Protein Science (1995), *4*:1658–1660. Cambridge University Press. Printed in the USA.

FOR THE RECORD

Crystallization and preliminary X-ray diffraction studies of the human adenovirus serotype 2 proteinase with peptide cofactor

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(RECEIVED April 25, 1995; ACCEPTED June 9, 1995)

Abstract: Recombinant human adenovirus serotype 2 proteinase (both native and selenomethionine-substituted) has been crystallized in the presence of the serotype 12, 11-residue peptide cofactor. The crystals (space group $P3_121$ or $P3_221$, one molecule per asymmetric unit, $a = b = 41.3$ Å, $c = 197.0$ Å) grew in solutions containing 20–40% 2-methyl-2,4-pentanediol (MPD), 0.1–0.2 M sodium citrate, and 0.1 M sodium HEPES, pH 5.0–7.5. Diffraction data (84% complete to 2.2 Å resolution with R_{merge} of 0.0335) have been measured from cryopreserved native enzyme crystals with the Argonne blue (1,024 × 1,024 pixel array) charge-coupled device detector at beamline X8C at the National Synchrotron Light Source (operated by Argonne National Laboratory's Structural Biology Center). Additionally, diffraction data from selenomethionine-substituted proteinase, 65% complete to 2.0 Å resolution with R_{merge} values ranging 0.05–0.07, have been collected at three X-ray energies at and near the selenium absorption edge. We have determined three of the six selenium sites and are initiating a structure solution by the method of multiwavelength anomalous diffraction phasing.

Keywords: cryocrystallography; crystallization; cysteine proteinase; proteinase/cofactor complex; selenomethionine; thiol proteinase; X-ray crystallography

Adenoviruses are non-enveloped, icosahedral, double-stranded DNA viruses responsible for a large variety of vertebrate diseases (Horwitz, 1985). Approximately 50 different serotypes of adenovirus are known to infect humans, causing ocular, gastrointestinal, and urinary diseases; significant epidemics of acute respiratory disease have been due to adenovirus (Ginsberg, 1984). Most adenovirus infections are self-limiting but infections in infants and immune-compromised individuals can be life-threatening. Virus capsids are assembled in the nucleus of the infected host cell during the course of infection. Late in an infection, the host cell intermediate filament proteins are cleaved, destroying the structural integrity of the host cell (Zhang & Schneider, 1994). Several viral precursor polypeptides must be cleaved, in a process known as maturation, for the virus to become infectious (Kräusslich & Wimmer, 1988; Webster et al., 1989). This proteolysis is not required for capsid assembly, however.

The enzyme responsible both for maturation and for proteolysis of host cell structural proteins is a virus-encoded 23-kDa cysteine endoproteinase (Weber & Tihanyi, 1994; Zhang & Schneider, 1994). Proteinases from 12 different adenovirus serotypes (infective in human, canine, murine, bovine, and avian hosts) have been sequenced. The amino acid sequences of these proteinases are highly conserved, with 17.6% identity and 54.6% similarity between those most distantly related, but they exhibit no homology with sequences of any other known proteinases (Rancourt et al., 1994). The proteinase is highly selective in its substrate recognition, binding to, and cleaving an elongated sequence of the form (M,L,I)XGX↓G or (M,L,I)XGG↓X (where X represents any residue) (Anderson, 1990; Dougherty & Semler, 1993; Webster et al., 1994). The enzyme from serotype 2 proteolytically processes six viral precursor polypeptides consistent with this sequence. To be enzymatically active, the proteinase must bind an 11-residue peptide cofactor that is cleaved by the proteinase from the C-terminus of the precursor to the hexon-associated protein, pVI, during maturation (Mangel et al., 1993; Webster et al., 1993). This peptide forms disulfide-linked dimers and it is proposed that it activates the proteinase via a thiol–disulfide bond interchange (Webster et al., 1993). Many proteinases are synthesized in an inactive form, but no other known proteinase requires a peptide cofactor for activity. Thus, the adenovirus proteinase represents a unique class of cysteine proteinases, and its molecular structure will be valuable for our understanding of its catalytic mechanism.

We chose to study the proteinase from adenovirus serotype 2 (Ad2), the most thoroughly characterized human adenovirus

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serotype. Its proteinase is 204 amino acid residues long (Anderson, 1990; Houde & Weber, 1990), including 8 cysteine residues. During synthesis of the proteinase, the N-terminal methionine is removed and the next residue, glycine, probably becomes acetylated (Anderson, 1990). In infected cells, the adenovirus proteinase is expressed in insufficient quantities for structural studies, but active recombinant proteinase, identical to the native enzyme except for the N-terminal acetyl group, has been expressed in *Escherichia coli* (Anderson, 1993). We report here the crystallization and preliminary X-ray diffraction studies of the Ad2 proteinase, bound to the peptide cofactor of serotype 12 (Ad12). The Ad2 and Ad12 peptide cofactors differ from each other by two amino acid substitutions (Ad2 peptide: GVQSLKRRRCF; Ad12 peptide: GVKSLKRRRCY) but are functionally interchangeable (Freimuth & Anderson, 1993). We chose the Ad12 peptide cofactor for these studies because it appears to yield a more active enzyme. The Ad2 proteinase is synthesized with seven methionine residues, but because the N-terminal methionine is cleaved in *E. coli*, our crystalline enzyme contains six methionines. We also report here the expression and purification of selenomethionine-substituted Ad2 proteinase and the crystallization and preliminary X-ray diffraction studies of this selenomethionine derivative with the Ad12 peptide cofactor.

Crystallization and X-ray diffraction results: Expression and purification of recombinant Ad2 proteinase were scaled up from a procedure described elsewhere (Anderson, 1993), with modifications for increasing the yield and purity. Briefly, *E. coli* BL21(DE3) cells harboring plasmid pT7AD23K8 were grown in TBY-rich medium to late-log phase, induced at 30 °C for 15–20 h, and lysed by freeze–thawing. The proteinase was purified from cell lysates to homogeneity in two steps: (1) ion-exchange chromatography and (2) metal (zinc) affinity chromatography. The proteinase was concentrated to 6 mg/mL in 10 mM Tris-HCl, pH 8, and 1 mM dithiothreitol (DTT).

Although we have not yet succeeded in crystallizing the Ad2 apo enzyme, we have crystallized Ad2 proteinase complexed with the Ad12 peptide cofactor by the hanging-drop vapor diffusion technique and the method of sparse matrix factorial sampling (Jancarik & Kim, 1991). Ad2 proteinase was mixed with a fourfold molar excess of lyophilized Ad12 peptide cofactor (the cofactor forms dimers in solution). Several screening conditions, both at room temperature and at 4 °C, yielded very small crystals. A single cluster of excellent crystals, good enough for diffraction analysis, grew from 40% 2-methyl-2,4-pentanediol (MPD), as the precipitating agent, in 0.2 M sodium citrate and 0.1 M sodium HEPES, pH 7.5. This solution had been incubated at 4 °C for 8 days and at room temperature for 7.5 months. Two crystals were retrieved from this cluster. Because the crystals grew from MPD (a cryosolvent), we could mount and freeze the crystals directly from the crystallization drop (Hope, 1988).

Diffraction data were measured with the Argonne blue (1,024 × 1,024 pixel array) charge-coupled device (CCD) detector (Strauss et al., 1990) at beamline X8C of the Brookhaven National Laboratory National Synchrotron Light Source, operated by the Argonne National Laboratory Structural Biology Center (Alkire et al., 1995). Data were collected, processed, scaled, and merged by programs MADNES (Messerschmidt & Pflugrath, 1987) and PROCOR (Kabsch, 1988). The crystal unit cell parameters are: $a = b = 41.3$ Å, $c = 197.0$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$. The data exhibit the systematic absence of all (00*l*) reflections except those with index $l = 3n$. The intensities exhibit the following relations: $I_{hkl} \neq I_{hk-l}$, $I_{hkl} \neq I_{khl}$, $I_{hk-l} = I_{khl}$. These observations are consistent with trigonal space group $P3_121$ or its enantiomorph, $P3_221$. Diffraction data from native crystals are 84% complete to 2.2 Å resolution, with an R_{merge} of 0.0335.

There can be only one molecule of the proteinase/peptide cofactor complex in each crystallographic asymmetric unit. The ratio (volume/protein molecular weight), V_m (Matthews, 1968), is 1.98 Å^3/Da in this crystal form, and the solvent content is approximately 38%, so there is insufficient room for more molecules in the unit cell. Although these values fall within the range expected for globular proteins, the solvent content of these crystals is lower than most.

Native crystals grew very slowly and have been difficult to reproduce. Efforts to increase the rate and reproducibility of crystallization have been unsuccessful. As a consequence, we have not yet succeeded in making heavy-atom derivatives of this crystal form. To circumvent this problem we have expressed, purified, and grown crystals of the selenomethionine-substituted proteinase in order to phase from the selenium atoms and solve the structure by the technique of multiwavelength anomalous diffraction (MAD) (Hendrickson et al., 1990).

Auxotrophic *E. coli* strain 834(DE3) cells were transformed with plasmid pT7AD23k8 containing the gene for the proteinase. Cells containing the constructs were grown in 2× M9-glucose minimal medium supplemented with $MgSO_4$, $FeSO_4$, amino acids (with selenomethionine in place of methionine), riboflavin, niacinimide, pyridoxine monohydrochloride, and thiamine (Leahy et al., 1994). Selenomethionine-substituted proteinase was purified to homogeneity by the protocol used for the native proteinase, with gel filtration through a Superdex 75 HR 10/30 (Pharmacia) FPLC column added as the final step. It was then concentrated to 6 mg/mL in 10 mM HEPES, pH 7.5, and 1 mM DTT, and stored at −80 °C. Successful incorporation of selenomethionine in the proteinase was confirmed by amino acid analysis. Unlike for the native proteinase, single crystals of selenomethionyl proteinase complexed with the peptide cofactor can be grown reproducibly within 2 weeks. We have observed that the quality of these crystals is very sensitive to the concentration of sodium citrate. Additionally, optimal crystallization conditions vary slightly for each batch of protein and vary with aging of the protein. Our best crystals grow at 4 °C, in 20–40% MPD and 0.10–0.15 M sodium citrate, pH 5.0–5.5.

We froze selenomethionyl proteinase/peptide cofactor complex crystals in liquid propane or liquid nitrogen directly from their growth droplets, in a cold room. Frozen crystals are stored in a liquid nitrogen storage dewar for later characterization and data collection at the synchrotron. These crystals exhibit a strong selenium fluorescence signal (Fig. 1A), are isomorphous with native crystals, and diffract well to at least 2.0 Å resolution. Diffraction data have been recorded at three wavelengths at and near the selenium absorption edge, using inverse beam geometry, with the Argonne blue CCD detector at beamline X8C of the National Synchrotron Light Source. For each of the three wavelengths, 65% of the complete 2.0-Å data have been measured with threefold redundancy; R_{merge} for each data set is in the range 0.05–0.07. We identified self-consistent Harker and cross-peaks in the anomalous difference Patterson map for three of the six selenium atoms (circular dots in Fig. 1B) and deter-

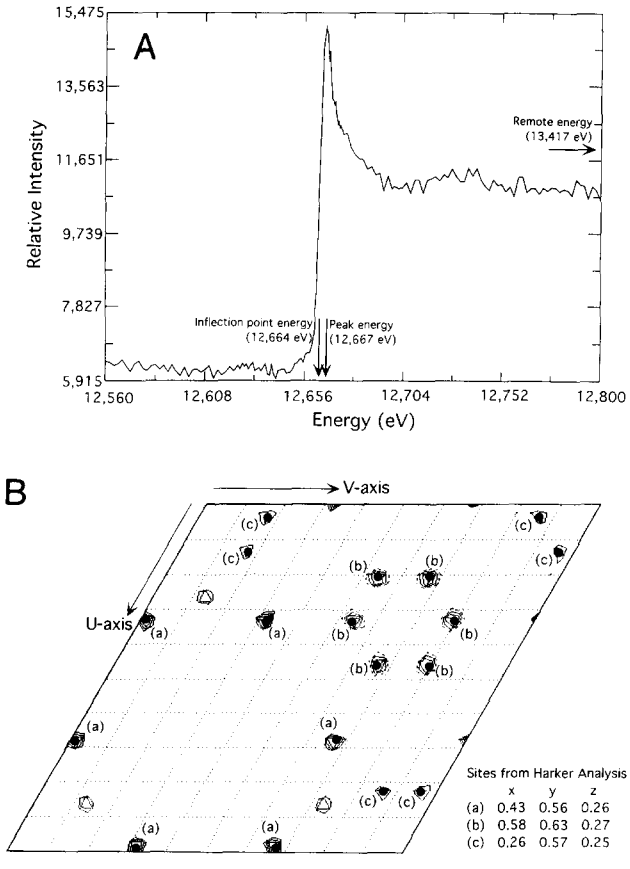

Fig. 1. A: Fluorescence spectrum from energy scan of crystals of selenomethionine-substituted Ad2 proteinase/Ad12 peptide cofactor. Diffraction data have been collected at the three indicated energies: 12,664.3 eV, 12,666.9 eV, and 13,416.9 eV. Nominal energy for selenium absorption edge is 12,658 eV ($\lambda = 0.980$ Å). **B:** Harker plane from difference anomalous Patterson map ($w = 1/3$), contoured at 1σ intervals starting at 1σ above the mean density. Map was calculated with all data lying between 6.0 Å and 2.3 Å resolution. Selenium difference anomalous Harker vectors from the three atoms initially interpreted from this Harker plane and the other Harker plane for this space group ($u = -v$) are indicated as solid circles.

mined that the space group is $P3_221$ (rather than its enantiomorph, $P3_121$). We then initiated heavy-atom isomorphous phase refinement with these Se sites, using program MLPHARE (Otwinowski, 1991). Examination of isomorphous difference and residual maps of the selenium derivative permitted us to find the remaining three selenium atoms. Single isomorphous replacement plus anomalous dispersion refinement yields a mean figure of merit of 65% for all data between 6 and 2.3 Å. The structure solution is now in progress.

Acknowledgment: This work is supported by the U.S. Department of Energy, Office of Health and Environmental Research, under contracts W-31-109-ENG-38 and DE-AC02-76CH00016. The Argonne National Laboratory Structural Biology Center at beamline X8C of the National Synchrotron Light Source is supported by USDOE contract W-31-109-ENG-38. L.J.K. is an Alexander Hollaender Distinguished Postdoctoral Fellow.

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