Improving catalytic properties of P450 BM3 haem domain electrodes by molecular Lego

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In this work the catalytic properties of a cytochrome P450 immobilised onto an electrode surface are improved by means of the molecular Lego approach.

Over the last few years a great effort has been focused on improving the electrochemical and catalytic response of cytochromes P450 (P450s) on electrode surfaces. This involved engineering both the electrode surface and the enzyme itself.[1–5] Several methods for the immobilization of P450s have been investigated, among which are the inclusion within conductive polymers or surfactant films[1,3–5] and the covalent bonding to the electrode surface *via* various self assembled monolayers (SAM).[2] The relevance of this field of research cannot be underestimated as it will lead to the construction of P450-bioelectrodes both for analytical (biosensors) and bioelectrocatalytic (bioreactors) purposes.[5,6]

In this work we have studied the electrochemical properties of the fatty acid monooxygenase cytochrome P450 BM3 haem domain (BMP) as well as its ability to catalyze reactions using an electrode as a source of electrons. BMP has already been successfully immobilized and fast electrode kinetics measured,[1,3] but its ability to hydroxylate organic substrates when immobilised has not yet been demonstrated. This point is essential because catalytic activity is fundamental for the correct function of a biosensor/biocatalyst based on this enzyme. As previously reported by Raner and co-workers,[7] cytochrome P450 BM3 catalyzes in solution the hydroxylation of *p*-nitrophenol to *p*-nitrocatechol in the presence of NADPH. We therefore used *p*-nitrophenol as a marker substrate to test the catalytic properties of the immobilised BMP, taking advantage of the changes in the spectroscopic properties in alkaline solution upon hydroxylation to *p*-nitrocatechol.[7]

The approach we used to achieve catalysis is the use of molecular Lego.[8,9] This protein engineering strategy was introduced by our laboratory to obtain efficient interaction of P450s on the electrode surface and to achieve self-sufficient, soluble and stable human P450s.[9] The idea is to assemble non-physiological redox modules, generating artificial redox chains, which could lead to new multi-domain constructs with improved electrochemical and catalytic properties. The redox modules, flavodoxin from *Desulfovibrio vulgaris* (FLD) and BM3 haem domain from *Bacillus megaterium* (BMP) were assembled at the genetic level generating the BMP–FLD fusion protein[8] (Fig. 1). The choice of flavodoxin was motivated by its high sequence similarity to the FMN binding domain of both P450 BM3 and the cytochrome P450 reductase of class II P450s. Furthermore it is interesting to observe that recently a P450–flavodoxin fusion protein was identified in the bacterium *Rhodococcus rhodochrous*.[10] The fusion protein showed the typical UV-vis spectra with the absorption peak at 450 nm when reduced and bubbled with CO (Fig. 2A). BMP–FLD was electrochemically characterized and its ability to hydroxylate *p*-nitrophenol while directly immobilized on the electrode surface was investigated and compared to that of the BMP on its own. The demonstration of the validity of this approach on the amenable bacterial system is key for its extension to more complex eukaryotic enzymes.[11]

Direct electrochemistry of BMP was investigated on glassy carbon (GC) electrodes using poly-(dimethyldiallylammonium chloride) (PDDA) for film assembly. The protein film was prepared mixing equal volumes of PDDA and protein (stock solution of 150 μM) before loading it onto the electrode surface. Cyclic voltammetry experiments were carried out at 25 °C, 100 mM potassium phosphate buffer (KPi) at pH 7 in strictly anaerobic conditions (<1.5 ppm oxygen). All potentials reported in this work are referred to NHE. Cyclic voltammograms revealed a redox

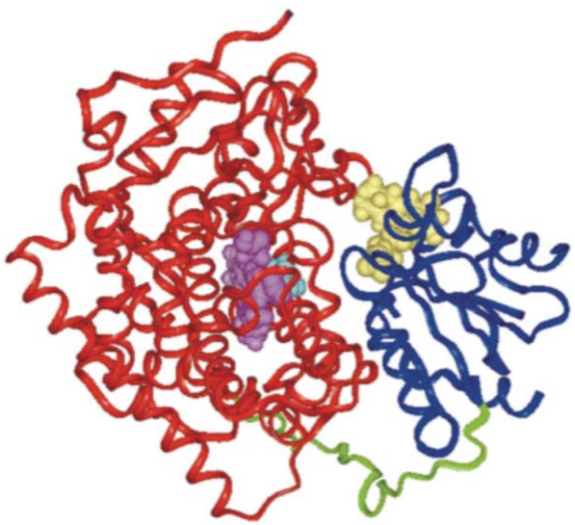

Fig. 1 3D model of P450 BMP–FLD. The BMP domain is shown in red, the FLD domain in blue, haem in pink, cysteine 400 in cyan, FMN in yellow and the loop connecting the two domains in green.

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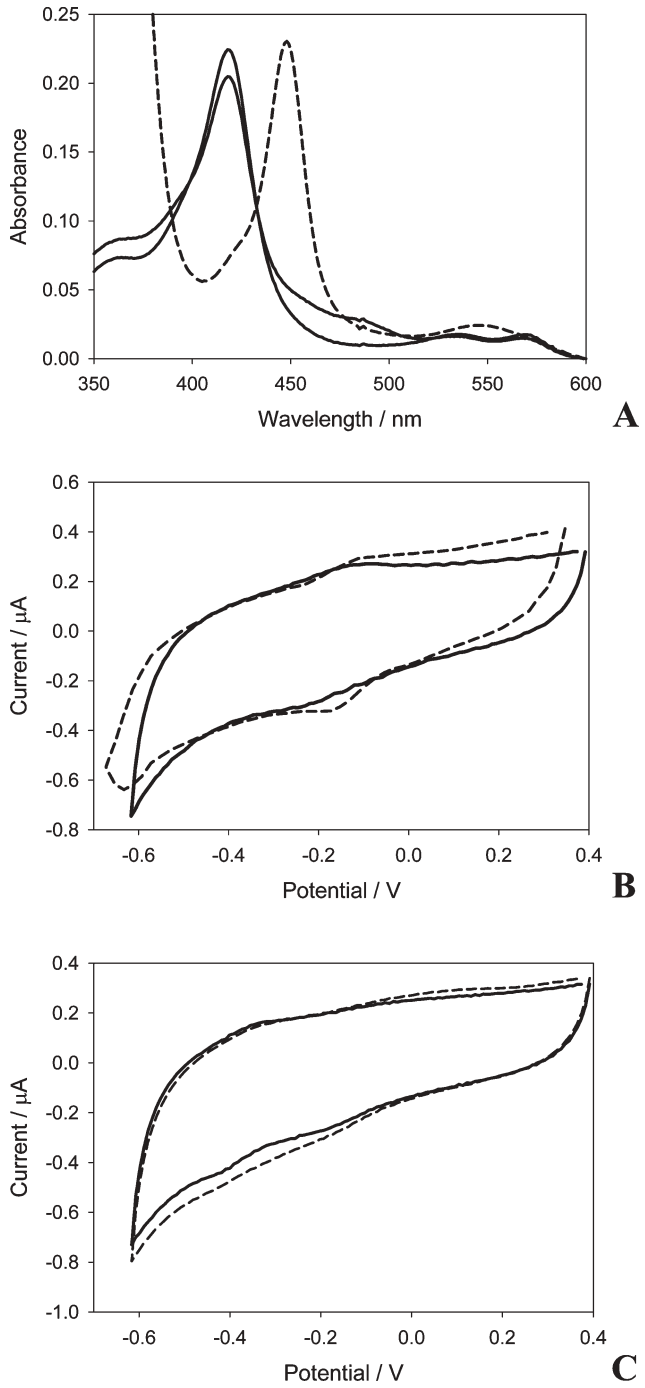

Fig. 2 Spectroscopic and electrochemical properties of P450 BMP and BMP–FLD fusion protein. **A.** UV-vis spectra of P450 BMP and P450 BMP–FLD. The spectrum of P450 BMP–FLD shows a shoulder at 458 nm due to the presence of the FMN co-factor. Reduction and CO bubbling of BMP–FLD results in the typical peak at 450 nm. **B.** Cyclic voltammogram of P450 BMP immobilized on PDDA modified GC electrode. The voltammogram was recorded in deoxygenated 100 mM KPi at pH 7 at 100 mV s^{-1}. **C.** Cyclic voltammogram of P450 BMP–FLD on PDDA modified GC electrode on 100 mM KPi at 100 mV s^{-1}. In all the figures the dashed line represents the signal obtained in the presence of CO.

couple centred at −167 mV (Fig. 2B), with the features of a reversible system. We have assigned this couple to the haem $Fe^{III/II}$ even though the reduction potential is not as positive as previously reported for this protein on electrodes[1–3] but it is closer to the one measured in solution. These differences are probably associated with the protein sensing diverse environments due to different immobilisation approaches. Voltammograms were taken at different scan rates from 50 mV s^{-1} to 60 V s^{-1}. Peak-to-peak separation did not vary significantly within the range considered. Instrumental limits did not allow higher scan rates, preventing the calculation of the k_{ET} using Laviron's approach.[12] Hill and co-workers measured a k_{ET} of 221 s^{-1} for BMP in similar conditions. In their case significant peak-to-peak separation was observed at 10 V s^{-1} (ref. 1) and because the same features were not observed here in a range that exceeded 10 V s^{-1} it indicates that in our system electron transfer is faster than 221 s^{-1}. Peak currents (i_{pc} and i_{pa}) were linearly dependent on the scan rate (v) as expected for immobilized electroactive species. In CO-saturated buffer both anodic and cathodic peaks shifted about +70 mV, in good agreement with previous findings for bacterial P450s (+35 to +80 mV[1,3,5]). No mid point potential shift was observed in the presence of substrate in keeping with Fleming *et al.*[1] This is probably due to the dehydration of the active site upon binding on to the electrode surface. Loss of the water molecule acting as the sixth ligand of the haem iron is known to induce the spin shift change typical of the substrate bound form. Catalysis studies were performed using 5 mM *p*-nitrophenol following its conversion to *p*-nitrocatechol observing the specific absorption at 515 nm in alkaline conditions. Concentrations were estimated using an extinction coefficient at 515 nm (ε_{515}) of 12.4 mM^{-1} cm^{-1}. Superoxide dismutase (SOD) and catalase were also added to avoid non-specific hydroxylation, such as *via* the Fenton reaction, and further oxidation of *p*-nitrocatechol.[7] The applied potential (−355 mV) was maintained for 90 min and the solution constantly stirred to allow constant oxygenation. While no *p*-nitrocatechol was observed in the absence of the protein, 0.34 ± 0.2 μM of product were detected in the presence of BMP under the same conditions (Table 1). This indicates that although fast electron transfer was observed from BMP, when on the electrode this enzyme alone is not using reducing equivalents for substrate hydroxylation.

We therefore investigated the effect of the fusion of the FLD electron transfer module in the framework of the molecular Lego approach. It is possible that direct contact of BMP with the electrode surface leads to an electron transfer faster than 221 s^{-1} but it does not result in an efficient catalytic performance. The advantage in having a BMP–FLD fusion lies in the ability of FLD to convey the electrons to the BMP active site: this hypothesis was put to the test here.

Spectroscopic analysis on pure BMP–FLD showed that both FMN and haem co-factors were present in a 1 : 1 ratio and that the protein shows the expected shift at 450 nm when reduced in the presence of CO (Fig. 2A). Immobilization on glassy carbon electrodes was achieved under the same conditions used for BMP (stock solution of 150 μM). The voltammograms in anaerobic conditions showed two redox couples centred at −376 and −130 mV as shown in Fig. 2C. Flavodoxin wild type contains one molecule of flavin mononucleotide (FMN). This co-factor undergoes sequential reduction from the quinone state to semiquinone (E_2) and from semiquinone to hydroquinone (E_1). The reduction potentials for these two semireactions have been determined by

Table 1 Redox and catalytic parameters for BMP and the BMP–FLD immobilized on glassy carbon electrodes modified with PDDA

Surface	$E_{1/2}$/ mV	ΔE/ mV	CO-shift/ mV	[*p*-nitrocatechol]/ μM
GC–PDDA–BMP	−167	60	+70	0.34 ± 0.2
GC–PDDA–BMP–FLD	−376	82	0	
	−130	126	+50	2 ± 0.37

redox potentiometry and cyclic voltammetry using protein both in solution and immobilized. Depending on the electrode configuration, the E_2 values were found to vary from −149 mV to −98 mV and the E_1 from −440 mV to −388 mV.[13,14] The mid point potential of the first redox couple of BMP–FLD (−376 mV) can therefore be assigned to the semiquinone to hydroquinone transition of the FMN. The second redox couple (−130 mV) is 37 mV more positive than that of the BMP alone and its peak-to-peak separation is wider. Given the range observed for the E_2 of FMN (−149 and −98 mV),[13,14] the larger peak-to-peak separation and shift observed for the immobilised BMP–FLD could account for the presence of two transitions with very close reduction potentials. Furthermore the intra-molecular electron transfer between the reduced flavodoxin semiquinone and the oxidised BMP[8] could be responsible for the more pronounced reduction peak compared to the oxidation one. On the other hand, when only BMP was immobilized the peak areas were comparable. The value of k_{ET} was not determined for BMP–FLD because of the inability to differentiate the two contributions to the redox peaks centred at −130 mV. Nevertheless, because the peaks tend to disappear at scan rates higher than 1 V s^{-1}, we suggest that the electron transfer rate is much lower in the case of BMP–FLD when compared to BMP.

In CO-saturated buffer the mid point potential of the second couple was found to shift by +50 mV showing a more pronounced feature for the oxidative peak than for the reductive one. This differential behaviour further supports the idea that two transitions are occurring in this potential range but only one is affected by the presence of CO. Since the FMN does not interact with CO, the E_1 and E_2 are not affected by its presence as shown by the redox couple at −376 mV.

When *p*-nitrophenol was added to BMP–FLD no shift in mid point potential was observed. When oxygenated buffer was used a catalytic current was observed. The turnover of *p*-nitrophenol was measured in the presence of SOD and catalase in the same conditions used for BMP. A potential of −355 mV was applied, keeping the FLD in the semiquinone and allowing intra-molecular ET. After 90 min, 2 ± 0.37 μM of *p*-nitrocatechol were formed, a quantity almost 6 times bigger than that obtained from BMP alone (Table 1).

The results presented in this communication demonstrate that protein engineering can lead to modular assemblies with improved bioelectrochemical/catalytic properties. In this case while BMP alone was found to be not very active toward the substrate even if a high k_{ET} was measured, the catalytic activity was improved by almost 6 times when the non-physiological redox partner FLD was fused to it. This could be due to a control role of the flavodoxin over the electron flow ensuring high coupling of the BMP. It is possible that in the case of BMP electrons are wasted in uncoupled reactions with the production of reactive oxygen species.[1,3] This is in keeping with the report by Gray and co-workers where BMP was shown to convert oxygen to either water or hydrogen peroxide depending on the method of immobilization on a carbon electrode.[3] The molecular Lego approach proved to be successful for the improvement of electrochemical and catalytic properties of P450 BMP suggesting that the same approach could be used for the production of new devices for nanobiotechnology.

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