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NADPH oxidase activity of cytochrome *P*-450 BM3 and its constituent reductase domain

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Abstract

Cytochrome *P*-450 BM3 from *Bacillus megaterium* catalyses NADPH oxidation in the absence of added substrate. This activity is also associated with the independently expressed flavin-containing reductase domain of the protein. The rates of these activities are more than two orders of magnitude lower than those in the presence of fatty acid *P*-450 substrates or artificial electron acceptors. Electrons derived from NADPH in this fashion are transferred onto oxygen, generating superoxide (O_2^-) anions. The formation of these active oxygen species is detectable by luminometry and the chemiluminescence can be inhibited through the addition of superoxide dismutase (but not catalase). This activity is reminiscent of the microbicidal NADPH oxidase activity associated with neutrophils and other leukocyte blood cell types. Diphenyliodonium, a potent inhibitor of the neutrophil NADPH oxidase, effectively inhibits fatty acid hydroxylase and electron transferase activities catalysed by *P*-450 BM3 and its reductase domain. CD studies on the native and NADPH-reduced *P*-450 BM3 and BM3 reductase indicate that no secondary structural alteration is caused by pre-incubation with the reductant. Therefore, the previously recognised reversible time-dependent inactivation of *P*-450 BM3 by NADPH may be attributed to the NADPH oxidase activity associated with the reductase domain of the enzyme.

Keywords: NADPH oxidase; Cytochrome *P*-450 BM3; Reductase domain; Oxygen free radical

1. Introduction

The cytochromes *P*-450 are a ubiquitous superfamily of enzymes which catalyse monooxygenation of a wide variety of organic compounds [1]. In mammals, these reactions may be necessary for the interconversions of endogenous metabolites, or may serve to increase the solubility of drugs and xenobiotics to facilitate their excretion. In microbes, activity of *P*-450s may be required for physiological biotransformations or to allow utilisation of unusual organic compounds as energy sources [2]. A number of *P*-450s from plants have been identified, with important functions in the biosynthesis of cuticles and plant hormones and detoxification of herbicides [3]. A large literature has also accumulated on the subject of the *P*-450s from fungi and yeasts [4].

Cytochrome *P*-450 BM3 is isolated from the Gram positive bacterium *Bacillus megaterium* and catalyses hydroxylation of long chain fatty acids, alcohols and amides as well as the epoxidation/hydroxylation of unsaturated fatty acids [5,6]. *P*-450 BM3 is unique among the *P*-450s in that it is 'catalytically self sufficient'. While activity of other *P*-450 systems relies on either one or two additional protein electron transferring components, *P*-450 BM3 requires only fatty acid substrate and NADPH for full activity [7]. The enzyme is comprised of an FAD-and FMN-containing NADPH-cytochrome *P*-450 reductase 'domain' fused at the C-terminal of the *P*-450 domain in a single polypeptide chain [8].

Like all bacterial *P*-450s, *P*-450 BM3 is a soluble enzyme. However, other bacterial *P*-450 systems are comprised of three components; the *P*-450, an iron-sulfur redoxin and a flavoprotein redoxin reductase. The best

Abbreviations: *P*-450, cytochrome *P*-450 linked monooxygenase; IPTG, isopropyl β-D-thiogalactopyranoside; Mops, morpholinopropanesulfate; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; CD, circular dichroism; EPR, electron paramagnetic resonance; SOD, superoxide dismutase; IDP, diphenyliodonium; GdnHCl, guanidinium hydrochloride; Cyt. *c*, cytochrome *c*.

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example of these 'class I' *P*-450s [9] is the *P*-450*cam* system from *Pseudomonas putida*, for which a high-resolution atomic structure has been determined [10]. *P*-450*cam* has been used as a structural model for the entire *P*-450 superfamily. 'Class II' systems are two-component (NADPH-cytochrome *P*-450 reductase and *P*-450) and contain members involved in drug and xenobiotic metabolism [1,9]. *P*-450 BM3 is an unusual example of a class II system, in which the reductase is linked to the *P*-450. The haem-containing domain of *P*-450 BM3 has been crystallised [9,11,12] and the first atomic structure of a class II *P*-450 was published recently [13]. Comparison of the structures of *P*-450*cam* and *P*-450 BM3 reveal that, while the overall fold (resembling a triangular prism) and secondary structure contents are similar, the helical arrangements in the haem domain of *P*-450 BM3 differ significantly from those in *P*-450*cam* and there are four major peptide insertions (of between 7 and 17 amino acids) within *P*-450 BM3 haem domain with respect to *P*-450*cam* [13]. However, *P*-450 BM3 may be a more accurate model for the mammalian class II systems, since it is much closer in size to the majority of them than is *P*-450*cam*, has much higher primary structure similarity to eukaryotic class IV *P*-450 enzymes than to any other bacterial *P*-450 and might also be expected to retain structural motifs required for interaction with the distinct redox partner [13–15].

The reductase domain of *P*-450 BM3 is one of a small, but evolutionarily strongly conserved, family of flavoprotein reductases which bind NADPH and contain both FAD and FMN prosthetic groups [16]. This family also contains eukaryotic *P*-450 reductase, the flavoprotein components of nitric oxide synthase from the rat [17] and sulfite reductase from *Salmonella typhimurium* [18,19]. These flavoproteins appear to have evolved from the fusion of genes encoding two distinct classes of flavoprotein, a ferredoxin-$NADP^+$ reductase (FNR)-like transhydrogenase and a flavodoxin-like electron transferase [16]. The transhydrogenase (FNR) subdomain of *P*-450 BM3 exhibits significant similarity to other NADH and NADPH reductases from the FNR 'family' of reductases, particularly in regions involved in binding FAD and dinucleotide cofactors [20,21]. These include the β-subunit of flavocytochrome *b*-245, a component of the microbicidal NADPH oxidase of phagocytes [21].

The NADPH oxidase is an electron transport system found in neutrophils and other phagocytic cells [22], the components of which have also been located in T and B lymphocytes [23]. Its function is to reduce oxygen with electrons from NADPH, producing superoxide radicals and resulting in the effective killing of pathogens ingested by the phagocytes. It appears that a membrane-bound flavocytochrome *b* contains the full electron transporting apparatus of the NADPH oxidase [24], but that cytosolic factors are involved in its regulation [21]. The flavocytochrome is composed of a 21 kDa α-subunit, which is probably the site of attachment of the haem moiety, and a β-subunit with an apparent molecular mass of approx. 92 kDa [21]. The β-subunit is a heavily glycosylated flavoprotein dehydrogenase [24], which has previously been reported to catalyse the production of superoxide independently of the α-subunit under non-physiological conditions [25].

In this report we investigate the ability of flavocytochrome *P*-450 BM3 and its isolated domains to act as NADPH oxidases, utilising certain experimental methods previously applied in studies of the phagocyte enzyme. We also investigate the susceptibility of activities of these proteins to an inhibitor (IDP) of the phagocyte enzyme, in order to identify the major oxidase site. Finally, we use enzymic and spectroscopic (CD) methods to investigate a previous theory that pre-incubation of *P*-450 BM3 with NADPH induces a time-dependent, reversible inactivation, and that this inactivation may involve a conformational change in the enzyme [7].

2. Materials and methods

2.1. E. coli strains, plasmid and bacteriophage vectors

E. coli strains TG1 (*sup*E, *hsd*Δ5, *thi*, Δ(*lac-pro*AB), F′ [*tra*D36, *pro*AB^+, *lac*I^q, *lac*ZΔM15]) [26] and XL-1 Blue (*sup*E44, *hsd*R17, *rec*A1, *gyr*A46, *thi*, *rel*A1, *lac*$^-$, F′ [*pro*AB^+, *lac*I^q, *lac*ZΔM15, Tn10(tet^r)]) [27] were used for plasmid propagation and the overexpression of the gene encoding cytochrome *P*-450 BM3 (*cyp*102) and the PCR-generated subgenes encoding its constituent reductase and haem 'domains'. Plasmid construct pJM23 was used for expression of *P*-450 BM3 in *E. coli*. The plasmid consists of a 5kb segment of *B. megaterium* chromosomal DNA (containing the *cyp*102 gene) cloned as a *Xba*I-*Eco*RI restriction fragment into vector pUC119 [28] and expression is from the *Bacillus* promoter [12]. Plasmid construct pJM27 was used for the expression of the flavoprotein reductase domain of *P*-450 BM3 (residues 473 to end) and consists of an approx. 1.8 kb PCR fragment of *cyp*102 cut with *Bam*HI (to generate cohesive ends from restriction enzyme sites engineered at 5′ ends of PCR primers) and cloned into vector p*tac*85 [29] under an inducible *tac* promoter [12]. Plasmid construct pJM20 was used for the expression of the haem-containing domain of *P*-450 BM3 (residues 1 to 472) and consists of an approx. 1.5 kb PCR fragment of *cyp*102 cut with *Eco*RI and *Bam*HI (to generate cohesive ends as with pJM27) and cloned into vector pUC118 [28] under the *lac* promoter [12].

2.2. Molecular biology techniques

DNA manipulations, bacterial transformations and other molecular techniques were performed by standard methods [30].

2.3. Expression and purification of intact cytochrome P-450 BM3 and its constituent domains

E. coli transformants carrying plasmids encoding wild-type *P*-450 BM3 and its constituent flavoprotein reductase domain were grown overnight to high cell density in Terrific Broth plus antibiotic (ampicillin) [31]. IPTG inducer (final concentration 25 μg/ml) was added to facilitate expression from plasmids pJM27 (encoding the reductase domain) and pJM20 (encoding the haem-containing domain). Wild-type *P*-450 BM3 was expressed from plasmid pJM23 under the control of its own promoter and without induction, as previously described [12]. A significant increase in the level of expression of intact *P*-450 BM3 (approx. 3-fold) was seen to occur if the culture of pJM23 transformants was grown to stationary phase, as has been previously reported [32].

30–35 g of wet cell pellets were the starting points for purification of the polypeptides. Following cell rupture by French pressure, polypeptides were purified to homogeneity by successive steps of ammonium sulfate precipitation, ion exchange chromatography on DEAE-Sephacel and either affinity chromatography on 2',5'-ADP-Sepharose (in the case of intact *P*-450 BM3 and its reductase domain) or by affinity for Bio-Gel HTP (DNA grade) hydroxyapatite (in the case of the haem domain) as previously described [12]. Purification of the polypeptides by gel filtration using Sephacryl S-300 HR was used as a final purification step, if required.

2.4. Spectroscopy, protein and enzyme assays

All UV-visible spectroscopy was performed on a Shimadzu 2100 spectrophotometer (Shimadzu, Kyoto, Japan). Protein concentrations were determined using the method of Bradford [33] and by the BCA technique [34] with BSA as standard. Cytochrome *P*-450 concentrations were estimated by the method of Omura and Sato [35] using $\epsilon = 91$ mM^{-1} cm^{-1} at 450 nm for the reduced plus CO adduct. Cytochrome *c* reduction was determined using $\epsilon = 21$ mM^{-1} cm^{-1} at 550 nm, as previously described, except that the buffer system used was 20 mM Mops (pH 7.4) containing 100 mM KCl [12]. NADPH-dependent fatty acid hydroxylation was measured at 30°C in the same buffer containing 0.5 mM sodium laurate and 0.2 mM NADPH, $\epsilon = 6.2$ mM^{-1} cm^{-1} at 340 nm. Catalase activity was measured at 30°C by monitoring the increase in absorbance at 240 nm in the same buffer system containing 10.5 mM hydrogen peroxide and 3 units (μmol/min under the conditions specified in the Sigma catalogue) of catalase. Xanthine oxidase activity was measured at 30°C as the superoxide-dependent rate of cytochrome *c* reduction in the same buffer system, as described by Kuthan et al. [36]. Deionised, double distilled water was used in all solutions. Rates shown are the means of at least three separate determinations, differing by less than 10%.

Circular dichroism spectra were recorded at 20°C on a JASCO J-600 spectropolarimeter. NADPH 'blanks' were also recorded in order to correct for its contribution in 'Enzyme plus NADPH' spectra. Molar ellipticity values were calculated using a value of 112 (calculated from amino acid sequences) for the mean residue weights. Analysis of the secondary structure content was undertaken by using the CONTIN procedure [37].

2.5. Luminometry

Luminometry was performed on an LKB Wallac 1250 display luminometer linked to a Datel Systems DPP Q7 printer. Assay systems contained 100 μl of enhancer (either 1 mM lucigenin [bis-*N*-methylacridinium nitrate] or 1–10 mM luminol [5-amino-2,3-dihydro-1,4-phthalazinedione]), 15 μl of 20 mM NADPH, 1–10 μl of enzyme and assay buffer (20 mM Mops [pH 7.4] + 100mM KCl) to 1 ml. Deionised, double distilled water was used for all solutions utilised in luminometry experiments.

2.6. Materials

Molecular biology reagents were purchased from Boehringer or United States Biochemicals. Radiochemicals were obtained from Amersham International. DEAE-Sephacel was purchased from Pharmacia-LKB. Diphenyliodonium chloride (IDP) was from Aldrich. All other reagents and enzymes were from Sigma.

3. Results

3.1. Substrate independent NADPH oxidation by P-450 BM3 and its reductase domain

P-450 BM3 catalyses rapid substrate-dependent NADPH oxidation. Rates as high as 77 s^{-1} (4620 mol/min per mol) have been reported for the hydroxylation of saturated fatty acids [7]. It has been observed that NADPH oxidation by *P*-450 BM3 also occurs in the absence of a substrate or added electron acceptor, albeit at a much lower rate. Substrate-independent oxidation of NADH occurs at an extremely slow rate (< 0.1 nmol/min per nmol enzyme). The NADPH oxidase property of *P*-450 BM3 is retained by its independently expressed reductase domain, but not by the haemoprotein domain. The rates are very similar for *P*-450 BM3 and its flavin domain (Table 1). The rates in the absence of substrate are approx. 10^3-fold lower than those in the presence of substrate. Extensive dialysis was performed to separate any possible substrate from the purified enzymes prior to initiation of the assays. The fact that haem iron is essentially all low spin in purified intact *P*-450 BM3 confirms the absence of fatty acid substrate [12]. Since NADPH oxidase activity was associated with the flavoprotein reductase domain (but not the haem-con-

Table 1
NADPH oxidation rates by *P*-450 BM3 and its constituent domains in the presence and absence of substrates and IDP

Enzyme	NADPH oxidation rates in presence/absence of substrates (nmol/min per nmol enzyme)		
	substrate absent	cytochrome *c*	lauric acid
BM3	2.15 (2.05)	3378	1191
BM3 + IDP	36.4 (0.85)	122	15.6
R dom.	2.1 (2.0)	2964	< 0.1
R dom. + IDP	39.2 (0.7)	49	< 0.1
P dom.	< 0.1	< 0.1	< 0.1
P dom. + IDP	< 0.1	< 0.1	< 0.1

For intact *P*-450 BM3 (BM3) and its reductase (R dom.) and *P*-450 (P dom.) domains, 'substrate absent' NADPH oxidation was measured by absorbance decrease at 340 nm (6.2 mM^{-1} cm^{-1}) at 30°C in a system containing enzyme, NADPH (0.2 mM) and assay buffer (20 mM Mops (pH 7.4) + 100 mM KCl) to 1 ml. 'Substrate present' rates were measured in the same buffers following addition of 25 μM cytochrome *c* (21mM^{-1} cm^{-1} at 550 nm) or 0.5 mM sodium laurate (6.2 mM^{-1} cm^{-1} at 340 nm). Final enzyme concentrations of approx. 40 nM ('substrate present') and 2 μM ('substrate absent') were used in the assays. The inhibitor IDP was added to a final concentration of 500 μM and the 'substrate absent' rates after 10 min are shown in parentheses.

taining *P*-450 domain), it appeared that in both the reductase domain and intact *P*-450 BM3 electrons from NADPH were passing onto the reductase domain to reduce the flavins (FAD and FMN; joint capacity of four reducing equivalents). However, the fact that (in systems containing 2 μM enzyme and 0.2 mM NADPH) slow substrate-independent NADPH oxidation was linear over periods of greater than 30 min indicated that the process was not completed by reduction of FAD and FMN. For both *P*-450 BM3 and its flavin domain, the enzymes oxidised more than 10-fold their molar equivalent of NADPH over this period. No oxidation of NADPH occurred in the absence of added enzyme and none was catalysed by the isolated haem domain of *P*-450 BM3. Using similar quantities of *P*-450 BM3 and cofactor, NADPH oxidation was seen to continue for more than 1 h (data not shown). It is evident that electrons must depart from flavin(s) of *P*-450 BM3 and BM3 reductase to an acceptor in solution. Oxygen is likely to be this acceptor and its reduction could lead to the generation of superoxide (O_2^-) anions and/or hydrogen peroxide (H_2O_2). In support of this conclusion, purging of buffer solutions with nitrogen for 2 min prior to initiation of the assays resulted in decreases of more than 65% in the rates of *P*-450 BM3-and BM3 reductase-catalysed substrate-independent NADPH oxidation, whereas the rate of cytochrome *c* reduction remained unchanged (data not shown).

3.2. Luminometric detection of active oxygen species

Assays were performed to detect products of oxygen activation using luminometry. In assay systems containing either intact *P*-450 BM3 or its reductase (flavin) domain and NADPH, the production of active oxygen species was detected by the luminescence of lucigenin. No such products were detected in experiments using the isolated *P*-450 (haem) domain (Fig. 1), indicating, again, that the flavoprotein reductase domain of *P*-450 BM3 provides the route by which electrons depart and that these electrons may be transferred directly from this domain onto O_2. The luminescence detected using luminol was found to be lower than that using lucigenin by a factor of approx. 20 (Fig. 2). There was no detectable stimulation of NADPH oxidation by lucigenin under the conditions employed in the luminometry assays. Therefore, it is not the case that direct reduction of lucigenin is the reason for the difference in luminescence seen between systems containing lucigenin and luminol. Since lucigenin luminescence can be decreased approx. 20-fold by the addition of superoxide dismutase (SOD), but not by catalase, it is clear that superoxide production through the reductase domain of *P*-450 BM3 is the main cause of lucigenin luminescence. Fig. 3 demonstrates the effect of addition of 250 units of SOD on *P*-450 BM3-mediated lucigenin luminescence. Addition of higher quantities of SOD further reduced the chemiluminescence. It is possible that residual luminescence (< 3% of original) may result from direct reduction of lucigenin. The lower level of luminescence detected using luminol can also be decreased to a similar extent using SOD (data not shown), indicating that O_2^- ions are mainly responsible for luminescence detected using both compounds. Assays of catalase (hydrogen peroxide breakdown) and SOD (inhibition of cytochrome *c* reduction in a xanthine/xanthine oxidase system) indicated that both en-

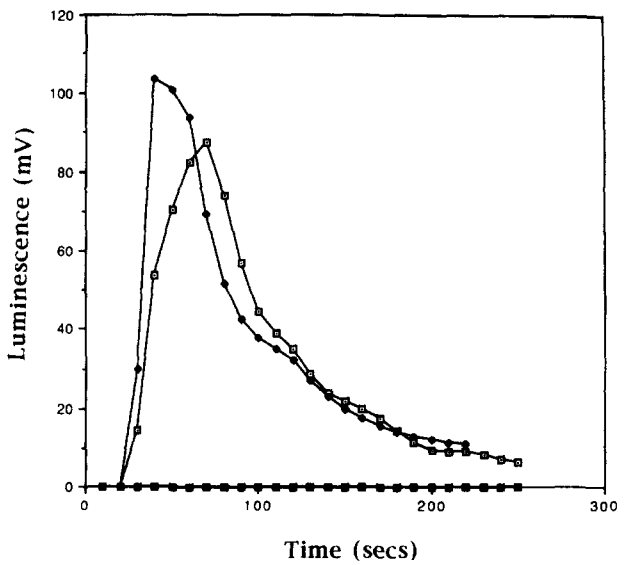

Fig. 1. Lucigenin chemiluminescence produced by *P*-450 BM3 and BM3 reductase catalysed NADPH oxidation. Luminescence of lucigenin (final concentration 100μM) was monitored at 30°C as described in the Materials and Methods section. Mixtures contained 1.0 μM enzyme and reactions were initiated by the addition of 0.3 mM NADPH. Symbols: (□) intact *P*-450 BM3, (■) *P*-450 domain, (◆) reductase domain.

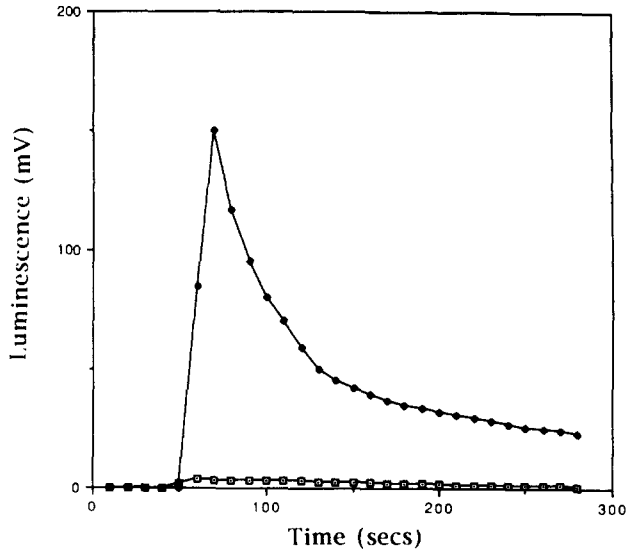

Fig. 2. Comparison of lucigenin and luminol chemiluminescence produced during NADPH oxidation. The relative intensity of luminescence produced by *P*-450 BM3 catalysed NADPH oxidation was measured in systems containing either 100 μM lucigenin (♦) or 100 μM luminol (□). Assays were performed at 30°C as described in the Materials and Methods section, using 2 μM *P*-450 BM3. A similar result was obtained when the reductase domain of *P*-450 BM3 was used for the same experiments (not shown).

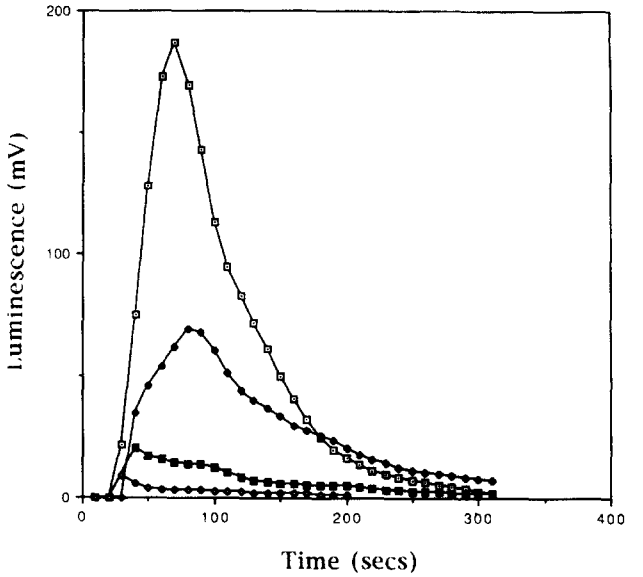

Fig. 4. Effect of diphenyliodonium on *P*-450 BM3 catalysed lucigenin chemiluminescence. Progressive inhibition of the burst of *P*-450 BM3 (2 μM) catalysed luminescence was achieved by the addition of increasing concentrations of diphenyliodonium chloride (IDP). Symbols: (□) no IDP added, (♦) 0.25 mM IDP, (■) 1 mM IDP and (◇) 2.5 mM IDP. Assays were performed at 30°C as described in the Materials and Methods section.

zymes retained > 90% of their activities in the presence of up to 100 μM luminol and lucigenin (data not shown). Mannitol (an inhibitor of hydroxyl radical formation) and various chelators of divalent iron (EDTA, 2,2'-dipyridyl, ethylenediamine di(*o*-hydroxyphenylacetic acid)) were without significant effect on the luminescence at concentrations up to 0.5 mM, again indicating that reductase-mediated generation of superoxide ions was the main basis of the luminescence detected (data not shown).

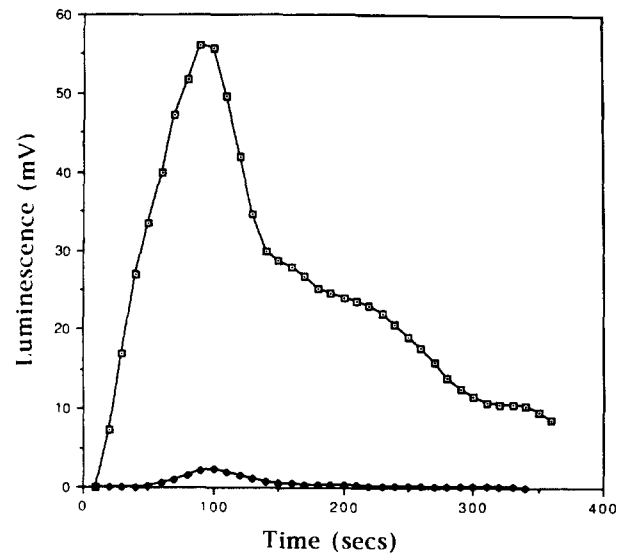

Fig. 3. Effect of superoxide dismutase on *P*-450 BM3 catalysed lucigenin chemiluminescence. The pronounced effect of addition of 250 units of SOD on the luminescence caused by *P*-450 BM3 (0.75 μM) mediated NADPH oxidation is shown (Symbols: (□) no SOD added, (♦) 250 units SOD added). Assays were performed at 30°C as described in the Materials and Methods section.

Experiments were performed to monitor NADPH oxidation during the catalytic processes of lauric acid hydroxylation (intact *P*-450 BM3) and cytochrome *c* reduction (*P*-450 BM3 and BM3 reductase) in the presence of 100 μM lucigenin. In all cases, initial rapid rates were quickly seen to slow considerably and activity came to a stop within approx. 45 s (data not shown). It would seem clear that the chemiluminescent ('activated') form of the compound causes inhibition of the activities of *P*-450 BM3 and its reductase. This would explain the rather short span of the chemiluminescent bursts seen in the experiments reported here.

A parallel experiment was performed in which slow, continuous consumption of oxygen was recorded using an oxygen electrode containing only assay buffer, NADPH (0.2 mM) and either *P*-450 BM3 or its reductase domain at a final concentration of 2 μM. The initial rate of oxygen consumption measured in both cases was 2 nmol oxygen consumed/min/nmol enzyme –very similar to the NADPH oxidation rates for these polypeptides (Table 1). No oxygen consumption was observed in the absence of enzyme, or when the haem domain of *P*-450 BM3 was used.

3.3. Inhibition of NADPH oxidase activity by diphenyliodonium chloride

Addition of sub-millimolar concentrations of IDP effectively inhibited *P*-450 BM3 catalysed sodium laurate hy-

droxylation and cytochrome *c* reduction, and also cytochrome *c* reduction catalysed by BM3 reductase (Table 1). The addition of IDP was also found to inhibit lucigenin chemiluminescence caused by the NADPH oxidase activity of *P*-450 BM3. Fig. 4 shows the progressive inhibitory effect of increasing concentration of IDP on the chemiluminescent burst caused by *P*-450 BM3-catalysed NADPH oxidation. Similar results were obtained using the BM3 reductase. This indicates that this domain carries the IDP-inhibitable site of *P*-450 BM3, which is likely to be one or both of the flavins. IDP does not appear to interact with the haem of *P*-450 BM3 in the presence or absence of NADPH, as evidenced by the lack of alteration in the haem region of the visible spectrum of *P*-450 BM3 on addition of 1 mM IDP (data not shown). In any case, interaction with *P*-450 haem would not be expected to inhibit the substrate independent NADPH oxidase activity associated with the flavoprotein domain of *P*-450 BM3. IDP did not affect luminescence of lugigenin caused by superoxide generated in a xanthine/xanthine oxidase system (data not shown).

Experiments in which the effect of IDP on substrate-independent NADPH oxidation were monitored revealed that the initial rate of *P*-450 BM3 and BM3 reductase mediated oxidation of NADPH is significantly increased in the presence of low concentrations of IDP (Table 1). This indicates that IDP is reduced by BM3 and its reductase. The IDP radical formed may then react back with flavin to inactivate the enzymes –as previously postulated for the mechanism of inhibition of the neutrophil NADPH oxidase by iodonium compounds [38], and as recently demonstrated for the inhibition of rat *P*-450 reductase [39]. Thus, while NADPH oxidation may initially be stimulated in the presence of IDP, superoxide anion production would be inhibited. This inhibition may occur initially due to competition between IDP and oxygen for electrons from the reduced flavin(s), but will later be due to inactivation of these redox centres by covalent linkage of IDP –a 'suicide substrate' for the BM3 reductase. Table 1 shows that while BM3 reductase-catalysed NADPH oxidation is relatively constant over a period of 10 min, the rate in the presence of 500 μM IDP decreases dramatically as the inhibitory IDP radical product is formed. As expected, 30 min pre-incubations of *P*-450 BM3 and its reductase with a 10-fold

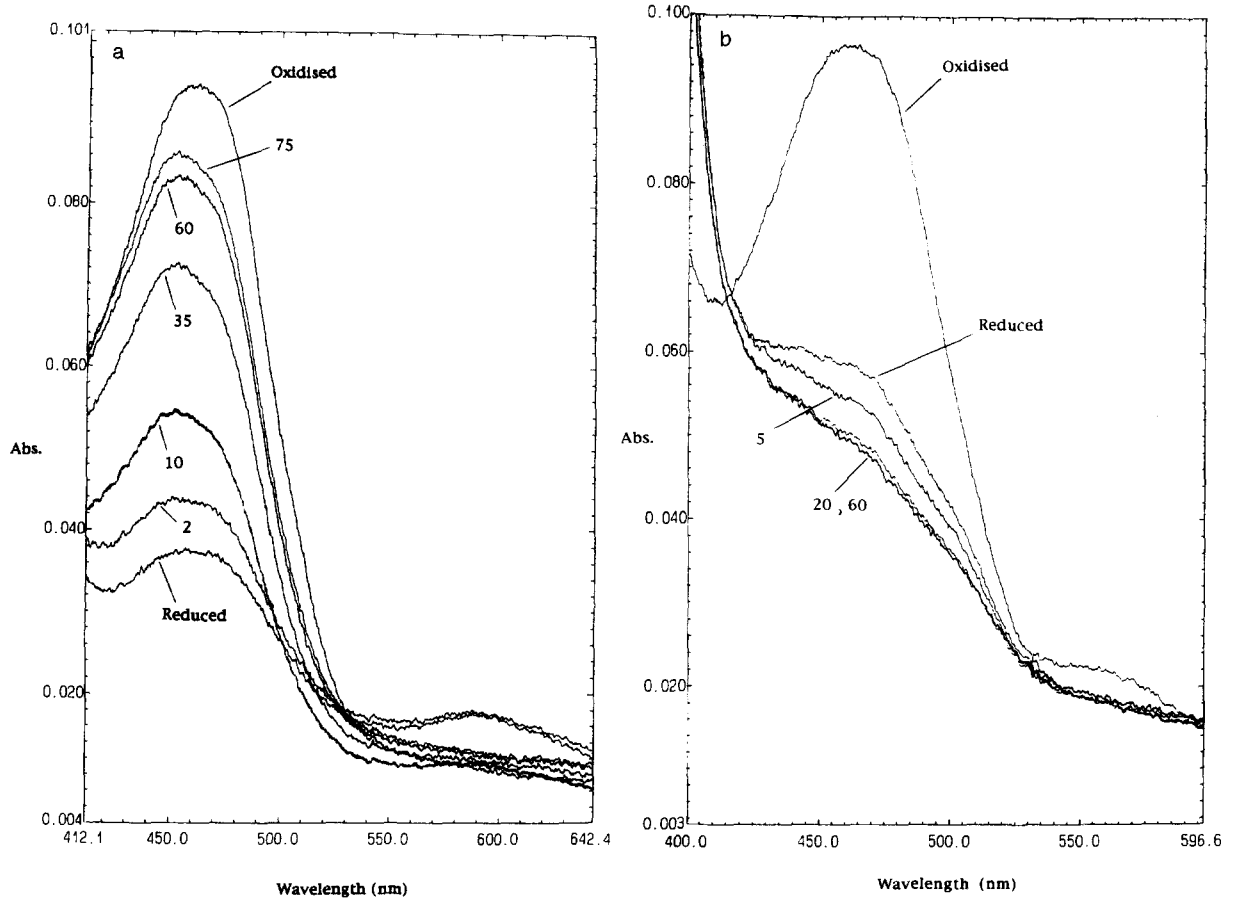

Fig. 5. Effect of diphenyliodonium on reoxidation of flavins in *P*-450 BM3 reductase domain. A solution of 6 μM BM3 reductase in assay buffer (20 mM Mops (pH 7.4) + 100 mM KCl) was reduced with 500 μM NADPH at 20°C. Visible spectra were recorded in the region of the major flavin absorbance band (approx. 460 nm) for the oxidised and reduced forms, and further spectra were taken at the time intervals indicated in minutes (a). (b) shows the inhibitory effect of addition of 1 mM IDP (at time zero) on the reoxidation of the flavins of BM3 reductase under the same conditions.

molar excess of IDP did not result in inactivation of the enzymes. Activity was retained when (200-fold) diluted samples of these mixtures were used in cytochrome *c* reduction assays. However, inclusion of a similar excess of NADPH in the pre-incubation buffer resulted in the loss of > 85% cytochrome *c* reducing ability, indicating that enzyme (and hence IDP) reduction is a prerequisite for inhibition (data not shown). As seen by visible spectroscopy, flavins of BM3 reductase reoxidise over time following the addition of an 80-fold molar excess of NADPH to the enzyme (Fig. 5a). Under the conditions used, reoxidation is complete after approx. 150 min –when the visible spectrum becomes virtually identical to the original oxidised spectrum in the region scanned. This reoxidation does not occur in the presence of 1 mM IDP (Fig. 5b) –providing further evidence for the flavins as the site of inhibition by IDP. Recently, we have shown that flavins can be dissociated from *P*-450 BM3 and its reductase by incubation with the denaturant guanidinium chloride at concentrations which do not irreversibly denature the enzymes (less than approx. 1.5 M). It is possible to regenerate more than 50% of the enzyme activity lost by NADPH/IDP treatment through incubation in approx. 0.5 M GdnHCl (to dissociate the modified flavins) and dilution to assay buffer containing 10-fold molar excess free FAD and FMN (Munro et al., unpublished data).

3.4. Effect of NADPH on the CD spectra of P-450 BM3 and its reductase domain

In view of the previous report [7] suggesting that time-dependent inactivation of *P*-450 BM3 on incubation with NADPH was associated with a conformational change in the protein, we monitored the effect of NADPH on the *P*-450 BM3 and BM3 reductase using CD. Fig. 6 shows the effect of enzyme incubation with NADPH on the far UV-CD spectra of *P*-450 BM3 (Fig. 6a) and BM3 reductase domain (Fig. 6b). Only very minor alterations in the spectra are observed following 90 min incubations with NADPH, indicating that the secondary structure of the enzymes is not significantly perturbed by the NADPH.

Fig. 7 shows the effect of the same incubations on the

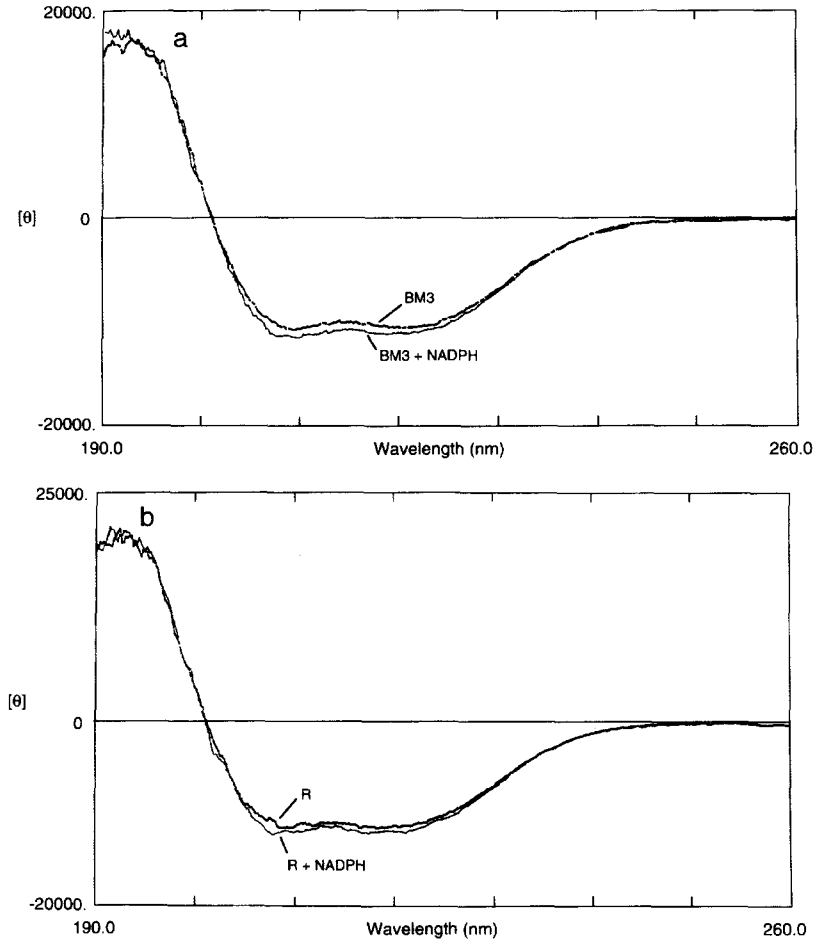

Fig. 6. Effect of NADPH on the far-UV-CD spectra of *P*-450 BM3 and BM3 reductase domain. Solutions of (a) *P*-450 BM3 and (b) BM3 reductase domain were at concentrations of 0.46 mg/ml. Each solution was mixed with NADPH (0.2 mM) and incubated at room temperature for approx. 90 min. CD spectra were collected before and after these incubations as described in the Materials and Methods section. The small spectral contributions due to NADPH were subtracted using appropriate blanks.

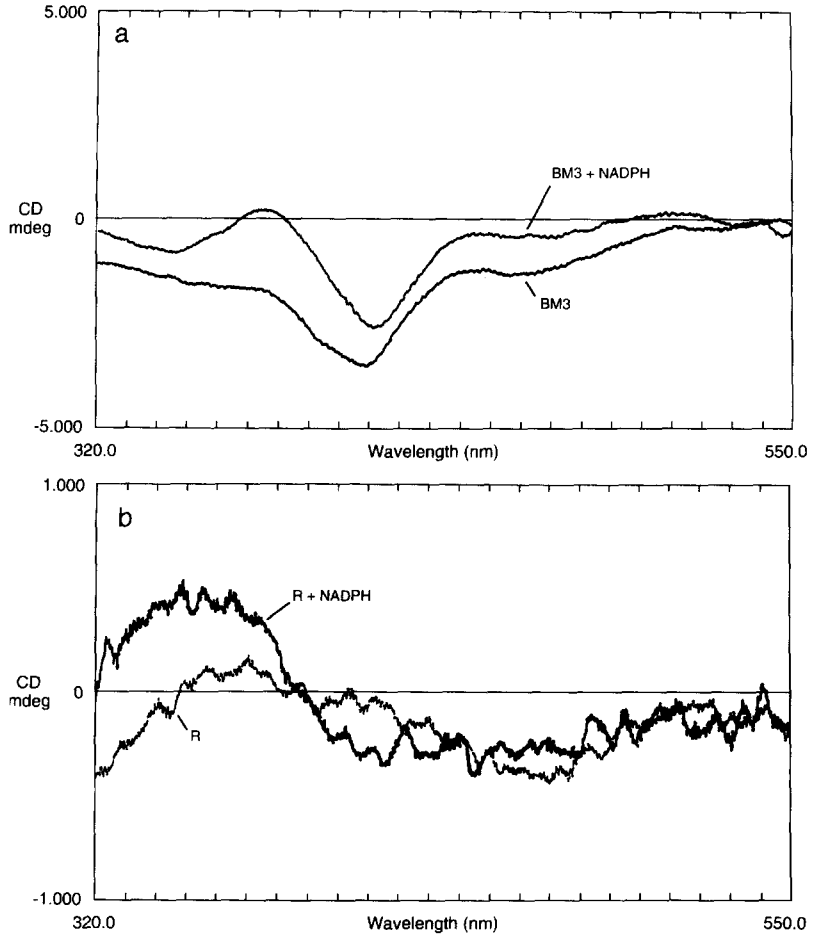

Fig. 7. Effect of NADPH on the visible-CD spectra of *P*-450 BM3 and BM3 reductase domain. Solutions of (a) *P*-450 BM3 and (b) BM3 reductase domain were at concentrations of 1.38 mg/ml. Each solution was mixed with NADPH (0.2 mM) and incubated at room temperature for approx. 60 min. CD spectra were collected before and after these incubations as described in the Materials and Methods section. The small spectral contributions due to NADPH were subtracted using appropriate blanks.

near UV-visible CD spectra of *P*-450 BM3 (Fig. 7a) and BM3 reductase (Fig. 7b). Clear differences in this region are seen to result from incubation with NADPH, and, although the signals from the BM3 reductase domain are weak, these can be largely ascribed to the alteration in signals from the reduced FAD and FMN chromophores.

4. Discussion

The results presented in this report clearly demonstrate the ability of cytochrome *P*-450 BM3 and its reductase domain to oxidise NADPH in the absence of fatty acid substrates or artificial electron acceptors, such as cytochrome c. Chemiluminescence experiments indicate that the electrons derived from NADPH oxidation are transferred onto oxygen to generate superoxide anions. Addition of superoxide dismutase inhibits this luminescence. Chemiluminescence has been widely used in the study of microbicidal NADPH oxidase of leukocytes, e.g., [23,40]. It has not been extensively used in the study of *P*-450 systems, but here we show that it provides a useful technique for detection of O_2^- and represents a convenient alternative to spectroscopic techniques (such as EPR and spin-trapping) [41] or the measurement of NADPH-dependent lipid peroxidation [42] previously used for the detection of O_2^- produced by mammalian *P*-450 reductase. Attempts to measure O_2^- production by following the superoxide dismutase-sensitive rate of cytochrome *c* reduction are made extremely difficult due to the high inherent cytochrome *c* reductase activity of *P*-450 reductase.

The reduction of O_2 to O_2^- is the function of the microbicidal NADPH oxidase of leukocytes and other white blood cell forms. The haemoprotein component of this complex flavocytochrome [24] appears vital for oxygen activation [43,20]. The low-potential type *b* haem in the NADPH oxidase (cytochrome *b*-245) is six coordinate, with two strong axial amino acid ligands to the haem iron [22]. This cytochrome is therefore dissimilar from the cytochromes *P*-450, which have a single axial haem ligand (a cysteine residue) and the second axial position vacant for the ligation and activation of oxygen during catalysis [44]. In the NADPH oxidase, electrons must be transferred

to O_2 without its ligation to haem iron. It has been proposed that this occurs by electron donation to O_2 at the haem edge [22] as has been proposed for reduction of ferric cytochrome *c* by O_2^- [45]. The cytochromes *P*-450 are also known to catalyse production of O_2^- through autoxidation of the oxygen-bound form, e.g. [46,47]. The cytochrome component of *P*-450 BM3 has little primary structure homology with the leucocyte haem enzyme, but there is much conservation of structure between the flavoprotein components of *P*-450 BM3 and the leukocyte oxidase [16]. Interestingly, the NADPH oxidase flavoprotein has also been reported to catalyse superoxide production independently of its cytochrome [25].

Diphenyliodonium (IDP) and its analogue diphenyleneiodonium (DPI) act as potent inhibitors of the NADPH oxidase system in various leukocyte cells [48,49] and in fibroblasts [50]. These iodonium compounds have been shown to react by abstraction of an electron from a nucleophile to form a radical, which then adds back to the nucleophile to form a covalent adduct [51]. The formation of DPI-and IDP-flavin adducts has been observed during inactivation of the neutrophil NADPH oxidase and reaction with flavins is postulated as the common mechanism by which IDP and its analogues act as inhibitors of various flavoenzymes [38]. Most recently, the formation of phenylated FMN has been demonstrated during inhibition of rat microsomal *P*-450 reductase by IDP [39]. We demonstrate here that IDP also inhibits fatty acid hydroxylase activity of *P*-450 BM3, and NADPH oxidase and cytochrome *c* reductase activities of *P*-450 BM3 and its reductase domain. IDP also inhibits lucigenin chemiluminescence caused by the NADPH oxidase activity of *P*-450 BM3 and its reductase. The inhibition is catalysis-dependent, indicating that reduction of IDP is a prerequisite for its action. The initial stimulation of substrate-independent NADPH oxidation seen in the presence of IDP is due to utilisation of IDP as a reducing substrate during the process of formation of its inhibitory form –as recently demonstrated with a mammalian *P*-450 reductase [39]. For both *P*-450 BM3 and the leukocyte NADPH oxidase, IDP inhibits superoxide production by inactivation of electron flow through the flavins. In the case of the leukocyte oxidase, this prevents electrons reaching the haem (the major site of O_2 activation). For *P*-450 BM3, the flavins themselves act as mediators of electron transfer to O_2. The capacity of flavoprotein electron transferases to generate superoxide anions has recently been reviewed [52].

The substrate-independent NADPH oxidation catalysed by *P*-450 BM3 and its reductase domain may provide explanations for unusual observations reported during previous studies of the enzyme. Firstly, Narhi and Fulco [7] observed a time-dependent inactivation of *P*-450 BM3 in the presence of NADPH and suggested that this might be due to an NADPH-induced conformational change from a 'high activity' to a 'low activity' form of the enzyme. Full activity was found to be restored by dialysis to remove the NADPH. Studies in our laboratories confirm the time-dependent inactivation effect of NADPH. However, our CD studies indicate that secondary structure of *P*-450 BM3 and its reductase domain are not significantly altered by incubation with 0.2 mM NADPH. Our other findings indicate that pre-incubation of *P*-450 BM3 with NADPH would result in oxidation of the cofactor at a rate approx. 10^3-fold lower than that seen in the presence of substrate. The generation of $NADP^+$ (a competitive inhibitor) may partially explain the inhibitory effect of *P*-450 BM3 pre-incubation with excess NADPH. Secondly, Peterson and Boddupalli [53] reported that between five and six electron equivalents per mole of enzyme are required to fully reduce *P*-450 BM3 with NADPH under an atmosphere of carbon monoxide, and that a sharp endpoint is not obtained in this titration. The necessity for excess NADPH may be due to its slow oxidation by the enzyme, using traces of oxygen remaining in solution as an electron acceptor.

In this report, we demonstrate the ability of the reductase domain of *P*-450 BM3 to generate O_2^- independently of its cytochrome and demonstrate the applicability of chemiluminescence techniques to its study. The reductase domain is highly homologous with mammalian hepatic *P*-450 reductase, which is responsible for the reduction of *P*-450s involved in drug and xenobiotic bioconversions. These studies strongly suggest that NADPH-cytochrome *P*-450 reductase enzymes may be a significant source of production of oxygen free radical species in vivo –particularly if there is imbalance between the relative availabilities of NADPH and *P*-450 substrates in the cell. Given the current interest in the roles of oxygen radical species in the development of such mammalian pathological states as inflammation, Parkinson's disease, atherosclerosis, cardiovascular disease and cancer, e.g., [54–58], this finding should merit further investigation.

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References

[1] Guengerich, F.P. (1991) J. Biol. Chem. 266, 10019–10022.
[2] Asperger, O. and Kleber, H.-P. (1991) in Microbial and Plant Cytochromes *P*-450: Biochemical Characteristics, Genetic Engineering and Practical Implications (Ruckpaul, K. and Rein, H., eds.), pp. 1–53, Taylor and Francis, London.
[3] Durst, F. (1991) in Microbial and Plant Cytochromes *P*-450: Biochemical Characteristics, Genetic Engineering and Practical Implications (Ruckpaul, K. and Rein, H., eds.), pp. 191–232, Taylor and Francis, London.
[4] Kappelli, O. (1986) Microbiol. Rev. 50, 244–258.
[5] Buchanan, J.F. and Fulco, A.J. (1978) Biochem. Biophys. Res. Commun. 85, 1254–1260.
[6] Ruettinger, R.T. and Fulco, A.J. (1981) J. Biol. Chem. 256, 5728–5734.

[7] Narhi, L.O. and Fulco, A.J. (1986) J. Biol. Chem. 261, 7160–7169.
[8] Narhi, L.O. and Fulco, A.J. (1987) J. Biol. Chem. 262, 6683–6690.
[9] Boddupalli, S.S., Hasemann, C.A., Ravichandran, K.G., Lu, J.-Y., Goldsmith, E., Deisenhofer, J. and Peterson, J.A. (1992) Proc. Natl. Acad. Sci. (USA) 89, 5567–5571.
[10] Poulos, T.L., Finzel, B.C. and Howard, A.J. (1987) J. Mol. Biol. 195, 687–700.
[11] Li, H., Darwish, K. and Poulos, T.L. (1991) J. Biol. Chem. 266, 11909–11914.
[12] Miles, J.S., Munro, A.W., Rospendowski, B.N., Smith, W.E., McKnight, J. and Thomson, A.J. (1992) Biochem. J. 288, 503–509.
[13] Ravichandran, K.G., Boddupalli, S.S., Hasemann, C.A., Peterson, J.A. and Deisenhofer, J.A. (1993) Science 261, 731–736.
[14] Hasemann, C.A., Kurumbail, R.G., Boddupalli, S.S., Peterson, J.A. and Deisenhofer, J. (1995) Structure 2, 41–62.
[15] Li, H. and Poulos, T.L. (1994) Structure 2, 461–464.
[16] Porter, T.D. (1991) Trends Biochem. Sci. 16, 154–158.
[17] Bredt, D.S., Hwang, P.M., Glatt, C.E., Lowenstein, C., Reed, R.R. and Snyder, S.H. (1991) Nature 351, 714–718.
[18] Faeder, E.J., Davis, P.S. and Siegel, L.M. (1974) J. Biol. Chem. 249, 1599–1609.
[19] Siegel, L.M. and Davis, P.S. (1974) J. Biol. Chem. 249, 1587–1598.
[20] Segal, A.W. (1989) J. Clin. Invest. 83, 1785–1793.
[21] Segal, A.W. and Abo, A. (1993) Trends Biochem. Sci. 18, 43–47.
[22] Isogai, Y., Izuka, T., Makino, Ryu, Iyanagi, T. and Yutaka, O. (1993) J. Biol. Chem. 268, 4025–4031.
[23] Dorseuil, O., Vazquez, A., Lang, P., Bertoglio, J., Gacon, G. and Leca, G. (1992) J. Biol. Chem. 267, 20540–20542.
[24] Segal, A.W., West, I., Wientjes, F., Nugent, J.H.A., Chavan, A.J., Haley, B., Garcia, R.C., Rosen, H. and Scrace, G. (1992) Biochem. J. 284, 781–788.
[25] Green, T.R. and Pratt, K.L. (1990) J. Biol. Chem. 265, 19324–19329.
[26] Gibson, T.J. (1984) PhD thesis, University of Cambridge, UK.
[27] Bullock, W.O., Fernandez, J.M. and Short, J.M. (1987) BioTechniques 5, 376–379.
[28] Vieira, J. and Messing, J. (1987) Methods Enzymol. 153, 3–11.
[29] Marsh, P. (1986) Nucl. Acids Res. 14, 3603.
[30] Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) in Molecular Cloning, A Laboratory Manual (2nd edition), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[31] Tartof, K.D. and Hobbs, C.A. (1987) Bethesda Res. Lab. Focus 9, 12.
[32] Narhi, L.O. and Fulco, A.J. (1982) J. Biol. Chem. 257, 2147–2150.
[33] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[34] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and D.C. Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[35] Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2379–2385.
[36] Kuthan, H., Ullrich, V. and Estabrook, R.W. (1982) Biochem. J. 203, 551–558.
[37] Provencher, S.W. and Glöckner, J. (1981) Biochemistry 20, 33–37.
[38] O'Donnell, V.B., Tew, D.G., Jones, O.T.G. and England, P.J. (1993) Biochem. J. 290, 41–49.
[39] Tew, D.G. (1993) Biochemistry 32, 10209–10215.
[40] Allen, R.C. (1986) Methods Enzymol. 133, 449–493.
[41] Bosterling, B. and Trudell, J.R. (1981) Biochem. Biophys. Res. Commun. 98, 569–575.
[42] Pederson and Aust, BBRC, 48, 789–795 (1972).
[43] Isogai, Y., Shiro, Y., Nasuda-Kouyama, A. and Izuka, T. (1991) J. Biol. Chem. 266, 13481–13484.
[44] Ruckpaul, K., Rein, H. and Blanck, J. (1989) pp. 1–65 in 'Basis and mechanisms of regulation of cytochrome *P*-450'. Edited by Ruckpaul, K. and H. Rein. Published by Taylor and Francis (London).
[45] Butler, J., Koppenol, W.H. and Margoliash, E. (1982) J. Biol. Chem. 257, 10747–10750.
[46] Sligar, S.G., Lipscomb, J.D., Debrunner, P.G. and Gunsalus, I.C. (1974) Biochem. Biophys. Res. Commun. 61, 290–296.
[47] Kuthan, H., Tsuji, H., Graf, H., Ullrich, V. Werringloer, J. and Estabrook, R.W. (1978) FEBS Lett. 91, 343–345.
[48] Cross, A.R. and Jones, O.T.G. (1986) Biochem. J. 237, 111–116.
[49] Hancock, J.T. and Jones, O.T.G. (1987) Biochem. J. 242, 103–107.
[50] Meier, B., Cross, A.R., Hancock, J.T., Kaup, F.J. and Jones, O.T.G. (1991) Biochem. J. 275, 241–245.
[51] Banks, D.F. (1966) Chem. Rev. 66, 243–266.
[52] Massey, V. (1994) J. Biol. Chem. 269, 22459–22462.
[53] Peterson, J.A. and Boddupalli, S.S. (1992) Arch. Biochem. Biophys. 294, 654–661.
[54] Barja, G. (1993) Free Rad. Res. Commun. 18, 63–70.
[55] Halliwell, B. and Gutteridge, J.M.C. (1989) in 'Free Radicals in Biology and Medicine' 2nd edition. Clarendon Press, Oxford.
[56] Byers, T. (1993) Nutr. Rev. 51, 333–345.
[57] Halliwell, B., Cross, C.E. and Gutteridge, J.M.C. (1992) J. Lab. Clin. Med. 119, 598–620.
[58] Halliwell, B. (1995) The Biochemist 17, 3–6.