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Crystallization and preliminary X-ray diffraction analysis of hydroxynitrile lyase from cassava (*Manihot esculenta*)

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Hydroxynitrile lyase from *M. esculenta* (cassava) was crystallized in two different crystal forms by the hanging-drop vapour-diffusion method. Crystals of form I were obtained from a mixture of polyethylene glycol 8000 and 2-methyl-2,4-pentanediol, and belong to the tetragonal space group $P4_12_12$ or its enantiomorph $P4_32_12$, with unit-cell parameters $a = b = 105.9$, $c = 188.9$ Å and with two molecules in the asymmetric unit. These crystals diffract to 2.9 Å with conventional X-ray sources and beyond 2.1 Å resolution with synchrotron radiation. The crystals are relatively sensitive to radiation damage and conditions for flash-cooling the crystals have been established. A complete native data set has been collected up to 2.2 Å resolution. Crystal form II has been obtained at pH 5.6 using lithium sulfate as a precipitant. The crystals apparently belong to the orthorhombic space group $P2_12_12$, with unit-cell parameters $a = 117.52$, $b = 127.09$ and $c = 78.08$ Å, have two molecules in the asymmetric unit and diffract to beyond 2.0 Å resolution. A complete native data set has been collected to 2.2 Å resolution.

1. Introduction

Hydroxynitrile lyase (HNL) from *Manihot esculenta* belongs to a family of enzymes catalyzing the stereospecific cleavage of a large number of aliphatic, aromatic and heterocyclic cyanohydrins into a corresponding carbonyl component and hydrogen cyanide (HCN) (Conn, 1981). The release of HCN is the final step in the cyanogenesis of higher plants and is thought to play an essential role in defense against infection by invading herbivores (Seigler, 1991; Nahrstedt, 1985). HNLs from *Prunus serotina* (Cheng & Poulton, 1993), *Sorghum bicolor* (Wajant *et al.*, 1994), *M. esculenta* (MeHNL; Hughes *et al.*, 1994), *Hevea brasiliensis* (HbHNL; Hasslacher *et al.*, 1996) and *Linum usitatissimum* (Trummler & Wajant, 1997) have been recently cloned and characterized. An extraordinary feature of the HNL enzyme family is the occurrence of diverse enzyme forms with respect to sequence similarities, the presence of cofactors and the existence of multiple homo- and heterodimeric isoforms (for a detailed review, see Wajant & Effenberger, 1996). It is supposed that HNLs have evolved independently several times from various distinct ancestral molecules (convergent evolution).

MeHNL consists of a single polypeptide chain of 258 amino acids and has a molecular mass of 28.5 kDa. The enzyme has been synthesized in a multimeric form in *Escherichia coli*, and shows full catalytic activity towards its native substrate acetone cyanohydrin (Wajant *et al.*, 1995). MeHNL shows a sequence identity of about 74% to the equivalent enzyme from *Hevea brasiliensis*, another *Euphorbiacea* species. However, there is no obvious sequence homology to other HNLs from different species. Recently, the structure of HbHNL was solved to 2.1 Å resolution (Wagner *et al.*, 1996), showing that the enzyme has a mixed α/β core domain with a three β-strand/three α-helix subdomain insertion. Although the structural studies of HbHNL have provided the first view of the active site, the catalytic mechanism of cyanogenesis is still the subject of controversial discussion. Based on the HbHNL structure, it has been suggested that the reaction involves an enzyme-bound intermediate, while recent site-directed mutagenesis experiments provided the first evidence for a non-enzyme-bound intermediate (Wajant & Pfizenmaier, 1996).

While under physiological conditions the conversion of acetone cyanohydrin into acetone and HCN is effectively irreversible, the isolated enzyme catalyzes the reaction also in the reverse direction. The stereoselective addition of HCN to carbonyl groups makes HNLs a useful tool for the biosynthesis of chiral cyanohydrins, which are important intermediates in the production of pharmaceuticals and fine chemicals (Wajant & Effenberger, 1996; Griengl *et al.*, 1997). MeHNL is of

special interest, since the enzyme shows full catalytic activity in organic solvents under rather low pH conditions. Given the biotechnological importance of this enzyme, an X-ray crystallographic study was initiated. We report here crystals of hydroxynitrile lyase from *M. esculenta* which are suitable for a detailed structure analysis.

2. Growth of form I crystals

Recombinant MeHNL was purified to homogeneity as described previously (Wajant *et al.*, 1996). For crystallization, the protein buffer was changed to 10 m*M* sodium acetate pH 5.4 by twice diluting and reconcentrating the enzyme in Centronic 10 tubes (Amicon). The final protein concentration was 15 mg ml^{-1}, determined by UV absorption at 280 nm. The crystallization conditions were screened by the hanging-drop vapour-diffusion method, using the sparse-matrix method (Jancarik & Kim, 1991). The droplets were prepared on siliconized cover slips by mixing 3 µl of the protein solution with 3 µl of the different precipitants and equilibrated over 0.7 ml reservoir solution at 293 K. Despite the use of a wide variety of buffers, precipitants and temperatures, all initial crystallization conditions were unsuccessful; however, precipitations obtained with polyethylene glycol (PEG) or 2-methyl-2,4-pentanediol (MPD) looked promising. Systematic variation of all components of the crystallization solutions gave crystal form I as shown in Fig. 1. The best crystals were grown from a mixture of 5% PEG 8000, 16% MPD in 100 m*M* sodium citrate pH 5.4. For crystallization, 5 µl of the precipitation buffer was mixed with 5 µl of a 30 mg ml^{-1} protein stock solution and equilibrated over 0.7 ml reservoir buffer. The bipyramidal crystals appeared within 3 d and reached a maximum size of 0.8 × 0.8 × 1 mm in two weeks.

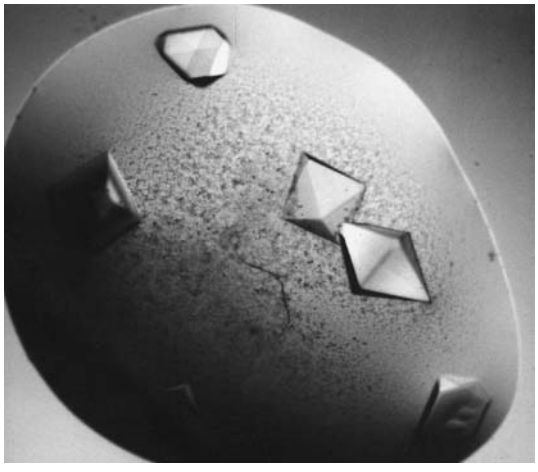

Figure 1
Photograph of tetragonal form I crystals of acetone cyanohydrin lyase from *M. esculenta* (MeHNL) obtained with PEG 8000 and MPD. The largest dimension of the crystals shown is 1.0 mm.

A single crystal with dimensions 0.8 × 0.8 × 0.7 mm was mounted in a glass capillary and a preliminary set of X-ray data was collected to 3.0 Å resolution using a MAR Research 18 cm image-plate system on a Rigaku rotating-anode generator operating at 44 kV and 110 mA at 293 K. The space group was determined using the autoindexing routine of *XDS* (Kabsch, 1988). Analysis of all data showed that the crystals belong to the tetragonal space group $P4_12_12$ or its enantiomorph $P4_32_12$, with $a = b = 105.9$ and $c = 188.9$ Å. The crystals diffract beyond 2.9 Å resolution using conventional Cu $K\alpha$ radiation, but are apparently sensitive to radiation damage. For this reason, conditions for flash-cooling were established by increasing the concentration of MPD as a cryoprotectant. The crystals were transferred stepwise to a final concentration of 6% PEG 8000 and 28% MPD in 100 m*M* sodium citrate pH 5.4. The flash-cooled crystals showed no loss of diffraction quality compared with the wet mounted crystals. X-ray data to 2.2 Å were collected from a crystal of form I using synchrotron radiation from beamline X11 at the EMBL Outstation at DESY, Hamburg (FRG) at a wavelength of 0.902 Å. Data were recorded with a MAR Research 30 cm image-plate detector using 1° oscillations with 10000 exposures per frame; a total of 64 images were collected from one crystal which was flash-frozen at 100 K as described above. Data were indexed and integrated using *DENZO* (Otwinowski, 1993) and scaled and reduced with the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994). The scaled intensities gave an $R_{\rm sym}$ factor of 8.0% and the completeness of the data set was 99.6% (18–2.2 Å) and 92.2% in the outermost resolution shell (2.27 to 2.20 Å).

Biochemical data suggest that the enzyme exists as a dimer in solution (Wajant, unpublished results). Assuming two molecules of hydroxynitrile lyase with a molecular weight of 28500 in the asymmetric unit, the volume per unit mass (V_m) is 4.3 Å^3 Da^{-1}, corresponding to a solvent content of 71%, which is a rather high value compared with a standard set of proteins (Matthews, 1968). Therefore, we have performed a rotation-function analysis to check the local symmetry of the molecules in the unit cell using the *X-PLOR* program suite (Brünger, 1992). The native Patterson map was calculated on a 1 Å grid using intensity data ($F^2 > 2\sigma$) in the resolution range 10–4 Å. The self-rotation function was calculated in a spherical polar angle coordinate system using an inner integration radius limit of 5 Å and an outer integration radius limit of 25 Å. The highest correlation values (σ) for the scans in ψ and φ were plotted against χ, clearly indicating the existence of a non-crystallographic twofold axes (data not shown). This packing model has been further verified by molecular-replacement calculations (space group $P4_12_12$) using the crystal structure of hydroxynitrile lyase from *Hevea brasiliensis* (Wagner *et al.*, 1996) as a search model.

3. Growth of form II crystals

The crystallization trials described above also examined the effect of different salts as precipitants and occasionally produced promising precipitations from buffers between pH 5 and 6. The most promising conditions were extensively screened by the hanging-drop vapour-diffusion method, and the best crystals were obtained from 1.2 *M* lithium sulfate in 100 m*M* sodium citrate pH 5.6. The droplets were prepared by mixing 4 µl of a 12 mg ml^{-1} protein solution in 10 m*M* sodium citrate pH 5.6 with 4 µl of the reservoir solution. Crystals produced in these experiments appeared within one to three months and grew to a maximum size of 0.2 × 0.1 × 0.3 mm, with a tendency to form clusters. The crystals were mechanically sensitive and for data collection they were transformed into a stabilization solution containing 100 m*M* sodium citrate pH 5.4, 1.4 *M* lithium sulfate and 20% glycerol and were immediately flash-cooled to 130 K. A set of X-ray intensity data was collected from a single crystal using a MAR Research 30 cm image-plate system on a Rigaku rotating-anode generator operating at 44 kV and 62 mA with Cu $K\alpha$ radiation. A total of 80 images of 1° rotation increments were taken (each taking 45 min) at a crystal-to-detector distance of 170 mm. The raw data were processed with *DENZO* (Otwinowski, 1993) and scaled and reduced with *AGROVATA*/*ROTAVATA* (Collaborative Computational Project, Number 4, 1994). A total of 171811 observations of 57893 unique reflections to 2.2 Å resolution were collected. The resulting data set is 96.9% complete with an overall $R_{\rm sym}$ on intensities

of 7.3%. The highest resolution shell (2.28–2.20 Å) is 98.7% complete with an R_{sym} of 13.3%. Examination of these data shows that the form II crystals belong to the orthorhombic system, with unit-cell parameters $a = 117.52, b = 127.09, c = 78.08$ Å. The data provide clear evidence that the 117.52 and 127.09 Å axes are twofold screws, while the 78.08 Å axis seems to be a pure dyad. However, not all reflections along this axis were recorded in this data set. These preliminary observations identify the space group as $P2_12_12$.

Given the difficulties in reproducing the form II crystals, efforts have been focused on the tetragonal crystal form I. The structure determination of these crystals, in combination with our ongoing mutagenesis and biotechnological studies, should help to yield a more detailed picture of the enzyme mechanism and the phylogenetic relationship between hydroxynitrile lyases from different species.

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