Communication

Crystallization of the Aspartylprotease from the Human Immunodeficiency Virus, HIV-1*

(Received for publication, November 16, 1988)

Brian M. McKeever*, Manuel A. Nava†, Paula M. D. Fitzgerald‡, James P. Springer*, Chih-Tai Leu*, Jill C. Heimbach†, Wayne K. Herbert*, Irving S. Sigal†, and Paul L. Darke*

From the *Department of Biophysical Chemistry, Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey 07065 and the ‡Department of Molecular Biology, Merck Sharp and Dohme Research Laboratories, West Point, Pennsylvania 19486

The aspartylprotease of the human immunodeficiency virus HIV-1 (NY5) has been crystallized in a form suitable for x-ray diffraction analysis. The crystals are tetragonal bipyramids and produce an x-ray diffraction pattern that exhibits the symmetry associated with space group P4_2_2_2 (or its enantiomorph, P4_2_2_2). The unit cell parameters are a = b = 50.3 Å, c = 106.8 Å, α = β = γ = 90°; measurable diffraction intensities are observed to a resolution of 2.5 Å. Density measurements indicate one molecule of 9,400 daltons/asymmetric unit. The symmetry of this space group could accommodate the proposed active dimer species of the protease if the 2-fold axis were coincident with one of the crystallographic 2-fold axes.

The human immunodeficiency virus, HIV-1,1 has been identified as the agent responsible for acquired immune deficiency syndrome, AIDS (1). As with other retroviruses, the HIV-1 genome exhibits a gag-pol-enc organization (2). The proteins encoded in these regions are initially translated as polyproteins that undergo posttranslational proteolytic cleavage to give the mature proteins needed to produce viable virus particles (3–7). In general, retroviruses encode within their genome a protease that is responsible for several of these cleavages. In the case of HIV-1, this protease has been identified as a 99-amino acid polypeptide that contains the triad Asp-Thr-Gly (8–12), a sequence that has been found to be characteristic of the active sites of aspartylproteases (13–15). Nine cleavage sites have been identified in the polyproteins of the three major open reading frames of HIV-1. The HIV-1 protease has been shown to be capable of cleaving peptides with sequences representing seven of the cleavage sites found in the gag and gag-pol products, but does not cleave peptides for the two cleavage sites in the env polyprotein (16). Deletions introduced into the murine leukemia virus have indicated a role for that viral protease in generating infectious virions (17, 18). Recently, a single nucleotide substitution in the HIV-1 viral genome, which replaced the putative active site aspartic acid residue of the HIV-1 protease with an asparagine residue, was found to eliminate the activity of this protease and to result in the production of non-infectious virions (19). The crucial role of the HIV-1 protease in the life cycle of the HIV-1 virus has made it a logical therapeutic target for the treatment of AIDS. To assist in the rational design of protease inhibitors, we have undertaken a structural investigation of the HIV-1 protease by single crystal x-ray diffraction methods.

MATERIALS AND METHODS

Purification—All chemicals are ACS reagent grade or better and are used without further purification. The DNA corresponding to the provirus of the NY5 strain of HIV-1 (20) was a generous gift from Dr. Malcolm Martin (National Institutes of Health-National Institute of Allergy and Infectious Diseases). Expression of HIV-1 protease in Escherichia coli was achieved as described in detail elsewhere (10). Briefly, the protease coding sequence was inserted into the plasmid pKK233 containing a modified trp promoter. The codon preceding the N-terminal Pro had been altered by site-directed mutagenesis to encode a Met, and a nonsense stop codon was inserted following the C-terminal Phe. Sequencing of the DNA demonstrated that the sequence was the same as that published for the BH1 isolate (3) with the exception that residue 37 is Asn in the NY5 isolate. Cells were cultivated in rich media to an A600 of 2.5–3.0, diaphragmed using cross-flow filtration, resuspended in minimal media, and induced by the addition of indoleacrylic acid to a final concentration of 200 μg/ml. After 2 h postinduction, the cells were harvested. Following centrifugation, 8 kg of cell paste was obtained from fermentations totaling 3500 liters.

Purification was performed in batches of 250 g of cell paste. Cells were lysed with lysozyme and treated with a Dounce homogenizer (10). After centrifugation, the supernatant was passed over a 2.2-liter DEAE-Sephadex A-25 column equilibrated in 50 mM Tris/HCl, pH 7.8, and eluted isocratically at 4 °C. Hydrolysis of the peptide Val-Ser-Gln-Thr-Pro-Ile-Val was used to detect the HIV-1 protease activity, as described previously (10). Active fractions were pooled, made 1.7 M in (NH₄)₂SO₄, applied to a Baker HiPropyl hydrophobic interaction column, and the bound protease eluted with a linear gradient of 1.7–0.0 M (NH₄)₂SO₄ in 50 mM CH₃COONa, pH 5.5, at 0 °C. Following dialysis, the pooled active fractions were applied to a Pharmacia LKB Biotechnology Inc. Mono-S column and the protease was eluted with a gradient of 0.0–0.5 M NaCl in 50 mM NaMES/MES, pH 6.0, at 25 °C. All buffers contained 10% glycerol, 5% ethylene glycol, 1 mM EDTA, and 1 mM DTT; in the CBX elution buffer the concentration of DTT was increased to 10 mM. The protein was further concentrated to 10–16 mg/ml at 4 °C, using Centricon-10 microconcentrators (Amicon), simultaneously exchanging the storage buffer for one consisting of 10 mM NaMES/MES, pH 5.0, 1 mM DTT, 1 mM Na₂EDTA, and 3 mM NaN₃ (buffer A). Gas phase sequencing demonstrated the anticipated N-terminal sequence beginning with Pro, the initial Met having been removed in E. coli (11).

Crystallization—Crystallization was carried out with the hanging-drop vapor diffusion technique (21) in 24-well Linbro cell culture plates. 2–10 μl of HIV-1 protease at 6 mg of protein/ml (diluted with buffer A) were mixed with an equal volume of a precipitating solution consisting of 100 mM imidazole/HCl (or NaMOPS/MOPS), pH 7.0, 250 mM NaCl, 10 mM DTT, and 3 mM NaN₃ on a siliconized glass cover slide. The cover slide was inverted and sealed over a 1-ml reservoir of the precipitating solution with silicone vacuum grease. The plate was stored at 4 °C for 4–7 days, after which time tetragonal bipyramidal crystals approximately 0.25 x 0.25 x 0.50 mm in size were observed. The crystals were transferred to the precipitating solution.
and used within 1–2 weeks; crystals stored for longer periods showed a complete loss of the diffraction pattern even though they physically appeared to remain intact. For x-ray intensity measurements, a single crystal was transferred at 4 °C to a disposable micro-plate cup containing 100 μl of the precipitating solution. This cup was placed inside a sealed styrofoam box, which was transferred to room temperature and allowed to stand undisturbed for at least 2 h. The crystal was then removed from solution and mounted in a thin-walled glass capillary containing a paper wick wetted with precipitating solution. Precipion and still x-ray diffraction photographs were obtained at room temperature on CEA Reflex-25 film using nickel-filtered, Cu Kα, radiation produced by a Rigaku RU-200 rotating anode generator running at 50 kV and 60 mA.

**Electrophoresis**—The protein was characterized electrophoretically using the Pharmacia PhastSystem. Precast 20% homogeneous SDS-polyacrylamide gels and buffer strips (Pharmacia) were employed. Samples were prepared by diluting 1 μl of stock protease solution to 1 mg/ml with a denaturing solution consisting of 25 mM Tris base, 162 mM glycine, 0.8% (w/w) SDS, 6.5% (v/v) 2-mercaptoethanol, 6.5% (v/v) glycerol, and 0.02% (w/v) bromphenol blue with the pH approximately 8.3. The samples were incubated at 100 °C for 2 min prior to use. To establish the presence of HIV-1 protease in the crystals, some crystals were removed from the experimental drops, thoroughly washed in precipitating solution, redissolved in buffer A by warming at 37 °C, and subjected to the same electrophoretic procedures as the stock protein solution. All gels were stained using either the Daiichi silver stain kit modified for use in the PhastSystem or Coomassie Brilliant Blue R-250.

**Density Measurements**—The density of the crystals was measured at 4 °C with a Ficoll 400 (Pharmacia) gradient (22, 23) calibrated with 5-μl droplets of buffer-saturated mixtures of hexane and bromoform. Densities for these standard solutions were accurately determined pycometrically. A linear gradient of the Ficoll medium was prepared in polycarbonate centrifuge bottles (Beckman) by continuously diluting a stock 60% (w/w) Ficoll 400 solution with the precipitating solution while loading the bottles. The bottles were then centrifuged for 3 h at 30,000 × g to smooth any discontinuities in the gradient. Small crystals of HIV-1 protease were placed in one of the tubes, the organic calibration droplets in the other, and the tubes were centrifuged for 5000 × g in a Beckman SW28, 30-tube rotor. A density calibration curve was constructed from the distances migrated by the calibration droplets from the top of the gradient solution. The final equilibrium positions of the HIV-1 protease crystals were plotted on this curve to obtain the average overall crystal density.

**RESULTS AND DISCUSSION**

The solubility of the HIV-1 protease is greatly effected by both temperature and pH. During concentration runs carried out at pH 5.0 and 4 °C, the protein would begin to precipitate as soon as its concentration reached 16–18 mg of protein/ml. This precipitate could be redissolved by resuspending the solid in a small amount of buffer A and letting it stand at room temperature for several minutes or in the cold room overnight. At pH 7.0, protein concentrations greater than 3–4 mg/ml were difficult to achieve, even at room temperature. These solubility properties were exploited in the crystallization of this enzyme.

Initial crystallization efforts were carried out around pH 5, the pH at which the protein exhibits maximal proteolytic activity. These trials, however, produced showers of rectangular plates which were useless for our purposes. Raising the pH of the precipitating solution produced a transition of crystal habits from plates at pH 5, to needles at about pH 6 to well formed, tetragonal bipyramids at pH 7 (Fig. 1). Raising the pH above 8 produced an amorphous precipitate, irrespective of temperature or protein concentration. While the plates would appear at any temperature between 4 °C and room temperature, the needles and bipyramids could only be grown in the cold. Analysis of the bipyramids by SDS-polyacrylamide gel electrophoresis produced a pattern nearly identical to that obtained with the stock protein solution from which they were grown (Fig. 2). At pH 7, crystallization was essentially quantitative for the protease and resulted in a degree of purification of the enzyme (note the pattern of the crystallization drop supernatant as compared with the solution and crystalline protein samples). Redissolved crystals also exhibited the expected level of enzymatic activity when used in the standard assay.²

Still x-ray photographs of HIV-1 protease crystals showed a measurable diffraction pattern to a resolution limit of approximately 2.5 Å. Precession photographs of the h0l (Fig. 3) and h00 zones showed that the crystals belonged to the tetragonal class and that the unit cell dimensions were a = b = 50.3 Å and c = 106.8 Å. The systematic absence of reflections with Miller indices of h00 ≠ 2n and 001 ≠ 4n indicated either space group P4₁2₁2 (No. 92) or its enantiomorph P4₃2₁2 (No. 96). The crystals were found to remain stable in the x-ray beam for up to 70 h.

Calculation of Matthew’s coefficient $V_m$, crystal volume/ unit molecular weight (24), yielded 3.06 Å³/dalton for a monomer of 10,000 daltons/asymmetric unit or 1.53 Å³/dalton if a dimer were to occupy the same space. Both of these values lie at the extremes of the distribution of values of $V_m$ found for crystalline proteins; to resolve the ambiguity, the density of the HIV-1 protease crystals was determined experimentally. An average value of 1.17 g/ml at 4 °C was found, which

² P. L. Darke, unpublished results.
Crystallization of HIV-1 Protease

Fig. 3: A 24-h exposure, $\mu = 10^8$ precession photograph of the h0l layer at a crystal to film distance of 100 mm

implies a total molecular mass (protein plus bound solvent plus free solvent) of 23,500 daltons for the contents of a single asymmetric unit. Assuming a partial specific volume of 0.74 cm$^3$/g, common for most globular proteins (22), the solvent content for the crystals was calculated to be approximately 60%. This led to a calculated protein molecular mass per asymmetric unit of these crystals containing a monomer. Although a $M$, 10,000 cut-off ultrafiltration membrane was used for concentrating the protein, virtually no leakage of the protease through the ultrafiltration membrane was observed. This provides evidence for the existence of a stable solution species with size characteristics greater than what would be expected for a globular protein of 10,000 daltons. If the mechanism of action of HIV-1 protease is the same as that proposed for the other aspartylproteases (25-27), then the enzymatically competent species of the HIV-1 protease is expected to be a dimer. This crystal form of HIV-1 protease can accommodate a dimeric enzyme if the dimer axis is colinear with a crystallographic 2-fold axis.

Acknowledgments—We thank Dale Lehman, Mary Mudri, and Ted Schaefer for assistance in the purification of the HIV-1 protease, Bob Maigetter and James Bailey for assistance with fermentation studies, and John Rodkey and Mohendra Sardana for protein sequencing.

REFERENCES
1. Broder, S., and Gallo, R. C. (1984) New Engl. J. Med. 311, 1292-1297
2. Dickson, C., Eisenman, R., Fan, H., Hunter, E., and Teich, N.

(1984) in RNA Tumor Viruses (Weiss, R., Teich, N., Varmus, H., and Coffin, J., eds) 2nd Ed, Revised, pp. 513-648, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
3. Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starich, B., Josephs, S. F., Doran, E. R., Rafaels, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Petteway, S. R., Jr., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Ghrayeb, J., Chang, N. T., Gallo, R. C., and Wong-Staal, F. (1985) Nature 313, 277-284
4. Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S., and Alizon, M. (1985) Cell 40, 9-17
5. Sanchez-Pescador, R., Power, M. D., Barr, P. J., Steimer, K. S., Stempen, M. M., Brown-Shimer, S. L., Gee, W. W., Renard, A., Randolph, A., Levy, J. A., Dina, D., and Luciw, P. A. (1985) Science 227, 484-492
6. Muesing, M. A., Smith, D. H., Cabraddilla, C. D., Benton, C. V., Lasky, L. A., and Capon, D. J. (1985) Nature 313, 450-458
7. Henderson, L. E., Copeland, T. D., Sowder, R. C., Schultz, A. M., and Oroszlan, S. (1988) in UCLA Symp. Mol. Cell. Biol. 71, 135-147
8. Debouck, C., Gorniak, J. G., Strickler, J. E., Meek, T. D., Metcalf, B. W., and Rosenberg, M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8903-8906
9. Graves, M. C., Lim, J. J., Heimer, E. P., and Kramer, R. A. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2449-2453
10. Darke, P. L., Luh, C.-T., Davis, L. J., Heimbach, J. C., Diehl, R. E., Hill, W. S., Dixon, R. A. F., and Sigal, I. S. (1988) J. Biol. Chem. 264, 2307-2312
11. Nutt, R. F., Brady, S. F., Darke, P. L., Ciccarone, T. M., Dylion Colton, C., Nutt, E. M., Rodkey, J. A., Bennett, C. D., Waxman, L. H., Sigal, I. S., Anderson, P. S., and Veber, D. F. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7129-7133
12. Schneider, J., and Kent, S. B. H. (1988) Cell 54, 363-368
13. Tob, H., Ono, M., Saigo, K., and Miyata, T. (1985) Nature 315, 691
14. Miller, R. H. (1987) Science 236, 722-725
15. Pearl, L. H., and Taylor, W. R. (1987) Nature 329, 351-354
16. Darke, P. L., Nutt, R. F., Brady, S. F., Garsky, V. M., Ciccarone, T. M., Leu, C.-T., Lamma, P. K., Friedinger, H. M., Veber, D. F., and Sigal, I. S. (1988) Biochem. Biophys. Res. Commun. 156, 297-303
17. Crawford, S., and Goff, S. P. (1985) J. Virol. 53, 899-907
18. Katoh, I., Yoshinaka, Y., Rein, A., Shibuya, M., Odaka, T., and Oroszlan, S. (1985) Virology 145, 280-292
19. Kohl, N. E., Emin, E. A., Schleif, W. A., Davis, L. J., Heimbach, J. C., Dixon, R. A. F., Scolnick, E. M., and Sigal, I. S. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4686-4690
20. Benn, S., Rutledge, R., Folks, T., Gold, J., Baker, L., McCormick, J., Fornino, P., Piot, P., Quinn, T., and Martin, M. (1985) Science 230, 949-951
21. McPherson, A. (1982) Preparation and Analysis of Protein Crystals, pp. 82-159, John Wiley & Sons, New York
22. Westbrook, E. M. (1985) Methods Enzymol. 114, 187-196
23. Matthews, B. W. (1985) Methods Enzymol. 114, 176-187
24. Matthews, B. W. (1968) J. Mol. Biol. 33, 491-497
25. James, M. N. G., Hsu, I.-N., and Delbaere, L. T. J. (1977) Nature 267, 808-813
26. Pearl, L. H. (1987) FEBS Lett. 214, 8-12
27. Suguna, K., Padlan, E. A., Smith, C. W., Carlson, W. D., and Davies, D. R. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7009-7013