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Cytochrome P450BM-3 reduces aldehydes to alcohols through a direct hydride transfer

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ABSTRACT

Cytochrome P450BM-3 catalyzed the reduction of lipophilic aldehydes to alcohols efficiently. A k_{cat} of ~25 min^{-1} was obtained for the reduction of methoxy benzaldehyde with wild type P450BM-3 protein which was higher than in the isolated reductase domain (BMR) alone and increased in specific P450-domain variants. The reduction was caused by a direct hydride transfer from preferentially *R*-NADP^{2}H to the carbonyl moiety of the substrate. Weak substrate-P450-binding of the aldehyde, turnover with the reductase domain alone, a deuterium incorporation in the product from NADP^{2}H but not D_2O, and no inhibition by imidazole suggests the reductase domain of P450BM-3 as the potential catalytic site. However, increased aldehyde reduction by P450 domain variants (P450BM-3 F87A T268A) may involve allosteric or redox mechanistic interactions between heme and reductase domains. This is a novel reduction of aldehydes by P450BM-3 involving a direct hydride transfer and could have implications for the metabolism of endogenous substrates or xenobiotics.

1. Introduction

Cytochrome P450BM-3 (CYP102A1) of *Bacillus megaterium* is an established model system to study mechanistic aspects of mammalian cytochrome P450-mediated metabolism of xenobiotics and endogenous compounds [1]. The natural fusion of the heme domain with the cytochrome P450BM-3 reductase domain (BMR) enables impressive catalytic activities reaching as high as 17,000 min^{-1} (k_{cat}) with arachidonic acid as a substrate [2]. The high catalytic activity is attributed to the tight alignment of the three functional domains, facilitating efficient hydride transfer from the cofactor NADPH to FAD, a subsequent reduction of FMN by a proton coupled electron transfer from FAD, and finally a one electron heme iron reduction through a highly reactive FMN intermediate to initiate the substrate oxidation cycle in the heme active site of P450BM-3 [3]. The mechanism of this cascade of redox reactions has been intensively studied and reviewed elsewhere [3,4]. Cytochrome P450BM-3 is known to hydroxylate various fatty acids, possibly the natural substrates [5], but reactions such as oxidations (allylic and benzylic), epoxidations (stereospecific), dealkylations, and even the oxidation of methane have also been studied. This manifold of reaction mechanisms have been of particular interest and found applications in biotechnology and in generating authentic drug metabolites [5–7].

We set out to investigate P450BM-3 wild type (WT) and several variants for their ability to oxidatively deformylate 1,2,3,4-tetrahydro-napthaline-2-carbaldehyde similar to studies performed by Roberts et al. [8]. Interestingly, no deformylated metabolites were observed instead the only metabolite formed was the primary alcohol a result of the direct reduction of the carbonyl moiety. Several aldehydes substrates were investigated including methoxy benzaldehyde (MBA) and the only metabolite formed was always the alcohol.

Usually, reductions of aldehydes are associated with carbonyl reductases such as aldo–keto reductase [9], and can be associated with NADPH dependent reductions in human liver microsomes [10]. Recently in reconstituted systems [11,12], a range of CYP-enzymes were able to reduce α,β-unsaturated fatty acids such as 4-hydroxy nonenal by a proposed radical mediated mechanism. In this report, we establish that P450BM-3, and an activity enhancing variant F87A/T268A, reduces aldehydes to alcohols and confirm that the reduction occurs through a direct hydride transfer from NADPH to the carbonyl.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, reagents were purchased from Sigma–Aldrich (St. Louis, MO) and culture media from Fisher Scientific

Abbreviations: P450, cytochrome P450; BM-3, *Bacillus megaterium* cytochrome P450 102A1; CPR, cytochrome P450 reductase; BMR, P450BM-3 reductase domain; BMP, heme domain of P450BM-3; MBA, *p*-methoxy-benzaldehyde; WT, wild type enzyme.

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(New Jersey, NJ). The P450BM-3 clone was a kind gift from Dr. Armand Fulco (University of CA at Los Angels). *S*-NADP^{2}H and *R*-NADP^{2}H were synthesized by a modified method of Ottolina et al. [13] and purified using an ion exchanger resin DE-25 and fractioned by a stepwise gradient of ammonium carbonate buffer up to 0.4 M to purify the reduced NADPH/^{2}H and lyophilization. Purity was determined by NMR spectroscopy.

2.2. Cloning and expression of WT and mutants of P450BM-3

The mutations F87A, T268A and F87A/T268A were introduced using a QuickChange Mutagenesis kit (Stratagene, La Jolla, CA) and mutations were confirmed through sequencing the entire gene. The truncated P450BMP (P450-domain only) and P450BMP T268A domain, and P450BM-3 reductase domain were generated according to [14,15]. P450BM-3 WT and mutants were expressed and purified by established methods [14–16]. Protein was expressed in *E. coli* BL21 DE3 cells in Terrific broth cultures (37 °C, 160 rpm) supplemented with 1 mM IPTG, 0.5 mM δ-aminolevulenic acid and ampicillin (100 mg L^{-1}) after 4 h for induction. Cells were harvested after 48 h by centrifugation. Following cell rupture using two passes through the micro fluidization machine (Micro DeBEE, B.E.E. Int., Easton, MA), the cell preparation was centrifuged for 1 h at 37 K rpm (Sorvall, Ti 50.2). The supernatant was directly loaded onto an affinity 2′,5′-ADP-sepharose 4B (General electric, Uppsala, SE) pre-equilibrated with buffer A (containing 100 mM potassium phosphate, pH 7.4, and 0.5 mM DTT). The column was washed with buffer B (containing 500 mM potassium phosphate, pH 7.4, and 0.5 mM DTT) for at least five column volumes. The protein was subsequently eluted with buffer A, containing 10 mM NADP, and dialyzed against buffer A (without DTT).

2.3. Characterization of P450BM-3 WT and variants

P450 CO-difference spectra, gel electrophoresis, pyridine hemochromogen analysis and Lowry protein determination assay were used to evaluate CYP-enzyme quality and quantity [17–20]. FMN and FAD content was measured fluorometrically as previously described [21] and quantified by standard addition method (addition of flavin standards to the sample). Cytochrome c reduction activity was measured in 50 mM KPi pH 7.4 with the addition of 100 μM cytochrome *c*, 100 μM NADPH and 1 nM enzyme, and the steepest slope of the assay was subtracted from a blank measurement [16].

2.4. Enzymatic assays (MBA reduction and lauric acid oxidation)

For substrate reduction assays, enzyme solutions were incubated in phosphate buffer (50 mM KPi, pH 7.4) with 100 μM substrate (dissolved in acetonitrile, final concentration 1%) and after 2 min pre-incubation initiated with NADPH (final concentration of 1 mM in 200 μL). After 5 min incubation at 37 °C, the incubations were extracted once with an equal volume of ethyl acetate, centrifuged at 10,000 rpm, and 80 μL of the organic layer derivatized with 20 μL of BSTFA for 4 h at 70 °C. One microliters of sample of silated solutions was injected onto a GC-column DB-1 (15 m, 0.2 mm, Agilent Technol., Santa Clara, CA) installed in a gas chromatograph (HP GC-17A) equipped with a mass selective detector (GCMS-QP5050), compounds separated using a temperature gradient program (40 °C, 1 min, 70 °C min^{-1} to 160 °C, 15 °C min^{-1} to 220 °C, 70 °C min^{-1}–290 °C, 2 min hold). Substrate and internal standard (2-hydroxymethyl naphthalene 20 mg L^{-1}) were detected at *m*/*z* 121 (122 for deuterium incorporation) and 141, respectively.

For the determination of lauric acid oxidation, 0.1 μM enzyme was incubated under the same conditions as above with 100 μM lauric acid and extracted with 3 × 2 mL ethyl acetate. Combined extracts were evaporated to dryness, resuspended and derivatized as mentioned above. Products of lauric acid hydroxylation separated on the same column, with a different temperature gradient as follows (100 °C, 1 min, 15 °C min^{-1} to 220 °C, 70 °C min^{-1} to 290 °C, 2 min hold) and detected at *m*/*z* 117, 131, 145, 345 [22]. Apparent Michaelis–Menten constants K_m and V_{max}, under linear conditions of time and protein, were derived after nonlinear regression analysis of the kinetic data using a simple enzyme kinetics model in SigmaPlot 2004 windows version 9.0 (Systat Software, Chicago, IL). Kinetic data is reported as the mean ± SD (computer generated).

2.5. Solvent isotope effect and incorporation of deuterium

For the measurement of the solvent isotope effect, the enzyme solution was repeatedly dialyzed (30 kDa cutoff membrane) against phosphate buffer in D_2O with the correction of changes to pH due to deuterium. Deuterium incorporation into MBA was measured by replacing NADPH with the pro-*R* and pro-*S* NADP^{2}H using the assay method above and followed the detection of *m*/*z* 122 for the mass increase due to the incorporation of deuterium at the benzylic position.

2.6. Assays for mechanistic characterization

Inhibition of the MBA-reduction by carbon monoxide or imidazole was measured by saturating the assay buffer with carbon monoxide or adding 300 μM imidazole respectively prior to initiating the assay as mentioned under kinetic assays. Reversibility of the MBA-reduction was measured by adding 1 mM $NADP^+$ and 300 μM methoxy-benzalcohol instead of MBA and NADPH into the assay solution and testing for the formation of NADPH spectrophotometrically at 340 nm and MBA via gas chromatography (*m*/*z* 137).

2.7. Binding spectra

For substrate binding experiments, the enzyme was dissolved in 50 mM Tris buffer pH 7.4 and the substrate added (in DMSO) to the appropriate final concentration (10 nM–100 μM). The increase of high spin state measured at 390 nm over the decrease in low spin state (420 nm) was measured against a blank with buffer, enzyme, and adjusted to the same DMSO concentration (difference spectra).

3. Results and discussion

3.1. Activity of aldehyde reduction in P450BM-3

During the expression of P450BM-3 and its variants, the incorporation of heme and/or both flavin prosthetic groups can be reduced, severely altering activity. A quality check of the proteins indicated that P450BM-3 F87A/T268A, P450BM-3 WT and BMR showed similar redox cofactor incorporation into the apoprotein (heme, FAD and FMN) with similar ratios of flavins to heme between proteins (Table 1). Cytochrome c reduction was similar in P450BM-3 WT and BMR but increased about 25% in P450BM-3 F87A/T268A.

As a model substrate to characterize the aldehyde reduction, *p*-methoxy benzaldehyde (MBA) was used. Aldehyde reduction to the corresponding alcohol was reproducible among several protein batches and the highest turnover achieved was 180 min^{-1} measured for P450BM-3 F87A/T268A variant in one expression. 4-Methoxybenzoic acid was not observed excluding the possibility of disproportionation as a mechanism to generate the methoxy benzyl alcohol from two aldehyde molecules, and no reduction was demonstrated in control assays in absence of NADPH or protein.

Table 1
Kinetics of *p*-methoxy-benzaldehyde reduction by WT and variants.

	Ratio heme:FAD	Ratio FAD:FMN	Cytochrome c reduction per FAD (min^{-1})	K_m (μM)	k_{cat} pmol (pmol P450 $min)^{-1}$
WT	1.05	0.75	2800 ± 500	150 ± 20	23 ± 1
F87A T268A	1.03	0.78	3800 ± 400	200 ± 20	38 ± 2
BMR domain	N/A	0.74	2700 ± 200	250 ± 30	9.9 ± 0.3

N/A not applicable.

A qualitative assessment of reduction activity towards different probe substrates demonstrated that P450BM-3 F87A/T268A was also able to reduce similar aromatic aldehydes with almost equal activity including naphthalene-2-carbaldehyde, 1,2,3,4-tetrahydro-naphthalene-2-carbaldhyde, 1-H-indole-3-carbaldehyde, and benzaldehyde, whereas activity for P450BM-3 WT and BMR were low (data not shown).

3.2. Kinetic evaluation of reduction in P450BM-3 variants

Kinetic evaluation of MBA-reduction demonstrated saturable kinetics invoking an enzyme catalyzed reaction (Supplementary material and Table 1). Several expressed P450BM-3 variants were additionally evaluated to assess a possible involvement of the separate domains of P450BM-3, and if the reaction can be enhanced through particular active site variants. E.g. P450BM-3 T268A is known to reduce the oxidative metabolism and potentially be involved in the peroxyradical cleavage process [23–25], whereas an F87A enlarges the active site [23]. Interestingly, the P450BM-3 F87A/T268A double mutant catalyzed the reduction with a twofold higher k_{cat} than P450BM-3 WT or BMR domain alone (Table 1). The K_m values for all tested enzymes were lowest for P450BM-3 WT, and highest for BMR domain. To assess the involvement of the heme and reductase domain separately, additional testing of (a) P450BM-3 protein variants carrying a single mutation within the P450 domain (F87 and T268), (b) truncated heme domain variants (BMP), and (c) BMR separately revealed that P450BM-3 F87A/T268A was the most active variant at a single concentration of 100 μM (Fig. 1). The truncated domain variants BMP WT and BMP T268A catalyzed the reduction only in trace amounts, and combining P450BM-3 WT or BMP T268A with BMR did not result in similar activities as the naturally fused domains. This indicates that both heme and reductase domains have to be fused to yield the most efficient aldehyde reduction activity, and P450 active site variants were able to enhance reductions. In contrast, activity of lauric acid oxidation was almost completely abolished in P450BM-3 F87A/T268A (5% residual activity) in comparison to P450BM-3 WT, while F87A and T268A retained about 20–80% oxidative activity. Thus, a reduced lauric acid oxidation might correspond to an increase rate of aldehyde reduction.

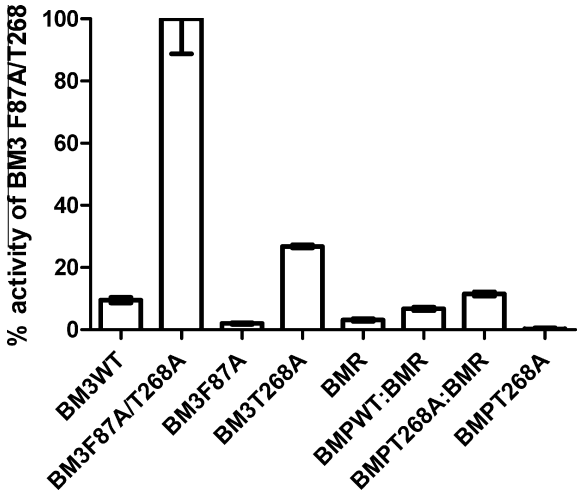

Fig. 1. Percentage activity of CYP450BM-3 WT and variants, BMR and combined enzyme domains (activity of CYP450BM-3 F87A/T268A for *p*-methoxy benzaldehyde (MBA) reduction set to 100%, reaction performed at 100 μM MBA).

3.3. Probing the aldehyde reduction mechanism

The reduction mechanism was investigated by several initial experiments. To transport electrons to the heme active site of P450BM-3, binding of a reductive equivalent such as NADPH and transfer of the pro-*R*-hydride onto FAD takes place [26]. A proton coupled electron transfer subsequently reduces the second cofactor FMN to a semiquinone or further hydroquinone within the FMN binding domain, which only moves towards the P450 domain and reduces the heme center in the first case but inhibits the transfer to the heme in the latter [1]. As previously reported, catalytic activity for cytochrome c reduction is about 3000 min^{-1} for P450BM-3 [16], consistent with our findings. The possibility that the hydride transfer from NADPH to FAD within the NADPH binding domain might not yield 100% efficiency would explain a 'hydride leaching'. In the case of MBA reduction, about 1% of the total cytochrome c activity would be transferred to a reducible substrate for P450BM-3 WT and the F87A T268A variant.

First we determined whether a hydride transfer from *pro-R*- or *pro-S*-$NADP^2H$ occurred. Indeed, a deuterium is incorporated from $NADP^2H$ into MBA evidenced by the increase of the signal for the mass fragment *m*/*z* 122 (from 121 fragment) and was large for pro-*R*-$NADP^2H$ (Fig. 2). This indicates a direct hydride transfer onto the substrate from $NADP^2H$ or potentially reduced flavin. Since the monitored fragment *m*/*z* 121 is derived following the cleavage of $O–Si(CH_3)_3$ and there are no other sites on MBA that can accept a hydride, this is direct evidence of a hydride transfer to the carbonyl. A direct analysis of the product by NMR was not feasible due to difficulty and low yields in the synthesis of pro-*R*-$NADP^2H$. A direct hydride transfer was confirmed in experiments conducted

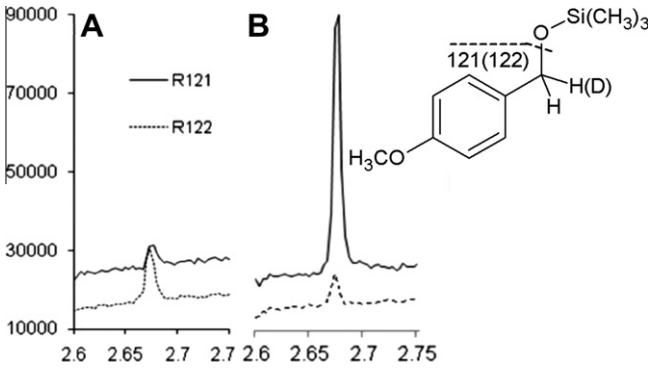

Fig. 2. Mass traces of extraction from MBA reduction using CYP450BM-3 F87A T268A and (A) 1 mM *R*-$NADP^2H$, and (B) 1 mM *S*-$NADP^2H$. R121 shows the mass trace of fragment of non-deuterated alcohol, R122 depicts the mass trace of deuterated alcohol. Inserted structure depicts the generation of fragment *m*/*z* 121 (122 after deuteration).

D_2O as solvent (see below) where the rate of reaction was reduced but no deuterium was incorporated in the product confirming that none of the protons on the product are solvent exchangeable and the increase of m/z of 1 (from 121 to 122) when reactions are conducted with *R*-NADP^{2}H can only occur through a hydride transfer from NADPH (either directly or via FAD) to the carbonyl of MBA. Estimating the ratio of mass increase from deuterium incorporation using the area of m/z 122 minus the naturally occurring isotope peak (area of m/z 122) from non-deuterated alcohol product generated using non-deuterated NADPH, and normalized over the total signal intensity (area of m/z 121 + m/z 122) indicates that 91 ± 16% of the area of m/z 122 derived from a hydride transfer originating from *pro-R*-NADP^{2}H. Using *pro-S*-NADP^{2}H, only an estimated 6.0 ± 0.5% of deuterium was incorporated according to this calculation, indicating the enantioselective abstraction of the *pro-R*-deuterium over *pro-S* as previously described for enzymes of the NADPH-oxidoreductase family [26]. The m/z 122 mass trace in Fig. 2B thus derives from the above mentioned isotope peak The purity of the produced NADP^{2}H enantiomers over NADP was >95% and the enantiopurity 85% and 94% for *pro-R*- and *pro-S*-NADP^{2}H respectively, according to NMR analysis (Supplementary material) assuming that the reaction might even yield a higher percentage of enantioselectivity.

Second, we determined the effect of D_2O on the aldehyde reduction possibly affecting protein–protein interactions and proton transfer mechanisms. Exchanging the aqueous buffer with D_2O based potassium phosphate buffer decreased MBA reduction by about 60% in P450BM-3 F87A/T268A (Fig. 3) but, and most importantly, without introducing a deuterium into the alcohol product. This could indicate that either protein–protein, domain interactions or proton transfer mechanisms involved in the reduction of flavins [27] were slowed down. Consequently, a reduction via the FMN hydroquinone cannot be excluded, as the mechanism of a hydride transfer is identical for both flavins. Interestingly, the high catalytic activity for cytochrome c reduction in the P450BM-3 F87A T268A variant in comparison to the WT seem to correlate with a high activity of a hydride transfer, which could be explained by a possible interference of the flavin semiquinone/hydroquinone state during reduction. A compromised heme one electron reduction and substrate oxidation can be caused by NADPH, if a flavin reduction occurs prior to substrate addition [3]. A similar 'feedback' in the redox cascade might have occurred during the reduction and CO binding to the heme domain, favoring a hydride 'leaching' to an easily reducible substrate.

Third, we tested the involvement of the heme domain of P450BM-3 and reversibility of the aldehyde reduction. Reduction of heme can be measured by the saturation of heme with carbon monoxide and subsequent reduction via NADPH to form the soret at 450 nm for the absorption heme–CO complex. Using P450BM-3 F87A/T268A after saturation of the assay buffer with carbon monoxide, activity of MBA reduction dropped to 30% of initial activity (Fig. 3), indicating that complexing ferrous heme with CO somehow interferes with the hydride transfer. Interestingly, 300 μM imidazole did not inhibit the reduction possibly indicating the importance of the heme spin state and reducibility. Aldehyde reduction was not reversible, as the incubation of MBA with NADP did not produce detectable amounts of aldehyde or NADPH.

Fourth, we conducted ligand binding experiments to test for aldehyde binding to the heme. The change of spin state was very low in P450BM-3 F87A/T268A and P450BM-3 WT and MBA caused an almost irrelevant reverse type I spectra with an estimated K_D of 0.11 μM. Ligand binding for P450BM-3 F87A/T268A and P450BM-3 WT was similar for lauric acid (type I, K_D 230 μM and 190 μM, respectively) leading to a significant shift in low to high spin heme iron. No significant heme spin state shift upon substrate binding indicates essentially no MBA binding in the P450 domain. This observation was substantiated by a high K_m for MBA for all proteins tested which was interestingly also much higher compared to substrates such as cytochrome c not binding in the heme domain [28]. Nevertheless, saturable binding kinetics indicate an actual substrate binding site, presumably close to the NADPH binding site or flavins which is necessary for hydride transfer.

Besides the hydride transfer from NADPH, an alternative possible mechanism could be speculated similar to the morpholine reductase directly transferring a hydride from a piperazine moiety of the flavins hydroquinone ($FADH_2$ or $FMNH_2$ in an 'overreduced' P450BM-3 [5]) to a reducible carbonyl [29] (Fig. 4). Previous reports propose a mechanism involving a reduction of aldehydes such as hydroxy nonenal by CYP450-enzymes through a radical formation and single electron reduction of the radical intermediate via the iron(II) species [11,12]. As the BMR domain alone was able to perform the reduction of MBA and the almost complete hydride transfer from *pro-R*-NADPH demonstrated a direct reduction by this domain, the described reaction seems unrelated to the radical mechanism. An increase in activity through an additional radical mechanism using P450BM-3 WT and mutants consisting of both P450 and reductase domain might be excluded and could possibly be caused by protein–protein interactions or a proton coupled electron transfer as evidenced by decreased turnover in D_2O solvent which is suggestive of proton movement.

The reduction of aldehydes by P450BM-3 through a direct hydride transfer has not been described previously and speaks to the versatility of this enzyme in facilitating oxidations and reductions. Interestingly, this reduction seems to be augmented in the fused P450 domains through a potential protein domain interaction probably based on redox kinetics or allosteric interactions.

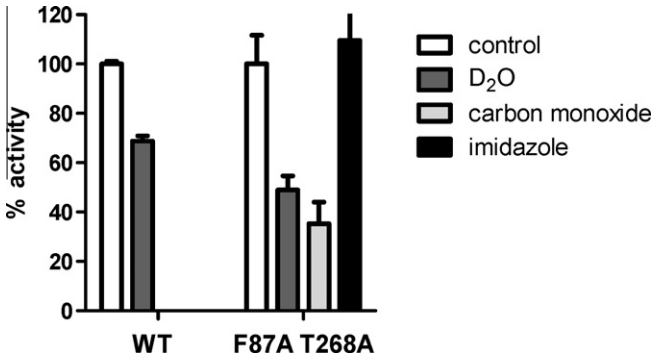

Fig. 3. Percentage activity of for *p*-methoxy benzaldehyde (MBA) reduction for CYP450BM-3 WT and CYP450BM-3 F87A/T268A using D_2O as a solvent and further testing inhibition by carbon monoxide and imidazole (control activity set to 100%, reaction performed at 100 μM MBA).

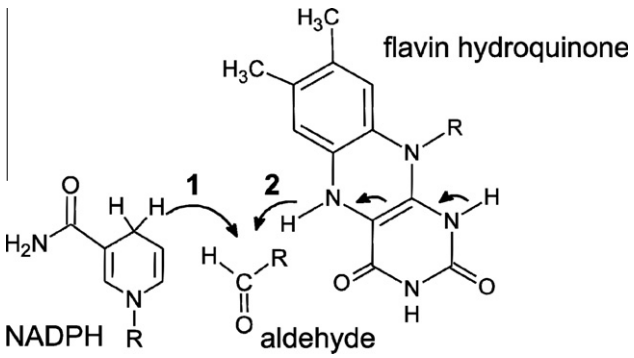

Fig. 4. Possible mechanism for the reduction of an aldehyde substrate by a hydride transfer from NADPH (1) or flavin hydroquinone (2).

Investigations are ongoing to determine, the importance of the heme domain and the two flavin domains for this reduction, and if this reaction has implication for other cytochrome P450 isozymes involved in the metabolism of xenobiotics or endogenous substrates.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.01.040.

References

[1] A.W. Munro, D.G. Leys, K.J. McLean, K.R. Marshall, T.W. Ost, S. Daff, C.S. Miles, S.K. Chapman, D.A. Lysek, C.C. Moser, C.C. Page, P.L. Dutton, P450 BM3: the very model of a modern flavocytochrome, Trends Biochem. Sci. 27 (2002) 250–257.
[2] M.A. Noble, C.S. Miles, S.K. Chapman, D.A. Lysek, A.C. MacKay, G.A. Reid, R.P. Hanzlik, A.W. Munro, Roles of key active-site residues in flavocytochrome P450 BM3, Biochem. J. 339 (Pt 2) (1999) 371–379.
[3] M.J.I. Paine, Electron transfer partners, in: P.R. Ortiz de Montellano (Ed.), Cytochrome P450: Structure, Mechanism, and Biochemistry, Springer, New York, 2006.
[4] H.M. Girvan, T.N. Waltham, R. Neeli, H.F. Collins, K.J. McLean, N.S. Scrutton, D. Leys, A.W. Munro, Flavocytochrome P450 BM3 and the origin of CYP102 fusion species, Biochem. Soc. Trans. 34 (2006) 1173–1177.
[5] C.J. Whitehouse, S.G. Bell, L.L. Wong, P450(BM3) (CYP102A1): connecting the dots, Chem. Soc. Rev. (2011).
[6] C.H. Yun, K.H. Kim, D.H. Kim, H.C. Jung, J.G. Pan, The bacterial P450 BM3: a prototype for a biocatalyst with human P450 activities, Trends Biotechnol. 25 (2007) 289–298.
[7] A.J. Warman, O. Roitel, R. Neeli, H.M. Girvan, H.E. Seward, S.A. Murray, K.J. McLean, M.G. Joyce, H. Toogood, R.A. Holt, D. Leys, N.S. Scrutton, A.W. Munro, Flavocytochrome P450 BM3: an update on structure and mechanism of a biotechnologically important enzyme, Biochem. Soc. Trans. 33 (2005) 747–753.
[8] E.S. Roberts, A.D. Vaz, M.J. Coon, Catalysis by cytochrome P-450 of an oxidative reaction in xenobiotic aldehyde metabolism: deformylation with olefin formation, Proc. Natl. Acad. Sci. USA 88 (1991) 8963–8966.
[9] O.A. Barski, S.M. Tipparaju, A. Bhatnagar, The aldo-keto reductase superfamily and its role in drug metabolism and detoxification, Drug Metab. Rev. 40 (2008) 553–624.
[10] C.S. Mazur, J.F. Kenneke, M.R. Goldsmith, C. Brown, Contrasting influence of NADPH and a NADPH-regenerating system on the metabolism of carbonyl-containing compounds in hepatic microsomes, Drug Metab. Dispos. 37 (2009) 1801–1805.
[11] I. Amunom, S. Srivastava, R.A. Prough, Aldehyde reduction by cytochrome P450, Curr. Protoc. Toxicol. (2011) Unit4 37. Chapter 4.
[12] I. Amunom, L.J. Dieter, V. Tamasi, J. Cai, D.J. Conklin, S. Srivastava, M.V. Martin, F.P. Guengerich, R.A. Prough, Cytochromes P450 catalyze the reduction of alpha, beta-unsaturated aldehydes, Chem. Res. Toxicol. 24 (2011) 1223–1230.
[13] G. Ottolina, S. Riva, G. Carrea, B. Danieli, A.F. Buckmann, Enzymatic synthesis of [4R-2H]NAD (P)H and [4S-2H]NAD(P)H and determination of the stereospecificity of 7 alpha- and 12 alpha hydroxysteroid dehydrogenase, Biochim. Biophys. Acta 998 (1989) 173–178.
[14] J.S. Miles, A.W. Munro, B.N. Rospendowski, W.E. Smith, J. McKnight, A.J. Thomson, Domains of the catalytically self-sufficient cytochrome P-450 BM-3. Genetic construction, overexpression, purification and spectroscopic characterization, Biochem. J. 288 (Pt 2) (1992) 503–509.
[15] A.W. Munro, S. Daff, J.R. Coggins, J.G. Lindsay, S.K. Chapman, Probing electron transfer in flavocytochrome P-450 BM3 and its component domains, Eur. J. Biochem. 239 (1996) 403–409.
[16] A.P. Jamakhandi, B.C. Jeffus, V.R. Dass, G.P. Miller, Thermal inactivation of the reductase domain of cytochrome P450 BM3, Arch. Biochem. Biophys. 439 (2005) 165–174.
[17] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[18] T. Omura, R. Sato, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature, J. Biol. Chem. 239 (1964) 2370–2378.
[19] T. Omura, R. Sato, The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties, J. Biol. Chem. 239 (1964) 2379–2385.
[20] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[21] D.H. Lang, C.K. Yeung, R.M. Peter, C. Ibarra, R. Gasser, K. Itagaki, R.M. Philpot, A.E. Rettie, Isoform specificity of trimethylamine N-oxygenation by human flavin-containing monooxygenase (FMO) and P450 enzymes: selective catalysis by FMO3, Biochem. Pharmacol. 56 (1998) 1005–1012.
[22] M.J. Cryle, J.J. De Voss, The role of the conserved threonine in P450 BM3 oxygen activation: substrate-determined hydroxylation activity of the Thr268Ala mutant, ChemBioChem 9 (2008) 261–266.
[23] I.G. Denisov, T.M. Makris, S.G. Sligar, I. Schlichting, Structure and chemistry of cytochrome P450, Chem. Rev. 105 (2005) 2253–2277.
[24] H. Hirao, D. Kumar, S. Shaik, On the identity and reactivity patterns of the "second oxidant" of the T252A mutant of cytochrome P450cam in the oxidation of 5-methylenenylcamphor, J. Inorg. Biochem. 100 (2006) 2054–2068.
[25] M. Sono, R. Perera, S. Jin, T.M. Makris, S.G. Sligar, T.A. Bryson, J.H. Dawson, The influence of substrate on the spectral properties of oxyferrous wild-type and T252A cytochrome P450-CAM, Arch. Biochem. Biophys. 436 (2005) 40–49.
[26] D.S. Sem, C.B. Kasper, Geometric relationship between the nicotinamide and isoalloxazine rings in NADPH-cytochrome P-450 oxidoreductase: implications for the classification of evolutionarily and functionally related flavoproteins, Biochemistry 31 (1992) 3391–3398.
[27] H.M. Girvan, A.J. Dunford, R. Neeli, I.S. Ekanem, T.N. Waltham, M.G. Joyce, D. Leys, R.A. Curtis, P. Williams, K. Fisher, M.W. Voice, A.W. Munro, Flavocytochrome P450 BM3 mutant W1046A is a NADH-dependent fatty acid hydroxylase: implications for the mechanism of electron transfer in the P450 BM3 dimer, Arch. Biochem. Biophys. 507 (2011) 75–85.
[28] A.L. Shen, C.B. Kasper, Differential contributions of NADPH-cytochrome P450 oxidoreductase FAD binding site residues to flavin binding and catalysis, J. Biol. Chem. 275 (2000) 41087–41091.
[29] M.J. Sutcliffe, L. Masgrau, A. Roujeinikova, L.O. Johannissen, P. Hothi, J. Basran, K.E. Ranaghan, A.J. Mulholland, D. Leys, N.S. Scrutton, Hydrogen tunnelling in enzyme-catalysed H-transfer reactions: flavoprotein and quinoprotein systems, Philos. Trans. Roy. Soc. Lond. B Biol. Sci. 361 (2006) 1375–1386.