Acta Cryst. (1996). D**52**, 591-593

Crystallization and preliminary X-ray diffraction studies of a hydroxynitrile lyase from *Hevea brasiliensis*

ULRIKE G. WAGNER,[a] MICHAEL SCHALL,[b] MEINHARD HASSLACHER,[c] MARIANNE HAYN,[b] HERFRIED GRIENGL,[d] HELMUT SCHWAB[b] AND CHRISTOPH KRATKY[a]* *at* [a]*Institut für Physikalische Chemie, Karl-Franzens-Universität Graz, A-8010 Graz, Austria,* [b]*Institut für Biochemie, Karl-Franzens-Universität Graz, A-8010 Graz, Austria,* [c]*Institut für Biotechnologie, Arbeitsgruppe Genetik, Erzherzog-Johann-Universität, A-8010 Graz, Austria, and* [d]*Institut für Organische Chemie, Erzherzog-Johann-Universität, A-8010 Graz, Austria. E-mail: kratky@bkfug.kfunigraz.ac.at*

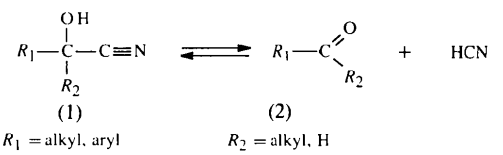
(*Received 25 September 1995; accepted 13 December 1995*)

Abstract

Crystals of the hydroxynitrile lyase from *Hevea brasiliensis* overexpressed in *Pichia pastoris* have been obtained by the hanging-drop technique at 294 K with ammonium sulfate and PEG 400 as precipitants. The crystals belong to the orthorhombic space group $C222_1$ with cell dimensions of a = 47.6, b = 106.8 and c = 128.2 Å. The crystals diffract to about 2.5 Å resolution on a rotating-anode X-ray source.

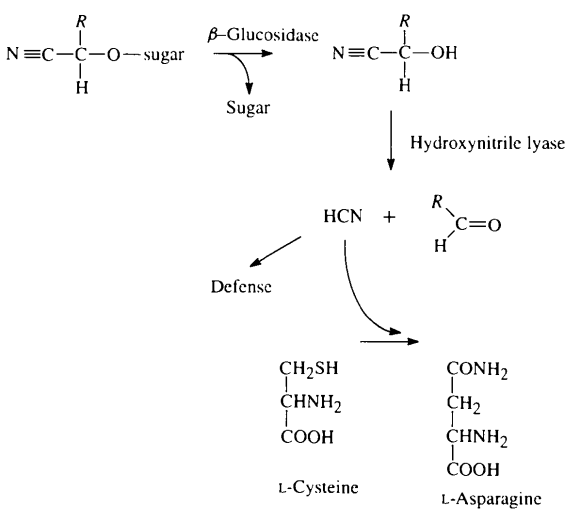
1. Introduction

Hydroxynitrile lyases (HNL's) catalyze the equilibrium between α-hydroxynitriles (cyanohydrins) (1) and the corresponding aldehydes (R_2 = H) or ketones ($R_2 \neq$ H) (2) and hydrogen cyanide:



Our interest in HNL is due to the following reasons.

(i) The biological function of HNL (see scheme below) is the cleavage of an α-hydroxynitrile (formed by β-glycosidase-mediated cleavage of a cyanogenic mono- or disaccharide) to yield hydrocyanic acid (HCN) and an aldehyde. Since both HCN and the aldehyde are toxic, the HNL reaction forms an efficient defense of the plant against herbivoral, fungal or animal attack (Nahrstedt, 1995). A second biological function of cyanogenesis rests with the utilization of the liberated HCN for the synthesis of the asparagine (Lieberei, Selmar & Biehl, 1985; Selmar, 1993).



(ii) Enzyme-catalyzed cyanohydrin formation has recently been recognized as a powerful tool for the synthesis of intermediates for the preparation of enantiopure pharmaceuticals (Kruse, 1992; Effenberger, 1994).

The cyanohydrin (1) is a chiral compound whenever R_1 and R_2 are different. Enantiomeric excesses above 99% have been achieved by enzyme-catalyzed formation of chiral (1). So far, enzymes with (*R*)-selectivity [isolated from *Prunus sp.* (Wajant & Mundry, 1993; Jansen, Woker & Kula, 1992; Smitskamp-Wilms, Brussee, van der Gen, van Scharrenburg & Sloothaak, 1991) and *Linum usitatissimum* (Xu, Singh & Conn, 1988; Albrecht, Jansen & Kula, 1993; Wajant, Riedel, Benz & Mundry, 1994)] and (*S*)-selectivity [from *Sorghum bicolor* (Jansen, Woker & Kula, 1992; Smitskamp-Wilms, Brussee, van der Gen, van Scharrenburg & Sloothaak, 1991; Wajant, Riedel, Benz & Mundry, 1994; Wajant, Mundry & Pfizenmaier, 1994), *Ximenia americana* (van Scharrenburg, Sloothaak, Kruse, Smitskamp-Wilms & Brussee, 1993; Bové & Conn, 1961), *Manihot esculenta* (Hughes, Carvalho & Hughes, 1994) and *Hevea brasiliensis* (Selmar, Lieberei, Biehl & Conn, 1989; Klempier, Griengl & Hayn, 1993; Hickel *et al.*, 1994)] have been investigated in more detail. Among all known hydroxynitrile lyases, the enzyme from almonds (*Prunus sp.*) is FAD-dependent, while all others require no coenzyme. The enzymes from *Prunus sp.* are typically single-chain proteins with molecular weights between 60 and 80 kDa. The other enzymes exist in homodimeric and heterodimeric states; molecular weights range from 80 to 110 kDa for the native enzyme, and from 22 to 42 kDa for the subunits. Several of these enzymes are also glycosylated. Owing to the importance of these enzymes, the majority of them have been cloned and sequenced. Recently, crystallization and preliminary X-ray diffraction studies were published for the hydroxynitrile lyase from almonds (Lauble, Müller, Schindelin, Förster & Effenberger, 1994). This prompts us to report on our structural studies of the hydroxynitrile lyase from the rubber-tree *Hevea brasiliensis*.

Compared to the HNL from almonds, the *Hevea brasiliensis* enzyme differs in several respects. Most importantly the *Hevea* enzyme does not have FAD as a cofactor and shows the opposite selectivity (*S*) to the enzyme from almonds.

No significant sequence homology could be found between HNL from *Hevea brasiliensis* and known protein strucures. A secondary structure prediction (Rost & Sander, 1993*a*,*b*, 1994)

and subsequent alignment with proteins of known three-dimensional structure showed maximum homology with haloalkane dehalogenase (Verschueren, Seljée, Rozeboom, Kalk & Dijkstra, 1993) and bromoperoxidase (Hecht, Sobek, Haag, Pfeifer & van Pée, 1994).

Thus, it is quite likely that the two known classes of HNL's – of which the enzymes from almonds and from *Hevea brasiliensis* are prototypical members – have evolved by convergent evolution from different precursors (Hickel, Griengl & Hasslacher, 1996), such as FAD-dependent oxidoreductases (almonds) and α/β-hydrolases (*Hevea*).

The HNL from *Hevea brasiliensis* is a non-glycosylated homodimeric protein with a monomer molecular weight of 30 kDa. It has an isoelctric point of 4.1 and shows maximum catalytic activity at a pH between 5.5 and 6.0. While its natural substrate is acetone cyanohydrin, it catalyzes cyanohydrin formation with a variety of aliphatic, aromatic and heterocyclic aldehydes (Klempier, Griengl & Hayn, 1993; Klempier, Pichler & Griengl, 1995). The broad range of substrates makes it an ideal candidate for biocatalytic applications.

2. Experimental

The recombinant hydroxynitrile lyase protein from *Hevea brasiliensis* was overexpressed in yeast (*Pichia pastoris*) and purified to homogeneity. Crystals were grown at 294 K using the hanging-drop vapor-diffusion method (McPherson, 1976) with Linbro multi-well tissue plates. Drops containing 3 µl of 9 mg ml^{-1} protein solution and 3 µl precipitant buffer were equilibrated against 700 µl precipitant buffer. The reservoir contained 2% PEG 400, 2.0 *M* ammonium sulfate in 0.1 *M* NaHepes (pH = 7.5). It took several weeks to obtain the first crystals. For subsequent trials we applied the macro-seeding technique (Stura & Wilson, 1992), which enabled us to obtain crystals in a few days. Without seeding, crystals did not grow spontaneously in the majority of drops.

Since crystals mounted with mother liquor in capillaries deteriorated in the X-ray beam at room temperature, we applied cryotemperature data-collection techniques. Crystals were transferred to a mixture of reservoir solution with 30%(*v*/*v*) glycerol. After soaking for about 1 min, the crystals were mounted in a loop made of polymeric fibre (Teng, 1990; Gamblin & Rodgers, 1993) and shock cooled by dumping into liquid nitrogen. At 98 K (temperature of the nitrogen stream near the crystal) the crystals were stable during data collection on a rotating-anode X-ray source (Cu $K\alpha$ radiation, 40 kV, 80 mA, 0.3 × 0.3 mm apparent focal spot size, graphite monochromator, collimator size 0.3 mm) equipped with a three-circle goniometer, a Siemens X-1000 area detector and a homemade N_2 gas-stream cryostat. A data set up to 2.4 Å resolution was collected at 100 K with a crystal-to-detector distance of 10 cm, using scans of 0.2° framewidth. The collected data set was indexed and processed with the program *XDS* (Blum, Metcalf, Harrison & Wiley, 1987; Kabsch, 1988*a*,*b*).

3. Results and discussion

The overexpressed hydroxynitrile lyase crystallizes with ammonium sulfate and PEG 400 as the precipitating agents at pH 7.5. Large crystals can only be obtained using macro-seeding techniques up to final sizes of about 0.4 × 0.4 × 0.08 mm. They diffract to 2.5 Å resolution. At cryogenic temperatures (100 K) the crystals are stable during collection of a complete data set (Fig. 1).

Table 1. *Data-collection statistics and completeness of the data set*

Number of measured reflections	41707
Number of unique reflections	11115
R_{sym}* (%)	7.1%

	Completeness (%)	
Resolution (Å)	$I > 0\sigma(I)$	$I > 3\sigma(I)$
20.0–4.98	99.1	97.9
4.98–3.93	99.2	97.9
3.96–3.46	99.3	97.6
3.46–3.15	99.0	96.8
3.15–2.92	93.3	89.8
2.92–2.75	90.5	85.7
2.75–2.61	83.7	78.3
2.61–2.50	67.9	61.4

* $R_{sym} = \sum_h \sum_i |I_{ih} - \langle I_h \rangle| / \sum_h \sum_i \langle I_h \rangle$, where $\langle I_h \rangle$ is the mean intensity of the i observations of reflection h.

The cell was identified to be orthorhombic with cell dimensions of a = 47.6, b = 106.8, c = 128.2 Å and systematic absences consistent with the symmetry of space group $C222_1$. The standard crystallographic merging R factor (on intensities) was 7.1%. The space-group symmetry, the unit-cell volume of 6.7×10^5 Å^3 and the molecular mass of 30 kDa for the protein leads to a V_m of 2.8 Å^3 Da^{-1} for a monomer in the asymmetric unit. This is in the expected range of 1.6–5.3 Å^3 Da^{-1} for V_m (Matthews, 1968), and the corresponding solvent content of 56% seems reasonable according to the mechanical stability of the crystal. A search for non-crystallographic symmetry yielded no pronounced peak in a self-rotation function (program *POLARRFN* from the *CCP*4 package; Collaborative

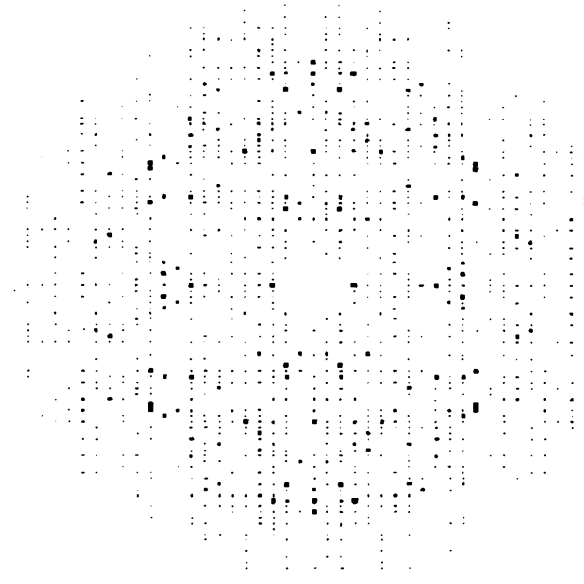


Fig. 1. Diffraction pattern of the 011 plane simulated from the *hkl* file by the program *HKLVIEW* of the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

Computational Project, Number 4, 1994). Since the protein occurs in solution in homodimeric state (molecular weight 60 kDa, Hickel, Griengl & Hasslacher, 1996), it is likely that in the crystal the two dimer halves are related by a crystallographic diad.

A complete native data set was collected from one crystal on a rotating anode at a wavelength of 1.54 Å. Table 1 lists the data-collection statistics and completeness of the data set after processing.

A search for heavy-atom derivatives is underway, since attempts to solve the structure by molecular replacement with the coordinates of haloalkane dehalogenase (Verschueren, Seljée, Rozeboom, Kalk & Dijkstra, 1993) have failed.

References

Albrecht, J., Jansen, I. & Kula, M.-R. (1993). *Biotechnol. Appl. Biochem.* **17**, 1991–203.

Blum, M., Metcalf, P., Harrison, S. C. & Wiley, D. C. (1987). *J. Appl. Cryst.* **21**, 235–242.

Bové, C. & Conn, E. E. (1961). *J. Biol. Chem.* **236**(1), 207–210.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Effenberger, F. (1994). *Angew. Chem.* **106**, 1609–1619.

Gamblin, S. & Rodgers, D. W. (1993). *Proceedings of the CCP4 Study Weekend*, pp. 28–32. Daresbury Laboratory, Warrington, England.

Hecht, H. J., Sobek, H., Haag, T., Pfeifer, O. & van Pée, K.-H. (1994). *Struct. Biol.* **1**, 532–537.

Hickel, A., Griengl, H., Gradnig, G., Sterk, H., Hayn, M. & Schall, M. (1994). *Österreichische Chemietage*, 28–30 September, Graz, Austria. Abstract 8.

Hickel, A., Griengl, H. & Hasslacher, M. (1996). *Plant Cell.* Submitted.

Hughes, F. J. P., Carvalho, De C. & Hughes, M. A. (1994). *Arch. Biochem. Biophys.* **311**, 496–502.

Jansen, I., Woker, R. & Kula, M.-R. (1992). *Biotechnol. Appl. Biochem.* **15**, 90–99.

Kabsch,W. (1988*a*). *J. Appl. Cryst.* **21**, 67–71.

Kabsch, W. (1988*b*). *J. Appl. Cryst.* **21**, 916–924.

Klempier, N., Griengl, H. & Hayn, M. (1993). *Tetrahedron Lett.* **34**, 4769–4772.

Klempier, N., Pichler, U. & Griengl, H. (1995). *Tetrahedron Assymmetry*, **6**(4), 845–848.

Kruse, C. G. (1992). *Chiral Cyanohydrines – Their Manufacture and Utility as Chiral Building Blocks. Chirality in Industry*, pp. 279–299. Chichester: John Wiley.

Lauble, H., Müller, K., Schindelin, H., Förster, S. & Effenberger, F. (1994). *Proteins Struct. Funct. Genet.* **19**, 343–347.

Lieberei, R., Selmar, D. & Biehl, B. (1985). *Pl. Syst. Evol.* **150**, 49–63.

Nahrstedt, A. (1985). *Pl. Syst. Evol.* **150**, 35–47.

McPherson, A. (1976). *Methods Biochem. Anal.* **23**, 249–345.

Matthews, B. M. (1968). *J. Mol. Biol.* **33**, 491–497.

Rost, B. & Sander, C. (1993*a*). *Proc. Natl Acad. Sci. USA*, **90**, 7558–7562.

Rost, B. & Sander, C. (1993*b*). *J. Mol. Biol.* **232**, 584–599.

Rost, B. & Sander, C. (1994). *Proteins*, **19**, 55–72.

Scharrenburg, G. J. M. van, Sloothaak, J. B., Kruse, C. G., Smitskamp-Wilms, E. & Brussee, J. (1993). *Ind. J. Chem.* **32b**, 16–19.

Selmar, D. (1993). *Apoplastic Occurrence of Cyanopenic Beta-Glycosidases and Consequences for the Metabolism of Cyanopenic Glycosides, ACS Symp. Ser.*, ch. 13, pp. 191–204. Washington, DC: American Chemical Society.

Selmar, D., Lieberei, R., Biehl, B. & Conn, E. E. (1989). *Physiol. Plant*, **75**, 97–101.

Smitskamp-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. (1992). *Crystallization of Nucleic Acids and Proteins. A Practical Approach*, edited by A. Ducruix & R. Giegé. New York: Oxford University Press.

Teng, T.-Y. (1990). *J. Appl. Cryst.* **23**, 387–391.

Verschueren, K. H. G., Seljée, F., Rozeboom, H. J., Kalk, K. H. & Dijkstra, B. W. (1993). *Nature (London)*, **363**, 693–698.

Wajant, H. & Mundry, K.-W. (1993). *Plant Sci.* **89**, 127–133.

Wajant, H., Mundry, K.-W. & Pfizenmaier, K. (1994). *Plant Mol. Biol.* **26**, 735.

Wajant, H., Riedel, D., Benz, S. & Mundry, K.-W. (1994). *Plant Sci.* **103**, 145–154.

Xu, L.-L., Singh, B. K. & Conn, E. E. (1988). *Arch. Biochem. Biophys.* **263**(2), 256–263.