mosquito® crystal
application note

crystal structure determination of a key processing enzyme in Huntington's disease

Huntingdon's disease

Huntington's disease (HD) is a neurodegenerative disorder caused by mutation of a single gene. The mutation is dominantly inherited with the disease affecting successive generations of afflicted families. HD has its onset usually in midlife, starting with a motor disorder such as chorea or the inability to sustain voluntary movements. The disease progresses with behavioural and cognitive symptoms, often manifested as delusional behaviour, poor concentration, apathy, depression, dementia, and ultimately premature death. Although to date there is no cure to the disease, discovery of the mutant huntingtin gene (mHtt) in 1993 has enabled significant progress to the understanding of the underlying disease mechanism. Studies aimed at developing a therapy are currently underway.

molecular basis of HD

The huntingtin protein is a widely expressed cytoplasmic protein, important for healthy neuronal function. In HD, a mutant of the huntingtin gene (mHtt) introduces polyglutamine (polyQ) expansions in the huntingtin protein. The production of mHtt and release of cleavage products or polyQ protein fragments have been demonstrated to be the cause of neuronal dysfunction and degeneration. Furthermore, the aggregation rate of the mutant huntingtin protein increases as the length of the polyQ region increases and is directly correlated to the time of disease onset [1]. A number of proteolytic enzymes, such as the protease caspases, have been identified that generate polyQ containing fragments.

BioFocus
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It is speculated that cleavage by the cysteine protease caspase-6, which is implicated in neuronal survival and apoptosis, is also linked to the generation of fragments that show neuronal toxicity [2]. It has therefore been postulated that inhibition of caspase-6 activity may prove to be neuroprotective in HD.

structure of caspase-6

Caspases are a family of cysteine proteases involved in apoptotic and inflammatory processes. Over 100 caspase structures have been reported in the Protein Data Bank since the mid-1990s, however, the structure of caspase-6 has only recently been determined [3]. The first crystal structure described the apo-enzyme in a non-canonical caspase conformation where the protein was in an inactivated state.

For the design of caspase-6-specific inhibitors, however, availability of crystal structures of the enzyme in its active conformation, as well as in the inhibitor-bound state, would be a huge advantage. In order to obtain such crystal structures, the CHDI Foundation, Inc. (Los Angeles, USA), a biomedical research foundation devoted to discovering Huntington's disease-modifying therapies, initiated a collaboration with BioFocus, a drug discovery partner, to crystallise the protein in a state amenable to drug design.

key benefits

- **reproducibility**
- **high accuracy of drop position and overlay**
- **screen lots of crystallisation space with minimal sample consumption**
- **fast, efficient hanging drop crystallisation setup**
- **optimisation screening with mosquito**

This application note describes the use of mosquito® Crystal to screen for and optimise crystallisation conditions to obtain the structure of caspase-6 in its active state and a caspase-6 inhibitor complex, consisting of caspase-6 and the non-specific and irreversible covalent caspase inhibitor, Z-VAD-FMK. The reproducibility and accuracy of mosquito Crystal during primary screen set-up and optimisation studies were crucial to obtaining well-diffracting crystals from a limited amount of sample in a time-efficient way.

methods: crystallisation screening & optimisation

crytallisation of apo-caspase-6

Purified apo-caspase-6 (8 mg/mL) in 20 mM sodium acetate pH 5.5, 50 mM NaCl and 0.5 mM tris(hydroxypropyl)phosphine was used for crystallisation screening with mosquito Crystal.

A study of the conditions required for crystallisation of apo-caspase-6 was carried out using extensive factorial screens covering a wide pH range. mosquito Crystal was employed to dispense 100 µL reservoir solution and 200 nL hanging drops (1:1 ratio) in 96-well format.

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This study identified several conditions having an acidic pH in common which yielded the previously described non-canonical crystal conformation [3] (Table 1a). In addition, factorial screens identified spherulites in the presence of 4 M sodium nitrate and 0.1 M Tris at pH 8.5 (Table 1b). Small crystals appeared when precipitant concentration and pH were adjusted to 3.8 M sodium nitrate and pH 7.4 respectively.

optimisation of apo-caspase-6 crystal formation

mosquito Crystal was also used to perform an additive screen, and 0.5% ethylene acetate was shown to improve crystal morphology. Addition of 5 mM tris(hydroxypropyl)phosphine, in combination with streak microseeding, yielded crystals of apo-caspase-6 suitable for data collection. Crystals grown in the presence of 3.3 M sodium nitrate, 0.1 M Tris pH 7.4, 0.5% ethyl acetate and 5 mM tris(hydroxypropyl) phosphine were cryo-protected by overlaying the crystallisation drop with 20% ethylene glycol, 3.5 M sodium nitrate and 0.1 M Tris pH 7.4 before flash-cooling in liquid nitrogen. A dataset was collected to a maximum resolution of 2.5 Å [4].

crystallisation of caspase-6 in complex with Z-VAD-FMK

Purified apo-caspase-6 was buffer-exchanged into 20 mM Tris pH 8.0, 50 mM NaCl and incubated with 100 µM inhibitory peptide Z-VAD-FMK, as described by Müller et al. [4]. The sample was concentrated to 11 mg/mL of protein and factorial crystallisation screens over a wide pH range were set up using mosquito Crystal in 96-well hanging drop format overlaying 200 nL reservoir solution on 200 nL protein.

Attempts to use near neutral pH crystallisation conditions which were successful for another caspase-6/inhibitory peptide complex Ac-VEID-CHO, PDB ID 3p4u) did not yield any crystals, even with cross-seeding (data not shown). However, small crystals or crystalline precipitate of the caspase-6 Z-VAD-FMK complex were observed exclusively at low pH, and larger, stacked plate-like crystals formed after 5 days in the presence of 10% v/v 2-propanol, 100 mM phosphate-citrate pH 4.2 and 200 mM lithium sulphate (Table 2).

optimisation of apo-caspase-6 Z-VAD-FMK complex crystal formation

Optimisation trials studying the variation around this hit condition, performed in 24-well plates using glass cover slides, returned badly stacked crystals. However, after switching to a 48-well format using the TTP Labtech 48-well sheets for set-up, the formation of single crystals was observed. Further optimisation involved crystal growth in 0.1 M lithium sulfate, 0.1 M sodium citrate pH 4.3 and 11% v/v 2-propanol at a protein concentration of 5.4 mg/mL in combination with streak microseeding and yielded crystals suitable for data collection (Table 2). Crystals were harvested by transfer into reservoir solution supplemented with 60% 2-propanol in a 70% 2-propanol atmosphere and flash-cooling in liquid nitrogen. Data was collected on beamline I04 at the diamond light source to 2.65 Å resolution [5].

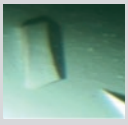
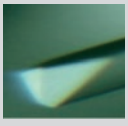
Protein, crystal form, diffraction and protein conformation	(a) apo-caspase-6 Acidic pH form 2.6 Å max. resolution non-canonical conformation (inactivated state)	(b) apo-caspase-6 physiological pH form 2.5 Å max. resolution canonical caspase conformation (active state)
Initial 96-well faxtorial screens	SaltRx 100+100 nL drops: Crystals in several conditions around pH 4.6	SaltRx screen 100+ 100 nL drops: Spherulites in the presence of sodium nitrate at pH 8.5
1st round of optimisation	24-well hanging drop format, 1+1 µL drops microseeding 	Optimisation in 96-well hanging drop format, 100+100 nL drops (in the presence of 3.8 M sodium nitrate, pH 7.4) 
2nd round of optimisation		Additive screen in 96-well format 100+100 nL drops 
3rd round of optimisation		24-well hanging drop set-up for optimsation by microseeding 

Table 1. Conditions for crystallisation of apo-caspase-6 in (a) acidic pH conditions and (b) at physiological pH. Hits were optimised by further screening and microseeding to produce crystals suitable for data collection as described by Müller et al. [5].

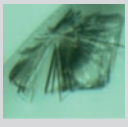
Protein, crystal form, diffraction and protein conformation	caspase-6 in complex with Z-VAD-FMK 2.7 Å max. resolution non-canonical conformation with inhibitor peptide bound
Initial 96-well faxtorial screens	ProPlex screen, Wizard screen I and II 100+100 nL drops: Crystals and crystalline precipitate in several conditions around pH 4.6 Stacked plates in the presence of 2-propanol and lithium sulphate at pH 4.2 
1st round of optimisation	Optimisation in 24-well hanging drop format, using glass cover slides No single crystals
2nd round of optimisation	Optimisation in 48-well hanging drop format, using TTP Labtech's 48-well sheets 
3rd round of optimisation	Optimisation in 48-well hanging drop format, using TTP Labtech's 48-well sheets and microseeding 

Table 2. Factorial screening revealed the formation of crystals and precipitates of caspase-6 in complex with the irreversible caspase inhibitor Z-VAD-FMK at low pH. Hits were optimised by further screening and microseeding to produce crystals suitable for data collection as described by Müller et al. [4].

results

structure of apo-caspase-6

The structure of apo-caspase-6 was solved by molecular replacement using the model of caspase-6 crystallised in the low pH crystal form (PDB-ID 2wdp). The low pH form of caspase-6 had been shown to adopt a non-canonical conformation. In contrast, inspection of the initial electron density maps for the apo-caspase-6 crystals that had been optimised at physiological pH revealed that the enzyme had crystallised in the canonical conformation. An overlay of the two different apo-caspase-6 conformations is shown in Fig 1.

structure of caspase-6 Z-VAD-FMK complex

The structure was solved by molecular replacement, using both crystal forms of apo-caspase-6 as search models. The non-canonical, low pH-model gave significantly better scores. Subsequent structure refinement showed that although adopting the non-canonical conformation, caspase-6 was still capable of inhibitor binding with a number of flexible loops around the ligand binding site adopting a unique conformation. Interestingly, when compared with the crystal structure of the caspase-6 Ac-VEID-CHO complex, the binding modes of the peptide within the non-canonical versus the canonical conformation are mutually exclusive (Fig 1b).

discussion

The automation of protein crystallography screening has contributed significantly to the understanding of enzyme activity and the rapid progress of crystal-based structural drug design.

In this study, mosquito Crystal was successfully employed to facilitate extensive crystallographic screening and optimisation of two forms ofapo-caspase-6 and caspase-6 in complex with Z-VAD-FMK. This research highlights that the reproducibility and accuracy of mosquito Crystal during primary screen set up and optimisation studies were crucial in obtaining well-diffracting crystals from a limited amount of sample in a time-efficient way. With the ability of mosquito Crystal to set up screens in under two minutes per plate, and with its positive displacement tip technology ensuring zero cross-contamination between samples, BioFocus was able to set-up an extensive factorial screen less than an hour after protein purification. This helped to significantly speed up the time to results, reducing the risk of protein degradation during set-up and the need for protein storage between purification and crystallisation. With highly accurate drop positioning and overlay, mosquito Crystal offered a clear experimental advantage in crystal screening and optimisation studies.

The work described in this study reveals that apo-caspase-6 is capable of adopting the canonical caspase conformation when crystallised at physiological pH. Caspase-6 had been shown to be inactive at pH below 5 [6], and the first crystal structure of apo-caspase-6, in which the protein adopts a non-canonical conformation had indeed been obtained at low pH.

More recently, Vaidya *et. al.* [7] reported increased stability of the non-canonical form of apo-caspase-6, compared to the active, canonical form and suggested that, in solution, the enzyme may feature a latent conformation that undergoes a transition upon ligand binding. The crystal structure of caspase-6 in complex with Z-VAD FMK shown in this study, however, demonstrates that caspase-6 in the non-canonical conformation is still capable of peptide binding, and that ligand binding is not sufficient to cause the transition of caspase-6 into the canonical caspase conformation (at least at acidic pH). Further studies into the relevance of the pH on the conformation of caspase-6 are anticipated to help understand the mechanisms that trigger the transition, and ultimately the significance of the non-canonical conformation for allosteric caspase-6 inhibitor design.

references

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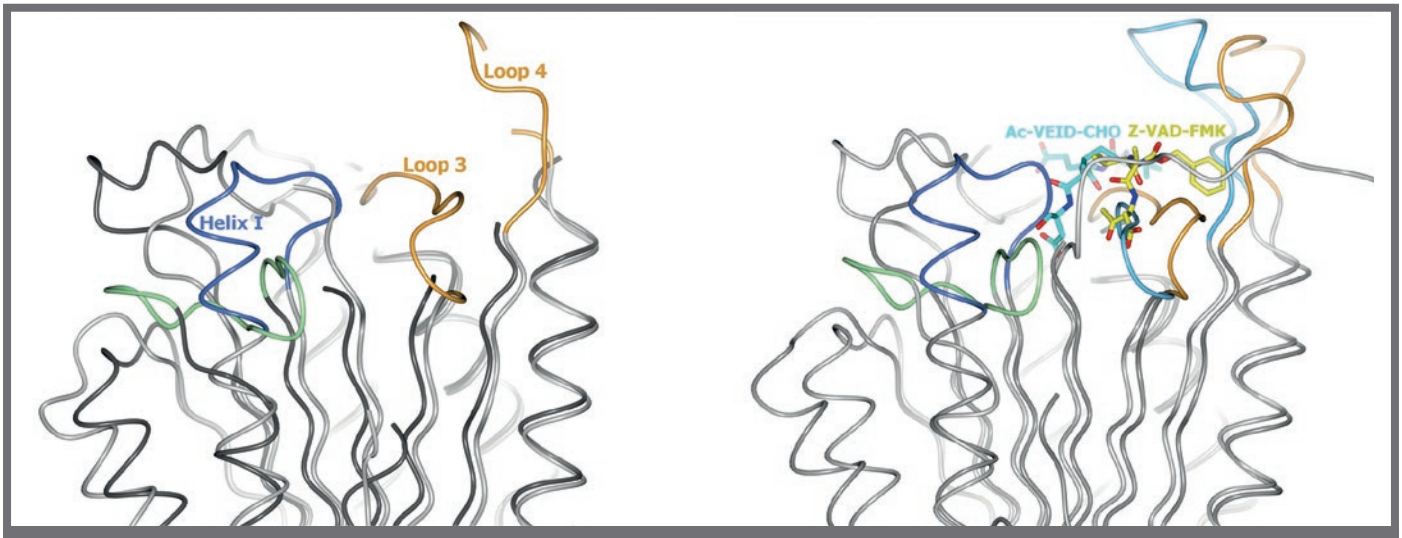

Fig 1. Overlay of the structure of:

(a). apo-caspase-6 in the canonical conformation obtained at physiological pH (dark grey) and in the non-canonical conformation crystallised at acidic pH (light grey). The loops L3 and L4 (orange) are disordered in the canonical form of the apo-enzyme. Residues 120-133 undergo conformational change at acidic pH, extending helix I by two turns (green/blue).

(b). caspase-6 in complex with Z-VAD-FMK, where the protein adopts the non-canonical conformation (peptide shown in yellow) with the crystal structure of the caspase-6/Ac-VEID-CHO complex, for which the protein is found in the canonical conformation (protein shown in light grey, loops highlighted in light blue and green, peptide shown in cyan). The binding modes of the peptides are mutually exclusive between caspase-6 in the canonical and non-canonical conformation.

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specifications

TTP Labtech's mosquito® crystal is a bench-top liquid handler which has been integrated with a wide array of other laboratory instrumentation, ranging from simple stacking robots through to complete plate preparation solutions, to achieve walk-away operation in many application areas.

Dispense range:	25 nL – 1200 nL
Plate capacity:	2 or 5
Plate format:	All SBS footprint plates and crystallisation plates
Min accessible volume:	10 nL
Dimensions:	390 mm x 470 mm x 690 mm (15.5" x 18.5" x 27") (w x d x h)
Weight:	27 kg (59 lbs)
Services:	110 V/220 V single phase 50.60 Hz
Noise:	64dBA peak noise during operation

designed for discovery

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