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A rational approach towards successful crystallization and crystal treatment of human cytomegalovirus protease and its inhibitor complex

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The crystallization and subsequent crystal treatment of both free human cytomegalovirus (hCMV) protease and its inhibitor complexes are reported. For free-enzyme crystals, diffraction was greatly improved by optimizing the crystallization conditions, raising the precipitant concentration in the reservoir and soaking the crystals in artificial mother liquors. Each of the six components in the final crystallization formula (16% PEG 4000, 0.1 *M* MES pH 6.0, 0.4 *M* LiCl, 10% glycerol, 5% *t*-butanol and 5 m*M* $Na_2S_2O_3$) plays a distinctive role and is indispensable. A synergistic effect of Na_2SO_4 and *t*-butanol on diffraction was observed and studied. A 2.0 Å multi-wavelength anomalous diffraction (MAD) data set was collected using a synchrotron-radiation source, leading to the elucidation of the three-dimensional structure of the enzyme. For the inhibitor-complex crystals, initial attempts with co-crystallization and soaking experiments at pH 6.0 did not produce conclusive results. Subsequently, experiments were designed to co-crystallize the complex at pH 8.0, the optimal pH for the enzyme and the inhibitor activity. Using 20–50 m*M* spermine in the crystallization buffer, crystals of two peptidomimetic inhibitor complexes were obtained at pH 7.5 and 8.0. Spermine was required for the inhibitor complexes to be crystallized at pH 8.0, possibly neutralizing net negative charges of hCMV protease owing to its acidic pI of 5.5. A 2.7 Å data set was collected from one of the inhibitor complexes and the structure was determined using the molecular-replacement method.

1. Introduction

Human cytomegalovirus (hCMV) is a member of the herpesvirus family that causes severe infection in immunocompromized individuals such as AIDS patients, organ transplant recipients and in newborns (Fields *et al.*, 1996). Herpesviruses encode a protease that cleaves the assembly protein precursor at a C-terminal site (called the maturation site) to release the mature assembly protein, a B-capsid protein. For a temperature-sensitive mutant of herpes simplex virus type 1, whose assembly protein precursor was not processed to its mature form, the progeny viral DNA was not packaged into virions, resulting in empty and non-infectious nucleocapsids. Therefore, the herpesvirus protease activity is essential for the successful packaging of infectious viruses (Gibson *et al.*, 1995). hCMV protease, similar in function to other herpesvirus proteases, is a promising target for anti-hCMV intervention. Crystal structures of hCMV protease and its inhibitor complexes should help in the design of better inhibitors.

The hCMV protease recognizes and cleaves the conserved sequence Ala-Ser/Ala. The enzyme is a single-chain protein consisting of 256 amino-acid residues, having two internal autocleavage sites at Ala143 and Ala209. Site-directed mutagenesis has shown that the conserved Ser132 is essential for enzymatic activity, indicating that the enzyme is a serine protease (Gibson *et al.*, 1995). The three-dimensional structure of hCMV protease has demonstrated that this enzyme has both a unique fold and a catalytic triad which is different from all other serine proteases (Tong *et al.*, 1996; Qiu *et al.*, 1996; Shieh *et al.*, 1996; Chen *et al.*, 1996). Compared with other known serine proteases, this enzyme is extremely slow, with a K_{cat}/K_m value of $0.027\ \mu M^{-1}\ min^{-1}$. Although a serine protease, it is readily inhibited by divalent zinc ions and very high concentrations of serine-protease inhibitors (Burck *et al.*, 1994). Through unknown mechanisms, both glycerol and Na_2SO_4 increase its activity, while organic solvent such as DMSO and *t*-butanol decrease its activity (Hall & Darke, 1995; Yamanaka *et al.*, 1995). A dimer is the active form of the enzyme (Darke *et al.*, 1996).

A series of peptidomimetic inhibitors were developed based on the substrate preference of hCMV protease (Ogilvie *et al.*, 1997). These inhibitors cover the P4 to P1′ residues of the cleavage site for the natural substrate of the enzyme. SAR results have shown that the side chains of the P4 and P3 positions prefer *t*-butyl groups; *N,N*-disubstituted asparagine is the best at the P2 position; the P1 position remains alanine.

We have recently solved the crystal structure of the inhibitor complex of the enzyme (Tong *et al.*, 1998). Here, we report (i) the development of a unique formula essential for both the crystallization of hCMV protease and the subsequent treatment of the crystals in order to improve their diffraction, (ii) a synergistic effect of Na_2SO_4 and *t*-butanol on the crystal diffraction and (iii) the crystallization of two hCMV protease complexes with the peptidomimetic inhibitors BILC 408 (C408) and BILC 821 (C821) at pH 8.0 and 7.5, respectively, which are the optimum pH conditions for hCMV protease activity (Burck *et al.*, 1994).

2. Materials and methods

The cloning, expression and purification of HCMV protease were performed as described previously (Bonneau *et al.*, 1997). All protein samples used in these studies contain the Ala143Gln mutation that eliminates one of the internal cleavage sites of the protease (Gibson *et al.*, 1995) and free-enzyme crystals were originally grown using this sample. Two additional mutations, Leu229Met and Thr181Met, were introduced in order to use the multiwavelength anomalous diffraction (MAD) method (Hendrickson, 1991) to obtain phase information; free-enzyme crystals were later obtained using a sample containing Se-Met with the triple mutation. The inhibitor complex was crystallized using samples with the triple mutation and regular methionine residues. The storage buffer was 20 m*M* acetate pH 5.0, 50 m*M* NaCl, 1 m*M* DTT, 0.1 m*M* EDTA. Isoelectric focusing experiments using both mini-gels and a column (Pharmacia Biotech) were carried out to characterize the enzyme preparation. The purity of the samples used for crystallization trials was estimated to be 98% or more by densitometry of Coomassie Blue stained SDS–PAGE gels. The concentration of the enzyme was estimated using the Bradford method or spectrophotometrically at A_{280}. Most crystallization experiments were conducted at room temperature using the hanging-drop technique (MacPherson, 1982); the microseeding technique (consecutive streaking of four drops) was used to obtain usable crystals for both the free enzyme and the inhibitor complex. Diffraction intensity data were measured at cryo-temperatures using an R-AXIS II imaging system mounted on a Rigaku X-ray generator or an image-plate/MAR detector at Brookhaven National Laboratory. The data were processed and scaled using the *DENZO*/*SCALEPACK* package (Otwinowski, 1993).

3. Results and discussion

3.1. IEF analysis

The pI of the hCMV protease sample was measured using an isoelectric focusing (IEF) gel (Fig. 1). Depending upon the amount of protein loaded (0.25–1.0 g), one to three major bands were focused between pH 5.3 and 5.7, which is close to the calculated value of 5.7 (from the *GCG* program). The nature of these multi-bands might be related to dimerization of the enzyme (discussed below). We tried to separate the bands using an isoelectric focusing column (Mono P, 5 ml) with pH gradient 5.65–4.90. Two peaks of equal intensity were eluted (monitored at A_{280}) when 100 µg of the sample was loaded on the column. The concentration of hCMV protease from either peak (about 1 ml for each) was about 4 µ*M*, close to its K_d (6.6 µ*M*) for dimerization (Darke *et al.*, 1996). This, along with the following observations, led us to believe that the two peaks corresponded to monomer and dimer: (i) from

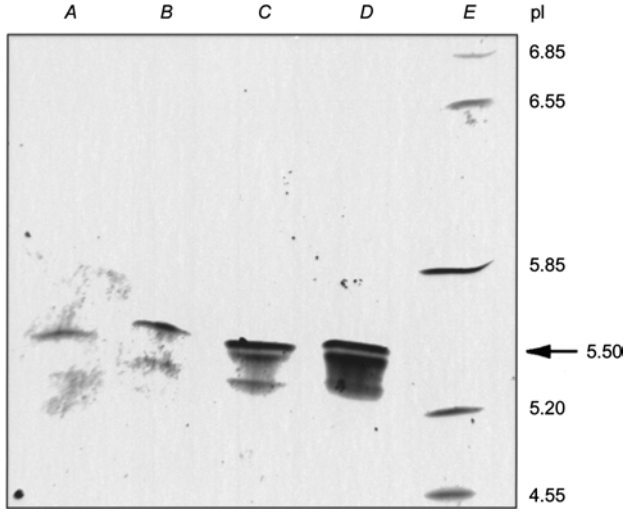

Figure 1
Result of isoelectric focusing of hCMV protease stained by Coomassie Brilliant Blue R-250. Lanes *A*, *B*, *C* and *D*, 0.25, 0.5, 1.0 and 2.0 µg of hCMV protease sample loaded, respectively; lane *E*, standard with pI indicated. The pI of hCMV protease is about 5.5.

the IEF column, the ratio between the intensity of the two peaks varied when different amounts of sample (50–200 µg) were loaded; (ii) the protein in both peaks had the same ladder of bands as the original sample; (iii) on the IEF gel, both the intensity and the number of bands varied when different amounts of protein sample were loaded (Fig. 1).

3.2. Crystallization of free enzyme

The result of the optimization process of crystallization is presented in Fig. 2. Initially, sparse-matrix crystallization screening (Jancarik & Kim, 1991) was conducted using commercial kits (Hampton Research) by mixing equal volumes (2 µl) of the protease sample (5.4 mg ml^{-1}) and reservoir solution. Small cubic crystals (0.01 × 0.01 × 0.01 mm) were obtained from a screening buffer composed of 25% *t*-butanol, 0.1 *M* Tris pH 8.5, 0.1 *M* $CaCl_2$, yet additional experiments designed to optimize this condition did not produce larger crystals. Small needle crystals (of dimensions 0.01 × 0.01 × 0.5 mm) were obtained from another screening buffer containing 20% PEG 4000, 0.1 *M* HEPES pH 7.5, 10% *i*-propanol (Fig. 2*a*) and four other similar screening buffers. Optimization of these conditions led to the 'three-component formula' of 16% PEG 4000, 0.1 *M* MES pH 6.0, 0.2 *M* LiCl. This formula, in conjunction with higher protein concentration (14 mg ml^{-1}), gave rise to crystals of dimensions 0.03 × 0.03 × 1.0 mm (Fig. 2*b*) and a tetragonal prismatic morphology.

Glycerol was evaluated in our crystallization trials since it is commonly used for protein crystallization and it is also known to increase the activity of hCMV protease. Crystals grew to dimensions of 0.05 × 0.05 × 1.0 mm using the three-component formula plus 10% glycerol ('four-component formula') and to 0.1 × 0.1 × 1.0 mm after the microseeding technique was used (Fig. 2*c*), which was essential to produce usable crystals in this work. These crystals diffracted to no better than 5 Å. In the hope of growing larger crystals, the four-component buffer was modified by including 5% *t*-butanol ('five-component formula'), as we had obtained small cubic crystals using *t*-butanol as precipitant. It was found that addition of 5% *t*-butanol improved both the appearance and the size of the crystals. These crystals diffracted to 4.0 Å horizontally (along the *a** or *b** axis) and 3.5 Å vertically (along the *c** axis) with

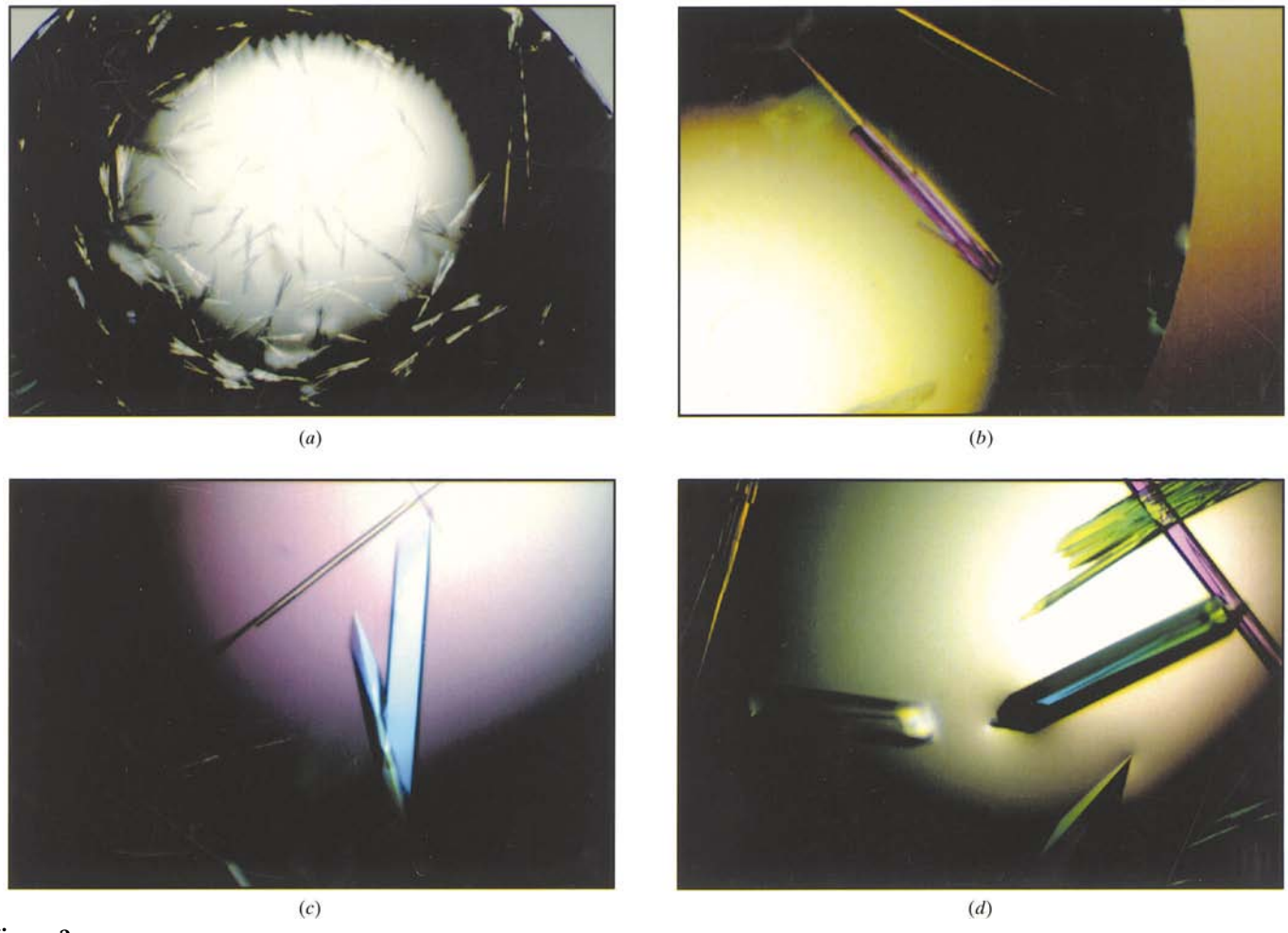

Figure 2
The progress of the crystallization of hCMV protease. (*a*) From sparse-matrix screening using buffer containing 20% PEG 4000, 0.1 *M* HEPES pH 7.5, 10% *i*-propanol; (*b*) from buffer containing 16% PEG 4000, 0.1 *M* MES pH 6.0, 0.4 *M* LiCl; (*c*) from buffer used in (*b*) with additional 10% glycerol; (*d*) from buffer used in (*c*) with 5% *t*-butanol.

Table 1
The effect of reservoir change and soaking on the diffraction of hCMV protease crystals.

	Five components	Six components/ reservoir change	Soaking in the first mother liquor	Soaking in the second mother liquor
Formula†	16% PEG 4000 0.4 *M* LiCl	16% PEG 4000 0.4 *M* LiCl 5 m*M* $Na_2S_2O_3$	30% PEG 4000 0.4 *M* LiCl 5 m*M* $Na_2S_2O_3$	30% PEG 4000 0.15 *M* Na_2SO_4 5 m*M* $Na_2S_2O_3$
Space group	Orthorhombic	Tetragonal	Tetragonal	Tetragonal
Unit-cell parameters (Å)	a = 71.2, b = 73.2, c = 216.0	a = 73.5, c = 216.0	a = 68.5, c = 211.5	a = 71.0, c = 208.8
Resolution (Å)	3.5	3.3	3.0	2.3

† All formulae contained the following additional components: 0.1 *M* MES pH 6.0, 10% glycerol, 5% *t*-butanol.

high mosaicity and split spots, which confused the autoindexing routines and led to the indexing of an orthorhombic unit cell with unit-cell parameters $a = 71.2$, $b = 73.2$, $c = 216.0$ Å.

3.3. Treatment of free enzyme crystals

Various treatments for improving crystal diffraction are shown in Table 1. The limited diffraction of the crystals (Table 1, column 2) and the split spots indicate molecular-packing disorder(s) inside the crystals. Therefore, we tried to dehydrate the crystals in order to induce tighter packing and improve diffraction. The reservoir buffer with the five-component formula was replaced by 24% PEG 4000, 10% glycerol and 5 m*M* DTT 2 d after setup and replaced again by 30% PEG 4000, 10% glycerol and 5 m*M* DTT after a further 2 d. The addition of 5 m*M* DTT was intended to protect the free cysteines in the enzyme during the reservoir change. Under those conditions, crystals now grew to dimensions of $0.12 \times 0.12 \times 1.0$ mm in one week. However, crystals became stuck to the thick film formed at the surface of the drop during this treatment and became unusable. This problem was solved by replacing 5 m*M* DTT with 5 m*M* $Na_2S_2O_3$. The 'six-component formula' (five components plus 5 m*M* $Na_2S_2O_3$) was finalized to accommodate the requirements for both crystallization of the enzyme and reservoir change. Crystals grown using the six-component buffer diffracted to 3.7 Å horizontally and 3.3 Å vertically after the reservoir change (Fig. 2*d*) and belong to tetragonal space group $P422$, with unit-cell parameters $a = b = 73.5$, $c = 216.4$ Å. More importantly, the mosaic spread was reduced and there were no split diffraction maxima. These crystals were further dehydrated by soaking in the six-component formula with 30% PEG 4000 (called LiCl mother liquor). The soaked crystals shrank by 10% in volume and diffracted to 3.2 Å horizontally and 2.9 Å vertically. This improvement in diffraction took place in 5 h and remained stable for as long as 10 d.

The presence of Na_2SO_4 at a concentration of 0.5 *M* or greater was reported to enhance the activity of herpes protease (Hall & Darke, 1995; Yamanaka *et al.*, 1995). It led us to speculate that this activation may result from a conformational change of the enzyme. An Na_2SO_4 mother liquor was therefore concocted using the formula of the LiCl mother liquor but replacing 0.4 *M* LiCl with 0.15 *M* Na_2SO_4 (equivalent in terms of ionic strength). Experimentally, crystals soaked in LiCl mother liquor for 5 h or longer were transferred into Na_2SO_4 mother liquor. The crystals soaked with Na_2SO_4 diffracted to 2.3 Å isotropically and to 2.0 Å using a synchrotron-radiation beam at Brookhaven National Laboratory. This improvement of diffraction, however, took 2 d to emerge and began to disappear rapidly after 3 d.

The synergistic effect of *t*-butanol and Na_2SO_4 on the diffraction was observed and investigated. Crystals soaked in the LiCl mother liquor diffracted to 3.0 Å with low mosaicity, but only to 3.5 Å with much higher mosaicity if *t*-butanol was omitted or added at a concentration of 1%; diffraction disappeared if the crystals were soaked with 10% *t*-butanol. Crystals soaked in the Na_2SO_4 mother liquor diffracted to 2.3 Å, but to no better than 4.0 Å if soaked in the Na_2SO_4 mother liquor without *t*-butanol. Sodium sulfate (Na_2SO_4) was as effective at 20, 50 and 100 m*M* as at 150 m*M*, but higher Na_2SO_4 concentrations could not be tested for soaking because of its solubility in the formula used. Crystals did not diffract if soaked without any salt (either LiCl or Na_2SO_4). It appears that both *t*-butanol (at the right concentration) and Na_2SO_4 are needed for the significant improvement of diffraction by soaking in the Na_2SO_4 artificial mother liquor.

3.4. Crystallization of the inhibitor complex at pH 8.0

At pH 6.0, numerous crystals obtained by soaking or co-crystallization with various inhibitors did not yield structures of the inhibitor complexes. We then tried to crystallize the inhibitor complexes at pH 8.0, since hCMV protease is most active at pH 8.0 but has little activity at pH 6.0 (Burck *et al.*, 1994). The protease is expected to be negatively charged at pH 8.0 owing to its acidic pI; the number of charges calculated by the program *GCG* is +3, −1 and −7 for the enzyme at pH 5, 6 and 8, respectively. Spermine was therefore tested in the crystallization buffer to neutralize the negative charges. This led to good quality crystals with C408 in 24% PEG 4000, 0.1 *M* Tris pH 8.0, 0.2 *M* NaCl, 10% glycerol, 20 m*M* spermine tetrachloride and 5 m*M* $Na_2S_2O_3$, with protease and inhibitor concentrations of 0.25 m*M* (7 mg ml^{-1}) and 2 m*M*, respectively. The crystals, with dimensions $0.02 \times 0.15 \times 0.7$ mm, were orthorhombic, with space group $P2_122_1$ and unit-cell parameters $a = 107.3$, $b = 52.8$, $c = 213.4$, and diffracted weakly to 3.0 Å when mounted directly from the drop. C408 is the most potent of the inhibitors used, with an IC_{50} value of 50 n*M*; inhibitor C821 (Fig. 3) is similar to C408. The presence of a *para*-iodo phenyl group in C821 allowed its binding to the enzyme to be investigated by crystallography. Crystals of the C821 complex of dimensions $0.04 \times 0.12 \times 0.8$ mm were obtained in a buffer composed of 18% PEG 4000, 0.1 *M*

Table 2
Effect of treatments on the crystal quality of hCMV protease complexed with C821.

	After reservoir change	After soaking
Unit-cell parameters (Å)	a = 109.9, b = 53.8, c = 213.4	a = 107.8, b = 53.4, c = 212.5
Resolution (Å)	3.0	2.7
R_{merge} (%)	7.4	7.8
Completeness (%)	88	96
Overall average intensity (last shell) $[I/s/(\chi^2)^{1/2}]$†	7.3 (2.34)	14.1 (3.67)

† Average intensity calculated in this way is independent of the χ^2 value.

HEPES pH 7.5, 0.2 *M* NaCl, 10% glycerol, 50 m*M* spermine tetrachloride, 5 m*M* $Na_2S_2O_3$. Without spermine, we were unable to obtain crystals suitable for diffraction studies.

The crystals of the C821 complex, however, did not diffract very well. In order to increase their diffraction power, we treated them in the same way as the free-enzyme crystals: reservoir change and soaking. The crystals of the C821 complex belong to an orthorhombic space group, with unit-cell parameters a = 109.3, b = 53.6, c = 213.1 Å after reservoir change. The diffraction of the crystals was anisotropic: with the a axis along the X-ray beam, they diffracted to about 4.5 Å and most spots were smeared; with the b axis along the X-ray beam, they diffracted to about 3.0 Å and most spots were sharp; diffraction gradually improved when rotating from the a to the b axis. A 3.0 Å data set starting from the b axis (Table 2) was collected. We then soaked these crystals sequentially in two artificial mother liquors containing 30% PEG 4000, 0.1 *M* HEPES pH 7.5, 10% glycerol, 5% *t*-butanol, 50 m*M* spermine tetrachloride, 5 m*M* $Na_2S_2O_3$ and 5% protein/inhibitor sample plus either 0.2 *M* NaCl or 60 m*M* Na_2SO_4. We added 5% *t*-butanol to the artificial mother liquors to make use of the synergistic effect; the presence of inhibitor was expected to prevent its dissociation from the complex. The C821 complex crystals (Fig. 4), after soaking, diffracted to 2.7 Å with sharp spots or 3.0 Å with smeared spots when the b axis or the a axis was aligned with X-ray beam, respectively. We collected a 2.7 Å data set of C821 complex crystals (Table 2) and determined its crystal structure (Tong *et al.*, 1998); the result did not reveal any direct binding of spermine to the protease.

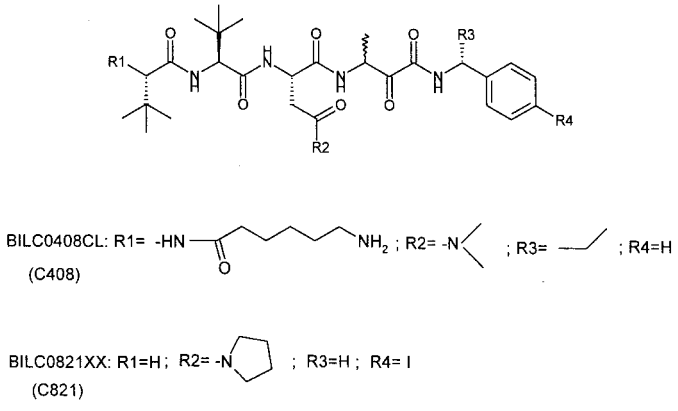

Figure 3
Chemical structure of the two peptidomimetic inhibitors of hCMV protease.

3.5. Comparison of crystallization conditions

Spermine is commonly used for crystallization of polynucleic acids (both DNAs and RNAs) and usually binds to DNA through charge–charge interactions, hydrophobic contacts and hydrogen bonds (Tari & Secco, 1995; Dautant *et al.*, 1995). It has also been used to improve the quality of crystals of proteins such as tropomyosin (Phillips *et al.*, 1987), tryptophan synthase $\alpha_2\beta_2$ complex (Ahmed *et al.*, 1985) and phosphorylase b (Oikonomakos *et al.*, 1985). In our case, however, it was used to obtain crystals otherwise difficult to produce at a desired pH.

Crystallization of hCMV protease at its optimal pH using solutions containing 28–33% polyethylene glycol monomethyl ether 550 (PEG MME 550), 50 m*M* HEPES pH 7.5, 50–200 m*M* NaCl and 10% glycerol has been reported (Shieh *et al.*, 1996). We have carried out complete factorial screening (Carter, 1990) at pH 6.5, 7.0, 7.5, 8.0 and PEG MME 550 concentrations of 45, 42, 39, 36, 30 and 24% using our sample in the presence of NaCl and glycerol. At these pHs, either with or without spermine, our sample precipitated in the drops within 2 d with 39–45% PEG MME 550 or stayed in solution for as long as a week with 24–36% PEG MME 550. Macroseeding the clear drops a week later did not produce any additional crystals. The different behavior of our sample and that of Shieh *et al.* (1996) during crystallization could be attributed to the different mutations introduced in the protease and/or the methods used for expression and purification.

4. Summary

The final crystallization formula with six components for native hCMV protease was developed through optimization and rationalization as follows: screening conditions → three component → four component → five component → six component.

The diffraction of the crystals was improved from 3.5 to 2.0 Å resolution by treatment of the crystals with a reservoir change and soaking in artificial mother liquors. The use of $Na_2S_2O_3$ rather than DTT made the reservoir change practical and it could be a useful alternate reducing agent for protein crystallization. Na_2SO_4 soaking removed anisotropy in the diffraction pattern. The presence of 5% *t*-butanol helps both crystallization and diffraction. The synergistic effect of Na_2SO_4 and *t*-butanol will promote the search for additives for crystallization and crystal treatment. This work has led to the successful determination of the crystal structure of hCMV protease at 2.0 Å (Tong *et al.*, 1996).

Although free hCMV protease was crystallized at pH 6.0, we failed to obtain crystals of inhibitor complex at this pH. At

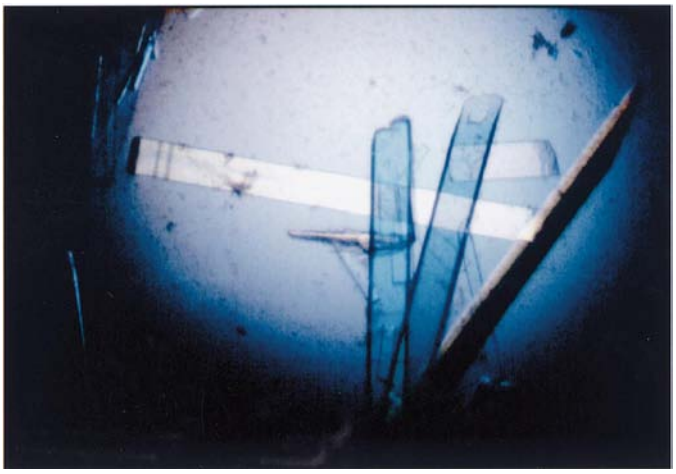

Figure 4
Crystals of hCMV protease complexed with peptidomimetic inhibitor C821 soaked in the first artificial mother liquor. These crystals grew as thin plates (0.04 × 0.12 × 0.8 mm) from buffer containing 18% PEG 4000, 0.1 *M* HEPES pH 7.5, 0.2 M NaCl, 10% glycerol, 50 m*M* spermine tetrachloride and 5 m*M* $Na_2S_2O_3$.

the optimal pH 8.0, the enzyme is negatively charged and very soluble owing to its acidic pI value of 5.5. We found that 20–50 m*M* spermine was required to crystallize the inhibitor complex of hCMV protease at its optimal pH. Crystals of two peptidomimetic inhibitor complexes of hCMV protease were obtained at a chosen pH and the structure of C821 complex were solved (Tong *et al.*, 1998) by the molecular-replacement method. The use of spermine for the crystallization of hCMV protease at a pH above its pI (presumably to neutralize net negative charges on protein surface) may prove generally applicable to other proteins.

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