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Benzene dioxygenase in *Pseudomonas putida*

Subunit composition and immuno-cross-reactivity with other aromatic dioxygenases

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The terminal oxygenase component of benzene dioxygenase from *Pseudomonas putida* strain ML2 was shown to contain two subunits, of M_r 54500 and 23500, by SDS/polyacrylamide-gel electrophoresis. The native M_r of the terminal oxygenase was estimated to be 168000 ± 4000. Polyclonal antibodies raised against each of the subunits cross-reacted with two polypeptides in cell-free extracts from toluene-grown *Pseudomonas putida* strain N.C.I.B. 11767. The M_r values of these polypeptides were similar to those reported for the subunits from the terminal dioxygenase component of toluene dioxygenase. These polypeptides were present only when this strain was grown on toluene. No cross-reactivity was observed with subunits of the naphthalene dioxygenase or benzoate dioxygenase systems.

INTRODUCTION

The aerobic catabolism of aromatic compounds by bacteria involves the initial dihydroxylation of the aromatic nucleus by dioxygenase enzymes to form *cis*-dihydrodiols [1]. A number of dioxygenases from different strains of *Pseudomonas putida* have been well characterized, for example those for benzene [2], toluene [3], naphthalene [4] and benzoate [5], and shown to be multicomponent in nature. They consist of one or two redox proteins which transfer electrons from NADH to a non-haem iron–sulphur protein, the terminal dioxygenase, which dihydroxylates the aromatic substrate. All four terminal dioxygenases show similar absorption spectra with absorption maxima around 450 and 550 nm. This is due to the (2Fe–2S) clusters which form the prosthetic group of these proteins. The dioxygenase component of the benzene(A_1), toluene(ISP_{Tol}), naphthalene(ISP_{Nap}) and benzoate dioxygenase enzyme systems are relatively large molecules (M_r 215000, 151000, 158000 and 201000 respectively). Polyacrylamide-gel electrophoresis in the presence of SDS has revealed the presence of two subunits with M_r of 52000 and 20800 in ISP_{Tol} [6], 55000 and 20000 in ISP_{Nap} [7], and 50000 and 20000 in benzoate dioxygenase [8]. Attempts to investigate the subunit composition of the benzene dioxygenase A_1 protein by a similar method have been inconclusive [9]. Ultracentrifuge measurements in the presence of SDS, however, have indicated the possibility of two subunits of similar M_r.

The present paper demonstrates that the benzene dioxygenase A_1 protein is also composed of two distinct subunits.

MATERIALS AND METHODS

Micro-organisms and growth conditions

The benzene-oxidizing strain of *Pseudomonas putida* (*P. putida* ML2) was kindly supplied by Dr. P. J. Geary of Shell Research Limited U.K. The naphthalene- and the toluene-oxidizing organisms were *Pseudomonas putida* N.C.I.B. 9816 and N.C.I.B. 11767 respectively.

P. putida ML2 was grown with benzene as the sole source of carbon by the method of Axcell & Geary [10], except that the potassium phosphate concentration in the medium was increased to 40 mM. Large-scale growth of this organism was also carried out as described by Axcell & Geary [10]. Medium (8.5 litres) was inoculated with 50 ml of overnight liquid culture, and cells were harvested in exponential growth phase (A_{660} 4.6). The cell paste was washed twice by resuspension in 25 mM-potassium phosphate, pH 7.2, and centrifuged at 8670 g for 30 min at 4 °C, yielding 12.3 g wet wt. of cells/litre of culture medium. The washed cell pastes were frozen at −20 °C until required.

P. putida N.C.I.B. 9816 was grown on a defined mineral medium as described by Ensley *et al.* [4]. Solid naphthalene was provided as sole carbon source (0.1%).

P. putida N.C.I.B. 11767 was grown in shake flasks on a defined mineral medium described by Jenkins & Dalton [11]. Carbon source, toluene, was placed in a glass test tube fitted into the rubber stopper. A small hole in the wall of the test tube allowed the escape of vaporized toluene into the culture vessel.

All three organisms were also grown on 10 mM-succinate as a non-inducing substrate. Growth media were inoculated with overnight liquid cultures. Cultures were incubated at 30 °C with shaking at 220 rev./min.

Preparation of cell-free extracts for enzyme-activity studies and immunoblotting

All operations were performed at 4 °C. Buffer A was 25 mM-sodium/potassium phosphate (pH 7.2) containing 0.1 mM-dithiothreitol. Frozen cells were thawed in buffer A (4 ml/g wet wt.). The suspension was subjected to the maximum output from an MSE 100 W ultrasonic disintegrator. Batches (8 ml) were treated for 5 × 30 s each with 1 min intervals; the probe was cooled between treatments. Cell debris and unbroken cells were removed by centrifugation at 17400 g for 20 min. The supernatant solution was centrifuged at 147000 g for 70 min to give a clear crude cell extract. Samples were stored at −20 °C. These and all the following centrifugation steps were carried out in a Beckman JA-10 or JA-20 rotor for the low-speed centrifugations and a

Beckman 70.1 Ti rotor for the high-speed centrifugations.

Enzyme assays

Enzyme activity in cell-free extracts was measured polarographically by using a Clark-type oxygen electrode at 30 °C in a total volume of 1.1 ml of 25 mM-sodium/potassium phosphate, pH 7.2. The assay mixture contained $FeSO_4$ (0.1, 0.1, 0 mM) and NADH (0.17, 0.33, 2.27 mM). The numbers in parentheses indicate the final concentrations used for assaying benzene, toluene and naphthalene dioxygenase activity respectively. In each assay, the cell-free extract was preincubated for 4 min in buffer in the reaction vessel before addition of NADH to determine the endogenous rate of oxygen uptake. When appropriate, the preincubation mixture also contained $FeSO_4$. After addition of NADH, the reaction was initiated by the addition of 1.1 μl of benzene, 37 μl of toluene-saturated buffer or 25 μl of 10 mM-naphthalene in 96% (v/v) ethanol. Controls were run with ethanol alone. Dissolved oxygen concentration at this temperature was taken to be 235 nmol/ml [12].

Purification of A_1 protein of benzene dioxygenase

The purification was carried out by the method of Crutcher & Geary [13] with some modifications. Cells (101 g wet wt.) were disrupted by two passages through a French pressure cell (Aminco, Silver Spring, MD, U.S.A.) at 27.5 MPa, and the mixture was centrifuged at 11300 *g* for 30 min. The pellet was resuspended in N_2-saturated buffer A and subjected (50 ml batches) to ultrasonic disruption as described above.

The mixture was centrifuged at 10900 *g* for 30 min. The supernatants from both steps were pooled and centrifuged at 41400 *g* for 1 h to give a supernatant cell-free extract (3.7 g of protein). In our hands, this procedure gave a higher yield of protein than a single French-press disruption.

A 40–70%-satd.-$(NH_4)_2SO_4$ protein fraction was used for the purification. This fraction was also used by Axcell & Geary in 1975 [2].

In the first gel-filtration step, Sephacryl S-200 (superfine grade) was substituted for Bio-Gel A0.5m, a modification made by Geary *et al.* in 1984 [9]. For the second gel-filtration step, Ultrogel AcA 34 was used instead of Sephadex G-150 (superfine grade).

This purification procedure gave an overall protein yield of 30 mg, with a specific activity of 58 units (nmol of O_2/min)/mg of protein.

Enzyme activity was assayed polarographically as described above, in a complementation type of assay with 183 and 185 μg of the proteins A_2 and B respectively. The volume of each of the three protein components used was 100 μl. Purified A_2 material from the Sephacryl S-200 column was used as source of A_2 protein, and a fraction eluted from the DEAE-cellulose column in the protein-B region was used as source of this ferredoxin protein.

Estimation of M_r of native A_1 protein

The apparent M_r of the native protein was measured by gel filtration on a Superose 12 column in the Pharmacia fast-protein-liquid-chromatography system. The elution buffer was 50 mM-Tris/HCl, pH 7.2, containing 5 mM-dithiothreitol or 0.1 M-KCl. An A_1-protein fraction from the Ultrogel AcA 34 step in the enzyme-purification procedure was used as source of the protein. The latter was dialysed overnight in the cold against the elution buffer, with two changes of buffer. Gel filtration was performed on freshly dialysed material at room temperature, at a flow rate of 0.2 ml/min. The column was calibrated with the following protein standards (M_r values in parentheses): bovine serum albumin (66000); yeast α-glucosidase (68500); yeast nucleoside-diphosphate kinase (102000); rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (144000); rabbit IgG (150000); pig heart fumarase (194000); sweet-potato β-amylase (200000); bovine liver catalase (250000); horse spleen apoferritin (443000).

Preparation of pure α and β subunits of A_1 protein

Purified A_1 protein (480 μg) was subjected to preparative SDS/polyacrylamide gel electrophoresis in 8% (w/v) acrylamide gel. After electrophoresis, proteins were detected by immersing the gel for 5 min in fresh Coomassie Brilliant Blue R-250 (0.25%) in methanol/water/acetic acid (5:4:1, by vol.).

Partially stained gel slices containing α and β proteins were excised, and the proteins were extracted by electroelution into 0.025 M-Tris/glycine, pH 8.3, containing 0.1% SDS. Electroelution was carried out at 50–60 V for 18 h at room temperature. Extracted samples were concentrated to a final volume of about 1 ml in a Minicon B15 concentration block at room temperature and stored at −20 °C.

Gel electrophoresis and estimation of subunit M_r

Electrophoresis in gels containing SDS was performed in a slab-gel apparatus (Protean double-slab-electrophoresis cell; Bio-Rad Laboratories, Richmond, CA, U.S.A.) by using the discontinuous system described by Laemmli [14], except that a 5%-acrylamide stacking gel was used. Proteins were boiled in sample buffer containing 6.6% (w/v) SDS and 5.5% (v/v) mercaptoethanol, for 3 min. Proteins were stained in Coomassie Brilliant Blue R-250 (0.25%) in methanol/water/acetic acid (5:4:1, by vol.) and destained by diffusion in 40% (v/v) methanol/5% (v/v) acetic acid. Standard protein markers used for calibration purposes were (M_r values in parentheses): bovine serum albumin (66000); ovalbumin (45000); bovine erythrocyte carbonic anhydrase (29000); β-lactoglobulin (18400); horse heart cytochrome *c* (12400).

Production of antisera against α and β subunits of benzene dioxygenase

The antibodies against the two subunits were elicited in separate young male New Zealand White rabbits: 2.3 and 3 ml of purified α and β protein solution, containing 0.2 and 0.08 mg of protein respectively, were mixed with an equal volume of Freund's complete adjuvant, and injected subcutaneously into several points on the back and the neck region. The rabbits received three further injections of immunogen within a period of 19 weeks: 180, 140 and 100 μg of α protein, and 91, 56 and 100 μg of β protein, was administered, at 3, 9 and 19 weeks after immunization respectively. In each case, Freund's incomplete adjuvant was used. Animals were bled just before and approx. 2 weeks after each injection. Serum was separated and stored in portions at −20 °C until required. Each antiserum was analysed by the immunosorbent assay method described below.

Enzyme-linked immunosorbent assay ('ELISA')

The titre of antiserum was monitored by non-competitive ELISA by the method of Brodeur *et al.* [15], with some modifications. Although antisera were raised to SDS-denatured protein A_1, antigen used in the ELISA was the native A_1 protein.

Antibody (IgG) purification

IgG was purified by $(NH_4)_2SO_4$ fractionation of antiserum, followed by affinity purification on a Protein A–Sepharose CL-4B column by the method specified by Pharmacia. Portions were stored at −20 °C until required.

Immunoblotting

Samples were separated on an SDS/12.5%-polyacrylamide gel as described above. Both the gel and the nitrocellulose membrane were equilibrated in transfer buffer [25 mM-Tris/192 mM-glycine, pH 8.3 containing 20% (v/v) methanol] for 45 min before electroblotting. All incubation steps, unless otherwise stated, were carried out at room temperature with gentle shaking. Polypeptides were electroblotted on to nitrocellulose membranes for 15 h at 30 V (0.2 A, for two gels), in transfer buffer followed by 50 min at 100 V (0.8–1.0 A). Electroblotting was performed in a Hoefer Scientific transfer cell (Biotech Instruments, Luton, Beds., U.K.) at room temperature, with cooling from a tap-water-circulation tube. The nitrocellulose membrane was incubated in 20 mM-Tris/HCl/500 mM-NaCl, pH 7.5, for 20 min and then blocked with 5% (w/v) skimmed milk in the same buffer for 90 min. The membrane was transfered from the blocking solution into antibody buffer (1% skimmed milk in the Tris/NaCl buffer) containing respectively 56 and 54 μg of anti-α or anti-β purified IgG fraction/ml. Binding of the first antibody was allowed to occur for 23 h at 4 °C.

The membrane was briefly rinsed with distilled water and then washed twice in Tris/NaCl buffer (for the membrane probed with anti-β IgG) or Tris/NaCl buffer containing 0.05% Tween-20 (for the membrane probed with anti-α IgG). Each washing step consisted of gentle agitation for 15 min. The binding of second antibody [Bio-Rad affinity-purified goat anti-(rabbit IgG)–horseradish peroxidase conjugate] and the development of the blot were performed by the protocols given by Bio-Rad.

Protein determinations

Protein concentration was determined by the method of Hess *et al.* [16], with bovine serum albumin as the standard. Samples containing SDS were precipitated by the method of Wessel & Flügge [17] before determination of protein content.

Benzene determination by h.p.l.c.

Benzene concentration in the polarographic assay of benzene dioxygenase was determined by h.p.l.c. After stimulation with benzene, samples were removed at intervals from the assay mixture through the injection port and applied to an Altex Ultrasphere-ODS column (Anachem). The column was eluted at a flow rate of 1.2 ml/min with 80% methanol in water. Detection was at 210 nm. Benzene (11.3 mM, in the assay buffer) was prepared immediately before use. Rates were corrected for the loss of benzene in the assay system in the absence of enzyme.

Materials

The following items were obtained from the sources indicated: dithiothreitol, NADH, ABTS [2,2′-azinodi-(3-ethylbenzthiazolinesulphonate)] (horseradish peroxidase enzyme substrate), NaF, H_2O_2, mercaptoethanol, protein markers and Coomassie Brilliant Blue R-250, Sigma; DEAE-cellulose, Whatman; Sephacryl S-200 (superfine) and Protein A–Sepharose CL-4B, Pharmacia; Ultrogel AcA 34, LKB; glycine, $(NH_4)_2SO_4$ (for enzyme work) and all reagents used for polyacrylamide-gel electrophoresis and gel-filtration chromatography (electrophoresis and AnalaR grades), BDH; Tween-20 and all materials and reagents used for immunoblotting, Bio-Rad, except methanol (AnalaR grade), Fisons, and

Table 1. Estimation of the M_r of A_1 protein

Experimental conditions are described in the Materials and methods section. Protein loading was 213–237 μg of A_1.

Elution buffer	[Mercaptoethanol] in sample buffer (M)	Estimated M_r
50 mM-Tris/HCl, pH 7.2, containing 0.1 M-KCl	None	168000
50 mM-Tris/HCl, pH 7.2, containing 5 mM-dithiothreitol	None	189000
	0.14	202000
	0.72	214000

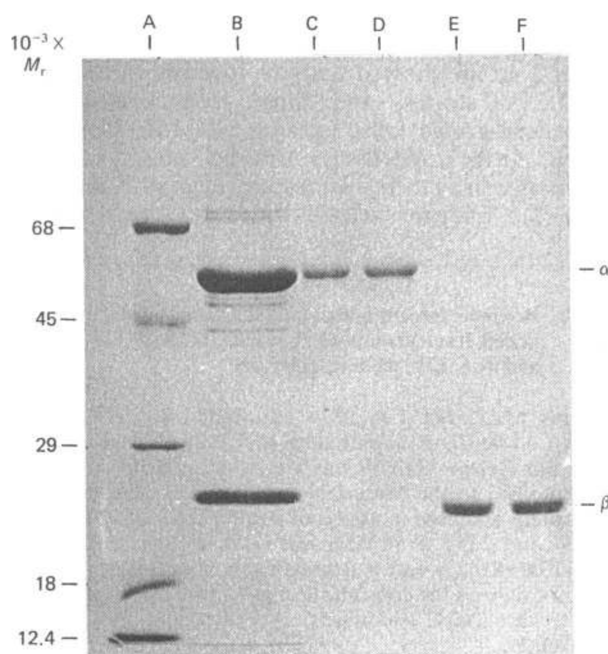

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified A_1 protein and its subunits

A 60 μg sample of protein A_1 was run in lane B. Lanes C and D (α subunit) and E and F (β subunit) each contained 4 μg of protein. The gel was 10% acrylamide. Lane A contained 5 μg of each of the following marker proteins: bovine serum albumin, ovalbumin, carbonic anhydrase, lactoglobulin and cytochrome *c*.

skimmed milk, Safeways supermarket; Freund's adjuvant, GIBCO; h.p.l.c.-grade methanol, Fisons.

RESULTS

Estimation of M_r of native A_1 protein

The M_r of the native protein was estimated under reducing and non-reducing conditions: Table 1 shows the values obtained under the conditions used.

Heterogeneous nature of the A_1 component of benzene dioxygenase

The subunit composition of the purified A_1 component of benzene dioxygenase was investigated by polyacrylamide-gel electrophoresis in the presence of 0.1% SDS. Two major bands were resolved, with M_r values of 54500 (S.D. 895; mean of 11 values) and 23500 (S.D. 576; mean of 11 values), which were called α and β respectively (Fig. 1, lane B). This lane was overloaded to demonstrate the presence of contaminating protein bands. The α and β components (Fig. 1, lanes C, D and E, F respectively) were purified by preparative SDS/polyacrylamide-gel electrophoresis from the A_1-protein preparation shown in Fig. 1 (lane B). From a typical purification, 120 μg of α and 60 μg of β were recovered. The α and β fractions, free of any detectable contaminating proteins, were used to immunize separate rabbits.

Substrate specificity

Benzene, toluene and naphthalene dioxygenase activities were assayed polarographically in cell-free extracts from *P. putida* strains ML2, N.C.I.B. 11767 and N.C.I.B. 9816 respectively. Extracts from benzene-grown cells of the ML2 strain showed activity towards all the three aromatic substrates, and those from toluene- and naphthalene-grown cells (strains N.C.I.B. 11767 and N.C.I.B. 9816 respectively) showed activity towards benzene as well as their own respective growth substrates (Table 2). Enzyme activity, however, was greatest towards the substrate on which the organism was grown. Toluene dioxygenase activity in *P. putida* N.C.I.B. 11767 was found to be inducible. Extracts of this organism grown on succinate showed no activity towards toluene, even when the assay system contained a high concentration of cell extract (11 mg). In contrast, the benzene-oxidizing activity of *P. putida* ML2, grown on succinate, was 0.9% of that observed in benzene-grown cells. Activity towards naphthalene in extracts of *P.*

Table 2. Benzene, toluene and naphthalene dioxygenase activities in cell-free extracts of *P. putida* ML2, N.C.I.B. 11767 and N.C.I.B. 9816 respectively

Strains ML2, N.C.I.B. 9816 and N.C.I.B. 11767 were grown on benzene, naphthalene and toluene respectively, and dioxygenase activity was measured polarographically as described in the Materials and methods section: 3 mg of protein was used in assays of strains ML2 and N.C.I.B. 11767 and 2 mg of protein was used in assays of strain N.C.I.B. 9816. A unit is defined as the amount of protein which catalyses the consumption of 1 nmol of oxygen/min under the assay conditions. Abbreviation: N.D., not determined.

Strain	Growth substrate	Dioxygenase activity (units/mg) with substrate:		
		Benzene	Toluene	Naphthalene
ML2	Benzene	20.03	12.09	2.21
N.C.I.B. 11767	Toluene	2.46	6.59	N.D.
N.C.I.B. 9816	Naphthalene	7.16	N.D.	23.50

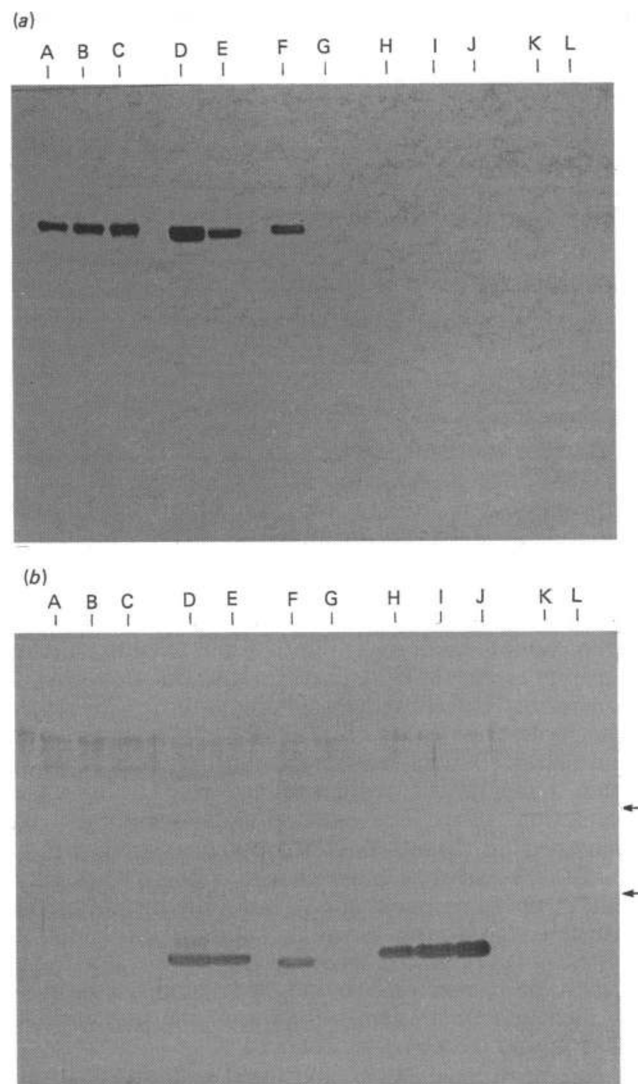

Fig. 2. Analysis of immunological cross-reactivity between benzene, toluene and naphthalene dioxygenase components in cell-free extracts

Samples were resolved in duplicate on 12.5%-acrylamide gels and electroblotted as described in the Materials and methods section. Lanes D–G, K and L each contained 25 μg of protein in cell-free extracts from *P. putida* ML2 grown on benzene (D) and succinate (E), *P. putida* N.C.I.B. 11767 grown on toluene (F) and succinate (G), or *P. putida* N.C.I.B. 9816 grown on naphthalene (K) and succinate (L). Lanes A–C contained 0.2, 0.3 and 0.4 μg of purified α-subunit protein respectively. Lanes H–J contained 0.1, 0.2 and 0.3 μg of purified β-subunit protein respectively. Blot (*a*) was probed with anti-α-subunit IgG, and blot (*b*) was probed with anti-β-subunit IgG.

putida N.C.I.B. 9816 grown on succinate was 2.4% of that in extracts from this organism grown on naphthalene.

The possibility exists that the oxygen uptake in these assays was due to substrate-dependent uncoupling of electron transport rather than true dioxygenase activity [18]. This was shown not to be the case, for two reasons.

(*a*) The rate of benzene utilization (measured by h.p.l.c.) was comparable with the rate of oxygen uptake in the oxygen electrode: with an initial benzene concentration of 11.3 mM, the oxygen uptake rate was 17.5 nmol/min per mg, compared with a benzene oxidation rate of 15.8 nmol/min per mg of protein; with 0.3 mM-benzene, these rates were 12.9 and 13.1 nmol/min per mg of protein for oxygen uptake and benzene oxidation respectively.

(*b*) There was a 1:1 stoichiometry between benzene added and oxygen consumed: for benzene additions of 11, 33 and 55 nmol, 15, 39 and 68 nmol of oxygen were consumed respectively.

Immunological cross-reactivity

Polyclonal antibodies raised against the separated subunits of benzene dioxygenase A_1 protein reacted with their respective antigens (Fig. 2, lanes A–C and H–J) in 'Western' blotting experiments: 0.2 μg of α subunit (Fig. 2*a*, lane A) and 0.1 μg of subunit β (Fig. 2*b*, lane H) could easily be detected. Antibodies were specific, since no inter-subunit cross-reactivity occurred between anti-α-subunit antibody and β polypeptide, or vice versa (Fig. 2*a*, lanes H–J, and Fig. 2*b*, lanes A–C, respectively). These specific antibodies could also detect antigen in cell extracts of the benzene-grown ML2 strain (Fig. 2, lane D). The two subunits of A_1 protein were present even when this strain was grown on succinate (Fig. 2, lane E).

The band width and intensity suggested that benzene-grown cells contained twice the amount of α and β protein that was present in succinate-grown cells.

The polyclonal anti-α-subunit and anti-β-subunit antibodies cross-reacted with polypeptides in cell extracts of strain N.C.I.B. 11767 grown on toluene (Fig. 2, lane F). The anti-α-subunit antibody cross-reacted with a single polypeptide of M_r approx. 55000. Anti-β-subunit antibody cross-reacted with a smaller polypeptide, of M_r 21000. These values correlate with the reported M_r values of components of ISP_{Tol} [6]. These polypeptides were absent when this organism was grown on succinate (Fig. 2, lane G).

The anti-α-subunit antibody did not react with any polypeptide in extracts from strain N.C.I.B. 9816 grown on naphthalene or succinate (Fig. 2*a*, lanes K and L). The anti-β-subunit antibody, however, showed a weak interaction with two polypeptides of M_r values intermediate between those of α and β subunits (Fig. 2*b*, lanes K and L; arrows indicate positions of these bands). These two bands were present in both succinate- and naphthalene-grown cells.

DISCUSSION

The soluble benzene dioxygenase enzyme system of *P. putida* ML2 catalyses the double hydroxylation of benzene to *cis*-1,2-dihydroxycyclohexa-3,5-diene. We have shown that this enzyme is also able to oxidize toluene and naphthalene.

This enzyme system consists of three components: an NADH-dependent flavoprotein (protein A_2, M_r 81000), a ferredoxin (protein B, M_r 12000) and a dioxygenase (protein A_1, M_r 215000) [9]. In the present study, the A_1 protein was shown to have a native M_r of 168000 ± 4000 and to consist of two distinct subunits by polyacrylamide-gel electrophoresis in the presence of 0.1% SDS. These two polypeptides, α and β, had M_r values of 54500 and 23500 respectively. In protein purification experiments, the ratio by weight of α to β subunits was always 2:1, giving a molar ratio of approx. 1:1. This subunit composition is similar to that of the terminal oxygenase component of other dioxygenases [6–8].

Immunological techniques were used to characterize the α and β subunits. Antisera were raised to SDS-denatured α and β subunits, producing polyclonal antibodies favouring sequence-related rather than structural specificity. The absence of immuno-cross-reactivity between anti-α-subunit polyclonal antibody and the β subunit, and vice versa (Fig. 2), provided evidence that α and β proteins were two distinct components.

Geary *et al.* [9] demonstrated the presence of two (2Fe–2S) clusters per molecule of A_1. In conjunction with the finding that the fully active A_1 molecule contains two additional Fe^{2+} atoms, they proposed that the active site of this protein contains (2Fe–2S)Fe^{2+} as the oxygen-activating unit. From these findings, together with the evidence for two distinct subunits in the A_1 protein with a molar ratio of 1:1, a subunit structure of $\alpha_2\beta_2$ may be proposed. This would result in a M_r of 156000 for the A_1 protein, which is similar to the reported values for toluene [6] and naphthalene [7] oxygenase components.

In the present study, the native M_r of this protein was estimated to be 168000 ± 4000. This is in close agreement with the predicted value, but is significantly less than the M_r of 215000 reported by Geary *et al.* [9]. The high value reported previously was estimated in the presence of dithiothreitol. We have shown in this study that dithiothreitol in the elution buffer gives an increased M_r (189000), which is elevated still further if the sample is treated with mercaptoethanol (to 214000). This is probably due to protein unfolding as a result of reduction of thiol cross-linkages. It should also be noted that there is a tendency for some terminal dioxygenases to aggregate [8].

The function of the two subunits is unknown, but, by analogy with other dioxygenases, the α subunit may contain the prosthetic group (2Fe–2S) [8] and the β subunit may be involved in substrate recognition [19].

The mechanism of regulation of benzene dioxygenase is unclear. It was observed that the ML2 strain grown on succinate contained approx. 50% of the amount of A_1 protein that was present when the organism was grown on benzene (Fig. 2, lanes D and E). This does not agree with the enzyme-activity data, which indicate that cells grown on succinate contain only 0.9% of the active enzyme present in benzene-grown cells. The reason for this high content of apparently inactive protein in succinate-grown cells requires investigation.

The degree of homology between the terminal oxygenase of benzene dioxygenase and other aromatic dioxygenases was studied immunologically. It was found that the anti-α-subunit and anti-β-subunit antibodies cross-reacted with two polypeptides in toluene-grown *P. putida* N.C.I.B. 11767. This strain has been shown to

contain toluene dioxygenase [11]. The M_r values of these polypeptides were very similar to those reported for the toluene dioxygenase components from other strains [6]. The absence of any cross-reacting bands in the succinate-grown cell extracts of *P. putida* N.C.I.B. 11767 indicates that in this organism ISP_{Tol} is induced by toluene. This was supported by the lack of enzyme activity towards toluene in extracts of succinate-grown cells. In contrast, no cross-reactivity was observed with naphthalene dioxygenase from *P. putida* N.C.I.B. 9816 grown on naphthalene or succinate. The terminal oxygenase component of naphthalene dioxygenase, ISP_{Nap}, is of M_r 158000 and is also composed of two subunits, α and β, with M_r values of 55000 and 20000 respectively; however, there are some properties which differ. In the naphthalene dioxygenase enzyme system, both NADH and NADPH can act as electron donors for its flavoprotein, which has iron as well as FAD as its prosthetic group [7]. This metalloflavoprotein can transfer electrons directly from NADH to cytochrome *c* in the absence of the other two components. In contrast with benzene and toluene dioxygenases, which require exogenous iron for optimum activity, naphthalene dioxygenase is not stimulated by exogenous iron [7]. In these respects, naphthalene dioxygenase is similar to the two-component benzoate dioxygenase [20–22] and 4-methoxybenzoate *O*-demethylase [18,23] enzyme systems.

In addition, it has been shown by Eich *et al.* [24] that antibodies against the oxygen-activating component of 4-methoxybenzoate *O*-demethylase did not inhibit benzene dioxygenase activity or show any antigenic relationship in double-radial-immunodiffusion tests. It is thus possible that at least two classes of oxygen-activating components may have developed independently of each other, with toluene and benzene dioxygenases forming a group separate from the naphthalene and benzoate dioxygenases.

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