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Crystallization of a non-B and a B mutant HIV protease

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HIV polymorphism is responsible for the selection of variant viruses resistant to inhibitors used in AIDS treatment. Knowledge of the mechanism of resistance of those viruses is determinant to the development of new inhibitors able to stop, or at least slow down, the disease's progress caused by new mutations. In this paper, the crystallization and preliminary crystallographic structure solution for two multi-resistant 99 amino acid HIV proteases, both isolated from Brazilian patients failing intensive anti-AIDS therapy are presented, *viz.* the subtype B mutant, with mutations Q7K, S37N, R41K, K45R, I54V, L63P, A71V, V82A and L90M, and the subtype F (wild type), naturally carrying mutations Q7K, I15V, E35D, M36I, S37N, R41K, R57K, D60E, Q61N, I62V, L63S, I64L and L89M, with respect to the B consensus sequence. Both proteins crystallized as a complex with the inhibitor TL-3 in space group $P6_122$. X-ray diffraction data were collected from these crystals to resolutions of 2.1 and 2.6 Å for the subtype B mutant and subtype F wild type, respectively, and the enzyme structures were solved by molecular replacement. The crystals of subtype F HIV protease are, to the best of the authors' knowledge, the first protein crystals obtained for a non-B HIV protease.

1. Introduction

Acquired immunodeficiency syndrome (AIDS) is a complex of symptoms and diseases resulting from human immunodeficiency virus (HIV) infection. HIV is a member of the *Lentivirus* genus, that includes simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV), among others. The HIV genome encodes for two large polyproteins, *gag* and *gag-pol*, that are subsequently processed by its 99-amino-acid homodimeric aspartyl protease (HIV PR) to render a mature HIV particle. The inhibition of this HIV PR leads to the production of immature non-infectious virus. However, the high polymorphism of HIV results in a rapid selection of viral variants resistant towards a specific inhibitor. Continuous *in vivo* HIV replication and the error prone nature of the viral reverse transcriptase are the driving forces for the generation of drug resistance (Caride *et al.*, 2001).

Almost all studies on HIV-1 drug susceptibility have been performed in developed countries, where subtype B still dominates the epidemic, but on a worldwide scale this is by far not a predominant HIV subtype. The Brazilian epidemic is characterized by multiple HIV-1 group M subtypes, primarily subtype B and subtype F, but also subtypes C and D. From the genes deposited in the HIV Sequence Database (http://www.hiv.lanl.gov/content/hiv-db/mainpage.html), 9.8% of Brazilian infections are of subtype F and 5.0% are of subtype C. Though these distributions represent the frequency in the HIV Database and not the population, it can give an idea of the distribution of subtypes in a given country.

In this work, we present the crystallization of two HIV-1 proteases isolated from Brazilian patients failing treatment. A subtype F wild type (*Fwt*) and a multi-resistant subtype B mutant (*Bmut*) HIV PR have calculated molecular weights of 10.6956 kDa and 10.8078 kDa, respectively, and both were crystallized as complexes with the *C*2-symmetric inhibitor TL-3 (Li *et al.*, 2000). This is, to our knowledge, the first report of crystals of a non-B subtype HIV-1 protease.

2. Material and methods

2.1. Expression and purification

The protease sequence of both viruses *Bmut* and *Fwt* carrying the point mutation Q7K were subcloned in pET11a and transformed into *E. coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene). The mutations, according to HXB2 consensus sequence, were Q7K, S37N, R41K, K45R, L63P, I54V, A71V, V82A and L90M (nine point mutations) for *Bmut*, and Q7K, I15V, E35D, M36I, S37N, R41K, R57K, D60E, Q61N, I62V, L63S, I64L and L89M (13 point mutations) for *Fwt*. The transformed

cells were grown at 310 K to an OD_{600} of 0.7 in LB medium supplemented with 1% glucose, 0.01% antifoam 204 (Sigma), 35 µg l^{-1} of chloramphenicol and 100 µg l^{-1} of ampicillin. IPTG was added to a final concentration of 1 m*M*. The cells were harvested after 4 h, re-suspended in lysis buffer (50 m*M* Tris–HCl pH 8.0, 5 m*M* EDTA, 5 m*M* DTT) and lysed using sonication. The inclusion bodies containing the protease were washed in lysis buffer containing 2 *M* urea and 1% Triton X-100, and then again in lysis buffer. The protease *Fwt* was solubilized during the washing procedure and was purified out of the urea/detergent solution. The washed inclusion bodies of *Bmut* were solubilized in buffer *A* (50 m*M* Tris–HCl pH 8.0, 8 *M* urea, 5 m*M* EDTA, 0.1% β-mercaptoethanol) and clarified by centrifugation (28 222*g*) before loading onto a Q Sepharose column (Amersham-Pharmacia), equilibrated with buffer *A*. The flow-through containing the protease was pooled out and was adjusted to 50 m*M* NaOAc pH 5.0 and loaded onto a HiTrap SP Sepharose column (Amersham-Pharmacia) equilibrated with buffer *B* (50 m*M* NaOAc, pH 5.0, 8 *M* urea, 2 m*M* EDTA, 0.1% β-mercaptoethanol). The bound proteins were eluted using a buffer *C* (buffer *B* + 1 *M* NaCl) gradient to a final concentration of 20% of this buffer.

Refoldings of pure proteins were carried out using three changes of refolding buffer (20 m*M* Na_2HPO4 pH 7.4, 100 m*M* NaCl, 2 m*M* EDTA, 0.1% β-mercapto ethanol) with the protein solution in a 6–8 kDa MWCO dialysis bag. After dialysis, the solution was clarified by centrifugation (28 222*g*), concentrated by ultrafiltration and stored at 353 K. The protein concentration was determined by the Bradford method.

2.2. Crystallization

The buffer of the *Bmut* protease was changed to 50 m*M* NaOAc pH 5.0, 2 m*M* EDTA and 2 m*M* DTT in two steps directly in the concentrator, and the protein was finally concentrated to 4.3 mg ml^{-1} (~0.4 m*M*). It was then mixed with two molar excesses of TL3 inhibitor (22 m*M* in DMF/DMSO 1:1) for 1 h. Protease (1 µl) was mixed with an equal volume of well solution in a hanging-drop crystallization experiment at 291 K. The initial screen was based on a variation of precipitants: ammonium sulfate (0.48–1.6 *M*), MPD [0–6%(*v*/*v*)] and PEG 3350 [0–15%(*w*/*v*)] or 8000 [0–25%(*w*/*v*)] containing 0.1 *M* sodium cacodylate at pH 5.6 and 6.2. Crystals appeared after 2 d and continued to grow for about two weeks in two conditions [0.1 *M* sodium cacodylate, pH 6.2, 0.26 *M* ammonium sulfate, 6%(*v*/*v*) MPD, 5.1%(*w*/*v*) PEG 3350 (condition 1), and 0.1 *M* sodium cacodylate pH 6.2, 0.32 *M* ammonium sulfate, 6%(*v*/*v*) MPD (condition 2)] rendering just one crystal per drop (see Fig. 1). The crystal sizes were 0.3 × 0.1 × 0.1 mm for condition 1 and 0.5 × 0.1 × 0.1 mm for condition 2.

Table 1
Crystallographic data and preliminary statistics for *Bmut* and *Fwt* HIV proteases.

Values relative to the last resolution shell are shown in parentheses when appropriate. The values for the second-best solution of molecular replacement are also shown in parentheses.

	Bmut	*Fwt*
Space group	$P6_122$	$P6_122$
Cell parameters (Å)	$a = b = 62.124$, $c = 84.309$	$a = b = 62.750$, $c = 82.410$
Resolution (Å)	53.45–2.10 (2.21–2.10)	54.23–2.60 (2.74–2.60)
No. of subunits in a.u.	1	1
V_M ($Å^3$ Da^{-1})	2.1	2.5
Completeness (%)	100 (100)	100 (100)
$I/\sigma(I)$	11.0 (2.0)	8.7 (2.0)
R_{merge} (%)	4.6 (38.1)	7.5 (39.0)
No. of unique reflections	6035	3258
Redundancy (%)	22	17.4
Molecular replacement†		
R factor (%)	40.6 (56.0)	41.5 (55.8)
F correlation (%)	63.8 (29.6)	62.5 (32.3)
I correlation (%)	68.7 (33.6)	67.2 (33.2)

† The resolution range 10–3 Å was used in the molecular replacement procedure for both *Bmut* and *Fwt*.

In a similar fashion, *Fwt* at a concentration of 4.9 mg ml^{-1} (~0.4 m*M*), in refolding buffer, was mixed with two molar excesses of TL3 inhibitor. Sitting drops with a 4 µl final volume were set up by mixing protein and well solution in equal proportions at 291 K. A shower of small rods (< 0.1 mm) grew using ammonium sulfate (0.64 *M* and 0.8 *M*) as precipitant. The number of single crystals per drop was decreased by lowering the temperature to 277 K (0.8 *M* ammonium sulfate), changing the protein-to-well-solution proportions to 2:1 and increasing the final volume of a sitting drop to 9 µl. Only two crystal rods appeared after approximately one week at 277 K with no further enlargement of the crystals. The crystallization box was then transferred to 291 K, which led to a further growth of crystals to a maximum size of 0.4 × 0.1 × 0.1 mm (Fig. 1).

2.3. Data collection and processing

The diffraction patterns for the *Bmut* crystal, grown in condition 1, and the one of the larger *Fwt* crystal were obtained using Cu $K\alpha$ radiation generated by a Rigaku ultraX 18 rotating-anode generator and recorded on a MAR 345 dtb image plate. Initial diffraction of the *Bmut* protein was significantly improved by three successive rounds of annealing, during which the crystal was transferred to a room-temperature cryosolution and then flash-cooled again (Fig. 2). The data were processed and scaled using *MOSFLM* 6.2.3 (Leslie, 1992) and *SCALA* 3.1.20 (Evans, 1997), respectively (Table 1). The solvent contents of the *Bmut* and *Fwt* crystals are close to 42%, with one protein subunit per asymmetric unit.

2.4. Molecular replacement

An HIV PR subtype B (wild type) X-ray structure solved at 1.09 Å resolution (PDB code 1kzk; Reiling *et al.*, 2002) was used as

Figure 1
Crystals of *Bmut* and *Fwt* proteases. (*a*) *Bmut* grown in condition 1 (used in data collection), (*b*) *Bmut* grown in condition 2, (*c*) *Fwt* initial screen obtained at 291 K using 0.8 *M* ammonium sulfate as precipitant, and (*d*) *Fwt* final crystals (used in data collection).

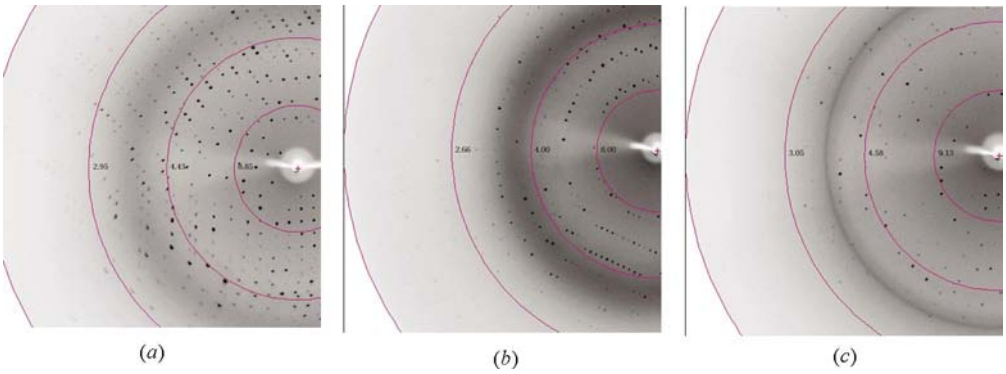

Figure 2
Diffraction patterns of the two proteases. (*a*) A *Bmut* crystal before annealing, (*b*) the same *Bmut* crystal after three cycles of annealing, and (*c*) an *Fwt* crystal.

the search model in the molecular replacement of both *Bmut* and *Fwt* proteases. The amino-acid sequence of the model is 92 and 88% identical to *Bmut* and *Fwt*, respectively. In both cases, an *AMoRe* (Navaza, 1994) translation function gave a clear solution in space group $P6_122$ (Table 1), whereas the same procedure with the data processed in $P6_522$ presented no contrast (data not shown).

3. Discussion

The production of purified protein in the amount required for crystallization screens is one of the key points in its structure determination. For that, the inclusion of the Q7K mutation, which is known to enhance protease stability (Rosé *et al.*, 1993), was crucial in attempts to obtain considerable expression levels of both *Bmut* and *Fwt* proteases. Concerning the crystallization experiments, it is important to notice the role of a multi-precipitant procedure in obtaining *Bmut* protease crystals (see, for example, Majeed *et al.*, 2003). Even though the initial screen included conditions with a single precipitant, the crystals appeared in the drops containing at least two precipitants of different classes: a salt, an organic solvent and a polymeric precipitant in condition 1, and a salt and an organic solvent in condition 2.

The molecular replacement approach for both *Bmut* and *Fwt* yielded clear solutions, which is not surprising given the high amino-acid sequence identity between the search model and multi-resistant B mutant and non-B proteases crystallized by us. Refinement of the crystallographic models is currently in progress. The structures, when complete, will help to explain the molecular basis of resistance of these protease variants to commercial inhibitors.

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References

Caride, E., Hertogs, K., Larder, B., Dehertogh, P., Brindeiro, R., Machado, E., de Sá, C. A. M., Eyer-Silva, W. A., Sion, F. S., Passioni, L. F. C., Menezes, J. A., Calazans, A. R. & Tanuri, A. (2001). *Virus Genes*, **23**, 193–202.
Evans, P. R. (1997). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **33**, 22–24.
Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **26.**
Li, M., Morris, G. M., Lee, T., Laco, G. S., Wong, C. H., Olson, J., Elder, J. H., Wlodawer, A. & Gustchina, A. (2000). *Proteins*, **38**, 29–40.
Majeed, S., Ofek, G., Belachew, A., Huang, C., Zhou, T. & Kwong, P. D. (2003). *Structure*, **11**, 1061–1070.
Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.
Reiling, K. K., Endres, N. F., Dauber, D. S., Craik, C. S. & Stroud, R. M. (2002). *Biochemistry*, **41**, 4582–4594.
Rosé, J. R., Salto, R. & Craik, C. S. (1993). *J. Biol. Chem.* **268**, 11939–11945.