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Cloning, Expression and Purification of Cindoxin, an Unusual Fmn-Containing Cytochrome P450 Redox Partner

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Cytochromes P450 (P450s) belong to a superfamily of haemoproteins that catalyse a remarkable variety of oxidative transformations. P450 catalysis generally requires that cognate redox proteins transfer electrons, derived ultimately from NAD(P)H, to the P450 for oxygen activation. $P450_{cin}$ (CYP176A1) is a bacterial P450 that is postulated to allow *Citrobacter braakii* to live on cineole as its sole carbon source by initiating cineole biodegradation. Here we report the cloning, expression, purification and characterisation of one of its postulated redox partners, cindoxin (Cdx), which has strong similarity to the FMN domain of cytochrome P450 reductase. Cindoxin reductase (CdR), which displays strong similarity to NADPH-dependent ferredoxin reductases, was unable to be expressed in a functional form. Mass spectrometric and HPLC analyses confirmed that the flavin cofactor of cindoxin was FMN. Redox potentiometric titrations were performed with cindoxin within the range $6 < pH < 8$; this enabled the quinone/semiquinone (E_1) and semiquinone/hydroquinone (E_2) redox potentials to be determined. Our results show that cindoxin might be somewhat different to other flavodoxins that interact with P450s, in which generally only one couple is important. Both redox states of cindoxin could be catalytically relevant. A catalytically active system was reconstituted in vitro with *E. coli* flavodoxin reductase (Fpr) acting as the terminal redox partner in the absence of CdR. Our results show that Cdx and Fpr support regio- and stereoselective $P450_{cin}$-catalysed cineole oxidation to (1*R*)-6β-hydroxycineole with turnover rates up to 1500 min^{-1}. This system is tightly coupled with 80% of NADPH reducing equivalents funnelled into substrate oxidation.

Introduction

The cytochromes P450 (P450s) are a superfamily of oxidative enzymes that catalyse an array of chemically impressive oxidative transformations. Their obligate heme cofactor enables them to activate molecular oxygen in order to catalyse oxidations. The reactions catalysed by P450s range from simple epoxidation and heteroatom oxidation to oxygen insertion into unactivated C–H bonds.[1] This latter reaction is considered the archetypal P450 reaction and is largely responsible for the range of biological functions associated with these enzymes ([Eq. (1)]).

$$RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+ \quad (1)$$

Such a hydroxylation reaction is carried out by the human P450s responsible for Phase I metabolism of xenobiotics, in which the molecule is at once made more hydrophilic and amenable to attachment of further polar moieties to aid excretion. Hydroxylation reactions also provide an activation point in the molecule to aid further transformation. This might be mediated by the P450 itself as seen in the biosynthesis of a variety of steroid hormones in which the hydroxylation is a prelude to further P450-mediated transformations that culminate in C–C bond cleavage.[1,2] Alternatively, the OH moiety might provide the focal point for the elaboration of a biodegradative pathway in which an essentially hydrocarbon substrate is utilised by a microorganism as a source of carbon and energy.

$P450_{cin}$ (CYP176A1), previously reported by us,[3] is shown here to be a P450 that initiates such a biodegradative cascade of reactions enabling *Citrobacter braakii* to live on cineole as its sole source of carbon and energy. Cineole (Scheme 1: **1**) is a monoterpene found in the essential oil of eucalypt trees and a relatively large amount is known about the chemistry of its biodegradation. A biodegradation pathway was proposed for cineole by Trudgill et al. based upon metabolites isolated from soil microbes.[4,5] It is thought that cineole is initially oxidised to a 6-hydroxycineole (Scheme 1) by a monooxygenase followed by further transformations to yield a highly functionalised hydroxyketo acid that can be incorporated into central metabolism. $P450_{cin}$ was tentatively postulated to catalyse the hydroxylation of cineole to a 6-hydroxycineole using *E. coli*'s endogenous flavodoxin/flavodoxin reductase system during initial overexpression studies and represents the first enzyme involved in cineole metabolism to be characterised.[3]

$P450_{cin}$ displays many of the characteristics associated with bacterial P450s: conversion from low to high spin in the presence of substrate and tight binding of the natural substrate, cineole. $P450_{cin}$ contains most of the conserved residues found in P450s with one notable difference: it lacks the almost uni-

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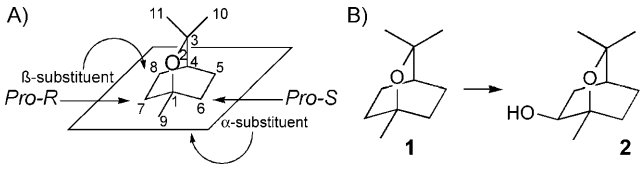

Scheme 1. A) The nomenclature of the hydroxycineoles is complex and there is no consistency in the literature for naming such compounds. To enable discussion of stereochemistry, we use the descriptors α and β. The α descriptor describes substituents below the plane passing through C5, C6, C7 and C8, while β describes substituents above this plane. Additionally, cineole is an achiral, meso compound and hydroxylation at either of the carbons adjacent to the C1 bridgehead leads to enantiomeric products and the creation of three new stereogenic centres at C1, C4 and C6. Therefore, we have defined the carbon atom that upon oxidation leads to the (*R*)-C1 isomer as the pro-*R* carbon and the carbon atom that leads to the (*S*)-C1 isomer as the pro-*S* carbon. B) Hydroxylation of cineole to (1*R*)-6β-hydroxycineole.

versally conserved threonine residue thought to be involved in dioxygen activation and instead has an asparagine at this position.[3] Mutagenic and crystallographic studies have suggested that this asparagine is not a functional replacement for threonine but rather, holds the substrate in place by donating a hydrogen bond to the ethereal oxygen of cineole.[6,7]

Another notable difference between $P450_{cin}$ and other bacterial biodegradative P450s, such as $P450_{cam}$ (CYP101A1), that was apparent upon sequencing of the CIN operon was that $P450_{cin}$ appeared to utilise a novel redox system for electron transfer. Historically, P450s were grouped into two major classes depending on the type of redox proteins they utilised. Electron transfer to bacterial and mitochondrial P450s from a pyridine nucleotide is usually mediated by two proteins, an FAD-containing ferredoxin reductase and an iron-sulfur protein, a so-called class I arrangement. In eukaryotes, however, electron transfer is generally mediated by a single flavoprotein that contains both an FAD and FMN domain, in a class II arrangement. However, as more P450 systems are isolated and characterised more redox systems are found that do not fit into this classical arrangement. There has recently been estimated to be ten different P450 classes although most of these are represented by single systems.[8] Analysis of the CIN operon reveals two open reading frames that encode potential redox partners for $P450_{cin}$. The first encodes *cinB*, apparently a 49 kDa, 452 aa protein that has strong similarity[3] with bovine adrenodoxin reductase (31% identity; 47% similarity), the FAD-containing ferredoxin reductase found in the class I adrenal mitochondrial system. The second (*cinC*) encodes a 16 kDa, 155 aa protein has strong similarity[3] with the FMN domain of cytochrome P450 reductase (CPR; 37% identity; 56% similarity), a class II reductase. The similarity of cindoxin (Cdx) to the FMN domain of CPR indicated its possible biological function. Although the arrangement of the P450 and reductase genes on the operon are identical to that found in class I bacterial systems,[9,10] the identity of the redox proteins is more like that found in a class II arrangement;[11] this results in the proposal that $P450_{cin}$ has an unusual, natural redox system intermediate between a class I and class II system.

We thus set out to isolate and characterise the redox proteins associated with the $P450_{cin}$ monooxygenase system involved in the biodegradation of cineole by *C. braakii*. We report here the cloning, overexpression and purification of an FMN-containing flavodoxin, trivially named Cdx, preliminary investigation of its putative reductase partner (Cdx reductase) and characterisation of the interaction of Cdx with $P450_{cin}$ in a reconstituted, catalytically active system. Concomitant with our work, an account of some of the redox chemistry of Cdx has been published by others[12] with material obtained from us. However, complete characterisation of the protein and its interaction with $P450_{cin}$ in a catalytic system has not been previously reported.

Results and Discussion

Expression, purification and isolation of Cdx

The *cinB* (Cdx reductase) and *cinC* (Cdx) genes were cloned into the expression vector pCW using a PCR-based protocol directly analogous to that described for $P450_{cin}$.[3] Expression of the *cinB* gene has so far proved impossible under the conditions evaluated. A wide range of expression conditions was tested including varying the expression temperature (22 to 30 °C), expression media and the use of chaperone systems, both specifically expressed and induced. The *cinB* gene displays strong similarity (47% similarity) to mitochondrial adrenodoxin reductase, the expression of which is greatly improved by the addition of the GroEL and GroES chaperones in *E. coli*.[13] Expression of pCW-CdR in 10% ethanol, which induces chaperone expression in *E. coli*,[14] was unsuccessful. Direct co-overexpression of the construct pGRO12, which encodes the GroEL and GroES genes, with pCW-CdR again yielded only inactive, unfolded protein that was observed in the pellet from cell lysates.

Expression of the *cinC* gene, however, was successful under conditions similar to those described for $P450_{cin}$.[3] As with $P450_{cin}$, expression of Cdx was very temperature sensitive and folded protein was not observed at temperatures above 30 °C. Purification of Cdx was achieved in two-steps involving anion exchange chromatography (Q Sepharose) followed by size-exclusion chromatography (G-75). This yielded ~500–2000 nmol of homogeneous protein (SDS-PAGE) per litre of original culture with an absorption ratio A_{456}/A_{280} of 0.67; this indicated that the Cdx is >95% pure based on the calculated molar extinction coefficient.

Confirmation that the purified protein was in fact the gene product of *cinC* was obtained by electrospray mass spectrometry which revealed a peak consistent with the calculated molecular weight (obs: 15966; calcd: 15964 Da) for the complete apo-protein retaining the N-terminal methionine (Figure 1). The second most abundant species (obs: 16419; calcd: 16419 Da) corresponds well to addition of a flavin mononucleotide cofactor (FMN, 456.34 Da). FMN was subsequently confirmed as the flavin cofactor by HPLC (vide infra). Presumably this species is the major species in vivo but the ionisation conditions of the mass spectrometer promotes loss of FMN. Finally,

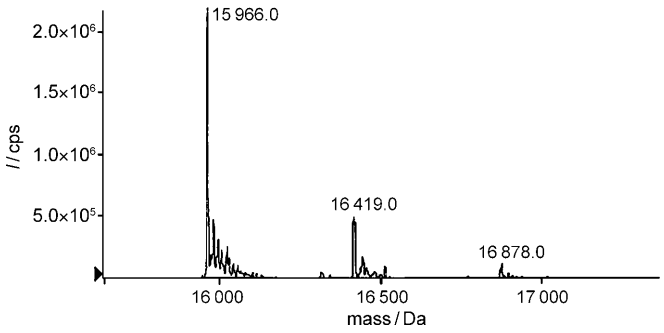

Figure 1. Electrospray mass spectrum of purified cindoxin (approximately 100 μM cindoxin dialysed against 5 mM ammonium acetate buffer). The most abundant species (15 966 Da) is consistent with the complete unprocessed apoprotein; the next (16 419 Da) is consistent with the holoprotein containing FMN; the least abundant species (16 878 Da) is consistent with the addition of a second FMN to cindoxin and is likely to be an artifact.

a very low abundance species (16 878 Da) was observed corresponding to a second addition of FMN to Cdx. This is more than likely an artifact in which free FMN is nonspecifically attached to Cdx during analysis given its sequence similarity to flavodoxins containing a single FMN[3] and our redox potentiometry results, which indicate the presence of only one set of redox couples.

Optical spectroscopy and redox potentiometry

The UV–visible absorption spectra of Cdx displayed the typical signature of a flavin-containing enzyme with absorbance maxima at 375, 456 and 480 nm (Figure 2-A). The protein stabilises a single electron reduced semiquinone form that exhibits a characteristic broad visible maximum around 600 nm (Figure 2-B), while the 456 nm maximum decreases to about half the intensity of the quinone form. The second electron reduction leads to the hydroquinone form that exhibits a weak absorption maximum around 420 nm (Figure 2-C).

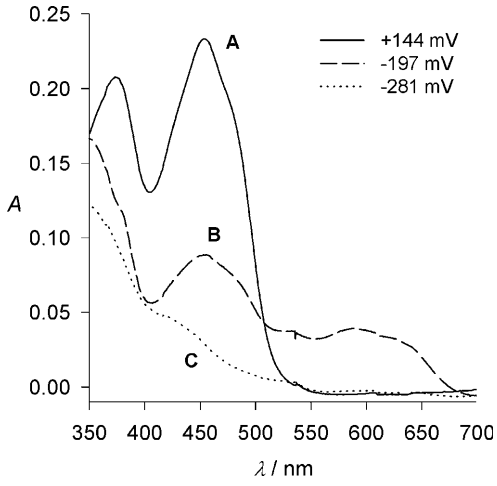

Figure 2. UV–visible spectra of cindoxin at pH 6.5 in its three oxidation states at the potentials shown (inset): A) fully oxidised quinone; B) semiquinone (single-electron reduced) form; and C) fully reduced hydroquinone.

Redox potentiometric titrations were performed with Cdx within the range $6 < pH < 8$ and this enabled the quinone/semiquinone (E_1) and semiquinone/hydroquinone (E_2) redox potentials to be determined. An example of the titration performed at pH 7.5 is shown in Figure 3, while all titration curves are given in the Supporting Information. The spectra were unaffected by pH within this range but a significant pH dependence of the quinone/semiquinone (E_1) redox couple was found (Table 1). This is indicative of proton coupled reduction of the quinone form, while the pH independence of E_2 indicates that no proton transfer occurs during the second electron transfer step. The pH dependence of a one electron/one proton transfer should be −59 mV per pH unit and the pK_a of the semiquinone N atom is typically too large to be determined that is, it is protonated at all pH values while the N atom is not protonated in the quinone form.[15] The variation of the quinone/semiquinone redox potential with pH is not as large (in magnitude) as this but the confidence limits of the determined potentials need to be considered and the data are not inconsistent with the expected $1e^-/1H^+$ transfer reaction within the uncertainties of the measurements.

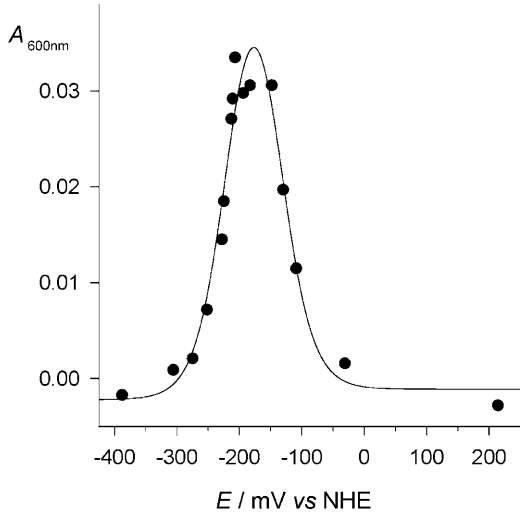

Figure 3. Potentiometric redox titration of cindoxin at pH 7.5. The data points represent the semiquinone absorption at each potential. The data were fit to Equation (2) (see the Experimental Section), and the fit is shown by the solid line (E_1 (Q/SQ) = 137(±9); E_2 (SQ/HQ) = −218(±9) mV vs. NHE See Table 1).

Our potentiometry results (Table 1) are in reasonable agreement with those reported by others for Cdx at pH 7.4 (E_1 =

Table 1. Redox potentials of cindoxin [mV vs. NHE] within the range $6 < pH < 8$ determined by redox potentiometry. The uncertainties represent the standard deviations from the experimental data fit to Equation (1).

pH	E_1 (quinone/semiquinone)	E_2 (semiquinone/hydroquinone)
6.0	−124(±8)	−188(±8)
6.5	−148(±11)	−224(±14)
7.0	−157(±6)	−222(±5)
7.5	−137(±9)	−218(±9)
8.0	−182(±10)	−222(±12)

-93 ± 17 mV; $E_2 = -226 \pm 5$ mV),[12] although no pH dependence was recorded. Clearly Cdx is quite different to other bacterial flavodoxins shown to interact with P450s,[16,17] in which the E_2 couple (hydroquinone/semiquinone) is so low that it is unlikely to be catalytically relevant. Interestingly, in the FMN domain of cytochrome P450 reductase, a quite different situation exists. Here, the semiquinone/hydroquinone couple is much higher making both accessible under physiological conditions ($E_1 = -43$ mV; $E_2 = -280$ mV).[18] Furthermore the FMN semiquinone is air stable and is thought to be incapable of participating in catalysis. Although significantly different redox potentials for substrate free $P450_{cin}$ have been reported,[12,19] values for substrate bound $P450_{cin}$ are more consistent (-182 mV[19] and -202 mV[12]). This value and those of Cdx might suggest that both redox states of Cdx could be catalytically relevant.

Cofactor analysis

Flavoproteins can incorporate either a flavin mononucleotide (FMN) cofactor or a flavin adenine dinucleotide (FAD) cofactor. Determination of the flavin cofactor used in Cdx was achieved by comparison with flavin standards separated by HPLC using the conditions of Brock et al.[20] The retention times of authentic FMN and FAD standards (15.2 min and 14.2 min, respectively) were compared to the retention time of the flavin moiety released from Cdx upon boiling (15.2 min). The retention time of the flavin cofactor identified it as FMN. The molar extinction coefficient of Cdx was determined to be $\varepsilon_{456} = 10\,800\ \text{M}^{-1}\,\text{cm}^{-1}$ by quantifying the amount of free flavin (using $\varepsilon_{456} = 12\,500\ \text{M}^{-1}\,\text{cm}^{-1}$)[21] extracted from a sample of holoprotein.[12]

Reconstituting $P450_{cin}$ catalysis

To reconstitute catalytic activity in vitro, $P450_{cin}$ required a suitable redox system to mediate the transfer of electrons from NADPH. Unfortunately, recombinant Cdx reductase from *cinB* was not available and without a suitable terminal redox partner, reconstitution of a catalytically active system is not possible. However, Waterman and Jenkins had reported that the endogenous flavodoxin/flavodoxin reductase system in *E. coli* is capable of supporting the activity of a variety of heterologously expressed P450s.[22] When $P450_{cin}$ was expressed in *E. coli* in the presence of cineole, a product was formed that was tentatively identified as 6-hydroxycineole (Scheme 1). This was attributed to the native *E. coli* reductase system mimicking the natural redox partners of $P450_{cin}$.[3] As a prelude to reconstitution experiments in vitro, we investigated electron transfer from *E. coli* NADPH-ferredoxin (flavodoxin) reductase (Fpr) to Cdx; these experiments were hoped to provide a means to achieving a catalytically active system for $P450_{cin}$. The reported redox values for Fpr ($E_1 = -308$ mV; $E_2 = -268$ mV)[17] suggested that it would be capable of a two electron reduction of Cdx. Electron transfer was monitored by UV–visible spectroscopy, with comparisons made at λ 370, 456 and 480 nm. The flavin of Cdx was shown to be fully reduced by the chemical reductant sodium dithionite but not by NADPH alone. This is not surprising because Cdx lacks an NADPH-binding domain. In the presence of NADPH and Fpr however, excess Cdx was shown to be reduced, unequivocally demonstrating electron transfer from Fpr to Cdx. Reconstitution of P450 activity, however, requires that electrons are delivered to the heme iron by the cognate redox proteins. This can be monitored by the addition of carbon monoxide to the ferrous P450, which yields a stable complex with a characteristic absorbance at 450 nm, and is a diagnostic test for the presence of P450 in a sample. The ability of the Fpr/Cdx redox couple to transfer electrons to $P450_{cin}$ was demonstrated by the production of a CO spectrum with a characteristic absorbance at 450 nm. In the absence of Cdx, a CO spectrum was not observed demonstrating that electrons are transferred in sequence from NADPH to Fpr to Cdx and finally to $P450_{cin}$.

Partial reduction of Cdx was performed to investigate electron transfer between Fpr and Cdx. The two proteins were mixed in a 1:10 (Fpr/Cdx) ratio, and this enabled the Cdx chromophore to be easily monitored without any substantial masking by Fpr. NADPH was added to provide enough electrons to reduce approximately half the Cdx to the hydroquinone or all of the Cdx to the semiquinone. Figure 4-A shows the initial spectrum of Cdx as a solid line and subsequent reduced spectra as dashed lines. Figure 4B shows the absorbance of key wavelengths over time. Upon the addition of NADPH the absorbance at 456 nm drops to ~50% of its initial value. Over time this value increases back towards the initial value. During this time, absorbance at 585 and 640 nm (indicative of flavin semiquinone) increases then decreases. This suggests an initial two-electron reduction followed by successive one electron oxidations via the semiquinone back to the fully oxidised form.

The Fpr-Cdx redox system was employed to support $P450_{cin}$ activity in vitro. Cineole was chosen as a substrate based on the history of $P450_{cin}$ isolation and its strong affinity for the enzyme ($K_D = 0.7\ \mu\text{M}$).[3] The relative ratios of P450 to redox partner play an important role in the overall rate of substrate oxidation and an empirical approach was taken to maximise the rate of turnover while conserving enzyme. The rate of substrate oxidation was determined by monitoring the rate of NADPH oxidation spectrophotometrically at 340 nm. Encouragingly, significant NADPH consumption was dependent on the presence of all three enzymes and cineole (Table 2; entries 1–5). The ratio of enzymes chosen as a starting point (P450/Fpr/Cdx 1:2:8) mirrors that used for the analogous enzymes employed in the well studied $P450_{cam}$ system.[23] These results are in harmony with our postulation that electron transfer proceeds strictly from NADPH to Fpr to Cdx to $P450_{cin}$. Reduced $P450_{cin}$ then exhibits significant catalytic activity only in the presence of its substrate, cineole. The low levels of background NADPH consumption seen in the absence of one component are presumably due to the formation of reduced oxygen species arising from molecular oxygen interacting with reduced flavin and/or heme prosthetic groups. These characteristics are shared by the well-studied $P450_{cam}$ system.[1]

With these results in hand, the ratio of the component enzymes of the system was optimised to produce maximum NADPH consumption in the presence of substrate. As the rate-

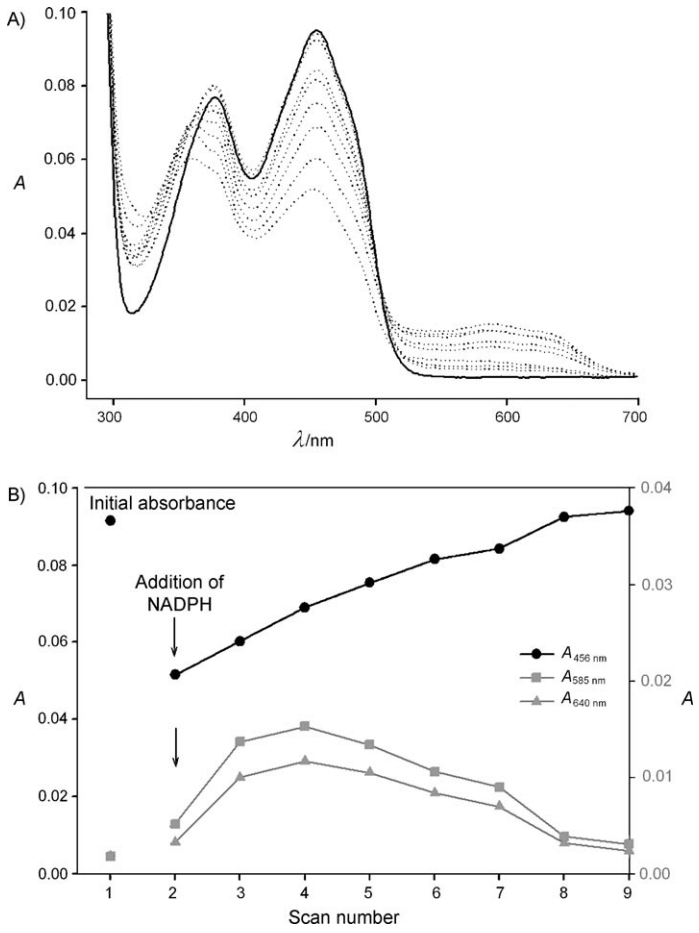

Figure 4. UV–visible spectra monitoring electron transfer from Fpr to Cdx. Scans were recorded every 30 s. A) The initial spectrum is shown as a solid line and subsequent spectra as dashed lines. NADPH (7.5 μM) was added to a solution of Cdx (12.5 μM) and Fpr (1.25 μM); this addition is sufficient to reduce either all of the cindoxin to the semiquinone or approximately half of the cindoxin to the hydroquinone. B) shows the behaviour of the absorbance at 456, 585 and 640 nm. Addition of NADPH results in the absorbance at 456 nm falling to ~50% of its original value before slowly increasing back to its initial point. The absorbance at 585 nm and 640 nm (indicative of flavin semiquinone) increases then decreases; this suggests an initial two electron reduction to the hydroquinone followed by consecutive one electron oxidations through the semiquinone to return to the fully oxidised quinone.

limiting step in $P450_{cam}$-mediated oxidation is the transfer of a second electron to the oxy-$P450_{cam}$–camphor complex,[24] we set about maximizing the concentration of reduced Cdx by increasing its concentration and that of Fpr. The results are shown in Table 2 (entries 6–14) and are in agreement with this hypothesis where the maximum rate of NADPH oxidation is seen at a ratio of 1:30:40 (there was no significant change in the amount of hydroxycineole produced at ratios of 1:10:20 and 1:30:40 compared with 1:2:8). In all subsequent experiments, a ratio of 1:2:8 was employed as a compromise between maximum rate and enzyme conservation.

The apparent kinetic constants (k_{cat} and K_M) for the interaction of Cdx with $P450_{cin}$ were determined by measuring the cineole-dependent NADPH-oxidation, and these values were found to be 32(±1) s^{-1} and 3(±0.3)μM, respectively. The concentration of Cdx was varied while all other components (Fpr, $P450_{cin}$ and cineole) were kept constant. The apparent k_{cat} for the Cdx/$P450_{cin}$ monooxygenase system was determined to be $32 \pm 1\ s^{-1}$, which is similar to that observed in the well-studied putidaredoxin/$P450_{cam}$ system ($k_{cat} = 30–32\ s^{-1}$)[25] and significantly faster than that of the $P450_{BioI}$ system with the flavodoxins YkuN and YkuP as redox partners (~100 min^{-1}).[16] The K_M for Cdx/$P450_{cin}$ was found to be 3.2 ± 0.3 μM. This is approximately ten times that observed in the putidaredoxin/$P450_{cam}$ system ($K_M = 0.33$ μM).[26] The K_M reported for $P450_{BioI}$ with YkuN and YkuP is two to seven times different (K_M = ca. 5–21 μM).[16]

Table 2. The NADPH consumption rates for the oxidation of cineole with varying concentrations of cindoxin.

	Ratio of enzymes (P450/Fpr/Cdx)	[P450] [μM]	[Fpr] [μM]	[Cdx] [μM]	NADPH consumption [μM min^{-1} per μM P450]
1	0:2:8	–	1	4	11
2	1:0:8	0.5	–	4	7
3	1:2:0	0.5	1	–	1
4	1:2:8[a]	0.5	1	4	11
5	1:2:8	0.5	1	4	218
6	1:1:1	0.5	0.5	0.5	21
7	1:1:8	0.5	0.5	4	97
8	1:2:4	0.5	1	2	148
9	1:4:8	0.5	2	4	307
10	1:4:16	0.5	2	8	449
11	1:10:20[b]	0.5	5	10	891
12	1:20:20	0.25	5	5	812
13	1:30:40[b]	0.25	7.5	10	1674
14	1:30:50	0.25	7.5	12.5	1570

[a] No cineole added. [b] At higher enzyme concentrations there was no appreciable change observed in the amount of product produced.

NADPH oxidation by P450s can result in the generation of oxidised products or unwanted side products (superoxide, hydrogen peroxide and water). This latter process is known as uncoupling. The products of the $P450_{cin}$ catalysed reaction (hydroxylated products and hydrogen peroxide) were identified and quantified. The regio- and stereoselectivity of the hydroxylation reaction was determined by comparison of the hydroxycineole from $P450_{cin}$-catalysed hydroxylation with synthetic standards.[27] (1*R*)-6β-hydroxycineole **2** was shown to be the sole cineole derived product upon $P450_{cin}$-mediated oxidation (Figure 5). Quantification of the products by GC showed that 80 ± 2% of the NADPH consumed by the reconstituted system was used in the hydroxylation of cineole and 12 ± 1% was uncoupled to hydrogen peroxide. The remaining 8 ± 3% might presumably be uncoupled to water.

Conclusions

The postulated redox partners for $P450_{cin}$ were cloned into expression vectors and overexpressed. High levels of the FMN-containing flavodoxin, trivially named Cdx, were obtained and purified to homogeneity but the presumably FAD-containing Cdx reductase was unable to be isolated in an active form. Despite this, a catalytically active system was reconstituted in vitro by substituting the Cdx reductase with *E. coli* flavodoxin

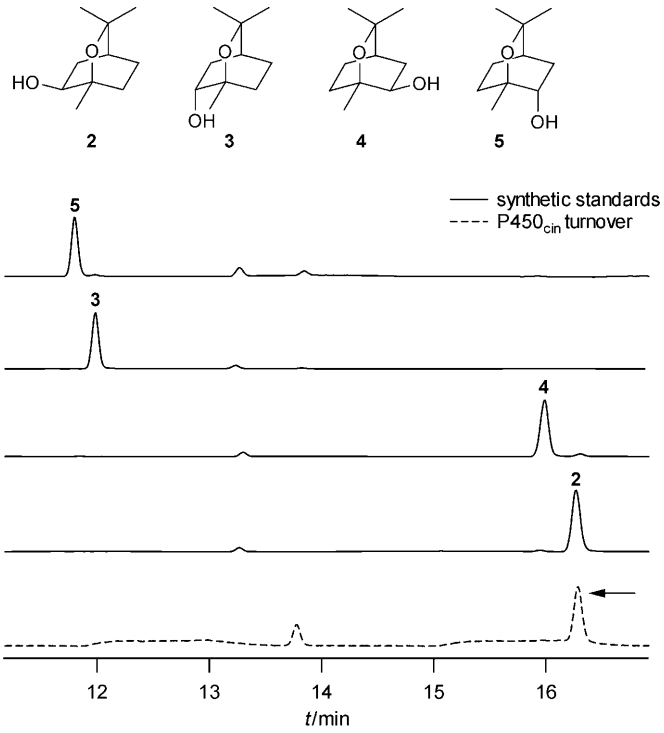

Figure 5. Enantioselective GC analysis of the trifluoroacetate esters of the four isomers of 6-hydroxycineole (**2**: (1*R*)-6β-hydroxycineole; **3**: (1*R*)-6α-hydroxycineole; **4**: (1*S*)-6α-hydroxycineole; **5**: (1*S*)-6β-hydroxycineole) and the product of $P450_{cin}$-catalysed hydroxylation of cineole. (1*R*)-6β-hydroxycineole **2** is the sole product of $P450_{cin}$-catalysed cineole oxidation.

reductase. This system oxidised cineole regio- and stereospecifically to (1*R*)-6β-hydroxycineole and was tightly coupled, converting approximately 80% of the NADPH consumed to product. Only 12% of the reducing equivalents were shuttled into hydrogen peroxide formation. Thus the $P450_{cin}$/Cdx system is experimentally shown to be the first class III[8] (or class IIc[28]) P450 in a recently proposed classification and might represent an ancestor of the typical eukaryotic class II system. We therefore believe that the enzymes encoded by the CIN operon are responsible for catalysing the hydroxylation of cineole to (1*R*)-6β-hydroxycineole, thus initiating a cascade of biodegradative reactions that enable *C. braakii* to live on cineole as its sole source of carbon and energy.

Experimental Section

Materials: Yeast extract and bactotryptone were obtained from Becton Dickinson (Cockeysville, MD). Reagent grade 1,8-cineole was obtained from Aldrich. Sodium dithionite (sodium hydrosulfite) was purchased from Aldrich. All other chemicals used were purchased from commercial sources and were of the highest grade generally available. The restriction enzymes were obtained from New England Biolabs, Inc. (Beverly, MA), and Boehringer Mannheim Corp. Biochemical Products (Indianapolis, IN) based on availability. *E. coli* strain DH5αF′ ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1, was obtained as frozen competent cells from Life Technologies. Inc. (Gaithersburg, MD).

Cloning of cinB-Cdx reductase (CdR): The PCR primers used were based on *cinB* sequencing information and incorporated suitable restriction sites for cloning (EcoRI and NdeI for the N-terminal primer: 5′-CAG AAT TCC ATA TGA GCA AGC TCC AAC AC-3′, and XbaI and EcoRI for the C-terminal primer: 5′-CCG AAT TCT AGA CCC ATA CAG AAT CAA TGC-3′). PCR (94 °C for 3 min; 30 cycles of 94 °C for 45 s, 52 °C for 1 min, 72 °C for 1 min 30 s) was performed using these primers with pC1 (a pUC vector containing the CIN operon; 1 μL, 1 mg mL^{-1}) as a template.[3] A single PCR product (~1.3 kbp) was isolated and cloned into pUC19 as an EcoRI insert. The orientation of the insert was selected for by screening clones with an NdeI/HindIII digest. The resulting plasmid was called pUC19-*cinB*. Any errors introduced from PCR were removed by replacing the internal sequence with wild-type sequence from pC1.[3] An internal BstBI site was mutated for use as a marker. An NgoMI/XhoI fragment (1.2 kb) was excised from pUC19-*cinB* and replaced with a similar fragment from pC1 containing wild-type sequence. The resultant vector was checked for the presence of the wild-type sequence with a BstBI digest. Terminal regions not replaced with wild-type sequence were checked by sequencing the ends with M13 forward and reverse primers. The intact gene was then cloned into pCW as an NdeI/HindIII fragment. The resultant plasmid was called pCW-CdR.

Cloning of cinC-cindoxin (Cdx): The putative flavodoxin gene *cinC* was excised from the 4 kb insert of pC1 by using PCR. The PCR primers used were based on *cinC* sequencing information and incorporated suitable restriction sites for cloning (EcoRI and NdeI for the N-terminal primer: 5′-ATG AAT TCC ATA TGA ACG CAT TGA TTC TC-3′, and XbaI and EcoRI for the C-terminal primer: 5′-ACG AAT TCT AGA GCT GGT CAG GAT ACT GC-3′). PCR (94 °C for 3 min; 30 cycles of 94 °C for 45 s, 52 °C for 1 min, 72 °C for 1 min) was performed using these primers with pC1 (1 μL, 1 mg mL^{-1}) as a template. A single ~450 bp PCR product was isolated and cloned into pUC19 as an EcoRI insert. The orientation of the insert was selected for by screening clones which dropped out a ~450 bp fragment in an NdeI/HindIII digest. The resulting plasmid was called pUC19-Cdx. The fidelity of the PCR insert of pUC19-Cdx was checked by sequencing using the M13 forward and reverse primers. The vector pUC19-Cdx was digested with NdeI/HindIII, dropping out a 450 bp insert which was cloned into similarly cut pCW to give pCW-Cdx.

Expression and purification of Cdx: *E. coli* (DH5α) cells freshly transformed with the expression vector pCW-Cdx were cultured overnight in 2×YT/Amp (2 mL, 50 μg mL^{-1} ampicillin) medium. This was then used to inoculate three Fernbach flasks (2.8 L) each containing TB/Amp (1 L, 50 μg mL^{-1} ampicillin) medium. The culture was incubated at 37 °C (~4.5 h) until an OD_{600} of 0.6 was attained. The cells were then cooled to 27.5 °C, induced with IPTG (1 mM final concentration) and incubated for 18 h. The cells were harvested through centrifugation (6000 *g*, 30 min) at 4 °C and then resuspended in a minimum volume of lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM KCl, 5% glycerol, 0.1 mM PMSF, 0.1 mM DTT and 0.5 mg mL^{-1} lysozyme). The cell suspension was stirred at 4 °C for 1 h, followed by sonication on ice (6×1 min intervals, 50% output). The lysate was centrifuged (15000 *g*, 1 h) to remove the cellular debris and the supernatant loaded directly onto a DEAE-ion exchange column which had been equilibrated in buffer A (50 mM Tris-HCl, pH 7.4, 50 mM KCl, 0.5 mM DTT) and washed with buffer A. Protein was eluted from the column by running a KCl gradient (50–500 mM) in buffer A. Fractions containing Cdx were eluted

at ~240 mM KCl as determined by the characteristic flavin absorbance at 456 nm. Pooled fractions were concentrated in an Amicon ultrafiltration cell containing a YM-10 membrane to a volume no greater than 3 mL. The concentrated protein was loaded onto a G-75 Sephadex gel filtration column equilibrated in buffer B (50 mM Tris, pH 7.4) and eluted with buffer B. Fractions containing Cdx were analysed by UV spectroscopy and combined to give an absorbance ratio A_{456}/A_{280} of 0.67 for purified Cdx. The purified protein was snap-frozen in liquid nitrogen and stored at −70 °C. Yields range from 500–2000 nmol pure Cdx per litre of culture.

Mass spectrum of Cdx: A sample of Cdx (1 mL, ~100 μM) was dialysed overnight in ammonium acetate (5 mM, 2×4 L). The sample was run on an Agilent 110 Binary HPLC system coupled to Applied Biosystems PE SCIEX QSTAR PULSAR QqTOF-MS using electrospray ionisation in positive ion mode. HPLC was performed on the sample (3 μL) using a C3 column (200 μL min^{-1}) according to the following protocol: 0% B (2 min), 0–70% B (20 min) 70% B (5 min) in which solvent B is acetonitrile/0.1% formic acid (90:10) and solvent A is formic acid (0.1%). Three species were observed at: 15966 Da which corresponds to the complete unprocessed apoprotein; 16419 Da which corresponds to the holoprotein; and 16878 Da which corresponds to the addition of a second FMN to Cdx and is likely an artifact.

Redox potentiometry: Redox titrations were performed in a Belle Technology Anaerobic box under an atmosphere of nitrogen ($[O_2] < 10$ ppm). The reductant was titanium (III) citrate (8 mM) and the oxidant was $K_2S_2O_8$ (5 mM). The solution potential was measured with a combination Pt/Ag–AgCl electrode and Hanna Model 8417 meter. The single electron redox mediators [Co(*trans*-diammac)]$(ClO_4)_3$, [Co(*cis*-diammac)]$(ClO_4)_3$, [Co(AMMEsar)]Cl_3, [Co(sep)]Cl_3, [Co(AMME-N_5S-sar)]Cl_3, [Co(CLME-N_4S_2-sar)]Cl_3, [Co-$((NMe_3)_2$sar)]Cl_5, [Fe(tacn)$_2$]Br_3 and Fe(NOTA)[29] were used all at 10 μM concentration to stabilise the solution potential and also to facilitate electron transfer with the reductant and oxidant. The buffers used were Tris (pH 8), phosphate ($6.5 < pH < 7.5$) and MES (pH 6, 50 mM) and with NaCl (10 mM).

Upon establishment of redox equilibrium, the UV–visible spectrum was measured within the glovebox with a Hitachi UV-mini spectrophotometer. Approximately 15 spectra were taken at different potentials and the absorption data (*Abs*) as a function of potential (*E*) fit were to [Eq. (2)][15,16]

$$Abs(E) = \frac{(A_{ox}10^{\frac{E-E_1}{59}} + A_{sq} + A_{hq}10^{\frac{E_2-E}{59}})}{1 + A_{ox}10^{\frac{E-E_1}{59}} + A_{hq}10^{\frac{E_2-E}{59}}} \quad (2)$$

in which A_{ox}, A_{red} and A_{sq} are the limiting absorbances of the oxidized, reduced and semiquinone form at a fixed wavelength. In this case the wavelength chosen (600 nm) corresponded to the semiquinone absorption maximum while A_{ox} and A_{hq} (hydroquinone form) ~0.

Calculation of molar extinction coefficient (ε) for Cdx: The molar extinction coefficient of Cdx was determined by firstly recording the absorbance at 456 nm of the purified flavodoxin (holoprotein). The holoprotein was then boiled at 100 °C for 5 min to release the FMN and centrifuged (10000 *g*, 10 min). The absorbance of the free FMN at 456 nm was measured and using an ε_{456} of 12500 M^{-1} cm^{-1},[21] the concentration of the original holoprotein was calculated. The molar extinction coefficient of Cdx was then determined using this concentration and the original A_{456} of the holoprotein. The molar extinction coefficient of Cdx was determined to be 10825 M^{-1} cm^{-1}.

Identification of the Cdx flavin by HPLC: Identification of the flavin of Cdx was achieved by boiling Cdx in the dark for 5 min, removing the precipitated protein by centrifugation and injecting the filtered supernatant (0.2 mm filter) onto a Shimadzu LC-10AT system using a C18 reversed-phase column (Partisil ODS-3 5U, length 250 mm, i.d. 4.6, Alltech) equilibrated in acetonitrile/0.05% phosphoric acid (10:90). The solvent was held at acetonitrile (10%) for 5 min before running a gradient (10–100% acetonitrile) over 20 min (flow rate of 1 mL min^{-1}). The retention time of the flavin from Cdx was compared to FMN and FAD standards. The flavin from Cdx (15.20 min) had a retention time that matched the FMN standard (15.17 min).

Expression and purification of *E. coli* flavodoxin reductase (Fpr): *E. coli* flavodoxin reductase was expressed and purified according to the procedure of Jenkins et al.[30] with a few modifications. In the final purification step the red Sepharose column was replaced with an S-300 Sephacryl gel filtration column, with the Fpr being eluted with buffer A. Fractions containing FpR were pooled and diluted (1:3) with buffer C (1 mM KH_2PO_4, pH 7.2, 10% glycerol). Fpr was then loaded onto a hydroxyapatite column equilibrated with Buffer D (10 mM KH_2PO_4, pH 7.2, 100 mM NaCl) and a phosphate gradient (10–300 mM KH_2PO_4) in Buffer D was used to elute the protein (1 mL min^{-1}). Purified protein was snap frozen in liquid nitrogen and stored at −80 °C.

Partial reduction of Cdx by Fpr: A solution of Cdx (12.5 nmol) and Fpr (1.25 nmol) was prepared in buffer B (1 mL, 50 mM Tris-HCl pH 7.4). The solution was added to a cuvette, purged with nitrogen, capped and a UV–visible spectrum (700–280 nm) was obtained. NADPH (7.5 nmol) was then added and a UV–visible spectrum (700–280 nm) recorded every 30 s for 5 min.

Generation of a $P450_{cin}$ CO-spectrum by using the Fpr-Cdx redox system: A solution of $P450_{cin}$ (2 μM), Fpr (4 μM) and NADPH (0.2 mM) in Buffer B was prepared and a UV–visible spectrum was recorded. CO was bubbled into the solution and a UV–visible spectrum revealed no change to the spectrum. However, upon the addition of Cdx (16 μM) a CO spectrum with an absorbance maximum at 450 nm was immediately produced showing that Cdx is required to transport electrons from NADPH to the P450.

Determination of NADPH oxidation rate in the reconstituted $P450_{cin}$ system: A solution containing purified $P450_{cin}$ (0.25–0.5 μM), varying concentrations of Cdx and *E. coli* Fpr, and cineole (excess, 6 mM), was prepared in buffer B and divided equally between two cuvettes. A baseline correction was performed at 340 nm and the reaction was initiated in the sample cuvette by the addition of a known amount of NADPH (~0.2 mM). The oxidation of NADPH was monitored by UV–visible spectroscopy at 340 nm over 5 min at room temperature and the rate of NADPH oxidation calculated. Background rates were measured in the same manner in the absence of $P450_{cin}$, Fpr, Cdx and cineole, respectively.

Kinetic analysis of Cdx-$P450_{cin}$ interaction: A solution containing purified $P450_{cin}$ (0.5 μM), *E. coli* FpR (15 μM), cineole (6 mM) and varying amounts of Cdx (0–20 μM) was prepared in buffer B and divided equally between two cuvettes. NADPH (~0.2 mM) was added to the sample cuvette and the solution mixed by inversion. The oxidation of NADPH was monitored by UV–visible spectroscopy at 340 nm over 5 min at room temperature (25 °C) and the initial reaction rates determined. The apparent K_M and V_{max} values were calculated by plotting the observed NADPH oxidation rates versus the concentration of Cdx and fitted to the Michaelis–Menten equation.

Determination of uncoupling in the reconstituted $P450_{cin}$ system: A solution containing purified $P450_{cin}$ (0.5 μM), Cdx (4 μM) and Fpr (1 μM) and cineole (1 mM) in Buffer B was prepared and divided equally between two cuvettes (1 mL each; reference and sample). A baseline correction was performed at 340 nm and the reaction was initiated in the sample cuvette by the addition of a known amount of NADPH (~0.2 mM). The consumption of NADPH was monitored by UV-visible spectroscopy at 340 nm over 5 min at room temperature to ensure all of the NADPH had been consumed and a solution of α-terpineol (250 μL, 1.08 mM) was added as an internal standard. The solution was extracted with ethyl acetate (500 μL), dried over $MgSO_4$ and analysed by GC/MS. The amount of hydroxycineole produced was determined from the ratio of the area under the (1*R*)-6β-hydroxycineole peak to the α-terpineol peak when compared to a standard curve generated from stock solutions of α-terpineol (1.08 mM) and (1*R*)-6β-hydroxycineole (2.33 mM).

Hydrogen peroxide formation assay: Hydrogen peroxide was quantified using an iron-thiocyanate assay as reported in the literature.[23]

Synthesis of 6-hydroxycineoles: The isomeric 6-hydroxycineoles **2** and **3** were prepared from enantio-enriched (−)-(*S*)-α-terpineol while **4** and **5** were prepared from (+)-(*R*)-α-terpineol according to the procedure described by Carman et al.[27] 1H NMR and 13C NMR were in agreement with the literature. **2** α_D+25.0 (0.5 in EtOH); **4** α_D −31.6 (0.3 in EtOH; lit. −26.0); **3** α_D −32.3 (2.3 in EtOH); **5** α_D+23.4 (0.6 in EtOH; lit. +30.2) .[27]

Identification of product of $P450_{cin}$ mediated oxidation of cineole: A solution (1 mL) containing purified $P450_{cin}$ (0.5 μM), Cdx (4 μM) and Fpr (1 μM), catalase (10–20 μg) and cineole (6 mM) was prepared in buffer B. Turnover was initiated by the addition of a known amount of NADPH (~0.2 mM) and the reaction monitored by UV–visible spectroscopy at 340 nm to ensure all of the NADPH had been consumed. The solution was extracted with ethyl acetate (0.5 mL) and analysed by GC/MS which showed a product consistent with 6-hydroxycineole by retention time and mass spectral fragmentation pattern. To determine the absolute stereochemistry of the oxidation, the 6-hydroxycineole was converted to its trifluoroacetate ester by adding several drops of trifluoroacetic anhydride to the alcohol in dichloromethane. The esterified hydroxycineole was analysed by GC on an enantioselective β-cyclodextrin column (temperature programme 120 °C for 2 min; 1 °C min^{-1} to 200 °C or 70 °C for 2 min; 4 °C min^{-1} to 130 °C) and compared with the trifluoroacetate derivatives of the synthetic standards.

Abbreviations: Amp: ampicillin, Cdx: cindoxin, CdR: cindoxin reductase, DTT: dithiothreitol, Fpr: *E. coli* flavodoxin reductase, IPTG: isopropyl-β-D-thiogalactopyranoside, P450: cytochrome P450, $P450_{cin}$: CYP176A1 from Citrobacter braakii, $P450_{cam}$: CYP101A1 from *Pseudomonas putida*; PMSF: phenylmethylsulphonyl fluoride, TB: terrific broth.

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