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Purification, crystallization and preliminary X-ray diffraction of anthocyanidin synthase from *Arabidopsis thaliana*

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† Since the submission of this paper Dr Andrea Prescott has died. Her contribution to plant biochemistry will be sadly missed.

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Anthocyanidin synthase (ANS) from *Arabidopsis thaliana* is a non-haem iron(II)-dependent dioxygenase reported to catalyse the conversion of leucoanthocyanidins to anthocyanidins. Anthocyanidins are precursors of anthocyanins, which are a major family of pigments in higher plants. ANS was crystallized by the vapour-diffusion method using polyethylene glycol as a precipitant. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 61.0, b = 73.2, c = 87.0 Å, and diffract to 2.4 Å using Cu $K\alpha$ radiation.

1. Introduction

Flavonoids are plant secondary metabolites whose roles include attracting pollinators, defending plants against pathogens, signalling in plant–microbe interactions, protecting plants against UV radiation and aiding seed dispersal (Strack & Wray, 1993). They exhibit various biomedical properties: they inhibit cell proliferation, are antioxidants and display antimutagenic, anti-inflammatory and anti-hypertensive effects (Hollman & Katan, 1999; Huang *et al.*, 1999). The anthocyanin subfamily of flavonoids cause pigmentation in plants and are one of the primary colourants in flowers.

Anthocyanidin synthase (ANS) catalyses one of the final steps in the flavonoid biosynthesis pathway of higher plants (Fig. 1) (Heller & Forkmann, 1993). Although there are reports on other enzymes involved in anthocyanin biosynthesis, until recently the only reported work on the ANS from *A. thaliana* has concerned the identification of its genetic locus and putative gene sequence (Pelletier *et al.*, 1997). ANS was predicted to be a 2-oxoglutarate (2-OG) dependent dioxygenase owing to the presence of conserved sequence motifs (*e.g.* H*X*D and R*X*S motifs, which are involved in iron and co-substrate binding, respectively; Prescott & Lloyd, 2000). In 1999, Saito and coworkers confirmed this proposal by expressing ANS from *Perilla frutescens* and demonstrating that it required iron and 2-oxoglutarate for catalysis (Saito *et al.*, 1999).

The biosynthetic pathway of flavonoids uses other non-haem dioxygenases which are closely related to ANS by sequence (*e.g.* FNS I, F3OH and FLS from Fig. 1). We are interested in how the flavonoid biosynthetic pathway has evolved using small changes in secondary structure to generate the different substrate and product selectivities which result in an array of flavonoid compounds from common intermediates.

Several enzymes of the non-haem dioxygenase family have been crystallized, including isopenicillin N synthase (IPNS) from *Aspergillus nidulans* (Roach *et al.*, 1995) and deacetoxycephalosporin C synthase (DAOCS; Valegard *et al.*, 1998) and clavaminic acid synthase (CAS; Zhang *et al.*, 2000) from *Streptomyces clavuligerus*. Of these enzymes, IPNS has the closest sequence similarity to ANS, with 23% sequence identity and 42% sequence similarity. Here, we describe the purification of ANS and its crystallization. We believe that this is the first reported crystallization of a 2-OG-dependent dioxygenase from higher eukaryotes.

2. Protein expression and purification

A cDNA encoding the *A. thaliana* ANS gene was cloned as an *Nde*I–*Bam*HI fragment into pET24a (Novagen) and transformed into *Escherichia coli* BL21(DE3) Gold cells. The cells were grown in a 30 l fermentor at 290 K using 2TY medium containing 30 µg ml^{-1} kanamycin. At an OD_{600} of 0.7, the cells were induced by the addition of 0.5 m*M* IPTG and growth continued at 290 K for 4 h. This gave an expression level that reached about 20% of the total soluble cellular protein (Fig. 2). Cultured cells were resuspended in lysis buffer [100 m*M* Tricine pH 7.3, 10%(*w*/*v*) glycerol, 10 m*M* DTT, 1 m*M* EDTA, 1 ml protease inhibitor cocktail (Sigma), 1 m*M* benzamidine, 0.4 mg ml^{-1} lysozyme, 0.08 mg ml^{-1} DNAse I) and homogenized with an ultrasonic processor. The solution was centrifuged at 35 000*g* for 20 min and the cell debris discarded. The supernatant was then passed through a 0.2 µm

Table 1
Data collection and processing statistics.

Values in parentheses indicate the value for the highest resolution shell (3.00–3.08, 2.40–2.46 and 2.70–2.77 Å, respectively).

	Rectangular aerobic crystal	Annealed aerobic crystal	Anaerobic crystal with $FeSO_4$, α-ketoglutarate and ascorbate
Resolution (Å)	3.0	2.4	2.7
Unit-cell parameters (Å)			
a	60.9	61.0	61.0
b	73.5	73.1	72.9
c	87.1	86.9	87.0
Total reflections	46074	156228	72855
Unique reflections	7636	15566	10880
R_{merge} (%)	12.4 (32.7)	7.7 (29.3)	6.7 (27.0)
Completeness (%)	93.8 (95.7)	99.1 (98.1)	98.4 (97.8)
$I/\sigma(I)$	8.6 (3.0)	15.6 (3.7)	14.8 (4.3)

filter before being loaded onto Q Sepharose FF resin in a Pharmacia 60/100 column. Elution was performed using a linear gradient of 0.1–1.0 *M* NaCl in a solution containing 50 m*M* Tricine pH 7.3, 1 m*M* EDTA and 1 m*M* DTT. Fractions containing ANS (125–150 mg) at ∼60–70% purity as judged by SDS–PAGE analysis (Fig. 2) were pooled. The buffer was exchanged into 50 m*M* MES pH 6.15, 10%(*w*/*v*) glycerol before using a Reactive Green 19 Dye resin column (Sigma), eluting isocratically with the loading buffer. Fractions containing ANS (55–65 mg) at ∼90–95% purity (by SDS–PAGE analysis) were pooled and concentrated. Prior to crystallization, the protein buffer was changed to 10 m*M* Tricine pH 7.3 using a PD-10 gel-filtration column (Pharmacia).

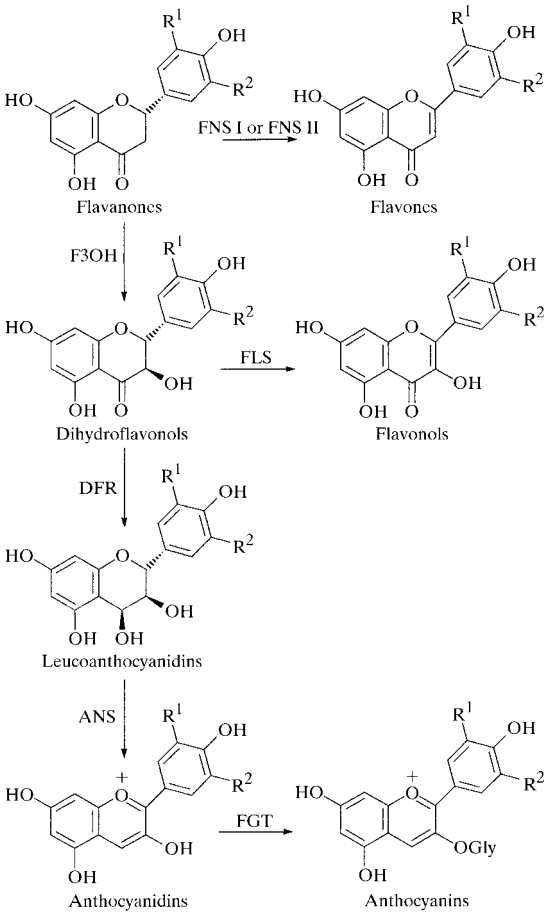

Figure 1
Later stages of the anthocyanin biosynthesis pathway (FNS I and FNS II, flavone synthases I and II, respectively; F3OH, flavanone 3β-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; FGT, flavonoid 3-*O*-glucosyltransferase). FNS I, F3OH, FLS and ANS are members of the 2-OG dependant dioxygenase family. Note that the nascent product of the ANS reaction may be a 2-flaven-3,4-diol (Heller & Forkmann, 1993).

3. Crystallization

Initial crystallization conditions for native ANS were screened for using the hanging-drop vapour-diffusion method. Droplets containing 2 µl of protein (35 mg ml^{-1}) and 2 µl of mother liquor were equilibrated against 0.5 ml of reservoir solution. Sparse-matrix screens from Molecular Dimensions were used at room temperature (290 K) under both aerobic and anaerobic conditions. Crystallization under anaerobic conditions was performed using an argon atmosphere (<0.5 ppm O_2) in a glove box (Belle Technology). After two weeks, a mixture of thin needles and rectangular-shaped crystals of various sizes were observed using apo protein under aerobic conditions from 30%(*w*/*v*) PEG 4000 with 100 m*M* sodium citrate (pH of stock solution = 5.6) and 200 m*M* ammonium acetate. The actual pH of the solution from the sparse-matrix screen was determined to be ∼6.2. The rectangular-shaped crystals were shown to belong to an orthorhombic space group ($P2_12_12_1$) and diffract to 2.9 Å.

Similar crystals could be obtained under anaerobic conditions using identical precipitation conditions with the addition of iron(II) and α-ketoglutarate; the time taken for crystallization to occur was reduced from two weeks to ∼16 h. Initially, only small thin needles or larger needles with extensive twinning could be produced and although these crystals belonged to the same space group as the rectangular-shaped aerobic apo-enzyme crystals, they diffracted significantly less well. Eventually, better conditions were found that produced larger single needles (∼500 × 50 × 50 µm) that diffracted to 2.7 Å in-house [30%(*w*/*v*) PEG 2000, 50 m*M* MES, 100 m*M* sodium citrate, 200 m*M* ammonium acetate, 5 m*M* $FeSO_4$, 10 m*M* potassium α-ketoglutarate and 10 m*M* sodium ascorbate pH 6.5].

4. X-ray diffraction analysis

The rectangular-shaped crystals from the initial aerobic conditions [30%(*w*/*v*) PEG 4000, 100 m*M* sodium citrate, 200 m*M* ammonium acetate pH 6.2] were transferred into a solution of mother liquor supplemented with 15%(*v*/*v*) glycerol and placed directly into a nitrogen stream at 100 K (Oxford Cryosystems Cryostream) using a nylon loop. X-ray diffraction data were collected using 30 min exposures on a MAR Research image-plate detector (345 mm). This was mounted on a Rigaku RU-200 rotating-anode generator operating at 3.9 kW with Cu $K\alpha$ radiation and equipped with Osmic mirrors. The largest crystals

Figure 2
SDS–PAGE analysis of the expression and purification of *E. coli* BL21(DE3) transformed with the plasmid containing the *A. thaliana* ANS gene [lanes 1 and 5, molecular-weight markers (BDH; 30, 42, 66 and 76 kDa); lane 2, crude cell extract after sonication; lanes 3 and 4, pooled ANS containing fractions after the Q Sepharose FF column and the Reactive Green 19 Dye column, respectively]. The molecular weight of ANS is 40.4 kDa. Further purification of ANS could be achieved by gel filtration, but was unnecessary for crystallization purposes.

diffracted weakly to a maximum of ~2.9 Å, but with one crystal good quality diffraction to 2.4 Å could be obtained by employing two successive rounds of 're-annealing' (Harp *et al.*, 1998; a simple protocol of blocking the nitrogen stream with a plastic credit card for ~6 s was employed). The raw data were processed with the programs *MOSFLM*, *SCALA* and *TRUNCATE* (*CCP*4 suite; Collaborative Computational Project, Number 4, 1994). A comparison of a data set collected on an unannealed crystal and the 2.4 Å data set (Table 1) demonstrated that little change in the unit-cell parameters had occurred during the re-annealing procedure. A 2.7 Å resolution data set was also collected on an anaerobic crystal [at 100 K using 15%(*v*/*v*) glycerol as a cryoprotectant] using the final optimized conditions (see above). The space group was identical ($P2_12_12_1$) and as with the annealed aerobic crystal there was little change in unit-cell parameters (Table 1). The solvent content of this crystal was 48.4% and there was apparently one molecule in the asymmetric unit, giving a Matthews coefficient (V_M) of 2.4 Å^3 Da^{-1}.

Molecular-replacement trials performed using *AMoRe* (Navaza, 1994) with X-ray structures of two bacterial non-haem iron(II)-dependent dioxygenases, isopenicillin N-synthase (PDB reference 1bk0; Roach *et al.*, 1997) and deacetoxycephalosporin C synthase (PDB reference 1dcs; Valegard *et al.*, 1998), as the starting models failed to identify a solution. Therefore, MIR (multiple isomorphous replacement) and MAD (multiwavelength anomalous diffraction) experiments are being planned to facilitate phasing. A search for heavy-atom derivatives and production of selenomethionine-substituted protein are both in progress.

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References

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Harp, J. M., Timm, D. E. & Bunick, G. J. (1998). *Acta Cryst.* D**54**, 622–628.
Heller, W. & Forkmann, G. (1993). *The Flavonoids: Advances in Research Since 1986*, edited by J. B. Harborne, pp. 499–535. London: Chapman & Hall.
Hollman, P. C. H. & Katan, M. B. (1999). *Food Chem. Toxicol.* **37**, 937–942.
Huang, Y. T., Hwang, J. J., Lee, P. P., Ke, F. C., Huang, J. H., Huang, C. J., Kandaswami, C., Middleton, E. & Lee, M. T. (1999). *Br. J. Pharmacol.* **128**, 999–1010.
Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.
Pelletier, M. K., Murrell, J. R. & Shirley, B. W. (1997). *Plant Physiol.* **113**, 1437–1445.
Prescott, A. G. & Lloyd, M. D. (2000). *Nat. Prod. Rep.* **17**, 367–383.
Roach, P. L., Clifton, I. J., Fulop, V., Harlos, K., Barton, G. J., Hajdu, J., Andersson, I., Schofield, C. J. & Baldwin, J. E. (1995). *Nature (London)*, **375**, 700–704.
Roach, P. L., Clifton, I. J., Hensgens, C. M., Shibata, N., Schofield, C. J., Hajdu, J. & Baldwin, J. E. (1997). *Nature (London)*, **387**, 827–830.
Saito, K., Kobayashi, M., Gong, Z., Tanaka, Y. & Yamazaki, M. (1999). *Plant J.* **17**, 181–189.
Strack, D. & Wray, V. (1993). *The Flavonoids: Advances in Research Since 1986*, edited by J. B. Harborne, pp. 1–22. London: Chapman & Hall.
Valegard, K., van Scheltinga, A. C., Lloyd, M. D., Hara, T., Ramaswamy, S., Perrakis, A., Thompson, A., Lee, H. J., Baldwin, J. E., Schofield, C. J., Hajdu, J. & Andersson, I. (1998). *Nature (London)*, **394**, 805–809.
Zhang, Z., Ren, J., Stammers, D. K., Baldwin, J. E., Harlos, K. & Schofield, C. J. (2000). *Nature Struct. Biol.* **7**, 127–133.