Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Purification, crystallization and preliminary X-ray crystallographic analysis of hydroxycinnamoyl-coenzyme A hydratase-lyase (HCHL), a crotonase homologue active in phenylpropanoid metabolism

Philip M. Leonard,[a] Caroline M. Marshall,[a] Eleanor J. Dodson,[a] Nicholas J. Walton[b] and Gideon Grogan[a]*

[a]York Structural Biology Laboratory, Department of Chemistry, University of York, Heslington, York YO10 5YW, England, and [b]Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, England

Correspondence e-mail: gg12@york.ac.uk

Received 13 August 2004
Accepted 30 September 2004

4-Hydroxycinnamoyl-coenzyme A hydratase-lyase (HCHL), also called feruloyl-CoA hydratase-lyase (FCHL), from *Pseudomonas fluorescens* strain AN103 is an enzyme of the crotonase superfamily that catalyses the one-step conversion of the CoA thioesters of 4-coumaric acid, caffeic acid and ferulic acid to the aromatic aldehydes 4-hydroxybenzaldehyde, protocatechuic aldehyde and vanillin, respectively. The reaction occurs *via* a hydration followed by a carbon–carbon bond-cleavage reaction. HCHL has been crystallized by the hanging-drop method of vapour diffusion using polyethylene glycol 20 000 Da as the precipitant. The crystals belong to the orthorhombic system, with proposed space group $P2_12_12$ and unit-cell parameters $a = 154.2$, $b = 167.5$, $c = 130.8$ Å. The V_M suggests that the asymmetric unit contains four trimers. Single-wavelength data collection has been undertaken and structure determination is under way by molecular replacement using data collected to 1.8 Å resolution. Determination of the structure of HCHL will provide insight into the catalytic mechanism of an unusual enzymatic reaction with relevance to the applications of the enzyme in metabolic engineering.

1. Introduction

4-Hydroxy-*trans*-cinnamic acids and their coenzyme A (CoA) thioesters are of major importance in plant metabolism as precursors of lignin, cell-wall phenolic components and defensive compounds (Dixon & Paiva, 1995). Ferulic acid (4-hydroxy-3-methoxy-*trans*-cinnamic acid) in particular is a widespread component of plant cell walls and its catabolism is essential to the overall biodegradation of plant-derived wastes.

Until comparatively recently, no biochemical mechanism for the degradation of cinnamic acids had been elucidated. The first biochemical and molecular-genetic characterization was reported from *Pseudomonas fluorescens* strain AN103, an organism selected for growth on ferulic acid as the sole carbon source. *P. fluorescens* AN103 was shown to degrade ferulic acid (**1**) *via* the pathway shown in Fig. 1, wherein vanillin occurs as an intermediate (Gasson *et al.*, 1998). (**1**) is first coupled to coenzyme A in an ATP-dependent reaction to form feruloyl-CoA (**2**). (**2**) is then hydrated to yield the intermediate 4-hydroxy-3-methoxyphenyl-β-hydroxypropionyl-CoA (HMPHP-CoA) (**3**), which subsequently undergoes C—C bond cleavage to yield the aldehyde vanillin (**4**) and acetyl-CoA. Both hydration and C—C bond-cleavage reactions were determined to be catalysed by the enzyme 4-hydroxycinnamoyl-coenzyme A hydratase-lyase (HCHL). The gene encoding HCHL was cloned and expressed and the HCHL protein was characterized biochemically (Mitra *et al.*, 1999), revealing it to be a homologue of the crotonase superfamily, an interesting group of low-sequence-similarity enzymes that are structurally homologous but catalyse a diverse range of chemical reactions (Holden *et al.*, 2001). The HCHL enzyme has a monomer molecular weight of 31 007 Da, with 273 amino acids per monomer. The two-step hydration/C—C bond cleavage catalysed by HCHL is a new addition to the range of reactions catalysed by the crotonase superfamily. Homologous genes encoding HCHL have more recently been reported from *Pseudomonas* spp. (Overhage *et al.*, 1999; Plaggenborg *et al.*, 2003), from *Amycolatopsis* sp. (Achterholt *et al.*, 2000) and (at much lower homology) from *Delftia acidovorans* (Plaggenborg *et al.*, 2001).

HCHL has been expressed heterologously in plants, where it causes a major diversion of the phenylpropanoid pathway and the formation of new products in large amounts, principally glucose conjugates of 4-hydroxybenzoic acid, a monomer for the production of liquid-crystal polymers (Mayer *et al.*, 2001; Mitra *et al.*, 2002; McQualter *et al.*, 2004). These are presumably formed from 4-hydroxybenzaldehyde produced by the action of HCHL on endogenous 4-coumaroyl-CoA. On the other hand, there was no detectable accumulation of the aroma

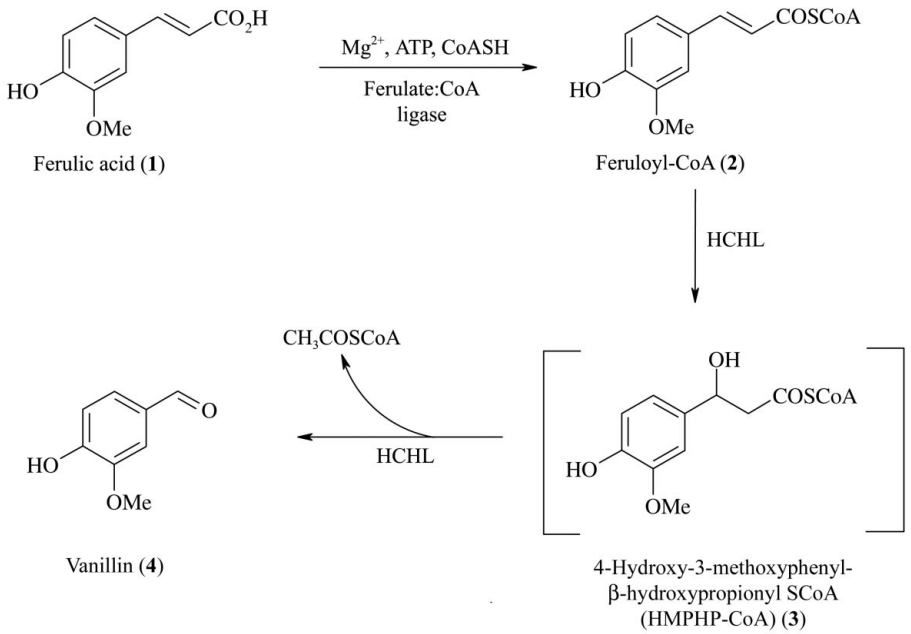

Figure 1
Metabolism of ferulic acid by *P. fluorescens* AN103. HCHL, hydroxycinnamoyl-coenzyme A hydratase-lyase.

compound vanillin even in the form of its glucoside.

Our interest in both the structural and mechanistic basis of C—C bond cleavage by crotonase homologues (Leonard & Grogan, 2004) and its applications in metabolic engineering prompted us to begin a study of the structure of HCHL.

2. Overexpression and purification

HCHL protein was purified from an overexpressing *Escherichia coli* strain BL21(DE3) which had been transformed with the plasmid pFI1039 encoding the gene for *P. fluorescens* biovar V strain AN103 HCHL (Gasson *et al.*, 1998), which had been constructed from the pSP72 plasmid (Novagen). Cells were grown in 4.5 l Luria–Bertani broth with 100 µg ml^{-1} ampicillin in a rotary shaker at 310 K until an OD_{600} of 0.5 was reached. HCHL expression was induced by the addition of 1 m*M* isopropyl-*β*-D-thiogalactopyranoside (IPTG) and cells were allowed to grow for a further 3.5 h. Induced cells were harvested by centrifugation and the cell pellet was resuspended in 30 ml 50 m*M* Tris–HCl pH 7.1, 1 m*M* dithiothreitol (DTT) and 20 µ*M* phenylmethylsulfonyl fluoride (PMSF) (buffer *A*) and disrupted by sonication. The homogenate was then centrifuged for 20 min at 12 000*g*, after which a 20–80%(*w*/*v*) ammonium sulfate cut was derived. The protein solution was taken to a concentration of 2 *M* ammonium sulfate and loaded onto a HiLoad 26/10 phenyl Sepharose column (Amersham), which was eluted with a decreasing ammonium sulfate concentration. Fractions were analysed by SDS–PAGE and those containing HCHL were pooled, concentrated and dialysed against 2 l buffer *A*. The protein solution was loaded onto a Mono Q anion-exchange column (Amersham) and eluted against a sodium chloride gradient (0–0.4 *M*). Fractions were analysed by SDS–PAGE and those containing HCHL were pooled, concentrated and loaded onto a HiLoad 16/60 Superdex 200 prep-grade gel-filtration column (Amersham) equilibrated with buffer *A*. Fractions were analysed by SDS–PAGE and those containing HCHL were pooled and concentrated to 10 mg ml^{-1}. The purity of preparation estimated by SDS–PAGE was close to 100%.

3. Crystallization and data collection

HCHL protein in buffer *A* was crystallized using the hanging-drop vapour-diffusion technique by mixing 1 µl protein solution with an equal volume of precipitant followed by equilibration at 290 K. Crystals were initially grown using the Clear Strategy screen from Molecular Dimensions Ltd (Brzozowski & Walton, 2001) condition No. 13. After optimization, crystals of maximum dimensions 0.3 × 0.2 × 0.05 mm (Fig. 2) grew within one week at a protein concentration of 10 mg ml^{-1} in 11%(*w*/*v*) PEG 20 000 with 8%(*v*/*v*) PEG 550 monomethyl ether, 0.8 *M* sodium formate, 0.2%(*v*/*v*) butane-1,4-diol in 0.05 *M* 2-(*N*-morpholino)-ethanesulfonic acid buffer pH 5.6.

A crystal of HCHL was flash-cooled prior to data collection by transferring it into a solution identical to the precipitant before positioning it in a cryostream (Oxford Cryosystems) at 120 K. The crystal was transferred to a liquid-nitrogen storage dewar and transported to the European Synchrotron Radiation Facility (ESRF), where data were collected on station ID14-EH1 at a single wavelength.

Table 1
Data-collection statistics for HCHL native data.

Values in parentheses refer to the highest resolution shell.

Beamline	ID14-EH1
Wavelength (Å)	0.934
Resolution (Å)	30–1.8 (1.85–1.8)
Unique reflections	308927 (25526)
Completeness (%)	100 (100)
R_{sym}† (%)	4.5 (33.2)
Multiplicity	4.2 (4.2)
$\langle I\rangle/\sigma(I)$	21.9 (3.3)

† $R_{sym} = \sum_{hkl}\sum_i |I_i - \langle I\rangle|/\sum_{hkl}\sum_i \langle I\rangle$, where I_i is the ith measurement and $\langle I\rangle$ is the weighted mean of all measurements of I. $\langle I\rangle/\sigma(I)$ indicates the average of the intensity divided by its average standard deviation.

4. Data analysis

The HCHL data set was autoindexed using the program *DENZO* (Otwinowski & Minor, 1997), indicating that the crystals have a primitive orthorhombic lattice with unit-cell parameters a = 130.8, b = 154.2, c = 167.5 Å and therefore a unit-cell volume of 3.38 × 10^6 Å^3. The data were processed and scaled using the *HKL* suite of programs (Table 1).

Crotonase-family enzymes are known to form trimeric disks with tight interactions between the three subunits (Mursula *et al.*, 2004); of those of known crystal structure, many form a hexamer with 32 symmetry in the crystal lattice through trimer dimerization. In many cases, all or part of the

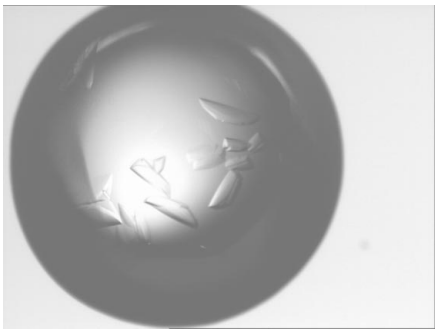

Figure 2
Crystals of hydroxycinnamoyl-coenzyme A hydratase-lyase.

hexamer is generated by crystal symmetry. For example, the structures of methylmalonyl-CoA decarboxylase (Benning *et al.*, 2000), dienoyl-CoA isomerase (Modis *et al.*, 1998) and 4-chlorobenzoyl-CoA dehalogenase (Benning *et al.*, 1996) all contain a trimer in the asymmetric unit and the structure of Δ^3-Δ^2-enoyl CoA isomerase (Mursula *et al.*, 2001) contains a monomer in the asymmetric unit. In this orthorhombic space group the threefold axis and at least two of the three twofold axes must be non-crystallographic. The molecular weight of an isolated molecule is 31 007 Da and that of the hexamer is 186 042 Da. The V_M is 2.3 Å^3 Da^{-1} for 12 molecules in the asymmetric unit. The native Patterson map calculated at 4 Å resolution contains a peak at $u = 0.34$, $v = 0.3$ and $w = 0.5$ which is 25% of the origin peak height, indicating that there is a strong non-crystallographic translation. It is therefore possible that the asymmetric unit contains four trimers, where we would expect two hexamers in each asymmetric unit or four independent trimers each with their partner trimer generated by the space-group twofold axis.

We would expect the self-rotation function to show a threefold rotation axis perpendicular to three twofold axes, with the twofold axes at 120° to each other. The self-rotation function calculated using *POLARRFN* (Collaborative Computational Project, Number 4, 1994) has a peak with 40% of the origin-peak height in the $\kappa = 120°$ section at $\omega = 90.0$, $\varphi = 63.8$, $\kappa = 119.5°$, indicating a threefold axis. The axis has direction cosines (0.4411, 0.8974, 0.0000). Two symmetry equivalents of the same peak are twofold axes with $\omega = 120.2$, $\varphi = 333.8$, $\kappa = 180.0°$ and $\omega = 120.2$, $\varphi = 153.8$, $\kappa = 180.0°$. The direction cosines of these twofold axes are (0.7753, −0.3811, −0.5037) and (−0.7753, 0.3811, −0.5037), respectively. Along with the crystallographic twofold axis along *c* with direction cosines (0.0, 0.0, 1.0), these form the required set.

The diffraction pattern was inspected using *HKLVIEW* (Collaborative Computational Project, Number 4, 1994), revealing that those reflections along 0*k*0 for which *k* was odd and those along 00*l* for which *l* was odd were systematically absent, indicating possible twofold screw axes along *k* and *l*. This was not the case along *h*00, indicating that the most likely space group was $P2_12_12$. For this reason *a*, *b* and *c* were reindexed to give revised unit-cell parameters $a = 154.2$, $b = 167.5$, $c = 130.8$ Å.

The structure of HCHL is currently being sought by molecular replacement using the structure of rat enoyl-CoA hydratase as a search model. The resulting model of HCHL will provide further information about the diverse reactions performed by members of the crotonase superfamily. The applications of the enzyme in metabolic engineering make it an exciting target for structural and functional analysis.

We would like to thank the Biotechnology and Biological Sciences Research Council (BBSRC) UK for funding. In addition, we would like to thank the Innovation, Priming and Research Fund, University of York for an award to GG. We would also like to thank the staff of the European Synchrotron Radiation Facility (ESRF) for provision of data-collection facilities and Drs John Payne and Arjan Narbad for producing plasmid pFI1039.

References

Achterholt, S., Priefert, H. & Steinbüchel, A. (2000). *Appl. Microbiol. Biotechnol.* **54**, 799–807.

Benning, M. M., Haller, T., Gerlt, J. A. & Holden, H. M. (2000). *Biochemistry*, **38**, 4630–4639.

Benning, M. M., Taylor, K. L., Liu, R. Q., Yang, G., Xiang, H., Wesenberg, G., Dunaway-Mariano, D. & Holden, H. M. (1996). *Biochemistry*, **35**, 8103–8109.

Brzozowski, A. M. & Walton, J. (2001). *J. Appl. Cryst.* **34**, 97–101.

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

Dixon, R. A. & Paiva, N. L. (1995). *Plant Cell*, **7**, 1085–1097.

Gasson, M. J., Kitamura, Y., McLauchlan, W. R., Narbad, A., Parr, A. J., Parsons, E. L. H., Payne, J., Rhodes, M. J. C. & Walton, N. J. (1998). *J. Biol. Chem.* **273**, 4163–4170.

Holden, H. M., Benning, M. M., Haller, T. & Gerlt, J. A. (2001). *Acc. Chem. Res.* **34**, 145–157.

Leonard, P. M. & Grogan, G. (2004). *J. Biol. Chem.* **279**, 31312–31317.

McQualter, R. B., Fong Chong, B., Meyer, K., Van Dyk, D. E., O'Shea, M. G., Walton, N. J., Viitanen, P. V. & Brumbley, S. M. (2004). In the press.

Mayer, M., Narbad, A., Parr, A. J., Parker, M. L., Walton, N., Mellon, F. A. & Michael, A. J. (2001). *Plant Cell*, **13**, 1669–1682.

Mitra, A., Kitamura, Y., Gasson, M. J., Narbad, A., Parr, A. J., Payne, J., Rhodes, M. J. C., Sewter, C. & Walton, N. J. (1999). *Arch. Biochem. Biophys.* **365**, 10–16.

Mitra, A., Mayer, M. J., Mellon, F. A., Michael, A. J., Narbad, A., Parr, A. J., Waldron, K. W. & Walton, N. J. (2002). *Planta*, **215**, 79–89.

Modis, Y., Filppula, S. A., Novikov, D. K., Norledge, B., Hiltunen, J. K. & Wierenga, R. K. (1998). *Structure*, **6**, 957–970.

Mursula, A. M., Hiltunen, J. K. & Wierenga, R. K. (2004). *FEBS Lett.* **557**, 81–87.

Mursula, A. M., van Aalten, D. M. F., Hiltunen, J. K. & Wierenga, R. K. (2001). *J. Mol. Biol.* **309**, 845–853.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Overhage, J., Priefert, H., Rabenhorst, J. & Steinbüchel, A. (1999). *Appl. Environ. Microbiol.* **65**, 4837–4847.

Plaggenborg, R., Overhage, J., Steinbüchel, A. & Priefert, H. (2003). *Appl. Microbiol. Biotechnol.* **61**, 528–535.

Plaggenborg, R., Steinbüchel, A. & Priefert, H. (2001). *FEMS Microbiol. Lett.* **205**, 9–16.