**β-Ketoadipate Enol-lactone Hydrolases I and II from Acinetobacter calcoaceticus***

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β-Ketoadipate enol-lactone hydrolase catalyzes a common step in the utilization of protocatechuate and cis,cis-muconate by bacteria. Either of the two compounds elicits the synthesis of an enol-lactone hydrolase in Acinetobacter. The enol-lactone hydrolase that is induced by each compound was purified, and the properties of the proteins were compared. Both enzymes appear to be dimers with molecular weights of approximately 25,000. The amino acid compositions of the enzymes differ, and the two proteins do not cross-react serologically. The NH₂-terminal amino acid residue of the protocatechuate-induced enol-lactone hydrolase (ELH I) is methionine and the NH₂-terminal amino acid residue of the cis,cis-muconate-induced enol-lactone hydrolase (ELH II) is proline. Therefore, ELH I and ELH II appear to be the products of different structural genes.

The serological specificity of ELH I and ELH II made it possible to demonstrate the mutually independent regulation of their synthesis in wild type cells and in constitutive mutant strains. The synthesis of ELH I is not impaired in mutant strains that cannot synthesize ELH II.

The rapid characterization of mutant strains that produce ELH I or ELH II constitutively was made possible by the development of pH indicator enzyme assays that were performed with toluenized cells. cis,trans-Muconate, which does not support the growth of Acinetobacter, elicits the synthesis of the enzymes that normally are induced by cis,cis-muconate to 20% of fully induced levels.

**β-Ketoadipate enol-lactone hydrolase (ELH)** plays an essential role in the utilization of many aromatic growth substrates via the β-ketoadipate pathway in bacteria. The benzenoid growth substrates are metabolized to either protocatechuate or catechol (Fig. 1); a convergent series of reactions converts the diphenols to a common intermediate, β-ketoadipate enol-lactone, which is cleaved to β-ketoadipate by the hydrolase (Fig. 1). Like the other enzymes of the β-ketoadipate pathway, the hydrolase is inducible, and the mechanism of its induction varies among different groups of bacteria (5–12). In most Pseudomonas species the enzyme is induced by its product, β-ketoadipate, and the synthesis of the hydrolase is controlled coordinately with that of carboxymuconate-lactonizing enzyme and carboxymuconolactone decarboxylase (4, 8, 10, 11). In representatives of Acinetobacter calcoaceticus either of two metabolites can induce the synthesis of β-ketoadipate enol-lactone hydrolase (Fig. 1). One activity, termed ELH I, is elicited by protocatechuate and is regulated coordinately with the synthesis of the other enzymes that participate in the conversion of protocatechuate to β-ketoadipyl-CoA. The other activity, termed ELH II, is induced by cis,cis-muconate in coordination with the enzymes that convert cis,cis-muconate to β-ketoadipyl-CoA (4–6, 13–15).

The existence of two inducers for β-ketoadipate enol-lactone hydrolase and the coordinate control of β-ketoadipate enol-lactone hydrolase activity with two different regulatory units of enzyme synthesis strongly suggested that there might be two structural genes for β-ketoadipate enol-lactone hydrolase in A. calcoaceticus and that each structural gene may be under separate regulatory control (5, 6). Further evidence indicating independent regulation of the synthesis of two β-ketoadipate enol-lactone hydrolase proteins came from the isolation of constitutive mutant strains of A. calcoaceticus (13, 15). One mutant class constitutively formed β-ketoadipate enol-lactone hydrolase along with other enzymes of the protocatechuate pathway (15). These organisms invariably lacked protocatechuate oxygenase, and thus it appeared that the constitutive enzyme synthesis in these strains was the consequence of the endogenous accumulation of the inducer protocatechuate. Mutant strains that were constitutive for β-ketoadipate enol-lactone hydrolase while forming a catalytically active protocatechuate oxygenase were not isolated. A second class of mutant strains constitutively formed β-ketoadipate enol-lactone hydrolase as well as the other enzymes that are induced by cis,cis-muconate in Acinetobacter (13). A third mutant class remained inducible for cis,cis-muconate-lactonizing enzyme.

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'The abbreviations used are: ELH, β-ketoadipate enol-lactone hydrolase; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
but synthesized \( \beta \)-ketoadipate enol-lactone hydrolase and the other enzymes that are induced by cis,cis-muconate at constitutive levels.

The first studies of the physical properties of \( \beta \)-ketoadipate enol-lactone hydrolase in *A. calcoaceticus* suggested that ELH I and ELH II differ substantially and, therefore, supported the view that they are the products of different structural genes (5). Filtration of crude extracts through columns of Sephadex G-200 indicated that the molecular weight of ELH I is 18,500, whereas that of ELH II is 82,600 (5). Moreover, the two \( \beta \)-ketoadipate enol-lactone hydrolase activities differed markedly in their sensitivity to thermal inactivation; ELH I in crude extracts was stable to heating to 47\(^\circ\), but ELH II lost more than one half of its activity after 5 min at that temperature (5).

The apparent differences in the physical properties of the two \( \beta \)-ketoadipate enol-lactone hydrolase proteins were not evident after they had been purified. Katagiri and Wheelis (16) showed that gel filtration of partially purified preparations of ELH I and ELH II gave molecular weights of 24,000 and 21,000, respectively. Furthermore, ELH II was shown not to be intrinsically thermostable but rather to be rendered sensitive to thermal inactivation by a component of crude extract (16). Katagiri and Wheelis (16) concluded that the possibility that the two enzymes were the products of a single structural gene could not be excluded on the basis of the physical evidence.

Subsequently, Wheelis and Katagiri\(^2\) prepared antisera against ELH I and showed that the antisera failed to cross-react with ELH II. The absence of shared serological determinants supported the notion that the two proteins are the products of different structural genes.

In this report we describe physical, chemical, and immunological studies that strongly favor the conclusion that the primary structures of ELH I and ELH II are coded by separate structural genes that are regulated independently.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains and Their Growth*—All of the experiments described in this report were conducted with mutant organisms derived from Juné’s (17) transformable *Acinetobacter calcoaceticus* strain BD 413 (17); this wild type organism bears the designation ADP1 in our collection. The properties of the mutant strains and the method of their selection are summarized in Tables I and II.

Strain ADP99, initially isolated because of its rough colony morphology on succinate agar plates, produces considerably less polysaccharide than the wild type organism and hence is relatively amenable to the operations involved in enzyme purification. Large cultures of strain ADP99 were grown at 30\(^\circ\) in a New Brunswick Fermacell CF-130 fermentor. Defined mineral growth medium (3) was supplemented with \( p \)-hydroxybenzoate in order to induce ELH I or with benzate in order to induce ELH II. The concentration of the carbon source was

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\(^2\) Unpublished observations.
maintained between 5 and 10 mM during growth. Cells were harvested in a refrigerated Sharples super centrifuge and stored at -20° until used for enzyme purification.

Small cultures were grown at 37° in 500-ml growth flasks containing 150 ml of the growth medium; constant aeration was provided by a Gyratory New Brunswick environmental incubator shaker. Carbon sources were prepared as 0.5 or 1.0 M stock solutions, filter sterilized, and added separately to the growth flasks to give a final concentration of 5 mM. Solidified medium was supplemented with 1% Ionagar (Oxoid). The small cultures were harvested by centrifugation at 3000 × g at 5°, washed twice with 25 ml of 20 mM Tris-HCl buffer, pH 8.0, containing 2 mM MgCl₂ and 1 mM EDTA, resuspended in 2 ml of the same buffer, and stored at -20° until extracts were prepared. Organisms in the 2-ml preparations were disrupted by 15 s of ultrasonication using a Branson Electronics Ultrasonifier equipped with a 1.2-cm Heat Systems Electronics probe.

Mutant strains ADP17, ADP23, and ADP27 (Table I) are spontaneous mutant strains that were selected on the basis of their inability to utilize cis-cis-muconate as a growth substrate (18). They proved to be genetically unstable, and secondary mutant strains that could utilize cis-cis-muconate rapidly overgrew the original mutant strains when cis-cis-muconate was added as an inducing substrate to growth media. cis,trans-Muconate does not serve as a growth substrate for the wild type strain but it does induce the enzymes of the catechol pathway to 20% of the level found in fully induced cells. Therefore, enzyme levels in the mutant strains were determined after growth with 10 mM glucose supplemented with 10 mM cis,trans-muconate because this combination of growth substrates does not place the mutant organisms at a selective disadvantage.

Growth with β-ketoadipate requires genetic expression of β-ketoadipate-succinyl-CoA transferase. Wild type cultures of A. calcoaceticus cannot grow at the expense of β-ketoadipate because it is not an inducer of transferase. Using β-ketoadipate as a selective growth substrate, Canovas and his associates (5, 13, 15) isolated a large set of mutant strains that synthesize transferase constitutively. Physiological examination revealed that the phenotypic acquisition of the ability to grow with β-ketoadipate could result from any one of a number of different regulatory mutations. Many of the transferase constitutive mutant strains also synthesized β-ketoadipate enol-lactone hydrolase constitutively; those that did not were constitutive for a transferase that was induced by growth with adipate in wild type cells and that appeared to use adipate rather than β-ketoadipate as a natural substrate (13). The regulatory mutant strains that did form β-ketoadipate enol-lactone hydrolase in the absence of inducer fell into three classes defined on the basis of the additional enzymes that were produced constitutively (5, 13, 15).

In the present study the characterization of mutant strains that grew with β-ketoadipate was greatly facilitated by the development of pH indicator assays, described below, that permit the semiquantitative estimation of the levels of cis,cis-muconate lactonizing enzyme, muconolactone isomerase, and β-ketoadipate enol-lactone hydrolase in toluenized cells. As summarized in Table II, application of the indicator assays to organisms selected for their ability to grow with β-ketoadipate permitted their rapid classification. Representatives of each mutant class described by Canovas et al. (5, 13, 15) were isolated; these are the organisms that represent Classes A, B, C, and E in Table II. In addition, a new class of mutant strains (Class D in Table II) that produces constitutively all of the enzymes of the protocatechuate pathway was identified.

**Selection of Transferase Constitutive Mutant Strains**—Isolated colonies of strain ADP1 were picked from succinate agar plates and placed in growth tubes containing 5 ml of growth medium supplemented with 10 mM glucose and 2 mM β-ketoadipate. The cultures became turbid after 12 hours of shaking at 37°. After 5 days a broth from each culture was transferred to a growth tube containing growth

| Constitutive mutant class | Strain | Properties determined with whole or toluenized cells | Properties determined with cell-free extracts |
|---------------------------|--------|------------------------------------------------------|---------------------------------|
|                           |        | Constitutive production of | Growth with p-hydroxybenzoate | Synthesis of PO* | Enzymes produced constitutivelya* |
|                           |        | MLE* | MI* | ELH* | + | + | CO, MLE, MI, ELH II, TR II |
|                           |        | + | + | + | + | + | CO, MI, ELH II, TR II |
| A | ADP96 | + | + | + | + | + | CMLE, CMD, ELH I, TR I |
| B | ADP98 | + | + | + | + | + | CMLE, CMD, ELH I, TR I |
| C | ADP6 | + | + | + | + | + | CMLE, CMD, ELH I, TR I |
| D | ADP87 | + | + | + | + | + | CMLE, CMD, ELH I, TR I |
| E* | ADP88 | + | + | + | + | + | CMLE, CMD, ELH I, TR I |

*Enzymes (see Fig. 1) are represented by the following abbreviations: PO, protocatechuate oxygenase; CMLE, β-carboxy-cis,cis-muconate-lactonizing enzyme; CMD, γ-carboxymuconolactone decarboxylase; ELH, β-ketoadipate enol-lactone hydrolase; TR, β-ketoadipate-succinyl-CoA transferase; CO, catechol oxygenase; MLE, cis,cis-muconate-lactonizing enzyme; MI, muconolactone isomerase.

*The identities of ELH I and ELH II were established by the serological methods described in this paper. The identities of TR I and TR II rest primarily on physiological criteria (5, 6, 13, 15) and must be regarded as tentative.

*Representatives of Class E were assumed to be constitutive for adipate-succinyl-CoA transferase (TR III, 13) and were eliminated after preliminary characterization of toluenized cells.
overnight; a loopful from each freshly grown culture was transferred to a tube containing 2 mm β-ketoadipate medium. Growth in this medium was always rapid, and after it occurred single colonies were isolated from the cultures on 10 mm succinate agar plates. A colony from each culture was picked and examined for the ability of the cells to grow on a 5 mm p-hydroxybenzoate agar plate. Cells from the colonies also were allowed to grow to stationary phase in 5 ml cultures containing 10 mm glucose. These cells were harvested by centrifugation for 10 min at 3000 g, washed twice with 0.85% NaCl, resuspended in 0.4 ml of 0.85% NaCl, and examined for the constitutive production of cis,cis-muconolactone-lactonizing enzyme, muconolactone isomerase, and β-ketoadipate enol-lactone hydrolase by the pH indicator assays described below.

Semiquantitative pH Indicator Assays for cis,cis-Muconate-lactonizing Enzyme, Muconolactone Isomerase, and β-Ketoadipate Enol-lactone Hydrolase in Toluenized Cells—The lactonization of cis,cis-muconate is accompanied by the uptake of a proton (Fig. 1), and consequently the activity of cis,cis-muconate-lactonizing enzyme can be monitored visually in the presence of cis,cis-muconate and phenol red. A solution containing the substrate and the indicator was adjusted to pH 5.5; in the presence of the enzyme the indicator solution changed from yellow to red. A drop of toluene was mixed with 1 ml of the cis,cis-muconate-lactonizing enzyme indicator assay mixture of Wu et al. (19) and 0.1 ml of a suspension of glucose-grown cells in saline was added. Cells that produced the lactonizing enzyme constitutively turned the indicator from yellow to red within 1 hour.

The interconversion of (+)-muconolactone and β-ketoadipate enol-lactone does not lead to a change in the pH of unbuffered medium, but the hydrolysis of the enol-lactone is accompanied by the release of a proton. The activity of muconolactone isomerase can be detected with the solution containing phenol red, (+)-muconolactone, and β-ketoadipate enol-lactone hydrolase. The solution is buffered weakly with EDTA which inhibits the acid-releasing conversion of (+)-muconolactone to cis,cis-muconate by the lactonizing enzyme. The indicator assay mix contained the following in a volume of 1.0 ml: 4 μmol of (+)-muconolactone, 0.2 units of β-ketoadipate enol-lactone hydrolase, 1.0 μmol of phenol red, 10 μmol of EDTA, and 20 μl of 0.1 M NH₄OH, a drop of toluene was mixed with the solution, and 0.1 ml of cell suspension was added. Uninduced wild type cells did not turn the solution from red to yellow in 2 hours; similar concentrations of cells that produced muconolactone isomerase constitutively turned the solution yellow within 20 min.

The activity of β-ketoadipate enol-lactone hydrolase was determined in a solution containing phenol red and (+)-muconolactone; muconolactone isomerase was added to form β-ketoadipate enol-lactone from the (+)-muconolactone. In a final volume of 1.0 ml the β-ketoadipate enol-lactone hydrolase indicator assay mix contained 4 μmol of (+)-muconolactone, 0.2 units of muconolactone isomerase, 1.0 μmol of EDTA, and 20 μg of phenol red. The assay was conducted in the same manner as the muconolactone isomerase indicator assay. Cells that were constitutive for β-ketoadipate enol-lactone hydrolase turned the solution yellow within 20 min after the start of the assay.

Enzyme Assays—Published spectrophotometric assays were used to determine the activities of cis,cis-muconate-lactonizing enzyme (20), muconolactone isomerase (20) β-carboxy-cis,cis-muconate-lactonizing enzyme (2), γ-carboxymuconolactone decarboxylase (2), β-ketoadipate enol-lactone hydrolase (2), and β-ketoadipate-succinyl CoA transferase (6). A unit of activity is defined as the amount of cell-free that removes 1.0 μmol of substrate per min under standard assay conditions. Protein concentrations were determined by the method of Lowry et al. (21).

Chemicals—Dimethyl sulfoxide, dithiothreitol, and thioglycolic acid were obtained from Sigma Chemical Co. Acrylamide, bisacrylamide, and ammonium persulfate were purchased from Bio-Rad Laboratories, Fuji Film Gel A-1.5 (100 to 200 mesh) and Bio-Gel P-100 (100 to 200 mesh) were obtained from Bio-Rad Laboratories. Dansyl chloride was purchased from Nutritional Biochemicals Corp. N-Methyl-N-nitro-N-nitrosoguanidine was obtained from Aldrich Chemical Corp. Enzymatic syntheses were used for the preparation of (+)-muconolactone (3) and β-carboxy-cis,cis-muconate (22). cis,cis-Muconate was synthesized by the persacetic acid oxidation of phenol (23). The procedure of Riegel and Lilienfeld (24) was used to prepare β-ketoadipic acid.

Coupled enzyme assays were performed with preparations of muconolactone isomerase (25) and β-ketoadipate enol-lactone hydrolase (2) that had been purified from extracts of Pseudomonas putida by published procedures.

Purification of ELH I from Extracts of p-Hydroxybenzoate-grown Cells—Except during heat treatment extracts were maintained at 5°C. Cells were harvested by centrifugation of DEAE-cellulose chromatography were similar to those described previously (1), except that protein was eluted from the DEAE-cellulose column with 6 liters of Buffer A (10 mM ethylene diamine dihydrochloride, 1 mM MnCl₂, adjusted to pH 7.4 with NaOH) containing NaCl in a linear gradient running from 0.035 to 0.35 M. The results of these and subsequent purification steps are summarized in Table III. β-Ketoadipate enol-lactone hydrolase co-eluted from the DEAE-cellulose column in fractions containing between 0.16 and 0.21 M NaCl; the column completely separated the enzymes from β-carboxy-cis,cis-muconate-lactonizing enzyme which was eluted in fractions containing between 0.12 and 0.16 M NaCl. The DEAE-cellulose eluate containing β-ketoadipate enol-lactone hydrolase activity (Step 3, Table III) was fractionated with ammonium sulfate by methods that have been described before (1). Extracts were brought to 50% of saturation with respect to ammonium sulfate by the addition of 291 g of the salt per liter of extract. Precipitated protein was removed by centrifugation and 125 g of ammonium sulfate were added per liter of supernatant liquid to bring it to 70% of saturation. Material precipitating between 50 and 70% of saturation with respect to ammonium sulfate was collected by centrifugation and dissolved in Buffer B (50 mM sodium phosphate, pH 7.8, and 100 μM dithiothreitol) (Step 4, Table III). This preparation was maintained at 45° for 30 min, cooled to 5°, and centrifuged at 35,000 × g for 15 min. The supernatant liquid (Step 5, Table III) was fractionated with ammonium sulfate; material precipitating between 50 and 70% of saturation with respect to ammonium sulfate was collected by centrifugation and dissolved in Buffer B (50 mM sodium phosphate, pH 7.8, and 100 μM dithiothreitol) (Step 6, Table III). This solution was passed through a Bio-Gel agarose A-1.5 column (2.5 × 100 cm) that had been equilibrated with Buffer B; the flow rate was maintained at 30 ml/hour, and 7-ml fractions were collected. β-Ketoadipate enol lactone hydrolase was eluted from the column between 490 and 540 ml of the eluate and was completely separated from γ-carboxymuconolactone decarboxylase by this step. Fractions containing the β-ketoadipate enol-lactone hydrolase activity (Step 7, Table III) were pooled, precipitated by

### Table III

| Step | Volume | Total Activity | Total Protein | Specific Activity | Recovery | Purification |
|------|--------|----------------|--------------|-----------------|---------|--------------|
| 1. Crude extract | 3600 ml | 45 units | 21.6 units/mg | 2.0 | 100 | 1.0 |
| 2. Dialed extract | 1100 ml | 41 | 20.5 | 2.0 | 95 | 1.0 |
| 3. First DEAE-cellulose eluate | 1000 ml | 34 | 8.4 | 4.0 | 90 | 2.0 |
| 4. First 50 to 70% saturated ammonium sulfate fraction | 100 ml | 27 | 3.0 | 9.0 | 63 | 4.0 |
| 5. Heat-treated extract | 1000 ml | 30 | 2.0 | 30 | 60 | 6.0 |
| 6. Second 50 to 70% saturated ammonium sulfate fraction | 80 ml | 90 | 0.73 | 27 | 49 | 13.5 |
| 7. Bio-Gel agarose eluate | 65 ml | 16 | 0.16 | 100 | 37 | 50 |
| 8. 0 to 70% saturated ammonium sulfate fraction | 4.0 | 14 | 0.14 | 100 | 30 | 5.0 |
| 9. Bio-Gel P-100 eluate | 26 ml | 11 | 0.08 | 90 | 3 | 13.0 |
| 10. Second DEAE-cellulose eluate | 14 ml | 8.0 | 0.0138 | 580 | 18 | 290 |
addition of ammonium sulfate to 70% of saturation, and dissolved in Buffer B (Step 8, Table III). The sample was passed through a Bio-Gel P-100 column (1.6 x 70 cm) that had been equilibrated with Buffer B; the flow rate was maintained at 30 ml/hour and 2.5-ml fractions were collected. Material eluting between 100 and 115 ml (Step 9, Table II) was equilibrated against Buffer C (5 mM sodium phosphate, pH 6.8, containing 100 μM di thiothreitol and 0.07 M NaCl) and applied to a DEAE-cellulose column (0.9 x 30 cm) that had been equilibrated with this buffer. The enzyme was eluted from the column with the same buffer containing NaCl in a linear gradient from 0.07 to 0.16 M over 500 ml; fractions of 3 ml were collected. α-Ketoadipate enol-lactone hydrolyase activity was eluted with a single peak of protein (Fig. 2); the specific activity of the enzyme was constant across the peak. Fractions containing the enzyme (Step 10, Table III) were pooled and precipitated by addition of ammonium sulfate to 70% of saturation. The precipitated protein was dissolved in Buffer B and stored at 5° in Buffer B containing ammonium sulfate at 40% of saturation.

**Purification of ELH II from Extracts of Benzoate-Grown Cells**

Purification procedures for ELH II are summarized in Table IV. The first seven steps were identical with those described for ELH I, except that the enzyme was precipitated from solutions containing ammonium sulfate between 45% of saturation (238 g/liter) and 65% of saturation (obtained by adding 123 g of ammonium sulfate to each liter of a 45% saturated ammonium sulfate solution). cis,cis-Muconate-lactonizing enzyme was completely separated from the β-ketoacidate enol-lactone hydrolase by the first DEAE-cellulose chromatography step; muconolactone isomerase eluted from the DEAE-cellulose column with the β-ketoacidate enol-lactone hydrolase by the first DEAE-cellulose chromatography step; muconolactone isomerase eluted from the DEAE-cellulose column with the β-ketoacidate enol-lactone hydrolase, the isomerase was separated by its precipitation from solutions containing ammonium sulfate at 45% of saturation. Eluate from the Bio-Gel agarose A-1.5 column was equilibrated against Buffer C and subjected to a second step of DEAE-cellulose chromatography by the procedure described for ELH I. Fractions from the second DEAE-cellulose column that contained ELH II were pooled and the protein was precipitated by the addition of ammonium sulfate to 65% of saturation. The precipitated protein was dissolved in Buffer B and ammonium sulfate was added to a final concentration of 40% of saturation (226 g/liter). After 1 month at 5° the preparation contained some needle-like crystals accompanied by a substantial amount of amorphous material.

**Immunological Techniques—Antibodies against ELH I and ELH II** were prepared by published procedures (25). The method of Stanier et al. (26) was used for the detection of serological cross-reaction on Ouchterlony double diffusion plates (27).

Normal rabbit antiserum inhibits β-ketoacidate enol-lactone hydrolyase activity. Therefore, the immunoglobulin fraction was separated from immune and normal rabbit serum by successive sodium sulfate fractionation (28) and was used for inhibition studies. Varying amounts of the immunoglobulin fraction against ELH I or ELH II were incubated with 0.15 unit of the enzyme in the assay mixture (lacking substrate) for 15 min. The enzyme assay then was initiated by adding the substrate to the incubation mixture. The immunoglobulin fraction from rabbits that had not been immunized did not inhibit β-ketoacidate enol-lactone hydrolase activity.

**Acrylamide Gel Electrophoresis**—Vertical gel electrophoresis (29) in 7% polyacrylamide gel was performed as described previously (1). Samples containing 50 or 100 μg of protein, 30% sucrose, and 5 μl of 0.05% bromphenol blue in a total volume of 0.1 ml were applied to each slot. A constant current of 85 ma was applied for 2 hours. The gels were stained with 0.1% Amido black in 7% acetic acid and destained with 7% acetic acid.

The molecular weights of β-ketoacidate enol-lactone hydrolyase subunits were estimated by sodium dodecyl sulfate gel electrophoresis (30) using previously described methods (1).

**Amino Acid Analysis**—Amino acid compositions were determined with a Beckman 120B amino acid analyzer by published procedures (31). The half-cystine content was determined as cysteic acid on the amino acid analyzer after 24 hours hydrolysis in the presence of 2% dimethyl sulfoxide (32). The amount of half-cystine in the enzymes also was measured as S-carboxymethylcysteine after the proteins were reduced and treated with iodoacetate (33). The tryptophan content of the enzymes was determined after hydrolysis in the presence of thioglycolic acid (34).

**NH₂-terminal Residues**—The NH₂-terminal amino acid residue of the β-ketoacidate enol-lactone hydrolase proteins were identified as their dansyl derivatives by the procedure of Weiner et al. (35).

### RESULTS

**Physical and Chemical Properties of Purified Enzymes**

**Purity of Enzymes** The specific activities of the most highly purified preparations of ELH I and ELH II were roughly similar: the second DEAE-cellulose eluate of ELH I (Step 10, Table III) possessed a specific activity of 580 units/mg of protein and the specific activity of crystalline ELH II (Step 9, Table IV) was 500 units/mg of protein. These enzyme preparations were used in all subsequent studies. Efforts to crystallize ELH I by the procedures used to crystallize ELH II were unsuccessful.

Each of the purified enzyme preparations migrated as a

### Table IV

| Step | Volume | Total activity | Total protein | Specific activity | Recovery | Purification |
|------|--------|----------------|---------------|------------------|----------|--------------|
| 1. Crude extract | 2.520 | 37.8 | 42.8 | 0.90 | 100 | 1.0 |
| 2. Dialyzed extract | 3.100 | 37.2 | 31.0 | 1.2 | 99 | 1.3 |
| 3. First DEAE-cellulose eluate | 1.550 | 32.5 | 7.7 | 4.2 | 87 | 4.7 |
| 4. First 45 to 65% saturated ammonium sulfate fraction | 1.00 | 25.2 | 2.52 | 10 | 67 | 11 |
| 5. Second 45 to 65% saturated ammonium sulfate fraction | 1.135 | 25.0 | 2.1 | 12 | 67 | 13 |
| 6. Second 45 to 65% saturated ammonium sulfate fraction | 0.89 | 18.0 | 0.77 | 25.4 | 48 | 26 |
| 7. Bio-Gel agarose eluate | 0.60 | 15.0 | 0.065 | 230 | 38 | 260 |
| 8. Second DEAE-cellulose eluate | 0.16 | 10.0 | 0.020 | 500 | 26 | 550 |
| 9. Crystallization | 0.4 | 5.0 | 0.010 | 500 | 13 | 550 |
bands. The most strongly stained band corresponded to a molecular weight of 12,000 and two fainter bands corresponded to a molecular weight of 25,000. The molecular weight of ELH II was estimated to be 26,000 by Robley C. Williams, Jr., 2 using meniscus depletion at sedimentation equilibrium in an ultra-centrifuge.

Molecular Weight and Subunit Molecular Weight Determinations—ELH I and ELH II eluted in the same fraction after filtration on a Bio-Gel agarose A-1.5 column. As shown in Fig. 4, the elution volumes of the enzymes corresponded to a molecular weight of 25,000. The molecular weight of ELH II was estimated to be 26,000 by Robley C. Williams, Jr., 3 using meniscus depletion at sedimentation equilibrium in an ultracentrifuge.

Sodium dodecyl sulfate gel electrophoresis of either β-ketoadipate enol-lactone hydrolase yielded three protein bands. The most strongly stained band corresponded to a molecular weight of 12,000 and two fainter bands corresponded to molecular weights of 25,000 and 52,000 (Fig. 5). Therefore, it appears that the β-ketoadipate enol-lactone hydrolase exist primarily as dimers, with molecular weights of approximately 25,000 but that the proteins may associate to form higher oligomers.

Amino Acid Compositions—the amino acid compositions of the two β-ketoadipate enol-lactone hydrolases are shown in Table V. Although the amino acid compositions generally are similar, there are some notable differences: ELH I subunits appear to have 3 more lysine and 4 more aspartyl residues than are present in subunits of ELH II, and ELH II subunits appear to contain 2 more phenylalanine residues than are present in subunits of ELH I.

NH₂-terminal Amino Acids—Dansylation followed by hydrolysis revealed that the NH₂-terminal amino acid residues of ELH I and ELH II are methionine and proline, respectively.

Serological Properties of Enzymes

Studies with Purified Enzymes—Antibodies prepared against ELH I formed a precipitin band with this enzyme but not with ELH II. Similarly, antisera prepared against ELH II gave rise to precipitin bands with ELH II but not with ELH I. The independent interaction of each enzyme with antisera prepared against it is shown in Fig. 6. For this photograph both antibody preparations were placed in the center well of the Ouchterlony plate; the spurs formed between the wells containing ELH I and the wells containing ELH II show that each protein contains different antigenic determinants. Differences in the serological properties of ELH I and ELH II also were revealed by analysis of the inhibition of their activity by the immunoglobulin fraction of antisera prepared against them (Fig. 7). As shown on the left side of Fig. 7, the activity of ELH II was not influenced by concentrations of the anti-ELH I immunoglobulin fraction that inhibited completely the activity of ELH I. The reciprocal experiment, depicted on the right side of Fig. 7, revealed that concentrations of the anti-ELH II immunoglobulin fraction that inhibited completely ELH II activity did not inhibit the activity of ELH I.

Serological Characterization of Cross-reacting Material against ELH I and ELH II in Extracts of Wild Type and Mutant Cells—The specificity of induction of ELH I and ELH II in wild type cells was demonstrated by analysis of the cross-reacting material formed by the organisms during growth with succinate, p-hydroxybenzoate, or benzoate. Extracts of succinate-grown cells did not contain significant β-ketoadipate enol-lactone hydrolase activity and did not form precipitin bands with antisera prepared against either ELH I or ELH II. Extracts of cells in which the enzymes of the protocatechuate pathway had been induced by growth with p-hydroxybenzoate contained material that cross-reacted with ELH I but not with ELH II. Extracts of benzoate-grown cells, which contained the enzymes of the catechol pathway, formed a precipitin band with antisera prepared against ELH II but not with antisera prepared against ELH I.

Further evidence for the independent genetic control of the synthesis of ELH I and ELH II came from the serological identification of the proteins formed by constitutive mutant strains during growth with succinate. As shown in Fig. 8, strains ADP96, ADP86, and ADP98 which produce constitutively the enzymes of the protocatechuate pathway also form cross-reacting material against ELH I in the absence of an exogenous inducer. Uninduced cultures of strains ADP98 and
Fig. 4. Molecular weight estimations of ELH I and ELH II on a Bio-Gel agarose A-1.5 column.

Fig. 5. Sodium dodecyl sulfate gel electrophoresis of ELH I and ELH II.

ADP98 which are constitutive for enzymes of the catechol pathway do not form ELH I cross-reacting material (Fig. 8) and do form ELH II cross-reacting material (Fig. 9). ELH II cross-reacting material was not found in the extracts of the mutant strains (ADP6, ADP86, and ADP88) that formed constitutively enzymes of the protocatechuate pathway (Fig. 9). Thus, the synthesis of ELH I appears to coincide precisely with the expression of genes for the protocatechuate pathway and the formation of ELH II is correlated strictly with the expression of genes for the catechol enzymes.

Growth in the presence of the inducer analog cis,trans-muconate elicits the synthesis of material that cross reacts with ELH II in the wild type strain ADP1 (Fig. 10). Mutant strains ADP17, ADP23, and ADP27 form ELH II and β-ketoadipate-succinyl CoA transferase at less than 10% of wild type levels when induced with cis,trans-muconate; the synthesis of other enzymes of the catechol pathway (catechol oxygenase, cis,cis-muconate-lactonizing enzyme, and muconolactone isomerase) appears to be unimpaired in these mutant strains. As shown in Fig. 10, the mutant strains do not form cross-reacting material for ELH II when induced with cis,trans-muconate. Thus, the mutations in these organisms appear to prevent the expression of the structural gene for ELH II. That these mutations do not impair the synthesis of ELH I is shown by the cross-reactions depicted in Fig. 11: p-hydroxybenzoate-grown (protocatechuate-induced) cultures of the three mutant strains contain material that forms a precipitin band with antisera prepared against ELH I. Therefore, mutations that render the ELH II structural gene dysfunctional do not appear to interfere with the expression of the ELH I structural gene.

Table V
Amino acid composition of enol-lactone hydrolases: amino acid residues per 12,000 daltons

| Amino acid                  | ELH I | ELH II |
|-----------------------------|-------|--------|
| Lysine                      | 5.8   | 3.1    |
| Histidine                   | 1.8   | 3.0    |
| Arginine                    | 3.0   | 3.3    |
| Aspartic acid               | 14.2  | 10.4   |
| Threonine                   | 5.7   | 6.3    |
| Serine                      | 5.9   | 5.1    |
| Glutamic acid*              | 13.8  | 14.0   |
| Proline                     | 3.9   | 4.0    |
| Glycine                     | 8.0   | 8.2    |
| Alanine                     | 12.0  | 13.3   |
| Cysteine                    | 1.5   | 1.6    |
| Valine                      | 5.7   | 6.2    |
| Methionine                  | 2.0   | 2.0    |
| Isoleucine                  | 6.0   | 6.3    |
| Leucine                     | 10.6  | 10.8   |
| Tyrosine                    | 2.5   | 3.0    |
| Phenylyalanine              | 3.7   | 5.7    |
| Tryptophan                  | 3.0   | 3.2    |

*The value represents the sum of the free acid and the amide.
DISCUSSION

The amino acid compositions of ELH I and ELH II differ by about 12 residues per subunit, corresponding to a minimum difference of 6 residues in the amino acid sequences of the proteins. Therefore the conclusion that the enzymes are the products of separate structural genes appears to be justified. Additional evidence showing that the proteins possess different structures comes from the failure of the enzymes to cross-react serologically and from the observation that the amino terminal amino acid of ELH I is methionine, whereas the amino terminal amino acid of ELH II is proline.

Herefore the inference that ELH I and ELH II are the products of separately regulated structural genes has rested almost entirely on measurements of the activity of the enzymes in crude extracts of wild type and mutant cells. The immunological specificity of the two proteins allowed them to be identified individually in crude extracts and thus permitted a more precise analysis of the factors that govern the synthesis of each enzyme. The serological evidence convincingly demonstrates that the syntheses of ELH I and ELH II are regulated independently and supports the conclusions drawn from earlier physiological studies (5, 6, 13-15). Cross-reacting material for ELH I but not for ELH II was found in extracts of p-hydroxybenzoate-grown wild type cells. Similarly, uninduced cultures of mutant strains that produce enzymes of the protocatechuate pathway constitutively formed material that cross-reacted with ELH I but not with ELH II. Therefore, the ELH activity associated with the expression of genes for the protocatechuate enzymes appears to be due primarily to ELH I. In contrast, the β-ketoadipate enol-lactone hydrolase activity that is elicited by the expression of genes for the catechol enzymes seems to be almost entirely attributable to ELH II. Material that cross-reacted with ELH II but not with ELH I was found in extracts of wild type cells in which enzymes of the catechol pathway had been induced. In addition, uninduced cultures of mutant strains that form enzymes of the catechol pathway constitutively contained material that cross-reacted with ELH II but not with ELH I.

Additional evidence for the independent control of the expression of the structural genes for ELH I and ELH II was provided by the properties of strains ADP17, ADP23, and ADP27. These organisms differ from the wild type in that they do not synthesize ELH II when grown in the presence of cis,trans-muconate; they appear to have undergone mutations with pleiotropic effects because they also are impaired in their ability to form β-ketoadipate-succinyl-CoA transferase when induced with cis,trans-muconate. Whatever the nature of the mutations that prevent the synthesis of ELH II, they do not appear to impair the synthesis of ELH I when the mutant strains are grown with p-hydroxybenzoate.

The physical properties of the purified preparations of ELH I and ELH II are quite similar: the proteins appear to be dimers with a molecular weight of approximately 25,000. The presence of bands corresponding to proteins with molecular weights of 52,000 after sodium dodecyl sulfate gel electrophoresis (Fig. 5) suggests that under some circumstances the β-ketoadipate enol-lactone hydrolase may aggregate to form oligomers higher than dimers. Canovas and Stanier (5) found that the molecular weights of ELH I and ELH II corresponded to 18,500 and 82,500, respectively, when estimations were made by the filtration of extracts of appropriately induced Acinetobacter cultures through Sephadex gels. Our results obtained with the purified proteins more closely resemble those of Katagiri and Wheelis (16) who repeated the experiments of Canovas and Stanier (5) and found molecular weights corresponding to...
24,000 and 21,000 for ELH I and ELH II, respectively. Katagiri and Wheelis (16) suggested that the discrepancy between their results and those of Cánovas and Stanier (5) might be due to differences in the state of aggregation of the β-ketoadipate enol-lactone hydrolase proteins in crude extracts. The results from sodium dodecyl sulfate gel electrophoresis indicate that under some circumstances the β-ketoadipate enol-lactone hydrolase proteins may aggregate to form higher oligomers and thus lend support to the suggestion of Katagiri and Wheelis (16).

**FIG. 8 (top left).** Ouchterlony double diffusion plate. The center well received antisera prepared against ELH I. The well labeled ELH I received 20 μg (about 10 units) of ELH I; other wells received 0.2 ml containing approximately 5 mg of protein) of crude extract derived from uninduced (succinate-grown) cultures of the designated strains. The characteristics of the mutant strains are summarized in Table II. Strains ADP96 (Class A) and ADP98 (Class B) produce constitutively enzymes of the catechol pathway; strains ADP6 (Class C), ADP87 (Class C), and ADP88 (Class D) produce constitutively enzymes of the protocatechuic pathway.

**FIG. 9 (top right).** Ouchterlony double diffusion plate. The center well received antisera prepared against ELH II. The well labeled ELH II received 20 μg (about 10 units) of ELH II; other wells received 0.2 ml containing approximately 5 mg of protein) of crude extract derived from uninduced (succinate-grown) cultures of the designated strains. The characteristics of the mutant strains are summarized in Table II and in the legend to Fig. 11.

**FIG. 10 (bottom left).** Ouchterlony double diffusion plate. The center well contained antisera prepared against ELH II. The wells labeled ELH I and ELH II contained 20 μg of a purified preparation of the indicated enzyme. The other wells contained crude extract (approximately 5 mg of protein) derived from cultures that had been induced for the catechol enzymes by growth with succinate in the presence of cis,trans-muconate. The wild type strain (ADP1) differed from the mutant strains (ADP17, ADP23, and ADP27) in that it formed cross-reacting material for ELH II when induced with cis,trans-muconate.

**FIG. 11 (bottom right).** Ouchterlony double diffusion plate. The center well contained antisera prepared against ELH I. The well labeled ELH II contained 20 μg of the purified enzyme. The other wells contained crude extracts (approximately 5 mg of protein) of the indicated strains. The wild type cells (strain ADP1) were grown with benzoate and therefore contained ELH II but not ELH I. Strain ADP6 produces ELH I constitutively and was grown with succinate. Strains ADP17, ADP23, and ADP27 were grown with p-hydroxybenzoate to demonstrate that they form cross-reacting material for ELH I despite the genetic block that prevents them from forming ELH II (Fig. 10).
The physical similarity of ELH I and ELH II raises the possibility that the structural genes for the isofunctional enzymes may be evolutionarily homologous. Comparison of the physical structures of enzymes that catalyze pairs of analogous reactions in the β-ketoadipate pathway of Pseudomonas putida has suggested that some of the proteins may share common ancestral genes; cis,cis-muconate-lactonizing enzyme and β-carboxy-cis,cis-muconate-lactonizing enzyme (36) possess similar physical properties as do muconolactone isomerase and γ-carboxymuconolactone decarboxylase (37).

Members of some sets of enzymes that catalyze identical biochemical reactions in a single bacterium possess quite different structures. Detailed analysis of the aspartokinase-homoserine dehydrogenase proteins elaborated by Escherichia coli has revealed that they differ substantially in molecular weight and subunit size (38-40), and the three proteins do not cross-react serologically (41). One of the two 6-phosphoglucuronate dehydrogenases from Pseudomonas multivorans has a subunit size of 40,000 and the other has a subunit size of 60,000 (42). Like the aspartokinases of E. coli, the 6-phosphoglucuronate dehydrogenases of P. multivorans catalyze the same biochemical reactions, but differ in their sensitivity to allosteric effectors (43). Hence, structural differences in the proteins that permitted the binding of different modulators would have been favored by selective forces.

In contrast to the foregoing examples, control of the enzymes of the β-ketoadipate pathway appears to be exerted solely through the regulation of enzyme synthesis. The maintenance of two structural genes for β-ketoadipate enol-lactone hydrolase in A. calcoaceticus permits one inducer, protocatechuate, to elicit the expression of one of the genes and another inducer, cis,cis-muconate, to elicit the expression of the other gene. In a closely analogous situation, P. putida elaborates two physically similar α-ketoglutarate semialdehyde dehydrogenases; one of the enzymes is induced by hydroxyproline (43) and the other is induced by glucarate (44). Insofar as is known, the activity of the dehydrogenases is not modified by allosteric modulators (44). The two enzymes share similar kinetics properties, molecular sizes, subunit sizes, and amino acid compositions; the proteins differ in their serological properties and comparison of tryptic peptides revealed that the amino acid sequences of the proteins must differ significantly (44). Regulation of the two malate synthases of E. coli also appears to be exerted primarily at the level of protein synthesis; one of the enzymes is repressed by pyruvate (45-47) and the other is induced by glycolate (48). The two enzymes are physically similar but possess different amino terminal amino acids (49).

It is worth noting that selective forces may favor mutations that result in variations in the primary structure of isofunctional enzymes that are regulated differently only at the level of induction. The structural gene for each isofunctional enzyme is regulated by a control mechanism that also governs the expression of structural genes for a different set of enzymes. For example, the structural gene for ELH I is expressed coordinately with genes for enzymes of the protocatechuate pathway and the structural gene for ELH II is expressed coordinately with genes for enzymes of the catechol pathway. Studies with constitutive mutant strains that produce one of the isofunctional enzymes constitutively have shown that the structural genes for coordinately induced enzymes are governed by a common regulatory gene, and in those cases for which evidence is available it appears that the genes that are under unified inductive control are linked within operons (15, 46, 47).

Recombinational events (which have been observed in A. calcoaceticus (17, 50) and P. putida (19, 51-57) as well as in the malate synthase systems of E. coli (46, 47)) are favored at sites where nucleotide sequences are similar. The presence of identical copies of nucleotide sequences coding for structural genes within a single chromosome could lead to intrachromosomal recombinational events causing either physiologically ineffective induction mechanisms or the loss of genetic material. Indeed Jackson and Yanofsky (58) have shown that transcriptional-duplication mutant strains of E. coli that bear two identical copies of trp genes are genetically unstable and lose one of the copies with a frequency of 10^-2. Accordingly it seems likely that mutations introducing changes into the nucleotide sequence for a structural gene for one of a set of homologous isozymes within an organism may be selectively advantageous if the mutations do not cause amino acid substitutions that impair the activity of the enzyme.

Two developments described in this paper should facilitate the exploration of the genetics of the β-ketoadipate pathway in A. calcoaceticus. One of these is the identification of cis,trans-muconate, which does not serve as a growth substrate for A. calcoaceticus strain ADP1, as a gratuitous inducer of the enzymes of the catechol pathway. The second is the pH indicator assays that allow the semiquantitative determination of the levels of cis,cis-muconate-lactonizing enzyme, muconolactone isomerase, and β-ketoadipate enol-lactone hydrolase in toluenized cells (Table II). The pH indicator screening procedure led to the identification of constitutive mutant strains of a type that had not been described previously (Class D, Table II) and has permitted the rapid categorization of all mutant strains that grow with β-ketoadipate into five phenotypic classes. Mutant strains in Class B produce catechol oxygenase, muconolactone isomerase, β-ketoadipate enol-lactone hydrolase, and β-ketoadipate-succinyl-CoA transferase constitutively, but do not form cis,cis-muconate-lactonizing enzyme in the absence of an inducer. In first describing mutant strains of this phenotype, Cánovas and Johnson (13) suggested that the organisms had undergone a mutation in a regulator gene that is unlinked to the regulator gene that governs the expression of cis,cis-muconate-lactonizing enzyme and concluded that at least two unlinked regulator genes govern the synthesis of the four enzymes that convert cis,cis-muconate to β-ketoadipyl-CoA. Subsequently, Morse and Yanofsky (59) reported a mutation within a structural gene that initiates transcription in the tryptophan operon of E. coli. Therefore, it is possible that the structural genes for the four enzymes that convert cis,cis-muconate to β-ketoadipyl-CoA lie within an operon in A. calcoaceticus and that a mutation within the structural gene for cis,cis muconate lactonizing enzyme could cause the transcription of other structural genes in the operon. Consequently, the properties of mutant strains in Class B (Table II) cannot be taken as evidence for the conclusion that two unlinked regulatory genes govern the expression of the enzymes that convert cis,cis-muconate to β-ketoadipyl-CoA in wild type Acinetobacter cells.

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