FEBS Letters 451 (1999) 342–346 FEBS 22070

Surface-modified mutants of cytochrome $P450_{cam}$: enzymatic properties and electrochemistry

Kenneth Kam-Wing Lo, Luet-Lok Wong, H. Allen O. Hill*

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

Received 19 April 1999

Abstract We report the electrochemistry of genetic variants of the haem monooxygenase cytochrome $P450_{cam}$. A surface cysteine-free mutant (abbreviated as SCF) was prepared in which the five surface cysteine residues Cys-58, Cys-85, Cys-136, Cys-148 and Cys-334 were changed to alanines. Four single surface cysteine mutants with an additional mutation, R72C, R112C, K344C or R364C, were also prepared. The haem spin-state equilibria, NADH turnover rates and camphor-hydroxylation properties, as well as the electrochemistry of these mutants are reported. The coupling of a redox-active label, *N*-ferrocenylmaleimide, to the single surface cysteine mutant SCF-K344C, and the electrochemistry of this modified mutant are also described.

Key words: Electrochemistry; Mutagenesis; $P450_{cam}$; Redox-active label; Surface cysteine

1. Introduction

Cytochrome P450 enzymes catalyse primarily the oxidation of un-activated carbon-hydrogen bonds to an alcohol functionality [1]. The best characterised of all P450 enzymes is cytochrome $P450_{cam}$ (CYP101) found in the soil bacterium *Pseudomonas putida* [2]. This enzyme catalyses the regio- and stereospecific hydroxylation of D-(+)-camphor to 5-*exo*-hydroxycamphor. The mechanism of the first electron-transfer step has been the focus of intense interest [3,4]. Most previous studies on the redox properties of the $P450_{cam}$ system were carried out using spectroscopic titration methods [5,6] but the direct electrochemistry of cytochrome $P450_{cam}$ system has been reported recently [7–9].

We have demonstrated the direct, un-promoted electrochemical response of $P450_{cam}$ at a bare edged-plane graphite (EPG) electrode [7], i.e. the electrochemistry is observed in the absence of mediators. This observation suggests that a specific pattern of positively charged surface amino acid residues favours an interaction between the enzyme and the electrode surface. Mutagenesis studies [10–13] and computer modelling [14,15] had suggested that the basic surface residues Arg-72, Arg-112, Lys-344 and Arg-364 of $P450_{cam}$ interact with acidic residues on the surface of the native redox partner, putidaredoxin, in the complex between these two proteins. These basic $P450_{cam}$ surface residues are on the proximal side of the haem and in the region of the enzyme where the haem is closest to the surface [16].

In order to investigate the role played by these residues on the electron transfer to $P450_{cam}$, and to facilitate its immobilisation on the electrode surface, we have carried out site-directed mutagenesis to modify the surface of this enzyme. Cysteine residues were chosen to replace these four basic residues based on the considerations that: (1) thiols show a strong affinity towards a gold surface and the genetic variants could be immobilised in a specific orientation [17,18], and (2) cysteines react readily with the maleimide moiety under physiological conditions and redox-active labels could be attached on the surface of the enzyme at a desired location.

The amino acid sequence of wild-type (WT) $P450_{cam}$ contains eight cysteine residues and cysteines 58, 85, 136, 148 and 334 are at or near the protein surface, with Cys-334 being the most exposed of the five. It has been shown that Cys-334 is responsible for the dimerisation of the WT enzyme via the formation of an intermolecular disulphide bond [19]. In the present work, these five cysteines were replaced by chemically inert alanines before new cysteine residues were introduced. We describe the enzymatic properties and electrochemistry of the surface cysteine-free $P450_{cam}$ mutant (C58A-C85A-C136A-C148A-C334A, abbreviated as SCF), and four single surface cysteine mutants, SCF-R72C, SCF-R112C, SCF-K344C and SCF-R364C. The incorporation of a new redox-active label, *N*-ferrocenylmaleimide, to SCF-K344C and the electrochemistry of this modified mutant are also reported.

2. Materials and methods

2.1. Mutagenesis and enzymes

General DNA manipulations followed standard methods [20]. Oligonucleotide-directed site-specific mutagenesis of the *camC* gene was carried out on an M13mp19 subclone by Kunkel's method [21] according to the Bio-Rad Mutagene kit (version 2). Mutation sites were identified by Sanger dideoxy chain termination DNA sequencing using the Sequenase Version 2 kit (Amersham International, UK). The entire sequences of the mutants were confirmed using an ABI 377 Prism DNA Sequencer at the DNA Sequencing Facility of the Department of Biochemistry of the University of Oxford.

The recombinant forms of cytochrome $P450_{cam}$ (both WT and mutants) [22] and the associated electron-transfer proteins, putidaredoxin reductase [23] and putidaredoxin [24], were expressed in *Escherichia coli*, and purified according to literature methods. Only SCF and SCF-K344C were expressed as the holoenzymes while SCF-R72C, SCF-R112C and SCF-R364C were expressed as the apo-forms. The reconstitution of these three mutants was carried out by addition of haemin to the lysate solution before the purification procedure [25]. All $P450_{cam}$ proteins were stored at −20°C in 40 mM phosphate buffer pH 7.4 containing 50% glycerol and 1 mM camphor. For the single surface cysteine mutants, 1 mM dithiothreitol was also present in order to prevent dimerisation of the protein molecules via disulphide bond formation. Glycerol, camphor and dithiothreitol were removed immediately prior to experiments by gel filtration on a Pharmacia PD-10 column equilibrated with 50 mM Tris-HCl, pH 7.4.

2.2. Activity assays

Activity assays were carried out at 303 K in a Cary 1E double beam

*Corresponding author. Fax: (44) (1865) 272990.
E-mail: allen.hill@chem.ox.ac.uk

PII: S0014-5793(99)00611-0

UV/Vis spectrophotometer equipped with a Peltier temperature control unit (±0.1°C). The incubation mixtures (2.5 ml) contained 50 mM Tris-HCl pH 7.4, 0.05 μM $P450_{cam}$, 16 μM putidaredoxin, 0.5 μM putidaredoxin reductase, 200 mM KCl, and 1 mM camphor. NADH was added to 400 μM to initiate the reaction, and monitored at 340 nm ($\varepsilon_{340\,nm} = 6.22\ mM^{-1}\ cm^{-1}$). Control reactions were performed as above, only without the $P450_{cam}$ component, to correct for the background NADH consumption rates due to the putidaredoxin-mediated air oxidation of NADH [26]. For gas chromatographic analysis, 2 ml of the incubation mixtures were extracted with 200 μl $CHCl_3$ and the aqueous and organic phases were separated by centrifugation (4000×*g*) at 4°C for 20 min. Ethyl benzene was used as the internal standard. The organic 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.) and a flame-ionisation detector. The column temperature was held at 60°C for 6 min and then increased to 120°C at a rate of 50°C/min. Under these conditions, the retention times of ethyl benzene, camphor and 5-*exo*-hydroxycamphor were 6.2, 11.3 and 18.7 min, respectively.

2.3. Redox-active label incorporation

2.3.1. Synthesis of N-ferrocenylmaleimide. The label *N*-ferrocenylmaleimide was synthesised according to a published method [27] except that aminoferrocene was used instead of 2-ferrocene-ethylamine. The product was recrystallised from ethyl acetate/petroleum ether (overall yield = 21%). FAB-MS: $m/z = 281$ $\{M^+\}$; ^1H NMR (500 MHz, in acetone-d_6) δ 6.95 (s, 2H, maleimide protons), δ 4.19–4.13 (m, 9H, ferrocenyl protons). Elemental analyses, found (%) C: 60.52, H: 4.56, N: 4.58; calculated (%) C: 59.82, H: 3.94, N: 4.98.

2.3.2. Labelling of SCF-K344C with N-ferrocenylmaleimide. *N*-Ferrocenylmaleimide (1 mg, 3.6 μmol) in 100 μl MeOH was slowly added to SCF-K344C (50 nmol) in 10 ml of buffer (50 mM Tris-HCl pH 7.4 containing 1 mM camphor and 300 mM KCl). The suspension was stirred at 4°C for 1 h and the solid residue removed by centrifugation. The supernatant was filtered through a sterile 0.22-μm filter and concentrated to 200 μl using a 10 000 MW cut-off centricon (Amicon) and centrifugation at 4°C. The solution was diluted with the same buffer to 1.5 ml and then reconcentrated to 200 μl. This washing process was repeated 10 times. Finally, the protein was eluted from a PD-10 column equilibrated with the same buffer to ensure the absence of any free labels.

2.4. Electrochemical studies

The electrochemical set-up for DC cyclic voltammetry experiments was described before [27,28]. The buffer was 50 mM Tris-HCl buffer pH 7.4 containing 1 mM D-(+)-camphor and 300 mM KCl. All electrochemical measurements were carried out in a glove box (Belle Technology) at oxygen levels below 1.5 ppm.

3. Results and discussion

3.1. Enzyme preparation and activity studies

The SCF mutant and single surface cysteine mutant SCF-K344C were expressed in medium yields relative to that of the WT enzyme. However, the other mutants were expressed as apoproteins and in low yields. This is in line with the low expression levels of the single mutants R72Q, R72E [10] and R112C [11]. Crystallographic studies of the WT enzyme revealed that the guanidino group of Arg-112 is hydrogen bonded to an oxygen atom of the 6-propionate of the haem prosthetic group [16]. The R112C mutation would disrupt this hydrogen bonding interaction and destabilise the protein [11].

The degree of exposure of the introduced cysteine residues was assessed by treatment with Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] [29]. The electronic absorption spectrum of the SCF mutant did not show any spectral changes, confirming the absence of exposed surface cysteines for this mutant. In contrast, all the other mutants reacted with Ellman's reagent, strongly suggesting that the additional cysteine moieties of SCF-R72C, SCF-R112C, SCF-K344C and SCF-R364C are exposed on the surface of the proteins.

Binding of camphor to $P450_{cam}$ results in the expulsion of all of the water molecules from the active site and the haem iron becomes five co-ordinate, concomitant with a change in its spin state from low spin to predominantly high spin [3,30]. The percentages of high spin haem of WT $P450_{cam}$ and the mutants in 50 mM Tris-HCl pH 7.4 buffer containing 1 mM camphor in the absence of potassium ions are summarised in Table 1. In the absence of potassium ions, the SCF mutant was only 17% high spin, much lower than that of the WT enzyme (68%). However, the five single site mutants C58A, C85A, C136A, C148A and C334A have been shown to exhibit very similar spin state equilibria to the WT enzyme [19]. Therefore, the altered spin state equilibrium of the SCF mutant is due to the cumulative effect of the five mutations. The increased high spin haem content of the SCF-R112C (30%) and SCF-R364C (52%) mutants implies that the surface residues Arg-112 and Arg-364 also affect the spin state equilibria of the enzyme. In the presence of ca 300 mM KCl, the haems of SCF, SCF-K344C and SCF-R364C were driven to essentially 100% high spin while the other two mutants required higher potassium ion concentrations (800 mM for SCF-R72C and 1.40 M for SCF-R112C).

The NADH turnover activities of the reconstituted $P450_{cam}$ system with camphor are given in Table 1. While the SCF mutant had a comparable NADH turnover rate to that of WT $P450_{cam}$, all the other mutants had lower activity. This observation indicates that the five native surface cysteines do not play an important role in the overall enzymatic properties

Table 1
Percentages of high spin haem, NADH turnover rates for camphor hydroxylation and electrochemical data of the WT $P450_{cam}$ and the mutants SCF, SCF-R72C, SCF-R112C, SCF-K344C and SCF-R364C

$P450_{cam}$	% high spin haem[a]	NADH turnover rate[b]	E_{pc}/mV[c]	E_{pa}/mV[c]	$E_{0.5}$/mV[c]
WT	68	34.4	−533	−361	−447
SCF	17	32.2	−530	−358	−444
SCF-R72C	18	22.9	−547	−344	−446
SCF-R112C	30	0.2	−512	−344	−428
SCF-K344C	19	24.5	−474	−393	−434
SCF-R364C	52	11.0	−533	−365	−449

[a]In 50 mM Tris-HCl pH 7.4 containing 1 mM camphor in the absence of potassium ions at 303 K.
[b]The turnover rates are determined in the presence of 200 mM KCl, and given as nmol of NADH consumed per nmol of $P450_{cam}$ per second at 303 K.
[c]Measurements were taken using an edge-plane graphite electrode in 50 mM Tris-HCl pH 7.4 containing 1 mM camphor and 300 mM KCl at 298 K. Scan rate = 10 V s^{-1}. All potentials are referred to SCE.

of the protein. In contrast, the four amino acid residues Arg-72, Arg-112, Lys-344 and Arg-364 are important in the NADH turnover activity. The NADH turnover rates of SCF-R72C and SCF-K344C were ca 30% lower than that of WT $P450_{cam}$. Reduced NADH consumption activities have also been observed for the single mutants R72Q, K344Q and K344E [10], in which the positive charge of Arg-72 and Lys-344 has been neutralised or reversed. It was proposed that the lower rate profiles result largely from perturbation of the putidaredoxin-$P450_{cam}$ association [10]. The NADH turnover rate of SCF-R112C was exceptionally slow, being only ca 0.7% of that of the WT enzyme. Similar observations have also been reported from the NADH and oxygen consumption assays for the single mutants in which Arg-112 was replaced by glutamic acid, glutamine [12], cysteine, methionine or tyrosine [13]. It was suggested that the positive charge of Arg-112 is crucial in binding to putidaredoxin and thereby maintaining the high catalytic activity of the enzyme [11–13].

Similar to the WT enzyme, the oxidation of camphor by these surface-modified mutants gave 5-*exo*-hydroxycamphor as the only product. The NADH consumed by WT $P450_{cam}$ is completely utilised for production formation, i.e. the coupling efficiency is 100%. All the mutants showed tight coupling ($>98\%$) except SCF-R112C, which showed a 31% coupling efficiency. The single mutants R112K, R112C, R112M and R112Y have been reported to exhibit coupling efficiencies ranging from 50 to 88% with the formation of hydrogen peroxide being the major decoupling pathway [13].

3.2. Electrochemistry

The electrochemistry of WT $P450_{cam}$ and the mutants was studied using cyclic voltammetry and the data are summarised in Table 1. The cyclic voltammogram of SCF-K344C is shown in Fig. 1. The cyclic voltammograms of the $P450_{cam}$ enzymes in camphor-containing buffer on a bare EPG electrode at a scan rate of 10 V s^{-1} revealed two waves with a mid-point potential ranging from −428 to −449 mV vs SCE. These values are in reasonable agreement with the literature value of −414 mV determined by redox titration methods [4]. Similar electrochemical responses were also detected in a buffer solution with an EPG electrode which had been in contact with the enzyme for 4–12 h at 4°C. The cathodic and anodic waves were symmetric and stable. At slow scan rates (ca <500 mV s^{-1}), the redox potentials exhibited an anodic shift and i_{pc} is larger than i_{pa}. For example, at a scan rate of 50 mV s^{-1}, SCF-K344C displayed a quasi-reversible couple with E_{pc} at −372 mV and E_{pa} at −214 mV ($E_{1/2} = -293$ mV) with $i_{pc}/i_{pa} = 4.9$. A decay in the faradaic current also occurred upon potential scanning. These findings suggest that the electrochemistry was dominated by oxygen reduction at a low scan rate. Although the buffer solutions were rigorously degassed and the electrochemical measurements were carried out under a highly anaerobic atmosphere (<1.5 ppm O_2), it is possible that the trace amount of oxygen could be sufficient to react with the ferrous haem after the first electron transfer step. However, at high scan rates, the oxygen in the solution at the electrode surface was consumed at the first few electrochemical scans and the diffusion of oxygen from the bulk is insufficiently fast and so the electrochemical signals essentially originate from the enzymes which are adsorbed on the electrode surface.

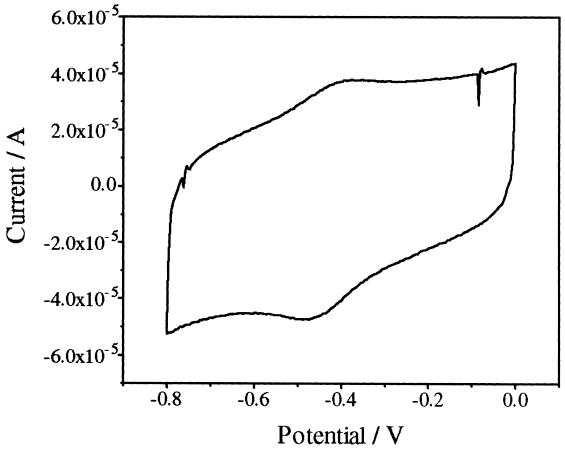

Fig. 1. Cyclic voltammogram on an edge-plane graphite electrode of SCF-K344C (15 μM) in 50 mM Tris-HCl pH 7.4 containing 1 mM D-(+)-camphor and 300 mM KCl at a scan rate of 10 V s^{-1} at 298 K.

On a bare gold electrode, the WT enzyme and the mutants showed quasi-reversible or irreversible electrochemistry at low scan rates (<100 mV s^{-1}). At higher scan rates, the cyclic voltammograms were similar to that of the background. At a scan rate of 5 mV s^{-1}, the cyclic voltammograms of the WT enzyme and the mutants showed a weak and sigmoidal-shaped cathodic wave with E_{pc} ranging from −240 to −273 mV. The anodic waves were of a smaller amplitude and occurred between −140 and −170 mV. The signals decayed gradually upon scanning. Although there are no surface cysteine residues on the SCF mutant, and the native surface cysteines of the WT enzyme are not close to the haem, the electrochemistry of the SCF mutant and the WT enzyme on a bare gold surface was indistinguishable from that of the single surface cysteine mutants. This suggests that the electrochemical signals arose, to a certain extent, from oxygen reduction (see above). In general, electrochemistry of redox-active proteins on an unmodified gold electrode is very difficult to achieve. For example, while the most extensively studied electron transfer protein, cytochrome *c*, showed reversible and persistent electrochemistry on a modified gold electrode, only transient electrochemical signals were observed on a bare gold electrode [31]. Nevertheless, the single surface cysteine mutant SCF-K344C occasionally showed a strong cathodic peak at −170 mV and a broader anodic signal at −50 mV at a scan rate of 20 mV s^{-1} on a bare gold electrode. Similar electrochemistry was not observed for the WT enzyme and the SCF mutant. The electrochemical signal did not show any decay upon electrochemical scanning and did not appear to be due to oxygen reduction. Similar electrochemical responses were also detected in a buffer solution on the same gold electrode which had been thoroughly rinsed with buffer, indicating that the electrochemistry was due to an adsorbed species on the gold surface. However, the details of the electrochemistry of these mutants on a bare gold surface require further clarification.

3.3. Labelling of SCF-K344C with N-ferrocenylmaleimide

We reported recently the incorporation of a redox-active *N*-(2-ferrocenylethyl)maleimide label onto the $P450_{cam}$ single site C334A mutant [27,28]. One of the two ferrocene-containing molecules was found to link to Cys-85 of the enzyme and

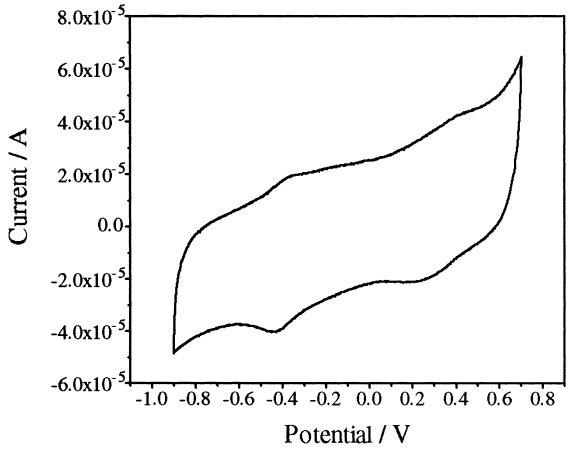

Fig. 2. Cyclic voltammogram on an edge-plane graphite electrode of SCF-K344C modified with *N*-ferrocenyl maleimide (12 μM) in 50 mM Tris-HCl pH 7.4 containing 1 mM D-(+)-camphor and 300 mM KCl at a scan rate of 10 V s^{-1} at 298 K.

occupied the camphor binding site in the substrate pocket as revealed by the X-ray crystal structure of the modified enzyme [28]. In the present work, we prepared another structurally related label, *N*-ferrocenylmaleimide, in which the ferrocene is directly connected to a maleimide group.

The conjugation between SCF-K344C and *N*-ferrocenylmaleimide was carried out in camphor-containing buffer and the reaction was monitored by electronic absorption spectroscopy. The Soret band remained at 392 nm throughout the reaction, indicating that the ferric haem remained high spin and camphor remained in the substrate binding pocket of the modified mutant. The modified mutant showed a NADH turnover rate of 24.4 s^{-1} with a 98% coupling towards camphor hydroxylation. These values are similar to those of the unmodified mutant SCF-K344C, suggesting that, surprisingly, the additional ferrocene moiety on the surface of the mutant did not significantly alter the enzymatic properties of the mutant.

The cyclic voltammogram of the *N*-ferrocenylmaleimide modified SCF-K344C mutant displayed two quasi-reversible redox couples with $E_{1/2} = -401$ mV and +333 mV vs SCE (Fig. 2). These couples exhibited adsorption behaviour and were assigned to the haem ferric/ferrous and ferrocenium/ferrocene redox reactions, respectively. A control reaction was also carried out using the SCF mutant and the ferrocene label with exactly the same reaction conditions and purification procedure. The final enzyme exhibited only a $P450_{cam}$ redox couple and no signals due to ferrocene, consistent with attachment of the ferrocene label specifically to SCF-K344C via the Cys-344 residue. The free ferrocene label itself showed a reversible couple with $E_{1/2} = +306$ mV vs SCE at an EPG electrode at a scan rate of 10 V s^{-1}. Upon conjugation, the reduction potentials of the haem and the label were increased by 33 and 27 mV, respectively. Despite the fact that the label was conjugated to a negatively charged protein molecule, an anodic shift in the redox potential was observed. The reason for the shifts in both the heme and ferrocene redox potentials is not known: however, the possibility of some interaction between the haem buried in the protein and the ferrocene reporter located on the surface of the mutant cannot be ruled out.

4. Conclusions

The present work describes the genetic engineering of cytochrome $P450_{cam}$ with the introduction of surface cysteine residues at specific locations. Although none of the mutation sites involved were in close proximity to the active site, the properties of the mutants noticeably differed from those of the WT enzyme. The haem spin state equilibrium, potassium ion binding and enzymatic activity were all affected by the cumulative effects of the five Cys-Ala mutations in the SCF mutant and the altered surface charge and polarity of the mutants. Quasi-reversible electrochemistry of all these mutants was achieved on EPG and gold electrodes. The coupling of a redox-active label on Cys-344 of the SCF-K344C mutant was also demonstrated. Anodic shifts were found in both the haem and ferrocene label of the modified mutant. Although the magnitude of the shifts was small, the label is on the surface of the mutant where the haem is closest to the surface, and interactions between these two iron centres may be present.

Acknowledgements: K.K.W.L. thanks the Croucher Foundation for a Post-Doctoral Fellowship.

References

[1] Ortiz de Montellano P.R. (Ed.) (1995) Cytochrome P450: Structure, Mechanism, and Biochemistry, 2nd edn., Plenum Press, New York.
[2] Yu, C.A. and Gunsalus, I.C. (1974) J. Biol. Chem. 249, 94–101.
[3] Sligar, S.G. (1976) Biochemistry 15, 5399–5406.
[4] Sligar, S.G. and Gunsalus, I.C. (1976) Proc. Natl. Acad. Sci. USA 73, 1078–1802.
[5] Peterson, J.A. (1971) Arch. Biochem. Biophys. 144, 678–693.
[6] Sligar, S.G., Cinti, D.L., Gibson, G.G. and Schenkman, J.B. (1979) Biochem. Biophys. Res. Commun. 90, 925–932.
[7] Kazlauskaite, J., Westlake, A.C.G., Wong, L.L. and Hill, H.A.O. (1996) Chem. Commun. 2189–2190.
[8] Zhang, Z., Nassar, A.E.F., Lu, Z., Schenkman, J.B. and Rusling, J.F. (1997) J. Chem. Soc. Faraday Trans. 93, 1769–1774.
[9] Lvov, Y.M., Lu, Z., Schenkman, J.B., Zu, X. and Rusling, J.F. (1998) J. Am. Chem. Soc. 120, 4073–4080.
[10] Stayton, P.S. and Sligar, S.G. (1990) Biochemistry 29, 7381–7386.
[11] Koga, H., Sagara, Y., Yaoi, T., Tsujimura, M., Nakamura, K., Sekimizu, K., Makino, R., Shimada, H., Ishimura, Y., Yura, K., Go, M., Ikeguchi, M. and Horiuchi, T. (1993) FEBS Lett. 331, 109–113.
[12] Nakamura, K., Horiuchi, T., Yasukochi, T., Sekimizu, K., Hara, T. and Sagara, Y. (1994) Biochim. Biophys. Acta 1207, 40–48.
[13] Unno, M., Shimada, H., Toba, Y., Makino, R. and Ishimura, Y. (1996) J. Biol. Chem. 271, 17869–17874.
[14] Stayton, P.S., Poulos, T.L. and Sligar, S.G. (1989) Biochemistry 28, 8201–8205.
[15] Pochapsky, T.C., Lyons, T.A., Kazanis, S., Arakaki, T. and Ratnaswamy, G. (1996) Biochimie 78, 723–733.
[16] Poulos, T.L., Finzel, B.C. and Howard, A.J. (1987) J. Mol. Biol. 195, 687–700.
[17] Vigmond, S.J., Iwakura, M., Mizutani, F. and Katsura, T. (1994) Langmuir 10, 2860–2862.
[18] Brizzolara, R.A., Boyd, J.L. and Tate, A.E. (1997) J. Vac. Sci. Technol. A 15, 773–778.
[19] Nickerson, D.P. and Wong, L.L. (1997) Protein Eng. 10, 1357–1361.
[20] Sambrook, J., Fritsch, E.F. and Maniatas, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[21] Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.
[22] Unger, B.P., Gunsalus, I.C. and Sligar, S.G. (1986) J. Biol. Chem. 261, 1158–1163.

[23] Yasukochi, T., Okada, O., Hara, T., Sagara, Y., Sekimizu, K. and Horiuchi, T. (1994) Biochim. Biophys. Acta 1204, 84–90.
[24] Peterson, J.A., Lorence, M.C. and Amarneh, B. (1990) J. Biol. Chem. 265, 6066–6073.
[25] Sligar, S.G., Filipovic, D. and Stayton, P.S. (1991) Methods Enzymol. 206, 31–49.
[26] Nickerson, D.P., Harford-Cross, C.F., Fulcher, S.R. and Wong, L.L. (1997) FEBS Lett. 405, 153–156.
[27] Di Gleria, K., Hill, H.A.O. and Wong, L.L. (1996) FEBS Lett. 390, 142–144.
[28] Di Gleria, K., Nickerson, D.P., Hill, H.A.O., Wong, L.L. and Fülöp, V. (1998) J. Am. Chem. Soc. 120, 46–52.
[29] Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70–77.
[30] Fisher, M.T. and Sligar, S.G. (1985) J. Am. Chem. Soc. 107, 5018–5019.
[31] Hill, H.A.O., Hunt, N.I. and Bond, A.M. (1997) J. Electroanal. Chem. 436, 17–25.