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An improved procedure for the preparation of X-ray diffraction-quality crystals of cytochrome $P450_{cam}$

DARREN NICKERSON,[a]* LUET-LOK WONG[a] AND ZIHE RAO[b,c] *at* [a]*Department of Chemistry, Inorganic Chemistry Laboratory, University of Oxford, Oxford OX1 3QR, England,* [b]*School of Life Sciences, Tsinghua University, Beijing 10084, People's Republic of China, and* [c]*Laboratory of Molecular Biophysics, University of Oxford, Oxford OX1 3QU, England.*
E-mail: darren.nickerson@icl.ox.ac.uk

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Abstract

A procedure for the crystallization of recombinant cytochrome $P450_{cam}$ has been developed which avoids the difficulties inherent in the glass-capillary free-interface diffusion method reported previously. The surface mutation, Cys334→Ala (C334A), originally designed to prevent dimer formation and thus improve routine handling of the enzyme, facilitates crystallization by the hanging-drop vapour-diffusion technique. Crystals of (C334A)$P450_{cam}$ grow within 48 h and diffract to beyond 1.2 Å at 100 K in-house on a Siemens multiwire area detector. Data have been collected from the camphor-bound form to 1.35 Å.

1. Introduction

Cytochrome P450 heme monooxygenases catalyse the oxidation of many endogenous and exogenous compounds (Ortiz de Montellano, 1995). These reactions are critical steps in the biosynthesis and degradation of steroids and other hormones, as well as in the metabolism of xenobiotics (Guengerich, 1991, 1995). Cytochrome $P450_{cam}$, from the soil bacterium *Pseudomonas putida* (Yu *et al.*, 1974; Gunsalus & Wagner, 1978), is by far the most thoroughly characterized member of the P450 superfamily. The catalytic mechanism has been studied in detail (Mueller *et al.*, 1995), and it was the first of the P450 enzymes to be structurally characterized (Poulos *et al.*, 1987), quickly becoming the model on which studies of other prokaryotic and eukaryotic P450 enzymes are based.

All P450 enzymes are thought to follow the same mechanism of substrate oxidation (Mueller *et al.*, 1995). The wide range of molecules oxidized by the enzymes and the pattern of reactivity is controlled primarily by the nature of the enzyme substrate pocket. It is possible, therefore, to use genetic engineering to alter the active site of a P450 enzyme such that it is reactive towards molecules which would not otherwise be attacked by the wild-type enzyme – a powerful approach which has potential applications in chemical synthesis and many areas of biotechnology. We have used site-directed mutagenesis to expand the substrate range of $P450_{cam}$ (Fowler *et al.*, 1994), to improve dramatically the activity towards the oxidation of molecules that are poor substrates for the wild-type (Nickerson *et al.*, 1997; Stevenson *et al.*, 1996), and to engineer the regioselectivity of substrate oxidation (England *et al.*, 1996). $P450_{cam}$ is a particularly attractive enzyme for such rational protein engineering studies because of the availability of the high-resolution crystal structure on which to base structural arguments, and this is currently an active area of research (Mueller *et al.*, 1995; Wong *et al.*, 1997).

The success of rational protein redesign depends critically on the availability of structural data. Active-site mutations may have significant effects on the local or even global structure of the enzyme, and the mode of substrate binding may not be what is predicted based simply on the structure of the wild-type. We have embarked upon a program of X-ray structure determination of the mutants of $P450_{cam}$ to gain new insight into the molecular recognition interactions between the enzyme and small-molecule substrates, but progress has been hampered by difficulties in crystallization of the enzyme. Diffraction-quality crystals of cytochrome $P450_{cam}$ were first obtained in 1982 (Poulos *et al.*, 1982) by the free-interface diffusion method (Salemme, 1972). The inherent difficulties in obtaining suitable crystals of $P450_{cam}$ by this method have been discussed (Poulos, 1986, 1996), and attempts to obtain X-ray quality crystals according to that procedure met with little success in our laboratory. We have reported a base mutation to $P450_{cam}$ in which the highly reactive surface cysteine responsible for dimerization of the enzyme has been substituted with an alanine by site-directed mutagenesis, and described the simplified handling of the mutant, especially at higher concentrations (Nickerson & Wong, 1998). We report here an improved protocol for the crystallization of cytochrome $P450_{cam}$, based upon this mutation, which reliably yields large single crystals diffracting to better than 1.2 Å resolution.

2. Materials and methods

2.1. *Protein purification and crystallization*

The preparation of the C334A mutant of cytochrome $P450_{cam}$ has been described elsewhere (Nickerson & Wong, 1998). The mutant and wild-type proteins were expressed in *Escherichia coli* as described previously (Unger *et al.*, 1986), and purified by anion-exchange chromatography on fast-flow DEAE sepharose, followed by FPLC (fast protein liquid chromatography) on a Resource-Q 6 ml column (Pharmacia). Fractions with UV absorbance ratios (A_{392}/A_{280}) greater than 1.6 were pooled (Gunsalus & Wagner, 1978) and concentrated to 30 μ*M* in 40 m*M* potassium phosphate pH 7.4, 150 m*M* KCl and 1 m*M* D-camphor. Dithiothreitol (1 m*M*) was added to the wild-type only, and both wild-type and mutant were stored at 277 K. Immediately prior to crystallization, 10 mg of each protein was freshly purified by FPLC and concentrated to 200 μl (50 mg ml^{-1}) in freshly prepared 50 m*M* potassium phosphate pH 7.0, 50 m*M* DTT and 250 m*M* KCl, using Centricon-30 centrifugal concentrators (Amicon). Crystallization was performed by the hanging-drop vapour-diffusion method at 289 K in Linbro plates (Flow Laboratories Inc., USA). Well solutions consisted of 50 m*M* potassium phos-

phate pH 7.0, 50 m*M* DTT, 250 m*M* KCl, and a fine gradient of ammonium sulfate concentrations between 38 and 43% saturation, and the drops contained 2 µl protein and 2 µl well solution.

2.2. *Data collection and processing*

Crystals were transferred to 1 ml of a soak solution consisting of 50 m*M* potassium phosphate pH 7.0, 250 m*M* KCl and 40% saturated ammonium sulfate. Several grains of solid camphor were added, and the crystals were allowed to soak for 24 h. Immediately prior to data collection, crystals were soaked for 30 s in a cryoprotecting solution consisting of 50 m*M* potassium phosphate pH 7.0, 250 m*M* KCl, 25% saturated ammonium sulfate, saturated D-camphor and 30% glycerol, captured in a natural fibre loop and flash frozen at 100 K in a stream of nitrogen gas. X-ray diffraction data were collected over the course of 10 d on a Siemens multiwire area detector mounted on a Rigaku RU-200H rotating anode operating at 59 kV and 70 mA, with a crystal-to-detector distance of 11.5 cm, an oscillation width of 0.25° per frame, detector swing angles of 0, 25 and 50° and exposure times between 60 and 300 s. The program *ASTRO* (Siemens, 1992) was used to guide the choice of crystal orientations which would optimize data completeness. Autoindexing and data reduction were performed using *XDS* (Kabsch, 1993), and the processed data were then scaled using the programs *ROTAVATA* and *AGROVATA* (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Single crystals of both wild-type and mutant cytochrome $P450_{cam}$ were apparent in the hanging drops of most conditions between 38 and 43% saturated ammonium sulfate within 24 h. Whereas free-interface diffusion has been reported to occasionally yield crystals of the first orthorhombic form of $P450_{cam}$ that is unsuitable for X-ray diffraction studies (Poulos, 1986), only the diffraction-quality second orthorhombic form described by Poulos *et al.* (1982) was obtained by vapour diffusion. Crystals of the wild-type protein were generally of low quality, being irregularly shaped and interspersed with a large amount of what appeared to be aggregated protein, and were not studied further. Those of the C334A mutant, however, were much improved both in terms of size and overall morphology, and were completely free of aggregate, growing to maximum dimensions of 0.25 × 0.36 × 0.09 mm within 48 h (Fig. 1). The optimal condition varied slightly between protein preparations, but the largest single crystals were normally obtained from well solutions containing between 40 and 41% saturated ammonium sulfate. Spectroscopic properties confirmed the formation of a thiolate complex with DTT, as expected in the absence of camphor (Poulos, 1996), and thus crystals were soaked in an artificial mother liquor containing saturating amounts of D-camphor for 24 h prior to data collection. Crystals of $P450_{cam}$ are susceptible to radiation damage while in the X-ray beam (Poulos *et al.*, 1985), and cryogenic data collection was employed to enable the collection of a complete data set from a single crystal. Once grown, crystals begin to lose diffracting power and so were flash-frozen immediately upon completion of the camphor soaks for data collection or for cryogenic storage.

Crystals of (C334A)$P450_{cam}$ routinely diffract to beyond 1.2 Å at 100 K. The extension of useful diffraction to such high resolution may well be due to a combination of factors. Firstly, the C334A mutation assures a consistently monomeric sample throughout the purification and crystallization procedure, and appears to prevent the formation of protein aggregates observed in comparable samples of the wild-type. We have also observed a rapid decrease in diffraction power upon ageing of crystals, and whereas crystal growth may take as many as 7 d by free interface diffusion, it is essentially complete within 36 h by vapour diffusion. In addition, the ability of cryogenic data collection to extend useful resolution limits is well known. Finally, minor alterations in intermolecular contacts or solvent structure may have occurred near Cys334, which is near to, but not contacting, another molecule according to the published coordinates of the wild-type enzyme.

Given the difficulty of achieving a reasonably complete data set with an area detector at high swing angles, data from a single camphor-soaked crystal were only collected to 1.35 Å. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell dimensions $a = 106.17$, $b = 103.02$, $c = 36.62$ Å. A total of 179 468 observations were merged to obtain 75 423 unique reflections which represent 85.3% of theoretically possible data, with an R_{merge} of 3.8% (Table 1).† The unit-cell dimensions compare well with those reported by Poulos *et al.*

† Data-collection statistics as a function of resolution have been deposited with the IUCr in Table *S*1. Free copies may be obtained through the Managing Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU (Reference: AD0025).

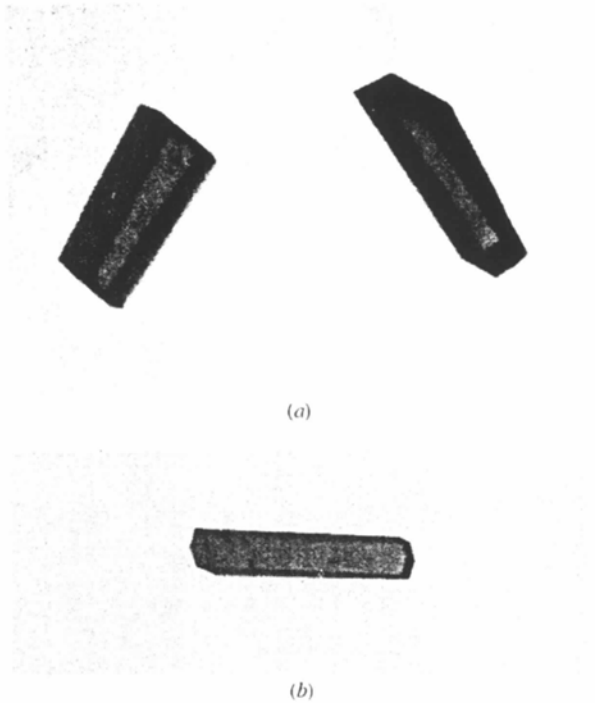

Fig. 1. (*a*), (*b*) Orthorhombic crystals of (C334A)$P450_{cam}$ with maximum dimensions of 0.25 × 0.36 × 0.09 mm.

Table 1. *Data-collection statistics*

Number of observations	179468
Number of unique reflections	75423
Resolution range (Å)	35.00–1.35
Highest resolution shell (Å)	1.39–1.35
Completeness overall (%)	85.3
Completeness in highest resolution shell (%)	63.7
$I/\sigma(I) > 3$ overall (%)	87.0
$I/\sigma(I) > 3$ in highest resolution shell (%)	64.8
R_{merge} overall (%)	3.8
R_{merge} in highest resolution shell (%)	21.5
Redundancy overall	2.4
Redundancy in highest resolution shell	1.6

(1987) of a = 108.67, b = 103.90, c = 36.38 Å, and molecular replacement will be carried out using their published coordinates as a search model followed by high-resolution refinement with *SHELX*97 (Sheldrick & Schneider, 1997). The superior quality of cryogenic data and 'freezing out' of dynamic processes, coupled with the extension of the resolution to 1.35 Å, may well provide additional insight into the structure and function of cytochrome $P450_{cam}$. More importantly, however, the crystallization procedure described herein should facilitate the structural characterization of genetic variants of the enzyme which has thus far been hindered by the inherent difficulty of published methods.

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