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Purification and Properties of α-Pinene Oxide Lyase from *Nocardia* sp. Strain P18.3

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α-Pinene oxide is an intermediate in the degradation of α-pinene by *Nocardia* sp. strain P18.3 and some *Pseudomonas* strains. The epoxide is cleaved by a lyase which catalyzes a concerted reaction in which both rings of the bicyclic structure are cleaved with the formation of *cis*-2-methyl-5-isopropylhexa-2,5-dienal. The enzyme has been purified to homogeneity from *Nocardia* sp. strain P18.3. It was induced by growth with α-pinene and constituted 6 to 7% of the soluble protein of cell extracts. The apparent molecular weight of the native enzyme was 50,000 by ultracentrifugal analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gave two dissimilar subunits with apparent molecular weights of 17,000 and 22,000. The enzyme was devoid of prosthetic groups, had no cofactor requirement, and had a broad pH activity range, a K_m for α-pinene oxide of 9 μM, and a turnover number of 15,000. Inhibitors included sulfhydryl reactive compounds, terpene epoxides, and pinane derivatives with substituent groups at carbon 3. A mechanism for the concerted reaction has been proposed in which decyclization is initiated by donation of a proton from the catalytic center to the oxygen of the epoxide with consequent destabilization. In vitro the enzyme was inactivated during catalysis, and a reactive cationic intermediate may be responsible for this phenomenon. The enzyme should be classified as a lyase EC 4.99.–.–.

α-Pinene oxide lyase has been implicated as the enzyme mediating cleavage of both carbocyclic rings of the bicyclic terpene structure when *Nocardia* sp. strain P18.3 and certain *Pseudomonas* strains are grown with α-pinene as the sole source of carbon (3). The epoxide has been shown to be formed by an NADH-linked α-pinene monooxygenase in *Pseudomonas fluorescens* (N. C. Floyd, A. Burfield, A. Magalhaes, P. M. Rhodes, and D. J. Best, EMBO Workshop, Geneva, 1986), and its involvement as an intermediate in α-pinene oxidation by the *Nocardia* strain is supported by circumstantial evidence (3). The lyase is specifically induced by growth of the organisms on α-pinene and is present at high specific activity (10 to 20 U/mg of protein) in cell extracts (3). The product of ring cleavage has been unequivocally identified as the branched-chain unsaturated aldehyde *cis*-2-methyl-5-isopropylhexa-2,5-dienal (3); in crude cell extract systems, the reaction does not require oxygen, added electron donors, acceptors, or other components.

In this paper we report the purification of α-pinene oxide lyase from *Nocardia* sp. strain P18.3 and some established properties of the enzyme, and we postulate a mechanism for concerted cleavage of both rings of the bicyclic substrate.

MATERIALS AND METHODS

Source and growth of organisms. *Nocardia* sp. strain P18.3, isolated from woodland soil, was maintained on nutrient agar slants and grown in liquid medium with α-pinene supplied in the vapor phase as described in the accompanying paper (3).

Cell extracts. Harvested cells were disrupted in the Hughes press and supernatant extracts were obtained as described in the accompanying paper (3).

Protein estimation. The protein contents of crude cell extracts and of bulk fractions obtained during lyase purification were measured by either the biuret method (2) or the Lowry et al. method (5) with crystalline bovine serum albumin as the standard. A rough indication of the protein content of column fractions was obtained by measuring absorbance at 280 nm. Typically, crude cell extracts contained 20 mg of protein per ml.

Preparation of terpene "solutions." Compounds of low water solubility were used as aqueous or buffered emulsions prepared as previously described (3). Emulsions of α-pinene oxide were routinely prepared in glycine-NaOH buffer (pH 9.0) to prevent spontaneous decomposition of the compound (6, 7).

Enzyme units. All enzyme activity is expressed as micromoles of product formed per minute.

Assay of α-pinene oxide lyase. The enzyme was assayed spectrophotometrically at 30°C by following the formation of the ring cleavage product 2-methyl-5-isopropylhexa-2,5-dienal at 235 nm. The initial product of ring cleavage is the *cis* isomer of the aldehyde (3), but assay in glycine-NaOH buffer at pH 9 (used to suppress spontaneous decomposition of the α-pinene oxide) results in a rapid isomerization of the product to the *trans* isomer. A typical assay contained, in 1 ml, 0.1 mmol of glycine-NaOH buffer (pH 9.0) and protein (0.025 to 0.15 U of activity). After preincubation at 30°C the reaction was initiated by the addition of 1 μmol of α-pinene oxide. Activities were calculated using the higher Σ_{235} of the *trans* isomer of 2-methyl-5-isopropylhexa-2,5-dienal (13,000).

Purification of α-pinene oxide lyase. All procedures were performed at 2 to 4°C.

(i) Step 1. Crude cell extract (15 ml) was diluted to a 70-ml volume with 21 mM Na^+-K^+-phosphate buffer (pH 7.1) and applied to a DEAE-cellulose column (2.5 by 11 cm). Unbound protein was removed by passage of 21 mM phosphate buffer through the column, and bound proteins were then displaced by passage of a linear gradient, constructed from 250 ml of 21 mM phosphate buffer (pH 7.1) and 250 ml of the same buffer containing 0.5 M KCl, through the column. Fractions (5 ml) were collected. Typically, fractions 61

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through 77 contained lyase activity and were pooled. The pooled fractions were concentrated by using an Amicon XM-50 Diaflo filter (Amicon Corp., Lexington, Mass.).

(ii) Step 2. The concentrated protein solution (typically 30 to 40 mg of protein in 5 ml of 21 mM phosphate buffer) was purified further by passage through a Sephadex G-100 column (2.5 by 80 cm), and 10.7-ml fractions were collected. Typically fractions 15 through 17 contained lyase activity. They were pooled and concentrated as in step 1.

(iii) Step 3. The final purification stage involved application of the concentrated protein solution (typically 8 to 10 mg of protein in 3 ml of 21 mM phosphate buffer) to a preparative polyacrylamide gel (10% acrylamide, 3 mm thick, 16 by 18 cm). Electrophoresis in the vertical mode in a Bio-Rad protean II apparatus (Bio-Rad Laboratories, Richmond, Calif.) was performed at a current of 70 mA until the tracking dye (bromophenol blue) was 5 mm from the bottom of the gel (10 h).

α-Pinene oxide lyase activity was detected by activity staining (3), the enzyme band was excised from the gel, and the protein was recovered by electroelution. The apparatus consisted of a small glass funnel with a closed length of dialysis tubing containing 1 to 2 ml of 21 mM phosphate buffer (pH 7.1) attached to the stem, which was sealed with a 5% acrylamide plug. Slices of the slab gel containing the lyase were placed upon the surface of the gel plug, buffer was added (dialysis bag in anodic buffer), and electrophoresis was carried out at 10 mA until the enzyme was translocated into the dialysis bag.

Analytical polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis under nondenaturing conditions was performed on 1.5-mm-thick slab gels according to the directions supplied by Bio-Rad. Sodium dodecyl sulfate-gel electrophoresis was carried out by the procedure of Laemmli (4). Gels were stained routinely with 0.25% Coomassie brilliant blue R-250 dissolved in ethanol-acetic acid-water (5:1:4) for 2 h and cleared by washing in ethanol-acetic acid-water (3:1:6).

Isoelectric focusing. Isoelectric focusing under denaturing conditions was performed with thin layers of a 5% polyacrylamide gel containing 2% ampholines (equal volumes of the pH 3.5 to 5.0 and pH 3.5 to 10 ranges), according to the directions supplied by Bio-Rad, and an LKB Multiphore 2117 electrophoresis unit (LKB, Bromma, Sweden). Gels were prefocused, and electrophoresis was carried out at 350 V for 6 h. Isoelectric focusing of the native enzyme was performed by the same procedure with urea and Triton X-100 omitted from the system.

Proteins were visualized by staining with 0.25% Coomassie brilliant blue R-250 for 15 h followed by clearing as described above. The pH gradient of the gels was established by soaking 2-mm slices from a 1-cm-wide gel strip in 0.5 ml of distilled water and measuring of the pH of each sample with an electrode.

Ultracentrifugal analysis. The molecular weight of the native enzyme was determined in a Beckman model E analytical ultracentrifuge by the method of Yphantis (8). The rotor speed was 25,900 rpm. One compartment of the double sector cell contained enzyme at an initial concentration of 0.5 mg/ml, whereas the other contained dialysate (42 mM Na^+-K^+-phosphate buffer, pH 7.1) as the reference. The enzyme was stable for the duration of the experiment (24 h).

Chemicals. Sources of terpenes were as described in the accompanying paper (3). Pinan-3-one was prepared as described by Brown and Garg (1). All other reagents were of analytical grade and supplied either by BDH Chemicals or Sigma (London) Chemical Co., both of Poole, Dorset, United Kingdom.

TABLE 1. Purification of α-pinene oxide lyase from *Nocardia* sp. strain P18.3

Step	Vol (ml)	Protein (mg)	Enzyme		Purification (fold)	% Recovery
			Total U	U/mg		
Crude extract	70	273	5,460	20	1	100
DEAE-cellulose	6.5	36	2,925	80	4	54
Sephadex G-100	7.5	9.2	1,980	214	11	36
Electrophoresis[a]	4.4	2.9	906	312	15	17

[a] Preparative polyacrylamide gel electrophoresis.

RESULTS

Purification of α-pinene oxide lyase. Preliminary investigations established that no significant purification was obtained either by ammonium sulfate fractionation or by preincubation of cell extracts at extremes of pH or elevated temperature. DEAE-cellulose chromatography resulted in a fourfold purification of the lyase, and passage through Sephadex G-100 yielded further purification with coincidence of lyase activity and a major protein peak. Preparative polyacrylamide gel electrophoresis yielded essentially pure enzyme, and the purification regime is shown in Table 1.

The homogeneity of material purified only 15-fold (Fig. 1) indicated that the enzyme constitutes some 6% of the extracted soluble cell protein. As a consequence of this the enzyme is easily located as one of the more heavily stained bands on nondenaturing polyacrylamide gels of crude cell extract.

Confirmation of enzyme purity. The purity of enzyme obtained by the routine procedure (Table 1) was further

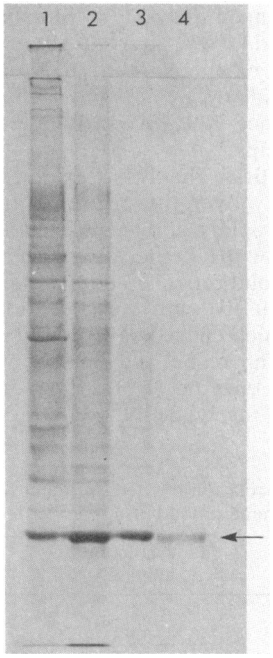

FIG. 1. Progress of α-pinene oxide lyase purification monitored by polyacrylamide gel electrophoresis. Samples from different purification steps were electrophoresed on 10% slab gels and stained with Coomassie brilliant blue. Lanes: 1, 150 μg of crude cell extract; 2, 75 μg of concentrated DEAE-cellulose fraction; 3, 50 μg of concentrated Sephadex G-100 fraction; 4, 50 μg of protein eluted from the preparative polyacrylamide gel. The protein band corresponding to lyase activity is indicated by the arrow.

investigated by nondenaturing polyacrylamide gel electrophoresis on 5, 10, 12, 15, and 22% polyacrylamide disc gels. In each case a single Coomassie brilliant blue-stained band was obtained. Electrophoresis on a 12 to 20% polyacrylamide linear gradient slab gel also gave a single stained protein band.

Molecular weight and subunit structure. Gel filtration on a column (2.5 by 84 cm) of Sephacryl S-200 gave an approximate M_r of 40,000; the standards used were cytochrome *c*, ovalbumin, malate dehydrogenase, lactate dehydrogenase, and fumarase. An accurate determination of the molecular weight of pure α-pinene oxide lyase was made by the Yphantis (9) method, which gave linear plots and an M_r of 50,000. The subunit composition was investigated by gel electrophoresis of the reduced denatured enzyme in the presence of 0.1% sodium dodecyl sulfate and 0.1% mercaptoethanol, which yielded two protein bands. A comparison of the distances migrated by the two subunits with the migration distances of selected polypeptide chains of known molecular weights in two separate experiments resulted, in each case, in calculated subunit molecular weights of 22,000 and 17,000. The results are equally compatible with a two (α, β)- or three (α, β, β)-subunit assembly forming the native enzyme, although in both cases the discrepancies revealed are rather large.

Isoelectric points the native enzyme and its subunits. Isoelectric focusing on nondenaturing gels gave a single major protein band with a pI of 4.0 with only traces of contaminating proteins present. Focusing runs of the dissociated enzyme in the presence of urea and Triton X-100 resulted in two protein bands with pIs of 3.9 and 4.5, confirming that the lyase is composed of two different subunits.

Catalytic requirements and stability. The native enzyme has no requirement for exogenous cofactors; it is active under anaerobic assay conditions and retains its catalytic activity after prolonged dialysis (48 h) or incubation at 4°C for extended periods (93% after 10 days). The absorbance spectrum of the enzyme showed only the peak due to aromatic amino acids (λ_{max}, 281 nm) with no distortions or additional absorbance indicative of the presence of nonprotein prosthetic groups.

Catalytic properties. The lyase exhibited no significant variation in activity over the pH range from 7.0 to 10.0. Activity declined rapidly above pH 10.0 and could not be tested reliably below pH 7.0 because of the instability of the substrate in acid solution. The activity was independent of the buffer system used, with the exception of Tris hydrochloride buffer, which caused severe inhibition at all tested pH values. β-Pinene oxide and limonene oxide were not substrates for the enzyme. A K_m for α-pinene oxide, in assays carried out in glycine-NaOH buffer at pH 9.0 was calculated as approximately 9 μM. Difficulties in preparing accurate solutions of the substrate, due to its low solubility and volatility, introduced inaccuracies into the assays, and these were compounded by inactivation of the enzyme during catalysis.

TABLE 2. Correlation between product formation and α-pinene oxide lyase inactivation[a]

α-Pinene oxide lyase added (pmol)	Product formed[b] (nmol)	Product/substrate molar ratio
1.3	16.1	12,400
2.6	32.9	12,700
5.2	59.5	11,400
6.5	78.0	12,000
7.8	86.9	11,100
10.4	116.0	11,200
13.0	143.0	11,000

[a] Assays were performed at pH 9.0 in glycine-NaOH buffer.
[b] Product formation at the end of the reaction was calculated from Σ_{235} 13,000 for the *trans* isomer of 2-methyl-5-isopropylhexa-2,5-dienal.

TABLE 3. Effects of terpenes on the activity of α-pinene oxide lyase from *Nocardia* sp. strain P18.3[a]

Addition	Concn (mM)	Relative activity (%)
No addition		100
β-Pinene oxide	0.013	47
β-Pinene oxide	0.11	13
β-Pinene oxide	2.8	1
Limonene oxide	2.8	68
Pinan-3-ol	2.8	12
Pinan-3-one	2.8	1
Carvone	2.8	1

[a] Assays were performed in glycine-NaOH buffer at pH 9.0 with 0.1 U of lyase and α-pinene oxide added at 0.56 mM.

In the presence of limited amounts of substrate and excess enzyme the formation of the reaction product, *cis*-2-methyl-5-isopropylhexa-2,5-dienal in buffers other than glycine-NaOH (*trans*-2-methyl-5-isopropylhexa-2,5-dienal in glycine-NaOH buffer [3]), was stoichiometric; 1 μmol was produced per μmol of α-pinene oxide introduced into the assay. However, when the amount of enzyme introduced into the assay was limited a direct relationship between the amount of enzyme added and the amount of product formed was obtained (Table 2). It was calculated that each molecule of the lyase produced, on average, 11,000 molecules of product before becoming catalytically inactive. This inactivation occurred at all tested pH values and was independent of the buffer system used. Addition of the reaction product or its *trans* isomer, isolated as described in the accompanying paper (3), to spectrophotometric assay systems did not cause marked inhibition of the reaction.

Inhibitors of α-pinene oxide lyase. Investigations of the substrate specificity of the lyase coupled with a futile search for possible intermediates between α-pinene oxide and the acyclic aldehyde revealed that a number of closely related terpene compounds were potent inhibitors of the enzyme (Table 3). These included β-pinene oxide and molecules in which the pinane skeleton is oxygenated at carbon 3. Studies with group-specific inhibitors showed marked sensitivity toward sulfhydryl active agents (Table 4) but no inhibition by a range of other tested compounds.

DISCUSSION

Floyd et al. (EMBO Workshop) reported that the metabolism of the bicyclic terpene α-pinene to an acyclic aldehyde by a strain of *P. fluorescens* required only two enzymic stages: the formation of α-pinene oxide by an NADH-dependent monooxygenase and decyclization by an enzyme that cleaved both rings of the terpene. It has been established that α-pinene oxide also plays a key role in the degradation of α-pinene by *Nocardia* sp. strain P18.3 and that cleavage of this epoxide is catalyzed by an induced lyase, which forms *cis*-2-methyl-5-isopropylhexa-2,5-dienal as the first detectable reaction product (3).

Purification of the lyase from the *Nocardia* strain was achieved in three steps (Table 1). The enzyme, induced by growth with α-pinene (3) is one of the quantitatively important proteins in the cell, constituting 6 to 7% of the soluble cell protein. Because *Nocardia* sp. strain P18.3 could only

TABLE 4. Effect of inhibitors on the activity of α-pinene oxide lyase[a]

Inhibitor[b]	Concn (mM)	Relative activity (%)
No addition		100
5,5'-Dithiobis-2-nitrobenzoate	0.5	1
p-Hydroxymercuribenzoate	0.44	1
Sodium arsenite	11.0	80
Iodoacetamide	0.24	95
KCN	12.4	95

[a] See footnote *a* of Table 3.

[b] The following compounds, at the concentrations indicated, did not cause any inhibition: tiron (0.3 mM), paraoxon (0.12 mM), phenylmethylsulfonyl fluoride (0.6 mM), sodium azide (11 mM).

be grown in Erlenmeyer flasks with α-pinene provided from a vapor tube (3), large crops of cells were difficult to produce. However the large amount of the lyase present in crude cell extract, coupled with acceptable recovery at each stage of purification, allowed useful amounts of pure enzyme to be obtained (Table 1). The specific activity of the lyase in crude extracts is high (10 to 20 U/mg of protein), and the pure enzyme has a turnover number of about 15,000.

Since the source of the α-pinene oxide in vivo is likely to be α-pinene from which it arises by monooxygenation (Floyd et al., EMBO Workshop) and monooxygenases typically have turnover numbers from 1,000 to 1,500, the level of α-pinene oxide lyase in cell extracts may seem excessive. A logical explanation for this can be drawn from the established instability of α-pinene oxide in aqueous solution at near-neutral pH (3, 6, 7); because of the chemical reactivity of the three-member epoxide ring, an ability to catalyze the rapid irreversible conversion of this compound into the *cis* aldehyde may be of significant advantage.

A proposed mechanism for the cleavage of the bicyclic epoxide leading to the acyclic aldehyde which does not involve any exogenous cofactors and has no identifiable stable intermediates is shown in Fig. 2. The most logical explanation that is compatible with the established properties is one in which the enzyme initiates a catalytic sequence involving a series of concerted rearrangements. We propose that the reaction starts by the addition of a proton, from a donor in the catalytic center, to the oxygen of the enzyme-bound epoxide (I). This destabilization of the epoxide (II) is followed by formation of a pinyl carbo-cation with a hydroxyl group at carbon 3 (III). Rearrangement of the pinyl carbo-cation to form the monocyclic carbo-cation (IV) is followed by a further rearrangement to the more stable tertiary carbo-cation (V). The series of concerted electron shifts may be initiated by a nucleophilic attack on the hydroxyl group by an unprotonated amino acid in the catalytic centre. The *cis* isomer of 2-methyl-5-isopropylhexa-2,5-dienal (VI), which we have shown to be the reaction product (3), would be specifically formed as a result of decyclization by the proposed mechanism. This mechanism is also compatible with some of the observed characteristics of the enzyme, including the high turnover number, failure of β-pinene oxide and limonene oxide to serve as substrates (they presumably bind to the catalytic center since they do act as inhibitors), and inhibition of the enzyme by pinan-3-ol and pinan-3-one (Table 3). The sensitivity of the enzyme to sulfhydryl reagents cannot be explained with certainty. The enzyme must also be able to effectively prevent the access of an OH^- nucleophile (which would terminate the rearrangement process) or the elimination of aproton at any intermediate stage, since these events would

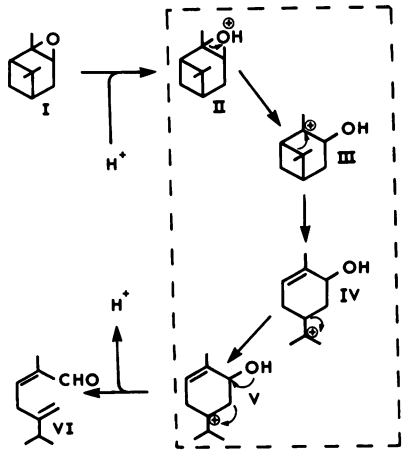

FIG. 2. Proposed scheme for the decyclization of α-pinene oxide by the lyase from *Nocardia* sp. strain P18.3.

lead to the consequent production of carveol, *trans*-sobrerol, and α-terpineol.

The inactivation of this thermally stable lyase during catalysis was unequivocally established in a variety of buffers and over a range of pH values. We have been unable to determine the cause, although it is not due to product inhibition. Because no additional substrates were involved, it was not possible to preincubate lyase and substrate before initiating catalysis. It is possible that there is a slow irreversible reaction between one of the reactive enzyme-bound intermediates (Fig. 2) and a group in the catalytic center that is essential for enzyme activity. This limited catalytic life may provide an explanation for the presence of a large amount of this very active enzyme in crude cell extracts.

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