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Crystal twinning of human MD-2 recognizing endotoxin cores of lipopolysaccharide

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Twinning of crystals causes overlapping of two or more reciprocal lattice points, and hence structure amplitudes for a single crystalline domain are hardly obtained from X-ray diffraction intensities. MD-2 protein forms a stable complex with Toll-like receptor 4 and recognizes bacterial lipopolysaccharide (LPS). Excessive immune responses activated by LPS cause septic shocks. Saccharide-trimmed human MD-2 crystallizes in the tetragonal form with apparent Laue symmetry of 4/*mmm*, and diffraction intensities from these crystals indicate crystal twinning. The crystal consists of two different domains, A and B. The c_A axis of domain A coincides with the c_B axis of domain B with a smaller lattice, and the a_A axis corresponds to the $(a_B + b_B)$ axis. This twinning severely imposes difficulty in structure determination. Through optimization of cryoprotectant, domain A was thoroughly transformed into domain B. The crystal containing only domain B is in space group $P4_12_12$ with one MD-2 molecule in the asymmetric unit. The structure of this form of MD-2 as well as its complex with antiendotoxic lipid IVa was successfully determined using the multiple isomorphous replacement method.

Keywords: crystal twinning; innate immunity; endotoxin.

1. Introduction

Crystal twinning is one of the most serious obstacles to structure determination. Two or more reciprocal lattice points of a twinned crystal overlap and hence structure amplitudes for a single crystalline domain are hardly obtained accurately (Parsons, 2003). Isomorphous replacement phasing requires accurate measurement of intensities from native and heavy-atom derivative crystals. Non-isomorphism often observed in twinned crystals involves different twinning fractions and makes it difficult to estimate accurate heavy-atom contribution. When the twinning fraction in a hemihedrally twinned crystal is not so high and the relative orientation between the twinned domains is known, detwinning procedures are applicable in some cases but introduce additional errors into the detwinned data (Yeates, 1997).

Human MD-2 is a 160 amino-acid glycoprotein with a 16 amino-acid secretion signal and two *N*-linked glycosylation sites (Gangloff & Gay, 2004). Secreted MD-2 forms a stable complex with Toll-like receptor 4 (TLR4) on the cell surface, and the complex recognizes lipopolysaccharide (LPS), also known as endotoxin, from gram-negative bacteria, leading to activation of innate immune responses (Nagai *et al.*, 2002; Poltorak *et al.*, 1998). Excessive responses against LPS frequently cause septic shocks, and hence MD-2 has been a therapeutic target (Visintin *et al.*, 2006). Recently, we have determined the crystal structures of human MD-2 itself and of its complex with antiendotoxic lipid IVa (Ohto *et al.*, 2007). The crystals of MD-2 and the complex are almost isomorphous and both show crystal twinning. Initial attempts to solve the structures using the twinned crystals failed. Here we report the crystal twinning and transformation into single crystals through optimization of cryoprotectant.

2. Materials and methods

2.1. Sample preparation

Details of protein expression, purification and crystallization have been described previously (Ohto *et al.*, 2007). In brief, human MD-2 was expressed in methyltropic yeast *Pichia pastoris*, and its monomeric form was purified to homogeneity through steps of chromatography and *N*-linked saccharide trimming which leaves one *N*-acetylglucosamine at each glycosylation site. Crystallization was performed using the hanging-drop vapor-diffusion method, and crystals of MD-2 itself and of the complex with lipid IVa were obtained.

2.2. Data collection and analyses of the data

Diffraction data sets were collected at 100 K on BL38B1 of SPring-8, Hyogo, Japan, using a Jupiter 210 CCD detector (Rigaku, Tokyo, Japan), and also by use of Cu $K\alpha$ radiation from an in-house rotating-anode generator (MacScience, Tokyo, Japan) equipped with an R-AXIS IV detector (Rigaku, Tokyo, Japan). The data sets were processed using the *HKL2000* package (Otwinowski & Minor, 1997). Analyses of diffraction intensities were carried out using *TRUNCATE* of the CCP4 suit (Collaborative Computational Project, Number 4, 1994). Detwinning was performed using *DETWIN* (Taylor & Leslie, 1998), and molecular replacement was performed using *MOLREP* (Vagin & Teplyakov, 1997).

3. Results and discussion

3.1. Analyses of twinned crystals

In the initial stage of data collection, crystals were transferred to a harvesting mother solution of 25% PEG 8000, 0.2 *M* Na-acetate, 0.1 *M* NaCl, 0.1 *M* Na-cacodylate pH 6.3, and then cryoprotected by the mother solution with addition of 10% glycerol. These crystals are called form 1 and a typical diffraction pattern is shown in Fig. 1(*a*). Diffraction intensities from this form 1 crystal showed apparent Laue symmetry of 4/*mmm* and cell parameters of a = 75 Å and c = 112 Å. Odd h numbered (h00) reflections showed weaker intensities than even h reflections, and intensity ratios of these reflections varied considerably among crystals. Reflections (00l) with $l \neq 4n$ (where n is an integer) were also observed, as were variations in intensity ratios. Because of this unusual property of diffraction patterns, the space group could not be determined definitely at this stage. Assuming that the space group is either $P4_1$ or $P4_12_12$, or its enantiomorph, an asymmetric unit contains four or two MD-2 molecules, respectively. In either case the Matthews coefficient (Matthews, 1968) corresponds to 2.3 Å^3 Da^{-1} with a solvent content of 0.56.

We examined intensity statistics to detect crystal twinning. The curves for cumulative intensity distributions of the form 1 crystals are shown in Fig. 2(*a*). The curves appear lower than the expected theoretical curves for single-crystal data for both centric and acentric reflections, and show sigmoidal properties which are typical of twinned crystals (Dauter, 2003). The values of the second moments of intensity (Fig. 2*b*) also suggest that the form 1 crystal is twinned. The mean value of the second moment of intensity is 1.77, while the expected values for a perfectly twinned crystal and for an untwinned one are 1.5 and 2.0, respectively (Dauter, 2003).

Attempts to solve the structure with the twinned data by use of the multiple isomorphous replacement (MIR) method failed in all of the probable space groups, and poor isomorphism among native crystals became apparent. The poor isomorphism presumably originated from differences in twinning fractions. Detwinning procedures seemed applicable although the procedures require twinning fractions not close to 0.5 and also operators relating two domains (Yeates, 1997). Assuming that two domains in point group 4 are related by twofold rotation along the a axis, apparent Laue symmetry of 4/*mmm* was explainable. However, detwinning with this assumption resulted in almost perfect twinning with twinning fractions close to 0.5. This perfect twinning was inconsistent with unusual systematic extinctions and obvious deviations from 4/*mmm* symmetry in the diffraction pattern. Hence, detwinning was unsuccessful.

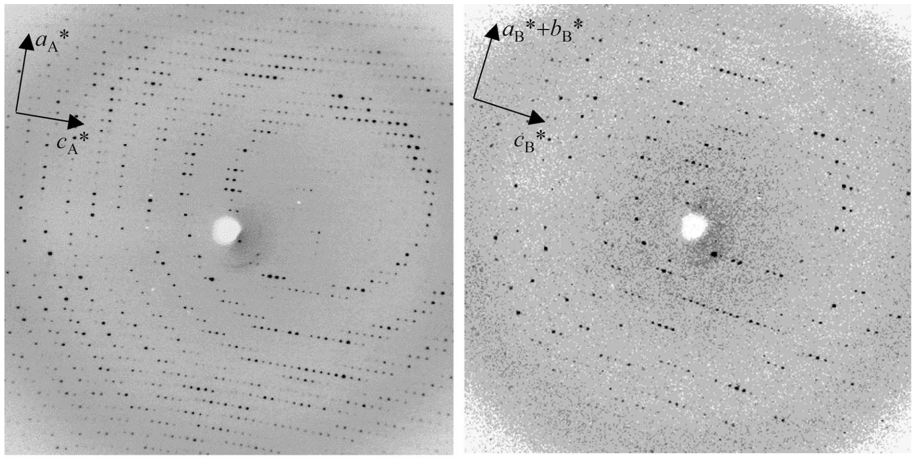

Figure 1
Typical diffraction patterns for MD-2 crystals. (*a*) Form 1 using 10% glycerol as a cryoprotectant. (*b*) Form 2 using 20% 2,3-butanediol as a cryoprotectant. Both patterns are from crystals in almost identical orientations, and axes for the reciprocal lattices are indicated.

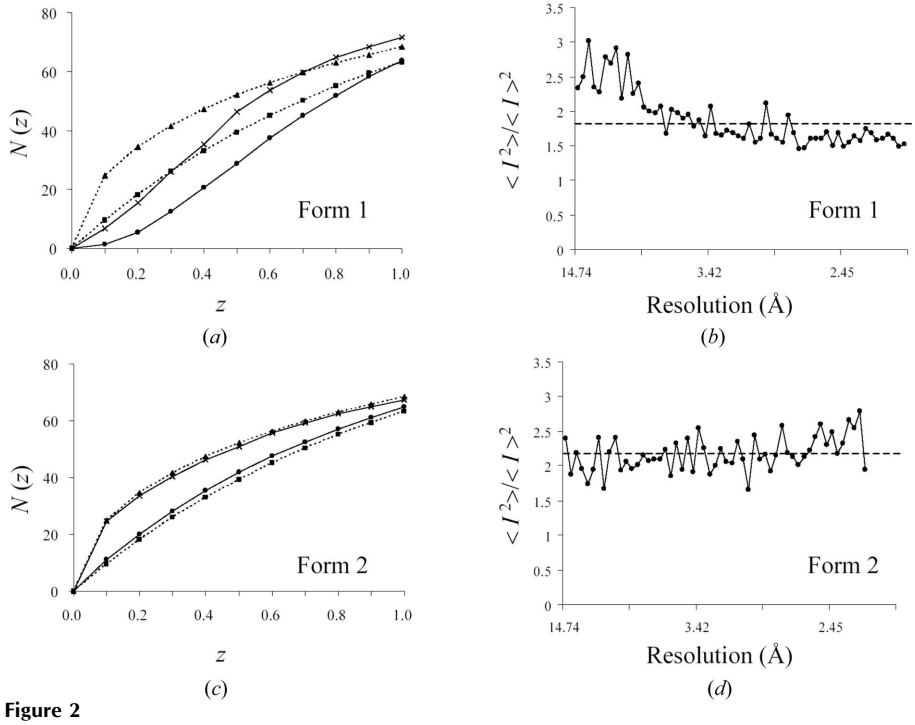

Figure 2
Analyses of diffraction intensities: (*a*) and (*b*) form 1; (*c*) and (*d*) form 2. (*a*) and (*c*) show plots for cumulative intensity (I) distribution for acentric and centric reflections, where z is $I/\langle I\rangle$ and $N(z)$ is the percentage of reflections with intensities less than or equal to z. Observed curves for acentric (dots) and centric (crosses) are drawn as solid lines, and the expected theoretical curves for the untwinned case for acentric (squares) and centric (triangles) are drawn as broken lines. (*b*) and (*d*) show plots of the second moments ($\langle I^2\rangle/\langle I\rangle^2$) of intensity, calculated using observed acentric data. The expected value for the untwinned case is 2.0, and that for the perfectly twinned case is 1.5. The mean value of the second moment is shown as a broken line.

Finally, after the crystal transformation followed by the structure determination of the transformed crystal, we judged that the form 1 crystal consisted of two different domains: domain A with the above-mentioned lattice constants and domain B with a smaller lattice as described below.

3.2. Transformation into single crystals

The form 1 crystals were reproducibly transformed into single crystals by soaking in a cryoprotectant solution at 277 K for one day.

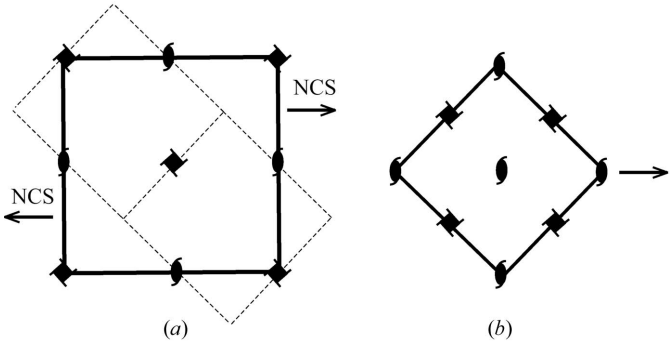

Figure 3
Schematic representation of two different lattices of the MD-2 crystals. (*a*) Domains A and B in the form 1 crystal. Domain A is supposedly in space group $P4_1$. Two lattices for domain B are shown with broken lines. (*b*) Domain B transformed from form 1. Crystal lattices are viewed along the *c* axis (crystallographic fourfold screw axis). The non-crystallographic twofold axis (NCS) is shown by an arrow in (*a*), and the corresponding crystallographic twofold axis is shown similarly in (*b*).

The optimum cryoprotectant contained 20% 2,3-butanediol instead of the 10% glycerol used previously. The resultant diffraction pattern is shown in Fig. 1(*b*), which is in almost the same crystal orientation as in Fig. 1(*a*). Reflections with $h = 2n + 1$ in the ($h0l$) zone in Fig. 1(*a*) disappeared in Fig. 1(*b*). The space group of this transformed crystal (form 2) was determined as $P4_12_12$ from Laue symmetry of 4/*mmm* and obvious systematic extinctions along the axial directions. Lattice parameters for this crystal are $a = 53$ Å and $c = 112$ Å. An asymmetric unit contains one MD-2 molecule, and the Matthews coefficient (Matthews, 1968) is 2.3 Å^3 Da^{-1}, which corresponds to a solvent content of 0.56. This value is identical to that for the twinned crystal (form 1). A lattice conversion by cryoprotectant has been reported for the orthorhombic form of a single crystal of actin (Govindasamy *et al.*, 2004). In that case the lattice parameters of the transformed crystal shrank by about 20% along the *c* axis.

The cumulative intensity distribution curves and the values of the second moments of intensity for the form 2 crystal are shown in Figs. 2(*c*) and 2(*d*). The curves are well fitted to the theoretical curves expected for single-crystal data, and the mean value of the second moment is 2.21. These suggest that the form 2 crystal is not twinned.

Using the data from the transformed crystals, determination of the structure of MD-2 using the MIR method was straightforward (Ohto *et al.*, 2007). The crystals of the lipid IVa complex were obtained under slightly different crystallization conditions and found to be isomorphous to the MD-2 crystals. They exhibited the same twinning and were similarly transformed into single crystals. The structure of the lipid IVa complex was solved using the molecular replacement method using the native MD-2 structure as a search model.

Fig. 3 shows schematic representations of the two different lattices of the MD-2 crystals viewed along the *c* axis. The initially obtained form 1 crystal (Fig. 3*a*) is a mixture of domain A with the lattice constants of form 1, and domain B with those of form 2, and hence shows twinning properties in the intensity statistics as shown in Figs. 2(*a*) and 2(*b*). The c_A axis of domain A coincides with the c_B axis of domain B, and the a_A axis corresponds to the ($a_B + b_B$) axis. This peculiar lattice configuration causes perfect overlaps of reflections from two domains. When soaked in the optimized cryoprotectant, domain A was supposed to be thoroughly transformed into domain B.

Packing of MD-2 molecules in a space group $P4_1$ lattice of form 1 was determined using the molecular replacement method using the observed data set up to 3.0 Å resolution, with the refined MD-2 structure as a search model, and is shown in Fig. 4. Four MD-2 molecules consisting of two pairs of dimers were contained in an

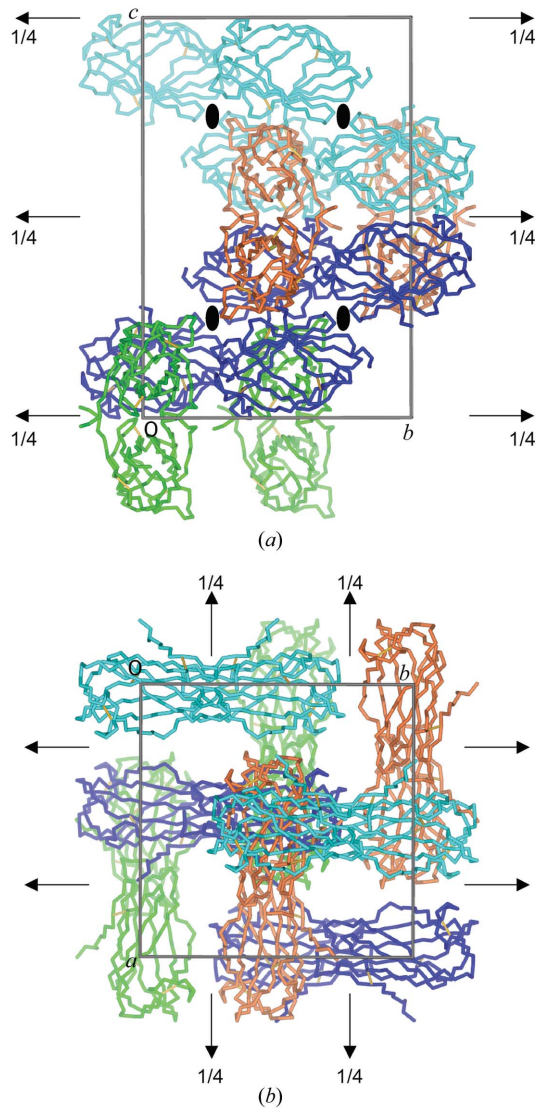

Figure 4
Packing of MD-2 molecules in the form 1 crystal. (*a*) View along the *a* axis. (*b*) View along the *c* axis. A total of 16 MD-2 molecules in a lattice are shown, and four MD-2 molecules in an asymmetric unit are drawn with almost identical colors in which front and back depths are represented with color deepness. Non-crystallographic twofold axes parallel to the *a* axis and to the *b* axis are indicated with black arrows and ellipsoids. The figures were prepared using program *Weblab Viewer* (Accelrys, CA, USA).

asymmetric unit. The twofold axis of non-crystallographic symmetry (NCS) is parallel to the a_A axis and relates a pair of MD-2 molecules. Two pairs are related by a diagonal translation of (0.5, 0.5, 0). The crystallographic twofold screw axis parallel to the c_A axis in domain A therefore mimics the fourfold screw axis, and this enables the lattice conversion of domain A to domain B. The NCS twofold axis in domain A corresponds to the crystallographic twofold axis in domain B, which is diagonal between the a_B and b_B axes.

The diffraction patterns and intensity statistics of the form 1 crystal suggested twinning of MD-2 crystals. Attempts to detwin the form 1 data indicated almost perfect twinning which suggests the existence of twofold symmetry perpendicular to the *c* axis. Furthermore, the packing of MD-2 molecules in form 1 corresponded well to that of form 2. Based on these findings, we conclude that the form 1 crystal consists of domains A and B. Since the arrangement of MD-2 molecules in form 1 is determined using the data set from twinned

crystals which contain contributions from both the domains, subtle structural differences between these forms still remain to be clarified.

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