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Catechol 1,2-Dioxygenase from *Acinetobacter calcoaceticus*: Purification and Properties

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Procedures for the purification of catechol 1,2-dioxygenase from extracts of *Acinetobacter calcoaceticus* strain ADP-96 are described. The purified enzyme was homogeneous as judged by ultracentrifugation and acrylamide gel electrophoresis. The enzyme contained 2 g-atoms of iron per mol of protein. The enzyme had a broad substrate specificity and catalyzed the oxidation of catechol, 4-methylcatechol, 3-methylcatechol, and 3-isopropyl catechol. The activity of the enzyme was inhibited by heavy metals, sulfhydryl inhibitors, and substrate analogues. The molecular weight of the enzyme was 85,000 as estimated by filtration on Bio-Gel agarose and 81,000 as estimated by sedimentation equilibrium analysis. The subunit size determined by sodium dodecyl sulfate-gel electrophoresis was 40,000. The amino terminal amino acid was methionine. The amino acid composition and spectral properties of 1,2-dioxygenase are also presented. Antisera prepared against the purified enzyme cross-reacted and inhibited enzyme activity in crude extracts from other strain of *A. calcoaceticus*, but failed to cross-react and inhibit isofunctional enzyme from organisms of the genera *Pseudomonas*, *Alcaligenes*, and *Nocardia*.

Catechol 1,2-dioxygenase (EC 1.13.1.1) (CO), a nonheme, trivalent, iron-containing enzyme, catalyzes the cleavage of the aromatic ring of catechol to *cis,cis*-muconate with the incorporation of 2 atoms of molecular oxygen into the substrate. It represents the initial enzyme of the β-ketoadipate pathway, a metabolic sequence used by microorganisms for the degradation of aromatic compounds (26). Enzymes of the β-ketoadipate pathway, including CO, are inducible in microorganisms. Comparative studies of the mechanisms of regulation in different bacterial genera indicate distinctive mechanisms of induction. Thus, regulation of enzymes of the β-ketoadipate pathway are different in *Acinetobacter* (2) and the fluorescent group of *Pseudomonas* (19). These two groups of organisms are taxonomically separated on the basis of morphology and deoxyribonucleic acid (DNA) content. Thus, organisms of the *Acinetobacter* genus are nonmotile coccobacilli with a DNA content ranging from 40 to 47% guanine plus cytosine (1) and are unlike the motile rod-shaped *Pseudomonas* species, which have a DNA content ranging from 58 to 69% guanine plus cytosine (14, 25).

CO has been purified from *Pseudomonas* species (10, 17) and *Brevibacterium fuscum* (18).

In our continuing effort to understand the nature of oxygenases, particularly the nonheme, trivalent, iron-containing dioxygenases, we have been investigating the properties of the protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus* (6, 7, 31; C. T. Hou, R. D. Schwartz, and M. L. Ohaus, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, RT-1, p. 275; C. T. Hou, M. L. Ohaus, and A. Felix, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, K156, p. 173). We have demonstrated that the organic substrate binding site is distinct from the iron-containing catalytic site (5). Recently we extended our work to CO from *A. calcoaceticus*.

In this report, we describe procedures for the purification of CO from *A. calcoaceticus* and describe some of its properties. We have also prepared antisera against the purified enzyme and present data on the immunological specificity of the enzyme from *A. calcoaceticus*. We hope that studies of these two nonheme, trivalent, iron-containing, aromatic ring fission enzymes will provide information on the essential active site conformation for intradiol cleavage of aromatic rings.

MATERIALS AND METHODS

Bacterial strain and its growth. Bacterial strain ADP-96 was derived from Juni's transformable *A. calcoaceticus* strain BD-413 and was isolated as described previously (21). Strain ADP-96 is a regulatory gene mutant that produces the enzyme CO constitutively in the absence of its inducer. Cultures

of strain ADP-96 were grown at 37 C in a 100-liter New Brunswick Fermacell model CF 130 fermenter in mineral medium (20) containing 10 mM sodium succinate as the sole carbon source. Cells were harvested with a refrigerated Sharples centrifuge and stored at −20 C until used.

Cultures of *P. aeruginosa* strain 45 (26), *Alcaligenes eutrophus* ATCC 17697, *Nocardia opaca* 17039, and *Acinetobacter calcoaceticus* ATCC 14987 were grown in 500 ml of mineral medium with 10 mM sodium benzoate as the sole carbon source to induce CO.

Chemicals. Benzoate and catechol were purchased from Matheson Coleman and Bell Co., Norwood, Ohio. 3-Isopropylcatechol, 4-nitrocatechol, 3-methylcatechol, 4-methylcatechol, 3-methoxycatechol, and protocatechualdehyde were obtained from Aldrich Chemical Co., Milwaukee, Wis. Pyrogallol and protocatechuate were purchased from Eastman Organic Chemical Co., Rochester, N.Y. Bio-Gel agarose A-1.5 was obtained from Bio-Rad Laboratories, Richmond, Calif. Ammonium sulfate (ultrapure) was obtained from Schwarz/Mann Co., Orangeburg, N.Y. α,α-Bipyridyl, *O*-phenanthroline, *p*-hydroxymercuribenzoate, tiron, dithiothreitol, and 5,5′-dithiobis-2-nitrobenzoic acid were obtained from Sigma Chemical Co., St. Louis, Mo.

Enzyme assay. Enzyme activity was measured routinely either spectrophotometrically, by measuring the increase in absorbance at 260 nm (A_{260}), or polarographically, by measuring oxygen uptake with an oxygen electrode. The assay system contained 0.6 μmol of catechol in 3.0 ml of 50 mM sodium phosphate buffer, pH 7.5. The reaction was started by addition of a suitable amount of enzyme. One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of *cis,cis*-muconic acid per min under the standard assay conditions. Protein concentrations were determined spectrophotometrically from the A_{280} and A_{260} (12) and by the method of Lowry et al. (13).

Purification of CO. Purification was carried out in 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 8.0 (buffer A), at about 4 C unless otherwise stated. Crude extract was was prepared from 500 g (wet weight) of frozen cells that were thawed at 4 C overnight before extraction. The cells were suspended in 1 liter of buffer A, placed in an ice bath, and stirred continuously with a magnetic stirrer. The cells were disrupted by 90 min of sonication with a Megason ultrasonic disintegrator. Every 5 min, 50 g of cracked ice was added to maintain the temperature of the cell suspension between 0 to 4 C during sonication. The sonicated cell suspension was centrifuged for 1 h at 15,000 × *g*. The supernatant liquid was termed the crude extract (step 1 in Table 1). Protamine sulfate solution (70 ml of 2% solution in 0.1 M Tris base) was added dropwise with constant stirring to 3.5 liters of crude extract. After standing for 30 min, the extract was centrifuged at 15,000 × *g* for 45 min. The supernatant solution (step 2 in Table 1) was fractionated with ammonium sulfate. Extracts were brought to 30% of saturation with respect to ammonium sulfate by the addition of 176 g of the salt per liter of extract. Precipitated protein was removed by centrifugation, and 162 g of ammonium sulfate was added per liter of supernatant liquid to bring it to 55% saturation. Material precipitating between 30 and 55% saturation was collected by centrifugation and dissolved in buffer A (step 3 in Table 1). This preparation was dialyzed overnight against 4 liters of buffer A, and the dialyzed material was applied to a diethylaminoethyl (DEAE)-cellulose column (5 by 40 cm) that had been equilibrated with buffer A. The sample was washed with 500 ml of buffer A and eluted with 3 liters of buffer A that contained NaCl in a linear gradient running from a concentration of 0 to 0.5 M. Fractions of 15 ml were collected at a flow rate of 75 ml/h. Fractions containing CO activity were pooled and were termed DEAE-cellulose eluate (step 4 in Table 1). The DEAE-cellulose eluate was brought to 45% saturation by addition of 277 g of ammonium sulfate per liter. Precipitated protein was removed, and an additional 65 g of ammonium sulfate was added per liter of supernatant liquid to bring it to 55% saturation. Material precipitating between 45 and 55% saturation with respect to ammonium sulfate was collected by centrifugation and dissolved in buffer A (step 5 in Table 1). This preparation was dialyzed overnight against buffer A, and 7-ml samples were passed through a Bio-Gel agarose A-1.5 column (2.5 by 100 cm) that had been equilibrated with buffer A. The flow rate was maintained at 30 ml/h, and fractions of 5 ml were collected. Fractions containing CO activity were pooled (step 6 in Table 1) and precipitated by bringing the solution to 55% saturation with respect to ammonium sulfate by addition of 351 g of the salt per liter of extract. The precipitated protein was dissolved in buffer A and dialyzed overnight against buffer A containing 0.1 M NaCl. In a volume of 15 ml, the enzyme was applied to a QAE-Sephadex column (2.5 by 50 cm) that had been equilibrated with buffer A containing 0.1 M NaCl. Protein was eluted from the column at a flow rate of 25 ml/h with buffer A containing NaCl

TABLE 1. *Purification of catechol 1,2-dioxygenase*

Step	Vol (ml)	Activity (U)	Protein (mg)	Sp act (U/mg)	Yield (%)
1. Crude extract	3,500	26,000	58,275	0.44	100
2. Protamine sulfate treatment	3,500	21,500	19,600	1.1	83
3. 30–55% saturated ammonium sulfate fraction	390	19,656	10,920	1.8	75
4. DEAE-cellulose eluate	600	16,287	2,333	7.0	62
5. 45–55% saturated ammonium sulfate fraction	20	14,518	1,220	11.9	56
6. Bio-Gel A-1.5 eluate	325	10,562	812	13.0	40
7. QAE-Sephadex chromatography	150	5,165	258	20.0	20

in stepwise increments of concentration. CO was retained on the column at 0.15 M NaCl and was eluted with 0.3 M NaCl. Fractions containing constant specific activity of enzyme were pooled (step 7 in Table 1) and precipitated by addition of ammonium sulfate to 55% saturation. The precipitated protein was dissolved in buffer A containing ammonium sulfate at 30% saturation and stored at 4 C.

Acrylamide gel electrophoresis. Vertical gel electrophoresis was performed as described by Raymond (23) in a 7% gel system. The gel buffer was 50 mM Tris-glycine, pH 7.5. The running and sample buffer was 50 mM Tris-glycine, pH 8.8. Samples containing 25, 50, or 100 μg of protein, 30% sucrose, and 5 μl of 0.05% bromophenol blue in a total volume of 0.1 ml were applied to the slots. A constant current of 300 V was applied for 2 h. The gels were stained with 0.1% amido black in 7% acetic acid-30% methanol and destained in the same solvent.

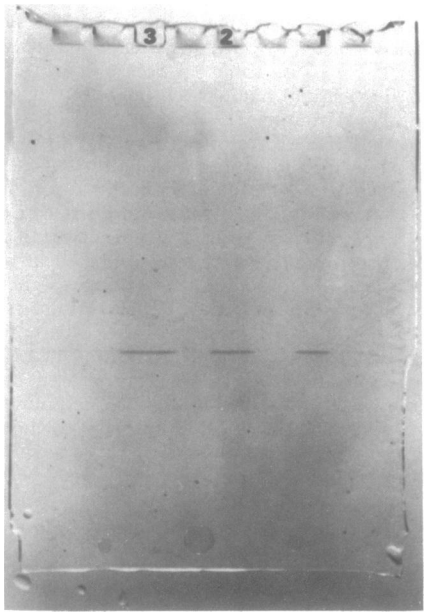

FIG. 1. *Vertical gel electrophoresis of CO. Slot 1 received 25 μg, slot received 50 μg, and slot 3 received 75 μg of enzyme protein.*

The molecular weights of CO subunits were estimated by sodium dodecyl sulfate-gel electrophoresis in a 10% gel system. The gel buffer was 50 mM Tris-glycine, pH 7.5, and the running buffer was 50 mM Tris-glycine, pH 8.8. The samples were prepared as described by Weber and Osborn (28) except that the proteins were equilibrated with 50 mM Tris-glycine buffer, pH 8.8. A constant current of 300 V was applied for 3 h. The detergent was removed by washing the gel slab with 7% acetic acid-30% methanol for 24 h. The gel was stained with 0.1% amido black in 7% acetic acid-30% methanol and destained in the same solvent.

Ultracentrifugation. Sedimentation velocities of protein solutions were measured in the Spinco model E ultracentrifuge at 42,000 rpm in buffer A. Sedimentation equilibrium experiments were carried out at a protein concentration of 6 mg/ml in 50 mM Tris-hydrochloride buffer, pH 8.0, under 4 C at 11,272 rpm according to the procedure of Yphantis (30).

Iron analysis. Iron analyses were carried out with a flameless atomic absorption spectrophotometer.

Amino acid analysis. Amino acid compositions were determined with a Beckman 120B amino acid analyzer. Acid hydrolysates were prepared as described by Moore and Stein (16).

Amino terminal residue. Amino terminal residue was determined by treating 2 mg of protein with dansyl chloride and performing chromatography of the hydrolyzed product on polyamide sheets according to the method of Weiner et al. (29).

Immunological techniques. Antisera against the purified CO (step 7 in Table 1) were prepared by published procedures (15). The method of Stanier et al. (26) was used for the detection of serological cross-reaction on Ouchterlony double-diffusion plates.

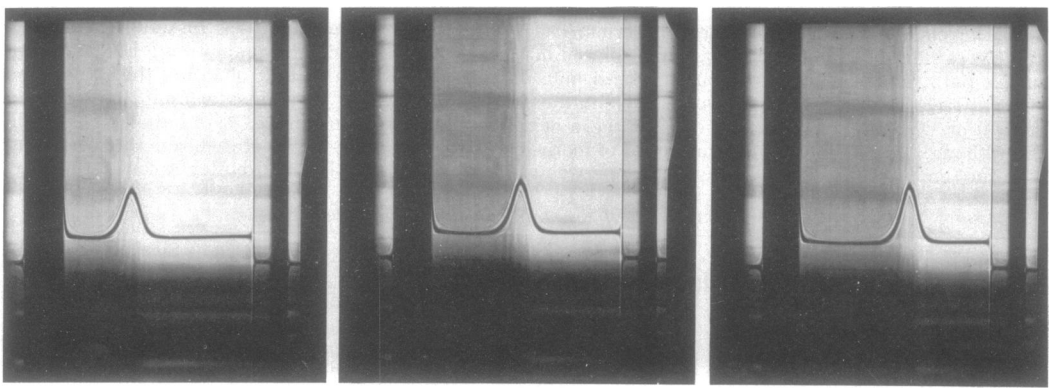

FIG. 2. *Ultracentrifugal pattern of the purified CO protein. Concentration was 5 mg/ml in 50 mM Tris-hydrochloride buffer, pH 8.0. The sedimentation was from right to left. The first photograph was taken 64 min after reaching 42,000 rpm at 4 C. Subsequent photographs were taken at intervals of 32 min.*

Inhibition studies. Serum from unimmunized rabbits absorbs strongly at 260 nm and interferes with the enzyme assay. Therefore, the immunoglobulin fraction was separated from immune and normal rabbit serum by sodium sulfate fractionation (9) and was used for inhibition studies. Varying amounts of the immunoglobulin fraction were incubated at room temperature with 0.15 unit of the enzyme in the assay mixture for 15 min. The enzyme assay was then initiated by addition of substrate to the incubation mixture. The immunoglobin fraction from rabbits that had not been immunized did not inhibit CO activity.

RESULTS

Purity of the enzyme. Purity of the enzyme preparation was examined by polyacrylamide gel electrophoresis and ultracentrifugation. The purified enzyme preparations migrated as a single protein band when subjected to electrophoresis on polyacrylamide gel (Fig. 1). By ultracentrifugal analysis, the Schlieren profiles of the CO revealed a single symmetrical peak (Fig. 2). The sedimentation velocities of the purified enzyme preparations were measured at three different protein concentrations. The observed dependence of sedimentation constant on protein concentration is shown in Fig. 3. The extrapolated sedimentation constant, $s_{20,w}$, was 2.93.

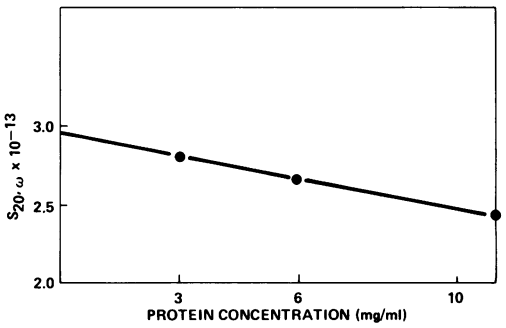

FIG. 3. *Sedimentation constant, $s_{20,w}$, of CO as a function of protein concentration.*

Substrate specificity. Substrate specificity of CO was studied by adding enzyme to air-saturated buffer containing 3 μmol of substrate analogues in an oxygen electrode vessel. The differences in the rate of oxygen uptake with and without enzyme were calculated. Among various compounds tested, 3-isopropylcatechol, 4-methylcatechol, and 3-methylcatechol were oxidized at rates 30, 18, and 12% that of catechol, respectively. The following compounds were not oxidized: 4-nitrocatechol; α-chloro-3,4-dihydroxyacetophenone; 3,4-dihydroxyphenylacetic acid; 3,4-dihydroxycinnamic acid; protocatechuic acid; protoacatechualdehyde; and pyrogallol.

Effect of pH. The effect of pH on enzyme activity and stability was examined. The enzyme had a broad pH optimum ranging from pH 7 to 9 (Fig. 4A). The enzyme gradually lost 15% of its activity over a period of 10 days upon storage at pH 7 to 9. Enzyme activity was completely lost in 6 days at pH 6 (Fig. 4B).

Effect of temperature. The effects of temperature on enzyme stability and activity were studied. The enzyme was relatively stable at

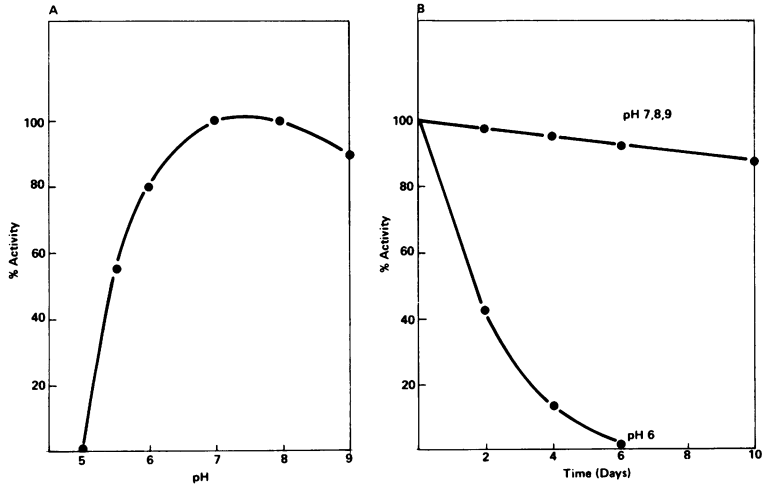

FIG. 4. *(A) Effect of pH on CO activity. pH 5.0, 5.5, 6.0, and 7.0, 50 mM potassium phosphate; pH 8.0 and 9.0, 50 mM Tris-hydrochloride. (B) Effect of pH on stability of CO. Enzyme was stored at 4 C in 1 ml of each buffer solution. After 2, 4, 6, and 10 days, the activity of a sample of the stored enzyme was determined spectrophotometrically.*

43 C and lost 18% of its activity after 30 min of incubation. On increasing the temperature to 47 C, 90% of the enzyme activity was lost after 30 min of incubation.

The optimum temperature for enzyme activity was 35 to 37 C. No catalytic activity was detected at 45 C.

Iron content. Qualitative analysis for metals by flameless atomic absorption spectrometry revealed that no metals other than iron were present in the enzyme. Two gram-atoms of iron were detected per mol of enzyme protein based on a molecular weight of 81,000.

Absorption spectrum. A concentrated solution of enzyme was distinctly red. The visible and ultraviolet absorption spectra of the purified enzyme preparations are shown in Fig. 5. The ultraviolet spectrum represents a typical absorption pattern of a protein solution with an A_{280}/A_{260} ratio of 1.42. In the visible range, a broad absorption between 390 and 600 nm with an absorption maximum at 440 nm was observed. The molar absorptivity at 440 nm was 2,860.

Inhibition studies. The effects of organic substrate analogues, metal ions, sulfhydryl agents, and iron-chelating agents as inhibitors of enzyme activity were determined (Table 2). Enzyme activity was inhibited to different extents by substrate analogues. Inhibition by methylcatechols and 3-isopropylcatechol may be due to competition as substrate for catalytic sites. 4-Nitrocatechol, although it is not a substrate, was found to be an effective inhibitor for CO. Among various metal ions and iron-chelating agents tested, Ag and Cu inhibited enzyme activity. Sulfhydryl compounds and reducing agents were not effective inhibitors of enzyme activity, except for *p*-chloromercuribenzoate.

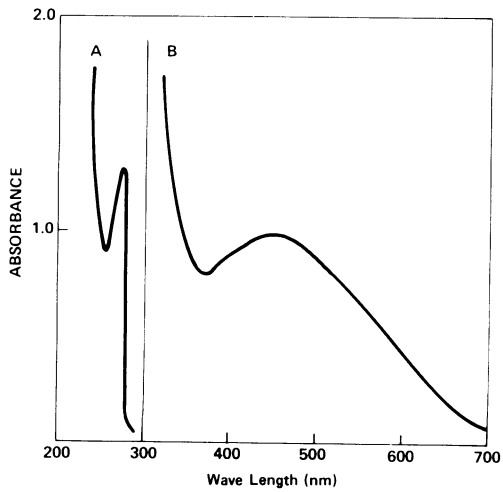

FIG. 5. *Absorption spectrum of CO. The concentrations of enzyme protein were 1 mg/ml in curve A and 14 mg/ml in curve B in 50 mM Tris-hydrochloride buffer, pH 8.0.*

Molecular weight and subunit size. The molecular weight of the enzyme was determined by filtration through a Bio-Gel agarose A-1.5 column and was estimated to be 85,000 (Fig. 6). Sedimentation equilibrium analysis in an ultracentrifuge according to the procedure of Yphantis (30) estimated the molecular weight of the enzyme to be 81,000.

Sodium dodecyl sulfate-gel electrophoresis yielded a value of 40,000 for the subunit size of CO (Fig. 7).

TABLE 2. *Inhibition studies*[a]

Inhibitors	Concn (mM)	Inhibition (%)
Substrate analogues:		
4-Methylcatechol	1.0	64
3-Methylcatechol	0.1	58
3-Isopropylcatechol	0.1	30
4-Nitrocatechol	0.1	92
3,4-Dihydroxyphenyl acetate	0.1	8
α-Chloro-3,4 dihydroxy-acetophenone	0.1	46
Benzoate	1.0	0
Phenol	1.0	64
Protocatechuate	0.1	0
Protocatechualdehyde	0.1	10
Metal ions and iron chelating agents:		
Sodium arsenite	1.0	6
$AgNO_3$	0.1	100
$CuSO_4$	0.1	91
$FeSO_4$	0.1	12
$FeCl_3$	0.1	0
O-phenanthroline	1.0	0
Tiron	0.1	0
α,α-Bipyridyl	1.0	0
Sulfhydryl agents and others:[b]		
PHMB	0.1	41
N-ethylmaleimide	0.1	3
Iodoacetamide	0.1	6
DTNB	0.1	0
Glutathione	0.1	4
EDTA	1.0	4
Mercaptoethanol	0.1	12
H_2O_2	0.1	9
DTT	0.1	6

[a] Assay system contained 14 μg of 0.05 M Tris-acetate, pH 7.5, enzyme, and an inhibitor as indicated, in a total volume of 2.95 ml. The reaction was started by the addition of 50 μl of catechol solution (0.6 μmol), and the rate of oxygen uptake was measured.

[b] Abbreviations: PHMB, *p*-hydroxymercuribenzoate; DTNB, 5,5-dithiobis-2-nitrobenzoic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

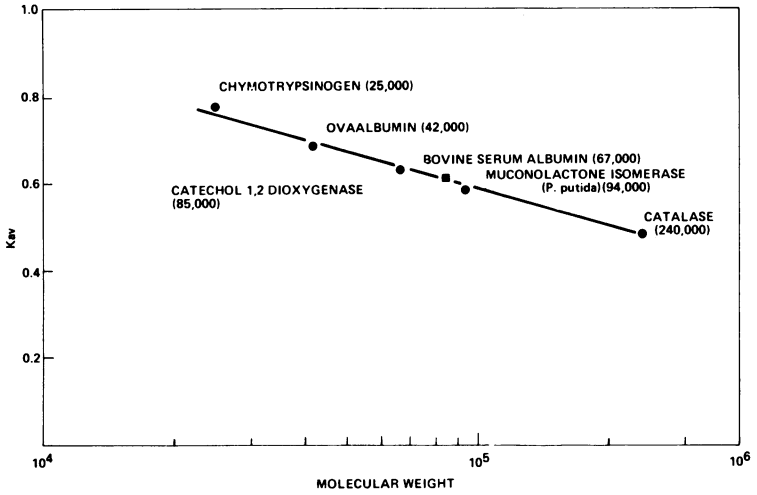

FIG. 6. *Molecular weight estimation of CO on a Bio-Gel agarose A-1.5 column.*

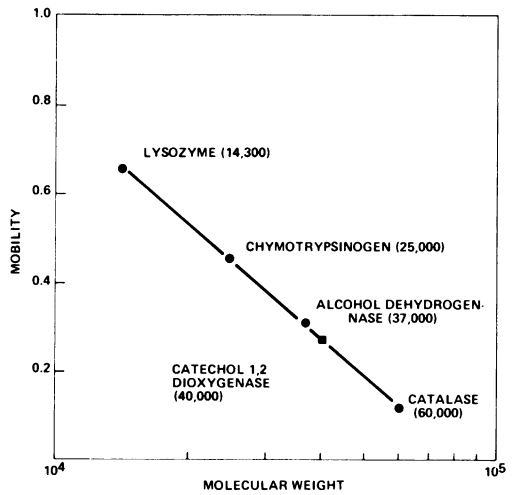

FIG. 7. *Sodium dodecyl sulfate-gel electrophoresis of CO.*

Amino acid composition. The amino acid content determined by hydrolysis of the enzyme is shown in Table 3. The values are expressed as number of residues per molecule, assuming a molecular weight of 81,000. The presence of three to four residues of half-cystine per molecule of enzyme was calculated.

Amino terminus. Dansylmethionine was the only α-amino-substituted amino acid recovered after dansylated CO was hydrolyzed.

Serological properties. Antisera prepared against the purified enzyme cross-reacted with other strains of *A. calcoaceticus*, but failed to cross-react with the isofunctional enzymes from strains of *Pseudomonas*, *Nocardia*, and *Alcaligenes*, when cell-free extracts of organisms were tested (Fig. 8).

TABLE 3. *Amino acid composition of Acinetobacter CO*

Amino acid	Residues per molecule after hydrolysis for:			Avg
	24 h	48 h	72 h	
Lysine	16.8	17.2	17.5	17.1
Histidine	19.8	19.4	19.6	19.6
Arginine	31.5	29.9	30.1	30.5
Aspartic acid	79.7	80.5	79.8	80.0
Threonine	27.9	24.0	21.0	31.3[a]
Serine	12.2	8.5	6.4	14.6[a]
Glutamic acid	63.8	61.9	61.6	62.4
Proline	32.6	32.2	27.8	34.1
Glycine	53.9	53.7	53.3	54.4
Alanine	53.5	52.6	52.4	54.0[a]
Half-cystine				3.4[b]
Valine	40.8	41.2	41.3	41.1
Methionine	7.3	6.5	5.7	8.3[a]
Isoleucine	28.3	27.9	27.7	28.5
Leucine	49.6	49.3	49.2	49.7
Tyrosine	23.4	9.9	6.7	31.4[a]
Phenylalanine	20.5	20.3	20.0	20.3
Tryptophan				9.4[c]

[a] These values were extrapolated to zero-time hydrolysis.

[b] Determined as cysteic acid on an amino acid analyzer after 24 h of hydrolysis in the presence of dimethyl sulfoxide (27).

[c] Estimated by spectrophotometric analyses in alkaline solution (4).

Similar results were obtained when inhibition of CO activity was studied by using the immunoglobulin fraction of the antisera. CO activity in cell-free extracts of *Pseudomonas*, *Nocardia*, and *Alcaligenes* were not inhibited by immunoglobulin. Partial inhibition of enzyme activity was observed with *A. calcoaceticus* ADP-96 (Fig. 9).

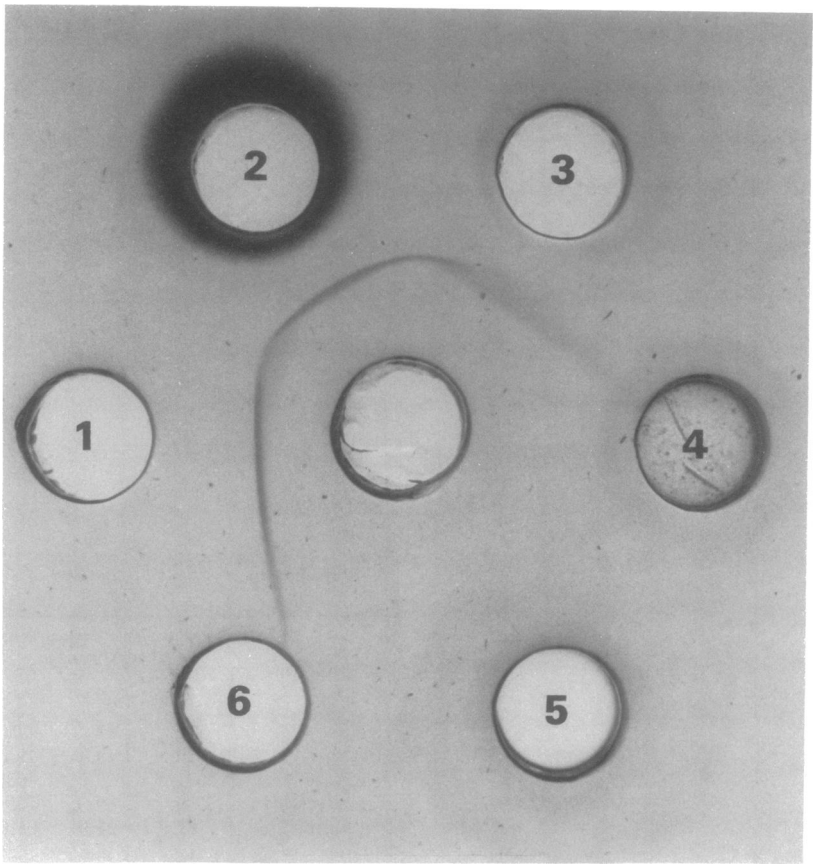

FIG. 8. *Ouchterlony double-diffusion plate. Central well contained immunoglobulin fraction of the antisera prepared against the purified enzyme. The outer wells contained cell-free extracts derived from induced (benzoate-grown) cultures of the designated organisms. (1) Purified CO; (2) Acinetobacter calcoaceticus, strain ADP-96; (3) A. calcoaceticus, strain ATCC 14987; (4) P. aeruginosa; (5) Alcaligenes eutrophus; (6) N. opaca.*

DISCUSSION

The purified enzyme preparation of CO has a red color with broad absorption between 390 and 650 nm. The enzyme contains 2 g-atoms of iron per 80,000 g of enzyme protein. The activity was not inhibited by typical chelating agents for ferrous ion, such as α,α-bipyridyl and *O*-phenanthroline. Inhibition by tiron, a ferric ion-chelating agent, was observed only after preincubation. Among various substrate anaologues tested as inhibitors of CO activity, 4-nitrocatechol was found to be the most effective inhibitor.

Difference in substrate specificity is one of the interesting characteristics noted among the isofunctional dioxygenases from various sources. CO from *Brevibacterium* (18) oxidized catechol, 4-methylcatechol, and 3-methylcatechol at the same rate, whereas the enzyme from *Pseudomonas* (9) oxidized catechol and 4-methylcatechol at the same rate and 3-methylcatechol at a rate 5% that of catechol. In contrast to *Brevibacterium* and *Pseudomonas*, the purified enzyme from *Acinetobacter* catalyzed oxidation of 4- and 3-methylcatechols at rates 18 and 12% that of catechol, respectively.

Recently, Fujiwara et al. (3) reported that the CO from *Pseudomonas* catalyzed the extradiol cleavage of 3-substituted catechols in addition to its known intradiol cleavage activity. In contrast, CO from *Brevibacterium* (3) catalyzed only intradiol cleavage of 3-substituted catechols. Our preliminary results indicated that the purified enzyme from *Acinetobacter* catalyzes both intradiol and extradiol cleavage of 3-substituted catechols (Hou et al., unpublished data).

The purified CO from *Acinetobacter* is similar in its molecular size, subunit composition,

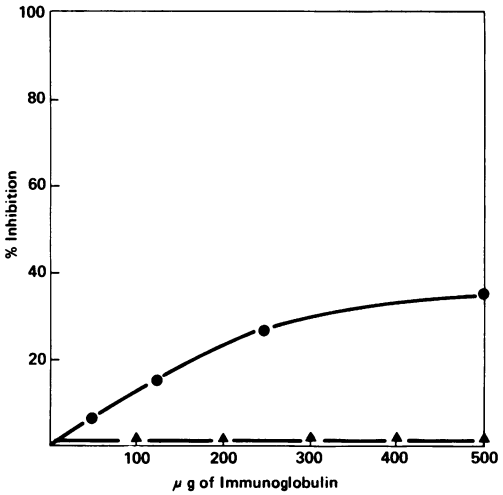

FIG. 9. *Inhibition of CO activity in cell-free extracts of various organisms by the immunoglobulin fraction from antisera prepared against the purified CO. The protein concentration of the immunoglobulin fraction was 5 mg/ml. The uninhibited activity was 0.15 unit. Symbols: ●, Acinetobacter calcoaceticus; ▲, P. aeruginosa, N. opaca, and Alcaligenes eutrophus.*

absorption spectrum, and iron content to the isofunctional enzyme from *Pseudomonas* (10). Amino acid analysis indicated that *Acinetobacter* CO has fewer residues per molecule in most of the amino acids in comparison with CO from *Pseudomonas*, except for tyrosine and tryptophan. However, the numbers of half-cystine, methionine, and proline residues are similar for these two isofunctional enzymes.

We first prepared antisera against the CO from *Acinetobacter* and demonstrated immunological specificity of this enzyme. Antisera prepared against *Acinetobacter* enzyme failed to cross-react or inhibit activity of the isofunctional enzyme in cell-free extracts of *Pseudomonas, Alcaligenes,* and *Nocardia*. In view of the similarity in properties of CO from *Acinetobacter* and *Pseudomonas*, their failure to cross-react serologically is surprising. Similar immunological specificity was observed by Patel et al. (21) with muconolactone isomerase, another enzyme of the β-ketoadipate pathway. The muconolactone isomerases from *Acinetobacter* and *Pseudomonas*, although similar in molecular size, subunit size, amino acid composition, and amino terminal amino acid sequences (12 of the first 15 residues are the same), were found to be serologically remote.

Similar kinetic and physical properties have been reported for certain isofunctional bacterial enzymes independently synthesized in response to different inducing compounds and yet are serologically highly specific. An example of such a system is α-ketoglutarate semialdehyde dehydrogenase in *Pseudomonas* (11), where one of the enzymes is induced by hydroxyproline and the other by glucarate. Similarly, in *Acinetobacter*, the isofunctional enzymes β-ketoadipate enol-lactone hydrolase I and II, induced independently by protocatechuate and *cis,cis*-muconate, respectively, are very similar in properties and yet are serologically specific (21).

It is interesting to observe that the CO activities in the cell-free extract and in the purified enzyme preparation from *Acinetobacter* were only partially inhibited by the immunoglobulin fraction.

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