Formation of flavin semiquinone during the reduction of P450 BM3 reductase domain with NADPH

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Cytochrome P450 BM3 (P450 102) from Bacillus megaterium is a unique bacterial P450, formed from the fusion of a fatty acid hydroxylase to a eukaryotic-like NADPH-cytochrome P450 reductase flavoprotein in a single (119 kDa) polypeptide chain (1, 2). It is an attractive model system for enzymological and structural studies due to its homology with mammalian drug metabolising P450 systems, and with nitric oxide synthase. The atomic structure of the haem (P450) domain the enzyme was determined recently (3).

The presence of three different coenzymes in the P450 BM3 molecule enables the dissection of the electron transfer pathway through the enzyme using techniques such as stopped-flow u.v.-visible spectrophotometry. In the P450 domain of the enzyme, absorbance changes at specific wavelengths are associated with the binding of fatty acid substrates, reduction of haem and ligation of various molecules (including carbon monoxide) to the haem iron.

Similarly, reduction of FAD and FMN coenzymes in the reductase domain of the enzyme results in the loss of their distinctive yellow colours as their visible spectra over a large wavelength range (approx. 500-400nm) are much diminished. Flavin reduction rates are also measurable by stopped flow spectrophotometry (4).

Reduction of flavins with the powerful reducing agent sodium dithionite facilitates the complete four electron transfer. However, this is not the case when the physiological reductant NADPH is used. The development of a distinct new absorbance band centred at approx. 600nm (Figure 1) is seen clearly when the reductase domain of P450 BM3 is reduced with a large excess of NADPH. If sufficient reductase domain is used (approx. 100μM or more) then a distinct blue colour is seen to develop rapidly in the solution. The species responsible is a flavin semiquinone. Since each molecule of NADPH transfers two electrons to the reductase, the accumulation of a three electron reduced form indicates that electron transfer between reductase molecules (intermolecular electron transfer) must occur. In aerobic conditions, the blue colour is lost over a period (the time of semiquinone decay depending on the amount of NADPH added) as electron transfer to aerobic oxygen is catalysed by the enzyme. Superoxide anions are produced during this process (5). The 600nm band is seen to disappear at quite an early stage during aerobic reoxidation of flavins. The fact that this species is not seen during reduction with sodium dithionite likely indicates that the reduction potential of the semiquinone is intermediate between those of NADPH and sodium dithionite.

References
4) Munro, A.W. et al. (unpublished data).

Figure 1 Visible absorption spectra of oxidised (Ox) P450 BM3 flavin domain (8μM) in 20mM Mops buffer (pH 7.4) containing 100mM KCl. Subsequently, the same enzyme was reduced (Red) by the addition of excess (200μM) NADPH.