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Purification and Characterization of Formyl-Coenzyme A Transferase from *Oxalobacter formigenes*

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Formyl-coenzyme A (formyl-CoA) transferase was purified from *Oxalobacter formigenes* by high-pressure liquid chromatography with hydrophobic interaction chromatography and by DEAE anion-exchange chromatography. The enzyme was a single entity on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel permeation chromatography (M_r, 44,000). It had an isoelectric point of 4.7. The enzyme catalyzed the transfer of CoA from formyl-CoA to either oxalate or succinate. Apparent K_m and V_{max} values, respectively, were 3.0 mM and 29.6 μmol/min per mg for formyl-CoA with an excess of succinate. The maximum specific activity was 2.15 μmol of CoA transferred from formyl-CoA to oxalate per min per mg of protein.

The only known organism that will degrade oxalate in the rumens of sheep is the anaerobic bacterium *Oxalobacter formigenes* (2, 8). This bacterium is of importance because its population in the rumens of sheep increases dramatically when sheep are slowly fed increasing levels of oxalate (3, 8, 11). The following are some unique features of this organism: (i) oxalate is the only known substrate used for its growth; (ii) oxalate is metabolized to form CO_2 and formate in approximately a 1:1 ratio; (iii) oxalate must be activated to oxalyl-coenzyme A (CoA) before the decarboxylation takes place (5); and (iv) ATP generation appears to involve an F_0F_1 ATPase driven by proton gradients generated by both intracellular proton consumption by the oxalyl-CoA decarboxylase reaction (2) and an electrogenic oxalate-formate antiporter in the cell membranes (4). The manner in which oxalate decarboxylation is coupled to energy production has not been fully explained. In the course of purifying and assaying oxalyl-CoA decarboxylase (5), it became apparent that there was a formyl-CoA transferase present in crude cell extracts that catalyzes the transfer of CoA from formyl-CoA to oxalate. This transferase activity was separated from the decarboxylase activity early in the purification scheme (5). Quayle (14) reported the presence of a formyl-CoA transferase in *Pseudomonas oxalaticus* but did not characterize the enzyme. Barker et al. (6) and Sly and Stadtman (15) reported on a CoA transphorase from *Clostridium kluyveri* but only partially purified the enzyme.

In this paper, we describe the purification of formyl-CoA transferase (EC 2.8.3.4) of *O. formigenes* to homogeneity and describe some of the properties of the enzyme.

MATERIALS AND METHODS

Organisms 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.

Enzyme purification. OxB cells were harvested by centrifugation (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 g of cells. Buffer B contained buffer A plus 1 mM phenylmethylsulfonyl fluoride. 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 (20,000 × *g* for 10 min). The supernatant was treated with 50 mg of protamine sulfate per g of cells to precipitate nucleic acids; the precipitate was removed by centrifugation (20,000 × *g* for 10 min). The enzymatically active protein fraction was precipitated with $(NH_4)_2SO_4$ between 26 and 80% saturation at 22°C. After centrifugation (20,000 × *g* for 10 min), the precipitate was dissolved in a minimal amount of buffer A. After filtration through a 0.2-μm-pore-size filter, this solution was injected in 2-ml portions (approximately 80 mg of protein) onto a preparative hydrophobic interaction chromatography column (Bio-Gel TSK Phenyl-5PW, 150 by 21.5 mm; Bio-Rad Laboratories, Richmond, Calif.). The initial column buffer C 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. At the time the sample was injected, a gradient elution with buffer D (0.1 M NaH_2PO_4, pH 6.7) was started by increasing the proportion of buffer D being pumped at 4%/min. At 10 min, the rate of the gradient was decreased to 1.33%/min, and at 40 min, the rate was again increased to 4%/min until 100% buffer D was pumped for 4 min. The gradient is shown in Fig. 1. The enzymatically active fractions (Fig. 1, shaded area) were combined, and the proteins were concentrated to approximately 4.8 mg/ml in an ultrafiltration cell (Amicon Corp., Lexington, Mass.) with a PM-10 membrane. To prepare the protein for the next purification step, the ionic strength was adjusted by stepwise additions of 20 mM NaH_2PO_4 (pH 6.7) containing 1 mM dithiothreitol to the ultrafiltration cell.

This protein solution was injected in 2-ml portions onto a DEAE anion-exchange column (Bio-Gel TSK DEAE-5PW, 75 by 7.5 mm; Bio-Rad Laboratories). The initial buffer was 20 mM NaH_2PO_4, pH 6.7, and the flow rate was 0.7 ml/min. Eluting buffer E was 400 mM NaH_2PO_4, pH 6.7, and was 20% at 5 min and 100% at 65 min. The eluted protein was monitored at 280 nm. The enzymatically active fractions were combined and concentrated to about 2.5 mg of protein per ml by ultrafiltration as described above. The buffer was exchanged in the ultrafiltration cell to make it 200 mM NaH_2PO_4, pH 6.7. This protein solution was put on the same

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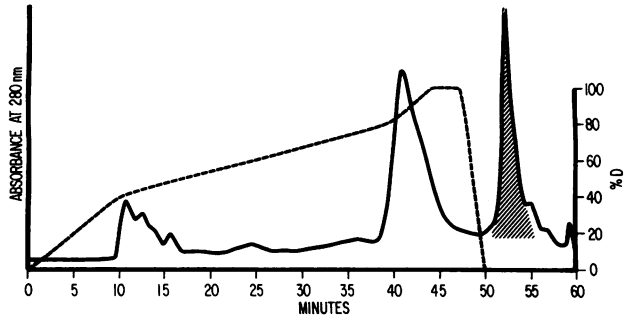

FIG. 1. Hydrophobic interaction chromatogram of redissolved $(NH_4)_2SO_4$-precipitated protein from *O. formigenes* on a Bio-Gel TSK Phenyl-5PW column starting at 1.5 M $(NH_4)_2SO_4$–0.1 M NaH_2PO_4, pH 6.7. Shaded area represents active fractions concentrated for next step in purification; the broken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

DEAE column again, but this time the initial buffer was 200 mM NaH_2PO_4 (pH 6.7), the elution buffer, F, was 400 mM NaH_2PO_4 (pH 6.7), and the elution was accomplished with a linear gradient to 100% F in 40 min. The flow rate was 0.7 ml/min. The enzymatically active fractions were combined and concentrated as described above to about 2.0 mg/ml. The purified enzyme was stored at −70°C.

Gel permeation chromatography. The purified enzyme solution was injected (20 μl) 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; Beckman Instruments, Inc., Berkeley, Calif.) in order to estimate the molecular weight of the enzyme. 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).

Electrophoresis. Fractions from the various chromatographic steps were examined by native and sodium dodecyl sulfate (SDS)-gradient gel electrophoresis in which the polyacrylamide concentration varied linearly from 8 to 25% (Phast System; Pharmacia, Inc., Piscataway, N.J.). The protein concentration of the samples was diluted to approximately 1 mg/ml, and for the SDS gels, the SDS was 1% and the mercaptoethanol was 0.01%. The samples were heated at 100°C for 5 min before 1 μl was applied to the gels. Molecular weight marker standards for the SDS gel electrophoresis were from kit MW-SDS-200 (Sigma Chemical Co., St. Louis, Mo.). 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 gels at pH 4.5 to 6.0 using the same equipment as described above. Reference isoelectric point markers consisted of an isoelectric point calibration kit with a pI range of 4.7 to 10.6 (BDH, Poole, England).

Enzyme activity assays. Thiocresyl formate was synthesized by the method of Bax and Stevens (7) and then converted to formyl-CoA by the method of Quayle (13) for preparing oxalyl-CoA. The formyl-CoA was adjusted to pH 5.5 and stored frozen until used. The concentration of formyl-CoA was determined by the hydroxylamine method of Hestrin (10), with acetyl-CoA as the standard and the value of 0.45 as the ratio found by Hestrin for the molar absorptivities of formyl hydroxamate and acetyl hydroxamate. The purity of the formyl-CoA was 95%. The formyl-CoA transferase activity was determined by estimating the amount of formate produced by measuring the reduction of

TABLE 1. Purification of formyl-CoA transferase from *O. formigenes*

Purification step[a]	Vol (ml)	Amt of protein (mg/ml)	Sp act (U/mg of protein)	Total activity (U)	Recovery (%)	Purification (fold)
Cell supernatant	15	45.2	0.069	46.4	100	
$(NH_4)_2SO_4$ treatment	10	19.8	0.108	21.4	46.1	1.5
Hydrophobic-interaction chromatography	4.5	4.8	0.460	10.0	21.6	6.7
First DEAE chromatography	2.5	2.5	0.707	4.4	9.5	10.2
Second DEAE chromatography	1.0	1.4	2.15	3.0	6.5	31.2

[a] Starting material, 1.7 g (wet weight) of cells.

NAD in the presence of formate dehydrogenase. The reaction mixture (300 μl) contained KH_2PO_4 (pH 6.8), 66 mM; formyl-CoA, 5.0 mM; potassium oxalate (pH 6.8), 10 mM; NAD, 0.83 mM; formate dehydrogenase, 0.25 IU (Boehringer Mannheim Biochemicals, Indianapolis, Ind.); and the amount of enzyme to be tested. The reaction was run at 25°C in a thermal cuvette holder; increases in A_{340} were recorded continuously (Response II Spectrophotometer; Gilford Instruments Laboratories, Inc., Oberlin, Ohio). The reaction was initiated by the addition of the formyl-CoA transferase. Blank values were determined by omitting the formyl-CoA transferase or the formyl-CoA or the oxalate. Omitting the formyl-CoA transferase produced the most reliable blank and was used in each subsequent determination. The pH optimum of the reaction was determined by adjusting the pH of the KH_2PO_4 between 4.0 and 8.0 in increments of 0.5 in the reaction mixture. The following enzyme activators and inhibitors were tested by incubating the compound with the enzyme for 10 min and then adding substrate: EDTA, 10 mM; Ca^{2+}, 10 mM; Mg^{2+}, 10 mM, thiamine PP_i, 1.0 mM; *N*-ethylmaleimide, 1.0 mM; *p*-chloromercuribenzoate, 1.0 mM. The following acceptors of CoA were tested: sodium succinate, 10 mM; sodium acetate, 10 mM; sodium malonate, 10 mM. The apparent K_m and V_{max} values for the reaction with formyl-CoA, oxalate, and succinate were determined from Lineweaver-Burke double-reciprocal plots at five substrate concentrations ranging from 0.5 to 15 mM. In each case, the substrate that was not being evaluated was present in excess (20 mM). Calculations of initial rates were made for measurements taken during the interval from 1 to 5 min. The formyl-CoA transferase was tested to see whether it would catalyze the transfer of CoA from succinyl-CoA or acetyl-CoA to oxalate by substituting succinyl-CoA or acetyl-CoA for formyl-CoA in the reaction mixture and adding oxalyl-CoA decarboxylase, thiamine PP_i (10 μM), and Mg^{2+} (1.0 mM).

Protein determination. Protein concentrations were determined by a modification of the Lowry method (12) with bovine serum albumin as the standard.

RESULTS

Purification of formyl-CoA transferase. A typical purification (Table 1) yielded a homogeneous protein that was purified 31.2-fold and had a specific activity of 2.15 U/mg of 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 formyl-CoA transferase makes up 0.2% of the soluble protein in the cell.

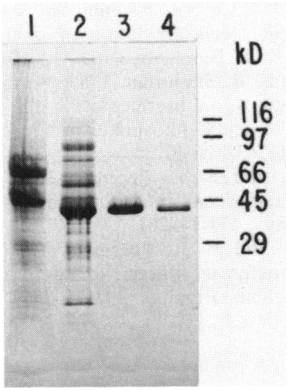

FIG. 2. SDS-polyacrylamide gel (8 to 25% acrylamide). Lanes: 1, enzyme active fraction after $(NH_4)_2SO_4$ precipitation; 2, enzyme active fraction after hydrophobic interaction chromatography; 3, enzyme active fraction after first DEAE chromatography; 4, purified formyl-CoA transferase after second DEAE chromatography. Molecular masses are shown on the right, in kilodaltons (kD).

Requirements for formyl-CoA transferase activity measurements. The pH optimum for enzyme activity was fairly broad, 6.5 to 7.5. The assay was run at pH 6.7 because formyl-CoA is less stable at basic pH. The enzyme requires either oxalate or succinate as acceptor for the CoA. Neither acetate nor malonate will act as an acceptor, because when they were tested at 10 mM concentrations, no activity above background was detected. None of the compounds tested had an activator or inhibitory activity except for the sulfhydryl reagents, i.e., *N*-ethylmaleimide, which inhibited the activity by 20%, and *p*-chloromercuribenzoate, which caused a precipitate to form and inhibited the activity by 91%. By substituting succinyl-CoA for formyl-CoA and adding excess oxalyl-CoA decarboxylase, it was possible to show that the enzyme would catalyze the transfer of CoA from succinyl-CoA to oxalate at a rate that was 11% of that observed when formyl-CoA was used in that same assay system. When acetyl-CoA was substituted for succinyl-CoA, no reaction occurred.

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 44,000 (±1,000) (Fig. 2). The gel permeation column chromatograph had a single peak, and when the elution time was compared with the calibration standards, the molecular weight was calculated to be 44,700 (±1,000). The functional unit of the enzyme is a monomer. The isoelectric point of the enzyme, determined by isoelectric focusing of the native enzyme, was calculated to be 4.7 by comparison with the calibration markers.

Kinetics. Values for Michaelis-Menten-type saturation kinetics were obtained for formyl-CoA as the donor molecule and for both oxalate and succinate as the acceptor molecules. The apparent kinetic constants K_m and V_{max} for the purified enzyme were, respectively, 3.0 mM and 29.6 μmol/min per mg for formyl-CoA, 5.1 mM and 6.4 μmol/min per mg for oxalate, and 2.3 mM and 19.2 μmol/min per mg for succinate. The correlation coefficient (r) for the best fit to a straight line was 0.99, and the standard errors for the K_m measurements were 0.5, 0.5, and 0.6, respectively, for the substrates.

DISCUSSION

This is the second enzyme purified from *O. formigenes* that has a role in oxalate metabolism by the organism. We think that the enzyme plays a significant role in conserving energy for the organism, because the oxalate must be activated by reaction with CoA before it can be decarboxylated by oxalyl-CoA decarboxylase (5). The organism has an oxalyl-CoA kinase enzyme system but requires ATP to function. By transferring the CoA from formyl-CoA back to oxalate, the organism saves that activation energy. Quayle (14) showed the presence of a formyl-CoA transferase in *P. oxalaticus*, but in that organism the formate is further metabolized by a formate dehydrogenase. He did not report the purification or further characterization of the enzyme, so it is not possible to compare the enzymes.

Other acyl-CoA transferases from bacterial systems such as *C. kluyveri* (6, 15) and *Escherichia coli* (9, 16, 17) have been characterized. In the case of *C. kluyveri*, the enzyme was described in terms of the formation of formyl-CoA from acetyl-CoA; and the reaction was found to be reversible. The enzyme from *O. formigenes* is different because it will not use acetyl-CoA as a substrate. In the case of *E. coli*, the CoA transferase has a molecular weight of 97,000 and consists of four subunits. Again, acetyl-CoA serves as a substrate for this enzyme, and those authors showed that the enzyme and acetyl-CoA will transport butyrate across isolated membrane vesicles even though only about 10% of the activity was found to be associated with the membrane fraction. It is not known whether the enzyme from *O. formigenes* will act as a transfer protein, but the enzyme was isolated from the cytoplasmic fraction. Studies to explore the possible role for formyl-CoA transferase as a transport protein are under way. If the formyl-CoA transferase acts as the transport protein that was studied by Anantharam et al. (4) and also catalyzes the transfer of CoA from formyl-CoA to oxalate, then this would be a significant savings in metabolic energy by this organism.

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