Relationships among Enzymes of the β-Ketoadipate Pathway. I. Properties of *cis,cis*-Muconate-Lactonizing Enzyme and Muconolactone Isomerase from *Pseudomonas putida*†

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ABSTRACT: Procedures for the efficient purification and crystallization of *cis,cis*-muconate-lactonizing enzyme and muconolactone isomerase from extracts of benzoate grown cultures of *Pseudomonas putida* are described. The amino acid compositions of the proteins are presented. The subunit size of *cis,cis*-muconate-lactonizing enzyme is approximately 40,000 daltons; the amino terminus of this enzyme appears to be threonine. Muconolactone isomerase is composed of subunits of about 12,000 daltons; the amino terminus of the isomerase is methionine. The relationship of these findings to former serological, physiological, and genetic studies of the enzymes is described.

cis,cis-Muconate-lactonizing enzyme and muconolactone isomerase catalyze sequential reactions in the microbial dissimilation of *cis,cis*-muconate *via* the β-ketoadipate pathway (Ornston and Stanier, 1966) (Figure 1). The enzymes are inducible in bacteria and the comparative study of the regulation of their synthesis in different bacterial genera has been the subject of intense investigation over the past several years (Ornston, 1966c; Canovas and Stanier, 1967; Johnson and Stanier, 1971; Ornston and Ornston, 1972; Stanier and Ornston, 1973). The regulatory mechanisms are quite dissimilar, but generally have been conserved within well defined bacterial groups. These observations have raised the possibility that the β-ketoadipate pathway evolved independently in different bacterial genera (Canovas *et al.*, 1967; Stanier and Ornston, 1973).

Genetic studies have shown that *catB* and *catC* (the structural genes for *cis,cis*-muconate-lactonizing enzyme and muconolactone isomerase, respectively) are tightly linked in *Pseudomonas aeruginosa* (Kemp and Hegeman, 1968) and in *Pseudomonas putida* (Wheelis and Stanier, 1970; Wheelis and Ornston, 1972; Wu *et al.*, 1972). The two enzymes are under strictly coordinate biosynthetic control in these species (Ornston, 1966c; Kemp and Hegeman, 1968). Furthermore, *catR*, a tightly linked regulatory gene, is required for the expression of *catB* and *catC* in *P. putida* (Wheelis and Ornston, 1972). These observations have suggested that *catB* and *catC* might be members of a complex operon in *Pseudomonas*.

cis,cis-Muconate-lactonizing enzyme and muconolactone isomerase have been purified only from a single bacterial strain, *P. putida*, biotype A, strain 90 (Ornston, 1966b). A broad serological survey conducted with antisera prepared against the pure enzymes demonstrated that cross reactivity with the homologous enzymes from related *Pseudomonas* species varied over a wide range, but generally the degree of cross-reaction correlated with the taxonomic distance of the examined strains from *P. putida*, biotype A (Stanier *et al.*, 1970).

β-Carboxy-*cis,cis*-muconate-lactonizing enzyme and γ-carboxymuconolactone decarboxylase (Figure 1) are inducible enzymes that catalyze reactions chemically analogous to those mediated by *cis,cis*-muconate-lactonizing enzyme and muconolactone isomerase, respectively (Ornston, 1966a). As shown in Figure 1, the substrates of the former pair of enzymes differ from those of the latter pair only in that they bear a carboxyl group that is lost as γ-carboxymuconolactone gives rise to β-ketoadipate enol lactone, an intermediate that is common to both pathways. The chemical analogy between the two reactions of the β-carboxy-*cis,cis*-muconate sequence and the two reactions of the *cis,cis*-muconate pathway has suggested that the enzymes catalyzing analogous reactions are homologous (Ornston and Stanier, 1966). Nevertheless, the specificity of the enzymes from *P. putida* is marked: the two enzymes unique to the β-carboxy-*cis,cis*-muconate sequence do not act upon intermediates in the *cis,cis*-muconate pathway and *cis,cis*-muconate-lactonizing enzyme and muconolactone isomerase are unreactive with β-carboxy-*cis,cis*-muconate and γ-carboxymuconolactone (Ornston, 1966a,b). Furthermore, the analogous enzymes are immunologically remote: antisera prepared against *cis,cis*-muconate-lactonizing enzyme do not react with β-carboxy-*cis,cis*-muconate-lactonizing enzyme and antisera against muconolactone isomerase do not react with γ-carboxymuconolactone decarboxylase (Stanier *et al.*, 1970).

Direct comparison of the primary structures of the enzymes of the β-ketoadipate pathway should permit quantitative assessment of their evolutionary relatedness. The ease with which this goal can be achieved depends to a large extent upon the feasibility with which the purified enzymes can be obtained and upon the size of the polypeptide subunits of the proteins. Published purification procedures for the enzymes of the β-ketoadipate pathway are time consuming and provide a low yield of purified protein (Ornston, 1966b). In this paper we present improved methods that permit the direct isolation of several hundred milligrams of both pure *cis,cis*-muconate-lactonizing enzyme and pure muconolactone isomerase from crude extracts of *P. putida*. We also

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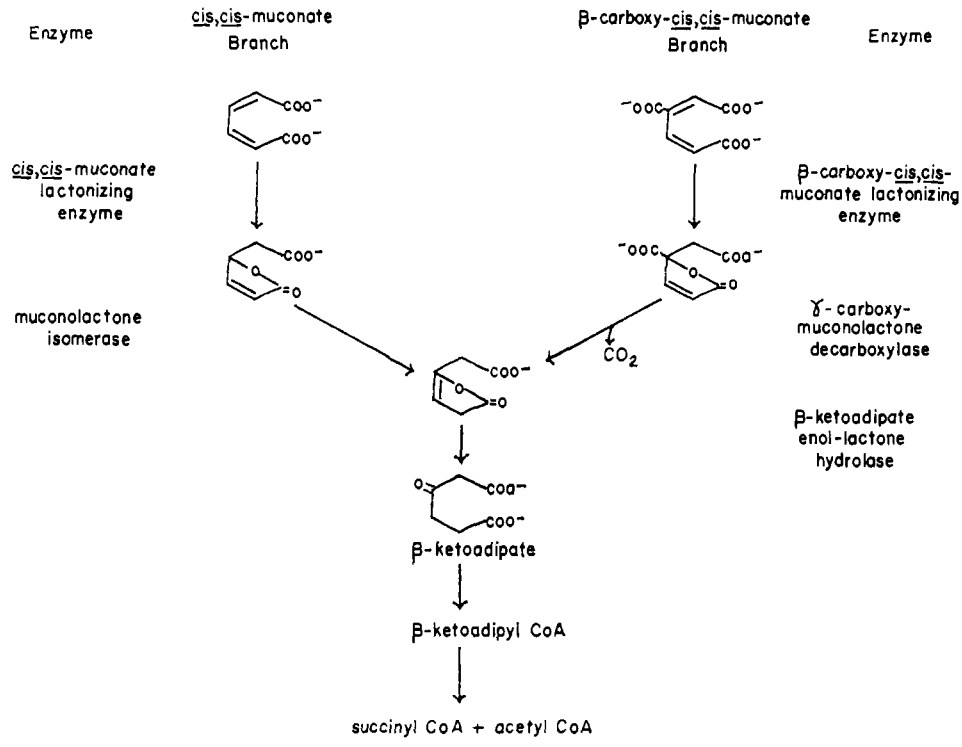

FIGURE 1: The β-ketoadipate pathway in bacteria.

describe some of the chemical and physical properties of the pure proteins. In the following paper (Patel *et al.*, 1973) we describe the purification of β-carboxy-*cis,cis*-muconate-lactonizing enzyme from extracts of *P. putida* and compare the physical and chemical properties of the two lactonizing enzymes. The third paper (Parke *et al.*, 1973) presents a procedure for the isolation of γ-carboxymuconolactone decarboxylase and compares some of the properties of this protein with those of muconolactone isomerase.

Experimental Section

Bacterial Strains. The bacterial strain employed was derived from *P. putida*, biotype A, strain 90 (Stanier *et al.*, 1966) (ATCC 12633). Large-scale growth of the wild-type strain with benzoate frequently led to lysis accompanied by the appearance of high titers (10^{11} plaque forming units (pfu)/ml) of a lytic bacteriophage designated *WOM*. Strain PRS2113 was selected as a *WOM*-resistant mutant of *P. putida* strain 90. Large-scale growth of strain PRS2113 was unimpeded by lysis and hence it was used as a source of enzyme preparations.

Growth of Cells. Cultures of strain PRS2113 were grown with aeration at 30° in defined mineral medium (Ornston and Stanier, 1966) supplemented with 10 mM sodium benzoate. The benzoate concentration was maintained between 2 and 10 mM during growth by periodic additions of a sterile 1.0 M sodium benzoate solution to the cultures; the pH was maintained between 6.5 and 7.5 by addition of sterile 1.0 M H_2SO_4. Six liters of benzoate-grown inoculum containing about 1 mg dry weight of cells/ml were grown in Fernbach flasks and added to 100 l. of growth medium in a New Brunswick Fermacell Model CF-130 fermentor. When the cultures in the fermentor reached a concentration of 1.5 mg dry weight of cells per milliliter, the cells were harvested in a Sharples refrigerated centrifuge. The packed cells were washed with 6 l. of buffer A (described below) before they were removed from the centrifuge and stored at −20° until used.

Buffers. Buffer A was 10 mM ethylenediamine dihydrochloride, 1 μM $MnCl_2$, adjusted to pH 7.3 with NaOH. Buffer B was 20 mM Tris-chloride–10 μM $MnCl_2$, at pH 7.4. The pH of the buffers was measured at 23°.

Chemicals. Schwarz-Mann provided *p*-chloromercuribenzoate, guanidine-HCl (Ultra Pure), and ammonium sulfate (Ultra Pure) for protein fractionation and crystallization. Whatman microgranular DE32 (DEAE) cellulose was precycled according to the manufacturer's instructions prior to use. Acrylamide, bisacrylamide, and ammonium persulfate were purchased from Canalco. Nutritional Biochemical Corp. provided 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) chloride.

Enzyme Assays. Published spectrophotometric assays (Ornston, 1966b) were used to determine the activities of *cis,cis*-muconate-lactonizing enzyme and muconolactone isomerase. A unit of activity is defined as the amount of enzyme that removes 1 μmol of substrate/min from the assay mixture under standard conditions (Ornston and Stanier, 1966). Protein concentrations were determined by the method of Lowry *et al.* (1951).

Separation of cis,cis-Muconate-Lactonizing Enzyme and Muconolactone Isomerase from Each Other. Prior to the separation of *cis,cis*-muconate-lactonizing enzyme and muconolactone isomerase from each other by DEAE-cellulose chromatography, the two enzymes were treated together in a single extract. The results of the procedure are summarized separately: the purification of the lactonizing enzyme in Table I and the purification of the isomerase in Table II.

Crude extracts were prepared from 800 to 900 g wet wt of frozen cells that were thawed in a 5° room overnight before extraction. The cells were suspended in 1 l. of buffer A, placed in an ice–water bath and stirred continuously with a

TABLE I: Purification of *cis,cis*-Muconate-Lactonizing Enzyme.

Step	Vol (ml)	Total Act. (Units × 10^{-3})	Total Protein (g)	Sp Act. (Units/mg)	Recov (%)	Purifcn (-fold)
1. Crude extract	3700	43.8	90.0	0.487	100	1.0
2. Dialysate	4000	52.0	85.0	0.612	118	1.25
3. DEAE-cellulose eluate	486	40.0	5.0	8.0	91	16.4
4. 30–40% saturated ammonium sulfate fraction	40	32.5	1.8	18.0	74	37.0
5. First crystallization	10.5	21.9	0.252	87.0	50	178.0
6. Second crystallization	10.0	20.0	0.222	90.0	46	182.0
7. Third crystallization	10.0	19.0	0.201	90.0	44	182.0

TABLE II: Purification of Muconolactone Isomerase.

Step	Vol (ml)	Total Act. (Units × 10^{-3})	Total Protein (g)	Sp Act. (Units/mg)	Recov (%)	Purifcn (-fold)
1. Crude extract	3700	243	90.0	2.70	100	1.0
2. Dialysate	4000	260	85.0	3.05	106	1.15
3. DEAE-cellulose eluate	1000	221	11.0	23.2	91	8.6
4. 50–60% saturated ammonium sulfate fraction	74	215	4.1	52.4	88	19.4
5. First crystallization	10	213	0.350	609.0	87	225.0
6. Second crystallization	6	208	0.240	860.0	85	318.0
7. Third crystallization	5	180	0.212	856.0	74	317.0

magnetic stirrer. The cells were disrupted by 45 min of sonication with a Branson Electronics Sonifier equipped with a 1.2-cm Heat Systems Electronics probe. Every 5 min 50 g of cracked ice was added to maintain the temperature of the preparation between 0 and 5° during sonication. The sonicated material was centrifuged for 1 hr at 15,000*g* in a Sorvall RC2-B refrigerated centrifuge to remove particulate matter. During this and all subsequent operations the temperature of the extract was maintained between 0 and 5° unless noted otherwise. The pellet was suspended in 1 l. of buffer A and centrifuged once more. The supernatant liquids from both centrifugations were pooled and were termed crude extract (step 1 in Tables I and II).

Solid ammonium sulfate was added to bring its concentration in the crude extract to 0.4 M; the pH was adjusted to 7.0 and the extract was allowed to stand for 1 hr. This salt treatment was essential for the sharp resolution of enzyme activities during chromatography on DEAE-cellulose (Meagher *et al.*, 1972). The ammonium sulfate was removed by dialyzing the extract against buffer A until the conductivity of the extract (determined with a Y.S.I. Model 31 conductivity bridge) was equal to that of buffer A. The dialyzed material was centrifuged to remove denatured protein; the supernatant liquid (step 2 in Tables I and II) was applied to a 5.0 × 100 cm DEAE-cellulose column that had been equilibrated with buffer A. A flow rate of 150 ml/hr was maintained with a Buchler polystaltic pump during chromatography. The sample was washed into the column with 500 ml of buffer A and eluted with 6 l. of buffer A that contained NaCl in a linear gradient running from a concentration of 0.0–0.2 M NaCl. Fractions of 21 ml were collected. As shown in Figure 2, *cis,cis*-muconate-lactonizing enzyme and muconolactone isomerase were almost completely separated from each other by this step. Tubes containing most of the lactonizing enzyme activity (fractions 74–96 in Figure 2) were pooled and were termed DEAE-cellulose eluate (step 3 in Table I). The pooled DEAE-cellulose eluate of muconolactone isomerase (fractions 97–145 in Figure 2) is represented as step 3 in Table II.

Crystallization of cis,cis-Muconate-Lactonizing Enzyme. The DEAE cellulose eluate containing *cis,cis*-muconate-lactonizing enzyme was stirred continuously as solid ammonium sulfate was added to bring its concentration to 30% of saturation at pH 7.0. After standing for 30 min, the preparation was centrifuged for 30 min at 15,000*g*. The supernatant liquid was brought to 40% of saturation with solid ammonium sulfate, allowed to stand for 30 min, and centrifuged once more. The pellet was dissolved in 100 ml of buffer B (30–40% saturated ammonium sulfate fraction, step 4 in Table I) and placed in a dialysis bag. The dialysis bag was placed in 1 l. of 20% saturated ammonium sulfate–10 μM $MnCl_2$ (pH 7.0) that was at room temperature and the entire preparation was then placed at 5°. After 12 hr, crystals began to form on the side of the dialysis bag; the ammonium sulfate solution was then stirred gently. Crystallization was complete in 72 hr. The crystals were collected by centrifugation at 15,000*g* for 15 min, resuspended in 20% saturated ammonium sulfate, and centrifuged once more. The precipitated crystals were dissolved in buffer B to form a final concentration of 1400 units/ml; denatured protein was removed by centrifugation. The supernatant liquid (first crystallization, step 5 in Table I) was placed in a dialysis bag and a second crystallization was achieved by the foregoing method against 10% saturated ammonium sulfate–10 μM $MnCl_2$ (pH 7.0).

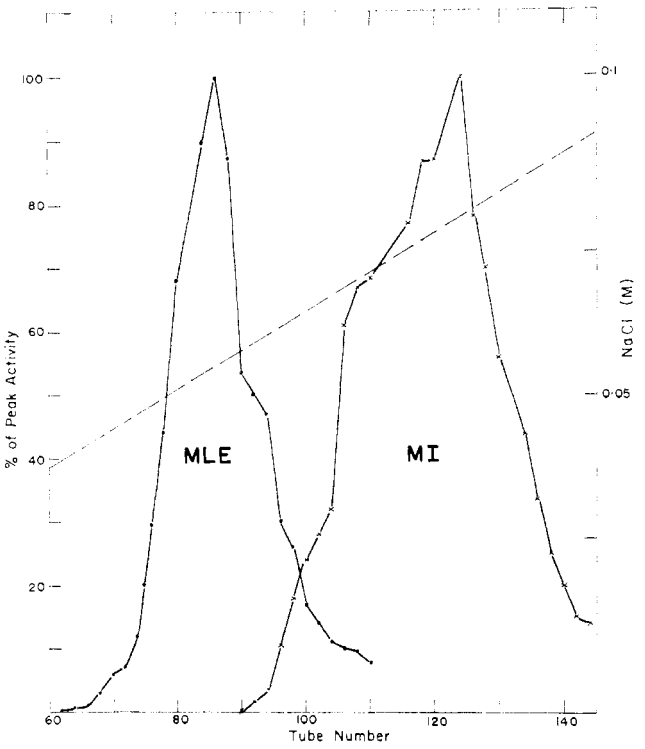

FIGURE 2: DEAE-cellulose chromatography of *cis,cis*-muconate-lactonizing enzyme (symbolized as MLE) and muconolactone isomerase (symbolized as MI). Tube 86, containing the peak fraction of *cis,cis*-muconate-lactonizing enzyme, contained 88 units of lactonizing enzyme/ml; tube 124, containing the peak fraction of muconolactone isomerase, contained 814 units of isomerase/ml.

The crystals were harvested by centrifugation and stored at 5° in 30% saturated ammonium sulfate–10 μM $MnCl_2$ (pH 7.0) (second crystallization, step 6, Table I). Further crystallization against 10% saturated ammonium sulfate did not increase the specific activity of the enzyme (third crystallization, step 7, Table I).

Crystallization of Muconolactone Isomerase. The DEAE-cellulose eluate containing muconolactone isomerase was subjected to ammonium sulfate fractionation. Material precipitating between 50 and 60% of saturation with ammonium sulfate was dissolved in 20 mM Tris-chloride (pH 7.4) at room temperature (50–60% saturated ammonium sulfate fraction, step 4, Table II). A dialysis bag containing the protein solution was placed in 1 l. of water that was 30% saturated with ammonium sulfate (pH 7.0). The entire preparation was placed at 5°; crystallization of muconolactone isomerase was complete in 72 hr. Crystals were collected by centrifugation, washed with 10 ml of cold 30% saturated ammonium sulfate, and dissolved in 20 ml of 20 mM Tris-chloride (pH 7.4) at room temperature. Denatured protein was removed by centrifugation. The supernatant liquid (first crystallization, step 5, Table II) was placed in a dialysis bag and muconolactone isomerase was recrystallized by the method used for the first crystallization except that the protein was allowed to equilibrate with 20% saturated ammonium sulfate (pH 7.0). The recrystallization was complete in 48 hr; the recovery of enzyme activity was 97% (step 6, Table II). A third crystallization against 10% saturated ammonium sulfate yielded crystals approximately 4 mm in length with a recovery of about 85% (step 6, Table II). This crystalline preparation appeared to be electrophoretically and serologically homogeneous, but was unstable when stored at 5° for several weeks. The crystals were stable when stored in the presence of 30% saturated ammonium sulfate–20 mM Tris-chloride (pH 7.4).

Both *cis,cis*-muconate-lactonizing enzyme and muconolactone isomerase were crystallized by extensive dialysis against distilled water at 5°. The enzymes were unstable in this state, however, and were converted to insoluble, inactive forms after a few weeks storage at 5°.

Storage of Enzymes as Microcrystals. In order to simplify the handling of the crystalline enzymes, they were converted to microcrystals. Crystals of the lactonizing enzyme were dissolved in buffer B and crystals of the isomerase were dissolved in 20 mM Tris-chloride (pH 7.4), respectively, at room temperature to a final concentration of 15–20 mg/ml. One-third volume of saturated ammonium sulfate was mixed with the solution of enzyme and then placed at 5°. Microcrystals of the enzymes appeared within hours. Both enzymes have been stored in this form at 5° for over a year without loss of activity.

Preparation of Antisera. Antisera were prepared against twice-crystallized *cis,cis*-muconate-lactonizing enzyme (step 6, Table I) and three-times-crystallized muconolactone isomerase (step 7, Table II). Young white male New Zealand rabbits were injected in both thighs with a solution containing 100 μg of crystalline enzyme in 0.5 ml of 0.85% NaCl emulsified with an equal volume of Freund's complete adjuvant (Difco). In subsequent injections the Freund's complete adjuvant was substituted with Freund's incomplete adjuvant (Difco). The second injection was given 60–90 days following the first. Two weeks later 50 ml of blood was removed from each rabbit by heart puncture. Subsequently the rabbits were bled weekly and injected biweekly.

Ouchterlony double diffusion plates (Wasserman and Levine, 1961) were prepared by the method of Stanier *et al.* (1970). The center well contained 0.1 ml of antiserum and the surrounding wells contained from 0.5 to 5 μg of antigen.

Acrylamide Gel Disc Electrophoresis. Disc electrophoresis was performed at room temperature using the method of Davis (1964). Gel columns (0.5 × 9.0 cm) contained 7% acrylamide, 0.07% bisacrylamide, 0.05% *N,N,N',N'*-tetramethylethylenediamine, 0.052% fresh ammonium persulfate, and 25 mM Tris-chloride (pH 7.5). Protein was prepared for electrophoresis by dialysis against 25 mM Tris-chloride (pH 8.9). This buffer was placed in both reservoirs and the anode was connected to the bottom reservoir. Samples containing 10 or 150 μg of protein, 30% sucrose, and 3 μl of 0.05% Bromophenol Blue in a volume of 0.1 ml or less were applied to the top of the gels. A constant current of 5 mA/gel was applied for 2 hr at which time the tracking dye had moved within 1 cm of the bottom of the gel. The gels were stained with 0.15% Amido Black in 7% acetic acid and destained electrophoretically. The migration of twice-crystallized lactonizing enzyme and three-times-crystallized isomerase in this electrophoretic system is shown in Figure 3.

In a second electrophoresis system 33 mM sodium phosphate buffer was employed. The gels were at pH 5.8 and the reservoirs contained buffer at pH 7.0. Other conditions of electrophoresis were identical with those described previously.

Sodium Dodecyl Sulfate Acrylamide Gel Electrophoresis. The molecular weights of the subunits of *cis,cis*-muconate-lactonizing enzyme and muconolactone isomerase were estimated by the sodium dodecyl sulfate gel electrophoresis system of Weber and Osborn (1969). The subunit size of the lactonizing enzyme was determined on gels containing 10%

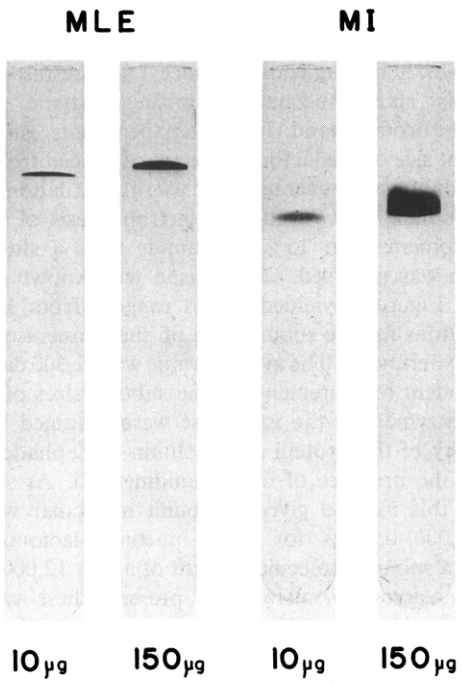

FIGURE 3: Disc gel electrophoresis of *cis,cis*-muconate-lactonizing enzyme (symbolized as MLE) (step 6, Table I) and of muconolactone isomerase (symbolized as MI) (step 7, Table II).

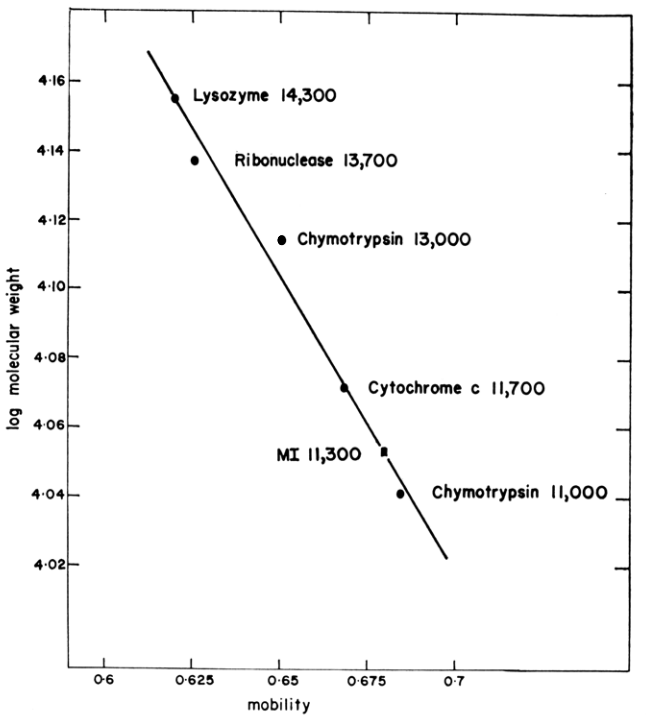

FIGURE 4: The migration of muconolactone isomerase subunits (symbolized as MI) during electrophoresis on 15% acrylamide gels in the presence of sodium dodecyl sulfate.

acrylamide and 0.06% bisacrylamide; the reservoirs contained 33 mM sodium phosphate buffer (pH 7.0).

The low molecular size of muconolactone isomerase subunits necessitated preparation of a tightly cross-linked gel of the following composition: 15% acrylamide, 0.36% bisacrylamide, 0.1% sodium dodecyl sulfate, 0.025% ammonium persulfate, 0.06% *N,N,N′,N′*-tetramethylethylenediamine, and 33 mM sodium phosphate (pH 7.0). The electrophoretic mobilities of standards used for the determination of the subunit size of muconolactone isomerase are shown in Figure 4.

Sodium dodecyl sulfate prevents the staining of protein with Amido Black. Therefore after electrophoresis the gels were shaken at 30° two times for 12 hr against 100 ml of 7% acetic acid–30% methanol in order to remove the detergent. The gels were then stained with 0.15% Amido Black in 7% acetic acid and destained electrophoretically.

Guanidine Hydrochloride Chromatography on Sephadex G-150. Independent estimates of the subunit size of muconolactone isomerase and *cis,cis*-muconate-lactonizing enzyme were obtained by a modification of the method of Fish *et al.* (1970). All work in 6 M guanidine-HCl was performed at 55° to further ensure complete denaturation (Tanford and Aune, 1970). Owing to the small size of the muconolactone isomerase subunit, Sephadex G-150 was substituted for agarose. A sample containing 2 mg of protein on 0.2 ml of 6 M guanidine-HCl, 0.1% dithiothreitol, and 50 mM Tris-chloride at pH 7.5 was applied to a 0.9 × 110 cm column of Sephadex G-150 that had been equilibrated with the same buffer except that 0.01% mercaptoethanol had been substituted for dithiothreitol. The protein was eluted from the column with the same buffer at a flow rate of 2.5 ml/hr; fractions of 0.75 ml were collected. Protein was measured as absorbance at 220 nm.

Recovery of Active Muconolactone Isomerase after Guanidine Hydrochloride Chromatography. After guanidine-HCl chromatography of muconolactone isomerase, fractions containing 2 mg of protein were pooled and dialyzed at 23° against 1 l. of 50 mM Tris-chloride buffer (pH 7.5), containing 0.1% dithiothreitol. After dialysis 1200 units of enzyme activity was recovered, corresponding to a recovery of 70% of the original enzyme activity. This preparation of muconolactone isomerase was precipitated and recrystallized by procedures described above. These crystals had a specific activity of 550 and were electrophoretically indistinguishable from native enzyme in the pH 8.9 disc gel system described previously.

Amino Acid Analysis. Amino acid composition was determined with a Beckman 120B amino acid analyzer after acid hydrolysis of 4-mg samples of protein according to the procedure of Moore and Stein (1963). Tryptophan was measured on the amino acid analyzer after hydrolysis in the presence of thioglycolic acid (Matsubara and Sasaki, 1969); absorbance of the proteins at 280 nm was used for an independent determination of tryptophan (Goodwin and Morton, 1946). The half-cystine content was determined as cysteic acid on the amino acid analyzer after performic acid oxidation (Hirs, 1967). The amount of cysteine in *cis,cis*-muconate-lactonizing enzyme was measured in the presence of 6 M urea–20 mM Tris-chloride (pH 7.9), with 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959). The cysteine of muconolactone isomerase, which did not react with 5,5′-dithiobis-(2-nitrobenzoic acid), was measured by titration of the protein with *p*-chloromercuribenzoate in the presence of 1.0 M chlorate–1.0 M Tris-chloride buffer at pH 7.0 according to the method of Boyer (1954).

Amino-Terminal Residues. The cyanate procedure of Stark and Smyth (1963) was used for a quantitative estimation of the amino-terminal residues. During the carbamylation of the proteins, 6 M guanidine-HCl was substituted for urea. The values for the amino-terminal residues represent the difference between the amino acids produced by carbamy-

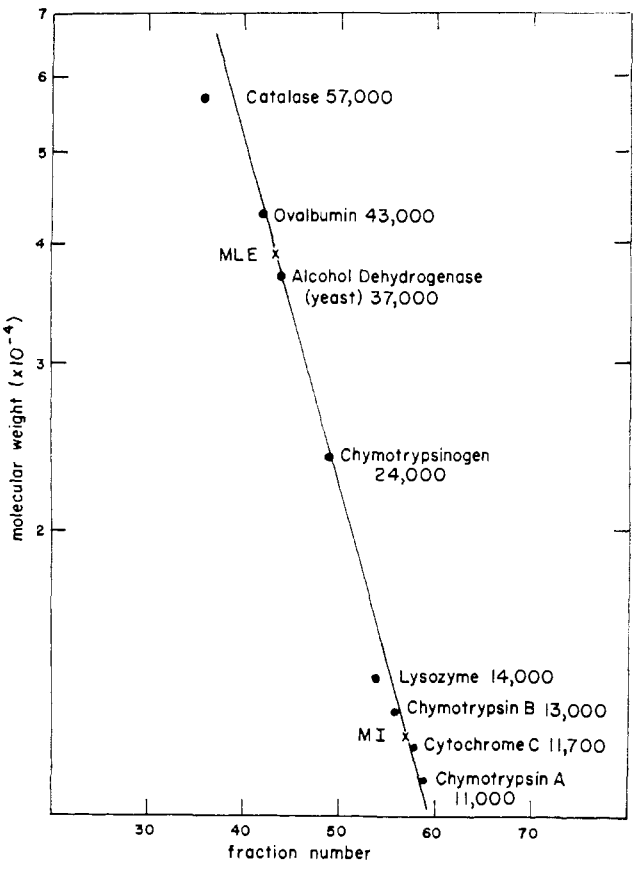

FIGURE 5: The elution of *cis,cis*-muconate-lactonizing enzyme (symbolized as MLE), muconolactone isomerase (symbolized as MI), and standard proteins from Sephadex G-150 columns after chromatography at 55° in the presence of 6 M guanidine hydrochloride.

lated and noncarbamylated protein. This difference was corrected on the basis of the reported yield of individual amino acids during hydrolysis (Stark and Smyth, 1963).

Amino-terminal residues were determined independently by treating 5–10 nmoles of whole protein with dansyl chloride and chromatography of the hydrolyzed product of the reaction on polyamide sheets (Wiener *et al.*, 1972).

Results

Criteria of Purity. Antisera against *cis,cis*-muconate-lactonizing enzyme gave a single sharp band when tested against the twice-crystallized enzyme on Ouchterlony plates; the precipitin band was confluent with a single band formed when crude extract containing the same amount of enzyme activity was placed in the outer well. Identical results were obtained when antisera against muconolactone isomerase were tested against the enzyme in crude extract and in purified form. These results indicate that the recrystallized lactonizing enzyme and isomerase, unlike earlier crystalline preparations of the enzymes (Ornston, 1966b), are immunologically pure.

As shown in Figure 3, the recrystallized lactonizing enzyme and isomerase revealed a single protein band after discontinuous electrophoresis at pH 8.9. Single bands also were observed when the disc electrophoresis was performed at pH 7.0. These results strongly indicate that the enzyme preparations were homogeneous. Further evidence of homogeneity came from the observation, described below, that the enzymes dissociated completely into subunits of well defined size when treated with denaturing agents.

Subunit Size. During sodium dodecyl sulfate disc gel electrophoresis *cis,cis*-muconate-lactonizing enzyme migrated as a single protein band. In three experiments estimates of the subunit size of the lactonizing enzyme ranged from 39,000 to 42,000 daltons; the average value was 41,000 daltons.

After sodium dodecyl sulfate electrophoresis of muconolactone isomerase on 15% acrylamide gels, a single band of protein was observed. Comparison with known proteins, shown in Figure 4, yielded values ranging from 11,000 to 11,400 daltons for the subunit size of the isomerase in three separate experiments. The average value was 11,300 daltons.

Independent measurements of the subunit sizes of the lactonizing enzyme and the isomerase were obtained by chromatography of the protein on a column of Sephadex G-150 at 55° in the presence of 6 M guanidine-HCl. As shown in Figure 5, this method gives a subunit molecular weight of about 40,000 daltons for *cis,cis*-muconate-lactonizing enzyme and a subunit molecular weight of about 12,000 daltons for muconolactone isomerase. At present, these values represent the most accurate estimate of the subunit sizes of the enzymes.

The small subunit size of muconolactone isomerase suggested that it might assume an active conformation if the guanidine-HCl were removed by dialysis. Indeed 70% of the original activity of the muconolactone isomerase was regained when the guanidine-HCl was removed in the presence of dithiothreitol and the resulting isomerase preparation was readily crystallized.

Amino Acid Composition. The amino acid composition of *cis,cis*-muconate-lactonizing enzyme is shown in Table III. All of the half-cystine in the lactonizing enzyme can be accounted for as cysteine (Table III).

The amino acid content of muconolactone isomerase is shown in Table III. The single half-cystine per 12,000 dalton subunit appears to be cysteine (Table III). Nearly integral values for the content of cysteine, tryptophan, phenylalanine, tyrosine, and glycine are obtained if the subunit size of the isomerase is assumed to be 12,000 daltons. Particularly striking in Table III is the relatively low content of the unsubstituted amino acid glycine (three residues per subunit), and the relatively high concentration of the cyclic amino acid proline (six residues per subunit).

Amino-Terminal End-Group Analysis. The cyanate method of Stark and Smyth (1963) did not yield a large quantity of any single amino acid from *cis,cis*-muconate-lactonizing enzyme. The data presented in Table IV show relatively high concentrations of threonine and of its breakdown product glycine. The only other amino acid recovered in significant quantity was glutamate, the most probable product of internal cleavage (Stark and Smyth, 1963). This information suggested that the amino-terminal end group of the lactonizing enzyme was threonine, but that much of this unstable amino acid is lost during hydrolysis of its hydantoin. Support for this conclusion came from the unambiguous identification of dansyl-threonine on chromatograms after treatment of the whole protein with dansyl chloride. A very faint spot of dansyl-glycine was also present on the chromatograms.

The data presented in Table IV clearly demonstrate that methionine is the amino-terminal residue of muconolactone isomerase. The cyanate procedure yielded 75% of the methionine (or its sulfoxides) predicted on the basis of a subunit size of 12,000 daltons. After treatment with dansyl chloride, muconolactone isomerase yielded dansyl-methionine con-

TABLE III: Amino Acid Compositions of *cis,cis*-Muconate-Lactonizing Enzyme and Muconolactone Isomerase.[a]

Amino Acid	*cis,cis*-Muconate-Lactonizing Enzyme		Muconolactone Isomerase	
	Calcd No. of μmol	No. of Residues/ 40,000 daltons	Calcd No. of μmol	No. of Residues/ 12,000 daltons
Lysine	0.122	12.7	0.157	5.5
Histidine	0.0698	7.2	0.178	6.2
Arginine	0.270	28.0	0.180	6.3
Aspartic acid	0.297	30.8	0.301	10.5
Threonine	0.170	17.6	0.114	3.9
Serine	0.173	18.0	0.140	4.8
Glutamic acid	0.374	38.8	0.271	9.5
Proline	0.146	15.2	0.187	6.5
Glycine	0.312	32.4	0.092	3.2
Alanine	0.404	41.9	0.215	7.5
Cysteine		2.06[b]		0.87[c]
Valine	0.200	20.8	0.206	7.2
Methionine	0.067	6.9	0.148	5.2
Isoleucine	0.270	28.0	0.0834	2.9
Leucine	0.450	46.7	0.362	12.6
Tyrosine	0.046	4.8	0.095	3.3
Phenylalanine	0.081	8.4	0.088	3.1
Tryptophan		4.07[d]		1.75[e]

[a] Hydrolysate derived from 0.4 mg of protein was applied to each column of the amino acid analyzer. [b] Average of 1.94 residues determined as cysteic acid after performic acid oxidation and 2.19 residues determined with 5,5′-dithiobis(2-nitrobenzoic acid). [c] Average of 0.90 residue determined as cysteic acid after performic acid oxidation and 0.84 residue determined by titration with *p*-chloromercuribenzoate. [d] Average of 4.4 residues determined as material absorbing at 280 nm and 3.73 residues determined after acid hydrolysis in the presence of thioglycolic acid. [e] Average of 2.07 residues determined as material absorbing at 280 nm and 1.42 residues determined after acid hydrolysis in the presence of thioglycolic acid.

taminated only with a barely detectable amount of dansylglycine.

Discussion

Influence of Subunit Composition on the Antigenic Determinants of cis,cis-Muconate-Lactonizing Enzyme and Muconolactone Isomerase. Mutations in *catB*, the structural gene for *cis,cis*-muconate-lactonizing enzyme, frequently have a dramatic effect on the antigenic determinants of the protein. For example, Wheelis and Ornston (1972) reported that 11 out of 12 *P. putida* strains bearing revertible *catB* mutations failed to form material that cross-reacted with antisera prepared against the wild-type *cis,cis*-muconate-lactonizing enzyme. Since it is improbable that all of the 11 cross-reacting material negative mutant strains carried nonsense mutations, it appears that the substitution of a single amino acid in the primary structure of the protein may alter its conformation to such an extent that its antigenic determinants are lost. Additional insight into the influence of the primary structure on the antigenic properties of *cis,cis*-muconate-lactonizing

TABLE IV: Amino-Terminal Residues of *cis,cis*-Muconate-Lactonizing Enzyme and Muconolactone Isomerase.

Amino Acid	Residues/ 40,000 Daltons of Lactonizing Enzyme	Residues/ 12,000 Daltons of Isomerase
Threonine	0.17	N.D.[a]
Glutamic acid	0.12	0.03
Glycine	0.055	0.01
Alanine	0.014	0.005
Methionine and sulfoxides thereof	N.D.[a]	0.75

[a] Not detected.

enzyme was gained by Condon and Ingraham (1967) who described the properties of a *cis,cis*-muconate-lactonizing enzyme formed by a *P. putida* mutant strain with a cold sensitive mutation in the *catB* gene. The structural gene mutation prevented the production of both active enzyme and cross-reacting material at 15°; at 30° the mutant strain formed a lactonizing enzyme that was catalytically and immunologically identical with the wild-type enzyme. If the rates of transcription and translation are the same in wild-type and mutant strains, the amino acid substitution in the mutant-lactonizing enzyme prevents the protein from assuming an antigenically active conformation at 15°.

As suggested by Condon and Ingraham (1967), the influence of a single mutation on the conformation of the lactonizing enzyme would be greatly amplified if the protein were composed of several subunits. Their proposal is supported by comparison of the 40,000 dalton subunit size with its reported molecular weight of 220,000 daltons (Ornston, 1966b): evidently the whole enzyme contains from four to six subunits. Extension of this line of reasoning to muconolactone isomerase, which appears to have eight subunits of 12,000 daltons in a protein of 93,000 daltons (Ornston, 1966b), leads to the prediction that mutational variants of this enzyme would vary more widely in serological response than those of the lactonizing enzyme. Indeed the available evidence, derived from the immunological survey of the two enzymes in homologous *Pseudomonas* strains by Stanier *et al.* (1970), indicates that the isomerase appears to have evolved more rapidly than the lactonizing enzyme. On the basis of present information, the apparent rate of variation in rates of evolution may be most readily attributed to differences in the subunit composition of the proteins.

Ratio of Polypeptide Subunits in Induced Cultures of P. putida. If the subunit size of *cis,cis*-muconate-lactonizing enzyme is taken as 40,000 daltons and the specific activity of the pure enzyme is 90 units/mg of protein, one unit of lactonizing enzyme corresponds to 0.28 nmol of polypeptide subunit. A subunit size of 12,000 daltons and a specific activity of 860 units/mg of protein for pure muconolactone isomerase lead to the conclusion that one unit of isomerase corresponds to 0.097 nmol of polypeptide subunit. The ratio of the activity of the isomerase to the lactonizing enzyme is 5.5 under a variety of inducing and repressing conditions in *P. putida* (Ornston, 1966c). On the basis of the foregoing data the ratio of polypeptide subunits can be calculated to be 1.9 isomerase subunits to 1.0 lactonizing enzyme subunit.

The strict coordinate control of the synthesis of the isomerase and of the lactonizing enzyme has suggested that their structural genes are components of a complex operon (Ornston, 1966c). Support for this proposal has come from the observations that the structural genes for the two enzymes are tightly linked (Wheelis and Stanier, 1970; Wu *et al.*, 1972) and that the activity of both genes is governed by *catR*, a regulatory gene that neighbors *catB*, the structural gene for the lactonizing enzyme (Wheelis and Ornston, 1972; Wu *et al.*, 1972).

In several cases evidence has been adduced to show that the structural genes within the operons of *Escherichia coli* produce equal amounts of polypeptide products (Morse *et al.*, 1968; Morse and Yanofsky, 1968; Wilson and Hogness, 1969). On the other hand, exceptions to this rule have been documented: appropriately induced cultures of *E. coli*, for example, translate the *lacZ* cistron about five times as frequently as the *lacA* cistron (Brown *et al.*, 1967). A general property of operons, noted by Wilson and Hogness (1969), is that the rate of translation of a cistron proximal to the operator is always equal to or greater than the rate of translation of distal cistrons. Application of this rule to the *catB* and *catC* genes of *P. putida* leads to the prediction that, if they do indeed comprise an operon, the *catC* gene (coding for muconolactone isomerase) is closer to the operator than the *catB* (*cis,cis*-muconate-lactonizing enzyme) structural gene.

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References

Boyer, P. D. (1954), *J. Amer. Chem. Soc. 76*, 4331.
Brown, J. L., Brown, D. M., and Zabin, I. (1967), *J. Biol. Chem. 242*, 4254.
Canovas, J. L., Ornston, L. N., and Stanier, R. Y. (1967), *Science 156*, 1695.
Canovas, J. L., and Stanier, R. Y. (1967), *Eur. J. Biochem. I*, 289.
Condon, S., and Ingraham, J. L. (1967), *J. Bacteriol. 94*, 1970.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.
Fish, W. W., Reynolds, J. A., and Tanford, C. (1970), *J. Biol. Chem. 245*, 5166.
Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.
Hirs, C. H. W. (1967), *Methods Enzymol. 11*, 197.
Johnson, B. F., and Stanier, R. Y. (1971), *J. Bacteriol. 107*, 476.
Kemp, M. B., and Hegeman, G. D. (1968), *J. Bacteriol. 96*, 1488.
Lowry, O. H., Rosebrough, N. J., Farr, A. C., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Matsubara, H., and Sasaki, R. M. (1969), *Biochem. Biophys. Res. Commun. 35*, 175.
Meagher, R. B., McCorkle, G. M., Ornston, M. K., and Ornston, L. N. (1972), *J. Bacteriol. 111*, 465.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.
Morse, D. E., Baker, R. F., and Yanofsky, C. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 1428.
Morse, D. E., and Yanofsky, C. (1968), *J. Mol. Biol. 38*, 477.
Ornston, L. N. (1966a), *J. Biol. Chem. 241*, 3787.
Ornston, L. N. (1966b), *J. Biol. Chem. 241*, 3795.
Ornston, L. N. (1966c), *J. Biol. Chem. 241*, 3800.
Ornston, L. N., and Stanier, R. Y. (1966), *J. Biol. Chem. 241*, 3776.
Ornston, M. K., and Ornston, L. N. (1972), *J. Gen. Microbiol. 73*, 455.
Parke, D., Meagher, R. B., and Ornston, L. N. (1973), *Biochemistry 12*, 3537.
Patel, R. N., Meagher, R. B., and Ornston, L. N. (1973), *Biochemistry 12*, 3531.
Stanier, R. Y., and Ornston, L. N. (1973), *Advan. Microb. Physiol. 9*, 89.
Stanier, R. Y., Palleroni, N. J., and Doudoroff, M. (1966), *J. Gen. Microbiol. 43*, 159.
Stanier, R. Y., Wachter, D., Gasser, C., and Wilson, A. C. (1970), *J. Bacteriol. 102*, 351.
Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.
Tanford, C., and Aune, K. C. (1970), *Biochemistry 9*, 206.
Wasserman, E., and Levine, L. (1961), *J. Immunol. 87*, 209.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Wheelis, M. L., and Ornston, L. N. (1972), *J. Bacteriol. 109*, 790.
Wheelis, M. L., and Stanier, R. Y. (1970), *Genetics 66*, 245.
Wiener, A. M., Platt, T., and Weber, K. (1972), *J. Biol. Chem. 247*, 3242.
Wilson, D. B., and Hogness, D. S. (1969), *J. Biol. Chem. 244*, 2143.
Wu, C.-H., Ornston, M. K., and Ornston, L. N. (1972), *J. Bacteriol. 109*, 796.