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addenda and errata

Retraction of articles by H. M. Krishna Murthy *et al.*

Two papers by H. M. Krishna Murthy *et al.* are retracted.

Two papers by H. M. Krishna Murthy *et al.* (Krishna Murthy *et al.*, 1999; Urs *et al.*, 1999) are retracted by the journal. This follows investigation by the University of Alabama at Birmingham, Alabama, USA, of structures deposited by H. M. Krishna Murthy. Krishna Murthy has noted that he is not in agreement with the retractions.

References

Krishna Murthy, H. M., Judge, K., DeLucas, L., Clum, S. & Padmanabhan, R. (1999). *Acta Cryst.* D**55**, 1370–1372.
Urs, U. K., Murali, R. & Krishna Murthy, H. M. (1999). *Acta Cryst.* D**55**, 1971–1977.

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Crystallization, characterization and measurement of MAD data on crystals of dengue virus NS3 serine protease complexed with mung-bean Bowman–Birk inhibitor

H. M. Krishna Murthy,[a]* K. Judge,[a] L. DeLucas,[a] S. Clum[b] and R. Padmanabhan[b]

[a]Center for Macromolecular Crystallography, University of Alabama at Birmingham, 79-THT, MCLM-244, 1918 University Boulevard, Birmingham, AL 35294-0005, USA, and [b]Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS 66160, USA

Correspondence e-mail: murthy@onyx.cmc.uab.edu

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Crystallization and preliminary characterization of the essential dengue virus NS3 serine protease complexed with a Bowman–Birk-type inhibitor from mung beans are reported. As the structure proved resistant to solution by molecular replacement and multiple isomorphous replacement methods, multi-wavelength anomalous diffraction data at the L_{III} edge of a holmium derivative have been measured. Promising Bijvoet and dispersive signals which are largely consistent with expected values have been extracted from the data. The structure, when determined, will provide a structural basis for the design, synthesis and evaluation of inhibitors of the protease for chemotherapy of dengue infections.

1. Introduction

Dengue viruses, members of Flaviviridae, are serious health threats to roughly 2.4 billion people living in infested areas (Gubler & Clark, 1995). They are the cause of the frequently fatal dengue fever and dengue hemorrhagic fever (Monath, 1994). Efforts towards combating the disease have hitherto concentrated on eliminating the mosquito vector(s) for the virus and the development of vaccines. This strategy has proved to be of limited efficacy and alternate strategies focusing on the pathogen are needed (Gubler & Clark, 1995; Monath, 1994). A two-component viral serine protease, with trypsin-like specificity, is essential for formation of infective dengue virions (Chambers *et al.*, 1990; Clum *et al.*, 1997; Falgout *et al.*, 1991, 1993; Irie *et al.*, 1989; Kapoor *et al.*, 1995; Preugschat *et al.*, 1990; Ramachandra *et al.*, 1996; Valle & Falgout, 1998; Zhang *et al.*, 1992; Zhang & Padmanabhan, 1993). The crystal structure of the catalytic component of the protease (Den2 protease) has recently been reported by us, and structural similarities to and differences from the analogous enzyme from the hepatitis C virus, which is not arthropod-borne, have been discussed (Krishna Murthy *et al.*, 1999).

Essential viral proteases, in general, have been excellent targets for chemotherapy, for example in case of HIV (Abdel-Meguid *et al.*, 1993, 1994; Krishna Murthy *et al.*, 1992; Palleja, 1998; Thompson *et al.*, 1994). Similarly, Den2 protease is a potential target in developing chemotherapeutic approaches for the treatment of dengue infections. One of the best ways of characterizing functional details of serine proteases is to define in detail their interaction with naturally occurring serine-protease inhibitors which function as substrates which are hydrolyzed, the products dissociating extremely slowly (Kraut, 1977; Laskowski & Kato, 1980; Steitz & Shulman, 1982). We report the crystallization and characterization of a ternary complex of Den2 protease with a naturally occurring serine protease inhibitor from the Bowman–Birk inhibitor family isolated from mung beans (MbBBi). Biochemical experiments have shown strong inhibition of Den2 protease by MbBBi and detailed kinetic characterization is in progress (M. Wetzel, S. Clum and R. Padmanabhan, unpublished results). Bowman–Birk family inhibitors have been isolated from various legumes and are double headed, capable of inhibiting two protease molecules simultaneously, with specificities determined by the amino-acid sequences at the inhibitor active sites (Ikenaka & Norioka, 1986; Laskowski & Kato, 1980). MbBBi has a Lys and an Arg, respectively, as P1 residues – using a standard nomenclature (Schechter & Berger, 1967) – on its two heads; the structure of the complex will thus afford the opportunity to characterize the similarities and differences between the interactions of Den2 protease with these two P1 residues. Structures of Bowman–Birk type inhibitors from several sources (Chen *et al.*, 1992; Suzuki *et al.*, 1987; Wei, 1983) and in complexes with proteases (Gaier *et al.*, 1981; Tsunogae *et al.*, 1986), including a trypsin complex with MbBBi (Lin *et al.*, 1993), have been reported. Efforts to determine the structure of the Den2 protease–MbBBi complex by molecular replacement using the known coordinates of Den2 protease and the inhibitor have not been successful. Similarly, attempts to obtain an MIR solution from a holmium derivative have not yielded positive results. We have therefore measured MAD data on the holmium derivative and

Table 1
MAD data-processing statistics.

Resolution†	$\langle I/\sigma(I)\rangle$‡	R_{sym}§	Completeness¶	Redundancy††	Bij. signal‡‡	Disp. signal§§
4.00	53.6 (40.7)	0.013 (0.026)	90.2 (98.1)	4.7 (5.4)	0.087 (0.041)	0.063 (0.047)
3.17	48.6 (42.1)	0.045 (0.035)	93.5 (89.4)	4.2 (4.7)	0.094 (0.067)	0.065 (0.041)
2.77	32.0 (37.7)	0.059 (0.063)	94.3 (97.8)	4.5 (4.1)	0.144 (0.078)	0.084 (0.067)
2.52	18.9 (22.5)	0.074 (0.088)	92.1 (91.7)	3.9 (3.9)	0.166 (0.074)	0.103 (0.066)
2.34	(11.8)	(0.111)	(83.5)	(3.2)		
2.20	(7.3)	(0.134)	(75.3)	(2.8)		
2.09	(6.1)	(0.156)	(76.9)	(3.1)		
All	38.2 (25.3)	0.048 (0.083)	92.5 (88.4)	4.3 (3.9)	0.123 (0.065)	0.079 (0.055)
Expected signals¶¶					0.104	0.074

† High-resolution limit of shell in Å; lowest resolution is 30 Å. ‡ Average signal-to-noise ratio; values for native data in parentheses. § $\sum(|I-\langle I\rangle|)/\sum I$, summed over equivalent observations excluding Bijvoet mates; values for native data (in parentheses) include Bijvoet mates. ¶ Percentage of accessible data in shell measured with $I > 3\sigma(I)$: values for native data in parentheses. †† Average number of observations for a reflection, excluding Bijvoet mates; values for native data (in parentheses) include Bijvoet mates. ‡‡ Bijvoet signal, $\sum(|F^+ - F^-|)/0.5\sum(F^+ + F^-)$ summed over acentric reflections; values for centric reflections are in parentheses. §§ Dispersive signal, $\sum(|F^{\lambda\mathrm{Inflex}} - F^{\lambda\mathrm{Remote}}|)/0.5(|F^{\lambda\mathrm{Inflex}} + F^{\lambda\mathrm{Remote}}|)$; in parentheses are $\sum(|F^{\lambda\mathrm{Inflex}} - F^{\lambda\mathrm{Peak}}|)/0.5(|F^{\lambda\mathrm{Inflex}} + F^{\lambda\mathrm{Peak}}|)$ ¶¶ Bijvoet signal: expected diffraction ratio between Bijvoet mates at λ^{Peak} calculated using formulae in Krishna Murthy *et al.* (1988), Hendrickson (1994) and Krishna Murthy (1996) assuming four Ho^{3+} per asymmetric unit. Dispersive signal: the same, calculated between $\lambda^{\mathrm{Inflex}}$ and $\lambda^{\mathrm{Remote}}$; note that both values are evaluated at zero scattering angle.

report here the presence of excellent Bijvoet and dispersive signals in the processed data. The observed signals are consistent with those expected (Hendrickson, 1994; Krishna Murthy *et al.*, 1988; Krishna Murthy, 1996) from the binding of four Ho^{3+} ions to each heterotrimer of the complex. The determination of this structure will lay the necessary structural foundation for understanding the enzymology of the protease at the atomic level. It will also provide the starting point for structure-assisted design of specific small-molecular inhibitors of the protease for evaluation as potential drugs.

2. Materials and methods

Purification of the unliganded Den2 protease, crystallization and structure solution have been reported (Krishna Murthy *et al.*, 1999). The inhibitor was extracted by previously reported methods (Chi *et al.*, 1982) and further purified by FPLC. The complex was prepared by mixing a solution of 0.2 mg ml^{-1} Den2 protease (in 20 m*M* Tris pH 7.8, 200 m*M* NaCl) with lyophilized MbBBi to a final inhibitor concentration of 0.038 mg ml^{-1}, giving a 2:1 molar ratio of enzyme to inhibitor. The complex was then concentrated by filtration to 4.7 mg ml^{-1} protein and crystallization experiments were carried out by vapor diffusion in hanging drops (McPherson, 1985). The best crystals were grown from 200 m*M* Tris pH 7.8, 150 m*M* NaCl, 40 m*M* LiCl, 1.7 m*M* $NiCl_2$ and 0.2% β-octyl glucoside (solution *A*) and 17% PEG 6000 in the wells of hanging-drop experiments, with the drops being made up from 2 µl protein mixed with an equal volume of well solution. Crystals grew to final dimensions of 0.16 × 0.18 × 0.35 mm over 4–8 weeks at 293 K. Crystals were characterized on a Siemens X1000 area-detector system using *XDS* for processing (Kabsch, 1988). Unit-cell parameters were determined to be $a = 75.5$, $b = 90.2$, $c = 98.8$ Å, $\alpha = \beta = \gamma = 90^\circ$. The space group was determined to be $P2_12_12_1$ from systematic absences. The holmium derivative was prepared by soaking crystals overnight in solution *A* supplemented with 20% PEG 6000 and 1 m*M* $HoCl_3$. Diffraction data used in molecular-replacement and MIR calculations were measured on the Siemens X1000 system and processed with *XDS*. The MIR calculations were performed using *SOLVE* (Terwilliger & Berendzen, 1996; Terwilliger *et al.*, 1987) and *MLPHARE* as implemented in *CCP*4 (Collaborative Computational Project, Number 4, 1994), and MR calculations were carried out using *AMoRe* (Navaza, 1994) and *POLARRFN* (Collaborative Computational Project, Number 4, 1994). Multiple-wavelength data on crystals soaked in Ho^{3+} were measured on beamline 17-ID of the Industrial Macromolecular Crystallography Association (IMCA) at the Advanced Photon Source (APS). The data were processed with *XGEN* (Howard, 1995) and analyzed using routines from *MADSYS* (Hendrickson, 1994). Data were measured at three wavelengths for the Ho^{3+} derivative using cryogenic techniques on a single-element 165 mm MAR Research CCD detector (Hope, 1990; Rogers, 1994). The wavelengths were chosen from inspection of a plot of f' and f'' produced using *CHOOCH* (Evans & Perrifer, 1996) and derived from X-ray fluorescence from the crystals measured as a function of wavelength. The three chosen wavelengths corresponded to the peak (1.53556 Å, λ^{Peak} in Table 1) of the Ho L_{III} edge, the inflexion point (1.53642 Å, $\lambda^{\mathrm{Inflex}}$) and the remote high-energy (1.52974 Å, $\lambda^{\mathrm{Remote}}$) point. The measured values for f' and f'' were −18.4 and 14.3, respectively, compared with estimated (Evans & Perrifer, 1996) values of −20.2 and 11.1 for an unliganded Ho^{3+} ion. Data were measured at each wavelength using the inverse-beam method; a 90° section of data and the 90° section 180° away from it were measured at a chosen wavelength before changing the wavelength and remeasuring the same 90° block at the new wavelength. The first 90° block was started at the inflexion point, followed by measurement of the same block at the peak and remote wavelengths; the sequence was repeated for each subsequent sector of data. Measurement of the entire data set took 7 h and the processed data showed no evidence of significant radiation damage. The measurements were in dose mode to compensate for beam decay. A hemisphere of data for the native crystals was also measured at 1.52974 Å using a similar protocol. Data at each wavelength were processed using *XGEN*, keeping Bijvoet mates separate throughout, except for the native set where they were merged.

3. Results and discussion

Assumption of one copy of the 2:1 protease–inhibitor complex in the asymmetric unit gave a reasonable value of 66% solvent content (Matthews, 1985). The assumption was confirmed by a self-rotation function calculated with native data. The calculation revealed a strong peak at $\omega = 159$, $\varphi = 143$ and $\kappa = 174^\circ$, consistent with the 2:1 complex assembly in the asymmetric unit displaying an approximate twofold rotational symmetry. In structures of Bowman–Birk inhibitors (Chen *et al.*, 1992) and their complexes (Lin *et al.*, 1993), it has been observed that the folding of the main chains of the two lobes of the inhibitor are related by approximate twofold rotational symmetry; amino-acid sequences, however, are not in general symmetric. Thus, in our complex structure, the approximate twofold non-crystallographic symmetry is expected to relate the two protease dimers closely, but will be less appropriate for the inhibitor, especially for the side chains. Cross-rotation function calculations, employing a variety of appropriate atomic models, carried out using *AMoRe* (Navaza, 1994), failed to produce a convincing solution.

Comparison of the unit-cell parameters of the Ho^{3+} derivative with those of native crystals did not reveal signs of gross non-

isomorphism; however, inspection of the distribution of intensity changes as a function of resolution indicated that significant non-isomorphism might be present. Nevertheless, attempts to phase the structure using MIR calculations were carried out to obtain low-resolution phases. Four heavy-atom binding sites were identified by *SOLVE* from a Bijvoet difference Patterson function of the Ho^{3+} derivative, and pairs of these could be related by the non-crystallographic symmetry transformation relating the two enzyme monomers in the asymmetric unit. Identical sites were identified from isomorphous difference Patterson functions; however, no useful phase information could be extracted beyond ~7 Å resolution. It was thus decided to measure MAD data on the Ho^{3+} derivative and use the structure of the derivative to determine that of the native complex through molecular replacement. Processing statistics, quality of data and signal levels are summarized in Table 1.

As diffraction data on native crystals of the complex extend to at least 2.1 Å, atomic details of the interaction of the inhibitor with the enzyme will be readily determinable. The structure of the complex, when determined, will form a important point of departure for design, synthesis and evaluation of specific inhibitors of Den2 protease. Because of sequence and inferred structural similarities among proteases of all orthropod-borne flaviviruses (Ryan *et al.*, 1998), the structure will be a useful model system for proteases of over 70 viruses. In addition, because Bowman–Birk-type inhibitors function as anti-carcinogenic agents owing to their inhibition of the proteases required for the establishment and spread of certain kinds of cancers (Kennedy, 1998*a*,*b*), the structure will also prove to be an excellent model until more specific structural information on these systems is obtained. MAD phasing calculations are currently in progress.

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