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Expression, crystallization and preliminary diffraction studies of the *Pseudomonas putida* cytochrome P450cam operon repressor CamR

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The *Pseudomonas putida cam* repressor (CamR) is a homodimeric protein that binds to the *camO* DNA operator to inhibit the transcription of the cytochrome P450cam operon *camDCAB*. CamR has two functional domains: a regulatory domain and a DNA-binding domain. The binding of the inducer D-camphor to the regulatory domain renders the DNA-binding domain unable to bind *camO*. Native CamR and its selenomethionyl derivative have been overproduced in *Escherichia coli* and purified. Native CamR was crystallized under the following conditions: (i) 12–14% PEG 4000, 50 m*M* Na PIPES, 0.1 *M* KCl, 1% glycerol pH 7.3 at 288 K with and without camphor and (ii) 1.6 *M* P_i, 50 m*M* Na PIPES, 2 m*M* camphor pH 6.7 at 278 K. The selenomethionyl derivative CamR did not crystallize under either of these conditions, but did crystallize using 12.5% PEG MME 550, 25 m*M* Na PIPES, 2.5 m*M* $MgCl_2$ pH 7.3 at 298 K. Preliminary X-ray diffraction studies revealed the space group to be orthorhombic ($P2_12_12$), with unit-cell parameters a = 48.0, b = 73.3, c = 105.7 Å. Native and selenomethionyl derivative data sets were collected to 3 Å resolution at SPring-8 and the Photon Factory.

1. Introduction

Pseudomonas putida PpG1 has a catabolism system for D-camphor that utilizes the gene products of the cytochrome P450cam hydroxylase operon (*camDCAB*). CamR is a homodimeric protein (MW 47.8 kDa) that negatively regulates the expression of both its own gene (*camR*) and *camDCAB* by binding to a single 26 bp operator (*camO*) (Aramaki, Koga *et al.*, 1993; Aramaki, Fujita *et al.*, 1994; Aramaki, Sagara *et al.*, 1995; Fujita *et al.*, 1993). In the presence of D-camphor, *camO* is released from CamR and these genes are divergently transcribed from overlapping promoters (Aramaki, Koga *et al.*, 1993; Aramaki, Fujita *et al.*, 1994; Aramaki, Sagara *et al.*, 1995; Fujita *et al.*, 1993). CamR contains two domains: the DNA-binding domain and the regulatory domain. The regulatory domain contains homology to two other proteins that bind D-camphor: P450cam and putidaredoxin reductase (which is found in the cytochrome P450cam operon). Binding of D-camphor by the regulatory domain modulates the conformation of the DNA-binding domain so as to reduce the affinity for the operator DNA *camO*. Two molecules of D-camphor can bind to one homodimeric CamR in a negative cooperative manner (Kabata *et al.*, in preparation). This is in contrast to the positive cooperativity of the *pur* repressor (PurR; Choi & Zalkin, 1992) and the noncooperativity of the *tet* repressor (TetR; Takahashi *et al.*, 1986, 1991) and the *trp* repressor (TrpR; Arvidson *et al.*, 1986; Schmitt *et al.*, 1995). The DNA-binding domain of CamR is thought to contain a helix–turn–helix (HTH) motif as a DNA-reading head (Aramaki, Yagi *et al.*, 1995; Aramaki, Sagara *et al.*, 1994; Harrison, 1991; Suzuki *et al.*, 1996). However, no structural information is available for CamR or its complexes with D-camphor and its cognate DNA. In order to clarify the structural basis for the D-camphor modulation of the *camO* DNA binding of CamR, we report here the expression, crystallization and preliminary X-ray diffraction studies for CamR.

2. Expression and purification

In the early stages of this work, a PL promoter system was used to express CamR (Aramaki, Sagara *et al.*, 1993). Subsequently, a more efficient system using the T7 promoter was constructed as follows. Plasmid pET21a (Novagen) was used as an expression vector for the production of CamR. The gene encoding CamR was inserted between the *Nde*I and *Hin*dIII restriction sites of pET21a under the control of the T7 promoter. The translational initiation codon of CamR was changed by insertion into the *Nde*I site from GTG to ATG. The resulting expression plasmid was designated pCAMR. *Escherichia coli* strain BL21(DE3)pLysS cells (Novagen) harbouring pCAMR were cultured at 310 K in LB media containing 100 mg l^{-1} ampicillin. When the OD_{600} reached 0.5–0.7, the culture was supplemented with 0.5 m*M* IPTG and 2 m*M* camphor to induce CamR expression. The cells were grown for a further 4 h before being harvested by centrifugation. The cell pellets were resuspended in lysis buffer (100 m*M* Tris–HCl pH 7.5, 2 m*M* EDTA) and disrupted by sonication. Ammonium sulfate was gradually added to the cell lysate to a final concentration of 0.25 g ml^{-1} and the solution was gently stirred for 30 min at 277 K. The solution was centrifuged and the resultant pellets were dissolved in 50 m*M* Tris–HCl pH 7.5, 1 m*M* EDTA, 10% glycerol. The dissolved fraction was dialyzed against 50 m*M* Tris–HCl pH 7.5, 1 m*M* EDTA, 10% glycerol and 50 m*M* NaCl. The dialysate was purified using two anion-exchange chromatography columns [DEAE Toyopearl (Tosoh Biosciences) and MonoQ (Amersham Biosciences)] with the elution conditions consisting of a linear gradient of NaCl from 0 to 2 *M* in 60 min at a flow rate of 1 ml min^{-1}. Further purification was performed by gel-filtration chromatography using a Superdex 75 column (Amersham Biosciences) previously equilibrated with 50 m*M* Tris–HCl pH 7.5, 1 m*M* DTT, 0.1 m*M* EDTA, 10% glycerol and 50 m*M* NaCl at a flow rate of 2 ml min^{-1}. The final yield of recombinant CamR was approximately 5–10 mg per litre of culture. The size and homogeneity of the protein were confirmed by SDS–PAGE (Fig. 1). The selenomethionyl CamR protein (SeMet CamR) was produced by *E. coli* B834 (DE3) cells harbouring pCAMR, which were cultured in minimal media containing selenomethionine. SeMet CamR was purified using the same method as was used for the native protein.

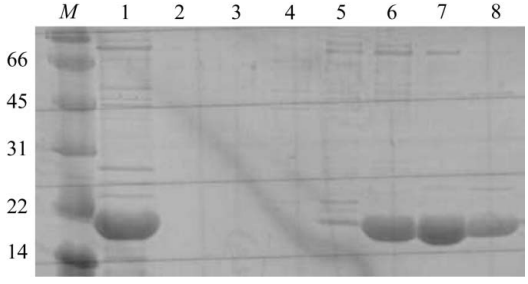
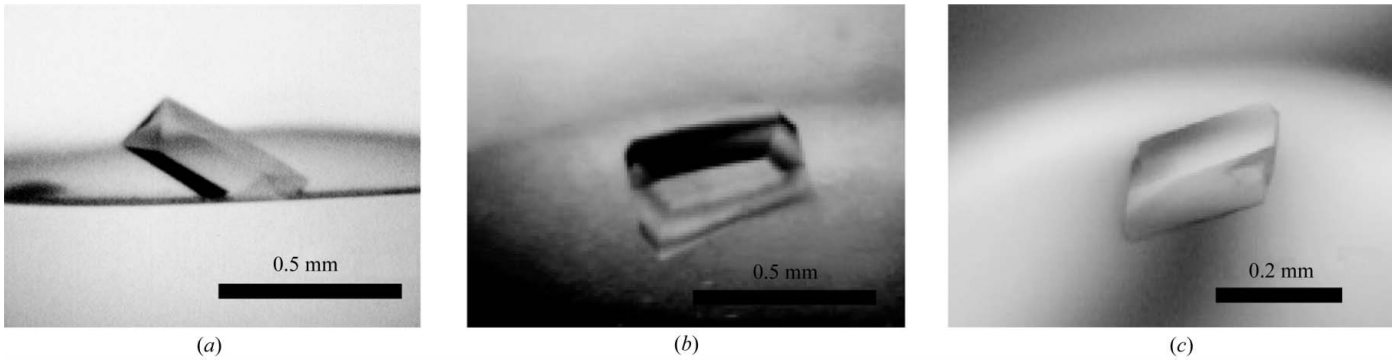


Figure 1
SDS–PAGE of each fraction of the gel filtration of native CamR protein (Superdex 75, Amersham Biosciences). Lane 1, fraction before being applied onto the Superdex 75 column. Lanes 2–8, eluted fractions 12, 15, 55, 56, 57, 58 and 59. Fractions 57–59 were used for crystallization. Lane *M*, molecular-weight standards in kDa.

3. Crystallization

The recombinant native CamR protein was concentrated to 15–20 mg ml^{-1} in 20 m*M* Tris–HCl pH 8. In the initial stages, screening of crystallization conditions for native CamR was performed using a locally constructed screen system (Shirakihara *et al.*, 1991) employing the microbatch method. In brief, precipitant, buffer (final concentration 50 m*M*), water and CamR were mixed, adding the protein last, to give a final volume of 5 µl. The mixture was placed in a small siliconized glass tube (inner diameter 2 mm, length 20 mm) and the tube was sealed tightly with Parafilm and incubated at 278 or 298 K. For promising conditions, a subsequent extensive search was made for the optimum conditions. The crystallization parameters that were varied included (i) the concentration of precipitants, (ii) the pH, (iii) the concentration and type of general additives such as glycerol and salts, (iv) the concentration and species of specific additives such as camphor and its derivatives, (v) the CamR concentration and (vi) the temperature. In the case of PEG, the effect of its molecular weight was also examined. The conditions for two crystal forms were optimized in this way.

The first of the two crystal forms (crystal form 1; Fig. 2*a*) was obtained with a 5 µl solution which contained 12–14% PEG 4000, 50 m*M* Na PIPES pH 7.3, 0.1 *M* KCl, 1% glycerol, 1–5 m*M* camphor, 7 mg ml^{-1} CamR and was incubated at 288 K. The parameters describing the conditions had to be strictly adhered to in order to obtain large crystals (0.5 × 0.4 × 0.3 mm), with the exception of the camphor concentration. Identical conditions without camphor produced similar-looking crystals. These crystals grew to maximum size after two months. The second crystal form (Fig. 2*b*) was obtained from a 5 µl solution which contained 1.6 *M* sodium/potassium phosphate, 50 m*M* Na PIPES pH 6.7, 2 m*M* camphor, 5 mg ml^{-1} CamR and was incubated at 288 K. Its growth period was similar to that of crystal form 1. The second crystal form was more tolerant towards variable quality CamR preparations. The better preparation obtained from the T7 promoter overexpression system was essential for



(*a*) (*b*) (*c*)

Figure 2
Crystals of CamR. (*a*) Crystals of native CamR grown in 12–14% PEG 4000, 50 m*M* Na PIPES, 0.1 *M* KCl, 1% glycerol pH 7.3 at 288 K with 2 m*M* camphor. (*b*) Crystals of native CamR grown in 1.6 *M* P_i, 50 m*M* Na PIPES, 2 m*M* camphor pH 6.7 at 278 K. (*c*) Crystals of the SeMet-derivative CamR grown in 12.5% PEG MME 550, 25 m*M* Na PIPES, 2.5 m*M* $MgCl_2$ pH 7.3 at 298 K.

Table 1
Data-collection statistics for SeMet CamR crystal.

Values in parentheses are for the highest resolution shell (3.16–3.0 Å).

Data sets	Edge	Peak	Remote
Wavelength (Å)	0.9794	0.9791	0.9879
Resolution (Å)	3.0	3.0	3.0
No. of observed reflections	46695	46653	46771
No. of unique reflections	7823	7804	7813
Redundancy	6.0 (5.7)	6.0 (5.7)	6.0 (5.7)
$\langle I/\sigma(I)\rangle$	4.5 (2.3)	4.9 (2.3)	4.7 (2.2)
$R_{\rm merge}$† (%)	14.8 (39.0)	14.4 (39.0)	14.7 (39.0)
Completeness (%)	98.8 (96.8)	98.7 (96.8)	98.7 (96.0)

† $R_{\rm merge} = \sum |I - \langle I\rangle| / \sum \langle I\rangle$.

formation of crystal form 1. The original PL promoter system produced CamR preparations of varied quality, some of which failed to reproduce the conditions for crystal form 1. In later stages, the microbatch method was replaced by the oil-batch method. In the oil-batch method, a crystallization drop (3–5 µl) was produced as described for the microbatch method. The drop was then placed at bottom of a well in an HLA plate (Nunc International), covered with silicone oil (9–11 µl) and incubated at an optimum temperature. This change did not bring about essential changes in the crystallization conditions for the two crystal forms. Crystals grown in this manner were harvested after one month for cryoprotection experiments, before they became too large. Crystals obtained from both conditions were suitable for cryoexperiments on addition of ethylene glycol (final conditions were 12–14% PEG 4000, 50 m*M* Na PIPES pH 7.3, 0.1 *M* KCl, 1% glycerol, 1–5 m*M* camphor, 20–25% ethylene glycol for crystal form 1 and 1.6 *M* sodium/potassium phosphate, 50 m*M* Na PIPES pH 6.7, 2 m*M* camphor, 20–25% ethylene glycol for crystal form 2).

The SeMet form of CamR did not crystallize under the conditions used for the native protein. Crystallization conditions were searched for using Crystal Screens I and II and Natrix (Hampton Research) by the oil-batch method in HLA plates (Nunc International). Typically, 1 µl of a 3–5 mg ml^{-1} protein solution in 20 m*M* Tris pH 8.0 was mixed in a 1:1 ratio with the crystallization screen solution and the resultant 2 µl drops were covered with 12 µl silicone oil and incubated at 288 or 298 K. Natrix 31 (30% PEG MME 550, 50 m*M* Na HEPES, 5 m*M* $MgCl_2$ pH 7.0 at 288 K) produced crystals. After optimization, again using the oil-batch method, highly ordered crystals were obtained in 12.5% PEG MME 550, 25 m*M* Na PIPES, 2.5 m*M* $MgCl_2$ pH 7.3 at 298 K. Cryoprotection was achieved by increasing the concentration of PEG MME 550 to 30%. Crystals appeared within 3 d and reached maximum size (0.2 × 0.2 × 0.3 mm) after one week (Fig. 2*c*).

4. X-ray analysis

An initial characterization of the native CamR crystals in both crystal forms was hampered by their sensitivity to temperature changes; mounting the crystals and performing diffraction experiments at ambient temperature gave rise to faint cracks in the crystals and resulted in weak and disordered diffraction patterns. Nevertheless, preliminary in-house X-ray characterization of capillary-mounted crystals revealed the fundamental diffraction parameters for the two crystal forms. Both crystal forms diffracted to 3 Å resolution and showed diffraction patterns consistent with a primitive orthorhombic lattice with unit-cell parameters a = 48.8, b = 77.5, c = 105.3 Å, $\alpha = \beta = \gamma = 90^\circ$. Once cryoconditions had been determined as described above, data collection became feasible for both crystal forms.

Preliminary characterization of both native and SeMet crystals at BL18A of the Photon Factory (Tsukuba, Japan) and BL40B2 of SPring-8 (Harima, Japan) indicated an orthorhombic space group. 3 Å resolution MAD data sets were collected using a SeMet CamR crystal utilizing the imaging-plate detector system at beamline BL18A. Diffraction data were autoindexed, integrated and corrected for Lorentz and polarization effects using the program *MOSFLM* (Collaborative Computational Project, Number 4, 1994). Scaling and merging of the data in *SCALA* revealed the space group to be $P2_12_12$ (detailed data statistics are summarized in Table 1) and allowed the unit-cell parameters to be refined to a = 48.0, b = 73.3 c = 105.7 Å, $\alpha = \beta = \gamma = 90^\circ$. For this space group, the unit-cell parameters are consistent with an asymmetric unit containing one CamR dimer (two CamR molecules) and 50% solvent. A full structure determination is in progress.

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