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Improved three-dimensional growth of manganese superoxide dismutase crystals on the International Space Station

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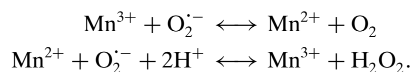
Manganese superoxide dismutase was crystallized in microgravity with 35 PCAM experiments (Protein Crystallization Apparatus for Microgravity) on the ISS (International Space Station) from 5 December 2001 to 19 April 2002. Crystals were very large in size and could easily be seen by eye. Crystals with 0.45 × 0.45 mm cross-sections and of up to 3 mm in length were obtained in several drops: an 80-fold increase in crystal volume compared with the largest earth-grown crystal. A smaller crystal (0.15 × 0.30 mm in cross-section and 1.6 mm in length) was soaked in cryoprotectant and placed in a cryoloop. Diffraction data were collected at 100 K at the BioCARS bending-magnet beamline. The space group was $C222_1$, with unit-cell parameters a = 100.64, b = 107.78, c = 179.82 Å. Diffraction spots to 1.26 Å resolution were observed. Unfortunately, the high-resolution diffraction degraded owing to radiation damage and the resolution limit for the complete data set was 1.35 Å. It is anticipated that increasing the crystal volume and diffraction limit through microgravity crystal growth will enable several types of technically challenging structure determinations.

1. Introduction

Superoxide dismutases (SODs) are important antioxidant enzymes that protect all living cells against toxic oxygen metabolites (for recent reviews, see McCord, 2002; Forsberg *et al.*, 2001). SODs contain Cu/Zn, Fe or Mn ions in their active sites. Humans have Cu/ZnSOD in the cytosol and extracellular spaces and MnSOD in the mitochondria. Mutations in SOD lead to degenerative diseases such as amyolateral sclerosis, diabetes and cancer. Bacteria have Fe and MnSOD. SODs are one of the fastest known enzymes, with a k_{cat}/K_M of $10^9\ M^{-1}\ s^{-1}$, and are rate-limited only by the diffusion of the substrate and products. MnSOD protects cells by dismuting two molecules of superoxide anion to form hydrogen peroxide and molecular oxygen *via* a cyclic oxidation–reduction reaction (Keele *et al.*, 1970).



$$Mn^{3+} + O_2^{\cdot-} \longleftrightarrow Mn^{2+} + O_2$$
$$Mn^{2+} + O_2^{\cdot-} + 2H^+ \longleftrightarrow Mn^{3+} + H_2O_2.$$

Structural data are needed to understand the enzymatic mechanism of MnSOD. The binding sites of the diatomic substrate and product as well as the source of the protons in the reaction are currently unknown. Towards this aim, room-temperature and 100 K diffraction data have been collected on earth-grown peroxide-soaked MnSOD crystals to define the mode of product binding to the active-site manganese. Peroxide density is visible at the obtained resolution (1.55 Å); however, it is difficult to interpret without an atomic resolution structure. Higher resolution data are needed to distinguish partially occupied peroxide or superoxide from hydroxide or water in order to resolve the true structural aspects of this mechanism. In order to observe product or substrate intermediates at atomic resolution, strong diffraction to better than 1.49 Å (the oxygen–oxygen bond distance in H_2O_2) for peroxide-soaked MnSOD and to 1.28 Å (the oxygen–oxygen bond distance in superoxide) for superoxide-soaked MnSOD would be desirable. To date, the highest resolution structure for earth-grown MnSOD is 1.55 Å (Borgstahl *et al.*, 2000).

In previous reports, large-volume crystals with low mosaicity resulted from microgravity growth (Snell *et al.*, 1995; Boggon *et al.*, 2000; Borgstahl *et al.*, 2001). For some proteins, an increase in diffraction resolution was observed (Kundrot *et al.*, 2001; Borgstahl *et al.*, 2001; Ng *et al.*, 2002). For example, a study on microgravity and earth-grown insulin crystals gave microgravity crystals that were on average 34 times larger, had sevenfold lower mosaicity and diffracted to significantly higher resolution than their earth-grown counterparts (Borgstahl *et al.*, 2001). This study on insulin statistically demonstrated that it was possible to grow very large crystal volumes in microgravity (>1 mm^3) without sacrificing mosaicity. There are several technically challenging structures for MnSOD to be determined involving

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neutron diffraction, Laue methods and the high- resolution cryotrapping mentioned above. For these studies, large-volume crystals with low mosaicity are either required or beneficial. The best earth-grown crystals to date are too small to make use of neutron techniques and do not diffract to the required resolution. Therefore, microgravity crystal-growth experiments were flown to determine if microgravity had a beneficial effect on native MnSOD crystal growth and could enable these studies. Preliminary results were positive and indicated a dramatic increase in crystal volume and an increase in diffraction resolution through microgravity growth.

2. Experimental methods and results

2.1. Purification and crystallization

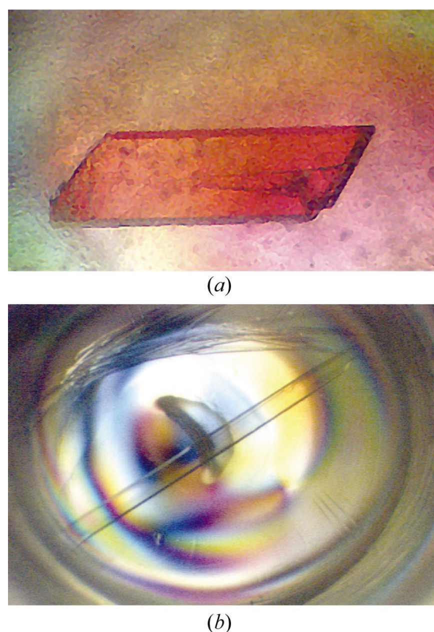
For overproduction of MnSOD, *Escherichia coli* strain OX326A.1 (pTTQA10) was used. This strain lacks endogenous Mn and FeSODs and harbors an ampicillin-resistant IPTG-inducible expression plasmid for *E. coli* MnSOD (Hopkin *et al.*, 1992; Steinman, 1992). Bacterial cultures were grown at 310 K in 8 l Terrific Broth supplemented with 20 mg $MnSO_4$ and 400 mg ampicillin in a VIRTI-culture fermentor (10 l air per minute, 300 rev min^{-1}). At mid-log stage, the culture was induced with 1 m*M* IPTG. After 4 h, cells were harvested, resuspended in 50 m*M* potassium phosphate pH 7.5, lysed by sonication and centrifuged.

The following rapid purification protocol was adapted from published procedures (Borgstahl *et al.*, 1992; Keele *et al.*, 1970; Beck *et al.*, 1988). The clarified lysate was heated to 333 K for 10 min and centrifuged. The supernatant was then dialyzed against 5 m*M* potassium phosphate pH 7.5, mixed with pre-equilibrated DE-52 resin and incubated on a rocking table at room temperature for 1 h. The filtrate was then dialyzed against 2.5 m*M* MES pH 5.5 and applied to a PerSeptive BioSystems CM/M POROS column. The column was thoroughly washed and eluted with a gradient of 2.5–500 m*M* MES pH 5.5. Very pure MnSOD eluted at approximately 200 m*M* MES. Peak fractions were pooled and dialyzed against 20 m*M* potassium phosphate pH 7.0. Approximately 15–20 mg of MnSOD was purified per litre of culture. Activity was monitored with a qualitative native polyacrylamide gel electrophoresis-based assay stained with *p*-nitroblue tetrazolium (Calbiochem).

Protein samples were concentrated to 18 and 20 mg ml^{-1}, respectively. Eight Bicine-buffered (50 m*M*, pH 8.5) precipitant solutions were prepared with varying PEG 6000 concentrations ranging from 20.5 to 24%. Seven potassium phosphate buffered (40 m*M*, pH 7.8) precipitant solutions were prepared with varying PEG 4000 concentrations ranging from 22.5 to 25%. Protein samples, precipitant solutions and loading instructions were shipped to New Century Pharmaceuticals Inc., AL, USA for loading into Protein Crystallization Apparatus for Microgravity (PCAM; Carter *et al.*, 1999*a*) trays on-site at Kennedy Space Center, FL, USA. Each chamber well was loaded with a 1:1 mixture of 10 µl of the protein solution and 10 µl of the precipitant solution. The chamber reservoirs were filled with 500 µl of precipitant solution each.

For temperature control, six PCAM cylinders were placed in an STES housing (single-locker thermal enclosure systems). Each PCAM cylinder contains nine stackable trays with seven sitting-drop wells per tray. Each PCAM well is surrounded by a donut-shaped reservoir, filled with an absorbent wick to absorb the precipitant solution. The trays are held in position by guide rods and separated from each other by an actuator plate held under tension by springs. An elastomer and adhesive is used to seal the trays. The actuator plate contacts a plunger that presses against the elastomer, separating the well from the reservoir. Upon entering microgravity, an astronaut retracts the plunger by rotating a shaft on the end of the cylinder clockwise. The seal is released and vapor diffusion between the reservoir and the well is allowed. Before the end of the mission, the astronaut rotates the shafts counter-clockwise to reseal the samples before return to earth (Carter *et al.*, 1999*a*; see also http://crystal.nasa.gov/technical/hardware/pcam.html).

Space shuttle flight STS-108 transported the PCAM experiments to the International Space Station (ISS). The launch was scheduled for 29 November 2001 and the PCAMs were loaded on 27 November 2001. Owing to delays, the actual launch was December 5, 2001 and the PCAMs arrived at the ISS on 7 December 2001. The experiments were activated on 8 December 2001 and were returned by space shuttle flight STS-110 on 19 April 2002. Crystals grew in 30 of 35 experiments. MnSOD crystals are typically orthorhombic plate-like needles in shape, with the longest axis coinciding with the *c* dimension. The best earth-grown crystals were about 1 mm in length, with a maximum thickness of approximately 0.15 mm. The earth crystals were plate-like, with a typical third dimension of only 0.02–0.05 mm. Crystals obtained in the PCAM microgravity experiment appeared in varying sizes from small needles to large three-dimensional crystals that were up to 0.45 × 0.45 mm thick and 3 mm long (Fig. 1*a*). The size of the largest crystals appeared to be limited by the 20 µl drop size (Fig. 1*b*). Remarkably, most microgravity crystals had equal growth in both the *a* and *b* directions. This was never observed for earth-grown crystals.



(*a*)

(*b*)

Figure 1
Examples of microgravity-grown MnSOD crystals in the PCAM crystallization chamber. (*a*) Crystal with dimensions 0.45 × 0.45 × 1.45 mm. The pink color is due to oxidized manganese in the active site. Earth-grown crystals typically grow as thin plates and are never thick enough to see the pink color. (*b*) An example of crystals limited in size to 3 mm in length by the drop volume. This photo was taken at lower magnification so that the entire drop volume could be seen.

2.2. Data collection

Diffraction data were collected on 26 April 2002 with a Quantum IV ADSC at Advanced Photon Source (APS) BioCARS beamline 14-BM-C for structure determination. The largest crystals proved difficult to manipulate with the standard cryo-crystallography tools available. A 0.15 × 0.30 × 1.60 mm crystal was briefly soaked in cryoprotectant solution containing 20% glycerol, mounted in a cryoloop and flash-cooled in a N_2 gas stream at 100 K. A wavelength of 0.9 Å was used. The unit-cell parameters limited the crystal-to-detector distance to 200 mm. At this distance, the crystals diffracted beyond the 1.80 Å resolution edge of the detector. As a result, the detector was translated vertically by 60 mm. Diffraction spots to 1.25 Å resolution were

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observed on the first few frames (Fig. 2), but degraded after a few frames owing to radiation damage. Therefore, the crystal was translated to a fresh position every 45° of data collection. An oscillation of 1° and an exposure time of 45 s were used. The detector was then lowered to a zero height offset and the same data swath recollected with 1.5 s exposures to complete the low-resolution shells. The diffraction data were indexed and integrated using the program *DENZO* and reduced and scaled with *SCALEPACK* (Otwinowski & Minor, 1997). The unit-cell parameters were determined to be a = 100.64, b = 107.78, c = 179.82 Å with space group $C222_1$ and did not change as a result of the microgravity crystallization conditions (Borgstahl *et al.*, 2000). The resolution limit of the diffraction data collected was 1.35 Å overall (with a linear $R_{\rm sym}$ of 0.046). Previously, a 1.55 Å resolution structure (with a linear $R_{\rm sym}$ of 0.070) was published using earth-grown crystals. A diffraction limit of ~1.3 Å was observed from this earth-grown crystal, but data at this resolution could not be measured owing to instrumental limitations (Borgstahl *et al.*, 2000). The higher $R_{\rm sym}$ for this earth-grown crystal indicates that the crystal was of lesser quality compared with the microgravity sample. Another indicator was that the diffraction limit of ~1.3 Å for the earth-grown crystal was observed from a 5 min X-ray exposure which overexposed the majority of the image, caused overloads on the majority of the reflections on the detector and was observed from only a single image at SSRL bending-magnet beamline 7-1. Therefore, the microgravity MnSOD crystals showed an improvement in diffraction resolution and data quality.

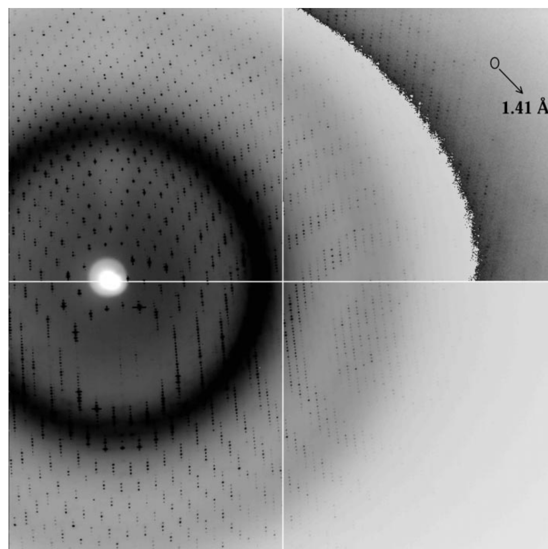

Figure 2
X-ray diffraction pattern from a microgravity MnSOD crystal at 100 K. The φ-rotation axis was vertical in this image. The resolution limit for the right edge was 1.39 Å and for the top right corner was 1.25 Å. The contrast was adjusted in the upper right quadrant so that some of the fainter high-resolution diffraction spots would be visible.

3. Discussion and concluding remarks

The goal with this research is to structurally understand the reaction mechanism of MnSOD. Two complementary approaches are planned: the first is to increase crystal quality in order to increase the structural resolution of the measured X-ray data and the second is to use neutrons to locate H-atom positions. Neutron studies currently require large crystals with volumes of ~1 mm^3 or more. Since these studies employ the Laue method, low-mosaicity crystals are also required to avoid reflection overlap, a serious problem for crystals with large unit cells. In this case, the crystal volume necessary for neutron studies has been produced and microgravity has been historically proven to produce low-mosaicity crystals (Snell *et al.*, 1995; Boggon *et al.*, 2000; Borgstahl *et al.*, 2001). It can be argued that an increased crystal volume also improves the resolution of the resultant diffraction data. This appears to be the case here when compared with the previous best resolution of 1.55 Å (Borgstahl *et al.*, 2000). The largest crystals could not be used for X-ray data collection at cryogenic temperatures owing to mounting difficulties. Techniques to enable this in the future are being developed.

Based on these early results from PCAM, more microgravity experiments are planned, with complete earth controls, for MnSOD. PCAM experiments, some with larger drop volumes to further enhance crystal volume, will be flown to enable the structure determinations described above. MnSOD crystal-growth experiments in the diffusion-controlled crystallization apparatus for microgravity (DCAM; Carter *et al.*, 1999*b*) are also under development. The vapor-diffusion method used in PCAM suffers from Maragoni convection. This causes flow in the drop and may have a negative effect on the resultant crystal quality (Marangoni, 1871; Chai *et al.*, 1992; Chayen *et al.*, 1997). Alternatively, the microgravity benefit to crystal volume seen in the PCAM experiments may have been aided by Maragoni convection, the flow resulting in more nutrient being available to the unsedimented crystal. It is anticipated that crystal size and crystal quality can be further improved by using the larger volumes and dialysis methods provided by the DCAM apparatus in microgravity. Finally, along with structural data collection, reflection-profile data will be collected from both microgravity and earth-grown crystals using superfine φ-slicing techniques to measure the true crystal mosaicity (Bellamy *et al.*, 2000).

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