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Crystallization and preliminary X-ray diffraction analysis of cytochrome P450$_{sca-2}$ from *Streptomyces carbophilus* involved in production of pravastatin sodium, a tissue-selective inhibitor of HMG-CoA reductase

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Cytochrome P450$_{sca-2}$ from *Streptomyces carbophilus* catalyzes the hydroxylation of ML-236B sodium salt to pravastatin sodium, a tissue-selective inhibitor of 3-hydroxy-3-methylglutaryl-coenzymeA (HMG-CoA) reductase. HMG-CoA reductase is a key enzyme in cholesterol biosynthesis. Crystals of the protein were obtained by the vapour-diffusion method, using ammonium sulfate as a precipitant. The crystals belong to the trigonal space group $P3_121$ (or its enantiomorph, $P3_221$) with unit-cell dimensions $a = 103.5$, $c = 79.8$ Å. Assuming the presence of one molecule in the asymmetric unit, the calculated value of V_m is 2.68 Å^3 Da^{-1}. A native data set was collected to a resolution of 2.2 Å.

1. Introduction

Cytochrome P450 is a member of the *b*-type haem proteins, which are involved in many processes including the biosynthesis of steroids, the metabolism of drugs and other xenobiotics, and the activation of procarcinogens (Guengerich, 1991; Ortiz de Montellano, 1995). Over 500 P450 genes have been identified in bacteria, yeast, fungi, plants and animals (Nelson *et al.*, 1996). P450s are divided into three classes according to their electron-transport system. Class 1 P450s require an FAD-dependent reductase and an iron–sulfur protein; most of the bacterial P450s and mitochondrial P450s belong to this class. Class 2 P450s require a reductase containing both FAD and FMN; microsomal P450s belong to this class. Class 3 P450s are catalytically self-sufficient; they require neither flavoprotein nor iron–sulfur protein. To date, crystal structures of five P450s have been determined. Three of the five belong to class 1: P450$_{cam}$ from *Pseudomonas putida* (camphor monooxygenase, CYP101; Poulos *et al.*, 1987), P450$_{terp}$ from *Pseudomonas* sp. (α-terpineol monooxygenase, CYP108; Hasemann *et al.*, 1994) and P450$_{eryF}$ from *Saccharopolyspora erythraea* (6-deoxyerythronolide B monooxygenase, CYP107A1; Cupp-Vickey & Poulos, 1995). One P450 belongs to class 2: the haemoprotein domain of P450$_{BM-3}$ from *Bacillus megaterium* (fatty acids monooxygenase, CYP102; Ravinchandran *et al.*, 1993). P450$_{BM-3}$ consists of a single polypeptide chain, containing the haem-containing P450 domain and the FAD/FMN-dependent reductase domain (Fulco, 1991). The last P450 belongs to class 3: P450$_{nor}$ from *Fusarium oxysporum* (nitric oxide reductase, CYP55A1; Park *et al.*, 1997).

Cytochrome P450$_{sca}$ from *S. carbophilus* catalyzes the hydroxylation of ML-236B sodium salt at the 6β position (ML-236B–Na) to give pravastatin sodium (pravastatin–Na), a cholesterol-lowering drug (Serizawa, Nakagawa *et al.*, 1983; Serizawa, Serizawa *et al.*, 1983; Serizawa & Matsuoka, 1991; Fig. 1). Pravastatin–Na is a tissue-selective inhibitor of HMG-CoA reductase (Tsujita *et al.*, 1986; Koga *et al.*, 1990), a key enzyme in cholesterol biosynthesis, and is produced by a two-step

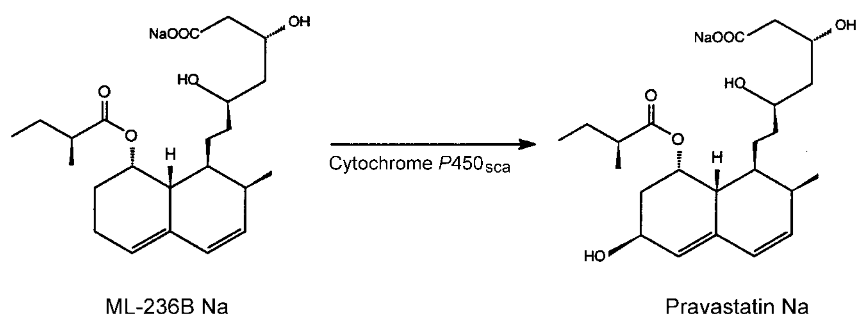

Figure 1
The reaction catalyzed by cytochrome P450$_{sca}$.

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fermentation process on an industrial scale. The first step is the production of ML-236B in *Penicillium citrinum* and the second is the hydroxylation of ML-236B–Na by *S. carbophilus*. Two isozymes were purified and designated, according to their order of elution upon hydroxyapatite chromatography, as $P450_{sca\text{-}1}$ and $P450_{sca\text{-}2}$ (Matsuoka *et al.*, 1989). The gene encoding $P450_{sca\text{-}2}$ was cloned and the amino-acid sequence was deduced (Watababe *et al.*, 1995). $P450_{sca\text{-}2}$ can be classified as a member of the CYP105 family based on its sequence similarity, as $P450_{sca\text{-}2}$ shared 79% sequence identity with $P450_{SU\text{-}1}$ from *S. griseolus*, a class 1 P450 (Omer *et al.*, 1990; O'Keefe *et al.*, 1991). An NADH P450 reductase containing both FAD and FMN was also purified from *S. carbophilus* as a redox partner of $P450_{sca\text{-}2}$ (Serizawa & Matsuoka, 1991). Although $P450_{sca\text{-}2}$ catalyzed the hydroxylation of ML-236B–Na with spinach-ferredoxin reductase and spinach ferredoxin, a reconstitution experiment with $P450_{sca\text{-}2}$ and with the reductase from *S. carbophilus* suggested the $P450_{sca}$ monooxygenase system consists of only two components. Therefore, the $P450_{sca}$ monooxygenase system resembles the class 2 P450s, based on its requirement for the FAD/FMN-dependent reductase.

ML-236B–Na is the first exogenous non-original compound discovered to be a substrate of $P450_{sca\text{-}2}$. The hydroxylation of ML-236B–Na is believed to be detoxification by $P450_{sca\text{-}2}$, as the minimal inhibitory concentration value of pravastatin–Na for *S. carbophilus* is much higher than that of ML-236B–Na (Matsuoka *et al.*, 1989). The hydroxylation ratio ($6\beta/6\alpha$) of $P450_{sca}$ is about 10 (Matsuoka *et al.*, 1989). We report here the crystallization and preliminary X-ray analysis of $P450_{sca\text{-}2}$ as a first step in the elucidation of the structural relationship to the substrate specificity and the regio-specific and stereospecific hydroxylation.

2. Purification and crystallization

$P450_{sca\text{-}2}$ was purified from *S. carbophilus* as described previously (Matsuoka *et al.*, 1989). Briefly, purification by ammonium sulfate precipitation was followed by application to DEAE-Toyopearl 650S (Tosoh, Japan), Q-Sepharose (Pharmacia, Sweden), Superdex 75 (Pharmacia, Sweden) and hydroxyapatite (DNA grade Bio-Gel HTP gel, Bio-Rad, USA) columns. The purified $P450_{sca\text{-}2}$ gave a single band on SDS–polyacrylamide gel electrophoresis stained with Coomassie brilliant blue. The buffer contained 20%(*v*/*v*) glycerol for all of the purification procedures.

Prior to crystallization, the glycerol concentration in the buffer was exchanged from 20%(*v*/*v*) to 10%(*v*/*v*). The purified protein solution was applied to a Mono Q (Pharmacia, Sweden) column pre-equilibrated with 50 m*M* Tris–HCl (pH 7.4), containing 1 m*M* dithiothreitol and 20%(*v*/*v*) glycerol. The column was washed with buffer *A* [50 m*M* Tris–HCl (pH 7.4), 1 m*M* dithiothreitol, 10%(*v*/*v*) glycerol] and eluted with a linear gradient of NaCl in buffer *A*. The pooled protein fractions were dialyzed against buffer *A* and concentrated by pressure-assisted filtration through a YM-30 membrane (Amicon, UK) to 10 mg ml^{-1}, as determined by the reduced carbon monoxide difference spectrum (Omura & Sato, 1964).

Crystals of $P450_{sca\text{-}2}$ were obtained at 277 K by the vapor-diffusion method (McPherson, 1982), using ammonium sulfate as a precipitant. 2 µl of protein solution was mixed with 2 µl of 2.0 *M* ammonium sulfate in buffer *B* [10%(*v*/*v*) glycerol, 0.1 *M* HEPES (pH 7.0)] and suspended over 1 ml of 1.5 *M* ammonium sulfate in buffer *B*. Small crystals appeared after a few weeks. In order to obtain larger crystals, the macroseeding technique (Stura & Wilson, 1990) was performed. Small crystals (typically 0.1 × 0.05 × 0.05 mm) were collected from the hanging drops, washed in 1.2 *M* ammonium sulfate in buffer *B* and placed into fresh solutions consisting of equal volumes of the protein solution and of 2.4 *M* ammonium sulfate in buffer *B*. This solution was then equilibrated against 1.2–1.5 *M* ammonium sulfate in buffer *B*. The dimensions of the macroseeded crystals reached 0.4 × 0.15 × 0.15 mm.

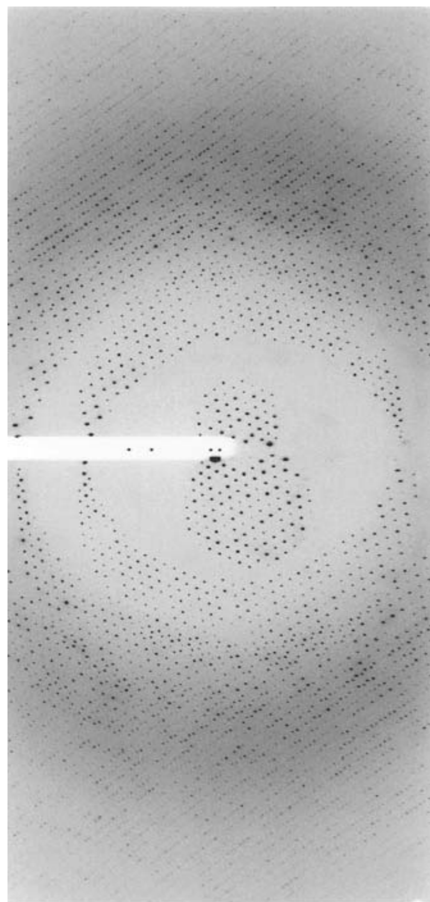

Figure 2
X-ray diffraction pattern from a single crystal of cytochrome $P450_{sca\text{-}2}$.

3. X-ray studies

A native data set was collected at 283 K from a single crystal mounted in a thin-walled glass-capillary tube at station BL-6A of the Photon Factory, Tsukuba, Japan. Diffraction data were measured using a Weissenberg camera and imaging plates as a detector (Sakabe, 1983) and were processed with *DENZO* and *SCALEPACK* (Otwinowski, 1993; Fig. 2). The crystals belong to trigonal space group $P3_121$ (or its enantiomorph $P3_221$) with unit-cell dimensions $a = 103.5$, $c = 79.8$ Å. Assuming the presence of one molecule in the asymmetric unit, the calculated value of crystal volume per protein mass (V_m) (Matthews, 1968) is 2.68 Å^3 Da^{-1}. This value corresponds to a solvent content of approximately 54%. A total of 109 889 observations were measured, and 22 673 independent reflections were obtained with an $R_{\rm merge}(I)$ of 0.077 and a completeness of 89.0% in the 40.0–2.2 Å resolution range. In the highest shell (2.28–2.2 Å), the completeness was not high (73.4%) because of limitation of the effective area of the detector and the $R_{\rm merge}(I)$ was large (0.379), presumably owing to radiation damage. Further experiments are necessary to collect data from crystals mounted with different crystal axes parallel to the spindle axis, as well as from crystals frozen at liquid-nitrogen temperature. Attempts to solve the structure by the molecular-replacement method using coordinates of previously determined P450s were unsuccessful. Solution of the structure using the heavy-atom isomorphous replacement method is in progress. The results of this study will elucidate the structural relationship to the substrate specificity and the stereospecific hydroxylation.

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References

Cupp-Vickey, J. R. & Poulos, T. L. (1995). *Nature Struct. Biol.* **2**, 144–153.

Fulco, A. J. (1991). *Annu. Rev. Pharmacol. Toxicol.* **31**, 177–203.

Guengerich, F. P. (1991). *J. Biol. Chem.* **266**, 10019–10022.

Hasemann, C. A., Ravinchandran, K. G., Peterson, J. A. & Deisenhofer, J. (1994). *J. Mol. Biol.* **236**, 1169–1185.

Koga, T., Shimada, Y., Kuroda, M., Tsujita, Y., Hasegawa, K. & Yamazaki, M. (1990). *Biochim. Biophys. Acta*, **1045**, 115–120.

McPherson, A. (1982). *Preparation and Analysis of Protein Crystals*, 1st ed., pp. 82–159. New York: John Wiley.

Matsuoka, T., Miyakoshi, S., Tanzawa, K., Hosobuchi, M., Nakahara, K. & Serizawa, N. (1989). *Eur. J. Biochem.* **184**, 707–713.

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

Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C. & Nebert, D. W. (1996). *Pharmacogenetics*, **6**, 1–42.

O'Keefe, D. P., Gibson, K. J., Emptage, M. H., Lenstra, R., Romesser, J. A., Little, P. J. & Omer, C. A. (1991). *Biochemistry*, **30**, 447–455.

Omer, C. A., Lenstra, R., Little, P. J., Dean, C., Tepperman, J. M., Leto, K. J., Romesser, J. A. & O'Keefe, D. P. (1990). *J. Bacteriol.* **172**, 3335–3345.

Omura, T. & Sato, R. (1964). *J. Biol. Chem.* **239**, 2370–2378.

Ortiz de Montellano, P. R. (1995). *Cytochrome P450. Structure, Mechanism and Biochemistry*, 2nd ed. New York: Plenum Press.

Otwinowski, Z. (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by L. Sawyer, N. W. Isaacs & S. Bailey, pp. 71–79. Warrington: Daresbury Laboratory.

Park, S.-Y., Shimizu, H., Adachi, A., Nakagawa, A., Tanaka, I., Nakahara, A., Shoun, H., Obayashi, E., Nakamura, H., Iizuka., T. & Shiro, Y. (1997). *Nature Struct. Biol.* **4**, 827–832.

Poulos, T. L., Finzel, B. C. & Howard, A. J. (1987). *J. Mol. Biol.* **195**, 687–700.

Ravinchandran, K. G., Boddupalli, S. S., Hasemann, C. A., Peterson, J. A. & Deisenhofer, J. (1993). *Science*, **261**, 731–736.

Sakabe, N. (1983). *J. Appl. Cryst.* **16**, 542–547.

Serizawa, N. & Matsuoka, T. (1991). *Biochim. Biophys. Acta*, **1084**, 35–40.

Serizawa, N., Nakagawa, K., Hamano, K., Tsujita, Y., Terahara, A. & Kuwano, H. (1983). *J. Antibiot.* **36**, 604–607.

Serizawa, N., Serizawa, S., Nakagawa, K., Furuya, K., Okazaki, T. & Terahara, A. (1983). *J. Antibiot.* **36**, 887–891.

Stura, E. A. & Wilson, I. A. (1990). *Methods*, **1**, 38–49.

Tsujita, Y., Kuroda, M., Shimada, Y., Tanzawa, K., Arai, M., Kaneko, I., Tanaka, M., Masuda, H., Tarumi, C., Watanabe, Y. & Fujii, S. (1986). *Biochim. Biophys. Acta*, **877**, 50–60.

Watababe, I., Nara, F. & Serizawa, N. (1995). *Gene*, **163**, 81–85.