Acta Cryst. (1996). D52, 887–889

Crystallographic studies and preliminary X-ray investigation of (*S*)-*p*-hydroxy-mandelonitrile lyase from *Sorghum bicolor* (*L.*)

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(*Received 11 December 1995; accepted 16 February 1996*)

Abstract

(*S*)-*p*-Hydroxy-mandelonitrile lyase from *Sorghum bicolor* has been crystallized in three different forms using the hanging-drop vapor-diffusion technique. Crystal form I is obtained from 1.4 *M* $(NH_4)_2SO_4$ in 100 m*M* Na-acetate, pH 4.6, and belongs to the orthorhombic space group $P2_12_12_1$. The cell dimensions are $a = 71.4$, $b = 95.8$, $c = 149.1$ Å. A complete set of diffraction data has been collected to 2.6 Å resolution. Form II crystals are grown from 500 m*M* Li_2SO_4 in 13% polyethylene glycol 8000. These crystals appear as hexagonal plates and diffract to 2.98 Å resolution but apparently are twinned. Cocrystallizing hydroxynitrile lyase with the inhibitor benzoic acid using 1.4 *M* $(NH_4)_2SO_4$ in 100 m*M* Na citrate, pH 5.4 as precipitant yields crystal form III, which belongs to the monoclinic space group $C2$ with $a = 150.7$, $b = 103.7$, $c = 90.6$ Å, $\beta = 101.3$. X-ray diffraction data were collected to 2.3 Å resolution.

1. Introduction

Cyanogenesis, the release of hydrogen cyanide from damaged tissues, has been described for many species of higher plants and is thought to be part of a defence mechanism against herbivores or fungi, respectively (Seigler, 1991; Nahrstedt, 1985). Hydroxynitrile lyases (HNL's) are involved in cyanogenesis through their ability to catalyze the stereospecific retro-addition of a large number of aliphatic, aromatic and heterocyclic cyanohydrines into toxic hydrogen cyanide and a corresponding carbonyl compound, respectively (Gerstner & Pfeil, 1972; Seigler, 1975).

Two groups of lyases from various angiosperms have been identified (Poulton, 1988). HNL's of group I contain a flavine-adenine dinucleotide (FAD) cofactor, are typically single-chain glycoproteins showing remarkable serological cross-reactivity (Gerstner & Pfeil, 1972) and catalyze exclusively the decomposition of *R*-cyanohydrines. The second group of lyases is FAD-cofactor independent and forms a more heterogeneous group of enzymes, distinct in molecular mass, subunit composition, glycoprotein nature, substrate specificity and stereoselectivity of the enzymatic reaction (Xu, Singh & Conn, 1988; Kuroki & Conn, 1989; Hughes, de Carvalho & Hughes, 1994; Wajant & Mundry, 1993). It has been suggested that this group of enzymes has originated by convergent evolution from different ancestral enzymes.

(*S*)-Hydroxynitrile lyase from *Sorghum bicolor* (*L.*) (Sb-HNL, E.C. 4.1.2.11) catalysing the dissociation of (*S*)-*p*-hydroxy-mandelonitrile into *p*-hydroxy-benzaldehyde and hydrogen cyanide (Bové & Conn, 1961) consists of two different polypeptide chains designated α (33 kDa) and β (22 kDa). The active enzyme form of Sb-HNL is a $\alpha_2\beta_2$ heterotetramer assembled as a dimer of $\alpha\beta$-dimers (Wajant & Mundry, 1993). The enzyme is *N*-glycosylated and exists in three different isoforms (Sb-HNL I, Sb-HNL II, Sb-HNL III). Differences between the isoenzymes may arise from variations in their primary structure and/or from heterogeneity in their carbohydrate contents.

Whereas the primary structure of Sb-HNL and other hydroxynitrile lyases fail to show significant homology, sequence comparison of Sb-HNL and wheat serine carboxypeptidase (CPD-W II) reveals 55% amino-acid identity and 73% similarity when conservative replacements are considered (Wajant, Mundry & Pfitzenmaier, 1994). Residues of CPD-W II identified in crystallographic studies (Bullock, Branchand & Remington, 1994) to be located in the active site are also conserved in Sb-HNL, implying that the catalytic mechanism of the two enzymes might be similar. In addition, residues of both subunits in CPD-W II that are involved in forming the $\alpha\beta$-dimer are conserved in Sb-HNL. A sequence alignment between the two enzymes also predicts a similar glycosylation pattern. Despite these common biochemical properties, hydroxynitrile lyase from *Sorghum* exerts no peptidase activity towards a standard set of potential substrates (H. Wajand, unpublished results).

In addition to its physiological role, Sb-HNL is important as a biocatalyst for the stereoselective synthesis of cyanohydrines (Effenberger, Ziegler & Förster, 1987; Effenberger, Hörsch, Förster & Ziegler, 1990). Chiral cyanohydrines can be transformed into optically active α-hydroxy acids, α-hydroxy ketones or β-ethanolamines, respectively, which are important building blocks for pharmaceuticals (Smitskamps-Wilms, Brussee, van der Gen, van Scharrenburg & Sloothaak, 1991).

In this paper we report crystal forms of hydroxynitrile lyase from *Sorghum* suitable for a high-resolution X-ray structure analysis. The determination of the three-dimensional crystal structure of Sb-HNL should help to understand how lyases of this type interact stereoselectively with substrates and in particular should clarify how sequence substitutions compared to carboxypeptidase II from wheat affect structure and function of this enzyme.

2. Material and methods

The inhibitor, benzoic acid, was obtained from Aldrich, all other chemicals and reagents used were of analytical grade. Protein concentrations were determined with the BCA protein assay (Pierce). (*S*)-*p*-hydroxy-mandelonitrile lyase activity was assayed according to Bové and Conn (Bové & Conn,

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1961). Sb-HNL was isolated and purified by affinity chromatography using monoclonal antibodies as described previously (Wajant, Böttiger & Mundry, 1993). A separation of the three Sb-HNL isoenzymes was obtained by additional ion-exchange chromatography. Sb-HNL was loaded onto a Q-Sepharose HP column (Pharmacia; 1.6 × 20 cm), equilibrated with 20 m*M* piperazine/acetate, pH 5.5 (buffer *A*). The isoenzymes were eluted from the column as single peaks by applying a 2 l linear sodium acetate gradient (0–500 m*M* CH_3COONa) in buffer *A*. Isoenzye I was eluted at 230 m*M* CH_3COONa, isoenzyme II at 265 m*M* CH_3COONa and isoenzyme III at 285 m*M* CH_3COONa, respectively (data not shown). Homogeneity of the isoenzyme preparations was checked by Coomassie-blue stained SDS–PAGE and isoelectric focusing (data not shown).

For crystallization the isoenzyme samples were dialysed against 10 m*M* Na-acetate, pH 4.6 and concentrated to 13 mg ml^{-1} using an Amicon concentrator with a YM10 membrane. Hydoxynitrile lyase–benzoic acid complex (Sb-HNL–BA) solutions were prepared by mixing nine volumes of 13 mg ml^{-1} protein stock solution with one volume of 100 m*M* benzoic acid in 100 m*M* sodium citrate, pH 5.4. Solutions of Sb-HNL–BA were incubated for 1 h at room temperature to allow binding of the inhibitor prior to exposure to precipitants in the crysallization procedure. The inhibitory effect of benzoic acid was shown for HNL from *Prunus lyonii* (Xu, Singh & Conn, 1986) and for Sb-HNL [S. Förster, unpublished results]. The crystallization experiments were performed at 293 K using the hanging-drop vapor-diffusion method (McPherson, 1976). Initial crystallization conditions were screened using the sparse-matrix sampling technique (Jancarik & Kim, 1991). Drops were prepared by mixing 4 µl of Sb-HNL or Sb-HNL–BA stock solution (see above), respectively, with equal volumes of the precipitant buffer. Drops were placed on siliconized cover slips and suspended over 0.7 ml of the corresponding buffer. For those initial conditions that gave crystals, a fine grid of parameter variations was applied for optimization. To improve crystal quality microseeding was also used (Stura & Wilson, 1990). For X-ray studies crystals were transferred to a stabilizing solution of the appropriate buffer and carefully washed. Crystals were mounted in glass capillaries which were sealed with epoxy resin.

All diffraction intensities were collected on a MAR image-plate detector using graphite-monochromatized Cu $K\alpha$ radiation produced with an Enraf–Nonius FR 571 rotating-anode generator operating at 45 kV and 80 mA. Crystal parameters were determined using the auto-indexing option of the *XDS* data processing package (Kabsch, 1988) and post refined during data evaluation using *IPMOSFLM* (Leslie, 1992). The three-dimensional data sets were collected in 1° oscillation steps, exposing every image for 20 min. For data collection of the orthorhombic form I, the crystal-to-detector distance was set to 130 mm, for data collection of the monoclinic form III to 110 mm, respectively. The data sets were processed with *IPMOSFLM* (Leslie, 1992) and statistics of the data are summarized in Table 1.

Table 1. *Crystallographic parameters and data-collection statistics*

Parameters	Form I	Form III
Space group	$P2_12_12_1$	$C2$
Unit cells (Å, °)	71.4 × 95.8 × 149.1	150.7 × 103.7 × 90.6, $\beta = 101.3$
Molecules per asymmetric unit	2	2
V_m (Å^3 Da^{-1})	2.32	3.16
Solvent content (%)	46.9	61.0
Max. resolution (Å)	2.2	2.1
Resolution of data collection (Å)	2.6	2.3
Resolution of last shell (Å)	2.69–2.60	2.38–2.30
Total observations	92854	100992
Unique reflections	30658	51747
Completeness (%)	97.4	88.7
Completeness of last shell (%)	90.4	82.9
Average $I/\sigma(I)$	5.8	8.5
Average $I/\sigma(I)$ in last shell	3.5	5.7
R_{sym} (I) for all data	0.087	0.059
R_{sym} (I) for last shell	0.170	0.114

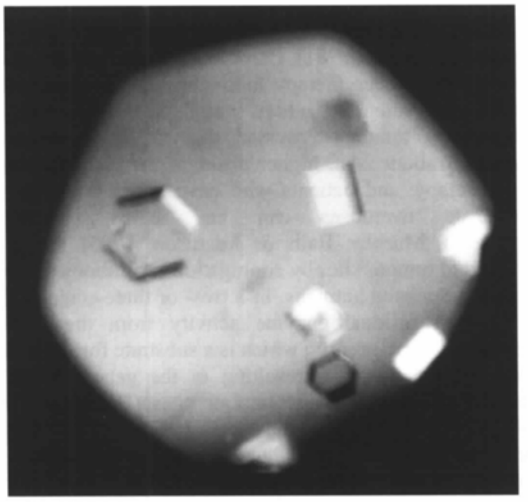
3. Results and discussion

Three different crystal forms of *Sorghum* hydroxynitrile lyase isoenzymes have been obtained. Form I crystals (Table 1) were obtained using a reservoir solution of 1.4 *M* $(NH_4)_2SO_4$ in 100 m*M* Na-acetate, pH 4.6. The crystals are well defined plates with maximum dimensions of 1.0 × 0.8 × 0.3 mm and appear frequently within a week. The unit-cell dimensions of the crystals were determined from 1139 reflexions using the auto-indexing routine of *XDS*. The data indicate that the crystals belong to the orthorhombic space group $P2_12_12_1$ with cell dimensions $a = 71.4$, $b = 95.8$, $c = 149.1$ Å after post refinement. These crystals diffract to about 2.2 Å resolution. With two molecules in the asymmetric unit, V_m is 2.32 Å^3 Da^{-1}, within the range observed for a standard set of proteins (Matthews, 1968). For this crystal form a native X-ray data set was collected to 2.6 Å resolution. Details of data processing are given in Table 1.

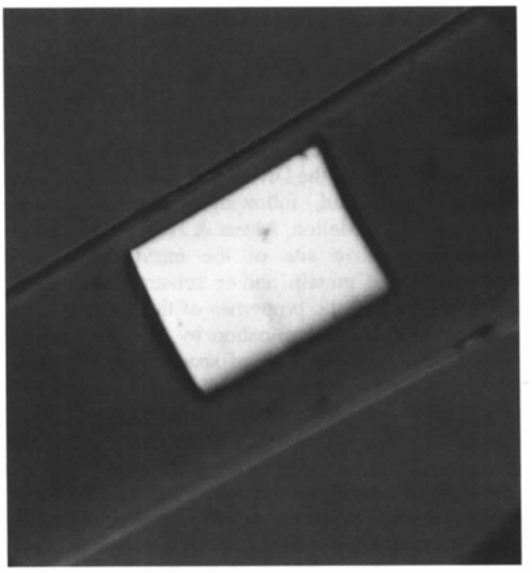
Form II crystals (Fig. 1*a*) were obtained using a reservoir solution of unbuffered 500 m*M* Li_2SO_4 in 13% PEG 8000. These crystals appear as hexagonal plates and grow to a maximum size of 0.6 × 0.5 × 0.5 mm within 10 d. Form II crystals show one very long axis. Even the setting of the crystal-to-detector distance to a maximum of 400 mm could not ensure that all diffraction spots in the low-resolution shell are well separated and only approximate unit-cell parameters could therefore be obtained. Using the auto-indexing option of *XDS*, the space group deduced from ten images collected over an angular range of 90° is rhombohedral $R3$ with cell parameters (hexagonal setting) $a = b = 70$, $c = 510$ Å. The translation of these parameters into the corresponding rhombohedral cell leads to $a' = 175$ Å and an unusually small angle of $\alpha' = 23.1°$. A more detailed examination of the images shows that the axes a and b can be clearly verified, while reflections along c^* are not perfectly arranged on a single lattice. One explanation would be that these reflections do not derive from one lattice and that there is a twinning along the c axis.

Form III crystals (Fig. 1*b*, Table 1) were obtained in the presence of the inhibitor benzoic acid, using 1.6 *M* $(NH_4)_2SO_4$ in 100 m*M* Na-citrate, pH 5.4, as precipitant. Crystals appear within a few days as clusters of small plates and show a pronounced tendency to interpenetrate. Using microseeding, crystal quality and yield were dramatically improved. Single crystals with dimensions up to 1.0 × 1.0 × 0.3 mm could be obtained within a week. The

crystallographic unit cell was determined from 2249 reflections using the *XDS* program. These crystals belong to the monoclinic space group $C2$ with cell dimensions $a = 150.7$, $b = 103.7$, $c = 90.6$ Å and $\beta = 101.3°$. Crystals diffract to 2.1 Å resolution. With two molecules of Sb-HNL-BA binary complex in the asymmetric unit V_m is 3.16 Å^3 Da^{-1}, corresponding to 61% solvent. A 2.3 Å resolution native data set has been collected for crystal form III. Statistics of the data processing are given in Table 1.



(*a*)



(*b*)

Fig. 1. Photographs of crystals of (*S*)-*p*-hydroxy mandelonitrile lyase of *Sorghum* (Sb-HNL). Crystal habits for (*a*) rhombohedral form II obtained with Li_2SO_4 and PEG 8000, and (*b*) monoclinic form III grown in presence of the inhibitor benzoic acid using $(NH_4)_2SO_4$, Na-citrate, pH 5.3 as precipitants. The largest dimension of crystals shown is 1.0 mm.

Based on the V_m values it is likely that for the orthorhombic and monoclinic crystal forms two molecules of $\alpha\beta$ Sb-HNL occupy the asymmetric unit, respectively. This interpretation is in agreement with gel-filtration chromatography data showing the existence of a heterotetramer $\alpha_2\beta_2$-Sb-HNL as the only active form of the enzyme (Wajant & Mundry, 1993).

The structure determination of the monoclinic hydroxynitrile lyase–benzoic acid complex by molecular replacement using the crystal structure of wheat serine carboxypeptidase II as a model is currently underway.

We thank Professor W. Saenger for use of data-collection facilities in Berlin. We also thank Professor U. Heinemann for his encouragement and support and for critical reading of the manuscript. This study was supported by grants from the Bundesministerium für Forschung under Technologie under A03U and A10U.

References

Bové, C. & Conn, E. E. (1961). *J. Biol. Chem.* **236**, 201–210.
Bullock, T. L., Branchand, B. & Remington, S. J. (1994). *Biochemistry*, **33**, 11127–11134.
Effenberger, F., Hörsch, B., Förster, S. & Ziegler, T. (1990). *Tetrahedron Lett.* **31**, 1249–1250.
Effenberger, F., Ziegler, T. & Förster, S. (1987). *Angew. Chem. Int. Ed. Engl.* **26**, 458–462.
Gerstner, E. & Pfeil, E. (1972). *Hoppe-Seylers Z. Physiol. Chem.* **353**, 271–286.
Hughes, J., de Carvalho, F. J. P. & Hughes, M. A. (1994). *Arch. Biochem. Biophys.* **311**, 496–502.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 40-411.
Kabsch, W. (1988). *J. Appl. Cryst.* **21**, 916–924.
Kuroki, G. W. & Conn, E. E. (1989). *Proc. Natl Acad. Sci. USA*, **86**, 6978–6981.
Leslie, A. G. W. (1992). *Jnt CCP4 and ESF-EACBM Newslett. Protein Crystallogr.* **26**, 10–14.
McPherson, A. (1976). *Methods Biochem. Anal.* **23**, 249–345.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Nahrstedt, A. (1985). *Pt. Syst. Evol.* **150**, 35–47.
Poulton, J. E. (1988). *Localization and Catabolism of Cyanogenic Glycosides*, in *Cyanide Compounds in Biology*, edited by D. Evered & S. Harnett, Ciba Foundation Symposium No. 140, pp. 67–91. Chichester, England: John Wiley.
Seigler, D. S. (1975). *Phytochemistry*, **14**, 9–29.
Seigler, D. S. (1991). *Cyanide and Cyanogenic Glycosides*, in *Herbivores, Their Interactions with Secondary Plant Metabolites*, edited by G. A Rosenthal & M. R. Berebaum, Vol. 1, pp. 35–77. San Diego: Academic Press.
Smitskamps-Wilms, E., Brussee, J., van der Gen, A., van Scharrenburg, G. J. M. & Sloothaak, J. B. (1991). *Recl. Trav. Chim. Pays-Bas*, **110**, 209–215.
Stura, E. A. & Wilson, I. A. (1990). *Analytical and Production Seeding Techniques*, in *Methods: A Companion to Methods in Enzymology*, edited by Ch. W. Cantor, Vol. 1, No. 1, pp. 38–49. San Diego: Academic Press.
Wajant, H., Böttiger, H. & Mundry, K.-W. (1993). *Biotechnol. Appl. Biochem.* **18**, 75–82.
Wajant, H. & Mundry, K.-W. (1993). *Plant Sci.* **89**, 127–133.
Wajant, H., Mundry, K.-W. & Pfizenmaier, K. (1994). *Plant Mol. Biol.* **26**, 735–746.
Xu, L. L., Singh, B. K. & Conn, E. E. (1986). *Arch. Biochem. Biophys.* **250**, 322–328.
Xu, L.-L., Singh, B. K. & Conn, E. E. (1988). *Arch. Biochem. Biophys.* **263**, 256–263.