Intergeneric Evolutionary Homology Revealed by the Study of Protocatechuate 3,4-Dioxygenase from *Azotobacter vinelandii*†

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ABSTRACT: Protocatechuate 3,4-dioxygenase (EC 1.13.1.3) was purified to homogeneity from extracts of *Azotobacter vinelandii*. The molecular weight of the oligomeric protein was estimated to be 510 000 by gel filtration and 480 000 by ultracentrifugation. The oligomer appears to be formed by association of equal amounts of nonidentical subunits which were estimated by sodium dodecyl sulfate gel electrophoresis to have respective molecular weights of 23 300 and 25 250. Ten gram-atoms of iron was associated with each mol of oligomer. Therefore, the enzyme appears to be a decamer with the structure 10($\alpha\beta$Fe). The amino acid composition of *Azotobacter* protocatechuate oxygenase closely resembles the amino acid compositions of protocatechuate 3,4-dioxygenases from *Pseudomonas aeruginosa* and *Thiobacillus* sp. These proteins from *P. aeruginosa* and *P. putida* are known to be formed by association of nonidentical subunits of a physical size similar to the subunits of the *Azotobacter* enzyme. Furthermore, antisera prepared against the *Azotobacter* oxygenase cross-reacted strongly with the isofunctional enzymes from the two fluorescent *Pseudomonas* species. A weak immunological cross-reaction was observed when the antisera were tested against protocatechuate 3,4-dioxygenase from *Acinetobacter calcoaceticus*. The results favor the conclusion that the bacterial protocatechuate 3,4-dioxygenases were derived from a common ancestral protein.

Protocatechuate 3,4-dioxygenase (EC 1.13.1.3) catalyzes the addition of molecular oxygen between vicinal hydroxyl groups on the aromatic ring of protocatechuate (Stanier & Ingraham, 1954). Chemically analogous intradiol cleavage of catechol is mediated by catechol 1,2-dioxygenase (EC 1.13.11.1; Kojima et al., 1967). The two enzymes usually are found together in microorganisms that utilize aromatic acids via the β-ketoadipate pathway. The respective oxygenases initiate a convergent set of metabolic sequences that give rise to the common intermediate β-ketoadipate and to the final end products succinate and acetyl-CoA (Stanier & Ornston, 1973; Ornston & Parke, 1977). Representatives of *Azotobacter* are metabolically exceptional in that they form protocatechuate 3,4-dioxygenase but not catechol 1,2-dioxygenase (Hardisson et al., 1969). Catechol is used by an entirely different pathway in these organisms (Sala-Trepat & Evans, 1971).

The structure of protocatechuate 3,4-dioxygenase has been studied in a number of laboratories (Fujisawa & Hayaishi, 1968; Fujisawa et al., 1972; Hou et al., 1976; Wells, 1972; Yoshida et al., 1976). The *Pseudomonas aeruginosa* enzyme has been reported to be an oligomer of 700 000 molecular weight formed by association of equal numbers of nonidentical subunits with molecular weights of 22 500 and 25 000 (Yoshida et al., 1976). Protocatechuate oxygenases from *Acinetobacter* (Hou et al., 1976) and *Thiobacillus* (Wells, 1972) possess similar physical properties and this evidence suggests that a single ancestral gene may have given rise to the protocatechuate oxygenases of these distant bacterial genera. On the other hand, another *Pseudomonas* isolate forms a protocatechuate oxygenase with an oligomeric molecular weight of 190 000 (Bull et al., 1979), considerably lower than that reported for the *P. aeruginosa* enzyme. Furthermore, antisera prepared against the *P. aeruginosa* protocatechuate oxygenase failed to cross-react with the isofunctional enzyme formed by representatives of other bacterial genera (Hou & Lillard, 1976). Therefore, the existing data leave the question of the evolutionary origin of the different protocatechuate oxygenases unresolved.

In this report we describe the physical, chemical, and serological properties of protocatechuate oxygenase derived from *Azotobacter*. The unique set of pathways used by members of this genus for the dissimilation of aromatic compounds suggests that their metabolic sequences evolved in isolation from those of other organisms. This inference is contradicted by the properties of *Azotobacter* protocatechuate oxygenase which are strikingly similar to those of isofunctional oxygenases from biologically distant bacterial strains. For example, serological comparisons conducted with *Azotobacter* protocatechuate oxygenase as the reference protein provide the first immunological evidence for a common ancestry for genes of the β-ketoadipate pathway of different bacterial genera. Yet, unlike other protocatechuate oxygenases that have been described, the *Azotobacter* enzyme appears to possess an unusual decameric structure. The available evidence concerning different protocatechuate oxygenase structures is reviewed under Discussion.

Experimental Procedures

Bacterial Strains. *Azotobacter vinelandii* strain OP was obtained from Paul E. Bishop, North Carolina State University, Raleigh, NC. *P. aeruginosa* strains OSU 64 and ATCC 14502 were provided by Paul Phibbs, Virginia Commonwealth University, Richmond, VA. *Acinetobacter calcoaceticus* strain BD413 was obtained from E. Juni, University of Michigan, Ann Arbor, MI, and is designated strain ADP-1 in our collection.

Media and Growth Conditions. Large-scale growth of *A. vinelandii* was accomplished at 30 °C in a New Brunswick Fermacell CF-130 fermenter containing 100 L of modified Burk's mineral medium (Strandberg & Wilson, 1967). Stock solutions of *p*-hydroxybenzoate were prepared as previously described (Meagher et al., 1972) and were added to culture

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media to a 10 mM concentration. Cells were harvested at late logarithmic phase in a refrigerated Sharples supercentrifuge at 4 °C and stored at −20 °C until use. For immunodiffusion studies, *Pseudomonas* and *Acinetobacter* strains were grown in 500-mL Erlenmeyer flasks containing 150 mL of mineral medium (Meagher et al., 1972) supplemented with either 10 mM *p*-hydroxybenzoate or 20 mM succinate. Cell-free extracts were prepared by ultrasonication using a Branson Electronics ultrasonifier as described elsewhere (Meagher et al., 1972).

Protocatechuate 3,4-Dioxygenase Assays. Protocatechuate 3,4-dioxygenase was assayed as described by Stanier & Ingraham (1954) with slight modifications. The assay mixture contained, in a total volume of 3 mL, 150 μmol of Tris(hydroxymethyl)aminomethane (Tris-HCl) buffer, pH 8.5, and 0.5 μmol of protocatechuate. The reaction was initiated by the addition of a suitable amount of enzyme, and the decrease in absorbance at 290 nm was recorded at 24 °C on a Gilford 2400 spectrophotometer. Alternatively, enzymatic activity was measured by monitoring oxygen uptake using a YSI Model 53 oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH). The assay system contained, in a final volume of 3 mL, 150 μmol of Tris-HCl buffer, pH 8.5, 1 μmol of protocatechuate, and a suitable amount of enzyme. The reaction was initiated by addition of substrate. One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of protocatechuate per min. Specific activity is expressed as units per milligram of protein. Protein concentrations were determined by the procedure of Lowry et al. (1951) using bovine serum albumin as a standard.

Purification of Protocatechuate 3,4-Dioxygenase. All purification procedures, unless otherwise stated, were performed between 0 and 4 °C. Buffers A, B, and C refer to 10 mM sodium–potassium phosphate, pH 7.5, containing 25 μM dithiothreitol, 50 mM Tris-HCl, pH 8.5, and 20 mM Tris-HCl, pH 7.5, respectively.

Cell pastes (100 g wet weight) were suspended in 50 mM sodium–potassium phosphate buffer, pH 7.5, and were disrupted by passage through an American Instrument continuous flow French pressure cell at 18 000 psi. The supernatant fraction obtained following centrifugation at 37000*g* at 4 °C for 25 min was used as the source of crude cell-free extract. The crude extract was brought to 45% saturation by the addition of solid ammonium sulfate and, following a 30-min equilibration period, protein was precipitated by centrifugation at 40000*g* for 20 min. Ammonium sulfate treatment was repeated on the supernatant fraction until 85% saturation was achieved. The protein pellet obtained after centrifugation was suspended in buffer A and dialyzed against two changes of the same buffer. The dialysate was loaded onto a DEAE-cellulose column (5 × 60 cm) previously equilibrated with buffer A. The column was washed with 2 volumes of the same buffer, after which a continuous linear gradient, constructed from 10 to 200 mM sodium–potassium phosphate in a total volume of 6 L, was applied. Fractions of 15 mL were collected at a flow rate of 60 mL/h. Fractions containing protocatechuate oxygenase, which eluted between 130 and 140 mM sodium–potassium phosphate, were pooled and incubated in a water bath until the temperature of the enzyme preparation reached 50 °C. The protein was maintained at this temperature for 15 min and then immediately cooled on ice. Denatured protein was removed by centrifugation at 37000*g* for 15 min. The supernatant fraction was further treated with ammonium sulfate, and the fraction precipitating between 45 and 75% saturation was collected by centrifugation and dialyzed against two changes of buffer B. The dialyzed protein was applied in 9-mL aliquots onto a Bio-Gel A1.5m column (2.5 × 90 cm) equilibrated with dialysis buffer B. Fractions of 3 mL were collected at a flow rate of 20 mL/h, and those containing protocatechuate oxygenase activity were pooled, concentrated by ammonium sulfate treatment (50–70%), and dialyzed against buffer C. The protein was then applied onto a second DEAE-cellulose column (0.9 × 25 cm), and protocatechuate oxygenase was eluted with 400 mL of buffer C containing a linear gradient constructed from 0 to 200 mM NaCl. Three-milliliter fractions were collected, and those possessing protocatechuate oxygenase activity were combined and concentrated by ammonium sulfate precipitation. The concentrated protein was dialyzed against buffer B and stored at 4 °C.

Polyacrylamide Gel Electrophoresis. The purified enzyme was analyzed by polyacrylamide gel electrophoresis by the general procedures of Davis (1964) using the gel and reservoir buffers of Laemmli (1970) but omitting the sodium dodecyl sulfate ($NaDodSO_4$). Protein samples (10–100 μg) were applied to 6% acrylamide gels, and electrophoresis was conducted at a constant current of 2 mA/gel until the tracking dye had migrated to the opposite end of the gel. Proteins were stained for 30–60 min in 0.25% Coomassie brilliant blue R 250 in 20% methanol–7.5% glacial acetic acid. Gels were destained in a Bio-Rad Model 155 destainer containing 20% methanol–7.5% glacial acetic acid.

$NaDodSO_4$ gel electrophoresis was performed as described by Weber & Osborn (1969). Protein samples in 10 mM sodium phosphate buffer, pH 7.2, containing 1% $NaDodSO_4$ and 1% β-mercaptoethanol were denatured by heating at 100 °C for 5 min. Proteins were applied to either 7.5, 10, or 12% gels, and electrophoresis was performed at a constant current of 6 mA/gel until the tracking dye had migrated to the opposite end of the gels. Staining and destaining were carried out as described above.

Isoelectric Focusing. Isoelectric focusing of the native enzyme was performed according to the procedures of Righetti & Drysdale (1971). Enzyme samples were mixed with glycerol and carrier ampholytes and layered onto 6% polyacrylamide gels (3 × 130 mm) containing 2% (w/v) carrier ampholytes (0.4%, pH 3.5–10; 1.6%, pH 4–8). The anodic and cathodic buffers were 10 mM phosphoric acid and 20 mM sodium hydroxide, respectively. Following electrofocusing for 8–10 h at a constant voltage of 400 V, the gels were removed and scanned at 480 nm by using a Gilford 2400 spectrophotometer. The gels were then sliced into 0.5-cm segments which were placed in vials containing 1 mL of degassed, double-distilled water. The pH of each segment was determined following overnight incubation at room temperature.

Ultracentrifuge Measurements. Ultracentrifuge measurements were made by using a Beckman-Spinco Model E ultracentrifuge equipped with Schlieren and interference optics. For both types of analysis, protein samples (2 mg/mL) were dialyzed against 50 mM Tris-HCl, pH 8.5, containing 5 mM β-mercaptoethanol for 18 h at 4 °C. The dialysate was used as the reference solution. Sedimentation velocity data were determined at 18 °C and 40 000 rpm by using a double sector cell fitted with sapphire windows. The molecular weight of the enzyme was determined by the sedimentation equilibrium method of Yphantis (1964) in a double sector interference cell fitted with sapphire windows. The centrifuge was operated at 9000 rpm at 20 °C.

Iron Analysis. For iron analysis, all reagents, standard solutions, and buffers were prepared by using double-distilled

water. A quantitative determination of iron was ascertained colorimetrically by the method of Fischer & Price (1964), as modified by Zabinski et al. (1972), and by atomic absorption using a Perkin-Elmer Model 503 atomic absorption spectrophotometer.

Absorption Spectra. Absorption spectra were determined with a Cary Model 219 recording spectrophotometer.

Amino Acid Analysis. The amino acid composition of the purified enzyme was determined on a Durrum 500 amino acid analyzer containing a computerized integration system. Protein samples (25 μg) were hydrolyzed at 105 °C in 6 N HCl in vacuo for 20, 48, 72, and 110 h. Cysteine was determined as cysteic acid after performic acid oxidation (Moore, 1963). Tryptophan was determined following hydrolytic treatment of the enzyme with 3 N mercaptoethanesulfonic acid (Hugli & Moore, 1972).

Immunological Techniques. Antiserum was prepared from New Zealand white rabbits by subcutaneous and intramuscular injections of homogeneous protocatechuate oxygenase (1 mg) emulsified in equal volumes of Freunds complete adjuvant (Difco). Two subsequent injections of 1 and 0.5 mg of enzyme emulsified in Freunds incomplete adjuvant were administered at 7-day intervals. Rabbits were bled on the third and fourth week after the initial injection. The immunoglobulin fraction was obtained by sodium sulfate precipitation (Kekwick, 1940). Immunodiffusion experiments were performed according to Ouchterlony (1953). The gel matrix contained 1% (w/v) Noble agar in Tris–barbital buffer, pH 8.0, at an ionic strength of 0.03. Plates were incubated at room temperature and photographed after 48 h. Immunoelectrophoresis was carried out on glass slides containing the same medium used for immunodiffusion. Electrophoresis was conducted in a Gelman apparatus (Ann Arbor, MI) at a constant current of 200 V for 4 h. Antiserum was then added to the central trough, and the slides were incubated overnight at room temperature in a humidity chamber.

Chemicals. Acrylamide, *N,N'*-methylenebis(acrylamide), $NaDodSO_4$, and molecular weight protein standards for $NaDodSO_4$ gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA. Carrier ampholytes were purchased from LKB Laboratories, Rockville, MD. Diethylaminoethylcellulose DE52 was obtained from Whatman Ltd., Maidstone, Kent, England. Sepharose CL 6B and gel filtration high molecular weight protein standards were purchased from Pharmacia Fine Chemicals Inc., Piscataway, NJ. All other chemicals and reagents were the best grade commercially available.

Results

Criteria for Purity. The procedure for the purification of protocatechuate 3,4-dioxygenase from *A. vinelandii* is summarized in Table I. The scheme resulted in a 27-fold purification of the enzyme with a 30% recovery. The apparent homogeneity of the enzyme was assessed by the following criteria. (1) The enzyme migrated as a single protein band when subjected to electrophoresis on polyacrylamide gels (pretreatment with β-mercaptoethanol was required for the dissociation of enzyme aggregates during electrophoresis). (2) The Schlieren profile of the enzyme during ultracentrifugation revealed a single symmetrical peak. (3) Immunoelectrophoresis of the purified enzyme produced a single precipitin arc with antisera prepared by using protocatechuate oxygenase as antigen.

Sedimentation and Diffusion Coefficients. From sedimentation velocity data, using a protein concentration of 2 mg/mL, the $s^0_{20,w}$ was determined as 20.9×10^{-13} S. The diffusion coefficient was calculated as 4.24×10^{-7} cm^2 s^{-1}.

Table I: Purification of Protocatechuate 3,4-Dioxygenase from *A. vinelandii*

	vol (mL)	total act.[a]	total protein (mg)	sp act.[b]	% recovery	purifn factor
crude extract	850	14 450	8245	1.75	100	1
45–85% ammonium sulfate fraction	898	15 266	6245	2.43	106	1.4
DEAE-cellulose eluate	385	9 433	801	11.78	65	6.7
45–75% ammonium sulfate fraction of heat-treated extract	28	9 415	426	22.12	65	12.6
Bio-Gel agarose eluate	126	6 846	161	42.52	47	24.3
50–70% ammonium sulfate fraction	13	5 688	130	43.75	39	25.0
second DEAE-cellulose eluate (concentrated)	18	4 286	92	46.69	30	26.7

[a] Units. [b] Units per milligram of protein.

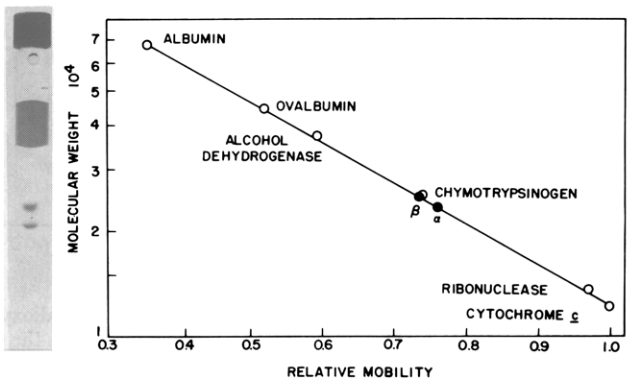

FIGURE 1: Subunit molecular weight determination of protocatechuate 3,4-dioxygenase. $NaDodSO_4$ gel electrophoresis was performed as described under Experimental Procedures. The subunits α and β are represented as closed circles.

Molecular Weight and Subunit Composition. The molecular weight estimated by gel filtration (Andrews, 1964) on Sepharose CL 6B was 510 000. Sedimentation equilibrium analysis yielded a molecular weight of 480 000 ± 3000 (mean and standard deviation of six determinations). A partial specific volume of 0.729, calculated from the amino acid composition, was used for sedimentation equilibrium calculations. For both techniques the enzyme was dialyzed against buffer containing β-mercaptoethanol.

$NaDodSO_4$ gel electrophoresis of the purified enzyme, denatured in the presence or absence of β-mercaptoethanol, resulted in the appearance of two protein bands with molecular weights of 25 250 and 23 300 (Figure 1). These results indicate that the enzyme is composed of nonidentical subunits in which disulfide linkages are not involved. Furthermore, when the enzyme was treated with 8 M urea and subjected to urea gel electrophoresis (Yoshida et al., 1976), two protein bands also were observed. Thus, the subunit composition of *Azotobacter* protocatechuate oxygenase is similar to those of other dioxygenases described which mediate intradiol cleavage of aromatic compounds.

Iron Content. Assuming a molecular weight of 480 000, the iron content of protocatechuate oxygenase as determined by atomic absorption spectrophotometry was 9.12 g-atoms/mol of enzyme. The colorimetric method yielded a value of 9.94 g-atoms/mol. From these results we conclude that the enzyme

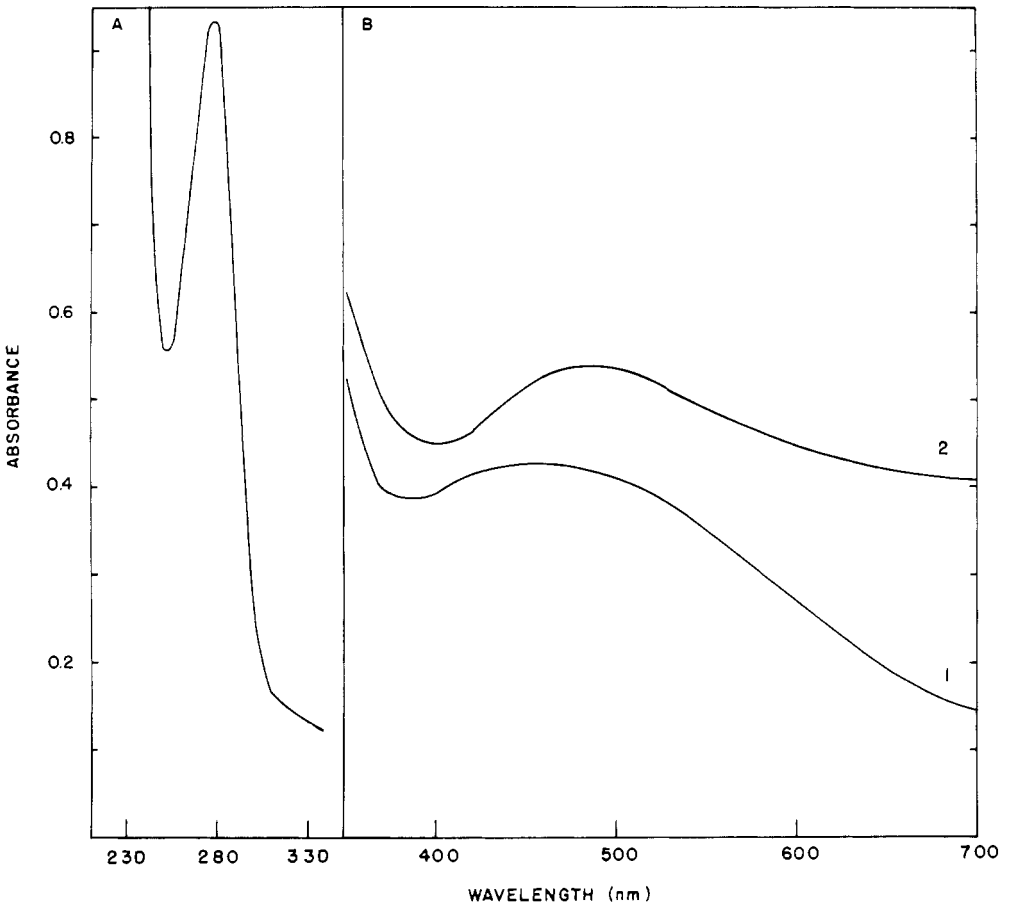

FIGURE 2: Absorption spectra of purified protocatechuate 3,4-dioxygenase. Protein concentrations were 0.6 mg/mL (panel A) and 6 mg/mL (panel B) in Tris-HCl, pH 8.5. Curve 1 of panel B represents the oxidized spectrum. For the reduced spectrum (curve 2), protocatechuate, 1 mM, was added under anaerobic conditions.

Table II: Amino Acid Composition of *Azotobacter* Protocatechuate 3,4-Dioxygenase[a]

amino acid	residues (M_r 480 000)	amino acid	residues (M_r 480 000)
Asp	436.4	Met	63.6
Thr	244.6[b]	Ile	245.4
Ser	206.4[b]	Leu	321.2
Glu	437.6	Tyr	181.1
Pro	265.9	Phe	174.3
Gly	363.8	His	118.1
Ala	323.5	Lys	185.3
Cys	47.6[c]	Arg	276.9
Val	314.4	Trp	64.0[d]

[a] The amino acid composition was determined on a Durrum 500 amino acid analyzer as described in the text. Glutamic acid and aspartic acid values represent the sum of the free acid and amide. [b] Extrapolated to 0-time hydrolysis. [c] Determined as cysteic acid after performic acid oxidation (Moore, 1963). [d] Determined following hydrolytic treatment of the enzyme with 3 N mercaptoethanesulfonic acid.

contains 10 g-atoms of iron per mol of enzyme.

Absorption Spectra. The absorption spectrum of protocatechuate oxygenase at pH 8.5 is shown in Figure 2. The ultraviolet spectrum represented a typical absorption pattern for a protein solution. The ratio of the absorbance at 280 nm to that at 260 nm was 1.43. In the visible region, the enzyme exhibited a broad absorption between 400 and 600 nm. The *molar* absorptivities for the M_r 480 000 oligomer at 280 and 480 nm were 779 000 and 33 900, respectively. Addition of protocatechuate under anaerobic conditions resulted in an increase in absorbancy and a shift in the spectrum to ~480 nm.

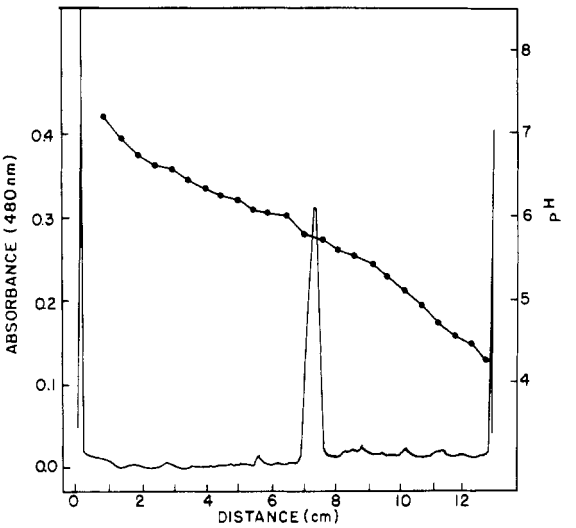

FIGURE 3: Isoelectric focusing of protocatechuate 3,4-dioxygenase. Electrofocusing in polyacrylamide gels was performed as described under Experimental Procedures. After focusing the gel was scanned at 480 nm (—), after which the gel was sectioned and the pH values (●) were determined.

Isoelectric Focusing. The procedure of Righetti & Drysdale (1971) was followed for isoelectric focusing. The absorptivity of the iron chromophore at 480 nm was used to locate protocatechuate oxygenase in the acrylamide gels after focusing (Figure 3). The isoelectric point was 5.87.

Amino Acid Composition. The amino acid composition of *Azotobacter* protocatechuate oxygenase is shown in Table II.

Serological Properties. Antisera prepared against *Azoto-*

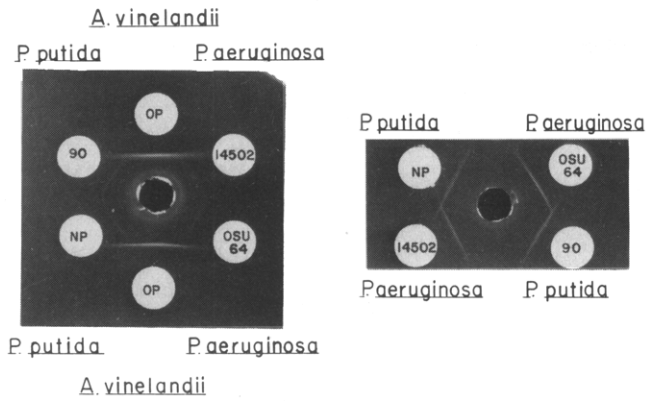

FIGURE 4: Ouchterlony double-diffusion plates. The center wells contained the immunoglobulin fraction of antisera prepared against *Azotobacter* protocatechuate oxygenase. The outer wells contained purified enzyme from *A. vinelandii* (strain OP) and crude extracts of *Pseudomonas* strains (90, NP, OSU 64, and ATCC 14502) which were grown on *p*-hydroxybenzoate.

bacter protocatechuate oxygenase formed a single precipitin band when tested on Ouchterlony double-diffusion plates with either purified enzyme or crude extracts of appropriately induced cells. The antisera also revealed strong cross-reaction when diffused against crude extracts containing protocatechuate oxygenase from either *P. aeruginosa* or *P. putida* (Figure 4). A spur was formed when the *Azotobacter* enzyme was placed in a well neighboring either of the *Pseudomonas* enzymes (Figure 4). Therefore, the *Azotobacter* oxygenase contains antigenic determinants that are not found in the *Pseudomonas* oxygenases. Intersecting spurs were formed when protocatechuate oxygenases from the two fluorescent *Pseudomonas* species were placed in neighboring wells (Figure 4). Hence, each *Pseudomonas* enzyme shares a unique set of antigenic determinants with the reference *Azotobacter* enzyme. The antiserum gave rise to a very weak precipitin band when tested against crude extracts containing *Acinetobacter* protocatechuate oxygenase.

Catalytic Properties. The pH optimum for the dioxygenase was found to be 8.5, and at this pH the enzyme was stable at 50 °C for 90 min. However, 90% of the enzymatic activity was lost after 10-min incubation at 60 °C.

The apparent K_m of protocatechuate oxygenase for its organic substrate, protocatechuate, was calculated from double-reciprocal plots (Lineweaver & Burk, 1934) as 1.8×10^{-5} M. The apparent K_m for oxygen, as measured polarographically in the presence of saturating amounts of protocatechuate (2 μmol), was 2.75×10^{-5} M.

The substrate specificity of the enzyme was studied polarographically by adding substrate analogues, 1 μmol, to air-saturated buffer solutions containing enzyme. As shown in Table III, protocatechuate oxygenase exhibited restricted specificity; in addition to protocatechuate (100%), only pyrogallol (10%), 3-methylcatechol (5%), and catechol (3%) were oxidized at rates that could be measured with the assay system used.

The effect of a variety of inhibitors on enzymatic activity was determined polarographically by adding the potential inhibitor (5 μmol) to assay mixtures containing protocatechuate, enzyme, and air-saturated buffer (Table IV). Protocatechuate oxygenase activity was inhibited by a number of substrate analogues, with those substituted in the "para" position being more potent inhibitors than "meta"-substituted compounds. The inhibition of enzymatic activity by protocatechualdehyde and 4-nitrocatechol was found to be competitive, and K_i values obtained for these inhibitors were 1.0 $\times 10^{-6}$ and 6×10^{-7} M, respectively. Ferrous ion chelators caused little or no inhibition. Tiron, a ferric iron chelator, inhibited activity after a preincubation period. The enzyme was not inhibited by sulfhydryl agents (Table IV).

Table III: Substrate Specificity of Protocatechuate 3,4-Dioxygenase from *A. vinelandii*[a]

compd	rel rate (%)
protocatechuate	100
pyrogallol	10[b]
3-methylcatechol	5
catechol	3
4-methylcatechol	1

[a] Each compound, 1 μmol, was added to a reaction mixture which contained 50 μmol of Tris-HCl, pH 8.5, and purified enzyme in a total volume of 3 mL. Oxygen consumption was measured with an oxygen electrode as described in the text. The relative rate of oxidation of the following compounds was less than 0.01%: 3-isopropylcatechol; 2,3-dihydroxybenzoate; 3,4-dimethoxyphenylacetic acid; *o*-aminophenol; phenol; caffeic acid; benzoate; *p*-hydroxybenzoate; *p*-(dimethylamino)benzaldehyde; 3,4-dihydroxyphenylacetic acid; 4-nitrocatechol; vanillic acid. [b] Rate of oxidation was determined at pH 7.5.

Table IV: Effect of Inhibitors on Protocatechuate 3,4-Dioxygenase[a]

substrate analogues[b]	% inhibn
4-nitrocatechol	100
protocatechualdehyde	95
3,4-dihydroxyphenylacetic acid	87
4-methylcatechol	66
p-hydroxybenzoate	65
2,3-dihydroxybenzoate	50
caffeic acid	36
catechol	33
p-(dimethylamino)benzaldehyde	25
iron chelators, sulfhydryl reagents, and others[b]	**% inhibn**
tiron	70[c]
H_2O_2	25
α,α-dipyridyl	16
o-phenanthroline	9

[a] Each reaction mixture contained 50 μmol of Tris-HCl, 5.0 μmol of inhibitor, and enzyme in a total volume of 3 mL. Reactions were initiated by the addition of 1.0 μmol of protocatechuate, and the relative rates of oxygen consumption were determined. [b] Substrate analogues and other compounds not inhibiting enzymatic activity included the following: 3-isopropylcatechol; 3-methylcatechol; 3,4-dimethylphenylacetic acid; *o*-aminophenol; phenol; benzoate; *p*-(hydroxymercuri)benzoate; iodoacetate; glutathione; dithiothreitol; β-mercaptoethanol; EDTA. [c] Preincubated with enzyme for 1 h before rate was determined.

Discussion

Throughout the Discussion we compare properties of protocatechuate 3,4-dioxygenases from various bacterial genera. Most of the relevant comparative data are summarized in Table V.

Kinetic Properties. To the extent that they have been determined, the kinetic properties of protocatechuate oxygenases from *Azotobacter*, *Pseudomonas*, and *Acinetobacter* are roughly constant. The optimal activities of the enzymes fall in a range between pH 8 and 9; the respective K_m values of the enzymes differ by no more than several-fold (Table V). The protocatechuate oxygenases from separate sources appear to differ to a slight extent in their thermal stability and in their sensitivity to various inhibitors.

Physical Structures. Like other well characterized dioxygenases, protocatechuate oxygenase contains two noni-

Table V: Comparison of Protocatechuate 3,4-Dioxygenases from Various Microorganisms

	A. vinelandii	*P. aeruginosa*[a]	*Pseudomonas* sp.[b]	*A. calcoaceticus*[c]	*Thiobacillus* A2[d]
pH optimum	8.5	8.0		8.5–9.0	8.2
K_m for protocatechuate	1.8×10^{-5} M	3.0×10^{-5} M		7.1×10^{-5} M	6.6×10^{-5} M
K_m for oxygen	2.8×10^{-5} M	4.3×10^{-5} M		5.9×10^{-5} M	3.8×10^{-5} M
$s_{20,w}$	20.9×10^{-13} S	19.4×10^{-13} S		19×10^{-13} S	18.8×10^{-13} S
$D_{20,w}$	4.2×10^{-7} cm^2 s^{-1}	2.5×10^{-7} cm^2 s^{-1}			
oligomeric mol wt					
gel filtration	510 000		190 000		
sedimentation equilibrium	480 000		190 000	677 000	660 000
sedimentation velocity		700 000			
subunit mol wt	23 300/25 250	22 500/25 000	22 000/24 000		21 000
Fe content (g-atoms/mol of oligomer)	10	8	3.9	7.3	5.75
$S\Delta Q$[e]	0	29		89	31
immunological cross-reaction with *Azotobacter* oxygenase	+	+	+	+ (weak)	ND[f]

[a] Fujisawa & Hayaishi (1968); Yoshida et al. (1976). [b] Bull et al. (1979). [c] Hou et al. (1976). [d] Wells (1972). [e] A measure of similarity of amino acid composition (Marchalonis & Weltman, 1971). Details are discussed in the text. [f] Not determined.

dentical subunits in apparently equal quantity (Figure 1). The combined molecular weight of the nonidentical subunits in the *Azotobacter* enzyme is 48 500. Since the molecular weight of the enzyme is ~480 000, the most direct interpretation of the data is that the oligomer contains 10α and 10β subunits. The enzyme also contains 10 iron atoms/480 000-dalton oligomer. Hence, based on the ratios of its chemical constituents, the enzyme may be described as a decamer with the structure 10($\alpha\beta$Fe).

The oligomeric molecular weight of 480 000 for the *Azotobacter* protocatechuate oxygenase is substantially less than the respective molecular weights of 700 000, 677 000, and 660 000 reported for the corresponding enzymes from *P. aeruginosa*, *A. calcoaceticus*, and *Thiobacillus* A2. Since the subunit sizes of the *Azotobacter*, *Pseudomonas*, and *Thiobacillus* enzymes are approximately the same, the oligomeric structures of the proteins appear to differ. Additional variation in oligomeric structure is demonstrated by *Pseudomonas* sp.[1] protocatechuate oxygenase which appears to be an $\alpha\beta$Fe tetramer. The fact that the protomers of the protocatechuate oxygenases self-associate to different degrees cannot be taken as evidence that the proteins are evolutionarily distant. London & Kline (1973) have shown that evolutionarily homologous enzymes may be elaborated in different organisms as dimers, tetramers, or hexamers. More striking than the diverse levels of protomer self-association is the apparent variation in the structure of the protomers themselves. Whereas the *Azotobacter* and *Pseudomonas* sp. oxygenases possess protomers that can be most simply described as $\alpha\beta$Fe, the protomer of the *P. aeruginosa* enzyme appears to be $\alpha_2\beta_2$Fe (Yoshida et al., 1976).

Amino Acid Compositions. Proteins with closely homologous primary structures must possess similar amino acid compositions whereas similar amino acid compositions in the proteins with widely divergent structures might be expected to be fortuitous and hence infrequent. This proposition was explored systematically by Marchalonis & Weltman (1971). Their measure of compositional relatedness, $S\Delta Q$, is the sum of the square of the difference in mole fraction of each amino acid that may be readily and quantitatively determined in a protein hydrolysate. In over 5000 pairwise comparisons of amino acid compositions, Marchalonis & Weltman (1971) found that an $S\Delta Q$ of less than 50 invariably reflected a structural affinity that was evident on comparison of amino acid sequences. Discernible similarities of amino acid sequence were found in 98% of the protein pairs with an $S\Delta Q$ of less than 100.

By use of the amino acid composition of *Azotobacter* protocatechuate oxygenase as a reference, $S\Delta Q$ values of 28, 31, and 89, respectively, were calculated on comparison with the amino acid composition of protocatechuate oxygenase from *P. aeruginosa*, *Thiobacillus* A2, and *A. calcoaceticus*. Hence, by the criterion of compositional relatedness, it appears that the *Azotobacter* protocatechuate oxygenase may possess considerable amino acid sequence homology with the isofunctional dioxygenases from *Pseudomonas* and *Thiobacillus*.

Immunological Comparisons. Serological techniques provide reasonably reliable estimates of amino acid sequence homology among proteins. Antisera prepared against *Azotobacter* protocatechuate oxygenase formed a precipitin band with the antigen, and heterologous cross-reaction was observed with crude extracts prepared from appropriately induced cultures of representatives of other bacterial genera. The immunological data show that the *Azotobacter* and *Pseudomonas* protocatechuate oxygenases share multiple antigenic determinants. Evolution of this degree of immunological similarity cannot be ascribed readily to convergent evolution, and we conclude that the *Azotobacter* and *Pseudomonas* enzymes share a common ancestral gene. As suggested by immunological cross-reaction (data not shown) and also by comparison of amino acid compositions, the enzyme from *Acinetobacter* appears to be distantly related to the other protocatechuate oxygenases.

The close similarity of *Azotobacter* protocatechuate oxygenase to those of fluorescent *Pseudomonas* species raises the possibility that additional evolutionary affinities will be revealed by comparison of genes for the β-ketoadipate pathway in these two bacterial groups. Fluorescent pseudomonads possess an unusual set of regulatory genes that allow β-ketoadipate to act as a product inducer of the three enzymes that give rise to it from β-carboxymuconate (Ornston & Parke, 1977). This induction pattern is not found in other pseudomonads that belong to the Acidovorans group (Ornston & Parke, 1977), and it is not found in *Acinetobacter* (Canovas et al., 1967). The inductive effect of β-ketoadipate is mimicked by the nonmetabolizable analogue adipate in *P. putida* (Parke & Ornston, 1976), and we have found that adipate is an effective inducer analogue for the three enzymes that give rise

[1] This bacterial strain has tentatively been identified as a representative of *Pseudomonas putida*, but some of the organism's properties are atypical of the species. Until the organism undergoes rigorous taxonomic scrutiny, it is best to refer to it as *Pseudomonas* sp.

to β-ketoadipate in *Azotobacter* (unpublished observations). Thus, the physiological evidence suggests that both structural and regulatory genes in *Azotobacter* and fluorescent *Pseudomonas* species may be evolutionarily homologous. In this regard, it should be noted that DNA from the two bacterial groups hybridizes to a substantial extent (DeLey & Park, 1966), and nearly identical amino acid sequences have been observed in comparison of ferredoxins (Hase et al., 1978) and cytochromes c_4 (Ambler & Murray, 1973) formed by members of the two taxa.

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