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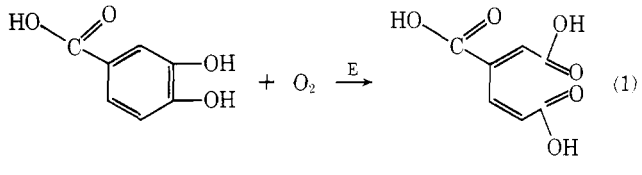
Protocatechuate 3,4-Dioxygenase from *Acinetobacter calcoaceticus*†

Ching T. Hou,* Marjorie O. Lillard, and Robert D. Schwartz

ABSTRACT: Protocatechuate 3,4-dioxygenase (PCD) from *p*-hydroxybenzoate-induced cells of *Acinetobacter calcoaceticus* was purified by heat and protamine sulfate treatment, ammonium sulfate fractionation, DEAE-cellulose, and Sephadex G-200 column chromatography. The enzyme appears to be homogeneous by ultracentrifugation and acrylamide gel electrophoresis. This is the first report of PCD purified from *Acinetobacter.* For comparison, crystalline *Pseudomonas* PCD was also obtained. The enzymes from *Acinetobacter* and *Pseudomonas* are quite similar in their molecular weight, molecular size, and iron content. The specific enzyme activity of PCD from *Acinetobacter* is about one-third of that from *Pseudomonas,* despite their similar iron content. Visible and circular dichroism spectra indicate some conformational differences between these two enzymes. Protocatechualdehyde, a competitive deadend inhibitor, binds *Pseudomonas* PCD more effectively than *Acinetobacter* PCD. *p*-Hydroxymercuribenzoate, specific for free -SH groups, inhibits only *Acinetobacter* PCD and shows no effect on *Pseudomonas* PCD. Amino acid analyses reveal very low proline and methionine content with higher lysine, glutamic acid, and isoleucine compositions for *Acinetobacter* PCD. Other properties, including active center conformation, were studied and discussed.

The oxidative attack on simple aromatic compounds by microorganisms leads to formation of catechol or protocatechuic acid as the last intermediate product with an aromatic structure. Protocatechuate 3,4-dioxygenase (PCD)[1] (protocatechuate:oxygen 3,4-oxidoreductase, EC 1.13.11.3), a nonheme trivalent iron-containing enzyme, catalyzes the conversion of protocatechuate to β-carboxy-*cis,cis*-muconic acid (eq 1).



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The enzyme activity has been reported from several sources (Stanier and Ingraham, 1954; Gross et al., 1956; Cain and Cartwright, 1960; Ornston, 1966). The protein isolated from *Pseudomonas aeruginosa* was first crystallized by Fujisawa and Hayaishi (1968). No other laboratories have reported the crystallization of PCD (personal communication with Drs. O. Hayaishi and H. Fujisawa). The valence state of the iron and other properties of the *Pseudomonas* PCD were also reported (Fujisawa et al., 1972a,b; Fujiwara and Nozaki, 1973).

In our continuing effort to understand the nature of oxygenases and the conformation of their active centers, we have been investigating the PCD induced in *Pseudomonas aeruginosa* (Zaborsky et al., 1975; Hou, 1975). We have demonstrated that the primary binding site for organic substrate is located in the amino acid residues of the enzyme protein and is distinct from the iron-containing catalytic site. Recently, we have isolated PCD from a completely different genus, the bacterium *Acinetobacter calcoaceticus.* We hope that a detailed comparison of these two enzymes might provide information on the conformation of the active sites and the essential characteristics required for the catalytic activity. We now report on the purification and physical-chemical properties of *Acinetobacter* PCD and compare them with the crystalline PCD from *Pseudomonas.*

Materials and Methods

Assay of PCD. The enzyme activity was assayed both spectrophotometrically and by measuring oxygen consumption. In the spectrophotometric assay, the decrease in absorbance at 290 nm (MacDonald et al., 1954) was measured with a Beckman automatic recording spectrophotometer Model DBG at 24 °C. The assay system contained, in a final volume of 3.0 ml, 1.2 μmol of protocatechuic acid, 150

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[1] Abbreviations used are: PCD, protocatechuate 3,4-dioxygenase; PC, pyrocatechase.

μmol of Tris-acetate buffer (pH 7.5), and a suitable amount of enzyme in a cuvette with a 10-mm light path. One unit of enzyme is defined as the amount that oxidizes 1 μmol of protocatechuic acid per minute at 24 °C. Specific activity is defined as the number of enzyme units per milligram of protein. In the oxygen consumption assay, the decrease in dissolved oxygen was recorded with a YSI oxygen monitor, Model 53 (Yellow Spring Instrument Co. Inc., Yellow Spring, Ohio), equipped with an A-25 strip-chart recorder (Varian Aerograph, Walnut Creek, Calif.). The assay system was the same as that used in the spectrophotometric assay. In all cases, the specific activities measured by these methods were identical.

Chemicals. Protocatechuic acid (PCA), pyrogallol, protocatechualdehyde, DL-β-3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylacetic acid, 2,5-dihydroxybenzoic acid, vanillic acid, *p*-hydroxymercuribenzoate, protamine sulfate Grade I, and 2-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, Mo.). α-Chloro-*p*-hydroxyacetophenone, α-chloro-3,4-dihydroxyacetophenone, 3,4-dimethoxyphenylacetic acid, 3-isopropylcatechol, 3,4-dihydroxycinnamic acid, 3-methylcatechol, 2,5-dimethylphenol, and 4-methylcatechol were products of Aldrich Chemical Co., Inc. (Milwaukee, Wis.). Phenol, 3,5-dihydroxybenzoic acid, and catechol were from Matheson Coleman and Bell (Norwood, Ohio). 2,4-Dibromoacetophenone and α-bromo-*p*-nitroacetophenone were purchased from Eastman Kodak Co. (Rochester, N.Y). *m*-Nitrophenol is a product of Fisher Scientific Co. (Fair Lawn, N.J.). Cyanogum 41 gelling agent and polyacrylamide gel-related chemicals were purchased from E-C Apparatus Corp., Miami, Fla.

Protein Determination. The protein content of the enzyme preparation was determined either from the absorbance at 280 and at 260 nm (Layne, 1957) or by the colorimetric method of Lowry et al. (1951) using crystalline bovine serum albumin as a reference standard.

Ultracentrifugation Studies. The sedimentation velocity was determined using a Spinco Model E ultracentrifuge. The enzyme was dissolved in 50 mM Tris-HCl buffer (pH 8.5). The centrifuge was operated at 42 000 rpm at 4 °C. Sedimentation equilibrium was carried out according to the procedure of Yphantis (1964) at 8000 rpm at 20 °C. All calculations were corrected to water at 20 °C and zero protein concentration (Schachman, 1957).

Analytical Gel Electrophoresis. Polyacrylamide gel electrophoresis (5% gel) was carried out at pH 8.5 with an E-C 470 Vertical gel system at 300 V. To each slot about 15 μg of protein (in 33% glycerol) was applied. The gels were stained with Amido Black and destained with E-C 489 electrophoretic destainer.

Spectral Studies. Absorption spectra of the enzyme were recorded with a Beckman Acta Model V. Circular dichroism (CD) spectra were obtained with a Durrum-Jasco J-20 recording spectropolarimeter purged with nitrogen (Japan Spectroscopic Co. Ltd., Tokyo, Japan). The following conditions were employed for obtaining spectra: scanning speed 5 nm/min; time constant, 1 s; scale 2 m°/cm; cell length, 0.5 or 10 mm; temperature, 25 °C.

Amino Acid Analysis. Amino acid analyses were performed with a Beckman Model 120B amino acid analyzer (Beckman Instruments, Inc., Mountainside, N.J.). Acid hydrolysates were prepared as described by Moore and Stein (1963).

PCD Purification. Acinetobacter calcoaceticus 80-1 was obtained from our culture collection. It is a clonal derivative of ATCC 14987. PCD was induced in 10-l. batch fermentations by growing the cells in P_i minimal salts medium supplemented with 0.3% *p*-hydroxybenzoic acid and 0.03% (wt/v) yeast extract at 30 °C and 2.5 l./min aeration rate (Abbott and Hou, 1973). PCD was purified by the following procedure performed at 4 °C except as noted. Frozen cells were thawed and suspended in three times their volume (wt/v) of 0.05 M Tris-HCl buffer (pH 8.5). The cells were disrupted by sonic oscillation using a Wave Energy Ultrasonic oscillator, Model W201 (Wave Energy System Inc., Newtown, Pa.), equipped with a refrigerated continuous probe and operated at 150 mV. The flow rate was 14 ml/min. The enzyme activity was recovered in the cell-free supernatant after centrifugation at 10 000*g* at 4 °C for 20 min. About 1350 ml of cell-free extract having a specific activity of 0.9 was obtained. The cell-free extracts were heated to 55 °C for 5 min in a bath maintained at 58 °C and then cooled in an ice bath. The resulting precipitate was removed by centrifugation. The precipitate was washed with 500 ml of 0.05 M Tris-HCl buffer (pH 8.5) and the washings were combined with the supernatant solution. To the supernatant, protamine sulfate solution in 0.1 M Tris base was added with magnetic stirring, using 0.1 g of protamine sulfate/g of protein. After 5 min of stirring, the precipitate was removed by centrifugation. The active supernatant solution was fractionated by the addition of solid ammonium sulfate. The protein fraction which precipitated between 30 and 50% saturation was collected and dissolved in a minimum volume of 0.05 M Tris-HCl buffer (pH 8.5). The enzyme was dialyzed against the same buffer overnight, then against water for 8 hr and finally against 0.05 M Tris-acetate buffer (pH 7.5) for 16 h. After dialysis, the soluble enzyme was separated from insoluble protein by centrifugation. DEAE-cellulose (medium mesh, obtained from Sigma Chemical Co.) was equilibrated with 0.05 M Tris-acetate buffer (pH 7.5) and packed in a 4 × 40 cm column at 4 °C. The enzyme fraction obtained from dialysis was applied to the column. The column was washed with 200 ml of buffer and the PCD was eluted with a continuous linear gradient formed from 800 ml of 0.05 M Tris-acetate buffer (pH 7.5) in the mixing bottle and 800 ml of 0.5 M NaCl in 0.05 M Tris-acetate buffer in the reservoir. The elution rate was 40 ml/h and 6.3-ml fractions were collected. The enzyme activity was eluted between fractions 110 and 150. The combined active fraction was washed and concentrated in an Amicon ultrafiltration unit using 0.05 M Tris-acetate buffer (pH 7.5) and membrane XM 100. The enzyme-containing solution (2.0 ml) was applied to a column (3 × 90 cm) of Sephadex G-200 (previously equilibrated with 0.05 M Tris-acetate buffer (pH 7.5)) and was eluted ascendingly with the same buffer at a rate of 12 ml/h. Fractions of 2.4 ml were collected. PCD was eluted at about fraction 100. Further purification by a column of Sepharose 4B shows no further increase in specific activity. A summary of the purification steps is given in Table I. The best fraction in Table I, specific activity of 20, was used throughout the experiments. Despite many attempts using various methods, it has not yet been possible to obtain *Acinetobacter* PCD in crystalline form.

PCD from *Pseudomonas aeruginosa* ATCC 23975 was purified and crystallized according to the procedure of Fujisawa and Hayaishi (1968). The scanning electron microscopy studies of *Pseudomonas* PCD have been reported (Hou et al., 1975b).

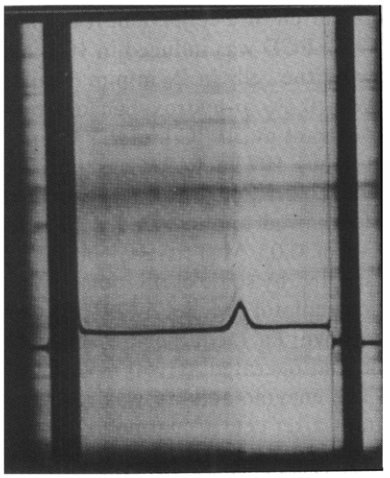

FIGURE 1: Ultracentrifugal pattern of purified PCD from *A. calcoaceticus*. The protein concentration was 5 mg/ml in 0.05 M Tris-HCl buffer (pH 8.5). The photographs were taken at intervals of 16 min after reaching 42 000 rpm at 4 °C. The progress of ultracentrifugation is shown from right to left.

Table I: Purification of PCD from *A. calcoaceticus*.

Step	Specific Activity (units/mg of Protein)	Protein (mg)	Total Activity (units)	Yield (%)
Crude extract	0.98	31 090	30 470	100
Heat treatment	2.07	10 300	21 320	70
Protamine treatment	2.51	7 960	19 980	66
Ammonium sulfate fractionation	7.46	1 590	11 860	39
Dialysis	5.93	1 060	6 290	21
DEAE-cellulose column	10.56	400	4 230	14
Sephadex G-200 (best fraction)	20.0	35	700	

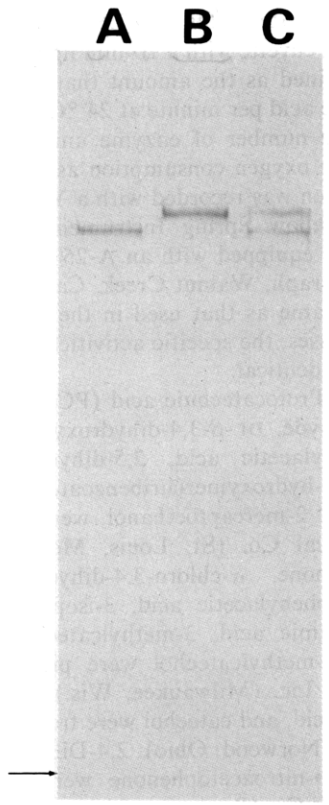

FIGURE 2: Gel electrophoretic pattern of PCD from different sources. About 15 μg of the enzyme was applied to each gel slot. Protein migrated to the anode (bottom) at a constant voltage of 300 for 2.5 h in 0.05 M Tris-glycine buffer (pH 8.5). Arrow indicates front marker. (A) *Acinetobacter* PCD (after Sephadex G-200 column); (B) *Pseudomonas* PCD; (C) coelectrophoresis of the two enzymes.

Results

Homogeneity. The homogeneity of highly purified enzyme preparation from *Acinetobacter* was examined by ultracentrifugation and acrylamide gel electrophoresis. The enzyme preparation was found to be homogeneous upon ultracentrifugation as shown in Figure 1. For purity and comparison, PCD from both sources were subjected to acrylamide gel electrophoresis. The results are shown in Figure 2. Enzymes from both sources have a single protein band (Figure 2A and B). When coelectrophoresed, two distinct protein bands are detected (Figure 2C). The PCD from *Acinetobacter* apparently possesses a greater negative charge than PCD from *Pseudomonas*.

Molecular Weight. The sedimentation velocity of the *Acinetobacter* PCD was measured at three different protein concentrations (6, 3, and 1.5 mg/ml). The dependence of sedimentation constant on protein concentration is not significant. The sedimentation constant, $s_{20,w}^0$, is 19 S.

Sedimentation equilibrium studies further confirmed that the *Acinetobacter* PCD was homogeneous. The molecular weight was calculated to be 677 000 taking the partial specific volume as 0.725.

Absorption Spectra. The visible and ultraviolet absorption spectra of PCD in pH 8.5 buffer are shown in Figure 3. For comparison, absorption spectra of *Pseudomonas* PCD are also shown in the figure. In the visible range, both enzymes showed a broad absorption between 400 and 500 nm; however, different absorption maxima were observed. The molar absorptivities are: 280 nm (795 000), 410 nm (27 900), and 450 nm (21 900) for *Acinetobacter* and 280 nm (816 000), 410 nm (26 600), and 450 nm (27 700) for *Pseudomonas* PCD.

Circular dichroism (CD) spectra of both enzymes are also compared and shown in Figure 4. Both enzymes showed a positive CD band between 250 and 300 nm, and a broad negative band around 475 nm. The CD bands, both in the aromatic region, 250–300 nm, and the visible region are identical for these two enzymes, with PCD from *Pseudomonas* having a greater intensity. At 380 nm, *Pseudomonas* PCD has a positive CD band, whereas *Acinetobacter* PCD exhibits a negative one. In the lower wavelength region, both enzymes exhibit a strong negative CD band with a maximum at 216 nm. From the value of the molar ellipticity at 222 nm, using the approximation method of Chen and Yang (1971) for estimating the α-helical content, relatively low helical contents of 0.85 and 7.24% were observed for *Pseudomonas* and *Acinetobacter* PCD, respectively.

Iron Content. Qualitative analysis for metals by flameless atomic absorption spectrophotometry revealed that no metals other than iron were present in PCD from *Acinetobacter*. Quantitative analysis of iron by atomic absorption spectrophotometry indicated 7.27 g-atoms of iron/mol of enzyme protein, based on a molecular weight of 677 000.

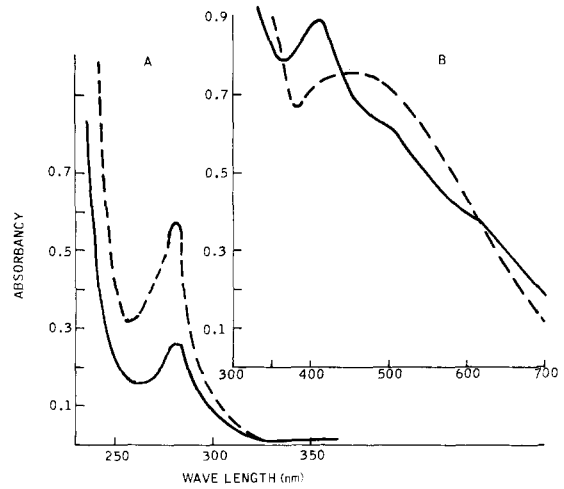

FIGURE 3: Absorption spectra of PCD. (—) Enzyme from *A. calcoaceticus;* the protein concentrations were: (A) 0.22 mg/ml; (B) 22.3 mg/ml. (- - -) Enzyme from *P. aerugenosa;* the protein concentrations were: (A) 0.48 mg/ml; (B) 19.2 mg/ml. Tris-HCl buffer, 0.05 M, pH 8.5, was used.

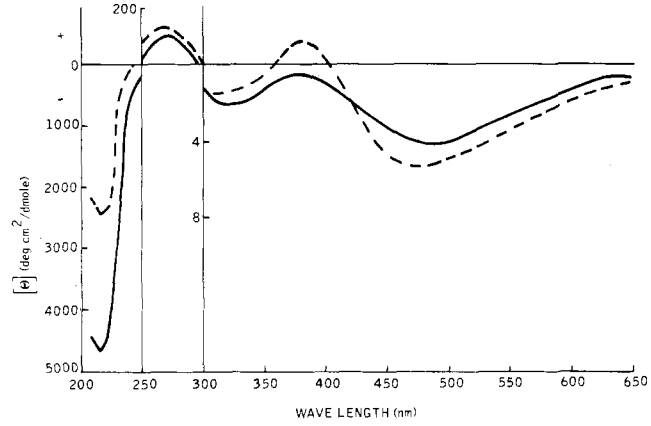

FIGURE 4: Circular dichroism spectra of PCD. The spectra were taken with a 10-mm cell (>250 nm), and a 0.5-mm cell (200-250 nm) at 25 °C in 0.05 M Tris-acetate buffer (pH 7.5). (—) Enzyme from *Acinetobacter;* (- - -) enzyme from *Pseudomonas*. The protein concentrations for *Acinetobacter* and *Pseudomonas* PCD are 22 and 25 mg/ml (300-650 nm), 0.35 and 0.35 mg/ml (250-300 nm), and 0.91 and 0.89 mg/ml (200-250 nm), respectively.

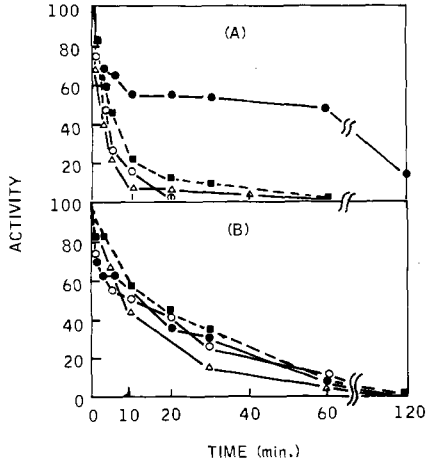

FIGURE 5: Stability of PCD from *Acinetobacter* and *Pseudomonas*. PCD was incubated at 60 °C for various times in different buffer solutions. (A) *Acinetobacter* PCD; (B) *Pseudomonas* PCD; (■- - -■), 0.05 M Tris-HCl (pH 9.5); (●—●) 0.05 M Tris-HCl (pH 8.5); (○—○) 0.05 M Tris-acetate (pH 7.0); (△—△) 0.05 M phosphate buffer (pH 6.0).

pH Optimum and PCD Stability. The optimum pH for *Acinetobacter* PCD activity is 8.5-9.0, slightly more alkaline than *Pseudomonas* PCD. The effect of pH on the stability of PCD was determined at 60 °C. The *Acinetobacter* PCD was found to be more stable than *Pseudomonas* PCD at pH 8.5. As can be seen from Figure 5, the *Acinetobacter* PCD retained about 50% of its activity, whereas *Pseudomonas* PCD retained less than 10% of its original activity, when incubated at 60 °C for 1 h. *Acinetobacter* PCD lost activity rapidly when incubated at 60 °C at pH values other than 8.5. This effect of pH was not seen with *Pseudomonas* PCD. At pH 8.5, at low temperature (4 °C), both enzymes retained full activity for 6 months.

Effect of Temperature on PCD Activity. Acinetobacter and *Pseudomonas* PCD exhibited the same optimum temperature for activity (35 °C) in 0.05 M Tris-HCl buffer (pH 8.5). The activation energies for *Acinetobacter* and *Pseudomonas* PCD, as calculated from the Arrhenius plots of velocity vs. the reciprocal of the absolute temperature, are 0.93 and 1.33 kcal, respectively. The reaction rate of *Pseudomonas* PCD is higher than that of the *Acinetobacter* enzyme, but only a slight difference was observed in their activation energies. This is due mostly to the difference in their preexponential factor A. ($\ln K = \ln A - (E_a/RT)$, where K is rate constant; E_a is activation energy; R is gas constant, and T is the absolute temperature.)

Michaelis Constant and Substrate Specificity. The K_m values of *Acinetobacter* PCD, calculated from a double reciprocal plot, are 7.14×10^{-5} M for PCA and 5.88×10^{-5} M for oxygen. Substrate specificity of the *Acinetobacter* PCD was studied by adding substrate analogues to air-saturated buffer solution in an electrode vessel and the rate of oxygen uptake recorded. The differences in rate of oxygen uptake with and without enzyme were calculated. The lowest detectable rate of reaction in this system with the amount of enzyme used was 0.5% of the rate of oxidation of PCA by the enzyme. The compounds tested (5 μmol/3 ml of reaction mixture) were: α-bromo-*p*-nitroacetophenone; α-chloro-3,4-dihydroxyacetophenone; chloranil; α-chloro-*p*-hydroxyacetophenone; catechol; 2,4-dibromoacetophenone; β-3,4-dihydroxyphenylalanine; 3,4-dihydroxyphenylacetic acid; 2,5-dihydroxybenzoic acid; 3,4-dihydroxycinnamic acid; *p*-dimethylaminobenzaldehyde; 2,5-dimethylphenol; 3,4-dimethoxyphenylacetic acid; 3-methylcatechol; 4-methylcatechol; 3-isopropylcatechol; *m*-nitrophenol; phenol; protocatechualdehyde; pyrogallol; and vanillic acid. Tris-HCl buffer, 0.05 M, pH 8.5, was used for all the assays except for pyrogallol which was carried out in a 0.05 M Tris-acetate buffer at pH 7.5 due to its instability in alkaline solution. None of the substrate analogues listed above were oxidized by the *Acinetobacter* PCD at rates detectable with this assay system. Therefore, *Acinetobacter* PCD shares with *Pseudomonas* PCD, the property of strict substrate specificity.

Inhibition studies were carried out with organic substrate analogues, metal ions, iron-chelating agents, and sulfhydryl agents. Each compound (6 μmol) was added with PCD to 0.05 M Tris-HCl buffer (pH 8.5) in a total volume of 3 ml. PCA (1.2 μmol) was then added and the rate of oxygen uptake was determined. The inhibition by organic substrate analogues, expressed as percent inhibition of oxidation of PCA, is shown in Table II. The 3-substituted cate-

Table II: Inhibition of *Acinetobacter* PCD Activity by Substrate Analogues.[a]

Compounds	Inhibition (%)
Substituted catechols	
Catechol	63
Protocatechualdehyde	54
4-Methylcatechol	59
α-Chloro-3,4-dihydroxyacetophenone	40
3,4-Dihydroxyphenylacetic acid	92
3,4-Dihydroxycinnamic acid	25
4-Nitrocatechol	100
3-Methylcatechol	0
3-Isopropylcatechol	0
Hydroxybenzoates	
Vanillic acid	0
2,5-Dihydroxybenzoate	32
3,5-Dihydroxybenzoate	0
Benzoic acid	0
Others	
3,4-Dimethoxyphenylacetic acid	0
α-Bromo-*p*-nitroacetophenone	0
m-Nitrophenol	56
o-Chloranil	51
Phenol	0
2,4-Dibromoacetophenone	0
p-Chloro-*p*-hydroxyacetophenone	0
p-Dimethylaminobenzaldehyde	0
2,5-Dimethylphenol	0
p-Hydroxymercuribenzoate	80

[a] In each case, 6 μmol of the compound was added with 4 μg of PCD to 0.05 M Tris-HCl buffer (pH 8.5) in a total volume of 3 ml. PCA, 20 μl (1.2 μmol), was then added and the rate of oxygen uptake determined.

Table III: Inhibition of *Acinetobacter* PCD by Metal Ions.[a]

Compounds	Inhibition (%)
Nickel chloride	43
Nickel ammonium sulfate	34
Cobalt nitrate	50
Ferric ammonium sulfate	0
Ferrous ammonium sulfate	30[b]
Cobaltous chloride	68
Nickelous nitrate	36
Cuprous chloride	68
Cupric sulfate	40
Molybdenum dichloride	0

[a] Assay conditions were the same as described in Table I. [b] Assayed spectrophotometrically.

Table IV: Amino Acid Composition of *A. calcoaceticus* PCD.[a]

Amino Acid	*Acinetobacter* PCD Hydrolysis for 20h	44h	72h	Calcd	*Pseudomonas* PCD[d]
Lysine	418	448	471	446	182
Histidine	167	174	184	175	194
Arginine	406	409	422	412	351
Aspartic acid	658	676	755	696	718
Threonine	271	216	187	300[b]	286
Serine	202	105	72	275[b]	205
Glutamic acid	700	821	839	787	513
Proline	11	11	11	11	425
Glycine	448	467	487	467	460
Alanine	522	543	569	545	477
Valine	283	295	305	294	269
Methionine	7	5	5	6	46
Isoleucine	569	561	579	570	364
Leucine	484	496	520	500	459
Tyrosine	109	70	43	130[b]	188
Phenylalanine	310	316	327	318	247
Half-cystine	55	36	45	46	96
Tryptophan				151[c]	158

[a] PCD was hydrolyzed with 6 N HCl at 110 °C for 20, 44, or 72 h. Analyses were performed on each hydrolysate as described in the text. The results are expressed on the basis of residues per 1 mol of enzyme. [b] These values were extrapolated to zero time hydrolysis. [c] Estimated by spectrophotometric analyses in alkaline solution (22). [d] Our data are similar to those of Fujisawa and Hayaishi (1968).

chols exhibited no enzyme inhibition. 4-Substituted catechols show greater inhibition, especially those having substituents with a carbon chain length of less than two. *Pseudomonas* PCD exhibits similar inhibition patterns, except for protocatechualdehyde and *p*-hydroxymercuribenzoate. The former compound, a competitive deadend inhibitor, seems to bind *Pseudomonas* PCD more effectively than *Acinetobacter* PCD. The latter compound, specific for free SH groups, inhibits only *Acinetobacter* PCD and shows no effect on *Pseudomonas* PCD.

Inhibition was also studied by varying inhibitor and PCA concentration at saturated dissolved oxygen. PCA concentrations between 0.5 and 1.8 times the K_m were used. Inhibitor concentrations were varied between 1 and 5 times the PCA concentration. PCD was added to start the reaction and the uptake of oxygen was measured. Data were analyzed by the least-squares method. The type of inhibition was defined as competitive, noncompetitive, or uncompetitive according to Cleland (1963). All of the inhibition caused by substrate analogues exhibits a competitive inhibition pattern. Noncompetitive inhibition was observed with *m*-nitrophenol, *o*-chloranil, and *p*-hydroxymercuribenzoate. The dissociation constant of the enzyme inhibitor complex, K_I, was determined from the following relationship:

$$K_M' = (1 + [I]/K_I)K_M$$

where K_M' is the apparent Michaelis constant in the presence of a concentration, [I], of an inhibitor; K_M is the Michaelis constant of the enzyme in the absence of inhibitor. The values for $K_I \times 10^5$ M for the enzymes from *Acinetobacter* and *Pseudomonas* respectively were: catechol, 38 and 13.2; 4-methylcatechol, 44 and 10.5; protocatechualdehyde, 7.3 and 1.4; α-chloro-3,4-dihydroxyacetophenone, 17 and 4.5; 3,4-dihydroxyphenylacetic acid, 4.1 and 1.0; 2,5-dihydroxybenzoic acid, 29 and 7.3; 4-nitrocatechol, 0.48 and 0.15. Higher dissociation constants were observed for the *Acinetobacter* PCD than for the *Pseudomonas* PCD.

The inhibition of PCD activity by transition metal ions (2 mM) is shown in Table III. *Acinetobacter* PCD was inhibited by nickel, nickelous, cobalt, cobaltous, cupric, cuprous, and ferrous ions but not inhibited by ferric and molybdenum. Other compounds tested (at five times the amount of substrate concentration) were: iodoacetamide, 5,5′-dithiobis(2-nitrobenzoate), mercaptoethanol, dithiothreitol, glutathione, *o*-phenanthroline, and H_2O_2. None exhibited inhibition of *Acinetobacter* PCD under the experimental conditions. Tiron inhibited the enzyme reaction only when it was preincubated with the enzyme for several hours. However, the iron in *Acinetobacter* PCD can be removed from the enzyme protein, as has been reported for *Pseudomonas* PCD,

in the presence of both *o*-phenanthroline and sodium dithionite under anaerobic conditions (Fujiwara and Nozaki, 1973; Hou, 1975).

Amino Acid Composition and Sulfhydryl Groups. The results of amino acid analyses are summarized in Table IV. For comparison, the amino acid composition of *Pseudomonas* PCD is also listed. These analyses indicate the presence of 46 residues of half-cystine per 677 000 g of *Acinetobacter* PCD. Of these, about 2 mol of sulfhydryl/mol of enzyme was readily titrated by *p*-hydroxymercuribenzoate according to the method of Boyer (1954). Surprisingly low contents of methionine and proline were noted. There are some quantitative similarities in amino acid composition between *Acinetobacter* and *Pseudomonas* PCD, e.g., histidine, arginine, aspartic acid, threonine, serine, glycine, alanine, leucine, tyrosine, phenylalanine, and tryptophan. The most abundant amino acid is glutamic acid for *Acinetobacter* PCD and aspartic acid for *Pseudomonas* PCD. Higher lysine, isoleucine, and glutamic acid contents were found in *Acinetobacter* PCD. About half the amount of cysteine was detected in PCD from *Acinetobacter* as in PCD from *Pseudomonas*.

Discussion

Acinetobacter is a completely different genus from *Pseudomonas*. The former is a nonmotile coccobacillus with DNA content ranging from 40 to 47% G + C. The latter is a motile rod-shaped bacterium, having a DNA content of 58–69% G + C.

In the present studies, the first homogeneous PCD from *Acinetobacter* was obtained and its properties were compared with those of PCD from *Pseudomonas*. The purification procedure leading to crystallization of PCD from *Pseudomonas* does not apply to PCD from *Acinetobacter*. Conformational differences in the PCD structure and/or compositional differences in the cell-free extracts of *Acinetobacter* might be the explanation. The visible and CD spectra of the enzyme from *Acinetobacter* differ from those for the *Pseudomonas* enzyme, as can be seen from Figures 3 and 4. This suggests some conformational differences between the two enzymes which may be responsible for the different specific activities. The possibility that the differences result from an artifact introduced during the purification procedure is quite unlikely, since different purification and crystallization procedures have been studied and all lead to the same final specific enzyme activity and no crystal formation.

The difference in molar ellipticity (positive and negative) at 380 nm for PCD isolated from different sources was noted. A similar phenomenon was reported for pyrocatechase (PC). The peak in PC isolated from *Pseudomonas arvilla* centered at 390 nm, and does not extend into the positive ellipticity, whereas PC isolated from *Brevibacterium fuscum* does have a positive band at 390 nm (Nagami and Miyake, 1972). The PCD preparations exhibit a difference in α-helical content, indicating a conformational difference. In the lower wavelength region, the CD of *Acinetobacter* is quite similar to that of PC, another trivalent nonheme dioxygenase (Nakazawa et al., 1969). PC exhibits a similar low α-helical content of about 6% ($[\theta]$ at 222 nm is −4120).

The PCD crystals from *Pseudomonas* obtained without sulfhydryl agent treatment showed globular aggregates which were uniform in size and shape. This preparation consisted of two to four components as revealed by ultracentrifugation and electrophoresis (Fujisawa and Hayaishi, 1968). However, the PCD from *Acinetobacter* obtained without incubation with sulfhydryl agent showed only one component, either by ultracentrifugation or by acrylamide gel electrophoresis.

Protocatechualdehyde, a deadend inhibitor for PCD and a structural analogue of the organic substrate, PCA, inhibits *Acinetobacter* PCD competitively with respect to PCA and noncompetitively with respect to oxygen. These results are consistent with an ordered bi-uni mechanism where the organic substrate first binds with PCD and then reacts with oxygen to form a ternary complex (Hou et al., 1975a). This finding is in agreement with the data from *Pseudomonas* PCD (Hori et al., 1973) and in contrast to the steroid dioxygenase data from *Nocardia* (Tai and Sih, 1970).

The K_I value of 4-nitrocatechol for *Pseudomonas* PCD was reported by Tyson (1975). It is hard to compare his data with ours since the K_m value for PCA in his report is about six- to tenfold lower than that reported by Fujisawa and Hayaishi (1968) and ours (Hou et al., 1975a).

It is interesting that *p*-hydroxymercuribenzoate, a thiol binding agent, inhibits *Acinetobacter* PCD but not *Pseudomonas* PCD. PCD from *Acinetobacter* contains fewer free sulfhydryl groups compared to that from *Pseudomonas*. This suggests that free sulfhydryl groups in *Acinetobacter* PCD are located at or near the active center of the enzyme. Iron analyses indicated 7–8 mol of iron/mol of enzyme. With fewer *p*-hydroxymercuribenzoate titratable sulfhydryl groups (2 mol/mol of enzyme), it is difficult to imagine that eight subunits are present in *Acinetobacter* PCD. Our efforts to determine the number of subunits by titration against PCD inhibitor are not conclusive. This is partly because protocatechualdehyde shows only 54% inhibition (Table II) and partly because it is not possible to follow the method described by Fujisawa et al. (1972a).

PCD from *Acinetobacter* retained full activity after incubation with either iodoacetamide, mercaptoethanol, glutathione, or dithiothreitol. These results are different from those found for *P. fluorescens* by Stanier and Ingraham (1954) and coincide with those found for *P. aeruginosa* by Fujisawa and Hayaishi (1968).

Amino acid analyses revealed a surprisingly low content of methionine and proline for *Acinetobacter* PCD. This suggests that about 90% of the methionine and the amino acid content of *Pseudomonas* PCD does not participate in the PCD activity and can be replaced. There are 274 residues more (per mole of PCD) of glutamic acid detected in PCD from *Acinetobacter*. The low isoelectric point of glutamic acid might contribute to the different mobility of PCD on gel electrophoresis. In comparing the specific enzyme activity of both PCD with their content of free sulfhydryl groups and cystines, a consistent relationship can be observed. PCD with high specific enzyme activity contains a greater number of free sulfhydryl groups.

Strict substrate specificity was also observed for *Acinetobacter* PCD. 4-Substituted catechols show greater inhibition, especially those having substituents with a carbon chain length of less than two. Since the substrate binding site of the iron-free apoenzyme also binds substrate analogues (Hou, 1975), an additional specific site might exist in the PCD active center. This site may be distinct from the substrate binding site, respond only to the carboxylic acid moiety, and be responsible for activating the iron-containing catalytic site.

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A Measurement of the Fraction of Chloroplast DNA Transcribed during Chloroplast Development in *Euglena gracilis*†

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ABSTRACT: The fraction of chloroplast DNA transcribed at different stages of chloroplast development in *Euglena gracilis* was measured by RNA–DNA hybridization. *Euglena* cells were grown in the dark in a heterotrophic medium to stationary phase and then transferred to the light. Chloroplast development was monitored by the increase in the cellular chlorophyll content in the absence of cell division. Total cell RNA was isolated at various stages of chloroplast development, and hybridized in a vast excess to [^{125}I]chloroplast DNA. The fraction of [^{125}I]chloroplast DNA in the form of a duplex was monitored by chromatography on hydroxylapatite columns. The amount of RNA–DNA hybrid in the duplex mixture was determined by correcting for the contribution of DNA–DNA renaturation under the same conditions. The fraction of chloroplast DNA transcribed was calculated by multiplying by two the amount of single-stranded DNA in the form of an RNA–DNA hybrid. Prior to the initiation of chloroplast development (i.e., in dark grown cells) the fraction of chloroplast DNA represented as RNA transcripts in the cell is 0.53. As chloroplast development proceeds, the fraction of the chloroplast DNA transcribed decreases to 0.47. Experiments in which mixtures of various RNA samples were hybridized to the chloroplast DNA indicate that there is a small portion of chloroplast DNA transcribed at later stages of chloroplast development which is not represented as transcripts at the onset of chloroplast development. Melting properties of the RNA–DNA hybrids show that the RNA–DNA duplexes are slightly less stable than renatured [^{125}I]chloroplast DNA.

The differentiation of proplastids into chloroplasts in the single cell alga *Euglena gracilis* offers a unique system for the study of transcriptional events during an intracellular developmental process. When *Euglena* is grown in the dark in a heterotrophic medium, it contains numerous proplastids which are the precursor bodies of chloroplasts. Illumination of these dark grown cells initiates chloroplast development, and within 48 hr a cell contains ten chloroplasts

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