Relationships among Enzymes of the β-Ketoadipate Pathway. II. Properties of Crystalline β-Carboxy-*cis,cis*-muconate-Lactonizing Enzyme from *Pseudomonas putida*†

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ABSTRACT: Procedures for the purification and crystallization of β-carboxy-*cis,cis*-muconate-lactonizing enzyme from extracts of *Pseudomonas putida* are described. Antisera prepared against the enzyme do not cross react with *cis,cis*-muconate-lactonizing enzyme purified from the same organism. Nevertheless, several lines of evidence indicate that the two lactonizing enzymes may be evolutionarily homologous: they possess similar molecular sizes (190,000 daltons), subunit sizes (40,000 daltons), crystalline structures, and amino-terminal sequences.

β-Carboxy-*cis,cis*-muconate (Figure 1) is a commonly occurring catabolite in microorganisms. It is produced by the oxygenative cleavage of protocatechuate (Stanier and Ingraham, 1954) and, in addition, can serve as a natural growth substrate for some bacterial species (Ornston and Ornston, 1972). As shown in Figure 1, β-carboxy-*cis,cis*-muconate is catabolized by either of two divergent metabolic routes. In one pathway, found in eucaryotes (Gross *et al.*, 1956; Cain *et al.*, 1968), the compound is lactonized to β-carboxymuconolactone which is converted to β-ketoadipate by a mechanism that has not yet been elucidated fully. In procaryotes the enzymatic lactonization of β-carboxy-*cis,cis*-muconate produces γ-carboxymuconolactone which is converted to β-ketoadipate by two enzymatic reactions (Ornston and Stanier, 1966).

The metabolic divergence at the level of β-carboxy-*cis,cis*-muconate suggests that the β-ketoadipate pathway evolved independently in eucaryotes and in procaryotes. If this is the case, the comparison of the lactonizing enzymes from the two biological groups may yield insight into the convergent evolutionary events that produced enzymes catalyzing analogous type reactions.

Further evidence of evolutionary divergence in the β-ketoadipate pathway comes from the comparative study of regulatory mechanisms in different bacterial genera: the synthesis of the enzymes is governed by distinctive mechanisms in *Acinetobacter calcoaceticus* (Canovas and Stanier, 1967), *Alcaligenes eutrophus* (Johnson and Stanier, 1971), *Nocardia opaca* (Rann and Cain, 1969), the fluorescent *Pseudomonas* group (Ornston, 1966c; Kemp and Hegeman, 1968), and the acidovorans *Pseudomonas* group (Ornston and Ornston, 1972). These observations have raised the possibility that the β-ketoadipate pathway may have evolved independently in different bacterial genera (Canovas *et al.*, 1967; Stanier and Ornston, 1973), a proposal that could be tested directly by comparison of the primary structures of the enzymes.

As noted in the foregoing paper (Meagher and Ornston, 1973), the three steps of the protocatechuate and catechol branches of the bacterial β-ketoadipate pathway are chemically analogous and may be catalyzed by homologous enzymes. Thus, for example, the *cis,cis*-muconate-lactonizing enzyme and the β-carboxy-*cis,cis*-muconate-lactonizing enzyme from a single bacterium may be produced by homologous structural genes. Evidence in favor of this view came from the observation that both lactonizing enzymes appeared to have similar molecular weights and that the two enzymes were inhibited similarly by polyanionic buffers (Ornston, 1966a,b). On the other hand, antisera prepared against *cis,cis*-muconate-lactonizing enzyme did not interact with β-carboxy-*cis,cis*-muconate-lactonizing enzyme (Stanier *et al.*, 1970), so the two enzymes appear to be serologically remote.

In the preceding paper we described some of the physical and chemical properties of *cis,cis*-muconate-lactonizing enzyme from *Pseudomonas putida*. In this paper we present procedures for the purification and crystallization of β-carboxy-*cis,cis*-muconate-lactonizing enzyme from *P. putida* and compare the properties of the two lactonizing enzymes from the same organism.

Experimental Section

Bacterial Strain and Its Growth. Enzymes of the β-carboxy-*cis,cis*-muconate pathway were induced in *Pseudomonas putida* strain PRS2105. This mutant organism, unlike its parental strain 90 (Stanier *et al.*, 1966), cannot form β-ketoadipyl-CoA–succinate transferase and consequently accumulates β-ketoadipate, the inducer of β-carboxy-*cis,cis*-muconate-lactonizing enzyme, when grown in the presence of *p*-hydroxybenzoate (Ornston, 1966c). The accumulation of inducer causes strain PRS2105 to synthesize high levels of the enzyme. Furthermore, strain PRS2105 cannot grow at the expense of protocatechuate and thus can be distinguished readily from contaminating organisms that might synthesize β-carboxy-*cis,cis*-muconate-lactonizing enzyme. Strain PRS2105 also is resistant to the lytic phage *WOM* (Meagher and Ornston, 1973).

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TABLE I: Purification of β-Carboxy-*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	14,000	426	451	0.94	100	1.0
2. Dialyzed extract	16,000	460	347	1.3	108	1.4
3. First DEAE cellulose eluate	5,400	318	16.3	5.0	74	5.3
4. First 35–55% saturated ammonium sulfate fraction	1,400	280	23.1	10.0	66	10.6
5. Heat-treated extract	1,525	276	4.35	63.0	65	67.0
6. Second 35–55% saturated ammonium sulfate fraction	50	263	2.6	100.0	61	105.0
7. Bio-Gel eluate	250	200	0.79	250.0	47	266.0
8. 0–25% saturated ammonium sulfate fraction	24	86.4	0.12	725.0	22	768.0
9. Second DEAE-cellulose eluate	64	51.2	0.064	800.0	14	848.0
10. Third 35–55% saturated ammonium sulfate fraction	5	40.0	0.050	800.0	9.4	848.0
11. Crystallization	5	32.0	0.040	800.0	7.5	848.0

Cultures were grown in a 100-l. New Brunswick Fermocell Model CF-130 fermentor under conditions described previously (Meagher and Ornston, 1973), except that the growth substrate was 20 mM succinate and 1 mM *p*-hydroxybenzoate. The accumulation of β-ketoadipate in the growth medium was monitored with the Rothera reaction (Evans, 1947).

Chemicals. β-Carboxy-*cis,cis*-muconate was synthesized enzymically following the procedure of Meagher *et al.* (1972). Bio-Gel Agarose A1.5m (100–200 mesh) was obtained from Bio-Rad Labs. Dimethyl sulfoxide was purchased from Sigma Chemical Co.

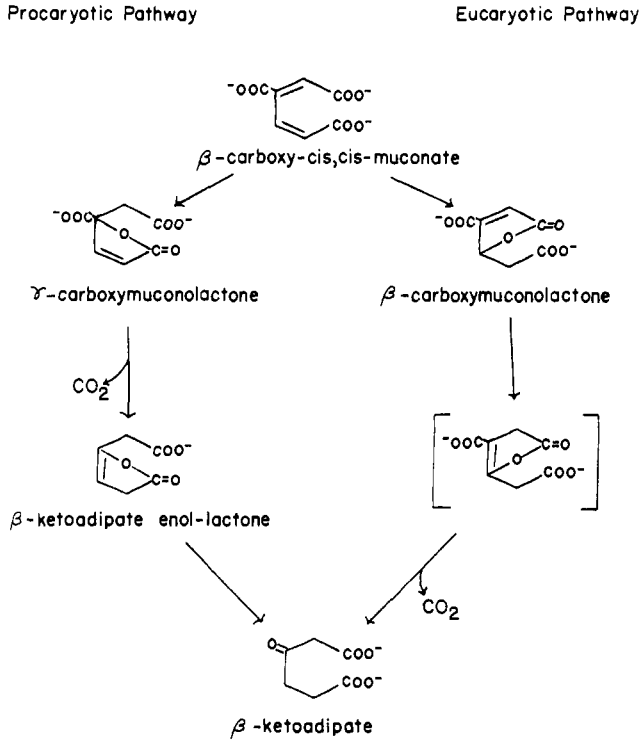

FIGURE 1: Pathways for the catabolism of β-carboxy-*cis,cis*-muconate in microorganisms. The compound shown in brackets is hypothetical.

Enzyme Assays. Published spectrophotometric assays (Ornston, 1966a) were used for the measurement of β-carboxy-*cis,cis*-muconate-lactonizing enzyme, γ-carboxymuconolactone decarboxylase, and β-ketoadipate enol lactone hydrolase activities. In all cases a unit of enzyme is defined as the amount of enzyme that removes 1 μmol of substrate from the assay mixture per min. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Purification of β-Carboxy-cis,cis-muconate-Lactonizing Enzyme. The preparation of extracts and DEAE-cellulose chromatography was identical with that described in the previous paper (Meagher and Ornston, 1973), except that the protein was eluted from the column with 6 l. of 10 mM ethylenediamine dihydrochloride–1 μM $MnCl_2$ (pH 7.3) (buffer A) containing NaCl in a linear gradient running from a concentration of 0.04–0.4 M. As shown in Figure 2, β-carboxy-*cis,cis*-muconate lactonizing enzyme, γ-carboxymuconolactone decarboxylase, and β-ketoadipate enol lactone hydrolase were separated from each other by DEAE-cellulose chromatography, so the procedure can be used as a starting step for the purification of all three enzymes from a single

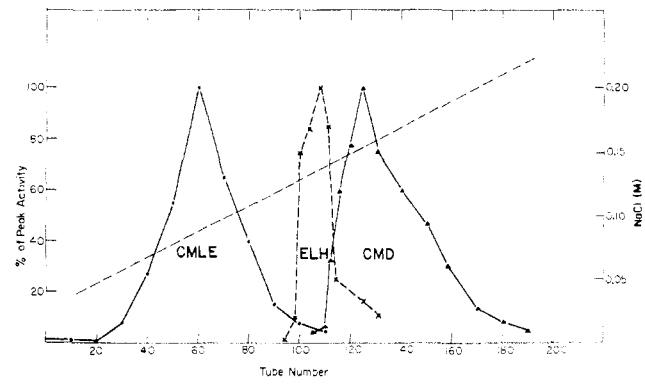

FIGURE 2: The elution of β-carboxy-*cis,cis*-muconate-lactonizing enzyme (CMLE), γ-carboxymuconolactone decarboxylase (CMD), and β-ketoadipate enol lactone hydrolase (ELH) from a column of DEAE-cellulose. The activity in the peak tubes was as follows: CMLE (205 units/ml); ELH (400 units/ml); CMD (360 units/ml). A linear gradient of NaCl was applied under conditions described in the text.

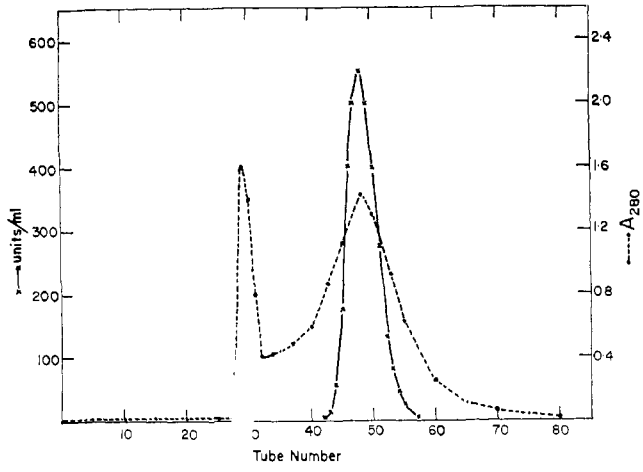

FIGURE 3: The elution of β-carboxy-*cis,cis*-muconate-lactonizing enzyme (CMLE) from a Bio-Gel agarose A1.5m column. Conditions of chromatography are described in the text.

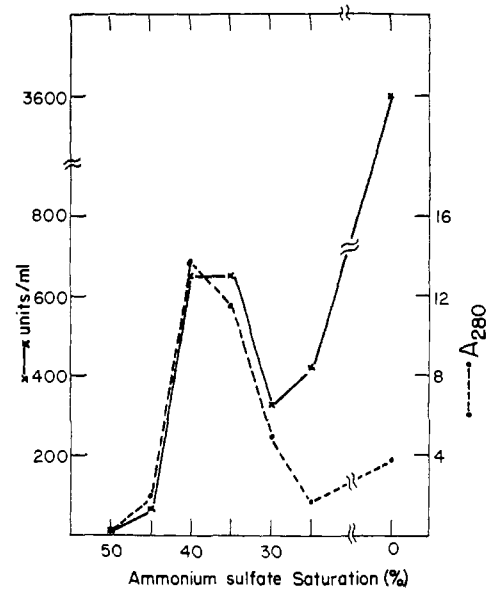

FIGURE 4: Fractionation of β-carboxy-*cis,cis*-muconate-lactonizing enzyme (CMLE) with ammonium sulfate. A solution containing 200,000 units of lactonizing enzyme (step 7 in Table I) was brought to 55% of saturation with respect to ammonium sulfate. The precipitated protein was extracted with 5-ml solutions containing decreasing concentrations of ammonium sulfate.

extract. Fractions containing β-carboxy-*cis,cis*-muconate-lactonizing enzyme activity were pooled and termed DEAE-cellulose eluate (step 3 in Table I).

Ammonium sulfate fractionation was performed by the general methods described before (Meagher and Ornston, 1973). Material precipitating between 35 and 55% of saturation with respect to ammonium sulfate (step 4 in Table I) was maintained at 55° for 30 min, cooled to 5°, and centrifuged at 35,000*g* for 15 min to remove denatured protein. The supernatant liquid (step 5 in Table I) was fractionated with solid ammonium sulfate. Material precipitating between 35 and 55% of stauration with respect to ammonium sulfate was dissolved in buffer A (step 6 in Table I) and 10-ml samples were passed through a 2.5 × 100 cm Bio-Gel agarose A1.5m column that had been equilibrated with buffer A; the flow rate was maintained at 30 ml/hr and 7-ml fractions were collected. The elution profile of the enzyme is shown in Figure 3. Fractions containing β-carboxy-*cis,cis*-muconate-lactonizing enzyme activity were pooled (step 7 in Table I).

The Bio-Gel eluate was precipitated by bringing it to 55% of saturation with respect to ammonium sulfate and was fractionated by a procedure similar to that described by Jacoby (1971) for the crystallization of enzymes. As shown in Figure 4, the lactonizing enzyme was extracted in two fractions: one between 30 and 40% of saturation with respect to ammonium sulfate and the other between 0 and 20% of saturation with ammonium sulfate. Material that was insoluble in 25% saturated ammonium sulfate (step 8 in Table I) was equilibrated with buffer A and applied in 16-ml fractions to a 0.9 × 30 cm DEAE-cellulose column that had been equilibrated with the same buffer. The enzyme was eluted with buffer A containing NaCl in a lineer gradient running from 0.0 to 0.3 M over 150 ml; fractions of 2 ml were collected. The β-carboxy-*cis,cis*-muconate-lactonizing enzyme was eluted with a single peak of protein; the specific activity of the enzyme was constant across the peak (Figure 5). Fractions containing the enzyme (step 9 in Table I) were pooled and precipitated by bringing it to 55% of saturation with respect to ammonium sulfate. The precipitated protein was dissolved in buffer A (step 10 in Table I) and stored in buffer A containing ammonium sulfate at 30% of saturation, pH 7.0 at 5°. After a month the enzyme crystallized as square plates (step 11 in Table I). Crystals of β-carboxy-*cis,cis*-muconate-lactonizing enzyme and of *cis,cis*-muconate-lactonizing enzyme are shown in Figure 6.

Immunological Techniques. Antisera against the crystalline β-carboxy-*cis,cis*-muconate-lactonizing enzyme were prepared by methods described in the previous paper (Meagher and Ornston, 1973). The method of Stanier *et al.* (1970) was used for detection of serological cross reaction on Ouchterlony double-diffsion plates.

Inhibition Studies. Normal rabbit serum inhibits the activities of the lactonizing enzymes. Therefore the immunoglobulin (IgG) fraction was separated from immune and normal rabbit serum by sodium sulfate precipitation (Kekwick, 1940) and was used for inhibition studies. Varying amounts of the immunoglobulin fraction against β-carboxy-*cis,cis*-muconate-lactonizing enzyme or *cis,cis*-muconate-lactonizing enzyme were incubated with one of the lacto-

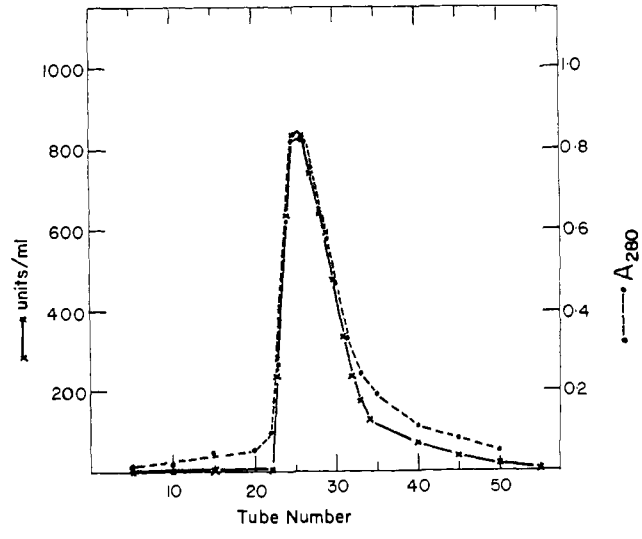

FIGURE 5: DEAE-cellulose chromatography of purified β-carboxy-*cis,cis*-muconate-lactonizing enzyme (step 8 in Table I).

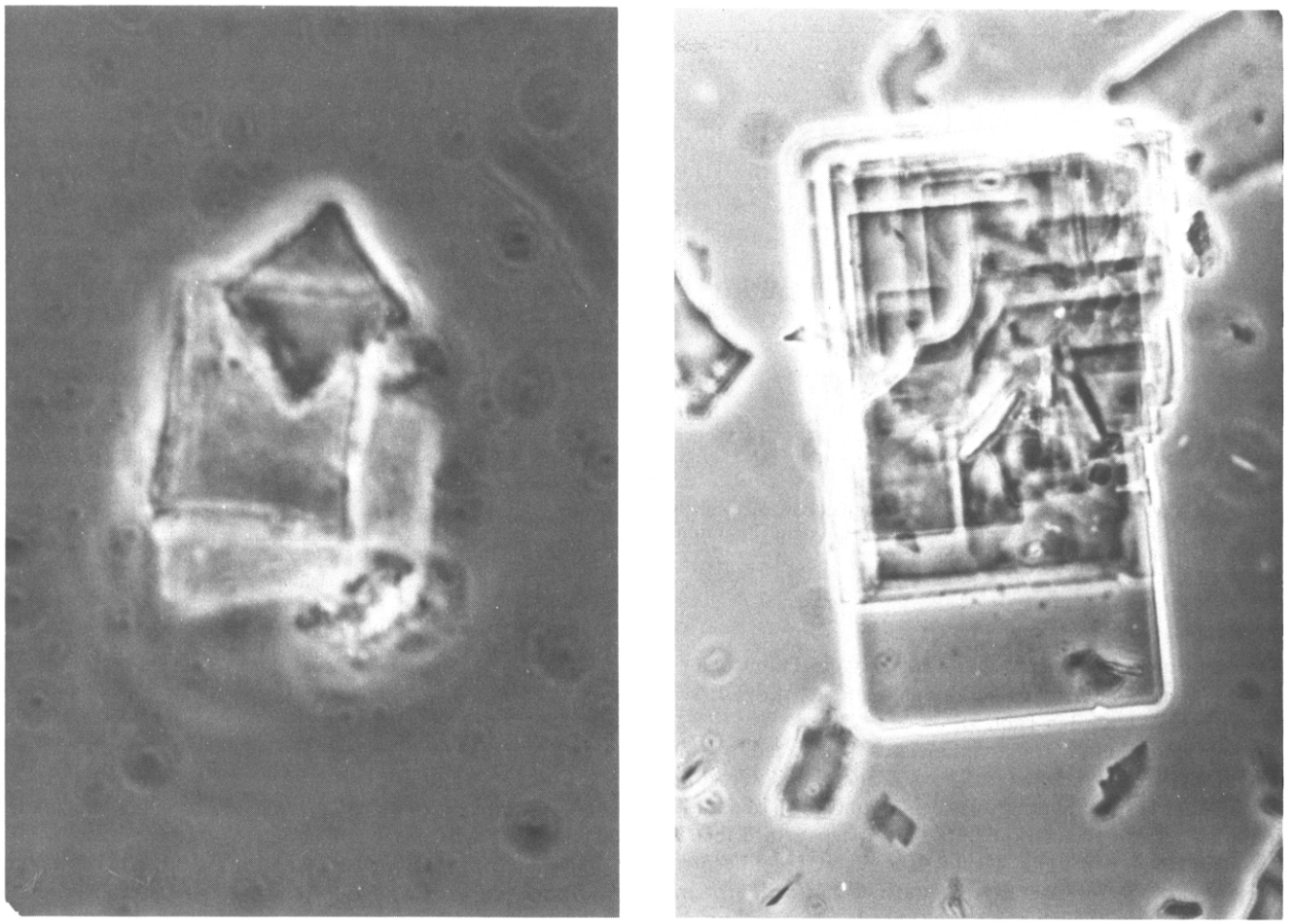

FIGURE 6: Crystalline preparations of β-carboxy-*cis*,*cis*-muconate-lactonizing enzyme (left) and *cis*,*cis*-muconate-lactonizing enzyme (right). Photomicrographs are shown at a magnification of 3000× (CMLE) and 850× (MLE).

nizing enzymes in the enzyme assay mixture (lacking substrate) for 15 min. The enzyme assay was then initiated by adding substrate to the incubation mixture. As a control, the lactonizing enzymes were incubated with the IgG fraction from rabbits that had not been immunized.

Acrylamide Gel Electrophoresis and Bio-Gel Agarose Chromatography. Disc gel electrophoresis (Davis, 1964) and sodium dodecyl sulfate electrophoresis (Weber and Osborn, 1969) were performed under conditions described in the preceding paper (Meagher and Ornston, 1973) except that 1 mM mercaptoethanol was present. The molecular weights of the two lactonizing enzymes were compared on Bio-Gel Agarose A1.5m columns.

Amino Acid Analysis. Amino acid contents were measured by the procedures described in the foregoing paper (Meagher and Ornston, 1973). In addition, the half-cystine content of β-carboxy-*cis*,*cis*-muconate-lactonizing enzyme was determined as cysteic acid on the amino acid analyzer after 24 hr of hydrolysis in the presence of dimethyl sulfoxide (Spencer and Wold, 1969). The free cysteine content was determined by titration with *p*-chloromercuribenzoate at pH 7.0 under conditions described in the previous paper (Meagher and Ornston, 1973).

Amino-Terminal Sequence. The sequence of amino acids at the amino termini of the lactonizing enzymes was determined on 5-mg samples of the pure enzymes using the sodium dodecyl sulfate–dansyl-Edman procedure of Wiener *et al.* (1972).

Results

Crystalline Shape. Photomicrographs of crystalline β-carboxy-*cis*,*cis*-muconate-lactonizing enzyme reveal thin and square plates; in this respect they are similar to crystals of *cis*,*cis*-muconate-lactonizing enzyme (Figure 6).

Serological Studies. Antisera against crystalline β-carboxy-*cis*,*cis*-muconate-lactonizing enzyme gave a single sharp precipitin line when tested against either the pure enzyme or crude extracts of induced cells. Thus the enzyme preparation appears to be homogeneous. No cross-reacting material was revealed when the antisera were tested against pure *cis*,*cis*-muconate-lactonizing enzyme from *P. putida*. These observations confirm and extend the conclusion of Stanier *et al.* (1970) that the two lactonizing enzymes are immunologically remote.

Further evidence in support of their conclusion came from measurement of the inhibition of enzyme activity by antisera prepared against the two lactonizing enzymes. As shown in Figure 7, *cis*,*cis*-muconate-lactonizing enzyme is not appreciably inhibited by antisera prepared against β-carboxy-*cis*,*cis*-muconate-lactonizing enzyme and β-carboxy-*cis*,*cis*-muconate-lactonizing enzyme is not inhibited by antisera prepared against *cis*,*cis*-muconate-lactonizing enzyme.

Disc Gel Electrophoresis. Crystalline β-carboxy-*cis*,*cis*-muconate-lactonizing enzyme migrated as a single band when subjected to disc gel electrophoresis (Figure 8). Although this observation supports the view that the enzyme

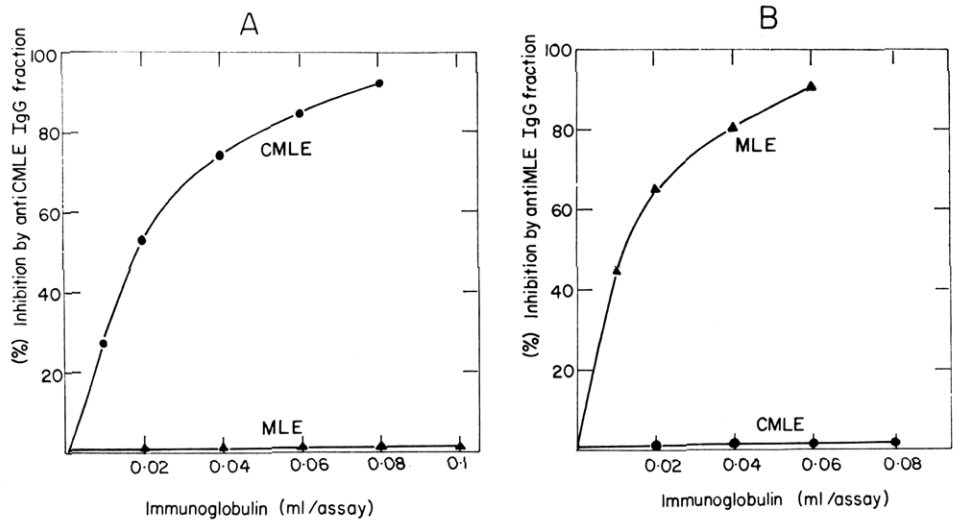

FIGURE 7: Inhibition of β-carboxy-*cis,cis*-muconate lactonizing enzyme (CMLE) and of *cis,cis*-muconate lactonizing enzyme (MLE). (A) By the IgG fraction from antisera prepared against CMLE. The protein concentration of the IgG fraction was 3.0 mg/ml. The uninhibited activity of CMLE was 0.35 unit; the uninhibited activity of MLE was 0.45 unit. (B) By the IgG fraction from antisera prepared against MLE. The protein concentration of the IgG fraction was 2.0 mg/ml. The uninhibited activities of the enzymes were as in part A.

preparation is homogeneous, it cannot be taken as conclusive evidence. It is worthy of note that a single protein band is observed after β-carboxy-*cis,cis*-muconate-lactonizing enzyme and *cis,cis*-muconate-lactonizing enzyme are subjected to electrophoresis on the same gel (Figure 8).

Molecular Weight. The molecular weights of the two lactonizing enzymes were indistinguishable (about 190,000 daltons) when compared on columns of Bio-Gel Agarose A1.5m.

Subunit Size. Estimation of the subunit sizes of the lactonizing enzymes by sodium dodecyl sulfate electrophoresis yielded values of 40,000 daltons.

Amino Acid Content. The amino acids yielded by timed hydrolysis of β-carboxy-*cis,cis*-muconate-lactonizing enzyme are shown in Table II. After performic acid oxidation 4.2 residues of cysteic acid were recovered per subunit of the protein. Two of these appear as cysteine after *p*-mercuribenzoate titration, so it appears that the protein possesses one disulfide bond per subunit. Unlike β-carboxy-*cis,cis*-muconate-lactonizing enzyme in crude extracts, the pure enzyme is insoluble in 25% saturated ammonium sulfate. It is possible that the difference in solubility properties is due to the formation of the disulfide bond during purification.

Amino-Terminal Sequence. The amino-terminal sequence of the two lactonizing enzymes is shown in Figure 9. Although the sequence of *cis,cis*-muconate-lactonizing enzyme is initiated by the dipeptide Thr-Ser, the amino termini of both lactonizing enzymes are quite hydrophobic. The amino acid sequences in the hydrophobic region are similar and, in the case of the peptide Val-Met, identical. The eighth residue in *cis,cis*-muconate-lactonizing enzyme appeared to be alanine and thus a tripeptide, Val-Met-Ala, may be shared by both lactonizing enzymes. Characterization of the dansyl derivative of the alanine, however, consistently was made ambiguous by the concomitant appearance of the dansyl derivative of isoleucine. Therefore the identity of the eighth residue of *cis,cis*-muconate-lactonizing enzyme is uncertain.

Discussion

Serological Evidence. All *Pseudomonas* species that employ the β-ketoadipate pathway for the dissimilation of aromatic compounds regulate the synthesis of the enzymes by a common mechanism. Characteristically, β-ketoadipate triggers the synthesis of the three enzymes that give rise to it from β-carboxy-*cis,cis*-muconate (Ornston, 1966c). Although many bacterial genera employ the β-ketoadipate pathway, this control system appears to be unique to *Pseudomonas*. The conservation of this control mechanism within the genus suggested to Canovas *et al.* (1967) that the pathway had a single evolutionary origin within the *Pseudomonas* genus. This conclusion was fully supported by a serological survey conducted by Stanier *et al.* (1970) who used complement fixation to determine the serological distance of *cis,cis*-muconate-lactonizing enzyme and muconolactone isomerase from homologous enzymes formed by other *Pseudomonas* species. Although the method was successful in determining

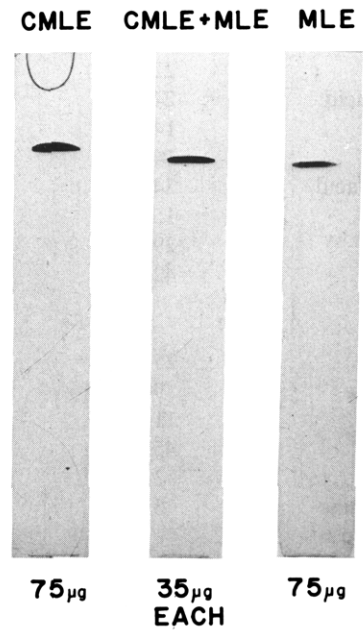

FIGURE 8: Disc gel electrophoresis of β-carboxy-*cis,cis*-muconate-lactonizing enzyme (CMLE, left) and of *cis,cis*-muconate-lactonizing enzyme (MLE, right). The middle gel contained equal amounts of CMLE and MLE.

Ile-Leu-Val-Met-Ala-·····
β-carboxy-*cis,cis*-muconate-lactonizing enzyme

Thr-Ser-Ala-Leu-Ile-Val-Met-Ala-Asx-Ala-·····
(Ile)
cis,cis-muconate-lactonizing enzyme

FIGURE 9: The amino-terminal sequences of β-carboxy-*cis,cis*-muconate-lactonizing enzyme (CMLE) and *cis,cis*-muconate-lactonizing enzyme (MLE). As noted in the text, the identity of the eighth residue is uncertain. It is either alanine, in which case the proteins share the common tripeptide Val-Met-Ala, or isoleucine, in which case only the dipeptide Val-Met is shared.

the serological relatedness of the enzymes from species closely related to *P. putida* biotype A, it failed to detect any immunological similarities between the *cis,cis*-muconate-lactonizing enzyme and the muconolactone isomerase of *P. putida* and the presumably homologous enzymes formed by the relatively distant *Pseudomonas* species *P. cepacia* (= *multivorans*). With the limits of the technique thus defined, it is not surprising that antisera prepared against the *P. putida* enzymes failed to react with the possibly homologous β-carboxy-*cis,cis*-muconate-lactonizing enzyme and γ-carboxymuconolactone decarboxylase from the same strain (Stanier *et al.*, 1970): the regulatory evidence indicates that any evolutionary divergence that yielded the analogous enzymes of the β-ketoadipate pathway must have preceded the divergence of *P. cepacia* and *P. putida*.

Our results confirm those of Stanier *et al.* (1970) and extend them to include the observation that antisera prepared against β-carboxy-*cis,cis*-muconate-lactonizing enzyme from *P. putida* strain 90 failed to react with *cis,cis*-muconate-lactonizing enzyme from the same strain. Thus the two lactonizing enzymes are serologically remote.

TABLE II: Amino Acid Composition of β-Carboxy-*cis,cis*-muconate-Lactonizing Enzyme.[a]

Amino Acid	Calcd No. of nmoles	No. of Residues/40,000 Daltons
Lysine	12.2	13.7
Histidine	9.9	11.1
Arginine	22.4	25.2
Aspartic acid	24.8	27.9
Threonine	14.8	16.6
Serine	14.3	16.1
Glutamic acid	34.2	38.4
Proline	15.2	17.1
Glycine	29.5	33.2
Alanine	42.7	48.0
Cystine		1.0[b]
Cysteine		2.1[c]
Valine	25.6	28.8
Methionine	9.3	10.4
Isoleucine	11.8	13.3
Leucine	45.0	50.7
Tyrosine	3.7	4.2
Phenylalanine	6.9	7.8
Tryptophan		4.3[d]

[a] Hydrolysate derived from 40 μg of protein was applied to each column of the amino acid analyzer. [b] ½(total half-cystine after dimethyl sulfoxide oxidation) − (cysteine). [c] Determined by *p*-mercuribenzoate titration. [d] Determined after 24-hr hydrolysis in the presence of thioglycolic acid (Matsubara and Sasaki, 1969).

TABLE III: Mole Fractions of Amino Acids in β-Carboxy-*cis,cis*-muconate-Lactonizing Enzyme, *cis,cis*-Muconate-Lactonizing Enzyme, and Crude Extract of *P. putida*.

Amino Acid	Mole Fraction (%) in β-Carboxy-*cis,cis*-muconate-Lactonizing Enzyme	Mole Fraction (%) in *cis,cis*-Muconate-Lactonizing Enzyme	Mole Fraction (%) in Crude Extract
Lysine	3.8	3.6	6.8
Histidine	3.1	2.0	2.3
Arginine	7.0	7.8	5.6
Aspartic acid	7.7	8.6	8.2
Threonine	4.6	4.9	4.6
Serine	4.4	5.0	4.9
Glutamic acid	10.6	10.8	10.0
Proline	4.7	4.2	4.9
Glycine	9.2	9.1	11.1
Alanine	13.2	11.7	11.5
Valine	7.9	5.8	7.3
Methionine	2.9	1.9	2.2
Isoleucine	3.7	7.8	5.3
Leucine	14.0	13.0	9.3
Tyrosine	1.2	1.3	2.3
Phenylalanine	2.2	2.3	3.8

Evolutionary Distance between the Lactonizing Enzymes. How great an evolutionary distance does the absence of immunological cross-reaction reflect? Comparison of the amino acid composition of the two lactonizing enzymes (Table III) reveals some marked discrepancies: for example, *cis,cis*-muconate-lactonizing enzyme contains twice as much isoleucine as β-carboxy-*cis,cis*-muconate-lactonizing enzyme. We expect that such differences reflect considerable variations in the primary sequences of the two proteins. On the other hand, the mole fraction of some amino acids is nearly identical in the two enzymes: the amount of lysine in the enzymes is considerably lower than that of crude extract and both enzymes contain a significantly high amount of leucine (Table III). The two lactonizing enzymes possess similar molecular weights, subunit sizes, and crystalline structures. These observations, coupled with those of the following paper (Parke *et al.*, 1973), suggest to us that enzymes of the catechol pathway (*cis,cis*-muconate-lactonizing enzyme and muconolactone isomerase) are encoded by a genetic segment that is homologous to the segment that codes for the analogous enzymes of the protocatechuate pathway (β-carboxy-*cis,cis*-muconate-lactonizing enzyme and γ-carboxymuconolactone decarboxylase). The putative homology must be ancient and we expect the primary structures of the proteins to contain widely divergent sequences. Nevertheless, we believe it likely that a sequence analysis more extensive than that depicted in Figure 9 will reveal demonstrably homologous regions of primary structure in the analogous enzymes.

An additional primary sequence that should be of interest for comparison is that of the β-carboxy-*cis,cis*-muconate-lactonizing enzyme formed by *Aspergillus niger*. A preliminary report (Thatcher and Cain, 1972) indicates that the eucary-

otic enzyme which gives rise to β-carboxymuconolactone (Figure 1) is now available in pure form. Comparison of the primary structures of the two β-carboxy-*cis,cis*-muconate-lactonizing enzymes may yield insight into convergent evolutionary forces that produced enzymes with similar activities

Acknowledgments

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References

Cain, R. B., Bilton, R. F., and Darrah, J. A. (1968), *Biochem. J. 108*, 797.
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. 1*, 289.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.
Evans, W. C. (1947), *Biochem. J. 41*, 373.
Gross, S. R., Gafford, R. S., and Tatum, E. L. (1956), *J. Biol. Chem. 219*, 781.
Jacoby, W. B. (1971), *Methods Enzymol. 22*, 248.
Johnson, B. F., and Stanier, R. Y. (1971), *J. Bacteriol. 107*, 476.
Kekwick, R. A. (1940), *Biochem. J. 34*, 1248.
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.
Meagher, R. B., and Ornston, L. N. (1973), *Biochemistry 12*, 3523.
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.
Rann, D. L., and Cain, R. B. (1969), *Biochem. J. 114*, 77p.
Spencer, R. L., and Wold, F. (1969), *Anal. Biochem. 32*, 185.
Stanier, R. Y., and Ingraham, J. L. (1954), *J. Biol. Chem. 210*, 799.
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.
Thatcher, D. R., and Cain, R. B. (1972), *Biochem. J. 127*, 32p.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Wiener, A. M., Platt, T., and Weber, K. (1972), *J. Biol. Chem. 247*, 3242.

Relationships among Enzymes of the β-Ketoadipate Pathway. III. Properties of Crystalline γ-Carboxymuconolactone Decarboxylase from *Pseudomonas putida*†

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ABSTRACT: Procedures for the purification and crystallization of γ-carboxymuconolactone decarboxylase from extracts of *Pseudomonas putida* are described. In several respects this enzyme is quite similar to muconolactone isomerase, an enzyme that catalyzes a reaction analogous to that of the decarboxylase. The proteins appear to have similar molecular sizes (93,000 daltons), subunit sizes (12,000 to 13,000 daltons), and crystalline structures. For these reasons it seems likely that they are the products of homologous structural genes.

γ-Carboxymuconolactone is chemically unstable. It decarboxylates spontaneously giving rise to β-ketoadipate enol lactone (Ornston and Stanier, 1966) (Figure 1). The reaction is catalyzed by γ-carboxymuconolactone decarboxylase, an inducible enzyme formed by many bacteria during the utilization of β-carboxy-*cis,cis*-muconate. The mechanism of the γ-carboxymuconolactone decarboxylase reaction is likely to be similar to that of muconolactone isomerase, another inducible bacterial enzyme that gives rise to β-ketoadipate enol lactone (Ornston and Stanier, 1966) (Figure 1). The first investigations of the two enzymes in *Pseudomonas putida* revealed that they both were thermostable and that they appeared to have molecular sizes corresponding to 93,000 daltons as estimated by gel filtration

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