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Crystallization and preliminary X-ray study of the 8-amino-7-oxopelargonate synthase from *Bacillus sphaericus*

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Abstract

The 8-amino-7-oxopelargonate synthase (AOPS) cloned from *Bacillus sphaericus*, overproduced in *Escherichia coli*, has been crystallized in the pyridoxal 5′-phosphate (PLP)-bound form at pH 7.5, using polyethylene glycol as the precipitant. One crystal form corresponds to a tetragonal space group, with unit-cell dimensions $a = b = 66$, $c = 181$ Å. These crystals do not diffract beyond 5 Å with conventional X-ray sources and cannot be used in the structure elucidation. A second crystal form is obtained when crystallization conditions are varied slightly by the addition of 0.2 *M* ammonium sulfate. The space group is $P2_12_12_1$, with unit-cell dimensions $a = 68.9$, $b = 85.5$, $c = 125.9$ Å, indicating the presence of two molecules in the asymmetric unit ($V_m = 2.26$ Å^3 Da^{-1}; 46% water). These crystals diffract X-rays up to 3.2 Å using in-house facilities and a preliminary data set has been collected. A second data set using the synchrotron radiation source W32 at LURE (Paris) has shown the crystals to diffract to at least 3 Å resolution, with good statistics. The structure determination of AOPS will provide a structural framework for the other α-amino ketone synthases for which no three-dimensional structure is yet available.

1. Introduction

The second of the five steps in the biotin (vitamin H) metabolic pathway in *Bacillus sphaericus* (Eisenberg, 1987; Yamada & Izumi, 1989) results in the transformation of pimeloyl-CoA into 8-amino-7-oxopelargonate (AOP) (Fig. 1). This reaction is catalyzed by 8-amino-7-oxopelargonate synthase (AOPS), a pyridoxal 5′-phosphate (PLP) dependent enzyme that has been detected in various microorganisms (Eisenberg & Star, 1968; Izumi, Morita, Tani & Ogata, 1973; Izumi, Sato, Tani & Ogata, 1973). The first purification to homogeneity of this enzyme cloned from *B. sphaericus* overproduced in *Escherichia coli*, and its preliminary characterization has been reported recently (Ploux & Marquet, 1992). Although the chemistry of PLP enzymes is well documented (Dolphin, Poulson & Avramovic, 1986), knowledge of the precise catalytic mechanism used by these synthases is still scant. Studies on the mechanism and the stereochemistry of 5-aminolevulinate synthase catalyzed reaction (Akhtar, Abboud, Barnard, Jordan & Zaman, 1976) have shown that the process is initiated by the rupture of the C2—HR bond of the external aldimine, leading to the PLP-stabilized carbanion, rather than by the rupture of the C2—COOH bond (Laghai & Jordan, 1976, 1977; Nandi, 1978). The decarboxylation would therefore occur after the acylation, and the overall transformation would proceed with net inversion of stereochemistry. However, the stereochemistry of each elementary step, being inversion or retention, remains to be established. Conflicting reports concerning the mechanism of serine palmitoyl-transferase have been published, the latest proposing a mechanism similar to that of 5-aminolevulinate synthase (Krisnangkura & Sweeley, 1976; Ploux, 1996). As part of a general project devoted to the understanding of the enzymology of biotin biosynthesis, as well as to potential development of industrial applications (microbial production of biotin, design of antimicrobial drugs and herbicides, and so on), a detailed characterization of 8-amino-7-oxopelargonate synthase (AOPS) has been undertaken. We report here the crystallization of AOPS and preliminary diffraction studies. Knowledge of the native three-dimensional structure of this enzyme will help define the catalytic mechanism and the design of specific inhibitors. Furthermore, it will be possible to use this structure as a model for other α-amino ketone synthases that share strong sequence similarities (Gloeckler *et al.*, 1990; Buede, Rinker-Schaffer, Pinto, Lester & Dickson, 1991) and for which no three-dimensional structure is yet available in the Protein Data Bank. To date, only one enzyme participating in the biotin synthesis pathway, the dethiobiotin synthase (DTBS), which is the penultimate enzyme of the pathway, has been structurally resolved (Alexeev, Baxter & Sawyer, 1994; Huang *et al.*, 1994).

2. Crystallization

The AOPS was purified to homogeneity from an *Escherichia coli* overproducing strain as reported previously (Ploux &

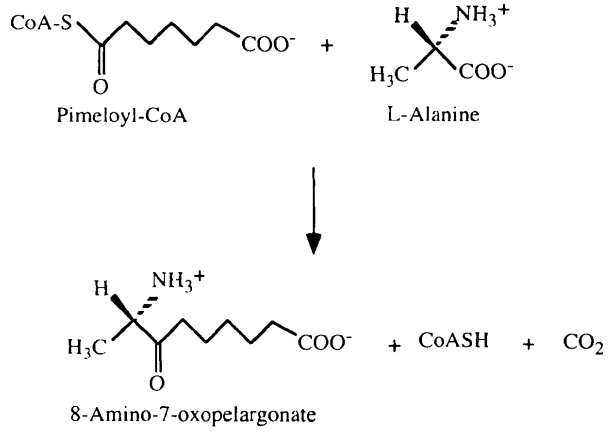

Fig. 1. Reaction catalyzed by the 8-amino-7-oxopelargonate synthase (AOPS) from *Bacillus sphaericus*.

Marquet, 1992). The PLP molecule was present at 100 m*M* in all the protein purification steps in order to form a complex stable with the enzyme *via* a covalent imine bound on Lys237. The PLP–protein complex remained stable in 10 m*M* Tris (hydroxymethyl)-aminomethane, HCL (pH 7.5) and 200 m*M* ammonium sulfate for one week at 277 K or several months when frozen at 253 K. Crystals of pure PLP–AOPS complex were obtained at 293 K using the vapour-diffusion technique with hanging drops (McPherson, 1990). Typically, 3 µl of the protein solution mentioned above ($c = 15$ mg ml^{-1}) was mixed with 3 µl of a reservoir solution containing the buffer, the precipitating agent and additives. The first crystal form (form 1) appeared above reservoir solution containing 10 m*M* MES (pH 6) in the presence of PEG 8000 as a precipitant agent in a concentration range of 4–10%(*w*/*v*). Subsequently a hypernucleation of prismatic crystals of dimensions $0.05 \times 0.05 \times 0.15$ mm occurred which, because of instability, tended to disappear after a few days. Microseeding techniques were used to reduce nucleation and increase the crystal size. A cat whisker was plunged into a solution of crushed crystals and passed through drops containing 2–8%(*w*/*v*) PEG 8000 (Stura & Wilson, 1991). Larger crystals were obtained under these conditions, stable enough to be submitted to an X-ray characterization.

The second crystal form (form 2) appeared when the drops were equilibrated over 100 m*M* MES pH 6.5, using 0.2 *M* ammonium sulfate and 22–26%(*w*/*v*) PEG 5000 monomethyl ether as the precipitating agents. Microcrystals appeared from the protein precipitate and were found to be stable and to grow best when the reservoir buffer was replaced with 100 m*M* Tris–HCl pH 7.5. In these conditions, crystals were visible within a few hours, resulting in a hypernucleation of thin and hollow rods. Over several PEG polymer size and various salts assayed in concentration ranges, the shape of the rod-like crystal could not be improved. Microseeding techniques, similar to those described above succeeded by lowering the PEG range to 18–22% and conduced after one week to proper rods of dimensions $0.1 \times 0.15 \times 0.8$ mm (Fig. 2). Unfortunately, this method still lacks in reproducibility and numerous microseeding experiments must still be performed in order to obtain crystals suitable for X-ray studies.

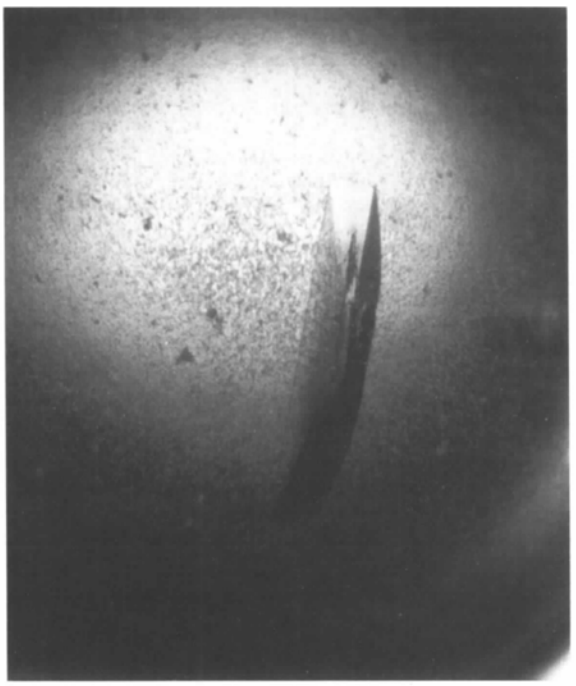

Fig. 2. Orthorhombic crystal of AOPS (space group $P2_12_12_1$ with cell parameters $a = 68.9$, $b = 85.5$, $c = 125.8$ Å).

3. X-ray diffraction data

Crystals of AOPS were characterized on an image-plate MAR-research detector (18 cm diameter), mounted on Rigaku RU200 rotating-anode source operating at 40 kV, 80 mA with Cu $K\alpha$ radiation. Crystals of form 1 did not diffract beyond 5 Å resolution and belong to the tetragonal space group $P422$, with unit-cell dimensions of $a = b = 66$, $c = 181$ Å. The poor diffraction quality of these crystals led to the abandonment of further investigations on this crystal form.

Under the same operating conditions, crystals of form 2 diffracted up to 3 Å. They belong to space group $P2_12_12_1$, with unit-cell dimensions $a = 68.9$, $b = 85.5$, $c = 125.9$ Å, indicating the presence of two molecules in the asymmetric unit (AU) ($V_m = 2.26$ Å^3 Da^{-1}; 46% estimated solvent volume) (Matthews, 1968). The two molecules per AU were confirmed by a self-rotation function calculated with the *AMoRe* software (Navaza, 1994), which clearly showed a single peak distinct from the origin. A preliminary set of X-ray intensity data was collected at 3.2 Å on our local detector. 1° frames were collected at a crystal-to-detector distance of 150 mm, with an exposure time of 1000 s frame^{-1}. The results of this data collection are summarized in Table 1. A second data set was collected at 3 Å on a synchrotron beamline using a 0.94 Å monochromatic radiation at a temperature of 277 K (DW32 LURE-DCI, Orsay, France) (Fourme & Frouin, 1992), using an image-plate MAR-research detector (18 cm diameter). Two crystals were used during the data collection due to radiation damage. For both crystals, 1° frames were collected at a crystal-to-detector distance of 270 mm with an exposure time of 60 s frame^{-1}. The result of this data collection is presented on Table 2. Both data sets were reduced with the *MARXDS* and *MARSCALE* packages (Kabsch, 1988*a*,*b*). The synchrotron beam source did not increase the diffraction limit of AOPS crystals beyond 3 Å. The shape of the reflections was significantly improved though, with excellent discrimination of reflections along the large axis. Unit-cell parameters were determined unambiguously at 3 Å, using *MARXDS*. As judged by R_{sym} and mean $I/\sigma(I)$, the quality of the X-ray data collected from synchrotron source was far better than X-ray data collected on the rotating anode.

4. Discussion

The addition of 0.2 *M* ammonium sulfate to our previous crystallization conditions resulted in a new crystal form suitable for X-ray studies. PEG, paired with the salt ammonium sulfate both in the drop and in the reservoir was found to be essential for protein stability and had a significant effect on crystal shape improvement. Luft & DeTitta (1995) observed that salts, by virtue of their enhanced osmotic properties, can have an important kinetic effect on the vapor-diffusion process,

Table 1. *Data collection and final refinement statistics of AOPS (Laboratoire de Cristallographie et Cristallisation des Macromolécules Biologique data)*

Resolution (Å)	Independent reflections*	Unique data (%)	Redundancy	R_{sym}† (%)	$I/\sigma(I)$ (average)
20–6.0	1560	75.6	7.4	9.73	36.26
6.0–5.0	1193	83.7	6.9	16.67	21.52
5.0–4.5	1072	86.3	6.6	16.41	21.22
4.5–4.0	1677	86.4	6.2	18.94	16.84
4.0–3.7	1492	87.6	5.4	24.14	10.47
3.7–3.4	2034	86.8	4.6	33.51	5.78
3.4–3.3	844	86.4	4.2	40.38	3.99
3.3–3.2	955	85.7	4.1	44.87	3.53
∞–3.2	10827	84.5	5.7	17.52	15.46

*84 rejected reflections during data processing. †$R_{sym} = \sum_{hkl}\sum_{ref}|I_{hkl} - \langle I_{hkl}\rangle|/\sum_{hkl}\sum_{ref}\langle I_{hkl}\rangle$.

Table 2. *Data collection and final refinement statistics of AOPS (DW32 LURE-DCI, Orsay data)*

Resolution (Å)	Independent reflections*	Unique data (%)	Redundancy	R_{sym}† (%)	$I/\sigma(I)$ (average)
29.–6.0	1748	84.7	3.8	4.59	42.43
6.0–5.0	1284	89.9	3.7	5.95	30.13
5.0–4.0	2849	89.5	3.3	6.87	24.60
4.0–3.6	2115	88.2	2.9	10.67	13.64
3.6–3.4	1406	85.1	2.7	16.25	8.29
3.4–3.2	1731	82.9	2.5	22.68	5.43
3.2–3.1	1026	81.7	2.5	31.27	3.23
3.1–3.0	1106	78.8	2.4	38.67	2.47
∞–3.0	13265	85.7	3.0	7.37	18.01

*425 rejected reflections during data processing. †$R_{sym} = \sum_{hkl}\sum_{ref}|I_{hkl} - \langle I_{hkl}\rangle|/\sum_{hkl}\sum_{ref}\langle I_{hkl}\rangle$.

resulting in an increase of the equilibration rate for aqueous PEG hanging drops. This observation may be one of the explanations concerning the crystal hypernucleation problem, which seems to be a difficult parameter to control due to a lack of reproducibility in our experiments. Giegé & Mikol (1989) noticed that the pH in crystallization drops is governed by that of the reservoir when the precipitant is ammonium sulfate, due to ammonia transfer between the two liquid phases, resulting in a lack of experiment reproducibility. To reduce this effect, we buffered the reservoirs at the same pH as that of the crystallization drops and we found an increase in the stability and reproducibility of the crystals. In addition, varying the protein to precipitant ratio, the reservoir volume and using microseeding techniques did not allow any dramatic decrease of the hypernucleation phenomenon. Experiments using glycerol as a cosolvent have been started, as this additive was found to reduce nucleation and influence crystal morphology (Sousa, 1995). Thus, we are currently working on the basis of different crystallization protocols including macroseeding, temperature and ionic strength variations with and without glycerol, in order to reduce hypernucleation and improve the crystal size and shape.

Since no three-dimensional structure of enzymes corresponding to the AOP synthase family is known, we will have to proceed by multiple isomorphous replacement techniques for the enzyme structure elucidation. The AOPS from *Bacillus sphaericus* contains two free cysteines, one of them reacting with 5,5′-dithiobis (2-nitrobenzoic acid, DTNB) (Ploux, 1996). We will attempt to fix mercury compounds onto this accessible cysteine residue, in order to obtain a useful heavy-atom derivative for use in a multiple isomorphous replacement procedure. This heavy-atom data search is in progress.

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References

Akhtar, M., Abboud, M. M., Barnard, G., Jordan, P. & Zaman, Z. (1976). *Philos. Trans. R. Soc. London Ser. B*, **273**, 117–136.

Alexeev, D., Baxter, R. L. & Sawyer, L. (1994). *Structure*, **2**, 1061–1072.

Buede, R., Rinker-Schaffer, C., Pinto, W. J., Lester, R. L. & Dickson, R. C. (1991). *J. Bacteriol.* **173**, 4325–4332.

Dolphin, D., Poulson, R. & Avramovic, O. (1986). In *Vitamin B6 Pyridoxal Phosphate*. New York: John Wiley.

Eisenberg, M. A. (1987). *Escherichia coli and Salmonella typhimurium*, Vol. 2, edited by F. C. Neidhardt, pp. 544–550. Washington DC: American Society for Microbiology.

Eisenberg, M. A. & Star, C. (1968). *J. Bacteriol.* **96**, 1291–1297.

Fourme, R. & Frouin, J. (1992). *Rev. Sci. Instrum.* **63**, 982–987.

Giegé, R. & Mikol, V. (1989). *Trends Biotechnol.* **7**(10), 277–282.

Gloeckler, R. Ohsawa, I., Speck, D., Ledoux, C., Bernard, S., Zinsius, M., Villeval, D., Kisou, T., Kamogawa, K. & Lemoine, Y. (1990). *Gene*, **87**, 63–70.

Huang, W., Lindqvist, Y., Schneider, G., Gibson, K. J., Flint, D. & Lorimer, G. (1994). *Structure*, **2**, 407–414.

Izumi, Y. Morita, H., Tani, Y. & Ogata, K. (1973). *Agr. Biol. Chem.* **37**, 1327–1333.

Izumi, Y., Sato, K., Tani, Y. & Ogata, K (1973). *Agr. Biol. Chem.* **37**, 1335–1340.

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

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

Krisnangkura, K. & Sweeley, C. C. (1976). *J. Biol. Chem.* **251**, 1597–1602.

Laghai, A. & Jordan, P. (1976). *Biochem. Soc. Trans.* **4**, 52.

Laghai, A. & Jordan, P. (1977). *Biochem. Soc. Trans.* **5**, 299–300.

Luft, J. R. & DeTitta, G. T. (1995). *Acta Cryst.* **D51**, 780–785.

McPherson, A. (1990). *Eur. J. Biochem.* **189**, 1–23.

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

Nandi, D. L. (1978). *J. Biol. Chem.* **253**, 8872–8877.

Navaza, J. (1994). *Acta Cryst.* **A50**, 157–163.

Ploux, O. (1996). In preparation.

Ploux, O. & Marquet, A. (1992). *Biochem. J.* **283**, 327–331.

Sousa, R. (1995). *Acta Cryst.* **D51**, 271–277.

Stura, E. A. & Wilson, I. A. (1991). *J. Cryst. Growth*, **110**, 270–282.

Yamada, H. & Izumi, Y. (1989). *Biotechnology of Vitamins, Pigments and Growth Factors*, edited by I. E. Vandamme, pp. 231–256. Essex: Elsevier.