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Purification and Characterization of Oxalyl-Coenzyme A Decarboxylase from *Oxalobacter formigenes*

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Oxalyl-coenzyme A (oxalyl-CoA) decarboxylase was purified from *Oxalobacter formigenes* by high-pressure liquid chromatography with hydrophobic interaction chromatography, DEAE anion-exchange chromatography, and gel permeation chromatography. The enzyme is made up of four identical subunits (M_r, 65,000) to give the active enzyme (M_r, 260,000). The enzyme catalyzed the thiamine PP_i-dependent decarboxylation of oxalyl-CoA to formate and carbon dioxide. Apparent K_m and V_{max} values, respectively, were 0.24 mM and 0.25 μmol/min for oxalyl-CoA and 1.1 pM and 0.14 μmol/min for thiamine pyrophosphate. The maximum specific activity was 13.5 μM oxalyl-CoA decarboxylated per min per mg of protein.

The rate of degradation of oxalate by ruminal bacteria is of concern because mortality rates may be high when ruminants are fed plants such as *Halogeton glomeratus* that contain large amounts of oxalate. Sheep can, however, be adapted to tolerate amounts of oxalate that would be lethal to nonadapted sheep (8). The adaptation phenomenon was explained by showing that increased levels of dietary oxalate select toward increased proportions of oxalate-degrading microbes in the rumen (3). An anaerobic, gram-negative, nonmotile, rod-shaped bacterium that degraded oxalate was isolated from sheep rumen contents (4), and similar isolates have been obtained from other anaerobic habitats (1, 16). This organism, which has been named *Oxalobacter formigenes*, metabolizes oxalate, producing approximately a 1:1 ratio of CO_2 and formate, and has no other known substrate for growth. Cell extracts degrade oxalate to CO_2 and formate but require an acyl coenzyme A (CoA) for activity. In this respect, *O. formigenes* extracts are similar to *Pseudomonas oxalaticus* extracts (11, 13–15), which also require oxalyl-CoA. Oxalate metabolism by *O. formigenes* differs from that of the aerobic *P. oxalaticus* because formate is an end product of the anaerobic metabolism by *O. formigenes*, whereas *P. oxalaticus* oxidizes formate to CO_2 via a NAD-linked formate dehydrogenase (15).

In this paper, we describe the purification of the oxalyl-CoA decarboxylase (EC 4.1.1.8) of *O. formigenes* to homogeneity and describe some of the properties of the enzyme.

MATERIALS AND METHODS

Organism and growth conditions. The strain of *O. formigenes* used was OxB, the type strain. It was grown under anaerobic conditions at 37°C, as previously described (1). The yield from 12 liters of prereduced medium was typically about 3.3 g (wet weight) of cells.

Protein purification. OxB cells were harvested by centrifugation in a centrifuge (The Sharples Corp., Philadelphia, Pa.). The cells were washed once in buffer A, which was made up of 0.1 M KH_2PO_4 and 1 mM dithiothreitol, pH 6.8. After centrifugation, the cell pellet was suspended in 4 ml of buffer B per gram of cells. Buffer B contained buffer A plus 1 mM phenylmethylsulfonyl fluoride, 10 mM $MgCl_2$, and 1 mM thiamine pyrophosphate (TPP). The cells were broken by passage three times at 20,000 lb/in^2 through a French pressure cell (American Instruments Co., Silver Spring, Md.). Cell debris was removed by centrifugation at 20,000 × *g* for 10 min. The supernatant was treated with 50 mg of protamine sulfate per g of cells to precipitate nucleic acids; these were removed by centrifugation (20,000 × *g* for 10 min). The enzymatically active protein fraction was precipitated at between 26 and 80% saturation with $(NH_4)_2SO_4$ at 20°C. After centrifugation at 20,000 × *g* for 10 min, the precipitate was dissolved in a minimal amount of buffer C (0.1 M NaH_2PO_4 [pH 6.7], 1 mM dithiothreitol, 10 mM $MgCl_2$, and 1 mM TPP). After centrifugation and filtration through a 0.2-μm-pore-size filter, this solution was injected in 2-ml portions (~80 mg of protein) into a preparative hydrophobic interaction chromatography column (Bio-Gel TSK Phenyl-5 PW, 150 by 21.5 mm; Bio-Rad Laboratories, Richmond, Calif.). The initial column buffer was 1.5 M $(NH_4)_2SO_4$ and 0.1 M NaH_2PO_4, pH 6.7. The flow rate was 4 ml/min, and the eluate was monitored at 280 nm. Elution buffer B was 0.1 M NaH_2PO_4, pH 6.7, and was 40% B at 10 min, 80% B at 40 min, and 100% B at 45 min and returned to 0% B from 48 to 50 min. The enzymatically active fractions (shaded area of peak I, Fig. 1) were combined, and the proteins were concentrated to ~11 mg/ml in an ultrafiltration cell (Amicon Corp., Lexington, Mass.) with an XM-100 membrane. Additions of 20 mM NaH_2PO_4, pH 6.7, with 1 mM dithiothreitol, 10 mM $MgCl_2$, and 1 mM TPP to the ultrafiltration cell adjusted the ionic strength of the protein solution for the next purification step.

This protein solution was injected into a preparative DEAE anion-exchange column (Bio-Gel TSK DEAE-5PW, 150 by 21.5 mm; Bio-Rad Laboratories). The initial buffer was 20 mM NaH_2PO_4, pH 6.7, and the flow rate was 4 ml/min. Eluting buffer B was 300 mM NaH_2PO_4, pH 6.7, and was 10% B at 5 min, 50% B at 40 min, and 100% B at 45 min. The eluted protein was monitored at 280 nm. The enzymatically active fractions were combined and concentrated to about 5 mg of protein per ml by ultrafiltration, as described above. The buffer was exchanged in the ultrafiltration cell to make it 0.1 M NaH_2PO_4 [pH 6.7]–1 mM dithiothreitol–1 mM TPP–10 mM $MgCl_2$.

This preparation was injected into a combined gel permeation chromatography column (Spherogel-TSK 4000 SW, 7.5 by 300 mm, plus Spherogel-TSK 3000 SW, 7.5 by 300 mm;

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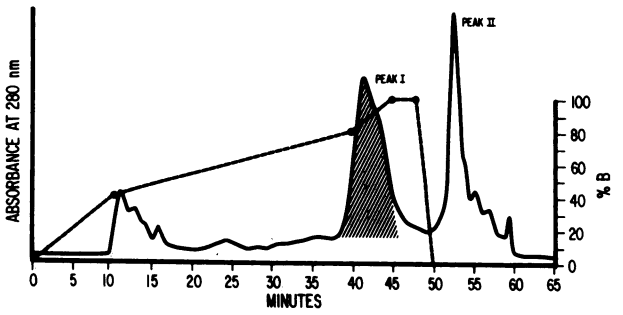

FIG. 1. Hydrophobic interaction chromatogram of redissolved $(NH_4)_2SO_4$-precipitated protein from *O. formigenes* on a Bio-Gel TSK Phenyl-5PW column starting at 1.5 M $(NH_4)_2SO_4$–0.1 M NaH_2PO_4, pH 6.7. Shaded area is active fractions concentrated for next step in purification; broken line is gradient of percent buffer B.

Beckman Instruments, Inc., Berkley, Calif.). The flow rate of the 0.1 M NaH_2PO_4 (pH 6.7) buffer was 0.5 ml/min. The column was calibrated by using a high-pressure liquid chromatography molecular weight marker kit (United States Biochemical Corp., Cleveland, Ohio). The enzymatically active fractions were combined and concentrated as described above to about 1.3 mg of protein per ml. The purified enzyme was stored frozen at −70°C.

Electrophoresis. Fractions from the various chromatographic steps were examined by native and sodium dodecyl sulfate-gradient gel electrophoresis in which the polyacrylamide varied from 8 to 25% (Phast System; Pharmacia, Inc., Piscataway, N.J.). Molecular weight markers for the native gel electrophoresis were the same as the high-pressure liquid chromatography molecular weight markers described above plus catalase (Pharmacia, Inc.), and molecular weight markers for the sodium dodecyl sulfate-gel electrophoresis were kit number MW-SDS-200 (Sigma Chemical Co., St. Louis, Mo.). A semipreparative native gradient gel electrophoresis was performed in order to locate the migration distance of the enzymatic activity. A precast 4 to 30% gradient gel was used (Pharmacia), and purified oxalyl-CoA decarboxylase was the sample. After electrophoresis, the gel was cut in half and one half was stained. The other half was cut into 18 4-mm strips. The strips were placed in Microfuge tubes (Beckman) with 500 μl of buffer C. The acrylamide was mashed and allowed to soak overnight at 4°C. Each sample was centrifuged, and the supernatant was assayed for enzyme activity and protein content. The gels were stained with Coomassie brilliant blue R-250. The isoelectric point of the purified enzyme was determined by performing an isoelectric focusing experiment with pH 4.5 to 6 gels with the same equipment. Reference isoelectric point markers consisted of an isoelectric point calibration kit with a pH range of 4.7 to 10.6 (BDH, Poole, England).

Enzyme activity assays. Assays of enzyme activity on crude enzyme preparations were initially performed by using [^{14}C]oxalate; the $^{14}CO_2$ released was measured (2). The reaction mixture (2 ml) contained (millimolar): KH_2PO_4 [pH 6.7], 50; $MgCl_2$, 5; TPP, 0.05; [^{14}C]oxalate, 8 (specific activity, 0.2 kBq/μmol); succinyl-CoA, 0.2; and an enzyme preparation. Enzyme activity decreased as enzyme purification proceeded, which suggested that the substrate for the decarboxylase might be oxalyl-CoA. Therefore, oxalyl-CoA was prepared as described by Quayle (11), and decarboxylase activity was determined by estimating the quantity of formate produced by measuring the reduction of NAD in the presence of formate dehydrogenase. The reaction mixture (300 μl) contained KH_2PO_4 [pH 6.7], 66 mM; TPP, 0.06 mM; $MgCl_2$, 6.6 mM; potassium oxalate [pH 6.7], 8.3 mM; oxalyl-CoA, 0.5 mM; NAD, 0.83 mM; formate dehydrogenase, 0.25 IU (Boehringer Mannheim Biochemicals, Indianapolis, Ind.); and an excess of acyl-CoA transferase. The latter was obtained from fractions 51 to 58 from the hydrophobic interaction chromatography column, and its purification and properties will be described elsewhere. The reaction was run at 30°C in a thermal cuvette holder; increases in A_{340} were recorded continuously (Response II Spectrophotometer; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The reaction was initiated by the addition of oxalyl-CoA decarboxylase. Blank values were determined by omitting enzyme or oxalyl-CoA. To show that oxalyl-CoA was converted to formyl-CoA by oxalyl-CoA decarboxylase, spectral changes that occurred during the decarboxylation reaction were determined by a method outlined by Quayle (12). The reaction mixture (1 ml) contained (millimolar) KH_2PO_4 [pH 6.7], 66; TPP, 0.06; $MgCl_2$, 6.6; and oxalyl-CoA, 0.5. The reference blank contained the same reagents, except 0.5 mM CoA was substituted for oxalyl-CoA. A 10-fold dilution of a portion of each solution was made, and a difference spectrum was obtained. Oxalyl-CoA decarboxylase (0.01 ml) was added to each tube and incubated for 10 min at 30°C. Another difference spectrum was obtained on a 1:10 dilution of the reaction mixtures. Parameters affecting decarboxylase activity in the assay system described were investigated by eliminating possible cofactors, CoA acceptors, and enzyme substrates from the reaction mixture. Succinate and acetate were used as CoA transferase acceptors, and succinyl-CoA was tested in the presence of oxalate as a possible enzyme substrate. The enzyme preparation used was from the DEAE chromatographic step of purification. It was dialyzed extensively against phosphate-buffered saline, pH 6.7, and 0.2 μg of protein was used in each assay. The specific activity (micromoles of NADH formed per min per mg of protein) was calculated for each assay. The apparent K_m and V_{max} values were determined for oxalyl-CoA and TPP from Lineweaver-Burke double-reciprocal plots for which the oxalyl-CoA concentration ranged from 0.1 to 1 mM and the TPP concentration ranged from 0.1 to 1 pM. For these measurements, sodium succinate was substituted for potassium oxalate in the reaction mixture. Calculations of initial rates were made for measurements taken during the interval of 1 to 5 min.

Protein determination. Protein concentrations were determined by a modification of the Lowry method (10) with bovine serum albumin as the standard.

RESULTS

Purification of oxalyl-CoA decarboxylase. Initial attempts to purify the enzyme were apparently not successful because the assay system used to measure enzyme activity was not complete. When fractions collected from the hydrophobic interaction chromatography column (Fig. 1) were assayed by the method involving $^{14}CO_2$ production from [^{14}C]oxalate, the only active fractions were in peak II, and these totaled less than 1% of the applied activity. Further purification steps led to complete loss of activity as measured with this assay system. When decarboxylase activity was measured as formate produced in the coupled enzyme system, the apparent recovery of activity in peak II (Fig. 1) increased to about 5%. Again, further purification led to apparent loss of activity. Then we realized that peak II contained mostly acyl-CoA transferase contaminated with a little oxalyl-CoA

TABLE 1. Purification of oxalyl-CoA decarboxylase from *O. formigenes*[a]

Purification step	Vol (ml)	Protein (mg/ml)	Sp act (U/mg of protein)	Total activity (U)	Recovery (%)	Purification (fold)
Broken cells	15	48.5	1.41	1,028	100	
Cell supernatant	13	50.1	1.30	844	82.2	
$(NH_4)_2SO_4$ treatment	8.2	40.9	1.57	525	51.1	1.1
Hydrophobic-interaction chromatography	8.1	11.1	3.15	283	27.6	2.2
DEAE chromatography	6.2	5.0	5.68	176	17.2	4.0
Gel permeation chromatography	6.6	1.3	13.5	115	11.3	9.5

[a] Starting material: 3.3 g (wet weight) of cells.

decarboxylase from peak I and that the assay system used the acyl-CoA transferase to transfer CoA from formyl-CoA to an acyl acceptor, producing the formate that was measured by the formate dehydrogenase coupled enzyme system. Subsequently, all assays for oxalyl-CoA decarboxylase contained an excess of peak II material that was free of oxalyl-CoA decarboxylase activity, along with the formate dehydrogenase. A typical purification (Table 1) yielded a homogeneous protein that was purified 9.5-fold and had a specific activity of 13.5 U/mg protein. By using values for the total enzyme activity in broken-cell preparations and the maximum specific activity for the purified fraction, it was calculated that oxalyl-CoA decarboxylase makes up 10.5% of the soluble protein in the cell.

The semipreparative electrophoresis indicated a single protein band when the gel was stained with Coomassie blue at 19 to 21 mm from the top. This corresponds to the enzyme activity found in gel slices 5 and 6 (Table 2). No protein bands or enzyme activity was found in any other portion of the gel.

Spectral changes during decarboxylation. The decarboxylation of oxalyl-CoA in the absence of acyl-CoA transferase is accompanied by a large decrease in absorption in the range of 260 to 270 nm and the appearance of a new maximum at 227 nm (data not shown).

Requirements for oxalyl-CoA decarboxylase activity measurements. Constituents essential for optimal enzyme activity are presented in Table 3. TPP can be almost completely removed from the enzyme by dialysis and is a necessary cofactor for activity. Magnesium addition enhances activity, but attempts to remove it by dialysis and to chelate it with EDTA reduced activity by about 24%. The acyl-CoA transferase is a necessary component, only because of the assay system used, and transfers the CoA from formyl-CoA to some other acyl acceptor. Oxalate and succinate can act as CoA acceptors, whereas acetate does not. The acyl-CoA transferase does not transfer CoA from succinyl-CoA to oxalate rapidly enough for succinyl-CoA to act as a pseudosubstrate when oxalate is present.

TABLE 2. Location of oxalyl-CoA decarboxylase in preparative electrophoresis gel

Gel slice no.	Enzyme activity (mM oxalyl-CoA/min)	Protein (μg/ml)
1	ND[a]	ND
2	0.01	ND
3	0.02	ND
4	0.09	2
5	1.03	25
6	0.92	22
7	0.01	ND
8	0.01	ND
9–18	ND	ND

[a] ND, Not detectable.

TABLE 3. Requirements for purified oxalyl-CoA decarboxylase activity as measured by formate formation

Reaction mixture	Reaction rate (μmol/min per mg of protein)	% of complete reaction
Complete[a]	5.33	100.0
Without acyl-CoA transferase	0.31	5.8
Without oxalate	1.02	19.1
Without oxalate, added succinate (10 mM)	5.01	94.0
Without oxalate, added acetate (10 mM)	1.00	18.8
Without TPP	0.25	4.7
Without Mg^{2+}	4.32	81.0
Without Mg^{2+}, added EDTA (10 mM)	4.05	76.0
Without oxalyl-CoA	0	0
Without oxalyl-CoA, added succinyl-CoA (0.5 mM)	0.10	1.2

[a] See Materials and Methods for complete reaction mixture.

Physicochemical properties of the enzyme. The purified protein migrated as a single band on sodium dodecyl sulfate-gradient polyacrylamide gels with a calculated molecular weight of 65,000 (±1,000) (Fig. 2B). The native protein migrated as a single band on the gradient polyacrylamide gel with a calculated molecular weight of 260,000 (±8,000) (Fig. 2A), which indicates that the enzyme is a tetramer. The elution time of the protein from the calibrated gel permeation chromatography column corresponded to a molecular weight of 243,000 (±5,000). The isoelectric point of the enzyme, determined by isoelectric focusing of the native enzyme, was

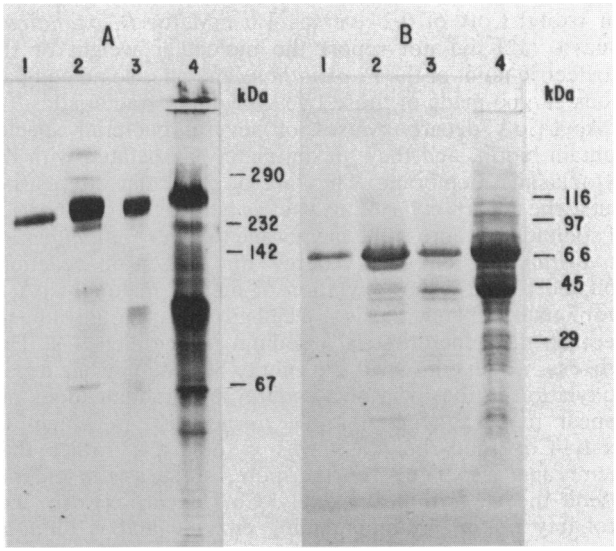

FIG. 2. Polyacrylamide gel electrophoresis (8 to 25% acrylamide). (A) Native gel. (B) Sodium dodecyl sulfate gel. Lanes: 1, purified oxalyl-CoA decarboxylase; 2, after DEAE chromatography; 3, after hydrophobic-interaction chromatography; 4, after $(NH_4)_2SO_4$ precipitation step. kDa, Kilodaltons.

calculated to be 4.90 by comparison with the calibration markers.

Kinetics. Values for Michaelis-Menten-type saturation kinetics were obtained with oxalyl-CoA and TPP. The apparent kinetic constants K_m and V_{max} for the purified enzyme were, respectively, 0.24 mM and 0.25 μmol/min for oxalyl-CoA and 1.1 pM and 140 nmol/min for TPP.

DISCUSSION

Oxalyl-CoA decarboxylase is an important enzyme because it is involved in the detoxification of oxalate in the intestinal tracts of animals. The potentially toxic compound oxalate is widely distributed in plants (9). Evidence exists that *O. formigenes* populates the gastrointestinal tracts of a diversity of herbivores (1), that the bacterial oxalyl-CoA decarboxylase degrades oxalate, and that oxalate toxicity is thus reduced. Recent evidence that the intestines of some healthy humans are not colonized by *O. formigenes* and that these individuals absorb more oxalate and may absorb less calcium than colonized persons (L. Doane, M. Liebman, and D. Caldwell, FASEB J. **2**:A429, 1988) adds further to the importance of the role of this oxalyl-CoA decarboxylase.

We have purified oxalyl-CoA decarboxylase from *O. formigenes*. In crude enzyme preparations, succinyl-CoA acted as a CoA donor with oxalate to initiate the decarboxylase reaction. In reactions with the purified enzyme, however, succinyl-CoA was not an adequate CoA donor even when the peak II acyl-CoA transferase was added. The spectral change that occurred when oxalyl-CoA was reacted with oxalyl-CoA decarboxylase was similar to the spectral change reported by Quayle (12) and supports the concept that formyl-CoA is formed in that reaction. The main function of the acyl-CoA transferase thus must be to transfer CoA from formyl-CoA to oxalate. TPP is a cofactor that is needed for enzyme activity, and Mg^{2+} is stimulatory.

The requirements of this enzyme for maximal activity suggest a similarity between it and the enzyme isolated from the aerobic organism *P. oxalaticus* by Quayle (12). Other similarities between our enzyme and the latter include a pH optimum of 6.7 versus 6.6 for *P. oxalaticus* and a K_m value for oxalyl CoA of 0.24 versus 1.0 mM for *P. oxalaticus*. Quayle (12) did not report the molecular weight or the isoelectric point of the *P. oxalaticus* enzyme, so no comparisons can be made of those two enzyme characteristics.

Acyl-CoA decarboxylases of several bacterial species contain biotin, and they are intimately associated with the cytoplasmic membrane. They are of particular interest because the decarboxylases also act as pumps for translocation of sodium ions across the membrane (6). In *Propionigenium modestum*, which gains energy for growth in reactions coupled to the decarboxylation of methyl malonyl-CoA to propionate, ATP synthesis and cell growth are totally dependent on the generation of a sodium ion gradient (7). This process, which conserves the energy liberated during decarboxylation in the form of a sodium ion gradient, does not appear to be functional in *O. formigenes*, in which the oxalyl-CoA decarboxylase was cytoplasmic rather than membrane associated. Furthermore, since avidin did not inhibit the *O. formigenes* oxalyl-CoA decarboxylase, it is probably not a biotin-containing enzyme and is different from the above-mentioned decarboxylases (A. Baetz, M. Allison, and H. Cook, unpublished data).

The mechanism for generation of ATP by *O. formigenes* has not been clearly defined. Aerobic oxalate degraders such as *P. oxalaticus* obtain energy from reactions coupled to the oxidation of formate produced from oxalate (5, 15). With *O. formigenes*, however, formate is an end product and is not appreciably oxidized. The Na^+ pump mechanism does not appear to function as an energy conservation mechanism, but both $\Delta\psi$ and ΔpH are generated when oxalate is degraded by cells (C. H. Kuhner, M. J. Allison, and P. A. Hartman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, K-36, p. 208). Further studies will be needed to define these mechanisms.

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