

SHORT COMMUNICATION

The dimerization of *Pseudomonas putida* cytochrome P450_{cam}: practical consequences and engineering of a monomeric enzyme

D.P. Nickerson and L.-L. Wong¹

Department of Chemistry, Inorganic Chemistry Laboratory,
South Parks Road, Oxford OX1 3QR, UK

¹To whom correspondence should be addressed

Cytochrome P450_{cam} dimerizes via the formation of an intermolecular disulfide bond, complicating the storage and handling of the enzyme, particularly at higher concentrations. The dimeric enzyme is 14% less active than the monomer and forms at a slow but significant rate even at 4°C ($k = 1.09 \times 10^{-3} \text{ mM}^{-1} \text{ h}^{-1}$). To eliminate any ambiguity introduced by dimer formation and to simplify handling and storage of the enzyme, site-directed mutagenesis was used to identify C334 as the single cysteine residue responsible for the formation of the disulfide linkage and to engineer a monomeric enzyme by substituting an alanine in its place. The C334A mutant is identical with the wild-type P450_{cam} monomer in terms of optical spectra, camphor binding and turnover activity, but shows no evidence of dimerization and aggregation even at millimolar concentrations. Preliminary ¹H NMR investigations also indicate a significant improvement in the quality of spectra obtained with this mutant. (C334A)P450_{cam} is therefore proposed as an alternative to the wild-type enzyme—a base mutant otherwise identical with the wild-type but with improved handling characteristics.

Keywords: cytochrome P450_{cam}/dimer/disulfide/monooxygenase/mutagenesis

Introduction

Cytochrome P450_{cam} (CYP101), isolated from the soil bacterium *Pseudomonas putida* (Gunsalus and Wagner, 1978; Sligar and Murray, 1986), is the best characterized of the P450 monooxygenases. The catalytic mechanism of P450_{cam} has been studied in detail (Mueller *et al.*, 1995) and the high-resolution crystal structure of the camphor-bound form has been determined (Poulos *et al.*, 1987). The *camC* gene encoding P450_{cam} has been cloned from *P. putida* and the protein has been overexpressed in *Escherichia coli* (Koga *et al.*, 1985; Unger *et al.*, 1986), simultaneously simplifying large-scale production and purification of the enzyme and allowing the preparation of site-directed mutants for detailed structure–function studies (Mueller *et al.*, 1995). Consequently, P450_{cam} has provided the most commonly used structural and mechanistic model for examining P450 enzymes found in eukaryotic and other bacterial systems.

Lipscomb *et al.* (1978) demonstrated that wild-type P450_{cam} showed a propensity to dimerize during protein purification and on freeze–thaw cycles. Since the dimerization reaction could be reversed by reducing agents such as β -mercaptoethanol, the involvement of intermolecular cysteine disulfide bonds was implicated. Surprisingly, the activity of the dimer was found to be indistinguishable from that of the monomer.

Titration of monomeric wild-type P450_{cam} with the thiol-specific alkylating reagent *N*-ethylmaleimide indicated the presence of four accessible cysteines, one of which was considerably more reactive than the others. It was concluded that dimerization involved one very reactive cysteine per P450_{cam} molecule, but it was not possible to identify the key residue, since neither the amino acid sequence nor crystal structure of P450_{cam} were known at the time.

The dimerization of wild-type P450_{cam} complicates both the purification and handling of the enzyme. It is often necessary to treat samples with a disulfide-reducing agent such as β -mercaptoethanol or dithiothreitol (DTT), followed by gel filtration to remove the thiol, immediately before experiments. Techniques which require high concentrations (millimolar range) of protein, such as the growth of protein crystals for X-ray diffraction or NMR and EPR, must contend with possible complications arising from dimer formation during the experiment. Inclusion of exogenous sulfhydryl reagents within experimental samples can lead to unexpected results, as in the case of the crystallization of P450_{cam} (Poulos *et al.*, 1982), in which the structure of the bis-thiolate DTT complex was unintentionally solved owing to the inclusion of this reagent in crystallization buffers (Poulos, 1997). Similar dimerization problems with yeast iso-1-cytochrome *c* from *Saccharomyces cerevisiae* and in the reconstitution of human myoglobin from a solubilized fusion protein have been overcome by site-directed mutagenesis. Indeed, the base mutants C107S of yeast iso-1-cytochrome *c* (Cutler *et al.*, 1987) and C110A of human myoglobin (Varadarajan *et al.*, 1989) are often referred to as the ‘wild-type’ proteins. More recently, site-directed mutagenesis has been used to prevent the formation of oligomeric aggregates of insulin at neutral pH, resulting in a mutant which remains monomeric at millimolar concentrations and is amenable to high-resolution NMR structural analysis (Olsen *et al.*, 1996). We have used site-directed mutagenesis to identify and replace the highly reactive cysteine residue of P450_{cam} with alanine in order to circumvent dimer formation and the consequent handling problems. The preparation of this mutant, its properties and preliminary NMR spectra which demonstrate the value of a strictly monomeric P450_{cam} variant are described here.

Materials and methods

General

All chemicals and buffers were of biotechnology grade or of the highest purity available and were purchased from Sigma or Boehringer Mannheim. UV–visible spectra were recorded on a Cary 1E dual-beam spectrophotometer. The crystallographic coordinates of camphor-bound cytochrome P450_{cam} were obtained from the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977).

General protein expression, purification and storage

The three proteins of the *P. putida* camphor hydroxylating system, namely P450_{cam} (both wild-type and mutants), putida-

redoxin and putidaredoxin reductase, were expressed in *E.coli* and purified according to literature methods (Unger *et al.*, 1986; Peterson *et al.*, 1990; Yasukochi *et al.*, 1994). The preparative-scale separation of monomeric and dimeric P450_{cam} was carried out by FPLC on a Resource Q 6 ml column (Pharmacia), by developing a gradient of 0 to 250 mM KCl over 30 column bed volumes in 40 mM phosphate (pH 7.4), 1 mM camphor. The dimer was detected on an analytical scale by gel filtration on a Superose-12 10/30 column (Pharmacia), eluting with 40 mM phosphate (pH 7.4), 1 mM camphor, at 0.5 ml/min and monitoring at 280 nm.

Kinetics of dimer formation

Monomeric P450_{cam} was freshly purified by anion-exchange chromatography and equilibrated into 40 mM phosphate (pH 7.4), 1 mM camphor. The protein solution was rapidly concentrated to 1.1 mM by high-pressure ultrafiltration (Amicon) and stored at 4°C. Aliquots were assayed for dimer content by analytical gel filtration at regular intervals and the percentage conversion to the dimeric form was calculated by integration of the chromatograms.

Dissociation constant determination and activity assays

All proteins were exchanged into 50 mM Tris-HCl buffer (pH 7.4) by gel filtration using PD-10 columns (Pharmacia) immediately prior to each experiment. Dissociation constants for the binding of *d*-camphor to 2 µM samples of ferric P450_{cam} were determined by the method of Peterson (1971). Enzymatic activities were assayed both by NADH consumption rates (UV-visible spectrophotometry) and product detection (gas chromatography). The NADH consumption rates were measured by monitoring the time course of the 340 nm absorption of NADH in 1.5 ml incubation mixtures (50 mM Tris-HCl, pH 7.4) containing 0.05 µM P450_{cam}, 16 µM putidaredoxin, 0.5 µM putidaredoxin reductase, 100 mM KCl, 1 mM camphor (added as a 1 M solution in ethanol) and 250 µM NADH (Gunsalus and Wagner, 1978). For gas chromatographic analysis, the incubation mixtures were extracted with 150 µl of chloroform and the aqueous and organic phases were separated by centrifugation (4000 *g*) at 4°C for 20 min. The chloroform extracts were analysed on a Fisons Scientific Instruments 8000 Series gas chromatograph equipped with a fused-silica DB-1 column (30 m × 0.25 mm i.d.) held isothermal at 120°C and a flame-ionization detector. The integrated areas of the 5-*exo*-hydroxycamphor peaks, the retention time of which was 11.9 min under these conditions, were compared.

Site-specific mutagenesis of P450_{cam}

General DNA manipulations followed standard methods (Sambrook *et al.*, 1989). Oligonucleotide-directed site-directed mutagenesis of the *camC* gene was carried out on an M13mp19 subclone by the method of Kunkel (1985) according to the Bio-Rad Mutagen kit. The mutagenic oligonucleotides were 5'-GTGGACTCGCGCCAACGGCGGA-3' (C58A), 5'-TTC-CAGCGAGGCCCGGTTTCATC-3' (C85A), 5'-GGAGCTGG-CCGCCTCGCTGATC-3' (C136A), 5'-GCAAGGACAGGC-CAACTTCACC-3' (C148A) and 5'-CGAAAACGCCGCC-CCGATGCAC-3' (C334A). Mutants were identified by Sanger dideoxy chain termination DNA sequencing using the Sequenase Version 2 kit (Amersham International, Amersham, UK).

¹H NMR spectroscopy

The 500 MHz ¹H NMR spectra of wild-type P450_{cam} and the C334A mutant were recorded on a Bruker AM500 spectro-

meter. Camphor was removed from stock samples by gel filtration on PD10 columns (Pharmacia) and then buffer-exchanged by ultrafiltration into 40 mM potassium phosphate (pH 7.4), 100 mM KCl in D₂O. The final concentrations of the samples were ~1.1 mM. ¹H NMR spectra were recorded at 308 K with solvent saturation and processed using identical parameters for spectral comparison.

Results

Kinetics of dimer formation

Monomeric and dimeric P450_{cam} were easily purified from aged samples of the wild-type protein which had been stored at 4°C, both on a preparative scale by anion exchange and on an analytical scale by size exclusion chromatography. The formation of dimer was monitored by analytical gel filtration of a 1.1 mM sample of the wild-type enzyme at periodic intervals over the course of 8 days. The second-order rate constant of dimerization was determined to be $1.09 \times 10^{-3} \text{ mM}^{-1} \text{ h}^{-1}$ at 4°C. At low protein concentrations (<10 µM) the rate of dimer formation was much reduced and it was possible to store the monomer at 4°C for several days without appreciable accumulation of the dimer.

Camphor binding and turnover by P450_{cam} monomer and dimer

Camphor was removed from dilute solutions of pure monomer and dimer by gel filtration and its binding constant to both proteins was measured by monitoring the formation of the five-coordinate high-spin ferric haem upon addition of aliquots of camphor to the proteins. The camphor binding constants thus determined for the monomer and dimer were 10.3 ± 1.1 and $10.8 \pm 1.3 \text{ µM}$ respectively. The NADH turnover rate of the dimer ($30.7 \pm 0.6 \text{ s}^{-1}$) was 14% lower than that of the monomer ($35.8 \pm 0.6 \text{ s}^{-1}$). Gas chromatographic analysis of the incubation products of both monomeric and dimeric samples indicated 100% coupling of NADH consumption to the formation of the single product 5-*exo*-hydroxycamphor.

Identification of solvent-accessible cysteines

The amino acid sequence of wild-type P450_{cam} contains eight cysteine residues (Haniu *et al.*, 1982; Unger *et al.*, 1986). Visual inspection of the three-dimensional structure of camphor-bound P450_{cam} identified cysteines 58, 85, 136, 148 and 334 as being at or near the surface of the protein, with C334 being the most exposed of the five. Cysteines 242 and 285 appeared to be relatively buried within helices I and K, respectively, while C357 was deep in the interior and bound to the haem iron. Mutants of P450_{cam} were therefore prepared in which the cysteine residues at positions 58, 85, 136, 148 and 334 were changed to structurally similar but chemically inactive alanines.

Characterization of the mutants

The electronic absorption spectra of freshly purified samples of each of the five mutants, both in the presence and absence of camphor, were identical with those of wild-type P450_{cam}. The camphor-bound ferrous carbonmonoxy forms all showed the 448 nm Sorét band characteristic of P450 enzymes. The camphor turnover activities of monomeric samples of each mutant were identical with the monomeric wild-type P450_{cam}, as measured by both NADH consumption and incubation product analysis. Samples of wild-type and mutant proteins were concentrated to 120 µM and stored at 4°C and aliquots were removed periodically for analysis. Within 24 h, the P450_{cam} dimer was detected by gel filtration chromatography

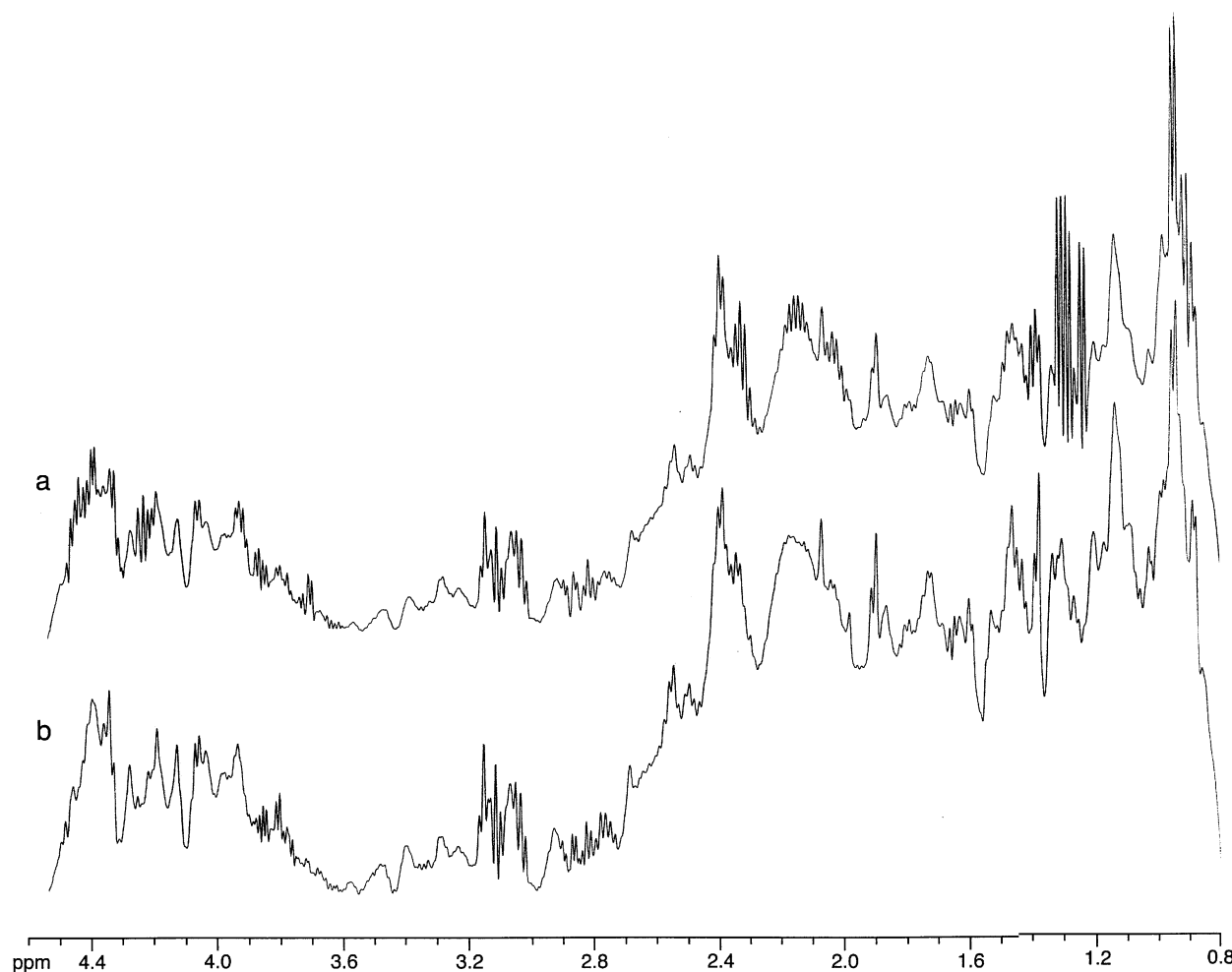


Fig. 1. One-dimensional 500 MHz ^1H NMR spectrum of (a) C334A and (b) wild-type P450_{cam} in 40 mM potassium phosphate buffer (pH 7.4) in D_2O at 308 K.

in the wild-type and C58A, C85A, C136A and C148A mutant proteins, but there was no evidence of dimer formation for the C334A mutant. The dimer continued to accumulate in the samples of the wild-type and the four other mutants while the C334A mutant remained monomeric after 3 months at 4°C. We therefore conclude that wild-type P450_{cam} dimerizes exclusively through C334.

(C334A)P450_{cam} was easily expressed and purified in large quantities in the absence of reducing agents and stock samples were concentrated to 1.1 mM and stored at 4°C. Whereas such concentrated preparations of freshly purified wild-type P450_{cam} would become turbid owing to the formation of aggregates, necessitating frequent filtration or centrifugation of the samples, stocks of the C334A mutant remained completely clear. The spectroscopic properties of this mutant were indistinguishable from the monomeric wild-type protein and the camphor dissociation constant and NADH turnover activity were also identical (Table I). Finally, camphor turnover by the C334A mutant remained 100% coupled to NADH consumption and produced only 5-*exo*-hydroxycamphor.

^1H NMR spectrum of (C334A)P450_{cam}

The 500 MHz ^1H NMR spectra of the diamagnetic, substrate-free forms of wild-type P450_{cam} and the C334A mutant were compared. The overall features of the two spectra and the chemical shifts of clearly resolved resonances were almost

Table I. Camphor dissociation constants (K_d) and activities of wild-type and mutant cytochromes P450_{cam}^a

	K_d (μM)	NADH turnover rate (s^{-1})
Wild-type monomer	10.3 ± 1.1	35.8 ± 0.6
Wild-type dimer	10.8 ± 1.3	30.7 ± 0.6
C334A	10.2 ± 0.6	35.9 ± 1.1

^aData given as mean $\pm$ standard deviation for $n \geq 5$.

identical. The linewidths of resonances in the aromatic regions were similar in the two spectra, but the resonances further upfield in the spectrum of the C334A mutant were sharper, with more resonances becoming resolved compared with the featureless envelopes in the spectrum of the wild-type (Figure 1).

Discussion

We sought to assess the extent to which the dimerization of P450_{cam} may complicate the biochemistry of this enzyme by undertaking a more complete characterization of the properties of the dimer and the rate of its formation *in vitro*. The separation of monomeric and dimeric enzyme was achieved by both anion-exchange and gel filtration chromatography, with the anion-exchange method being the most suitable for

preparative-scale separations, while analytical gel filtration, with very consistent peak shape and retention times, proved to be the best choice for a more quantitative measure of dimer content. The second-order rate constant of dimerization was $1.09 \times 10^{-3} \text{ mM}^{-1} \text{ h}^{-1}$ at 4°C , representing a conversion of nearly 20% of the 1.1 mM sample to the dimeric form within 8 days.

The accessibility of each of the eight cysteine residues of P450_{cam} was assessed by inspection of the crystallographic coordinates and suggested that of the five cysteines near the protein surface (58, 85, 136, 148 and 334), C334 was the most likely candidate for disulfide bond formation. Nevertheless, each of the five most exposed cysteines was substituted with alanine by site-directed mutagenesis and the properties of the mutants were examined. When concentrated samples of these and the wild-type were assayed for dimer formation, the C334 mutant was the only protein to remain monomeric even at high concentrations ($>1 \text{ mM}$) over an extended period. The camphor binding constant and oxidation activity of this mutant were also identical with those of the wild-type monomer (Table I). The successful engineering of a strictly monomeric P450_{cam} enzyme also had an unexpected benefit: while the wild-type P450_{cam} showed a tendency to aggregate, especially at moderate concentrations ($>100 \mu\text{M}$), the mutant did not show any evidence of aggregation even at the highest concentrations studied ($>1 \text{ mM}$). This property of the mutant is very valuable in all protein manipulations.

High-resolution NMR has been used to assign tentatively several paramagnetically shifted methyl resonances near the haem moiety of base adducts of P450_{cam}. The two-dimensional methods required were made possible by the sharper linewidths of the adducts compared with other low-spin forms (Banci *et al.*, 1994). Our preliminary one-dimensional ^1H NMR spectroscopic investigations on the low-spin camphor-free P450_{cam} showed that the prevention of dimer formation by the C334A mutation might be beneficial for obtaining spectra of higher resolution than is possible with the wild-type. Whilst the resonances in the aromatic region were only slightly sharpened, primarily because NMR linewidths for these side-chains depend mainly on the rates of aromatic ring flips (Veitch and Williams, 1990), resonances in the other regions of the spectrum were significantly sharper, with more resonances being resolved in the mutant. This might arise from the absence of any dimer in the concentrated sample of the mutant, thus removing the broader resonances due to the dimer which has a slower tumbling rate. We are investigating the possibility of obtaining higher resolution two-dimensional NMR spectra with the C334A mutant.

The camphor binding constant of P450_{cam} monomer and dimer were identical within experimental error (Table I), suggesting that the substrate retains equal access to the active-site in each form. This is consistent with the location of the substrate access channel proposed by Poulos *et al.* (1986), since the hydrophobic corridor defined by the B' helix and the F–G loop are well-removed from the protein–protein interface in the vicinity of C334. The dimer showed the $>96\%$ shift of the haem spin-state to high spin upon camphor binding observed for the monomer and 5-*exo*-hydroxycamphor was the only product and thus camphor binding and the C–H bond activation steps were not perturbed by dimerization.

The dimer was found to retain 86% of the activity of the monomer. This small difference in activity is inconsistent with a change in the rate-determining step which, under the

experimental conditions, is the first electron transfer from reduced putidaredoxin to P450_{cam} (Hintz *et al.*, 1982). That product release has not become rate limiting implies that the product exit channel is unobstructed in the dimer. This is in accord with the proposal that the hydroxylated product may leave the active-site via a hydrophilic channel comprised of several ordered water molecules near the haem propionate groups (Poulos *et al.*, 1987), since this region is also well removed from the interface between the two P450_{cam} molecules. It is more likely that the slight, but significant, reduction in the activity of the dimer arises from perturbations of the P450_{cam}–putidaredoxin binding interaction. Cysteine 334 is not far removed from the proposed interface between the two proteins (Stayton *et al.*, 1989; Pochapsky *et al.*, 1996) and dimerization via the thiol side-chain would be expected to interfere with the protein–protein interactions.

In conclusion, we have shown that C334 is the surface cysteine residue involved in dimerization of wild-type P450_{cam} via intermolecular disulfide bond formation. The position of this residue in the three-dimensional structure gave rise to a dimeric form in which the structural elements generally held to be most critical to P450_{cam} catalysis had been retained. The C334A mutation ensures the presence of a single P450_{cam} species in solution at all concentrations and also appears to prevent protein aggregation—both highly desirable in protein chemistry investigations. We have now incorporated this base mutation in our mutagenesis work on this important haem monooxygenase enzyme.

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