Diketocamphane enantiomer-specific 'Baeyer–Villiger' monooxygenases from camphor-grown *Pseudomonas putida* ATCC 17453

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Pseudomonas putida ATCC 17453 grew with either (+)- or (–)-camphor as sole carbon source. Enantiomer-specific 'biological Baeyer–Villiger' monooxygenases were synthesized irrespective of the camphor isomer used for growth. The two enzymes are probably the products of separate genes but showed many similarities. Each consisted of two electrophoretically identical subunits, bound flavin mononucleotide (FMN) non-covalently and accepted electrons from an induced NADH dehydrogenase which interacted with the FMN bound to the oxygenating component. They showed minor differences in M_r , with 3,6-diketocamphane 1,6-monooxygenase being the smaller enzyme. Isoelectric focussing showed the two enzymes to have different acidic pI values. Polyclonal antibodies raised against 3,6-diketocamphane 1,6-monooxygenase also cross-reacted with 2,5-diketocamphane 1,2-monooxygenase and its subunits.

Introduction

Camphor exists in two enantiomeric forms; the (+)-isomer occurs primarily in the wood of *Cinnanocum camphora* and, although the (–)-isomer is less widely distributed it occurs in a number of plant essential oils. The racemate also occurs naturally and is found in the essential oil of *Crysanthemum sinense* (Pelter & Harper, 1969). *Pseudomonas putida* ATCC 17453 will grow with either isomer of camphor as sole source of carbon (LeGall *et al.*, 1963). Although numerous papers and reviews have been written concerning the metabolism of (+)-camphor by *P. putida* ATCC 17453 knowledge concerning the pathways and enzymology of (+)- and (–)-camphor oxidation is incomplete. Reactions leading to cleavage of both carbocyclic rings of the bicyclic monoterpene have been elucidated for the degradation of (+)-camphor (Conrad *et al.*, 1961, 1965*a, b*; Ougham *et al.*, 1983) and the metabolism of (–)-camphor has been shown to proceed through a parallel enantiomeric sequence of intermediates, that were transiently accumulated in culture medium (Chapman *et al.*, 1964; Gunsalus & Marshall, 1971). The established reaction sequences are shown in Fig. 1. The genes that code for these catabolic enzymes are carried on the 165 MDa

transmissible CAM plasmid (Rheinwald *et al.*, 1973; Palchaudhuri & Chakrabarty, 1976; Ougham *et al.*, 1983). Isobutyrate has been implicated as a common catabolic intermediate close to central metabolism and the genes that code for isobutyrate metabolism and essential anaplerotic and amphibolic pathways are chromosomal (Rheinwald *et al.*, 1973).

The established enzymology of ring cleavage has some interesting complications. The initial monooxygenase, a cytochrome-P450-containing enzyme complex, is induced by growth with either isomer of camphor (Gunsalus & Marshall, 1971). It forms 5-*exo*-hydroxycamphor from (+)-camphor and hydroxylates (–)-camphor in the corresponding enantiomeric position. Two discrete hydroxycamphor dehydrogenases have been reported, each with a preference for the hydroxycamphor derived from the camphor isomer used for growth but neither showing absolute specificity (Gunsalus & Marshall, 1971). Ring oxygen insertion into 2,5-diketocamphane, formed from (+)-camphor, is catalysed by a 1,2-monooxygenase that is a very loose complex of two proteins, an NADH dehydrogenase and a flavin mononucleotide (FMN)-containing oxygenating component. Both proteins of this biological 'Baeyer–Villiger' oxygenase have been purified to homogeneity (Trudgill *et al.*, 1966; Taylor & Trudgill, 1986). In complete contrast to the hydroxylase and the two dehydrogenases, this oxygenase is absolutely specific for (+)-camphor and substituted (+)-camphor derivatives, such as 2,5-diketocamphane. It is inactive with (–)-camphor and

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Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; FPLC, fast protein liquid chromatography.

Fig. 1. Early reactions in the catabolism of camphor isomers by *Pseudomonas putida* ATCC 17453. Compounds are: a, (+)-camphor; b, 5-*exo*-hydroxycamphor; c, 2,5-diketocamphane; d, (–)-camphor; e, 3,6-diketocamphane; f and g, enantiomeric unstable lactones produced by oxygenation of the diketocamphanes. Enzymes are: 1, camphor *exo*-hydroxylase; 2a, 2b, *exo*-hydroxycamphor dehydrogenases; 3, 2,5-diketocamphane 1,2-monooxygenase; 4, 3,6-diketocamphane 1,6-monooxygenase (the monooxygenases are also able to oxygenate the respective camphor isomers).

other ketocamphanes that do not possess a keto group at the 2-position.

It had been assumed, on the basis of the ability of the organism to grow with (–)-camphor, the spectrum of accumulated metabolites, the specificity of the 2,5-diketocamphane 1,2-monooxygenase and from genetic studies in which mutants, unable to grow with (+)-camphor but able to grow with (–)-camphor and the enantiomer of 2-oxo- Δ^3 -4,5,5-trimethylcyclopentenyl-acetic acid derived from (+)-camphor, that an iso-functional enzyme specific for (–)-camphor and 3,6-diketocamphane is also synthesized by the organism (LeGall *et al.*, 1963; Gunsalus & Marshall, 1971; Rheinwald *et al.*, 1973). In this paper we report the purification and characterization of this enantiomeric 'Baeyer–Villiger' monooxygenase and compare it with 2,5-diketocamphane 1,2-monooxygenase.

Methods

Bacterial strains. *Pseudomonas putida* C1B (ATCC 17453) was obtained from the American Type Culture Collection, Rockville, MD, USA, maintained on nutrient agar slopes at 4 °C and subcultured at monthly intervals.

Growth conditions. Cultures were grown aerobically at 30 °C. Basal media contained (g l^{–1}): KH₂PO₄, 2; Na₂HPO₄, 4; (NH₄)₂SO₄, 1; and 4 ml trace metal stock solution (Rosenberger & Elsdon, 1960). Camphor was added at 2.5 g l^{–1} before autoclaving. Some loss of camphor occurred during autoclaving and the resulting saturated medium contained approximately 1.5 g l^{–1}. Cultures were usually grown according to the following procedure: 20 ml of medium in a 100 ml conical flask was inoculated from a slope and grown on an orbital shaker (150 r.p.m.) for 24 h. Subsequently, 10% inocula were transferred sequentially to 50 ml and 500 ml volumes of media, in 250 ml and 2 l conical flasks, respectively, at 12 h intervals. Two 500 ml cultures were used to inoculate 10 l of medium in a New Brunswick Microform laboratory fermenter which was stirred at 400 r.p.m. and aerated with sterile air at a rate of 3 l min^{–1}. Growth was monitored at 580 nm and further additions of camphor (0.5 g l^{–1}) made at readings of approximately 1.6 and 2.0. Cells were harvested by continuous flow centrifugation when the OD₅₈₀ reading reached approximately 2.5. For growth with (+)-camphor as carbon source larger batches of cells were obtained by using the 1 l fermenter culture to inoculate 30 l of non-sterile medium in a 50-plastic vessel which was stirred at 700 r.p.m. and aerated with sterile air at a rate of 7 l min^{–1}. Growth was monitored at 580 nm and further additions of (+)-camphor (1 g l^{–1}) were made at readings of 3.0 and 4.0. Cells were harvested when the optical density value exceeded 5.5 and while the cultures were still in the exponential phase of growth, typically after 5 h. Cell pastes were resuspended in an equal volume of 42 mM-phosphate buffer (pH 7.1) and stored at –20 °C until required.

Preparation of cell extracts. Cell suspensions were thawed at room temperature and disrupted by ultrasonic disintegration at 23 KHz with a probe amplitude of 18 μ m (MSE Soniprep 150). Each 100 ml portion was disrupted for eight 30 s periods, interspersed with 60 s of cooling on ice. Broken cell suspensions were centrifuged (48000 g, 3 °C, 45 min). The protein content of extracts was measured by the method of Gornall *et al.* (1949) and they were stored at –20 °C until required.

Buffers. The 42 mM-sodium/potassium phosphate buffer used routinely was prepared by dissolving 4 g Na₂HPO₄ and 2 g KH₂PO₄ in 1 l distilled water, giving a pH of 7.1. Phosphate buffers of different molarities were made by diluting this buffer as required. Other buffers were made by adjustment of a solution of the appropriate compound to the required pH with 1 M-NaOH or 1 M-HCl and diluting to the required concentration.

Enzyme assays. NADH dehydrogenase was assayed by following the rate of NADH oxidation when the enzyme fraction, containing 0.02–0.2 U of enzyme, was added to a 1 cm light-path cuvette that contained, in a final volume of 1 ml; 0.15 μ mol NADH, 25 nmol FMN and 40 μ mol phosphate buffer (pH 7.1).

Diketocamphane monooxygenase activities were routinely assayed either by measuring substrate-stimulated NADH oxidation spectrophotometrically or by measuring oxygen consumption polarographically. A typical reaction system contained, in a total volume of 3 ml: 0.5 μ mol NADH, 120 μ mol phosphate buffer (pH 7.1) and 0.025–0.25 U of oxygenase activity. After the endogenous rate was measured the reaction was started by the addition of 1 μ mol (+)- or (–)-camphor as appropriate. Purification of the oxygenase proteins yielded preparations that were progressively deficient in the NADH dehydrogenase component and, occasionally, in FMN and it was necessary to add these components to the standard assays. As the

quantities required varied with the degree of purification and assay situation they are indicated at the appropriate points in the text.

Enzyme units. One enzyme unit (U) is defined as the consumption of 1 μmol of substrate or the formation of 1 μmol product min^{-1} . All assays were done at 30 °C.

Protein measurement. The protein content of crude cell extracts was routinely measured by the modified biuret method of Gornall *et al.* (1949). A crude estimate of the protein content of column fractions was made by measuring A_{280} . For accurate measurement of the protein content of pooled column fractions and purified enzymes the modified biuret assay or the tannin-gum-arabic turbidometric assay (Mejbaum-Katzenellenbogen & Dobryszewska (1959) was used, depending upon the amount of material available.

Enzyme purification. The soluble NADH dehydrogenase was purified from (+)-camphor-grown *P. putida* ATCC 17453 according to the procedure of Trudgill *et al.* (1966) and had a specific activity of 300 U (mg protein^{-1}).

3,6-Diketocamphane 1,6-monooxygenase was purified from (–)-camphor-grown cells by a modification of the procedure described previously (Taylor & Trudgill, 1986) for the purification of 2,5-diketocamphane monooxygenase. The only significant modification was the substitution of fast protein liquid chromatography (FPLC) on a Hiload 16/10 Q Sepharose column for the Polyanion 17 column used previously. All procedures, with the exception of FPLC, were carried out at 4 °C.

Step 1. Cell extract, prepared from approximately 65 g of (–)-camphor-grown cells, was treated with saturated $(\text{NH}_4)_2\text{SO}_4$ to bring it to 50% saturation. After stirring at 4 °C for 30 min the precipitate was removed by centrifuging at 27000 g and the pellet discarded. The clear yellow supernatant was then brought to 75% saturation by addition of solid $(\text{NH}_4)_2\text{SO}_4$ and again stirred and centrifuged. The pellet was dissolved in 150 ml 21 mM-phosphate buffer (pH 7.1) and dialysed overnight against two changes (5 l each) of the same buffer.

Step 2. The dialysed solution was applied to a DEAE-cellulose column (11 $\times$ 2.5 cm) equilibrated with the same buffer. The column was washed with 4 column volumes of buffer and then developed by passage of a linear KCl gradient (300 ml, 0–0.4 M-KCl in phosphate buffer). The flow rate was 1 ml min^{-1} and 3–4 ml fractions were collected. Diketocamphane oxygenase-containing fractions were pooled as appropriate and dialysed against two changes (2 l each) of 50 mM-Tris/HCl buffer (pH 7.7) for a total of 7 h.

Step 3. FPLC was carried out on a Hiload 16/10 Q Sepharose column (Pharmacia Biosystems). Sequential chromatographic runs were performed in which the column load did not exceed 20 mg protein. Enzymes were eluted from the column by the passage of a linear KCl gradient (250 ml, 0–0.5 M-KCl in the Tris/HCl buffer) and 3 ml fractions collected. Fractions containing enzyme were pooled and stored at –20 °C until required.

2,5-Diketocamphane 1,2-monooxygenase from (+)-camphor-grown *P. putida* ATCC 17453 was purified using the same sequence of steps, scaled up to process the volume of extract obtained from 250 g of cells

PAGE. This was done under non-denaturing conditions on 1.5 mm-thick 6, 10 or 12.5% (w/v) polyacrylamide minigels (80 $\times$ 60 mm) according to the procedure of Davies (1964). SDS slab gel electrophoresis was done according to the procedure of Laemmli (1970) on 11, 12.5 and 15% (w/v) gels. Gels were stained with 0.2% (w/v) Coomassie brilliant blue R250 in ethanol/water/glacial acetic acid (9:9:2 by vol.) for at least 1 h and destained in water/ethanol/glacial acetic acid (13:5:2 by vol.).

Isoelectric focussing. Isoelectric focussing was carried out on 2 mm thick polyacrylamide gels over a pH range from 3 to 9 in an LKB Multiphor 2117 electrophoresis unit essentially as described by Vesterberg (1972). Myoglobin was used as a marker protein. After

focussing gels were fixed and washed in methanol/glacial acetic acid/water (5:1:4 by vol.) and stained with Coomassie brilliant blue R250

M_r determination. The M_r value of native 3,6-diketocamphane 1,6-monooxygenase was determined in the ultracentrifuge by the method of Yphantis (1964). A double sector cell was used with the enzyme solution in one compartment and the diffusate, 42 mM-phosphate buffer (pH 7.1) in the other. The rotor speed was 21740 r.p.m. and the initial protein concentration 0.5 mg ml^{-1} . The enzyme was stable for the duration of the experiment.

GLC. A Carol-Erba HRGC 5300 Mega series instrument fitted with an SE-34 column (0.32 mm $\times$ 30 m) with a film thickness of 0.25 mm (Alltech) was used. Carrier gas flow rate was 1.5 ml min^{-1} and the temperature programme 45–80 °C at 4 °C min^{-1} ; 80–150 °C at 25 °C min^{-1} and 150–200 °C at the maximum rate (approx. 30 °C min^{-1}).

Oxygen uptake measurement. O_2 consumption by cell suspensions or by cell extracts was determined by conventional Warburg manometry or with a Clark-type O_2 electrode in an agitated vessel maintained at 30 °C (Yellow Springs Instrument Co.).

Preparation of antibodies. A total of 150 μg of electrophoretically homogeneous 3,6-diketocamphane 1,6-monooxygenase from *P. putida* ATCC 17453, emulsified in Freund's complete adjuvant was injected subcutaneously at multiple sites into an adult female rabbit, followed by a similar injection in Freund's incomplete adjuvant after 3 weeks. A trial bleed of the animal was performed 2 weeks after the second injection and the full bleed 1 week later. The IgG fraction of the antiserum was partially purified by acidification (pH 4.5), ammonium sulphate precipitation and heat treatment (50 °C) according to the procedure of McKinney & Parkinson (1987). A sample of pre-immune serum was taken from the rabbit before immunization.

ELISA. ELISA were done on microtitre well plates with alkaline phosphatase-labelled goat anti-rabbit immunoglobulin (Bio-Rad) and *p*-nitrophenyl phosphate as substrate. Reactions were stopped by the addition of 50 μl 3 M-NaOH. A_{405} values were read on a Bio-Rad model 2550 plate reader.

Immunodiffusion. Ouchterlony double immunodiffusion analysis was done in 3 mm-thick gels made from PBS (1^{-1} : 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 , 0.2 g KH_2PO_4) solidified with 1.5% (w/v) purified agar. Centre wells were typically filled with 30 μl of control serum, test serum or purified antiserum and 10–25 μg of antigenic protein in 30 μl of solution placed in the outer wells. After 18 h incubation at 20 °C, plates were visually examined for precipitin lines.

Immunoblotting (Western blotting). After separation by SDS-PAGE the proteins were electrophoretically transferred at a constant voltage of 100 V for 60 min from the polyacrylamide gel onto a nitrocellulose sheet in a Bio-Rad Trans Blot electrophoretic transfer cell. Blotting buffer was composed of 25 mM-Tris, 192 mM-glycine and 20% (v/v) methanol, pH 8.3.

Rabbit antibodies against 3,6-diketocamphane 1,6-monooxygenase were used for antigen detection. After incubation overnight at 4 °C in PBS containing 3% bovine serum albumin, the nitrocellulose sheet was washed in PBS, then incubated for 1 h at room temperature with rabbit antibody solution diluted 2×10^3 -fold. The sheet was again washed in PBS, incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad) diluted 2×10^3 -fold in PBS for 1 h, washed again and developed with 5-bromo-4-chloro-3-indolyl phosphate/-nitroblue tetrazolium reagent (Bio-Rad).

Chemicals. (+)-Camphor, (–)-camphor (95 and 99% purity) were obtained from Sigma; DEAE-cellulose (DE-52) from Whatman and other chemicals were generally Analar grade from Merck. Gas chromatographic analysis of the 95% (–)-camphor confirmed that, as

reported by the manufacturers, the impurity was fenchone. Goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate and the substrate kit were obtained from Bio-Rad.

Results

Pseudomonas putida ATCC 17453 was capable of growth with either isomer of camphor as sole source of carbon. Growth rates and growth yields showed no significant differences. The oxidation of camphor was inducible and, irrespective of the isomeric configuration of the camphor used for growth, both isomers of camphor were oxidized rapidly without an initial lag phase. Addition of chloramphenicol ($50 \mu\text{g ml}^{-1}$) had no effect upon this oxidation pattern.

Fig. 2. Chromatography of NADH dehydrogenase (△), and (a) 3,6-diketocamphane 1,6-monooxygenase (▲) and (b) 2,5-diketocamphane 1,2-monooxygenase (▲) on DEAE-cellulose. (a). Protein (○) from the $(\text{NH}_4)_2\text{SO}_4$ fractionation (1200 mg) was loaded onto a 2.5×10 cm DEAE-cellulose column which was then developed as described in Methods and 3.8 ml fractions were collected. (b). Protein (○) from the $(\text{NH}_4)_2\text{SO}_4$ fractionation (4700 mg) was loaded onto a 5×10 cm DEAE-cellulose column which was then developed as described in Methods and 10 ml fractions were collected. Gradient sizes were adjusted to be directly proportional to the column bed volumes.

Enzyme assays

Difficulties attended the assay of the NADH-linked 2,5-diketocamphane 1,2-monooxygenase from *P. putida* ATCC 17453. The enzyme is a loose complex of two proteins with a dissociation constant of approx 50 nM (Taylor & Trudgill, 1986) and it is not possible to perform spectrophotometric assays with crude cell extract because of the presence of soluble NADH oxidases and free flavins that compete for NADH. Spectrophotometric assays can be employed subsequent to $(\text{NH}_4)_2\text{SO}_4$ fractionation. As purification of the oxygenating component progresses it becomes NADH dehydrogenase-depleted and it is necessary to add NADH dehydrogenase to display activity of fractions containing the oxygenase. In the absence of information to the contrary the assumption that a similar situation would appertain to oxygenation of (–)-camphor (and 3,6-diketocamphane) was suggested by the knowledge that a high level of the soluble NADH dehydrogenase was also induced by growth on (–)-camphor (Gunsalus *et al.*, 1965). This proved to be a useful starting point. Availability of substrates also presented a problem. 2,5-Diketocamphane 1,2-monooxygenase is also active towards (+)-camphor as a substrate (Conrad *et al.* 1965; Taylor & Trudgill, 1986) but is inactive towards (–)-camphor. The enzyme may be conveniently assayed with (+)-camphor which has the advantage of ensuring that the biological Baeyer-Villiger oxygenase is not confused with the NAD-linked 5-hydroxycamphor dehydrogenases. 3,6-Diketocamphane was not available to us and, since it has been reported that, in relatively crude protein preparations, (–)-camphor serves as a substrate for biological Baeyer-Villiger oxygenation (Gunsalus *et al.*, 1965), this terpene ketone was used as a substrate in the search for the enantiomer-specific oxygenase.

Purification of 3,6-diketocamphane 1,6-monooxygenase and 2,5-diketocamphane 1,2 monooxygenase

Preliminary purification trials with the 3,6-diketocamphane 1,6-monooxygenase exploited the regime already established for 2,5-diketocamphane 1,2-monooxygenase purification from (+)-camphor-grown *P. putida* (Taylor & Trudgill, 1986). $(\text{NH}_4)_2\text{SO}_4$ fractionation showed that, as with 2,5-diketocamphane 1,2-monooxygenase, an NADH-dependent oxygenase, active with (–)-camphor, was selectively precipitated in the 50–75% saturation fraction. In contrast, DEAE-cellulose chromatography yielded fractions with low oxygenase activity when (–)-camphor was used as substrate in the absence of added NADH dehydrogenase. As with the purification of 2,5-diketocamphane 1,2-monooxygenase from (+)-camphor-grown cells, some of the soluble NADH dehydrogenase carried over into the

Table 1. Purification of 2,5- and 3,6-diketocamphane monooxygenases from *P. putida* ATCC 17453

Fractions were assayed for monooxygenase activity with (+)- or (–)-camphor as substrate, as appropriate. Typically 0.15–0.4 U NADH dehydrogenase was added to fractions deficient in this enzyme and FMN was supplied at 5–10 μ M to assays of fractions from FPLC columns. (a), 3,6-Diketocamphane monooxygenase from extract of (–)-camphor-grown cells; (b), 2,5-diketocamphane monooxygenase from extract of (+)-camphor-grown cells.

Stage	Total protein (mg)	Total activity* (U)	Specific activity*† [U (mg protein) ^{–1}]	Yield† (%)	Purification (-fold)
(a)					
Crude extract	8664	ND	ND	ND	–
(NH ₄) ₂ SO ₄ fractionation	1260	17.8	0.014	100	1
DEAE-cellulose chromatography	73	4.5	0.062	25.3	4.4
Q Sepharose	16	3.9	0.244	21.9	17.4
(b)					
Crude extract	30228	ND	ND	ND	–
(NH ₄) ₂ SO ₄ fractionation	4762	84	0.018	100	1
DEAE-cellulose chromatography	133	22	0.165	26.2	9.2
Q-Sepharose	49	16.4	0.335	19.5	18.6

ND, Not determined, see Methods.

* Not the maximum activity, not extrapolated to ∞ protein concentrations.

† (NH₄)₂SO₄ fraction used as 100% yield and specific activity reference point.

50–75% saturation fraction and was eluted as a distinct fraction on the DEAE-cellulose column. However, when NADH dehydrogenase (0.4 U), purified from the 0–50% saturation (NH₄)₂SO₄ fraction from extract of (+)-camphor-grown cells, according to the procedure of Trudgill *et al.* (1966), was added to assays, fractions with activity towards (–)-camphor were revealed (Fig. 2a).

A clear distinction between this enzyme and 2,5-diketocamphane 1,2-monooxygenase was immediately apparent, since the oxygenase and residual NADH dehydrogenase were separated from each other with only a margin of overlap. In contrast, chromatography of a 50–75% (NH₄)₂SO₄ saturation protein fraction from (+)-camphor-grown cells on DEAE-cellulose under closely similar conditions always resulted in significant overlap of the NADH dehydrogenase and 2,5-diketocamphane oxygenase peaks so that, although this oxygenating complex was not fully assembled, significant activity was observed (Fig. 2b).

Purification of 3,6-diketocamphane 1,6-monooxygenase was completed by FPLC on a Q-Sepharose column (Pharmacia-LKB). This step introduced an additional complication by stripping flavin from the enzyme and, in addition to purified NADH dehydrogenase, FMN had also to be added to assays for oxygenase activity to be revealed [flavin–adenine dinucleotide (FAD) was ineffective]. The 2,5-diketocamphane 1,2-monooxygenase from (+)-camphor-grown cells was eluted at a different position on the gradient

and it also lost its flavin component which is known to be FMN (Taylor & Trudgill, 1986) on this particular column.

Preliminary native PAGE analysis revealed that the 2,5-diketocamphane 1,2-monooxygenase still contained some impurities and a portion of it was therefore subjected to a clean-up step by FPLC on a Superose 12 HR 10/30 column (Pharmacia-LKB) for use in immunological studies. Table 1 shows typical purification results for the two enzymes.

Product of oxygenation of (–)-camphor by 3,6-diketocamphane monooxygenase

Previous proposals that (+)- and (–)-camphor are metabolized by converging enantiomeric reaction sequences were supported by the isolation of the same hydroxycamphor and diketocamphane intermediates, in their respective enantiomeric forms from cultures growing on the respective camphor isomers (Gunsalus & Marshall, 1971). Purified 3,6-diketocamphane monooxygenase (0.2 U) was incubated with 3 μ mol (–)-camphor in the presence of 750 nmol NADH, 7.5 nmol FMN and 1.2 U NADH dehydrogenase [purified from (+)-camphor grown *P. putida*] in phosphate buffer, pH 7.1, for 8 min at 30 °C. The product was extracted with diethyl ether and analysed by GLC. Because of the inclusion of excess camphor a significant portion remained at the end of the reaction (retention time

Fig. 3. Analysis of a mixture of (1) 3,6-diketocamphane 1,6-monooxygenase (24 μ g) and (2) 2,5-diketocamphane 1,2-monooxygenase (52 μ g) by FPLC on a Mono Q column. Enzymes were eluted by the passage of a linear gradient from 0 to 0.5 M-KCl in 50 mM-Tris/HCl buffer (---) through the column. Flow rate, 1 ml min⁻¹; fraction size, 1 ml.

10.92 min) to act as an internal marker for the reaction product (retention time 19.04 min), giving relative retention times of 1:1.74. When an identical experiment was done with (+)-camphor and the 2,5-diketocamphane 1,2-monooxygenase the (+)-camphor had a retention time of 10.86 min and the reaction product, 18.98 min, giving relative retention times of 1:1.75. This confirmed that the oxygenase purified from (–)-camphor grown cells was indeed the 3,6-diketocamphane 1,6-monooxygenase proposed previously (Gunsalus & Marshall, 1971).

Properties of 3,6-diketocamphane 1,6-monooxygenase and comparison with 2,5-diketocamphane 1,2-monooxygenase

(i) Analytical FPLC

The pure 3,6-diketocamphane 1,6-monooxygenase was subjected to FPLC on a Mono Q column and eluted as a sharp, symmetrical protein peak quite distinct from the 2,5-diketocamphane 1,2-monooxygenase, clearly showing the two enzymes to be discrete proteins (Fig. 3).

(ii) PAGE

Native PAGE of the 3,6-diketocamphane 1,6-monooxygenase gave a single protein band that migrated

Fig. 4. A montage of SDS-PAGE and Western blotting of purified subunits of 3,6-diketocamphane 1,6-monooxygenase and 2,5-diketocamphane 1,2-monooxygenase separated on a 12.5% polyacrylamide gel according to the procedure of Laemmli (1970). Lanes: 1, M_r (29000–205000; Sigma); 2, 2,5-diketocamphane monooxygenase (7 μ g); 3, 3,6-diketocamphane 1,6-monooxygenase (7 μ g) all stained with Coomassie brilliant blue; 4, Western blot of 2,5-diketocamphane 1,2-monooxygenase subunit probed with a 1:2000 dilution of partially purified antibody raised to 3,6-diketocamphane 1,6-monooxygenase; 5, Western blot of 3,6-diketocamphane 1,6-monooxygenase probed with the same antibody preparation. Blots were developed with a 1:2000 dilution of goat anti-rabbit IgG, conjugated to alkaline phosphatase, as described in Methods.

ahead of the 2,5-diketocamphane 1,2-monooxygenase. This latter enzyme migrated as two adjacent protein bands as reported previously (Yu & Gunsalus, 1969; Taylor & Trudgill, 1986). SDS-PAGE gave a single protein band for each enzyme on gels ranging from 11 to 15% acrylamide (Fig. 4). A small difference in M_r was apparent and, by reference to the marker proteins, the 3,6-diketocamphane 1,6-monooxygenase subunit has an M_r of $40\,300 \pm 800$ (average of 3 runs), some 3000–4000 smaller than the subunit of the 2,5-diketocamphane 1,2-monooxygenase.

(iii) pI values

Isoelectric focussing gave a pI value of 5.5 for the 3,6-diketocamphane 1,6-monooxygenase while the value for 2,5-diketocamphane 1,2-monooxygenase was lower at 4.6.

(iv) M_r of native 3,6-diketocamphane 1,6-monooxygenase

The M_r of the native enzyme was determined to be 76000 by ultracentrifugal analysis from Yphantis (1964) plots

of results taken from four separate sets of plate readings. This value too was smaller than the 78000 reported for the 2,5-diketocamphane 1,2-monooxygenase (Taylor & Trudgill, 1986).

3,6-Diketocamphane 1,6-monooxygenase; electron donors and electron carriers

The purified enzyme exhibited a λ_{max} at 282 nm and no evidence for the presence of any remaining flavin prosthetic group. The active oxygenating complex was reconstituted by the addition of FMN but FAD was ineffective. The oxygenase was inactive with (+)-camphor. Unfortunately neither 2,5- nor 3,6-diketocamphane was available for testing as an alternative substrate.

The purified NADH dehydrogenase from (+)-camphor-grown cells was an efficient electron donor and dictated the absolute specificity of the reconstituted complex for NADH.

Previous suggestions that the same soluble NADH dehydrogenase is synthesized irrespective of the camphor isomer used as carbon source (Gunsalus *et al.*, 1965) are supported by these studies. Similar induced levels of NADH dehydrogenase were found in crude extracts of (+)- and (–)-camphor-grown cells. Closely similar behaviour during $(\text{NH}_4)_2\text{SO}_4$ fractionation and DEAE-cellulose chromatography, and identical elution patterns when enzyme from either source was subjected to FPLC on the Mono Q column all suggest that a common dehydrogenase serves to provide electrons to either 2,5- or 3,6-diketocamphane monooxygenase.

Immunological studies

(i) Immunodiffusion

In immunodiffusion experiments on Ouchterlony plates antibodies raised against purified 3,6-diketocamphane 1,6-monooxygenase gave fairly weak precipitin bands with the 3,6-diketocamphane oxygenase but, although a range of combinations of antibody and 2,5-diketocamphane monooxygenase was used, no precipitin bands were seen at any time with the latter enzyme.

(ii) ELISA

Interaction of the antibody with the two enzyme proteins was then explored in a more quantitative manner by ELISA. In a typical assay, PVC wells were coated with 2 μg antigen (3,6-diketocamphane monooxygenase or 2,5-diketocamphane monooxygenase), drained, washed with PBS followed by addition of 50 μl of control (pre-immune) serum, immune serum or partially purified antibody, over a dilution range from 10^1 to $> 10^4$. Wells were again drained, washed, 200 μl of a 10^3 dilution of

the goat anti-rabbit alkaline phosphatase conjugate added, incubated for 2 h, drained, washed and substrate added. Reactions were stopped after 30 min at 37 °C and the A_{405} of each well measured against the appropriate control. Control absorbance values did not exceed 0.01. The interaction of antibodies raised against 3,6-diketocamphane monooxygenase was almost identical with both antigens over the concentration range investigated.

(iii) Western blotting

Immunoblotting analysis was performed for two reasons: first, to confirm the close relationship between the two enantiomeric isofunctional enzymes, that was suggested on the basis of subunit size, holoenzyme structure, flavin specificity and use of a common NADH dehydrogenase; and second, to establish whether or not the two electrophoretically distinct forms of 2,5-diketocamphane monooxygenase both cross-reacted with the antibody. Accordingly, immunoblotting analysis was performed with both enzymes run on native and SDS-polyacrylamide gels. Dilutions of partially purified antibody in excess of 10^3 were necessary to suppress non-specific background reaction and under these conditions the 3,6-diketocamphane monooxygenase, the two bands of the 2,5-diketocamphane 1,2-monooxygenase (native gels) and the subunit band of each enzyme (SDS gels) all cross-reacted with the antibody. Results for an SDS gel are shown in Fig. 4.

(iv) Enzyme induction patterns

Although we decided to adopt a 'single isomer' approach to cell growth for enzyme isolation in that each enzyme was initially isolated from cells grown on the respective camphor enantiomer, the whole cell oxidation studies suggested a less discriminatory situation; cells grown on either isomer of camphor being capable of the immediate rapid oxidation of both. Cultures were grown on (+)- and 95% pure (–)-camphor as described in Methods. Equal quantities of cells were disrupted and, following centrifugation, equal amounts of protein were subjected to chromatography on separate indentical DEAE-cellulose columns. Fractions from both columns were assayed for 2,5-diketocamphane monooxygenase and 3,6-diketocamphane monooxygenase, and active fractions pooled. Pool volumes were measured and a sample from each assayed for its respective monooxygenase in the presence of excess NADH dehydrogenase and an optimum amount of FMN. Because of the number of steps involved and the difficulties of optimizing assays it was difficult to obtain precise values for oxygenase component activity. However, the results shown for the partially purified enzymes in Table 2 clearly demonstrated the induction of both monooxygenases irrespective of the camphor isomer used for

Table 2. Activities of 2,5-diketocamphane and 3,6-diketocamphane monooxygenases obtained by DEAE-cellulose chromatography of extracts of (+)- and (-)-camphor-grown *P. putida* ATCC 17453

Crude cell extracts (4 g protein) obtained from cells grown on (+)- or (-)-camphor were subjected to DEAE-cellulose chromatography on separate columns (20 × 2.5 cm) which were washed with 300 ml 21 mM-phosphate buffer (pH 7.1) and developed with a linear gradient from 0 to 0.4 M-KCl in 600 ml of the same buffer. Fractions of 6 ml were collected, 2,5- and 3,6-diketocamphane monooxygenases assayed, pooled separately, and each pool assayed in the presence of NADH dehydrogenase (1.6 U) and FMN (1.5–4 µM).

Growth substrate	Enzyme	Volume pooled (ml)	Total activity (U)	Activity ratio
(+)-Camphor	2,5-diketocamphane monooxygenase	21.9	29.6	
(+)-Camphor	3,6-diketocamphane monooxygenase	36.5	8.4	3.5:1
(-)-Camphor	2,5-diketocamphane monooxygenase	30.0	54.3	
(-)-Camphor	3,6-diketocamphane monooxygenase	36.0	15.9	3.4:1

growth. Neither enzyme was detectable when extract of succinate-grown cells was similarly processed.

Discussion

Growth of *P. putida* ATCC 17453 on (-)-camphor, followed by (NH₄)₂SO₄ fractionation of the resultant cell extract, confirmed the enantiomeric specificity of the Baeyer-Villiger monooxygenase induced by growth on this isomer. Although 3,6-diketocamphane was not available it is logical to presume that, as is the case for 2,5-diketocamphane 1,2-monooxygenase, a second keto group (in this case in the 3-position) is not a requirement and that the ability to distinguish 3,6-diketocamphane from the 2,5- derivative is conferred by the position of the carbonyl group adjacent to the most substituted bridgehead. The two enzymes are closely related in size and subunit structure. The holoenzymes can be distinguished by ion exchange purification procedures, reflecting their different pI values, and by non-denaturing PAGE. SDS-PAGE of the 3,6-diketocamphane 1,6-monooxygenase demonstrated that it consists of two electrophoretically identical subunits which are about 3 kDa smaller than the pair from which the 2,5-diketocamphane monooxygenase is constructed (Taylor & Trudgill, 1986). Given the difficulties of assay optimization, the specific activities of the pure proteins under identical assay conditions showed consistent differences (Table 1). The lower specific activity of the 3,6-diketocamphane 1,6-monooxygenase was also re-

flected in relative recoveries of each enzyme although, paradoxically, (-)-camphor appeared to be the better inducer (Table 2). The two enzymes are very similar in other respects. Each binds flavin in a non-covalent manner; each apoenzyme is specifically reactivated by FMN and both can make use of the NADH dehydrogenase (EC 1.6.8.1) induced by growth with (+)-camphor as the electron donor. Similarly, the induction of an NADH dehydrogenase during growth on (-)-camphor that can donate electrons to both oxygenases and behaves in an identical manner when subjected to FPLC on a Mono-Q column suggests that both oxygenases use a common NADH oxidase. It has been suggested that the weak association of 2,5-diketocamphane 1,2-monooxygenase and the NADH dehydrogenase is through the FMN, for which they both have affinity (Taylor & Trudgill, 1986). 2,5-Diketocamphane 1,2-monooxygenase can be purified essentially with the FMN intact, by avoiding FPLC ion exchange columns but, as the NADH dehydrogenase has a rather weak binding affinity (K_D 4.5 µM) for FMN (Trudgill *et al.*, 1966), a link through the prosthetic group would explain the fragility of the oxygenating complexes formed by both the enantiomeric oxygenases with the NADH dehydrogenase.

Immunological investigations again stressed the close relationship between the two proteins. Ouchterlony double diffusion, immunoprecipitation experiments gave a weak single precipitin line with antibody and antigen under a variety of conditions. Failure to detect any precipitation with 2,5-diketocamphane 1,2-monooxygenase is puzzling since ELISAs showed no major differences in binding of antigen or 2,5-diketocamphane 1,2-monooxygenase to the antibody and, in the immunoblotting experiments, both native enzymes and the subunits bound the antibody.

Although these experiments have established some of the more fundamental characteristics of these two enantiomer-specific isofunctional biological Baeyer-Villiger monooxygenases, a precise understanding of their evolutionary and structural relationship, and the significance of differences in overall charge and molecular mass which make them able to distinguish and select enantiomeric camphor molecules must await more detailed study, including sequence determination, subunit assembly and binding site structure.

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