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Short-Chain Acyl-CoA-Dependent Production of Oxalate from Oxaloacetate by *Burkholderia glumae*, a Plant Pathogen Which Causes Grain Rot and Seedling Rot of Rice *via* the Oxalate Production

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In *Burkholderia glumae* (formerly named *Pseudomonas glumae*), isolated as the causal agent of grain rot and seedling rot of rice, oxalate was produced from oxaloacetate in the presence of short-chain acyl-CoA such as acetyl-CoA and propionyl-CoA. Upon purification, the enzyme responsible was separated into two fractions (tentatively named fractions II and III), both of which were required for the acyl-CoA-dependent production of oxalate. In conjugation with the oxalate production from oxaloacetate catalyzed by fractions II and III, acetyl-CoA used as the acyl-CoA substrate was consumed and equivalent amounts of CoASH and acetoacetate were formed. The isotope incorporation pattern indicated that the two carbon atoms of oxalate are both derived from oxaloacetate, and among the four carbon atoms of acetoacetate two are from oxaloacetate and two from acetyl-CoA. When the reaction was carried out with fraction II alone, a decrease in acetyl-CoA and an equivalent level of net utilization of oxaloacetate were observed without appreciable formation of CoASH, acetoacetate or oxalate. It appears that in the oxalate production from oxaloacetate and acetyl-CoA, fraction II catalyzes condensation of the two substrates to form an intermediate which is split into oxalate and acetoacetate by fraction III being accompanied by the release of CoASH.

Key words: *Burkholderia glumae*, oxalate production, oxaloacetate, plant pathogenesis *via* oxalic acid, short-chain acyl-CoA.

Oxalate is widely synthesized by microorganisms, plants and even mammals, and is thought to have a variety of physiological or pathological actions which differ with the life form. In man, oxalate is an apparently useless end product of metabolism and is excreted into the urine. When oxalate is overproduced such as in the case of primary hyperoxaluria type 1, it is toxic because its calcium salt is hardly soluble in aqueous solutions at neutral pH, calcium oxalate deposits being formed in the kidneys, bone and other tissues (*1*). In plants, the recent discovery that germin is actually an oxalate oxidase suggests an important role for oxalate in seed germination (*2*), in addition to its still ambiguous general role. Oxalic acid is also a common metabolic product found in the culture fluid of several fungi (*3-9*), and in some of them it has been implicated in plant pathogeneses (*5-9*).

The mechanisms of oxalate production in various life forms fall into two main classes, one from glyoxylate and the other from oxaloacetate as immediate precursors. The oxalate production from glyoxylate in guinea-pig liver was shown to be predominantly catalyzed by lactate dehydrogenase (*10*), and in mammalian liver glyoxylate is thought to be formed mainly from glycolate through the action of glycolate oxidase, although the origin of the glycolate has not yet been fully determined. In microorganisms and non-green tissues of plants, on the other hand, a likely precursor of glyoxylate is, in many cases, isocitrate, and the conversion of glyoxylate to oxalate is catalyzed by various types of glyoxylate dehydrogenase or oxidase (*11-15*). With respect to the oxalate production from oxaloacetate, on the other hand, the hydrolytic cleavage catalyzed by oxaloacetase yielding oxalate and acetate is the only mechanism so far reported. This enzyme has been partially purified from *Aspergillus niger* (*16, 17*), and in addition, extracts of spinach leaves and red beet root tissue were shown to contain an enzyme which converts oxaloacetate to oxalate and acetate (*18*), suggesting that oxaloacetase is also responsible, at least in part, for the oxalate production in green and non-green plant tissues.

Burkholderia glumae (formerly named *Pseudomonas glumae* [*19*]), which was studied in this work, was isolated as the causal agent of grain rot and seedling rot of rice (*20*). When the bacteria were cultured in a hemisynthetic agar medium containing potato extract, peptone, glucose, and 0.1% $CaCl_2$, numerous calcium oxalate crystals were observed in the colonies (*21*). In addition, among the total 200 isolates collected from various localities in Japan, all the 180 virulent isolates produced such crystals, while the

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20 avirulent isolates did not (*21*), suggesting the involvement of oxalate production in the pathogenesis.

In this paper we show that oxalate is produced from oxaloacetate in *B. glumae*, but through a mechanism which is entirely different from that catalyzed by oxaloacetase. The mechanism disclosed is unique in that a short-chain acyl-CoA is involved in the oxalate production from oxaloacetate, and when acetyl-CoA is the acyl-CoA substrate acetoacetate and CoASH are formed together with oxalate as the reaction products.

EXPERIMENTAL PROCEDURES

Materials—Propionyl-CoA carboxylase was purified from bovine liver acetone powder according to Lane and Halenz (*22*) up to the step of DEAE-cellulose column chromatography. Li_3 acetyl-CoA and Tween 80 were purchased from Wako Pure Chemical Industries (Osaka); Li methylmalonyl-CoA, Li propionyl-CoA, Na succinyl-CoA, palmitoyl-CoA, CoASH and β-hydroxybutyrate dehydrogenase from Sigma (St. Louis, USA); malate dehydrogenase, lactate dehydrogenase, acetate kinase, pyruvate kinase, citrate synthase, glutamate dehydrogenase, oxalate decarboxylase, and formate dehydrogenase from Boehringer Mannheim (Germany); *N*-[Tris(hydroxymethyl)methyl]glycine (Tricine) from Nacalai Tesque (Kyoto); peptone from Difco (Detroit, USA); Toyopearl HW-55 from Toyo Soda Manufacturing (Tokyo); Sephadex G-25 (coarse) and Sephadex G-50 (fine) from Pharmacia (Uppsala, Sweden); DE52 from Whatman International (Maidstone, England); silicic acid (100 mesh powder) from Mallinckrodt Chemicals (Paris, KY, USA); and Centriplus-10 from Amicon (Beverly, MA, USA). [1-^{14}C]acetyl-CoA (55 mCi/mmol), L-[U-^{14}C]aspartic acid (160 mCi/mmol), and [1-^{14}C]acetic acid sodium salt (55 mCi/mmol) were products of American Radiolabeled Chemicals (St. Louis), Moravek Biochemicals (Brea, CA, USA), and NEN Life Science Products (Boston, USA), respectively. The sources of the reagents used for the oxalate determination with oxalate oxidase were described previously (*23*). All other chemicals were of reagent grade.

Growth of Bacteria and Preparation of a Cell-Free Extract—*B. glumae* was grown in a hemisynthetic growth medium usually for approximately 40 h at 37°C with constant mechanical shaking. To prepare 1 liter of the growth medium, 200 g of about 2-mm thick potato slices was extracted with 1 liter of H_2O by boiling for 15 min, and after cooling the extract was filtered through 4 layers of gauze. To the potato extract were then added 10 g peptone, 10 g glucose, 0.5 g KH_2PO_4, 3 g Na_2HPO_4-$12H_2O$ and 3 g NaCl, and after the pH has been adjusted to 6.8 the total volume was brought to 1 liter with H_2O. Cell growth was followed by measuring the absorbance at 600 nm (A_{600}). Oxalate production was also monitored at intervals of 5-15 h or at the end of culture by a spectrophotometric method involving oxalate oxidase (*23*) as described below. The harvested bacteria were stored at −80°C. For preparation of a cell-free extract, cells were thawed in 3 volumes (3 ml/g wet weight) or 7 volumes of 50 mM Hepes/NaOH-0.01% (v/v) Tween 80 (pH 7.0), and then disrupted twice in a French Pressure Cell Press (SLM Instruments, Urbana, IL, USA) using a 20 K manual-fill cell at a gauge pressure of 620 psi (cell pressure, about 10,000 psi), followed by centrifugation at 100,000×*g* for 60 min.

All the colonies of pathogenic wild type *B. glumae* were shown to synthesize large amounts of oxalate and greenish-yellow pigments including toxoflavin (*24*). The wild type *B. glumae* used in this study was derived from a colony named 1A. One of the mutants, named 1C, lacking the ability to synthesize oxalate and the pigments as well as the pathogenecity was used as a control.

Determination of Oxalate—Oxalate was usually determined spectrophotometrically with oxalate oxidase as described previously (*23*), except that an automated microplate reader (Type 340; BIO-TEK Instruments, Winooski, VT, USA) was used instead of a spectrophotometer, and the increase in absorbance was determined at 562 nm. Under the conditions used in this study, millimolar extinction coefficient (ε_{mM}) of the oxalate determination was approximately 16 when no interfering substances were present in the assay system.

^{14}C-oxalate was determined as the $^{14}CO_2$ evolved on incubation with oxalate oxidase. For this purpose, samples containing ^{14}C-oxalate were incubated, after treatment with stearate-deactivated charcoal (*23*), with 50 mU of oxalate oxidase in a test tube (diameter, 1.6 cm), to which a counting vial had been connected *via* a thick rubber tube. The vial contained a filter paper strip (Whatman No. 1, 8.0×1.5 cm), to which 0.1 ml of 25% β-phenethylamine in methanol had been added. After overnight incubation at pH 3.3 and 37°C with vigorous shaking (150-160 strokes/min), 10 ml of an emulsion scintillator (15 g DPO, 1.8 g dimethyl-POPOP, and 1 liter Triton X-100, made up to 3 liters with toluene) was added to each vial and then the radioactivity was counted. Under the conditions used, the counting efficiency for the $^{14}CO_2$ determination was 71%.

Where indicated, oxalate was also determined by the oxalate decarboxylase-formate dehydrogenase method essentially according to Beutler *et al.* (*25*). In this assay, a control incubation containing 25 nmol of standard oxalate in addition to the sample was run simultaneously to measure the efficiency of the oxalate determination. Since formate dehydrogenase uses not only NAD but also O_2 as an electron acceptor (*26*), ε_{mM} at 340 nm of the standard oxalate was below 6.22, and ranged from 4.94 to 6.1 (average, 5.57). The concentration of oxalate formed in the original reaction mixture was calculated from the ε_{mM} of the standard oxalate added to each sample. In preliminary experiments, the formation of NADH, as measured as the increase in A_{340}, was proportional to the amount of standard oxalate added.

Determination of Enzymic Production of Oxalate from Oxaloacetate—Initially, the enzyme activity responsible for the formation of oxalate from oxaloacetate was measured under the conditions (assay system 1) under which oxaloacetase from *A. niger* had been studied (*17*). The reaction was carried out at 37°C in a reaction mixture (1.0 ml) consisting of 100 mM Tris/HCl (pH 8.0) or Hepes/NaOH (pH 7.0), 300 μM $MnCl_2$, 1 mM (2.5 mM where indicated) oxaloacetate, and the enzyme, and stopped by immersing the reaction tubes in a boiling water-bath for 5 min. After denatured proteins were removed by brief centrifugation the supernatant was treated with 80 mg of stearate-deactivated charcoal per ml, and the concentration of oxalate was determined with oxalate oxidase (*23*).

After the requirement of short-chain acyl-CoA for the

oxalate production from oxaloacetate had been disclosed, the enzyme activity was determined in assay system 2, in which the reaction was carried out for 5 min at 37°C or for 10 min at 26°C in an incubation mixture (usually 400 μl) consisting of 100 mM Hepes/NaOH (pH 7.0), 50 μM EDTA, 350 μM $CoCl_2$, 360 μM aetyl-CoA or propionyl-CoA, 1.25 mM oxaloacetate, and the enzyme. The reaction was stopped and oxalate formed was determined as above.

Determination of Other Enzyme Activities—The activities of malate dehydrogenase (*27*), NADP-isocitrate dehydrogenase (*28*), and isocitrate lyase (*29*) were assayed essentially according to the references cited, except that glyoxylate formed through the action of isocitrate lyase was determined with lactate dehydrogenase. The reactions for determination of the alanine:glyoxylate (AGT) and glutamate:glyoxylate (GGT) aminotransferase activities were carried out at 30°C in an incubation mixture comprising 100 mM Tricine/NaOH (pH 8.0), 1 mM EDTA, 50 μM pyridoxal 5-phosphate, and 5 mM glyoxylate with 125 mM L-alanine or 100 mM L-glutamate as the amino acid substrate. After deproteinization with $HClO_4$, pyruvate formed from L-alanine through the AGT activity was determined with lactate dehydrogenase, and α-ketoglutarate formed from L-glutamate through the GGT activity with glutamate dehydrogenase. All assays were carried out under conditions under which the enzyme reactions proceeded linearly with respect to both the incubation time and the amount of enzyme added.

One unit of each enzyme was defined as the activity which catalyzed the formation of 1 μmol of reaction product in 1 min.

Determination of Acetone—Acetone was determined spectrophotometrically at 440 nm after conversion to its 2,4-dinitrophenylhydrazone derivative and extraction into CCl_4. When the formation of acetone was also to be determined, the reaction for the oxalate production was carried out in a Thunberg tube, and immediately after initiation of the reaction the tube was capped through a taper ground glass joint to prevent loss of acetone by evaporation. After termination of the reaction, the reaction tube was cooled in an ice-bath and the cap was replaced by a sidearm. Then the reaction mixture was frozen on the inner surface of the tube, and acetone was transferred together with H_2O by evacuation to the sidearm, which was kept in liquid nitrogen. Acetone in the distillate (usually 600 μl) was converted to its 2,4-dinitrophenylhydrazone in a reaction mixture of about 3 ml essentially according to the method of Ariga (*30*), and extracted into 1.2 ml of CCl_4 by vigorous shaking for 10 min at room temperature as described by Greenberg and Lester (*31*). The CCl_4 layer was washed twice with 2.5 ml each of H_2O and once with 1.8 ml of 0.5 N NaOH, and then the A_{440} of the CCl_4 layer was determined. In this method, the reaction of acetone with 2,4-dinitrophenylhydrazine may not reach completion because the hydrazone has been reported to be easily dissociable (*30*), and some loss of acetone may occur by volatilization during the procedure. Therefore, a standard curve for acetone determination was prepared using known amounts of acetone, which were subjected, in parallel with the sample, to the same procedure in every acetone determinations. The linearity of the acetone determination including all the procedures was fairly good and 100 nmol of acetone in the original reaction mixture gave a reading at 440 nm of about 0.11. When the formation of ^{14}C-acetone was to be measured, 400 μl of the CCl_4 layer was evaporated to dryness in a counting vial in the presence of 200 μl of 2.5 mM 2,4-dinitrophenylhydrazine in CCl_4, and then the radioactivity was determined with 10 ml of an emulsion scintillator.

Other Methods—Partitioning chromatography on a silicic acid column was performed essentially according to the method of Varner (*32*), except that a longer column (0.8 cm I.D. × 60 cm L) was used. CoASH and acetyl-CoA were determined by means of the reaction with 5,5′-dithiobis-(2-nitrobenzoate) (DTNB) before and after incubation with citrate synthase, respectively (*33*). Acetate was determined after separation by evaporation from interfering substances according to the principle proposed by Bartley (*34*). To 420 μl of a sample in a Thunberg tube were added 100 μl of 2.5 M citric acid and a trace amount of Na [1-^{14}C]-acetate (3.64 nmol, 444 kdpm), and after the mixture had been frozen on the inner surface of the tube, acetic acid was transferred together with H_2O by evacuation to a sidearm, which was kept in a dry ice-ethanol bath. The concentration of acetate in the transferred solution was then determined using acetate kinase with pyruvate kinase and lactate dehydrogenase as indicator enzymes, and corrected for the recovery of [1-^{14}C]acetic acid in the transferred solution. Enzymic methods with NAD^+ or NADH (*35*) were employed for the determination of oxaloacetate, acetoacetate, β-hydroxybutyrate and pyruvate. Protein was determined by means of the Biuret reaction (*36*).

RESULTS

Oxalate Production in Culture—When wild type *B. glumae* (strain 1A) was cultured in a hemisynthetic medium containing potato extract, peptone and glucose, an enormous amount of oxalate was produced and accumulated in the medium, reaching 18 mM after 66 h (Fig. 1). The oxalate production was accompanied by a decrease in the pH of the culture medium from 7.2 to 6.2. Amino acids initially contained in the culture medium at a sum total concentration of approximately 15 mM were almost completely exhausted by 20 h, except that lysine was consumed from about 1 mM to only 0.66 mM. The NH_3 concentration rose to 4.6 mM during this period. Then glucose began to be consumed, suggesting that *B. glumae* preferentially utilizes amino acids over glucose. On the other hand, a mutant strain, 1C, lacking pathogenecity failed to produce oxalate and grew without the accompanying decrease in pH. The mutant also consumed amino acids initially and then glucose. Considerable production of oxalate was also observed when wild type 1A was cultured without glucose, and on the omission of potato extract and peptone the oxalate concentrations after a 20-h culture were 72 and 37%, respectively, of that attained in the presence of both potato extract and peptone.

Then the ability of TCA-cycle intermediates, amino acids, sugars, glycolate and glyoxylate, to support the oxalate production was examined. For this purpose, 270 mg wet weight of washed *B. glumae* 1A was cultured at 37°C for 7 h in 50 ml of Ayers, Rupp and Johnson's culture medium (1.0 g of $NH_4H_2PO_4$, 0.2 g each of KCl, and $MgSO_4$-$7H_2O$/liter, pH 6.8) in the presence of 10 mM test compounds. When cells were incubated in the absence of carbon sources

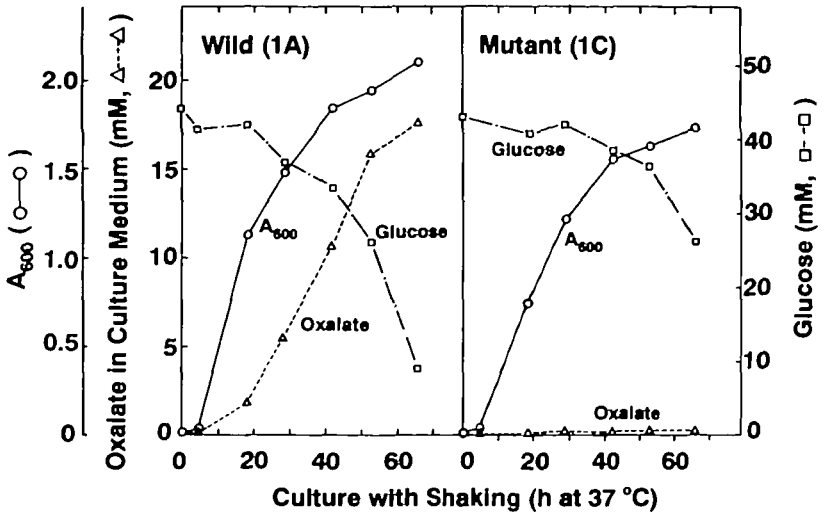

Fig. 1. **Oxalate production by *B. glumae* in culture.** Wild type *B. glumae* 1A and its mutant, 1C, were separately cultured in 300 ml of a hemi-synthetic potato extract-peptone-glucose medium at 37°C with shaking (90 strokes/min) as described under "EXPERIMENTAL PROCEDURES." At 0-time (immediately after the addition of 1.5 ml of a precultured bacterial suspension), and after 4.5, 18, 28.5, 42, 52.5, and 66 h 10 ml of the culture medium was withdrawn, and an 1-ml aliquot was subjected to A_{600} and pH determination. To the remaining 9 ml, 0.19 ml of 6 N HCl was added, and then a 1-ml aliquot was removed for the determination of glucose with a glucose assay kit (mutarotase-glucose oxidase method, Glucose CII Testwako, Wako Pure Chemical Industries). The remaining acidified medium was made to 39 mM with respect to EDTA, neutralized to pH 5.6 with NaOH, treated with stearate-deactivated charcoal, and then used or the determination of oxalate with oxalate oxidase.

the A_{600} decreased slightly with minimal production of oxalate (0.03 and 0.22 mM at 0-time and after 7 h). This small level of oxalate production probably represents oxalogenesis from endogenous substrates. Citrate, malate, succinate, L-glutamate, and L-serine each caused an appreciable level of oxalate production (1.2-1.7 mM after 7 h), and isocitrate, acetate, hydroxypyruvate and glucose were also effective (0.4-0.7 mM after 7 h). Glycine and xylitol were without effect, and the effect of glycolate was marginal. Oxalate was also produced from glyoxylate (0.6 mM after 7 h), but in this case oxalogenesis was also observed with 1C (0.35 mM after 7 h), and the combination of glyoxylate and acetate was more effective than glyoxylate or acetate alone, especially for 1A (1A: 1.3 mM, 1C: 0.5 mM, after 7 h). When oxalate was used as the substrate, no consumption occurred in either 1A or 1C, suggesting that oxalate is not used by *B. glumae* and that the inability of 1C to accumulate oxalate is not due to its degradation. The results obtained with cells in culture thus suggested that *B. glumae* 1A produces oxalate from a TCA-cycle intermediate and then excretes it into the culture medium.

Oxalate Production by a Cell-Free Extract—When cell-free extracts of *B. glumae* 1A were incubated with 2.5 mM oxaloacetate under the conditions (assay system 1) under which oxaloacetase from *A. niger* had been studied (*17*), a small but significant amount of oxalate was produced, but the extract of 1C failed to produce oxalate under the same conditions. The extract of 1A also produced oxalate from L-malate but only in the presence of NAD and its generating system (1 mM NAD, 20 mM pyruvate, and 125 μg/ml lactate dehydrogenase), suggesting that oxaloacetate is the immediate precursor of oxalate in *B. glumae*.

With respect to other enzyme activities examined, the extracts of 1A and 1C exhibited comparable activities. Both 1A and 1C showed very high malate dehydrogenase activity (1A: 32.2, 1C: 19.5 U/mg protein), and moderate NADP-isocitrate dehydrogenase activity (1A: 3.7, 1C: 2.9 U/mg protein), suggesting that the TCA-cycle actively operates in *B. glumae*. Isocitrate lyase, a glyoxylate cycle enzyme, was also detectable, its levels in 1A and 1C being 0.18 and 0.28 U/mg protein, respectively. The alanine:glyoxylate (1A: 0.08, 1C: 0.09 U/mg protein) and glutamate:glyoxylate (1A: 0.16, 1C: 0.18 U/mg protein) aminotransferase activities were also comparable. Although mutant 1C was capable of producing a small amount of oxalate when cultured in the presence of glyoxylate as the sole carbon source, its inability to produce oxalate in the hemisynthetic potato extract-peptone medium may not be due to the lack of the glyoxylate supply *via* the glyoxylate cycle, because its isocitrate lyase activity was comparable to that of wild type 1A.

Dependency of the Oxalate Production from Oxaloacetate on Short-Chain Acyl-CoA—The oxalate-producing activity in assay system 1 exhibited a broad pH optimum of 6.8-8.0, and the reaction proceeded linearly for 5-6 min. It is noteworthy, however, that the initial velocity *vs.* enzyme concentration curve was concave, especially in the range where small amounts of cell-free extract were used. Under these conditions, 100 μl (protein: *ca.* 1 mg) of a cell-free extract of *B. glumae* 1A catalyzed the formation of approximately 10 nmol of oxalate per min at 37°C.

In trials to purify the enzyme, the activity was lost with almost every kind of purification procedure, including dialysis and gel filtration, although the enzyme was quite stable when kept in an ice-bath or at −20°C. Then we found that the enzyme absolutely requires small molecule(s) for its activity. In the experiments shown in Fig. 2, a cell-free extract was filtered through a Centriplus-10 to obtain a small molecule fraction (SMF, $M_r < 10{,}000$), and the extract remaining on the filter was passed through a Sephadex G-25 column to free it from small molecules. The large molecule fraction (LMF) thus obtained did not show any detectable activity by itself, but its activity was restored on addition of the small molecule fraction. On a survey for the activator in the small molecule fraction, we found that acetyl-CoA and propionyl-CoA augment the enzyme activity profoundly. CoASH, palmitoyl-CoA, and other TCA-cycle intermediates, including citrate, isocitrate, α-ketoglutarate, succinate, fumarate and malate, could not substitute for acetyl-CoA or propionyl-CoA.

Purification of the Enzyme Responsible for the Acetyl-CoA-Dependent Production of Oxalate from Oxaloacetate—We then attempted to purify the enzyme, monitoring its activity as the oxalate production from oxaloacetate in the presence of acetyl-CoA (assay system 2). All manipulations for the purification were carried out at 2-4°C. In a

representative purification run, frozen cells (5 g) were thawed in 35 ml of 50 mM Hepes-0.01% (v/v) Tween 80 (pH 7.0) and then disrupted in a French Pressure Cell Press as described under "EXPERIMENTAL PROCEDURES." After centrifugation (100,000 × *g*/60 min), the supernatant (cell extract, 35 ml) was brought to 30% saturation with respect to $(NH_4)_2SO_4$ and the resultant precipitate was removed by centrifugation. To the supernatant was added additional $(NH_4)_2SO_4$ to a final concentration of 55% saturation and then the precipitated protein was dissolved in 5 ml of 20 mM Hepes-0.01% Tween 80-0.1 M KCl (pH 7.0). The ammonium sulfate fraction (8 ml) was then applied to a Toyopearl HW-55 gel filtration column (2.5 × 44 cm) from which the enzyme was eluted just behind the elution position of ferritin (M_r = 440,000), indicating that the molecular weight of the enzyme is approximately 400,000. The active fractions were combined and dialyzed overnight against a large excess of 20 mM Hepes-0.01% Tween 80 (pH 7.0). The resulting dialyzed Toyopearl fraction amounted to 23 ml, of which 11.5 ml was adjusted to pH 7.5 and applied to a DE52 column (1 × 12 cm), which had been equilibrated with 20 mM Hepes-0.01% Tween 80 (pH 7.5). The column was washed with 50 ml of the equilibration buffer and then the enzyme was eluted with a linear gradient of KCl, from 0 to 200 mM, in 10 column bed volumes of the equilibration buffer, followed by further elution with the equilibration buffer containing 200 mM KCl and the buffer containing 500 mM KCl. As shown in Fig. 3, four protein peaks were eluted from the DE52 column, and the enzyme activity producing oxalate in the presence of acetyl-CoA was detected in peak II when the reaction for the assay was stopped by immersing the reaction tubes in a boiling water-bath for 5 min. A summary of the purification is presented in Table I.

When the enzyme reaction was stopped with $HClO_4$

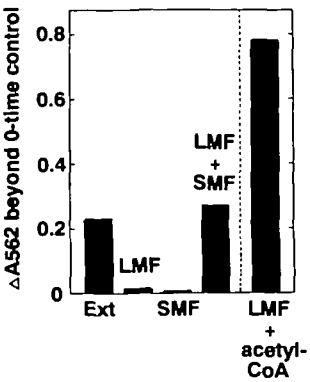

Fig. 2. **Acetyl-CoA-dependent formation of oxalate from oxaloacetate.** A cell-free extract of *B. glumae* 1A was separated into a filtrate (small molecule fraction, SMF) and a concentrated extract by ultrafiltration through a Centriplus 10. The extract concentrated on the filter was then subjected to gel-filtration through a Sephadex G-25 column (1.5 × 45 cm), the combined void volume fractions being named the large molecule fraction (LMF). The reaction for oxalate production was carried out in assay system 1 (buffer: Hepes/NaOH, pH 7.0) for 6 min with either 100 μl of cell-free extract, 200 μl of LMF, 200 μl of SMF, or a combination of them. Where indicated acetyl-CoA was included at a final concentration of 0.9 mM. The oxalate formed was determined with oxalate oxidase. Ext: cell-free extract.

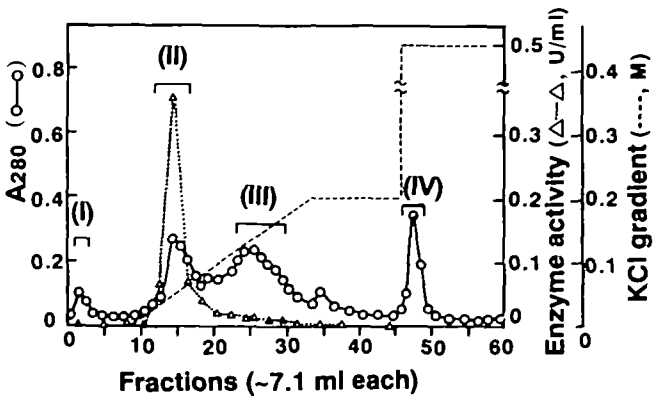

Fig. 3. **DE52 column chromatography.** The experimental details are given in the text. The fractions indicated by brackets, for peaks I, II, III, and IV, were combined, respectively.

TABLE I. **Purification of the enzyme responsible for the acetyl-CoA-dependent conversion of oxaloacetate to oxalate.**

Enzyme preparation	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (mU/mg)	Yield (%)
Cell extract	35	38	453	84	100
$(NH_4)_2SO_4$ (30-55%)	8	35	363	96	92
Toyopearl (M_r ~400 k)	23	17	99	172	45
DE52* (Peak II)	36	9	8	1,125	23.7

Starting material: 5 g wet weight of *B. glumae* 1 A. *From 11.5 ml of the Toyopearl eluate.

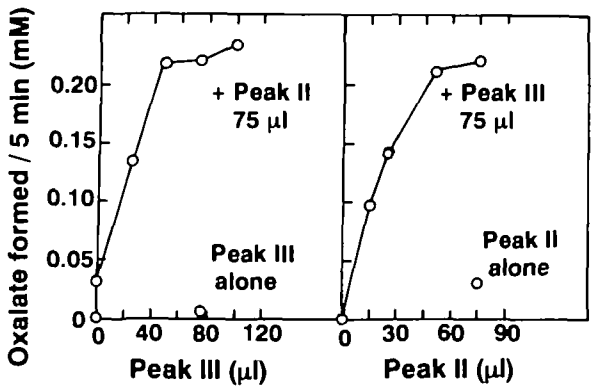

Fig. 4. **Oxalate formation through the combined actions of the peak II and peak III materials.** The reaction for the formation of oxalate from oxaloacetate in the presence of acetyl-CoA was carried out for 5 min in assay system 2 (reaction volume, 400 μl), except that various amounts of the combined peak III material in the presence of 75 μl of the combined peak II material (left panel) or various amounts of the combined peak II material in the presence of 75 μl of the combined peak III material (right panel) were used as the enzyme as indicated, and terminated by adding $HClO_4$ to a final concentration of 0.5 mM. The acidified reaction mixtures were neutralized with KOH, adjusted to a final volume of 800 μl with H_2O, and then centrifuged in the cold to remove the denatured protein and $KClO_4$ precipitates. Then the oxalate concentration in the neutralized supernatants was determined by the oxalate decarboxylase-formate dehydrogenase method (*25*), because the oxalate oxidase method is not applicable when $HClO_4$ is used for deproteinization.

(final, 0.5 mM) and oxalate was determined by the oxalate decarboxylase-formate dehydrogenase method (*25*), however, oxalate formed with the combined peak II material was much lower than that detected when the reaction was stopped by heating, and the combined peak II and peak III materials were both required for the efficient formation of oxalate. As shown in Fig. 4, the oxalate production in the presence of a saturating amount of the combined peak II material was dependent on the amount of the combined peak III material added, and *vice versa*. Essentially the same results were obtained when the enzyme reaction was stopped by adding HCl to a final concentration of 0.2 mM and oxalate was determined, after neutralization, with oxalate oxidase. The activity of the combined peak II material in the presence of a saturating amount of the combined peak III material and that of the combined peak III material in the presence of a saturating amount of the combined peak II material were both completely lost on 5-min boiling, suggesting that two different proteins are involved in the acetyl-CoA-dependent formation of oxalate from oxaloacetate. We believe at present that these two fractions are enzymes, but for the sake of safety the combined peak II and peak III materials are hereafter referred to as fraction II and fraction III, respectively.

It was also observed that a cell-free extract of avirulent mutant 1C did not catalyze the acetyl-CoA-dependent production of oxalate from oxaloacetate even in the presence of a saturating amount of the partially purified fraction II or fraction III, suggesting that both fractions II and III are missing in this mutant.

Metal Requirement—The eluate from a Toyopearl HW-55 column exhibited appreciable activity even when it was dialyzed for 44 h against 200 mM EDTA (pH 7.0) and then separated from EDTA by passage through a Sephadex G-50 (fine) column, but the activity of the EDTA-treated desalted enzyme decreased considerably when 50 μM EDTA was included in the reaction mixture. Therefore, the metal requirement of the enzyme was examined in the presence of 50 μM EDTA. As shown in Fig. 5, Co^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} augmented the activity when their concentrations exceeded 50 μM, although higher concentrations of Ni^{2+} and Zn^{2+} were rather inhibitory. Fe^{2+} and Cd^{2+} were also effective, but Mg^{2+}, Ca^{2+}, and Ba^{2+} were without effect, and Cu^{2+} was inhibitory. Among the effective metals Co^{2+} was the most effective, as judged from both the extent of activation and the apparent K_a value.

Other Properties—The enzyme purified up to the step of Toyopearl chromatography was specific to oxaloacetate. Other organic acids, including citrate, isocitrate, α-ketoglutarate, succinate, malate, pyruvate, fumarate, *cis*-aconitate, lactate, acetate, glycolate, glyoxylate, and formate, did not act as substrates and did not inhibit the reaction with oxaloacetate when included in the reaction mixture to a final concentration of 1.0 or 1.25 mM, except that 1.25 mM glyoxylate caused about 80% inhibition. The apparent K_m for oxaloacetate determined in the presence of 360 μM acetyl-CoA was 0.18-0.19 mM. With respect to acyl-CoA, on the other hand, propionyl-CoA was more effective than acetyl-CoA. When determined in the presence of 1.25 mM oxaloacetate, the apparent K_m values for propionyl-CoA and acetyl-CoA were about 90 and 60 μM, respectively, but the apparent V_{max} value attained with propionyl-CoA was about twice that attained with acetyl-CoA. No activity was detectable when acetyl- or propionyl-CoA was replaced by CoASH, succinyl-CoA, or palmitoyl-CoA, and the stimulation of the enzyme activity observed

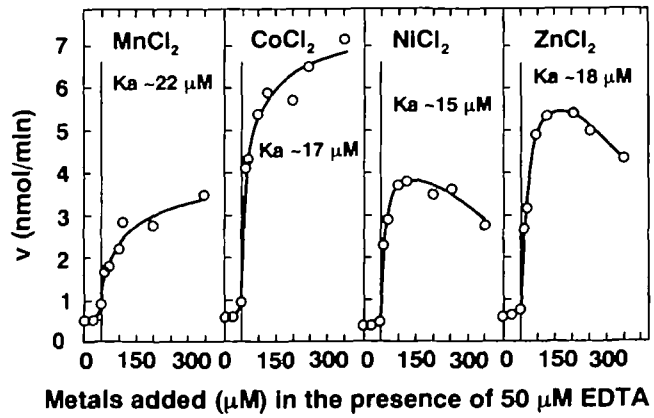

Fig. 5. **Requirement for metals.** Two milliliters of the Toyopearl eluate was first passed through a Sephadex G-50 (fine) column (1.6× 44 cm) with 20 mM Hepes-0.01% Tween 80-0.1 M KCl (pH 7.0) as the equilibration and elution buffer. The Sephadex effluent (5.0 ml) was then subjected to dialysis for 44 h against 500 ml of 200 mM EDTA (pH 7.0), followed by second Sephadex G-50 column chromatography to free it from EDTA. The reactions for demonstration of the metal requirement were carried out with the EDTA-treated desalted Toyopearl eluate in the presence of 50 μM EDTA and the indicated concentrations of various metals. For calculation of the apparent K_a values for metals the metal concentration was corrected for 50 μM EDTA, based on the binding of EDTA with divalent metals in a molar ratio of 1:1.

TABLE II. **Stoichiometry of the reaction.** The reaction was carried out in assay system 2 at 26°C for 10 min with 150 μl each of fraction II (*ca.* 52 mU) and/or fraction III (*ca.* 50 mU) in a total volume of 800 μl. A control incubation involved the same ingredients except that oxaloacetate was omitted, and in another control incubation, 20 mM Hepes-0.01% Tween 80 (pH 7.0) was added in place of the enzymes. For termination of the reaction, a 550-μl aliquot of each reaction mixture was transferred to a test tube containing 10 μl of 11.6 N HCl (final HCl, 0.19 N), and at the same time the reaction tube containing the remaining reaction mixture was immersed in a boiling water-bath for 5 min. After centrifugation, the acid-stopped reaction mixture was neutralized and then immediately used to determine acetoacetate, oxaloacetate/pyruvate and CoASH/acetyl-CoA. Then oxalate in the acid-stopped and heat-stopped reaction mixtures was determined, after charcoal treatment, with oxalate oxidase.

Enzymes	Net utilization of OAA*	OAA-dependent utilization of acetyl-CoA	OAA-dependent production of			
			AcAc	CoASH	oxalate	
					(HCl)	(Boiling)
	(μM in reaction mixture)		(μM in reaction mixture)			
Fractions II & III	173	159	162	136	124	135
Fraction II alone	143	121	6	13	47	129
Fraction III alone	35	56	20	40	21	7

*Corrected for the decarboxylation to form pyruvate. OAA: oxaloacetate, AcAc: acetoacetate.

with methylmalonyl-CoA was at most 13% that of acetyl-CoA.

Stoichiometry of the Reaction and Identification of Another Product—We examined, using dialyzed Toyopearl eluate or a combination of fractions II and III, whether or not acetyl-CoA is utilized, and found that in conjugation with the formation of oxalate from oxaloacetate a stoichiometric amount of acetyl-CoA is converted to CoASH. The conversion of acetyl-CoA to CoASH was absolutely dependent on the presence of both the enzyme and oxaloacetate. We examined the formation of citrate, acetate and acetone, but citrate was not produced in a detectable quantity and the amounts of the latter two detected were far below the stoichiometric ones, if at all. We then found that the formation of a nearly stoichiometric amount of acetoacetate was coupled with the acetyl-CoA-dependent formation of oxalate from oxaloacetate. When both fractions II and III were added, equivalent amounts of CoASH, oxalate and acetoacetate were formed with the concomitant consumption of oxaloacetate and acetyl-CoA, as shown in Table II. In this experiment, more than the stoichiometric amounts of oxaloacetate and acetyl-CoA were consumed, but in another experiment carried out with the Toyopearl eluate the decrease in the concentration of oxaloacetate corrected for its spontaneous decarboxylation to pyruvate was 158 μM and approximately the same amount (155 μM) of oxalate was formed. In still another experiment, in which the net utilization of oxaloacetate was 161 μM, the disappearance of acetyl-CoA and production of CoASH amounted to 157 μM and 149 μM, respectively. Considering all these results we concluded that in the reaction of oxaloacetate with acetyl-CoA catalyzed by fractions II and III, equivalent quantities of oxalate, acetoacetate and CoASH are formed.

In order to identify the reaction products and gain an insight into the reaction mechanism the incorporation patterns of ^{14}C into the reaction products were examined using [*U*-^{14}C]oxaloacetate or [1-^{14}C]acetyl-CoA as a radioactive substrate. In this experiment, [*U*-^{14}C]oxaloacetate was generated *in situ* from L-[U-^{14}C]aspartate with aspartate aminotransferase during the incubation at 37°C for 5 min with fractions II and III in the presence of non-radioactive acetyl-CoA. When [1-^{14}C]acetyl-CoA was the radioactive substrate, non-radioactive oxaloacetate was generated from L-aspartate in the same way and the incubation was carried out under the same conditions. The reactions were terminated, and at the same time the acetoacetate and pyruvate formed were converted to more stable β-hydroxybutyrate and lactate, respectively, by adding NADH, malate dehydrogenase, β-hydroxybutyrate dehydrogenase, and lactate dehydrogenase, and after an additional 15-min incubation, all the reactions were stopped by immersing the reaction tubes into a boiling water-bath. A portion of each deproteinized reaction mixture was subjected to partitioning chromatography on a silicic acid column to separate the β-hydroxybutyrate formed. The other portion of the deproteinized reaction mixture was treated with stearate-deactivated charcoal and then incubated with oxalate oxidase for the conversion of ^{14}C-oxalate to $^{14}CO_2$. As shown in Fig. 6, radioactive β-hydroxybutyrate was formed from both L-[*U*-^{14}C]aspartate and [1-^{14}C]acetyl-CoA. In either case, the radioactivity detected in the β-hydroxybutyrate fraction was almost completely coincident with the elution position of carrier β-hydroxybuty-

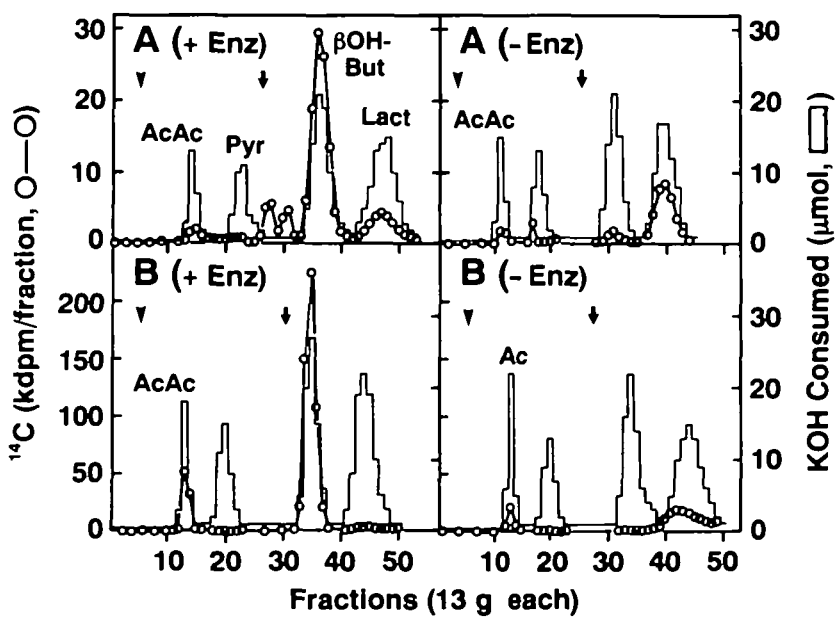

Fig. 6. **Incorporation of ^{14}C from [*U*-^{14}C]oxaloacetate (A) and [1-^{14}C]acetyl-CoA (B) into β-hydroxybutyrate.** The reaction mixture (1,200 μl) contained 5 mM α-ketoglutarate, *ca.* 6 U of aspartate aminotransferase (GOT), 210 μl each of fraction II (*ca.* 72 mU) and fraction III (*ca.* 70 mU), and substrates, in addition to the buffer, EDTA and $CoCl_2$. The substrates were 1 mM L-[*U*-^{14}C]aspartate (2,293 kdpm) plus 360 μM acetyl-CoA in A, and 1 mM L-aspartate plus 360 μM [1-^{14}C]acetyl-CoA (2,093 kdpm) in B. In the minus-enzyme controls (−Enz), fractions II and III were replaced by 20 mM Hepes-0.01% Tween 80 (pH 7.0). The reaction was started by the addition of GOT, and after 5-min incubation at 37°C *ca.* 15 U of malate dehydrogenase was added together with *ca.* 13.5 U of lactate dehydrogenase, *ca.* 0.9 U of β-hydroxybutyrate dehydrogenase and 2 μmol of NADH, followed by an additional 15-min incubation at 37°C. The reactions were finally terminated by immersing the reaction tubes in a boiling water-bath for 5 min, and a 750-μl aliquot of each deproteinized reaction mixture was made to 0.63 N with respect to H_2SO_4 and then subjected to partitioning chromatography on a silicic acid column together with non-radioactive acetoacetate (AcAc, 24 μmol) or acetate (Ac, 24 μmol) where indicated, and pyruvate (Pyr, 25 μmol), β-hydroxybutyrate (βOH-But, 50 μmol), and lactate (Lact, 50 μmol) as carriers. Elution was carried out with chloroform, and 5% (CB5) and 8% (CB8) *n*-butanol in chloroform, each of which had been equilibrated against 0.5 N H_2SO_4. Arrowheads and arrows indicate that the solvent was changed from chloroform to CB5, and from CB5 to CB8, respectively. The carrier organic acids in the eluate were detected by titration with 10 mM KOH to the phenol red end-point and at the same time the organic acids were quantitatively extracted as their K-salts into the aqueous phase by vigorous agitation. The ^{14}C radioactivity in the aqueous phase was then determined using a 300-μl aliquot. The sums of the ^{14}C-radioactivity detected in the β-hydroxybutyrate fraction were +Enz: 100.7 kdpm and −Enz: 5.3 kdpm in A, and +Enz: 525.1 kdpm and −Enz: 8.3 kdpm in B.

rate, and the formation of ^{14}C-β-hydroxybutyrate was dependent on the presence of the enzymes (fractions II and III). Besides β-hydroxybutyrate, ^{14}C-lactate was formed from L-[*U*-^{14}C]aspartate but not from [1-^{14}C]acetyl-CoA, regardless of the presence or absence of the enzymes, reflecting the nonenzymic decarboxylation of [*U*-^{14}C]oxaloacetate to ^{14}C-pyruvate.

When [1-^{14}C]acetyl-CoA was the radioactive substrate, ^{14}C was also detected just behind the carrier acetoacetate, *i.e.*, the position at which acetate was eluted under the conditions used. However, this radioactive compound was also formed in the absence of the enzymes, being eluted in good coincidence with the elution position of the carrier acetate (Fig. 6B, -Enz). In addition, the radioactivity in the acetate fraction was very low and less than one-tenth that of the ^{14}C-β-hydroxybutyrate formed.

In contrast to the formation of ^{14}C-β-hydroxybutyrate, ^{14}C-oxalate was formed from L-[*U*-^{14}C]aspartate but not from [1-^{14}C]acetyl-CoA. It was thus suggested that the two carbon atoms of oxalate are both derived from oxaloacetate, and among the four carbon atoms of acetoacetate two are derived from oxaloacetate and two from acetyl-CoA. Indeed, when the amounts of ^{14}C-β-hydroxybutyrate and ^{14}C-oxalate formed from L-[*U*-^{14}C]aspartate and that of ^{14}C-β-hydroxybutyrate formed from [1-^{14}C]acetyl-CoA were calculated based on the above assumption, they were all shown to be about the same (Table III). It was thus suggested that during the course of oxalate production from oxaloacetate and acetyl-CoA the two substrates combine to form a six-carbon intermediate which is then split into acetoacetate and oxalate, with the release of CoASH somewhere in the reaction sequence. The formation of a very small quantity of ^{14}C-acetate, if at all, together with the inability to measure the acetate formation in the stoichiometry experiment clearly distinguishes the oxalate production in *B. glumae* from that catalyzed by oxaloacetase, which hydrolyzes oxaloacetate to oxalate and acetate (*16, 17*).

TABLE III. **Summary of the ^{14}C-incorporation experiment.** The amount of ^{14}C-oxalate formed in experiment in Fig. 6 was estimated as the $^{14}CO_2$ evolved on incubation of the deproteinized reaction mixture with oxalate oxidase as described under "EXPERIMENTAL PROCEDURES." The charcoal-treated samples were subjected to the reaction with oxalate oxidase in the absence or presence of standard [*U*-^{14}C]oxalate (48.6 kdpm), and $^{14}CO_2$ evolved in the absence of standard ^{14}C-oxalate was corrected for its recovery as $^{14}CO_2$. The enzymic formation of ^{14}C-β-hydroxybutyrate and ^{14}C-oxalate from L-[*U*-^{14}C]aspartate was calculated assuming that two ^{14}C atoms each of L-[*U*-^{14}C]aspartate (478 dpm/nano-atom C) are incorporated into β-hydroxybutyrate and oxalate, respectively. The enzymic formation of ^{14}C-β-hydroxybutyrate from [1-^{14}C]acetyl-CoA was calculated assuming that the specific radioactivity of the product is the same as that of the radioactive substrate (4,760 dpm/nmol). The radioactive compound eluted from the silicic acid column just behind the carrier acetoacetate or together with the carrier acetate was tentatively regarded as acetate, and the amount of it formed from L-[*U*-^{14}C]aspartate was calculated from the specific radioactivity of the two carbon atoms of L-[*U*-^{14}C]aspartate. When [1-^{14}C]acetyl-CoA was the radioactive substrate the specific radioactivity of ^{14}C-acetate formed was assumed to be the same as that of the substrate.

Radioactive substrate	Enzymic formation of radioactive		
	β-hydroxybutyrate (nmol)	oxalate	acetate
A. L-[*U*-^{14}C]Asp	214	232	4
B. [1-^{14}C]Acetyl-CoA	232	<1	23

Reaction with Fraction II or Fraction III Alone—The reaction of oxaloacetate and acetyl-CoA in the presence of fraction II or fraction III alone was analyzed 4 times, once with incubation at 26°C for 10 min and 3 times with incubation at 37°C for 5 min, representative results being presented in Table II. In all of the 4 trials, a decrease in acetyl-CoA and a roughly equivalent level of net utilization of oxaloacetate were observed in the presence of fraction II alone, although the extent of the decrease varied from 32 to 76% of that observed in the presence of both fractions II and III. The decreases in acetyl-CoA and oxaloacetate catalyzed by fraction II alone were not accompanied by the formation of CoASH, acetoacetate or oxalate, as long as the reaction was stopped with acid and the reaction products were determined promptly, suggesting that fraction II catalyzes the reaction of oxaloacetate with acetyl-CoA to form a hypothetical six-carbon intermediate to which CoASH is still bound. The oxalate formation was evident when the reaction with fraction II alone was stopped by heating, and in one experiment shown in Table II a small amount of oxalate was detected even when the reaction was stopped with HCl. In another set of experiments carried out for other purposes and in which the manipulation of samples took some time, more oxalate and CoASH were detected in the neutralized HCl-stopped reaction mixture, suggesting that non-enzymic formation of oxalate and CoASH from the hypothetical six-carbon intermediate may occur fairly rapidly at neutral pH.

Low levels of oxaloacetate-dependent utilization of acetyl-CoA and formation of CoASH were also observed in the reaction with fraction III alone, but in this case oxalate production was not evident even when the reaction was stopped by heating (Table II). The reaction with fraction III alone may be, at least in part, due to a small amount of fraction II contained in the fraction III preparation used (Fig. 3).

In another set of experiments, [*U*-^{14}C]oxaloacetate generated *in situ* from L-[*U*-^{14}C]aspartate was incubated at 26°C for 10 min with either fraction II (*ca.* 38 mU) alone, fraction III (*ca.* 36 mU) alone, or both fractions II and III in the presence of 360 μM acetyl-CoA. The reaction was stopped and at the same time acetoacetate and pyruvate formed were converted to more stable β-hydroxybutyrate and lactate, respectively, by adding excess NADH, malate dehydrogenase, β-hydroxybutyrate dehydrogenase, and lactate dehydrogenase. After an additional 5-min incubation at 26°C, a portion of the reaction mixture was made acidic with HCl and the other portion was subjected to 5-min heating to finally stop the reaction. Then the formation of ^{14}C-labeled CO_2, acetone and oxalate was determined. In this experiment, the amount of ^{14}C-oxalate formed in the presence of both fractions II and III was 99 nmol and 85 nmol (165 and 142 μM in the reaction mixture) when the reaction was stopped by heating and with HCl, respectively, indicating that the reaction proceeded well. When the reaction with fraction II alone was stopped by heating the ^{14}C-oxalate formed amounted to 40 nmol (67 μM). Under these conditions, neither $^{14}CO_2$ nor acetone was formed to any detectable extent no matter whether the reaction was carried out with fraction II alone, fraction III alone or both fractions II and III, or whether the

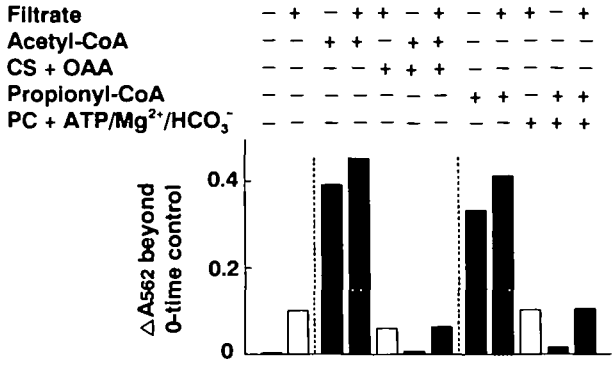

Fig. 7. **Effect of the ultrafiltrate after removal of acetyl-CoA and propionyl-CoA.** An ultrafiltrate (M_r < 10,000, small molecule fraction) was prepared from a cell-free extract of *B. glumae* as described in the legend to Fig. 2. The preincubation mixture (500 μl) for removal of acetyl-CoA comprised 50 mM Hepes (pH 7.0), 4 mM oxaloacetate, 50 μg (*ca.* 5 U) of citrate synthase, and either 225 μl of ultrafiltrate, 135 nmol of acetyl-CoA or a combination of them. The preincubation mixture (500 μl) for removal of propionyl-CoA comprised 50 mM Hepes (pH 7.0), 4 mM $MgCl_2$, 2 mM ATP, 50 mM $KHCO_3$, 42 mU of propionyl-CoA carboxylase, and either 225 μl of ultrafiltrate, *ca.* 135 nmol of propionyl-CoA or a combination of them. Where indicated, citrate synthase/oxaloacetate and propionyl carboxylase/Mg^{2+}-ATP/HCO_3^- were omitted from the preincubation mixture. After preincubation at 30°C for 20 min the mixture was cooled in an ice-bath and then a 200-μl aliquot was subjected to incubation with 60 μl each of fractions II and III (fraction II: *ca.* 30 mU, fraction III: *ca.* 20 mU) in 400 μl of 100 mM Hepes (pH 7.0) containing 50 μM EDTA, 350 μM $CoCl_2$, and 1.25 mM oxaloacetate. The reaction for oxalate production was carried out at 37°C for 5 min and terminated by immersing the reaction tube in a boiling water-bath for 5 min. Then oxalate in the deproteinized reaction mixture was determined with oxalate oxidase. CS: citrate synthase, OAA: oxaloacetate, PC: propionyl-CoA carboxylase.

reaction was stopped by heating or with HCl.

Effect of the Ultrafiltrate after Removal of Acetyl-CoA and Propionyl-CoA—The short-chain acyl-CoA-dependent oxalate production from oxaloacetate in *B. glumae* was disclosed starting from the finding that a cell extract of this plant pathogen failed, after dialysis or gel-filtration, to produce oxalate from oxaloacetate unless an ultrafiltrate of the extract was added. Then we found that the oxalate production absolutely requires a short-chain acyl-CoA such as acetyl-CoA and propionyl-CoA (*cf.* Fig. 2), and analyzed the reaction mechanism using acetyl-CoA as the acyl-CoA substrate. To ask whether the endogenous cosubstrate in the ultrafiltrate is solely acetyl-CoA and/or propionyl-CoA we then examined the ability of an ultrafiltrate to support the oxalate production from oxaloacetate after removal of acetyl-CoA or propionyl-CoA. Acetyl-CoA was exhausted by preincubation with a large excess of citrate synthase in the presence of oxaloacetate, and propionyl-CoA was removed with propionyl-CoA carboxylase in the presence of Mg^{2+}-ATP and bicarbonate. As shown in Fig. 7, the ability of the ultrafiltrate to support the oxalate production by fractions II and III was reduced to only about 60% on preincubation with a citrate synthase and oxaloacetate, although the effect of acetyl-CoA added to the ultrafiltrate was completely removed under the same conditions. When the ultrafiltrate was preincubated with propionyl-CoA carboxylase in the presence of Mg^{2+}-ATP and bicarbonate, no apparent decrease was observed in the ultrafiltrate-dependent oxalate production under conditions in which propionyl-CoA added to the ultrafiltrate was exhausted. It is therefore possible that for the oxalogenesis from oxaloacetate, *B. glumae* contains, in addition to acetyl-CoA, some other endogenous cosubstrate(s), which remains to be identified.

DISCUSSION

A new reaction for oxalogenesis from oxaloacetate was found in *B. glumae*, a plant pathogen which causes grain rot and seeding rot of rice *via* the production of oxalate. The reaction requires a short-chain acyl-CoA such as acetyl-CoA and propionyl-CoA as a cosubstrate, and two enzymes tentatively named fraction II and fraction III. Available evidence suggests that when acetyl-CoA is the acyl-CoA substrate fraction II catalyzes the aldol condensation of the carbonyl carbon of acetyl-CoA and the methylene carbon of oxaloacetate to form a six-carbon intermediate to which CoASH is still bound, and then the intermediate is hydrolyzed by fraction III to oxalate and acetoacetate, being accompanied by the release of CoASH (Fig. 8). The proposed mechanism for oxalate production from oxaloacetate in *B. glumae* is based on the following observations: (i) when the reaction was carried out in the presence of both fractions II and III the disappearance of acetyl-CoA was paralleled by almost equivalent levels of net utilization of oxaloacetate (utilization corrected for pyruvate formation), and formation of oxalate, acetoacetate and CoASH (Table II); (ii) neither acetate, citrate, acetone nor CO_2 was formed in the reaction; (iii) an isotope incorporation experiment showed that the origin of the two carbon atoms of oxalate is oxaloacetate, and among the four carbon atoms of acetoacetate two are derived from oxaloacetate and acetyl-CoA, respectively (Fig. 6 and Table II). The rearrangement of carbon atomes can not occur unless oxaloacetate and acetyl-CoA once combine to form a six-carbon intermediate which is then split into acetoacetate and oxalate, with the release of CoASH somewhere in the reaction sequence; (iv) Among possible combinations of acetyl-CoA and oxaloacetate only the synthesis of a C-C bond between the methylene carbon of oxaloacetate and the carbonyl carbon of acetyl-CoA gives rise to the formation of a six-carbon intermediate which can be cleaved to oxalate and acetoacetate; (v) when the reaction was carried out in the presence of fraction II alone acetyl-CoA and oxaloacetate were consumed, but the amounts of oxalate, acetoacetate and CoASH formed were negligible or far below stoichiometric ones as long as the reaction was stopped with acid and the products were determined promptly (Table II). Thus all the data available at present suggest the reaction sequence shown in Fig. 8, although the structure of the proposed intermediate is unusual and unprecedented in enzymology. The proposed reaction catalyzed by fraction II is apparently similar to those catalyzed by acetyl-CoA acyltransferase [EC 2.3.1.16], glycine acetyltransferase [EC 2.3.1.29], δ-aminolevulinate synthase [EC 2.3.1.37], and so on, but in these reactions CoASH is released concomitantly with the C-C bond formation between the carbonyl carbon of acyl-CoA and the methylene carbon of another substrate. In the case of the oxalate production from acetyl-CoA and oxaloacetate in *B. glumae*, on the other hand, it is suggested that the acetyl-CoA-derived

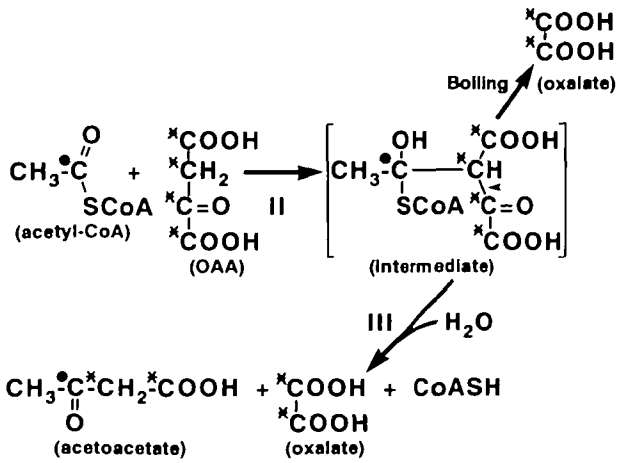

Fig. 8. **Proposed reaction mechanism for the formation of acetoacetate and oxalate from acetyl-CoA and oxaloacetate.** When propionyl-CoA is the acyl-CoA substrate the formation of propionylacetate and oxalate through a seven-carbon intermediate is expected. ● and * represent ^{14}C derived from [1-^{14}C]acetyl-CoA and [*U*-^{14}C]oxaloacetate, respectively. The arrowhead indicates the carbon-carbon bond which is expected to be hydrolytically cleaved by fraction III or non-enzymatically on heating.

CoASH moiety is retained in the intermediate until it is hydrolyzed to form oxalate enzymatically by fraction III or non-enzymatically. We understand at present that heating the reaction mixture accelerates the non-enzymatic conversion of the intermediate to oxalate. The proposed intermediate has the β-keto acid structure, nevertheless no decarboxylation was detectable, other than that associated with the non-enzymic formation of pyruvate from oxaloacetate, under the conditions used. The structure of the intermediate has to be directly identified, and how the CoASH moiety and the carboxyl group derived from the γ-carboxyl group of oxaloacetate are stabilized in the intermediate needs to be explained in the future.

The condensation of oxaloacetate with acetyl-CoA catalyzed by citrate synthase (*37*) is well known. In this case, the methyl group of acetyl-CoA is joined to the carbonyl of oxaloacetate to form citryl-CoA, and in order for this to occur a proton is known to be abstracted from the methyl of acetyl-CoA, forming its enolate anion, which then attacks the carbonyl of oxaloacetate. In the proposed mechanism for the oxalate production from oxaloacetate and acetyl-CoA shown in Fig. 8, on the other hand, the carbonyl of acetyl-CoA and the methylene group of oxaloacetate are joined through a new C-C bond. It is probable that the reaction is initiated by abstraction of a proton from the methylene group of oxaloacetate forming its carbanion, which then attacks the carbonyl of acetyl-CoA. In this sense fraction II may belong to the enolase superfamily, the common initial step of the catalysis of which is the abstraction of the α-proton of a carboxylic acid (*38*).

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