Multiple native flavin reductases in camphor-metabolizing *Pseudomonas putida* NCIMB 10007: functional interaction with two-component diketocamphane monoxygenase isoenzymes

Andrew Willetts¹ and David R. Kelly²

¹Department of Biological Sciences, University of Exeter, Exeter EX4 4QG, UK
²Department of Chemistry, University of Wales College of Cardiff, Cardiff CF1 3TB, UK

Although they have been studied for nearly 50 years, the source of the FMNH₂ needed for effective biooxidation by the 2,5- and 3,6-diketocamphane monoxygenase (DKCMO) isoenzymes induced by the growth of *Pseudomonas putida* NCIMB 10007 (ATCC 17453) on camphor remains incompletely characterized. Prior studies have focussed exclusively on enzymes present in cells harvested during late-exponential-phase growth despite considerable circumstantial evidence that the flavin reductase (FR) component of these multicomponent monoxygenases is subject to growth-phase-dependent variation. In this study, a number of alternative FMNH₂-generating activities, including both conventional FRs and enzymes also able to serve as ferric reductases, were isolated from camphor-grown cells, and the relative level, and hence potential contribution, of these various proteins shown to vary considerably depending on the point of harvest of NCIMB 10007 within exponential-phase growth. While two constitutive monomeric ferric reductases (molecular masses 27.0 and 28.5 kDa) were found to be the major relevant sources of FMNH₂ during the initial stages of growth on camphor-based media, a significant subsequent contribution throughout the mid- to late-exponential phases of growth was also made by the camphor-induced homodimeric 37.0 kDa FR Fred, recently reported to serve such a role exclusively. The possible involvement of camphor-induced putidaredoxin reductase (51.0 kDa) as a contributory activity was also investigated and considered. Studies with highly purified preparations of the isofunctional DKCMOs confirmed the potential of the various reductases to function effectively as sources of the requisite FMNH₂ to both monoxygenases at different times throughout growth on camphor.

INTRODUCTION

Type 2 Baeyer–Villiger monoxygenases (type 2 BVMOs; Willetts, 1997) are a subgroup of the NAD(P)H : FMN-dependent two-component monoxygenases (TCMOs; Ellis, 2010). They can alternatively be classed as a subgroup of the NAD(P)H : FMN-dependent class C flavoprotein monoxygenases (van Berkel et al., 2006). They are so named because they bioxygenate carbonyl-containing substrates to corresponding lactones/esters or carboxylic acids, outcomes equivalent to the peracid-catalysed Baeyer–Villiger chemical oxidation. These biochemical features distinguish them both from other NAD(P)H : FMN-dependent TCMOs, and from the NAD(P) : FAD-dependent classes D, E and F TCMOs (van Berkel et al., 2006).

Known type 2 BVMOs include the luciferases from various bioluminescent bacteria (Hastings et al., 1985), 1,8-cineole monoxygenase from *Rhodococcus* sp. C1 (Williams et al., 1989), and 2,5- and 3,6-diketocamphane monoxygenase (DKCMO), which are two enantiocomplementary isoenzymes that catalyse a key lactone-forming step in the degradation of the (+)- and (−)-camphor antipodes, respectively, in *Pseudomonas putida* NCIMB 10007 (=ATCC 17435=⇒C1B; Conrad et al., 1965a; Taylor & Trudgill, 1986; Jones et al., 1993; Fig. S1, available in the online Supplementary Material). Although the considerable potential of some of these enzymes to serve as effective biocatalysts for the generation of valuable chiral products of high purity was recognized and exploited some time ago (Gagnon et al., 1995; Villa & Willetts, 1997; Beecher & Willetts, 1998), it is only the recent successful recombinant expression of the genes for both isoenzymic...
DKCMOs from NCIMB 10007 in Escherichia coli (Kadow et al., 2011, 2012; Iwaki et al., 2013) that has rekindled specific interest in these particular type 2 BVMOs.

A characteristic feature of both isoenzymic DKCMOs and 1,8-cineole monooxygenase that they share with some, but not all, other FMN-dependent TCMOs is that they distribute the flavin nucleotide- and nicotinamide nucleotide-dependent tasks between a homodimeric monooxygenase component and a separate flavin reductase (FR) with unbound FMN, the flavin thus effectively serving as a second substrate to transfer reducing power between the functionally distinct subunits. In such FMN-dependent TCMO systems that have been studied in sufficient detail (Valton et al., 2004), there is a distinct affinity for each component for a particular redox form of the flavin. Typically, the component acting as the FR includes an active site that can accommodate both a reduced pyridine nucleotide and the flavin substrate, and has a higher affinity for oxidized flavin, while the biooxygenating component has a complementary active site that has a higher affinity for the reduced flavin: the relative affinities of each component ensure that the flavin moiety once reduced is rapidly transferred within the multimeric complex (Ellis, 2010). In the specific case of the DKCMOs, both isoenzymes have been reported to exploit separate copies of the same monomeric 36 kDa flavin-reducing component referred to as ‘NADH dehydrogenase’ (Gunsalus et al., 1965a). A unique feature of the DKCMOs from camphor-grown NCIMB 10007 that distinguishes them from all other TCMOs characterized to date, including other class C flavin monooxygenases such as various bacterial luciferases (Meighen, 1991), class D flavoprotein monooxygenases such as 4-hydroxyphenylacetate-3-monoxygenase from Pseudomonas aeruginosa (Meighen, 1991), class E flavin monooxygenases such as various bacterial luciferases, class F flavoprotein monooxygenases such as 4-hydroxyphenylacetate-3-monooxygenase from E. coli W (Prieto et al., 1996; Galán et al., 2000) and Acinetobacter baumannii (Chaiyen et al., 2001; Sucharitakul et al., 2006), and styrene monooxygenases from various Pseudomonas and Rhodococcus species (O’Leary et al., 2002) concerns the extent to which the participating components can reasonably be expected to exhibit functional dependence on each other. The relevant genes encoding both the biooxygenating and the reductase components of these previously studied multicomponent monooxygenases are located in the same operon or proximal DNA, and hence are likely to have some evolved dedicated functional interdependence. However, this is most unlikely to be so for the DKCMO isoenzymes in the native strain of NCIMB 10007, where the relevant biooxygenating subunit genes are located on the CAM plasmid, which encodes no relevant dedicated reductase (Kadow et al., 2011, 2012; Iwaki et al., 2013), being instead dependent on exploiting one or more surrogate chromosomally encoded reductases, such as the monomeric 36 kDa NADH dehydrogenase (Trudgill et al., 1966; Taylor & Trudgill, 1986; Jones et al., 1993), although significantly the purified enzyme had greater NADH : (acceptor) oxidoreductase activity with artificial electron acceptors such as methylene blue (Gunsalus et al., 1965a). Although the molecular mass of this monomeric protein as assessed by its sedimentation characteristics (Trudgill et al., 1966) was reported to be 36.0 kDa (range 32.0–38.0 kDa), analysis of the amino acid complement of the purified protein suggested that its true molecular mass was higher, at approximately 43 kDa (Gunsalus et al., 1965b). However, the validity of an exclusive functional relationship between this particular FR and the biooxygenating subunits of the two DKCMO isoenzymes has been questioned. Firstly, the equivalent FR subunits isolated from other class C flavoprotein monooxygenases, including various bacterial luciferases, proved to be effective alternative entities in transferring reducing power transfer to both DKCMO isoenzymes (McGhie, 1998). Secondly, and more indirectly, various FRs each with its own significantly different molecular mass and reductive activity can be detected in association with the equivalent type 2 BVMO 1,8-cineole monooxygenase isolated from Rhodococcus sp. C1 harvested at different times throughout the growth of this bacterium on 1,8-cineole (Williams, 1991). Two recent developments have challenged further the long-standing functional relationship of the DKCMOs and the monomeric 36 kDa NADH dehydrogenase first proposed by Gunsalus et al. (1965a). Recombinant expression studies by Kadow et al. (2011, 2012, 2014) demonstrated that the genes encoding the biooxygenating subunits of both the 2,5- and 3,6-DKCMO isoenzymes were functional when expressed in E. coli by co-opting Fre, a native FR from the surrogate strain. Of even greater relevance, it has been shown that the biooxygenating subunits of both DKCMO isoenzymes can perform highly efficient lactone-forming biotransformations in association with a camphor-induced homodimeric FR (2 × 18 kDa) detected in the cells of NCIMB 10007 (ATCC 17435) entering into the stationary phase of growth in strict contrast to the monomeric 36 kDa NADH dehydrogenase reported previously in equivalent cells (vide infra). Further, the chromosomal genes encoding the relevant monocistrionic approximately 18 kDa FR subunit when assembled as dedicated tandem plasmid constructs with the relevant CAM plasmid-encoded genes for the biooxygenating subunits of the DKCMO isoenzymes (FR × 2.5-DKCMO and FR × 3.6-DKCMO) were successfully expressed in E. coli to yield highly active lactone-forming multimers (Iwaki et al., 2013).

A further element of doubt has been introduced by the recognition that many Pseudomonas strains, including P. putida, harbour multiple FMN : NAD(P) reductases (Fpr) within their genomes (Fontecave et al., 1994; Schröder et al., 2003). Fprs, which serve an essential role as ferric reductases to satisfy the obligate requirement of bacteria for Fe(II), are also known to serve a disparate range of additional reductive roles in these micro-organisms, including action as efficient FRs able to reduce free flavin cofactors
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(Fontecave *et al.*, 1987; Lee *et al.*, 2007; Yeom *et al.*, 2009; Yeom & Park, 2012). Thus one or more functional Fpr present in NCIMB 10007 could potentially serve as a source of FMN$^2-$ for the isoenzymic DKCMOs in camphor-grown cells. Because an intracellular source of Fe(II) is an obligate requirement for aerobic micro-organisms to serve key roles such as in functioning cytochromes, Fprs are considered to be constitutive activities (Schröder *et al.*, 2003). However, FMN : NAD(P)H-dependent FprA and FMN : NADH-dependent FprB in *P. putida* KT2440 both show some additional elements of adaptive response to mild osmotic shock, including the relative availability of ferric and ferrous ions when growing in defined minimal media (Yeom *et al.*, 2009). Fprs, like Frs, are typically small monomeric proteins (molecular mass 10–40 kDa), and although the two groups show comparatively little sequence similarity (Nivièrie *et al.*, 1996), they do share a common ability to act as NADH : (acceptor) oxidoreductases (diaphorases) with artificial electron acceptors such as methylene blue and blue tetrazolium (Moody & Dailey, 1985; Lee *et al.*, 2007).

In the light of these observations, and the ongoing uncertainty about the nature of the one or more Frs able to furnish reducing power to the biooxygenating subunits of the two isoenzymic DKCMOs in camphor-grown *P. putida* NCIMB 10007, the aims of the work reported here were: (i) to identify the number and nature of enzymes in camphor-grown NCIMB 10007 able to serve as Frs donating reducing power to 2,5- and 3,6-DKCMO, and to establish which, if any, are functionally redundant *in vivo*; (ii) to establish whether the functionally active FMN$^2-$-donating activities are subject to growth phase-dependent variability during proliferation of the bacterium in various camphor-based minimal media either supplemented or depleted with respect to Fe(II) and Fe(III).

**METHODS**

**Bacterial strains and culture conditions.** *P. putida* NCIMB 10007 (ATCC 17453) was maintained and cultured on a basal salts medium supplemented with either (+)-, (−)- or (rac)-camphor as the principal organic carbon source as fully detailed previously (Beecher & Willetts, 1998).

**Purification and characterization of DKCMO isoenzymes.** Samples of highly purified biooxygenating subunits of both enantiospecific DKCMO isoenzymes were prepared at 4 °C using an LKB Biological FPLC system and the activity determined by GC using a Shimadzu $^{14}$C system fitted with a Lipodex D chiral column as fully detailed previously (Gagnon *et al.*, 1995).

**Purification and detection of FMN-reductase activities.** All purification procedures were conducted at 4 °C using an LKB Biological FPLC system. Separate four-stage and three-stage schedules were progressively developed to provide, respectively, effective small-scale and preparative-scale purification strategies (Fig. S5, Table S1). FR activity was assayed anaerobically in a reaction mixture (1 ml) containing Tris/HCl buffer (60 mM, pH 7.6), 0.075 mM NADH, 0.02 mM FMN and 0.5 mM KCN to block any terminal oxidase activity. The reaction was initiated by the addition of an appropriate aliquot of protein and the decrease in absorbance at 340 nm monitored to completion against an equivalent blank containing all components except FMN. NADH : (acceptor) oxidoreductase activity (diaphorase) was assayed in a similar way except that FMN was replaced with 0.1 mM methylene blue, and the decrease in absorbance monitored at 660 nm. Enzyme activities were calculated using published absorption coefficients.

**Biocatalytic reactions with combinations of purified enzymes.** Reactions with the various combinations of purified enzymes shown in Table 1 were carried out in reaction mixtures (1 ml) containing Tris/HCl buffer (60 mM, pH 7.6), 0.1 mM NADH, 0.02 mM FMN and 1 mM of the relevant camphor antipode. In each case 0.5 mg of the relevant pure DKCMO was used and 0–50 mU of the various tested concentrated (ultrafiltration) purified candidate Frs. The amount of camphor remaining after 90 min at 25 °C was determined by GC, as fully detailed previously (Beecher *et al.*, 1996). Each biotransformation was replicated 10-fold with the same or equivalent concentrated purified enzyme preparations, the results averaged and the variance recorded.

**N-terminal sequencing.** Purified proteins separated by either SDS-PAGE or native PAGE were transferred to a PVDF membrane (Problott; Applied Biosystems) using a semi-dry blotting apparatus (Pharmacia). N-terminal sequencing was performed by Edman degradation (gas-phase) by the BBSRC-funded sequencing service at the University of Aberdeen.

**RESULTS**

**Growth of *P. putida* NCIMB 10007 on various camphor-based minimal media.**

For many years, studies on the relevant enzyme complement of camphor-grown *P. putida* NCIMB 10007 (ATCC 17435) have been predicated on activities isolated from early stationary-phase cells (Taylor & Trudgill, 1986; Jones *et al.*, 1993). This despite the fact that prior studies of the first two committed enzymes in the relevant degradative pathway (camphor 5-monooxygenase and the 5-hydroxy camphor dehydrogenase isoenzymes; Fig. S1) revealed significant variations in specific activity throughout different phases of camphor-based growth (Gunsalus *et al.*, 1965b, 1967), and it was known that many, if not all, of the committed enzymes are subject to transcriptional and/or translational regulatory control (Gunsalus & Marshall, 1971; Hartline & Gunsalus, 1971). Consequently, consensus outcomes from studies undertaken to characterize the growth of NCIMB 10007 on (+)-, (−)- and (rac)-camphor (Grogan, 1995; Beecher, 1997; McGhie, 1998; representative results shown in Fig. S2) were used to delineate sampling times relevant to examining changes in the specific activities of 2,5-DKCMO, 3,6-DKCMO and ‘total’ NADH : FMN FR. Cell-free extracts were prepared from biomass harvested at various representative stages during growth on (+)- and (−)-camphor compared to equivalent changes in biomass grown on succinate (representative results shown in Fig. 1a, b). Several interesting outcomes were revealed consistently by these replicated (×18) studies. Firstly, growth on (+)-camphor consistently exhibited a less extensive lag phase followed by a considerably higher molar growth yield during exponential phase than equivalent growth on (−)-camphor, although the overall rate of growth was very similar.
Table 1. Activation of purified preparations of the 2,5- and 3,6-DKCMO isoenzymes by aliquots of various concentrated (ultrafiltration) candidate FRs from camphor-grown P. putida NCIMB 10007

Reactions with the various combinations of purified enzymes were carried out in reaction mixtures (1 ml) containing Tris/HCl buffer (60 mM, pH 7.6), 0.1 mM NADH, 0.02 mM FMN and 1 mM of the relevant camphor antipode. In each case 0.5 mg of the relevant pure DKCMO was used and 0–50 mU of the various tested candidate FRs. The amount of camphor remaining after 90 min at 25 °C was determined by GC as fully detailed previously (Beecher et al., 1996). In each case, mean data and maximum variability recorded from 10 replicate runs are shown.

<table>
<thead>
<tr>
<th>Enzyme/FR</th>
<th>(+)-Camphor remaining (%)</th>
<th>(–)-Camphor remaining (%)</th>
</tr>
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<tbody>
<tr>
<td>2,5-DKCMO + no addition</td>
<td>94 (±0.5)</td>
<td>99 (±0.3)</td>
</tr>
<tr>
<td>2,5-DKCMO + 15 mU 51.0 kDa FR</td>
<td>83 (±1.4)</td>
<td>96 (±0.1)</td>
</tr>
<tr>
<td>2,5-DKCMO + 50 mU 51.0 kDa FR</td>
<td>65 (±2.5)</td>
<td>99 (±0.2)</td>
</tr>
<tr>
<td>2,5-DKCMO + 15 mU 37.0 kDa FR</td>
<td>79 (±1.7)</td>
<td>98 (±0.4)</td>
</tr>
<tr>
<td>2,5-DKCMO + 50 mU 37.0 kDa FR</td>
<td>63 (±2.2)</td>
<td>98 (±0.2)</td>
</tr>
<tr>
<td>2,5-DKCMO + 15 mU 27.0–28.5 kDa FR mix</td>
<td>75 (±1.7)</td>
<td>97 (±0.4)</td>
</tr>
<tr>
<td>2,5-DKCMO + 50 mU 27.0–28.5 kDa FR mix</td>
<td>59 (±2.9)</td>
<td>96 (±0.3)</td>
</tr>
<tr>
<td>3,6-DKCMO + no addition</td>
<td>98 (±0.2)</td>
<td>92 (±0.6)</td>
</tr>
<tr>
<td>3,6-DKCMO + 15 mU 51.0 kDa FR</td>
<td>99 (±0.2)</td>
<td>81 (±1.4)</td>
</tr>
<tr>
<td>3,6-DKCMO + 50 mU 51.0 kDa FR</td>
<td>98 (±0.2)</td>
<td>67 (±2.9)</td>
</tr>
<tr>
<td>3,6-DKCMO + 15 mU 37.0 kDa FR</td>
<td>99 (±0.3)</td>
<td>80 (±0.9)</td>
</tr>
<tr>
<td>3,6-DKCMO + 50 mU 37.0 kDa FR</td>
<td>98 (±0.1)</td>
<td>62 (±2.2)</td>
</tr>
<tr>
<td>3,6-DKCMO + 15 mU 27.0–28.5 kDa FR mix</td>
<td>97 (±0.4)</td>
<td>79 (±1.8)</td>
</tr>
<tr>
<td>3,6-DKCMO + 50 mU 27.0–28.5 kDa FR mix</td>
<td>99 (±0.2)</td>
<td>56 (±3.0)</td>
</tr>
</tbody>
</table>

Fig. 1. (a) Specific activity of 2,5-DKCMO, 3,6-DKCMO throughout the growth of P. putida NCIMB 10007 on either (+)-camphor, (–)-camphor or succinate as sole carbon source. (b) Specific activity of total FR throughout the growth of P. putida NCIMB 10007 on either (+)-camphor, (–)-camphor or succinate as sole carbon source.

Throughout exponential-phase growth on either camphor antipode. Secondly, as confirmed by the equivalent levels present in succinate-grown biomass, both DKCMO isoenzymes and total FR are all subject to significant transcriptionally and/or translationally regulated changes in specific activity throughout the various phases of growth on both (+)- and (–)-camphor. These changes were most noticeable for 2,5-DKCMO during growth on (+)-camphor, the enantiocomplementary antipode. Lesser but still highly significant changes occurred both for 3,6-DKCMO during growth on enantiocomplementary (–)-camphor, and for total FR during growth on either camphor antipode. Thirdly, although the two DKCMO isoenzymes are known to be highly selective towards their respective antipodes when serving as substrates for biooxygenation (Gagnon et al., 1995; Beecher, 1997; Kadow et al., 2014; Iwaki et al., 2013), and the samples of (+)- and (–)-camphor used were of the highest commercial grade available, there was a small but significant element of ‘cross-inducibility’ of the enantiomerically redundant DKCMO isozyme by both camphor antipodes.

Influence of Fe(III) and Fe(II) on the specific activities of camphor-related enzymes during growth of P. putida NCIMB 10007 on (rac)-camphor-based minimal media

Because of both the known effects of trace levels of iron salts in growth media on the reductive activities of bacteria...
such as Clostridium pasteurianum (Knight & Hardy, 1967) and Peptostreptococcus elsdenii (Mayhew & Massey, 1969), and the reported ability of the multiple FMN:NAD(P)H ferric reductases of various Pseudomonas strains to serve additional roles as FRs (Fontecave et al., 1994; Schröder et al., 2003; Lee et al., 2007), the influence on total FR activity of changes in the Fe(II) and/or Fe(III) complements of the trace elements solution used to supplement growth of NCIMB 10007 on (rac)-camphor-based minimal media was investigated. The results obtained (Fig. S3; mean data used) from five separate replicated experiments consistently demonstrated a number of different trends. Firstly, doubling the level of added Fe(II) alone from 50 to 100 μM promoted a uniform drop in the overall level of total FR activity throughout all tested phases of growth. Secondly, adding 50 μM Fe(III) in the co-presence of 50 μM Fe (II) consistently resulted in a 10–15% increase in total FR activity at all sampled points throughout the growth curve. Thirdly, when the addition of 50 μM Fe(III) was combined with the omission of any supplementary Fe(II) plus the addition of 5 μM 2',2'-dipyridyl to chelate out any potential sources of Fe(II) present in other media components, this resulted in a 14–20% increase in total FR activity uniform throughout all tested phases of the growth curve. Attempts to establish the effect of the combined omission of both Fe(II) and Fe(III) were essentially precluded by the very poor resultant growth of NCIMB 10007 on this doubly depleted camphor-based minimal medium, thereby making enzyme assay data inconsistent and unreliable.

Analysis and characterization of the total FR titre in (rac)-camphor-grown P. putida NCIMB 10007

The significant growth-dependent differences in the total FR titre recorded in camphor-grown NCIMB 10007 were investigated more fully by developing appropriate purification protocols. Initial selective precipitation using saturated ammonium sulfate solution confirmed that the total FR complement in biomass harvested at any point during exponential growth could be recovered consistently as a major fraction representing approximately 70% of the total FR titre that salted out in the 30–50% cut, with the bulk of the residual activity (25%) concentrated in the 50–70% cut. For comparative purposes, dialysed and concentrated (ultrafiltration) preparations of FR-active fractions salted out using a combined 30–70% cut were generated from biomass harvested in the early exponential (OD₅₀₀ 0.6), mid-exponential (OD₅₀₀ 1.0) and late-exponential (OD₅₀₀ 1.4) phases of growth on (rac)-camphor minimal medium supplemented with 50 μM Fe(II). An effective four-stage small-scale chromatography (FPLC) protocol employing successive anionic exchange and affinity resins to isolate NADH-dependent activities to the exclusion of most other major protein types was developed to ensure maximum potential separation of the various NADH:FR-active proteins in the three salted out samples prepared from five equivalent separately grown samples of biomass. All final-stage fractions from each sample were then tested for FMN:NADH reductase activity and protein content (representative results shown in Fig. 2a, c, e). Fractions found to contain FR activity were concentrated (ultrafiltration), the retentates run on SDS-PAGE with standard protein markers and the resolved gels then stained (representative results shown in Fig. 2b, d, f). It was apparent that FR activity in each of the three different salted out samples was associated with more than one protein band and, as suggested by previous results (vide infra), that there were both quantitative and qualitative differences in the distribution of FR activity between various different proteins depending on which stage of growth on camphor-based minimal medium biomass of NCIMB 10007 was harvested. The major FR activities appeared to be associated with two constitutive proteins located principally in fractions 17–22 with denatured molecular mass values of approximately 27.0 and 29.0 kDa, one inducible protein located principally in fractions 11–14 with a molecular mass of approximately 50.0 kDa, and a second inducible protein located principally in fractions 17–21 with a molecular mass of approximately 17.0–18.0 kDa. The data obtained in these studies for late-exponential phase cells of camphor-grown NCIMB 10007 (Fig. 2f) are similar to those presented elsewhere for an equivalent biomass sample (McGhie, 1998), although the significance of the multiple FR-positive activities observed was not recognized at the time. For further analysis, the discrete 17.0–18.0 kDa band present in fraction 17 prepared from each tested growth phase was electroblotted onto a PVDF membrane and N-terminal sequence determination performed (Fig. 3). For comparison, also included are the N-terminal sequence of the monomeric subunit (approximate molecular mass 18 kDa) of Fred (Iwaki et al., 2013), the homodimer characterized as an active FR in early stationary-phase camphor-grown ATCC 17435 (NCIMB 10007), and of Fre, a surrogate FR from E. coli BL21(DE3) shown to be able to transfer reducing power from NADH to both DKCMO isoenzymes in recombinant constructs (Kadow et al., 2014).

To provide sufficient material to undertake more accurate characterization of the equivalent native FR activities, a different three-stage preparative scale purification was developed which used a HiLoad Superdex 200 16/60 column pre-calibrated with standard marker proteins (Fig. S4) as the final separation step. All final-stage fractions from each series of samples were then tested for FMN:NADH reductase activity and protein content (representative results shown in Fig. 4a, c, e) and the molecular mass of the protein(s) present in active fractions assessed by comparison with Fig. S4. Because of apparent discrepancies between these outcomes and those obtained for equivalent samples by SDS-PAGE (Fig. 2b, d, f), samples of all relevant FR-active final-stage fractions were concentrated (ultrafiltration), the retentates run on native PAGE with standard protein markers and the resolved gels then stained (representative results shown in Fig. 4b, d). Again, for further analysis, the discrete 52.5 and 51.0 kDa bands present, respectively, in fractions 7 and 8 prepared from early exponential-phase-growth cells were electroblotted onto...
PVDF membranes and N-terminal sequence determinations performed (Fig. 3).

The combined outcomes of these various repeated separation and characterization studies consistently displayed several interesting features. Firstly, with the exception of the significant 17.0–18.0 kDa denatured protein activity detected after SDS-PAGE, which was deemed to be the monomeric subunit of a homodimer corresponding to the 37.0 kDa native activity recovered from preparative-scale chromatography, all other FR-active proteins detected in
17.0–18.0 kDa FR subunit (this study)  
18.0 kDa subunit of Fred (ATCC 17453)  
28.0 kDa FMN-reductase Fre E.coli BL21(DE3)  
51.0 kDa FR-active protein (NCIMB 10007)  
Putidaredoxin reductase (ATCC 17453)  
52.5 kDa protein FR-inactive protein (NCIMB 10007)  
Cytochrome P<sub>450</sub>MO (ATCC 17453)

**Fig. 3.** Comparative sequence data for some proteins purified from camphor-grown *P. putida* NCIMB 10007 in this study and relevant proteins purified from either camphor-grown *P. putida* ATCC 17453 (NCIMB 10007) by Iwaki *et al.* (2013) or *E. coli* BL21(DE3) by Kadow *et al.* (2014). Identical amino acid residues are underlined and shown in bold; equivalent amino acid residues are italicized.

the tested samples were apparently monomeric as equivalent denatured and native activities were identified consistently by both purification protocols. Secondly, as confirmed by the similar relevant quantitative data resulting from both purification protocols, whereas some of the FR-positive activities, such as the 27.0 and 28.5 kDa proteins, appeared to be constitutively expressed throughout exponential-phase growth of NCIMB 10007 on camphor-based minimal medium, other significant contributory activities, such as the 37.0 kDa (2 × 17.0–18.0 kDa denatured activity on SDS-PAGE gels) and the 51.0 kDa native proteins were inducible to some extent, and were present at significantly higher specific activity as growth progressed into and beyond mid-exponential phase. Thirdly, while the major contributors to total FR activity at all stages of exponential-phase growth were the 27.0, 28.5, 37.0 and 51.0 kDa native proteins, other relatively minor activities (the approximately 41.0, 45.0 and 47.5 kDa proteins from the SDS-PAGE gels) are likely to represent functionally redundant FR activities, at least with respect to the DKCMO isoenzymes during growth of NCIMB 10007 on a camphor-based minimal medium. Conversely, the ability of the concentrated (ultrafiltration) major FR contributory activities resulting from the preparative-scale purification (the 27.0 plus 28.5 kDa ‘mix’, 37.0 and 51.0 kDa native proteins) to facilitate dose-dependent biooxyg enation by aliquots of highly purified 2,5- and 3,6-DKCMO oxygen-dependent subunits prepared as described previously (Beecher & Willetts, 1998) was consistently demonstrated using replicate (×10) linked enzyme assays (mean data shown in Table 1). Fourthly, there was no evidence that an FR-active NADH oxidase/NADH dehydrogenase characterized as a 36.0 kDa monomer by its sedimentation characteristics and reported by Trudgill and colleagues as the major donor of FMNH<sub>2</sub> to the DKCMO isoenzymes in camphor-grown NCIMB 10007 (Trudgill *et al.*, 1966; Taylor & Trudgill, 1986; Jones *et al.*, 1993) played any significant role in this respect. Even allowing for the possibility that the true molecular mass of the protein reported by Trudgill and colleagues was 43.0 kDa as indicated by its amino acid complement (Gunsalus *et al.*, 1965b), and as such might correspond to either the 41.0 or 45.0 kDa monomeric activities detected in this study, neither of these possible equivalent FR activities played any significant role in servicing the required FMNH<sub>2</sub> for the DKCMO isoenzymes of camphor-grown NCIMB 10007.

When compared to other relevant studies, it seemed most likely that the camphor-inducible homodimeric 37.0 kDa FR (17.0–18.0 kDa denatured protein on SDS-PAGE gels) detected in this study corresponded to Fred, the homodimer with a reported subunit molecular mass of approximately 18.0 kDa characterized as an active FR in early stationary-phase camphor-grown ATCC 17435 (NCIMB 10007) by Iwaki *et al.* (2013). This likelihood was given further credence both by the comparative sequence data (Fig. 3) and by confirming that the equivalent activity was undetectable in succinate-grown NCIMB 10007 biomass (data not shown). Similarly, the 27.0 and 28.5 kDa FR activities recorded in this study were considered likely to be equivalent to the 28.8 kDa NADH-dependent (FprB) and the 29.6 kDa NAD(P)H-dependent (FprA) ferric reductases reported in *P. putida* KT2440 (Lee *et al.*, 2007) and shown to be highly active FMH : NADH reductases (Yeom *et al.*, 2009). Although insufficient discrete sequence data for these two activities from NCIMB 10007 could be obtained to allow meaningful comparison (data not shown), it was highly significant that both the 27.0 and 28.5 kDa activities in this camphor-grown *P. putida* strain, like FprA and FprB in *P. putida* KT2440, were inducible above a significant threshold constitutive level by modifying the Fe(II) and Fe(III) complement of the growth medium to favour ferric reductase
Fig. 4. (a) Separation of FR active proteins from OD_{500} 0.6 camphor-grown cells of NCIMB 10007 on the pre-calibrated HiLoad 16/60 Superdex 200 gel-filtration column (Fig. S4). Corresponding molecular masses for relevant fractions are: fraction 7, 52.5 kDa; fraction 8, 51.0 kDa; fraction 14, 37.0 kDa; fraction 19, 28.5 kDa; fraction 20, 27.0 kDa. (b) Native PAGE gel of concentrated selected fractions from (a). Standard marker proteins (S) were: BSA, 67 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin, 21 kDa; RNase, 14 kDa. (c) Separation of FR active proteins from OD_{500} 1.0 camphor-grown cells of NCIMB 10007 on the pre-calibrated HiLoad 16/60 Superdex 200 gel-filtration column (Fig. S4). Corresponding molecular masses for relevant fractions are: fraction 7, 52.5 kDa; fraction 8, 51.0 kDa; fraction 14, 37.0 kDa; fraction 19, 28.5 kDa; fraction 20, 27.0 kDa. (d) Native PAGE gel of concentrated selected fractions from (c). Standard marker proteins (S) were as for (b). (e) Separation of FR active proteins from OD_{500} 1.4 camphor-grown cells of NCIMB 10007 on the pre-calibrated HiLoad 16/60 Superdex 200 gel-filtration column (Fig. S4). Corresponding molecular masses for relevant fractions are: fraction 7, 52.5 kDa; fraction 8, 51.0 kDa; fraction 14, 37.0 kDa; fraction 19, 28.5 kDa; fraction 20, 27.0 kDa. The native PAGE gel of concentrated selected fractions from (e) (late-exponential phase, OD_{500} 1.4) was very similar to (d), the equivalent gel for mid-exponential-phase fractions (OD_{500} 1.0).
activity (vide infra; Fig. S3). Also significant was that both the 27.0 and 28.5 kDa activities from camphor-grown NCIMB 10007 acted very effectively as NADH: (acceptor) oxidoreductases with various artificial electron acceptors, including methylene blue and nitro blue tetrazolium, a characteristic shared with FprA and FprB (Lee et al., 2007).

Somewhat surprisingly, the major contributory FR-active protein detected in camphor-grown NCIMB 10007 with a molecular mass of 50.0−51.0 kDa was consistent on sequence comparison data (Fig. 3) with putidaredoxin reductase (Pdr), the NADH:FMN-dependent subunit of the camphor-5-monoxygenase complex (P450cam), the initiating enzyme activity in the established pathway of camphor degradation in NCIMB 10007 (Hedegaard & Gunsalus, 1965; Trudgill, 1978; Fig. S1). The 52.5 kDa protein, which was inactive as a FR, was consistent on sequence comparison data (Fig. 3) with the cytochrome P450cam (CPY101) biooxygenating subunit of the camphor-5-monoxygenase complex. Similar co-purification phenomena of both DKCMO isoenzymes with one or more subunits of camphor-5-monoxygenase have been reported elsewhere (Gunsalus et al., 1967; Taylor & Trudgill, 1986; Grogan, 1995; Beecher, 1997; McGhie, 1998; Iwaki et al., 2013). The fact that Pdr and cytochrome P450MO were both totally absent in succinate-grown NCIMB 10007 (data not shown) supported these conclusions.

**DISCUSSION**

The 2,5- and 3,6-DKCMO isoenzymes induced by the growth of *P. putida* NCIMB 10007 on camphor, like other type 2 BVMOs, such as the luciferases from various bioluminescent bacteria (Hastings et al., 1985), are TCMOs (Ellis, 2010) that act as a loosely associated multimeric complex able to source the FMNH₂ needed for effective biooxidation by free diffusion from more than one FR present during growth. However, whereas in the case of *Vibrio fischeri* ATCC 7744 it is Fase1, one of three different characterized FRs present in this bioluminescent bacterium, that serves a predominant (90%) role with Fre and LuxG, the other two FRs, in effect being functionally redundant (Zenno & Saigo, 1994), in camphor-grown NCIMB 10007 there is considerable potential for functional multiplicity because the potential to generate FMNH₂ is distributed much more evenly between three, and possibly four, different FRs. For *P. putida* NCIMB 10007 this may represent an evolved solution to the problem posed by reduced flavin-dependent aerobic growth. The reduction of free flavins by reduced pyridine nucleotides is not an efficient reaction. The kinetics are slow unless non-physiological concentrations of both reactants are present (Gaudu et al., 1994). Consequently in many cases living organisms dependent on aerobic growth contain multiple FRs different in enzyme nature and molecular mass (Fieschi et al., 1995). It is possible that in depth studies of the stoichiometry of the various alternative candidate FRs may yield some insight into their relative functional importance for each separate isoenzyme. In this respect, it is interesting to note the recent discovery (Iwaki et al., 2013) that the CAM plasmid of ATCC 17435 (NCIMB 10007) carries two isofunctional 2,5-DKCMO-encoding genes (camE25-1 and camE25-2) but only a single 3,6-DKCMO-encoding gene (camE36).

In NCIMB 10007, a basal level of FR activity at all stages of aerobic growth is represented by the constitutive elements of the 27.5 and 28.5 kDa ferric reductases equivalent to FprA and FprB in *P. putida* KTT2440 (Yeom et al., 2009), and these two enzymes probably serve as the major sources of FMNH₂ to the DKCMO isoenzymes during the initial phases of growth on camphor-based minimal media when these two activities account for 80−90% of the total FR titre. These two constitutive FR activities are then potentially supplemented by two additional camphor-induced quanta – Fred, a homodimeric 37.0 kDa FR recently identified in camphor-grown NCIMB 10007 (Iwaki et al., 2013), and putidaredoxin reductase (Pdr), a protein first reported in camphor-grown *P. putida* nearly 50 years ago (Conrad et al., 1965b). Whereas camphor-induced Fred has been recognized previously to serve such a DKCMO-supporting role (Iwaki et al., 2013), the widely accepted role of Pdr in camphor-grown *P. putida* is as the supplier of FMNH₂ to the haem moiety of the cytochrome P450cam biooxygenating subunit of the multimeric camphor-5-monoxygenase complex (Unger et al., 1986), the camphor-induced hydroxylase that initiates the catalytic pathway for the assimilation of both (+)- and (−)-camphor (Gunsalus et al., 1967; Sokatch, 1986; Fig. S1). Whether *in vivo* most, if not all, of any available Pdr will be committed exclusively to servicing the multimeric camphor-5-monoxygenase complex in camphor-grown NCIMB 10007 is a moot point. Similarly, although from mid-exponential phase onwards the relative levels of activity attributable to these four reductase-positive proteins when assayed as purified entities suggest that they each have the potential to contribute significantly towards the total FR titre (Fig. 4c, e), this does not exclude the possibility that one or more of these activities is functionally redundant with respect specifically to one or both DKCMO isoenzymes, as has been suggested in the cases of the equivalent multiplicity of FRs present in the luciferase-dependent bioluminescent bacteria *V. fischeri* (Zenno & Saigo, 1994) and *Vibrio harveyi* (Campbell & Baldwin, 2009).

The apparent potential of Pdr to serve a significant, albeit possibly gratuitous, role as a supplier of FMNH₂ to the DKCMO isoenzymes, which being TCMOs deploy a non-integral flavin cofactor effectively as a substrate shuttle (Valton et al., 2004), is counter intuitive on two counts. Firstly, Pdr has a well-established role in the camphor-5-monoxygenase multimeric complex, and secondly Pdr is known to successively transfer reducing power from NADH initially to FAD and then to FMN with both flavin coenzymes being integrally incorporated within the tertiary structure of Pdr (Peterson et al., 1990), as is the case for a number of other FAD plus FMN-dependent flavodoxin
reductases (Hall et al., 2001). However, it may not be insignificant that both Fred, the homodimeric FR recently proposed as a major supplier of FMNH$_2$ to the DKCMO isoenzymes of camphor-grown NCIMB 10007 (Iwaki et al., 2013), and the monomeric NADH oxidase/dehydrogenase originally reported to serve such a role (Trudgill et al., 1966; Taylor & Trudgill, 1986; Jones et al., 1993) have been shown to possess separate FAD- and FMN-specific binding sites. Further, initial research in the 1960s (Gunsalus et al., 1966; Taylor & Trudgill, 1986; Jones et al., 1993) prior to the more extensive characterization of the enzymes of the camphor-degradation pathway in subsequent years (Trudgill, 1978; Ougham et al., 1983) also resulted in some speculation of such a dual role for Pdr based primarily on the ability of the flavoprotein to interact gratuitously with the biooxygenating subunits of the DKCMO isoenzymes as well as cytochrome P450cam.

Among the more interesting elements of inducible enzyme expression characterized in this study are firstly the effects that resulted from changes to different levels of iron salts in the trace element composition of the camphor-based minimal medium, and secondly the effects observed on changing the single camphor antipode used as the sole carbon source. Thus, the approximately 14–20% increase over the basal constitutive level of the 27.5 and 28.5 kDa reductase activities promoted by the combination of replacing Fe(II) with 50 μM Fe(III), with the concomitant potential to increase the biooxygenating activity of both DKCMO isoenzymes, may help to explain the inconsistent and contradictory early reports of the involvement of some form of iron in the activity of the DKCMO isoenzymes of NCIMB 10007, which possibly resulted indirectly from inconsistencies between using corn steep liquor [relatively high Fe(II) content] and yeast extract [relatively low (FeII) content] as a supplement to help promote growth (Bradshaw et al., 1959; Conrad et al., 1965a, b; Trudgill et al., 1966; Yu & Gunsalus, 1969). The significant levels of ‘cross-inducibility’ of the two DKCMO isoenzymes by each camphor antipode acting as the sole source of carbon in the growth medium reflect similar unexplained data reported elsewhere (Taylor & Trudgill, 1986), and contrast sharply with the very high degree of enantioselectivity shown by both enzymes when biooxygenizing the relevant antipode as a substrate (Beecher et al., 1996; Beecher, 1997; Beecher & Willetts, 1998; Iwaki et al., 2013). It is possible that these discrepancies may reflect differences in the relative specificity of the relevant repressor proteins (relatively low) and the corresponding enzymes (relatively high). Because of the extent of the cross-inducibility recorded for both isoenzymes, the phenomenon is unlikely to be due simply to the presence of the relevant complementary isomer as a minor impurity in the samples of camphor used, which were the highest commercial grades available (95–97% pure for both antipodes). One further potentially influential factor that may be relevant to both the observed cross-inducibility and the disproportionate levels of induction of 2,5-DKCMO by (+)-camphor and 3,6-DKCMO by (−)-camphor (Fig. 1a) is the recent discovery of the duplex nature of the 2,5-DKCMO-encoding genes within the CAM plasmid (Iwaki et al., 2013), which could be studied further by relevant strain construction.

One issue that should be addressed is why this study, in agreement with another relevant recent study (Iwaki et al., 2013), failed to find any evidence for a role for a monomeric 36.0 kDa NADH oxidase/dehydrogenase reported by Trudgill and colleagues as the source of FMNH$_2$ for the DKCMO isoenzymes in camphor-grown NCIMB 10007 (Trudgill et al., 1966; Taylor & Trudgill, 1986; Jones et al., 1993). The same activity was reported to comprise a single polypeptide chain with a molecular mass of 43.0 kDa based on its amino acid complement (Gunsalus et al., 1965b; Trudgill et al., 1966). Interestingly, a recent CODEHOP PCR-based search using FR-specific motifs of a DNA preparation from NCIMB 10007 that included the CAM plasmid-encoded information failed to find any evidence for a gene corresponding to a putative monomeric FR with a predicted molecular mass of either approximately 36.0 or 43.0 kDa corresponding to the probe motifs used (Kadow, 2012). The most likely possibility is that the activity reported to be ‘a single polypeptide chain with an M of 36 000 that will bind one molecule of FMN’ (Taylor & Trudgill, 1986) corresponds to the homodimeric 37.0 kDa FR activity detected in this and the recently reported study of Fred (Iwaki et al., 2013). Interestingly, kinetic data obtained by Trudgill’s group from an equilibrium dialysis study with an equimolar mixture of FMN and FAD (Trudgill et al., 1966) indicated the involvement of a protein with a binding site for each flavin cofactor and an estimated molecular mass of 17.5 kDa, data entirely consistent with the characteristics reported for the monomeric subunit of Fred (Iwaki et al., 2013). Although this 1966 publication preceded the introduction of SDS-PAGE as a technique to assist protein characterization (Shapiro et al., 1967), significantly, there is no reference to, or data showing, the outcome of SDS-PAGE gel preparations of the purified activity in other subsequent relevant publications by this group (Trudgill, 1978; Ougham et al., 1983; Taylor & Trudgill, 1986; Jones et al., 1993).

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Flavin reductases in camphor-metabolizing P. putida


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