin 7.5SR6 to a hyperbolic equation to illustrate the increase in velocity over the recorded time course.

Under this experimental setup the readout obtained with AMC substrate was more stable than the one obtained with the corresponding ACP substrate. For consistency we used AMC substrates for all subsequent experiments including the substrate hydrolysis under initial velocity conditions (see above and Table 1).

Analytical ultracentrifugation

Analytical ultracentrifugation sedimentation velocity experiments were conducted using a Beckman ProteomeLab XL-I with an An-50 Ti analytical rotor. All data acquired from these experiments were obtained using the UV/Visible absorbance detection system of the ultracentrifuge and double sector 12-mm
charcoal-filled Epon centrepieces with quartz windows. In a first run the absorption was measured at 280 nm ($A_{280} = 0.8$ to 0.9, corresponding to a protein concentration of 1 mg/ml) and in a second run at 235 nm (with concentrations of 0.5 and 0.25 mg/ml). The experiments were conducted at 4°C at a speed of 40000 rev/min. The results from 300 scans were analysed using the LAMM equation of the program SEDFIT [17]. All experiments were performed in 25 mM phosphate buffer, pH 6 (or pH 5.0), containing 100 mM NaCl and 1 mM TCEP.

**Crystallization, data collection and structure determination**

Prior to crystallization the monodispersity of the protein sample was assessed at 23°C by DLS (dynamic light scattering) using a ProteinSolutions DynaPro instrument (Wyatt Technology). Initial crystals of active caspase-6 were obtained using the SaltRx crystal screen (Hampton Research) on a 96-well sitting drop vapour diffusion Intelli-Plate (Art Robbins Instruments) at room temperature (23°C) against a reservoir of 1.5 M ammonium chloride and 0.1 M sodium acetate at pH 4.6. The protein concentration was 12 mg/ml and 0.3 μl of both protein and reservoir were used (a ratio of 1:1). The crystals were used to produce a seed stock [18].

Using the seed stock in a 1:5000 dilution, crystals of ligand-free active caspase-6 were obtained within 2–3 days by hanging drop vapour diffusion on a Linbro plate (Hampton Research) at room temperature against a reservoir containing 1 M ammonium citrate and 0.1 M sodium acetate, pH 4.5. The protein concentration was 12 mg/ml and 2μl of protein and reservoir and 0.5 μl of seed stock were used (a protein/reservoir/seed stock mixture of ratio 4:4:1).

Diffraction datasets were collected on the X10SA PXII beamline at the SLS (Swiss Light Source) in Villigen, Switzerland. The crystals belong to the monoclinic space group P21, and diffraction to a maximum resolution of 1.95 Å (1 Å = 0.1 nm). The data were processed and scaled with XDS/XSCALE. The structure was determined by molecular replacement using MolRep [19] and the caspase-7 structure (one monomer from PDB entry 1K86) as a search model. The structure was refined by iterative cycles of refinement using CNX (Accelrys) as part of the APRV graphical interface [20] and visual inspection using the Coot program [21]. Non-crystallographic symmetry restraints were used during refinement. In the final model a considerable number of terminal residues at the N- and C-termini of both large and small subunits (Table 2) were not defined by electron density and omitted. Details of data and refinement statistics are presented in Table 2.

The final structure is similar to that of other caspsases. Superimposition of monomer A of the final co-ordinate sets with caspase-1 (PDB entry 1ICE) yields an RMSD (root mean square deviation) of 1.39 Å for 171-Cα positions using lsqman [22]. Similar comparisons with caspase-3 (PDB entry 1CP3) and -7 (PDB entry 1K88) yields RMSDs of 1.17 Å (177-Cα positions) and 1.19 Å (179-Cα positions) respectively.

**RESULTS AND DISCUSSION**

**Expression and purification of active caspase-6**

The human gene CASP6 encodes caspase-6 (Swiss-Prot entry P55212; ENZYME entry EC 3.4.22.59), a 293-amino-acid long polypeptide. This gene product consists of a 23-amino-acid pro-peptide and a caspase domain. During proteolytic maturation of caspase-6, Met1–Asp23 and Asn180–Asp193 in the inter-domain linker are removed. Consequently, the active caspase-6 is a two-chain molecule consisting of the p18 and p11 subunits. Caspase-3 and -7, also caspases with a so-called ‘short pro-peptide’, have been expressed and crystallized successfully using constructs starting at the first residue of the large subunit, p18, without the pro-peptide. Similarly, caspase-6 constructs used in this study start at the Ala18 residue and are referred to as caspase-6-(24–293), or simply caspase-6. Throughout the manuscript residues are numbered according to the caspase-6 sequence. The structure-based sequence alignment (Figure 1) allows the identification of the corresponding amino acid numbers in caspase-1.

Human caspase-6 was expressed in *E. coli* as a soluble and active protease, and purified using standard protocols [23]. All of the caspase-6 preparations used in this study were greater than 95% pure according to SDS/PAGE (Nufage 4–15% Bis-Tris gel used according to the supplier, Invitrogen). Furthermore, LC-MS analysis of the final products (Supplementary Figures S1A and S1B) verified that they mainly contained two polypeptide chains corresponding to amino acids 24–179 (the large p18 subunit) and amino acids 194–293 (the small p11 subunit), in other words all polypeptide chains have been processed at both cleavage sites 179 and 193 (Figure 1) and the inter-domain linker is fully removed. Auto-processing during expression in *E. coli* is also observed for other human caspsases such as caspases-3 and -7 [23]. Preparations of the catalytically inactive active-site mutant, caspase-6-C163A, were an exception and can be purified as a single-chain molecule.

However, the excision of the inter-domain linker peptide, at residues 180–193, reaches completion only during purification of caspase-6 and not during expression in *E. coli*. SDS/PAGE (Nufage 4–15% Bis-Tris gel used according to the supplier, Invitrogen) of the eluate from the first purification step indicates the presence of two bands for the large subunit at around 18 kDa (results not shown). According to LC-MS analysis,
Figure 1  Structure-based alignment of caspase-1, -3, -6 and -7

The sequences shown correspond to the protein used in the respective crystallization experiment. Caspase-1, PDB entry 1ICE; caspase-3, PDB entry 1C3P; caspase-7, PDB entry 1K86; caspase-6, residues 24–293. The amino acid numbering on the top of the alignment refers to the sequence of full-length caspase-6; the one on the bottom to the sequence of caspase-1. Secondary structure elements shown on top of the alignment refer to the caspase-6 structure. Residues highlighted with boxes are parts of the protein that were used in the respective crystallization experiment but that were not visible in the electron density. The linker regions that are removed during processing are highlighted in light grey. Conserved residues are highlighted in bold and a dark grey box.

The consensus sequence shown in the bottom line was generated by aligning sequences of known human caspases [caspase-1 (P29466), -2 (P42575), -3 (P42574), -4 (P49662), -5 (P51878), -6 (P55212), -7 (P55210), -8 (Q14790), -9 (P55211), -10 (Q92851) and -14 (P31944); the number in brackets is the Swiss-Prot accession number]. The catalytic dyad residues (cysteine and histidine) are highlighted by asterisks.

active caspase-6 batches, purified using a preliminary two-step purification protocol, contain the large subunit 24–179 and a larger species with a mass matching the sequence 24–193 (Supplementary Figure S1B). After the introduction of an additional purification step, the polypeptide comprising residues 24–193 was no longer observed. The improved purity is probably not due to improved separation, but rather a result of the extended purification time allowing the enzyme to fully auto-process. The small subunit always exists as one species (comprising residues 194–293) on SDS/PAGE (NuPage 4–15% Bis-Tris gel used according to the supplier, Invitrogen) and in LC-MS analysis; a polypeptide comprising residues 180–293 (by cleavage at 179 and not 193) has never been observed. This could indicate that the caspase-6 zymogen is first cleaved at 193, which in turn makes the 179 site accessible. However, it is also possible that both sites are equally accessible and processing at position 193 is simply much more efficient. Indeed, the sequence around this site (190–193; Thr-Glu-Val-Asp) presents a better substrate than the ones around 179 (176–179; Asp-Val-Val-Asp) (Table 1).

To benchmark the caspase-6 batches produced against published results we determined the specific activity using Ac-DEVD-AFC and Ac-DEVD-AMC as substrates (for details see Table 1, Supplementary Table S1 and Figure 2). The respective values of $37 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($K_m = 87 \mu M$ and $k_{cat} = 3.2 \text{s}^{-1}$) and $44 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($K_m = 126 \mu M$ and $k_{cat} = 5.5 \text{s}^{-1}$) are consistent with the published values for the cleavage of Ac-DEVD-pNA ($K_m = 180 \mu M$ and $k_{cat} = 0.4 \text{s}^{-1}$ [24]). The observed 10-fold difference in $k_{cat}$ is probably attributable to the different chromophore employed.
Next we studied the cleavage of more specific caspase-6 substrates (Table 1, Supplementary Table S1 and Figure 2). Using a combinatorial library, Val-Glu-His-Asp (VEHD) had been determined as the optimal tetra-peptidic caspase-6 substrate [13]. According to these data the P3 position is very selective for a glutamic acid residue, whereas both the P2 and P4 position are less restrictive and accept different aliphatic side chains, as well as threonine and, in the case of P2, some aromatic side chains. Ac-TEVD-AMC (the caspase-6 inter-domain cleavage site), Ac-VEHD-AMC (the optimal substrate according to [13]) and Ac-VEID-AMC (marketed as a caspase-6 specific substrate) were selected as potentially optimal tetra-peptidic substrates. All three substrates are cleaved efficiently with specific activities of around $200 \times 10^3 \text{M}^{-1} \cdot \text{s}^{-1}$. We have no explanation for the unusual Michaelis–Menten constants (the high $K_m$ and highest observed substrate turnover; Table 1) for Ac-VEHD-AMC, which were confirmed in subsequent experiments.

Finally, we explored how the cleavage efficiencies for these optimal caspase-6 substrates compare with substrates based on the cleavage sequences in Htt, a putative natural substrate of caspase-6 [14]. On the basis of the putative caspase-3 (Htt residue 552) and caspase-6 (Htt residue 586) cleavage sites, Ac-DLND-AMC and Ac-IVLD-AMC were selected. As expected from the published sub-site mapping study [13], Ac-DLND-AMC is only cleaved very poorly. However, Ac-IVLD-AMC is also a poor caspase-6 substrate compared with the best substrates tested in the present study, underscoring the importance of glutamic acid at position P3. As often encountered in protease research, the cleavage site in a putative natural substrate does not necessarily correspond to the optimal sequence of a small peptide.

## Oligomeric state of caspase-6

Apoptotic caspases are classified as initiators, such as caspases-8 and -9, or effectors, such as caspases-3 and -7 and this classification correlates with the oligomeric state in solution. Inactive initiator caspases are monomeric, and activated by dimerization on a protein scaffold, elicited by a signalling event [25,26]. In contrast, effector caspases are obligate dimers and activated by limited proteolysis [27]. Based on its high homology to the ‘archetypical’ effectors caspase-3 and caspase-7 (with 41% and 38% sequence identity and 58% and 54% sequence homology respectively to caspase-6), and additional in vitro and in vivo data [28,29], caspase-6 has been classified as an effector caspase, implying that it is an obligate dimer and activated by proteolytic cleavage.

To further our understanding of the putative effector caspase-6, we decided to characterize its biochemical properties in solution. Surprisingly, the initial size-exclusion chromatographic analysis of caspase-6 revealed that the retention time of the zymogen was consistent with the size of a dimeric caspase species, whereas the retention times of the two-chain forms (inhibited and apo- enzymes) was consistent with the size of a monomeric caspase species (see Supplementary Figure S3 available at http://www.BiochemJ.org/bj/423/bj4230429add.htm). When individual
fractions were tested for their proteolytic activity, the highest activity was found in the fractions with the highest protein concentrations, in other words protein elution and activity profiles matched (see Supplementary Figure S4 available at http://www.BiochemJ.org/bj/423/bj4230429add.htm). These observations are clearly inconsistent with current models of effector caspase activation. However, the size-exclusion retention time is dependent upon the molecular effective hydrated radius, dictated by the molecular shape, as well as non-specific interactions with the column matrix, and therefore is an error-prone method for the determination of molecular mass. Thus we proceeded to determine the mass of the different caspase-6 forms by employing two, more direct, biophysical methods. First, we coupled our chromatographic system with a static light-scattering detector to determine the molecular masses by MALS, a technique that has been used previously to determine the oligomeric state of caspase-9 [26]. The results showed that the caspase-6 zymogen, active caspase-6 and caspase-6 inhibited with Ac-LEHD-CMK are all dimeric in the eluate (Figure 2A). The estimated masses were 70500 Da, 63070 Da and 63800 Da respectively.

Subsequently, we employed analytical ultracentrifugation. Figure 2(B) shows the sedimentation velocity distributions of 15 μM caspase-6 zymogen, active caspase-6 and caspase-6–Ac-LEHD-CMK. Active caspase-6 and caspase-6–Ac-LEHD-CMK were equivalent, under the assay conditions, whereas the caspase-6 zymogen was slightly faster in sedimentation. All three proteins exhibited monophasic behaviour and were dimers (estimated masses of about 58 kDa) despite the slight differences in their sedimentation velocities. In the next set of experiments, we varied the concentration of active caspase-6 and caspase-6 zymogen from 32 to 8 μM (see Supplementary Figure S5 available at http://www.BiochemJ.org/bj/423/bj4230429add.htm). A lower concentration could not be used as it was beyond the detection limits of our instrument. However, even at the lowest concentration, active caspase-6 behaved as a dimer and exhibited monophasic behaviour. Thus the K_d of the caspase-6 dimer is significantly below 10 μM. The anomalous behaviour of caspase-6–CT163A zymogen, e.g. the higher apparent mass by size-exclusion chromatography and the broader distribution curves in the MALS and sedimentation velocity experiments, might indicate a high intrinsic mobility of this species.

Finally, to estimate the K_d of the caspase-6 dimer, we determined the proteolytic activity of caspase-6 as a function of protein concentration. Figure 3 shows that the relationship between the turnover of the substrate Ac-DEVD-AFC and caspase-6 concentration is linear within the range from 1 pM to 20 nM enzyme. Given that active caspases are obligate dimers, these results indicate that caspase-6 remains dimeric even at concentrations below 1 nM. However, they do not permit the exact determination of a K_d, the determination of an exact K_d for monomer–dimer equilibriums by sedimentation equilibrium experiments is only possible where concentrations for both species are significantly populated. Given the apparent high stability, and by analogy to caspase-3 [30], such an experiment was beyond the sensitivity limit of the optical detection methods available to us.

Together, these results provide strong evidence that the caspase-6 used in the present study is dimeric in solution, in both the zymogen and active states, and is consistent with the existing classification of caspase-6 as an effector caspase.

Overall structure
The crystals diffracting to a maximum resolution of 1.95 Å contain two caspase dimers, each composed of two small and two large subunits in the asymmetric unit. On the basis of our biochemical results, and by analogy with other caspases, we conclude that in solution caspase-6 exists as a dimer and consequently one dimer represents one functional biological unit. The two caspase dimers, as well as each monomer within each dimer, are largely identical. However the second dimer of our model, consisting of the subunits C and D (see co-ordinates), has fewer disordered residues than the A and B dimer (Table 2). Therefore in the following description we refer to the C and D dimer.

The dimeric structure is consistent with that of all other caspases, with the exception of the caspase-9–BIR3 complex [31]. Caspase-6 consists of a central 12-stranded β-sheet (Figure 4), which is flanked by ten α-helices. Thus the dimer forms one spherical molecular entity that is slightly elongated along the central β-sheet. The two active sites are both on the same face of the dimer, roughly 30 Å apart, and are surrounded by four surface-loops. No ligand is bound in either active site.

In monomer D, the polypeptide chain of the small subunit can be traced in the electron density, starting at residue 199, and occupies the central cavity (red features in Figures 4 and 5A). Five residues of the N-terminus of the small subunit, as well as 14 residues of the C-terminus of the large subunit, remain invisible. A similar number of residues are disordered in the other three monomers of the asymmetric unit.

The active site of caspase-6 is in a latent conformation
There are several notable differences around the active site that are unique to caspase-6 compared with all other caspases. The active site of caspases is usually flanked to the south (in the ‘standard orientation’) by a short anti-parallel β-sheet (sheets 3′ and 3″ in Figures 5B and 5C) and the histidine residue of the catalytic dyad is located N-terminal of this β-sheet. In caspase-6 this anti-parallel β-sheet is not formed. Instead these residues are part of a three-turn extension of the central helix D. The active site histidine residue is now located on a turn connecting strand 3 with helix D (Figure 5A). In addition, helix B is also elongated by one turn.

These unique structural features contribute to a dramatic misalignment of the catalytic machinery, as well as the substrate...
conformation, the Arg220 peptide bond is rotated and points into strand 1 and helix B is located in loop L1. In all caspase structures (Leu61–Arg65 and His126–Ile136), as well as the parts of the inter-domain linker (loops L2 and L2′), there are highly conserved structure elements, that are different from other caspases, are the elongated helices B and D. These two residues determine the primary formation of helices B and D are coupled. A highly conserved unusual conformation and how this conformation is stabilized. A detailed analysis of the structure reveals that the extended conformation has only been observed in caspase-6. No residues that prohibit the canonical caspase-fold could be identified in the caspase-6 primary sequence. Nevertheless, this conformation seems to be stabilized by several interactions involving residues unique to caspase-6 (His52 with Asp96; His53 with Asp131; Arg54 with Asp131; His121 with Glu53). In addition, helix D contacts neighbouring molecules within the asymmetric unit, which might further stabilize this conformation, however, we do not think that rearrangements in two secondary structure elements can solely be caused by crystal contacts.

In caspase zymogens, the so-called L2 loop (Figure 4) connecting the large and the small sub-unit is uncleaved and occupies the central cavity. Activation by limited proteolysis separates this loop into L2 (C-terminus of the large subunit) and L2′ (N-terminus of the small subunit). However, in ligand-free caspase-6, as well as in caspase-1 and -7 [27,33], L2′ is not fully expelled from the central cavity. Thus it is not properly positioned to contribute to the formation of the loop-bundle, L2–L2′–L4. Only when the loop-bundle forms does the active site cysteine residue shift to a position close to the active site and the catalytic machinery fully mature, a mechanism well-described for caspase-7 [27]. For this feature, the latent state of caspase-6 therefore parallels that of caspase-7. However, as described above, in the ligand-free form of caspase-6, not only is the active site cysteine residue (located in the large subunit) misaligned, but so are elements in the small subunit, such as the active site histidine and residues required for substrate recognition. Thus activation of latent caspase-6 not only requires formation of the loop-bundle, but also partial unwinding of helices B and D, and the formation of a short anti-parallel β-sheet between strand 3 and helix D. These changes in secondary structure may contribute to the formation of the loop-bundle as, in the structure of ligand-bound caspase-7, residues on loop L2 interact with residues located between strand 3 and strand 3′ through a network of hydrogen bonds between the backbone atoms [34].

**Significance of the latent conformation for substrate turnover**

Latent conformations of the cleaved species have been described for both the initiator caspase-1 and the effector caspase-7 [27,33]. However, there is virtually no understanding of the latent conformation’s biological significance, although it has gained attention in the past as it is fundamental to the concept of allosteric caspase inhibition [35,36]. Specifically, it is unknown whether the latent state exists in solution and, if so, whether it influences substrate turnover. Therefore we sought to capture evidence of the latent conformation in the solution-state by enzymatic methods.

First, we analysed the early time points of our enzyme progress curves but did not notice a lag-phase. This is not surprising as all of our enzymatic characterization was performed under Michaelis–Menten conditions, where the substrate concentration is significantly higher than the enzyme concentration. Furthermore, the instrument used for routine enzyme characterization does not permit monitoring the very early phase of substrate turnover. Next, we analysed substrate cleavage under pre-steady-state conditions using a stopped-flow instrument. Under the assay conditions employed, a lag time of approx. 0.05 s was detectable at only the highest protein concentration (Figure 6). The short lag time indicates that the energetic difference between latent

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**Figure 4 Overall structure of the caspase-6 dimer in its ligand-free form**

The approximate dimensions of the caspase-6 dimer are 40 × 60 × 65 Å. The two large subunits are represented in grey, the two small subunits are represented in blue. The residues of the catalytic dyad (Cys163 (Cys285) and His121 (His237)), the conserved specificity determining residues Arg220 (Arg341), Arg64 (Arg166) and Gln161 (Gln283), as well as the His219 (Trp340) are highlighted as sticks. In brackets is the corresponding residue using the caspase-1 numbering.

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Figure 5  The active site of caspase-6

(A) Close-up of caspase-6 active site. Residues Arg64 (Arg166), His121 (His237), Gln161 (Gln283), Cys163 (Cys285), Trp217 (Trp338), His219 (Trp340) and Arg220 (Arg341) are shown as sticks. In brackets are the corresponding residues using the caspase-1 numbering system. Comparison of (B) ligand-free caspases-6 and -7 (PDB entry 1K86) active sites, with the (C) ligand-free caspase-6 and ligand-bound caspase-7 (PDB entry 1F1J) active sites. The same caspase-6 residues as in (A) and, in addition, the equivalent caspase-7 (yellow and green; labels in italics) are shown as sticks. In (D) the positional shifts of the Cα carbon atoms are indicated.

Figure 6  Pre-steady-state kinetics of substrate hydrolysis by caspase-6

(A) Progress curves recorded using 1, 5, 10 and 15 μM caspase-6 (traces from bottom to top) and 10 μM of the substrate Ac-DEVD-AMC. (B and C) First derivative of the progress curves recorded with 10 and 15 μM caspase-6 respectively. The solid lines represent the fit of the data to a hyperbolic function to illustrate the increase in velocity within the first 20 ms of the kinetic. The progress curves obtained with 1 and 5 μM caspase-6 showed a constant initial velocity.
and active conformations is very small and that, in solution, both the latent and the fully mature conformations are present. As the latency observed in the structure is not paralleled by latency in vitro, it seems unlikely, based on these results, that the latent conformation has evolved to regulate caspase activity. However, in vitro enzymology is an insufficient tool to predict intracellular proteolysis where macromolecular substrates are cleaved in a highly viscous environment. A better understanding of the significance of caspase latency would require a more detailed kinetic analysis of caspase-6 activity (or caspases in general), and more knowledge about the physiological substrates and their intracellular concentrations.

Conclusions

HD is an autosomal dominant disorder that is characterized by the progressive death of the GABAergic striatal medium spiny neurons. HD results from the expansion of an N-terminal polyglutamine stretch of the Htt protein beyond a threshold of approx. 36 amino acids. Currently, there are no disease-modifying therapies available for HD. Furthermore, well-validated molecular targets, besides the Htt protein itself, are only emerging recently, as the lethal pathophysiological processes attributable to expansion of the polyglutamine tract remains unknown. Specifically, the neuronal growth factor FGF-2 (fibroblast growth factor-2) [37] and BDNF (brain-derived neurotrophic factor) [38] have been discussed as possible targets for the treatment of HD.

Studies of post-mortem brain tissue indicate that polyglutamine-expanded Htt is an in vivo proteolytic target. Analysis of the neuronal aggregates, characteristic of the HD pathophysiology, demonstrates that they contain a heterogeneous mixture of various N-terminal Htt fragments, and it is their formation that has been hypothesized to be the lethal pathophysiological process [39,40]. In vivo Htt appears to be proteolysed by caspases, calpains and aspartyl proteases [41–43]. However, the most striking observation is that the deletion of a putative ‘caspase-6’ cleavage site in a polyglutamine-expanded Htt transgene ameliorates the otherwise observed motor behavioural phenotype and shortened lifespan of mice harbouring the transgene [11]. These observations are consistent with the ‘toxic fragment hypothesis’ – that the pathophysiology of HD results from the proteolytic processing of mutant Htt [44]. The toxic fragment hypothesis does not specify the mechanism whereby the N-terminal Htt fragment is toxic.

Thus caspase-6 is a potential molecular target for HD drug discovery in particular, and anti-neurodegenerative drug discovery in general, given the observation that it plays an effector role in APP-mediated DR6 signalling. The structure presented in this paper will further the understanding of caspase-6 function and might accelerate the development of disease-modifying therapeutics. In particular, the structural data may enable the development of non-peptidic active site inhibitors, or allosteric non-active site inhibitors [35,36], which need to be bio-available across the blood–brain barrier and capable of slowing these devastating diseases. The description of a novel, latent caspase conformation shows that especially the latter strategy is possible in principle, if ligands that bind to and stabilize the latent conformation can be identified.

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REFERENCES

SUPPLEMENTARY ONLINE DATA

The crystal structure of caspase-6, a selective effector of axonal degeneration

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1 To whom correspondence should be addressed (email martin.renatus@novartis.com). The structural co-ordinates reported will appear in the Protein Data Bank under entry code 2wdp.
Table S1  Substrate cleavage by caspase-6 under initial velocity conditions

The exponential fit is calculated by fitting the kinetic data to eqn (3) (see main paper), \([S]_0 = 1\) and 5 \(\mu\)M, \(|E| = 100\) or 10 \(\mu\)M. See the Experimental section in the main paper for more details. Substrates 9, 10 and 11 are based on the optimal substrate VEHD (according to \([13]\)) but contain suboptimal residues (P2, P3 and P4 respectively). Mean values are of at least two independent measurements. SEM, standard deviation of the mean. nd, not determined.

\(^a\)The experimental conditions \((|S|_0 \ll 0.1 \times K_m)\) required for the use of eqn (3) cannot be satisfied.

\(^b\)The substrate turnover is too low under experimental conditions to allow use of eqn (3).

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Figure S1  LC-MS analysis of active two-chain caspase-6

(A) The protein was purified using the optimized three-step purification protocol. The LC trace shows that large and small subunits are present in equivalent amounts. The fraction corresponding to the large subunit contains one polypeptide, the measured mass matches the theoretical mass of the caspase-6 residues 24–179. The mass of the small subunit matches the polypeptide 194–293. The numbering refers to full-length caspase-6 (including the pro-peptide). (B) The protein was purified using a two-step protocol omitting the hydrophobic interaction chromatography step. The LC trace shows that large and small subunits are present in equivalent amounts. The fraction corresponding to the large subunit contains two masses which match polypeptide chains 24–179 and 24–193, whereas the small subunit consists of only one species matching the polypeptide 194–293. The numbering refers to full-length caspase-6 (including the pro-peptide). (C) LC-MS analysis of same protein batch as in (B) after incubation with the covalent irreversible binding inhibitor Ac-LEHD-CMK. The active site Cys163 is located in the large subunit. Both the 24–193 and the 24–179 species have reacted with the peptidic inhibitor, as indicated by the mass-shift of approx. + 550 Da. This mass shift corresponds to the mass of Ac-LEHD-CMK excluding the mass of one molar equivalent of chloride ions. The fact that the double-processed (at residues 179 and 193), as well as the single-processed (at residue 193) caspase-6 molecules can react with an irreversible inhibitor is a first hint that cleavage at position 193 is sufficient for caspase-6 activation. However, our results cannot quantify the relative activities of the two species, as the elucidation of caspase-6 activation is beyond the scope of the present study.
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Figure S2  Substrate hydrolysis by caspase-6 under initial velocity conditions

(A) Hydrolysis of substrates Ac-DEVD-AMC, Ac-VEID-AMC, Ac-TEVD-AMC and Ac-DVVD-AMC. (B) Hydrolysis of substrates Ac-IVLD-AMC, Ac-DLND-AMC, Ac-VEQD-AMC, Ac-VLHD-AMC and Ac-WEHD-AMC. Substrate turnover was measured at 23°C. The assay buffer was 200 mM Hepes, pH 7.4, containing 1 mM EDTA, 20 mM DTT and 0.05 % CHAPS. The Michaelis constant, \( K_m \), was obtained from measurements conducted using at least two different enzyme concentrations and various substrate concentrations up to 200 \( \mu \text{M} \). The solid lines are fits using eqn (1) (see main paper). Each point represents an average of four replicates. For each substrate one representative curve is shown.

Figure S3  Analytical size-exclusion chromatography of caspase-6

Samples (100 \( \mu \text{l} \) at 1 mg/ml) were applied onto a Superdex 75 HR 10/30 column in 50 mM sodium acetate buffer, pH 5.5, containing 50 mM NaCl. Caspase-6-C163A zymogen (-----) elutes at 11.6 ml, the caspase-6–Ac-LEHD-CMK complex (solid line) at 12.7 ml and the active ligand-free caspase-6 (- - - - -) at 12.8 ml. The inset shows the calibration curve of the column. Molecular mass of standard proteins (of 158 kDa, 44 kDa, 17 kDa and 1.350 kDa) in kDa (log units) was plotted against the retention volume. Using the function of the regression curve, the estimated molecular masses are 59.4 kDa, 34.5 kDa and 32.8 kDa respectively.

Figure S4  Preparative size-exclusion chromatography

Approx. 30 mg of protein in a volume of 5 ml (buffer as eluted from a phenyl-Sephrose column) were loaded onto Superdex 75pg HL 16/60. The activity of each 5 ml peak fraction was tested using Ac-DEVD-AFC as a substrate in assay buffer consisting of 200 mM Hepes, pH 7.4, containing 1 mM EDTA, 20 mM DTT and 0.05 % CHAPS.
**Figure S5  Sedimentation velocity in analytical ultracentrifugation experiments**

Sedimentation velocity of caspase-6-C163A zymogen and active caspase-6 two-chain at different concentrations and two different pH values. See the main paper for experimental details.