Defluorination of 4-fluorophenol by Cytochrome P450BM3-F87G: Activation by long Chain Fatty Aldehydes.

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Abstract:

Cytochrome $P450_{BM3}$-F87G catalyzed the oxidative defluorination of 4-fluorophenol, followed by reduction of the resulting benzoquinone to hydroquinone via the NADPH P450-reductase activity of the enzyme. The k_{cat} and K_m for this reaction were 71 ± 5 min^{-1} and 9.5 ± 1.3 mM, respectively. Co-incubation of the reaction mixture with long chain aldehydes stimulated the defluorination reaction, with the 2,3-unsaturated aldehyde, 2-decenal producing a 12-fold increase in catalytic efficiency. At 150 μM aldehyde, k_{cat} increased to 158 ± 4, while K_m decreased to 1.8 ± 0.2. The effects of catalase, glutathione and ascorbate on the reaction were all consistent with a direct oxygen insertion mechanism, as opposed to a radical mechanism. The study demonstrates the potential use of $P450_{BM3}$ mutants in oxidative defluorination reactions, and characterizes the novel stimulatory action of straight chain aldehydes on this activity.

Keywords: aldehydes | cytochrome p450 | defluorination | fluorophenol | biotechnology

Article:

Introduction

The impact of halogenated phenolic compounds on the environment and human health represents a major concern as a result of their widespread industrial use and known chemical toxicities (Harvey et al. 2002; Meharg et al. 2000). The increased use of fluorinated compounds in large scale agrochemical and pharmaceutical production has resulted in considerable interest in the metabolism of these compounds (Key et al. 1997; Rietjens and Vervoort 1992). Aerobic

metabolism of halophenols by a variety of bacterial or fungal peroxidases has been studied, and a number of important chemical features of the process have been elucidated (Murphy 2007; Osman et al. 1997; Szatkowski et al. 2011). For example, the reaction involving dehaloperoxidase from Amphitrite ornata is thought to proceed via a free radical mechanism resulting from the sequential transfer of two electrons from the substrate to the activated ferro-heme intermediate. The radical nature of the reaction apparently leads to the formation of multiple products. Likewise, the thiol-heme ligated peroxidase, chloroperoxidase, has been examined with respect to its ability to metabolize several different fluorophenol substrates (Osborne et al. 2006). Nearly all of the products of this oxidative reaction can be rationalized in terms of a fluorophenoxy radical intermediate (Osborne et al. 2009).

Cytochrome P450 enzymes have been implicated in dehalogenation reactions as well. Ohe et al. (1997) described the rat liver microsome-catalyzed substitution of a variety of substituted phenol derivatives, including fluoro- and chlorophenol, and attributed this to the microsomal cytochrome P450 enzymes. Vatsis and Coon (2002) reported the defluorination of 4-fluorophenol by several purified mammalian cytochrome P450 enzymes, where the observed product was the hydroquinone. These authors concluded that the reaction may ultimately involve an electrophilic attack of the hydroperoxo-P450 intermediate on the aromatic ring system of the substrate, resulting in the initial formation of the benzoquinone product, which is rapidly reduced to the hydroquinone by the NADPH-cytochrome P450 reductase that is present in the reaction. We report here the rapid dehalogenation of 4-fluorophenol by a mutant form of a cytosolic bacterial P450 enzyme, cytochrome P450BM3-F87G (BM3-F87G), and the significant stimulation of this activity in the presence of long chain fatty aldehydes.

Materials and methods

Materials

BM3-F87G was expressed in E.coli and purified by DEAE and Q-sepharose chromatography according to published protocols (Raner et al. 2000). The concentration of enzyme was determined using the reduced vs reduced CO difference spectrum with $\varepsilon 450 = 96{,}000\ M^{-1}\ cm^{-1}$. All chemical reagents were purchased from Sigma-Aldrich unless otherwise specified.

Metabolism of halophenols

BM3-F87G was incubated in the presence of 4-fluorophenol or 4-chlorophenol (1–20 mM) and 1 mM NADPH in 100 mM potassium phosphate buffer (pH 7.4) for 5–60 s, in 200 μl. The reactions were quenched with the addition of 400 μl 3 % (w/v) perchloric acid and placed on ice

for 10 min. Samples were centrifuged to remove precipitated protein and 500 µl of the supernatant for each reaction was analysed by HPLC as described previously (Raner et al. 2002). Samples were injected in an initial mobile phase consisting of H2O/acetonitrile/trifluoroacetic acid (94.9:5:0.1, by vol) at 1.5 ml min−1. After 4 min, the mobile phase was switched to acetonitrile/trifluoroacetic acid (99.9:0.1, by vol.) for 4 min, followed by re-equilibration in the initial mobile phase for an additional 4 min. The column used was a 4.6 × 150 mm C18 RP column (5 µm pore size). Hydroquinone was detected at 290 nm, and typical retention times of ~2.5–3.5 min were observed. Standard hydroquinone samples were run at the end of each set of experiments as a control. When present, the aldehyde compounds were initially dissolved in water (300–1.4 mM stock solutions) immediately prior to their use. A hydroquinone standard curve was prepared from 1 to 40 µM to calculate reaction velocities.

Analysis of glutathione conjugates

Reactions including 4-fluorophenol (20 mM), BM3-F87G (0.86 µM) and NADPH (1 mM) were carried out in the presence of 2 mM glutathione in 1 ml, and the reaction products were immediately subjected to HPLC analysis as described above. Additional peaks in the chromatogram resulting from glutathione addition were isolated by collecting samples directly from the HPLC and the UV/Vis absorption spectra were recorded between 250 and 500 nm.

Other catalytic assays

The assays for p-nitrophenoxydodecanoic acid de-alkylase and p-nitrophenol hydroxylase have been described in detail elsewhere (Schwaneberg et al. 1999; Raner et al. 2002). In brief, reactions with p-nitrophenoxydodecanoic acid were carried out at pH 8.2 in the presence of 45 µM substrate and 1 mM NADPH in a 1 ml cuvette at 25 °C. The reaction was followed at 410 nm and reaction velocities were calculated using the ε410 of 17.5 mM−1 for the nitrophenol product. Oxidation of p-nitrophenol to 4-nitrocatechol was carried out in 100 mM potassium phosphate buffer (pH 7.0) using 6 mM p-nitrophenol, 1 mM NADPH and varying concentrations of 2-decenal (50–200 µM) in 200 µl. Reactions were quenched and analyzed by HPLC using the published method for 4-nitrocatechol analysis (Raner et al. 2002). The conversion of benzoquinone to hydroquine by BM3-F87G was demonstrated by incubating 50 mM benzoquinone with 0.50 µM F87G in 100 µM phosphate buffer, pH 7.4 and 1 mM NADPH for 5–30 s at 37 °C. Benzoquinone reduction was monitored using HPLC as described.

Results and discussion

Purified BM3-F87G catalyzed the NADPH-dependent dehalogenation of 4-fluorophenol and 4-chlorophenol to give the product hydroquinone, presumably according to the reaction shown in Fig. 1. Although no benzoquinone product was detected, exogenously added benzoquinone was reduced to the hydroquinone in the presence of the cytochrome BM3-F87G and NADPH (data not shown). The reactions were linear with enzyme concentration and time (up to 20 s) and NADPH concentrations above 1 mM did not influence the reaction rate. Both reactions followed Michaelis–Menten kinetics and the observed K m and k cat were 9.5 ± 1.3 mM, and 71 ± 5 min−1, respectively for 4-fluorophenol and 4 ± 1 mM and 11.3 ± 3 min−1 for chlorophenol. These k cat values were higher than those observed for either rabbit P450 2E1 or 2B4 reported by Vatsis and Coon (2002), consistent with the generally high rate of catalysis associated with P450BM3, compared with the microsomal P450 s. Uncoupling of NADPH oxidation from product formation in P450 catalysis can lead to elevated levels of H2O2 and other reactive oxygen species, therefore to assess the possible role these species may have in generating the hydroquinone product, several control experiments were carried out. The inclusion of catalase (an enzyme that removes H2O2 via disproportionation to H2O and O2) in the reaction had very little effect on the observed hydroquinone formation (Table 1), suggesting that uncoupled H2O2 formation was not responsible for hydroquinone production. The presence of 2 mM ascorbate (a radical scavenger) had a significant effect on the reaction, yielding an 80 % increase in product formation (Table 1). The source of this increased activity is unknown; however we suggest that it may result from protection of the enzyme from free radical-mediated inactivation or enhanced reduction of the benzoquinone formed in the reaction to the product hydroquinone. Studies using horseradish peroxidase and microperoxidase demonstrated that ascorbate had the exact opposite effect on the defluorination reaction catalyzed by these enzymes, presumably via reduction of the 1-electron oxidized phenoxy radical generated in the reaction back to the starting material (Osman et al. 1997). Our data clearly demonstrates that a free phenoxy radical is not released from the active site in the reaction involving BM3-F87G. This does not preclude its formation during catalysis, as it may simply mean ascorbate does not have access to such an intermediate, if it forms, in the P450 active site. It is, however, a distinguishing feature of a P450-type oxidation as opposed to a peroxidase-type reaction.

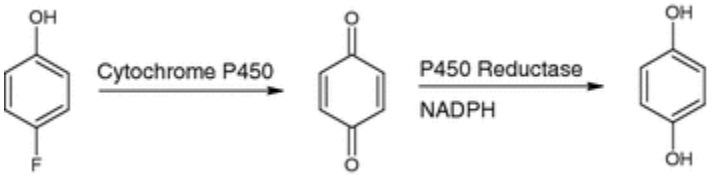

Fig. 1

Reactions catalyzed by BM3-F87G in the presence of 4-fluorophenol and NADPH

Table 1

Activity data for the BM3-F87G-catalyzed defluorination of 4-fluorophenol in the presence of 2-decenal, catalase, ascorbate and glutathione

Reaction	**Turnover number (min^{-1})**
Control	63.5 ± 7
+Catalase (100 U)	45 ± 4
+Ascorbate (2 mM)	109 ± 29
+GSH (2 mM)	9.0 ± 2.8
Decenal (75 μM)	118 ± 7
Decenal (75 μM) + Catalase (100 U)	89 ± 5
Decenal (75 μM) + Ascorbate (2 mM)	204 ± 19
Decenal (75 μM) + GSH (2 mM)	8.8 ± 3

Turnover numbers are based on three independent trials and standard deviations are reported

Interestingly, the inclusion of glutathione (an alternative radical scavenger) almost completely eliminated the hydroquinone product (Table 1). In these reactions, an additional product peak was observed when glutathione was present (Fig. 2). This new product peak appeared at 4.4 min in the chromatogram, while the hydroquinone peak at 2.5 min was dramatically reduced. Lau et al. (1988) reported that glutathione would react directly with benzoquinone to form multiple glutathione conjugates that could be separated by HPLC, and characterized by their UV/Vis absorption spectrum. We therefore isolated the unknown product peak generated in the presence of glutathione by semi-preparative HPLC, and subjected it to UV/Vis spectrophotometric analysis (Fig. 2; inset). Based on the chromatographic behavior and measured λmax at ~305 nm, we suggest that the 2-(glutathion-S-yl) hydroquinone conjugate was the primary product in the reaction. This result indicates the effect of glutathione was not to inhibit the formation of benzoquinone; rather it simply sequestered the benzoquinone from subsequent reduction to the hydroquinone by forming the glutathione conjugate. The Michaelis–Menten behavior of the reaction, along with the behavior of the reaction in the presence of glutathione, catalase and ascorbic acid, strongly suggest that the reaction proceeds via the well-characterized cytochrome

P450 oxygenation-type reaction, followed by NADPH-supported reduction of the benzoquinone product to the hydroquinone.

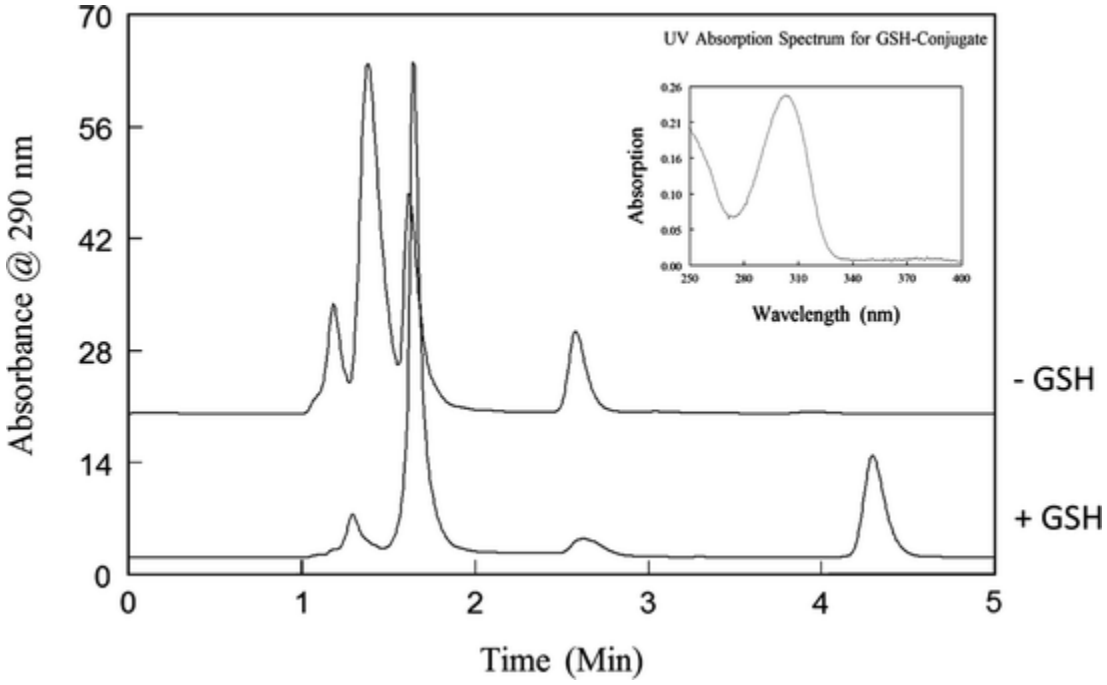

Fig. 2

HPLC chromatogram for BM3-F87G-catalyzed defluorination of 4-fluorophenol in the absence (A) and presence (B) of 2.0 mM glutathione. The additional peak at 4.4 min in chromatogram (A) was isolated and the absorption spectrum was recorded between 250 and 400 nm and is shown in the inset

Long-chain aliphatic aldehydes were initially included in the reaction mixture as potential inhibitors of the BM3-F87G activity, in a further attempt to establish that the 4-fluorophenol was reacting in the active site. Contrary to our expectations, these aldehydes produced a stimulatory effect on the defluorination that was dependent on aldehyde concentration and aldehyde structure. For example, straight chain saturated aldehydes showed increased stimulatory activity with increasing chain length, up to an optimal chain length of 10-carbons (Fig. 3). At 150 μM, pentanal and hexanal had no effect on the rate of hydroquinone formation by BM3-F87G. For decanal, the same aldehyde concentration produced a three-fold increase in the activity of the enzyme. Aldehydes with longer chain lengths than 10 still produced a stimulatory effect, however further increases in stimulation were not observed, possibly due to solubility issues related to these compounds. Control experiments in which NADPH was not included in the incubation mixture were also carried out, and no product was observed, demonstrating that the enhanced activity was enzyme turnover dependent, and not a direct chemical effect of the aldehyde on the 4-fluorophenol substrate.

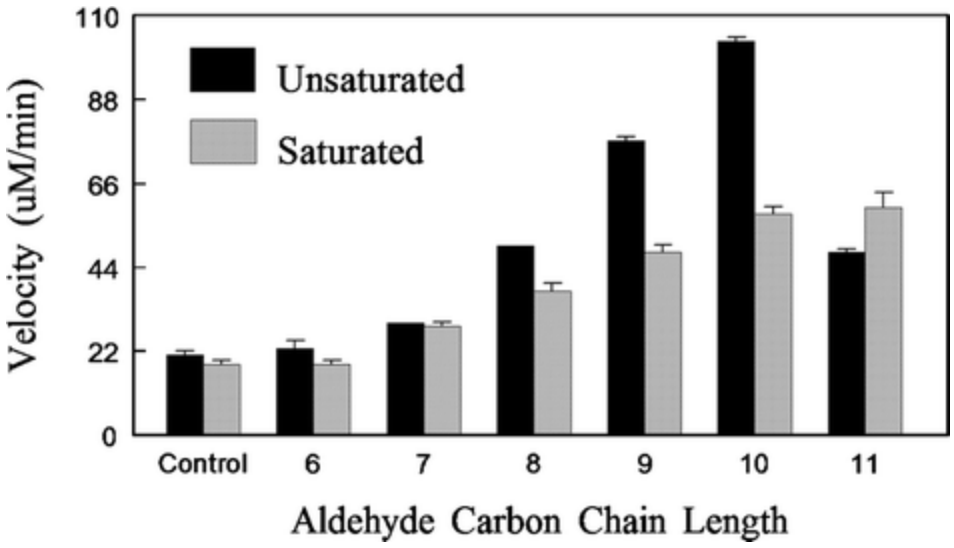

Fig. 3

Bar graph indicating the activity of BM3-F87G in the defluorination of 4-fluorophenol in the presence of saturated and 2,3-unsaturated fatty aldehydes of increasing chain length. All aldehydes were present at 25 μg ml^{-1}, and reactions were carried out for 20 s at 37 °C. All reactions were carried out in duplicate, and the values reported represent the average of the two independent trials

To assess the structural features of the aldehyde contributing to this activation effect, a number of different aldehyde derivatives were examined with regard to their effects on hydroquinone formation. Introduction of a double bond in the 2,3-position of the aldehyde further stimulated the activity of the enzyme, and again the overall effect was dependent on carbon chain length. As indicated in Fig. 3, activity was increased by a factor of 5 when 2-decenal at 150 μM was included in the reaction mixture, whereas 2-pentenal and 2-hexenal yielded no such induction. Where induction was observed, equivalent molar concentrations of the 2,3-unsaturated aldehyde had a greater stimulatory effect than its saturated counterpart, identifying the 2,3-unsaturation as a structural feature influencing induction. The one exception was 2-undecenal, where a reduction in the stimulatory effect was observed upon increasing the chain length from 10 to 11. This may result from steric effects in the active site associated with the additional carbon atom. The effect of the aldehyde structure was further examined using a series of aromatic and branched aldehydes as inducers (Fig. 4). Only straight chain aldehydes appeared to enhance the defluorination reaction, owing, most likely, to steric effects within the BM3 active site. This observation suggests that stimulation requires simultaneous aldehyde and substrate binding.

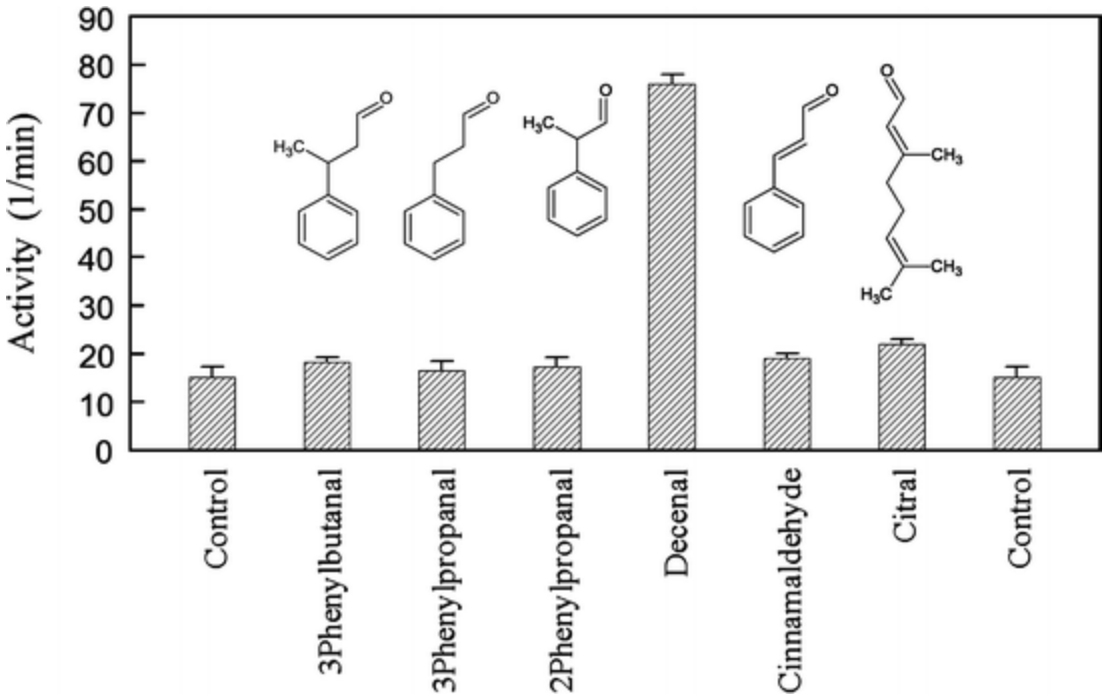

Fig. 4

Bar graph showing the effects of different aromatic aldehyde on the rate of defluorination of 4-fluorophenol, catalyzed by P450BM3-F87G. All aldehydes were present at 100 μM final concentration in the reaction, and the substrate concentration was 12 mM. Reactions were carried out in duplicate, and the values reported represent the average of the two independent trials

To ensure that the aldehyde was not simply promoting the uncoupling of the BM3 enzyme, thereby generating reactive oxygen species capable of initiating a free radical mechanism leading to benzoquinone formation, several control experiments were carried out. The effects of glutathione, catalase, and ascorbic acid in the presence of 2-decenal were examined and the general effects were found to be identical to those observed in the absence of 2-decenal (Table 1). In other words, catalase resulted almost no change in activity, ascorbic acid appeared to enhance activity, yielding an 80 % increase, and glutathione almost completely eliminated the hydroquinone as a product, generating in its place, what appeared to be the 2-(glutathion-S-yl)-hydroquinone at an enhanced rate compared to controls. These results indicate that the mechanism associated with BM3-catalyzed defluorination does not appear to be altered by the presence of the aldehyde, rather the aldehyde simply improves the efficiency of the enzyme in catalysis.

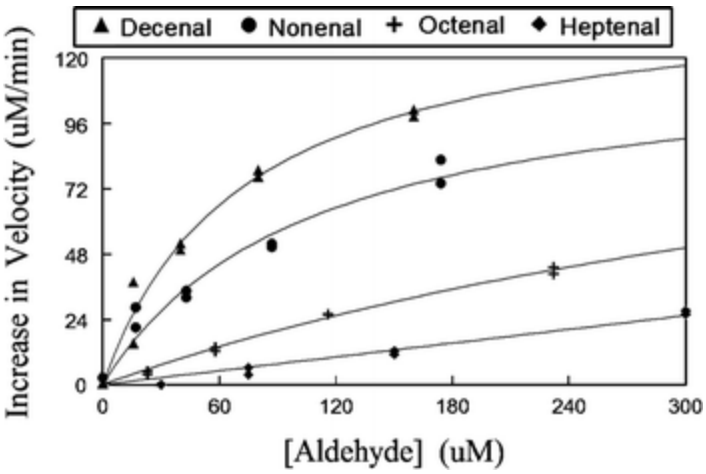

Fig. 5

Graph showing the increase in catalytic activity of the BM3-F87G toward 4-fluorophenol as a function of increasing concentrations of different 2,3-unsaturated aldehydes. All increases were measured relative to the activity in the absence of added aldehyde. A constant substrate concentration of 12 mM was used throughout. All experiments were carried out in duplicate

We examined the saturation behavior of the activation for 2,3-unsaturated aldehydes containing 7–10 carbon atoms by plotting the increase in velocity as a function of aldehyde concentration (Fig. 5). The data was then fit to the equation for a rectangular hyperbola (Eq. [1]). In this equation, ΔV is the increase in velocity in the presence of the aldehyde, ΔV max is the apparent maximum change in velocity, K d act is the apparent dissociation constant for activation,

$$\Updelta V = \frac{\Updelta V_{\max} \times [A]}{K_{\mathrm{d}}^{\mathrm{act}} + [A]}$$

and [*A*] is the concentration of aldehyde. The data is consistent with a simple binding equilibrium, in which binding of the aldehyde stimulated activity of the enzyme. Values for $K_{\mathrm{d}}^{\mathrm{act}}$ were calculated and compared for each aldehydes, and as expected, 2-decenal had the lowest $K_{\mathrm{d}}^{\mathrm{act}}$ at 72 ± 15 μM, followed by 2-nonenal, 2-ocetenal and 2-heptenal, with $K_{\mathrm{d}}^{\mathrm{act}}$ values of 143 ± 30, 563 ± 200 and >1,500 μM, respectively. This suggests more efficient binding for the longer chain aldehydes. Since 2-decenal appeared to be the most potent activator, the effects of varying concentrations on the Michaelis–Menten parameters V_{max} and K_{m} for 4-fluorophenol were evaluated (Supplementary Fig. 1). Increasing concentrations of 2-decenal had a significant effect on the observed catalytic efficiency ($K_{\mathrm{cat}}/K_{\mathrm{m}}$) for the reaction, which was relatively linear over the range of aldehyde concentrations used (Fig. 6). A 12-fold increase in catalytic efficiency between 0 and 150 μM 2-decenal was observed. The effect on K_{cat} and K_{m} individually was more complex. Interestingly, the K_{m} for 4-fluorophenol decreased from 9.5 ± 1 to 1.8 ± 0.2 mM over the range of 0–75 μM 2-decenal,

while K_{cat} increased only from 71 ± 4 to 92 ± 2 min^{-1}. Increasing the 2-decenal concentration from 75 to 150 μM caused no further decrease in K_m, however K_{cat} increased from 92 ± 2 to 158 ± 4 min^{-1} over this range of concentrations. The significance of this complex activation behavior in terms of the mechanism is currently not understood, however it may suggest direct chemical interactions occur between substrate and aldehyde during catalysis.

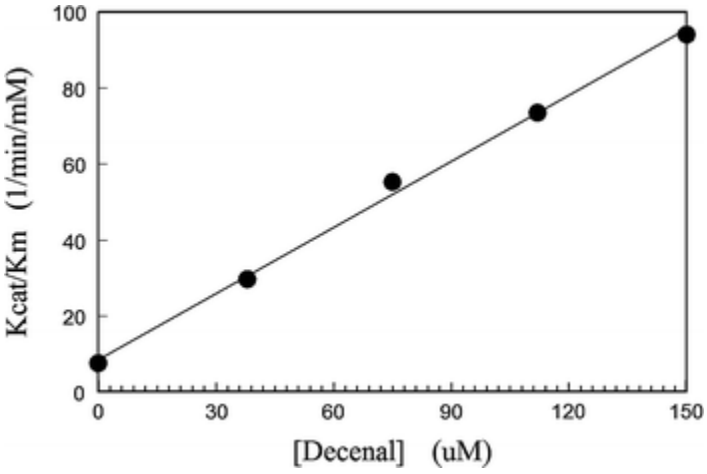

Fig. 6

Secondary plot showing the effect of increasing aldehyde concentration on catalytic efficiency (K cat/K m) for the reaction

Finally, the effect of the aldehydes on other reactions catalyzed by BM3-F87G was probed using the substrates p-nitrophenol and p-nitrophenoxydodecanoic acid. Prior studies have shown that both of these substrates can be oxidized by the BM3-F87G enzyme (Raner et al. 2002; Schwaneberg et al. 1999). In contrast to the defluorination of 4-fluorophenol, the effect of 2-decenal on the dealkylation of the substrate p-phenoxydodecanoic acid was to inhibit activity by ~80 %. The observed k cat in the absence of 2-decenal was 102 ± 2 min−1, whereas inclusion of 150 μM 2-decenal resulted in a k cat of 21 ± 5 min−1. This inhibition is most likely steric in nature, since the p-nitrophenoxydodecanoic acid would be expected to occupy a large portion of the active site that could otherwise accommodate the long chain fatty aldehyde. The oxidation of p-nitrophenol, on the other hand, was stimulated by 2-decenal, in much the same way as the defluorination reaction (data not shown). Saturation was observed with increasing aldehyde concentration, yielding a K d act of 350 ± 45 μM, which was considerably higher than that observed for 4-fluorophenol activation. The larger size of the nitro substituent in the p-position of the substrate may result in more steric interference in the active site, lowering the efficiency of aldehyde binding.

In summary, we have shown that BM3-F87G catalyzes the efficient defluorination of 4-fluorophenol and that the activity can be stimulated by the inclusion of long chain aldehydes. Activation increases with carbon chain length and 2,3-unsaturation in the aldehyde. Activation

involves effects on both K m and K cat for the 4-fluorophenol substrate, and oxidation of other p-substituted phenolic substrates can be stimulated as well. The high catalytic activity associated with the BM3 enzyme and the ability to significantly stimulate that activity makes this an ideal system to explore for potential applications in bioremediation involving fluorinated phenolic compounds.

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