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

We thank May K. Ornston for her helpful suggestions and assistance.

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*†

Donna Parke, Richard B. Meagher, and L. Nicholas Ornston*

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

† From the Department of Biology, Yale University, New Haven, Connecticut 06520. *Received March 23, 1973*. This work was supported by Grant GM18566-01 from the U. S. Public Health Service. Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research. D. P. is a predoctoral trainee supported by Public Health Service Grant HD-00032-10 from the National Institute of Child Health and Human Development and R. B. M. was a predoctoral trainee supported by Public Health Service Training Grant GM397-11 from the National Institute of General Medical Sciences.

TABLE I: Purification of γ-Carboxymuconolactone Decarboxylase.

Step	Vol (ml)	Total Act. (Units)	Total Protein (g)	Sp Act. (Units/mg)	Recov (%)	Purifcn (-fold)
1. Crude extract	11,177	1,155,570	363.7	3.2	100	1.0
2. Dialyzed extract	12,896	1,063,426	276.6	3.8	92	1.2
3. DEAE-cellulose eluate	3,029	637,507	29.6	21.5	55	6.7
4. 45–65% saturated ammonium sulfate fraction	438	495,350	14.7	33.7	43	10.5
5. Heat-treated extract	884	481,780	2.2	218.0	42	68.1
6. Bio-Gel eluate	495	387,552	0.67	579.3	33	181.0
7. QAE-Sephadex eluate	87	208,800	0.11	1951.4	18	609.8
8. 0–70% saturated ammonium sulfate fraction	7	190,467	0.10	1943.5	16.5	607.3
9. Crystallization	3.2	185,025	0.086	2139.0	16	668.4

(Ornston, 1966a,b). For these reasons, it was suggested that the structural genes for the enzymes may be evolutionarily homologous.

The test for homology must be the comparison of primary sequences, a task that requires substantial quantities of pure proteins and is simplified if the subunit sizes of the proteins are relatively small. In the first paper of this series (Meagher and Ornston, 1973) we described the purification of muconolactone isomerase and reported that it possesses subunits of about 12,000 daltons. In this paper we present a procedure for obtaining homogeneous preparations of γ-carboxymuconolactone decarboxylase and present evidence indicating that the subunit size of this protein is approximately 13,000 daltons.

Experimental Section

Bacterial Growth. Pseudomonas putida strain PRS2105 was grown under conditions described in the previous paper (Patel *et al.*, 1973).

Buffers. Buffer A was 10 mM ethylenediamine dihydrochloride–1 μM $MnCl_2$, adjusted to pH 7.3 at 23° with NaOH.

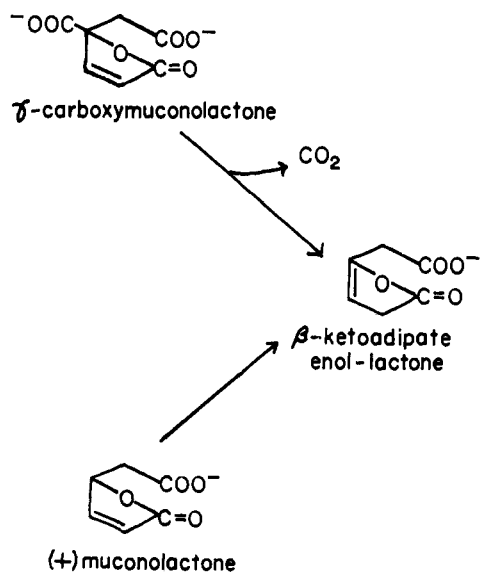

FIGURE 1: Convergent reactions for the transformation of γ-carboxymuconolactone and muconolactone to β-ketoadipate enol lactone.

Buffer B was prepared by mixing Na_2HPO_4 and KH_2PO_4 to a final phosphate concentration of 20 mM and a final pH of 7.0 at 23°. Buffer C contained 20 mM Tris-chloride and 0.1 M NaCl at a final pH of 7.2 at 5°.

Chemicals. Hydrazine was purchased from Pierce Chemical Co. and quaternary aminoethyl- (QAE) Sephadex A-50 from Pharmacia Fine Chemicals. Sigma provided *p*-toluenesulfonic acid, and 3-(2-aminoethyl)indole was obtained from Matheson, Coleman & Bell.

Enzyme Assay. Published spectrophotometric procedures were used for the determination of the activity of γ-carboxymuconolactone decarboxylase (Ornston, 1966a). During the first four steps of purification (Table I) the conversion of the substrate to β-ketoadipate was measured in the presence of excess β-ketoadipate enol lactone hydrolase; $\Delta\epsilon_{230}$ for this reaction is 5170 M^{-1} cm^{-1}. Heat treatment (step 5 in Table I) destroys hydrolase activity. After this step the conversion of γ-carboxymuconolactone to β-ketoadipate enol lactone, for which $\Delta\epsilon_{230}$ is 2730 M^{-1} cm^{-1}, was measured in the absence of the hydrolase. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Purification of γ-Carboxymuconolactone Decarboxylase. The preparation of crude extract (step 1), ammonium sulfate treatment (step 2), and DEAE-cellulose chromatography (step 3) were described in the preceding paper (Patel *et al.*, 1973). The results of these and subsequent purification steps are summarized in Table I. The portion of the DEAE-cellulose eluate containing γ-carboxymuconolactone decarboxylase activity (step 3, Table I) was subjected to ammonium sulfate fractionation by previously described methods (Meagher and Ornston, 1973); material precipitating between 45% and 65% of saturation with respect to ammonium sulfate was dialyzed against buffer B and adjusted to a protein concentration of 20 mg/ml (step 4, Table I). The extract was maintained at 50° for 30 min and cooled to 5°, and solid ammonium sulfate was added to 30% of saturation. Precipitated protein was removed by centrifugation, the pellet was washed with buffer B that was 30% saturated with respect to ammonium sulfate, and the supernatant fractions were pooled (step 5 in Table I). Ammonium sulfate was added to 75% of saturation and the protein that it precipitated was collected by centrifugation, dialyzed against buffer B containing 0.1 M NaCl, and applied in 50 ml fractions to 5.0 × 100 cm columns of Bio-Gel Agarose A1.5m that had been equilibrated with the same buffer. The buffer was used to elute the protein at a flow rate of 60 ml/hr; fractions of 10 ml were

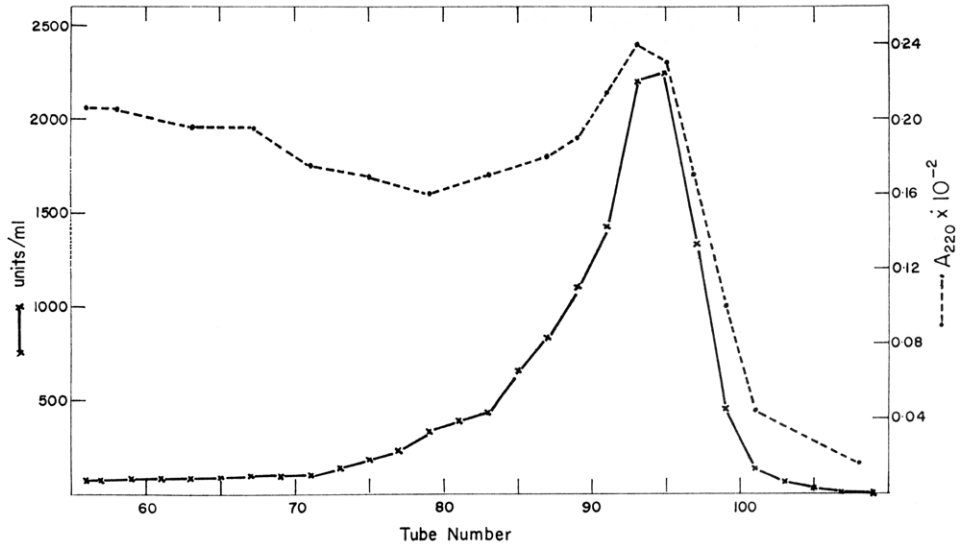

FIGURE 2: The elution of γ-carboxymuconolactone decarboxylase from a Bio-Gel Agarose A1.5m column. Conditions of chromatography are described in the text.

collected. The elution profile of the enzyme is shown in Figure 2. Fractions containing γ-carboxymuconolactone decarboxylase activity were pooled (step 6, Table I); the enzyme was precipitated by adding ammonium sulfate to 75% of saturation, collected by centrifugation and dialyzed against buffer C. In a volume of 39 ml, the enzyme was applied to a 2.6 × 24.5 cm QAE-Sephadex column that had been equilibrated with buffer C. Protein was eluted from the column at a flow rate of 15 ml/hr with buffer C containing NaCl in stepwise increments of concentration: γ-carboxymuconolactone decarboxylase was retained on the column at a NaCl concentration of 0.13 M and was eluted with buffer contain-

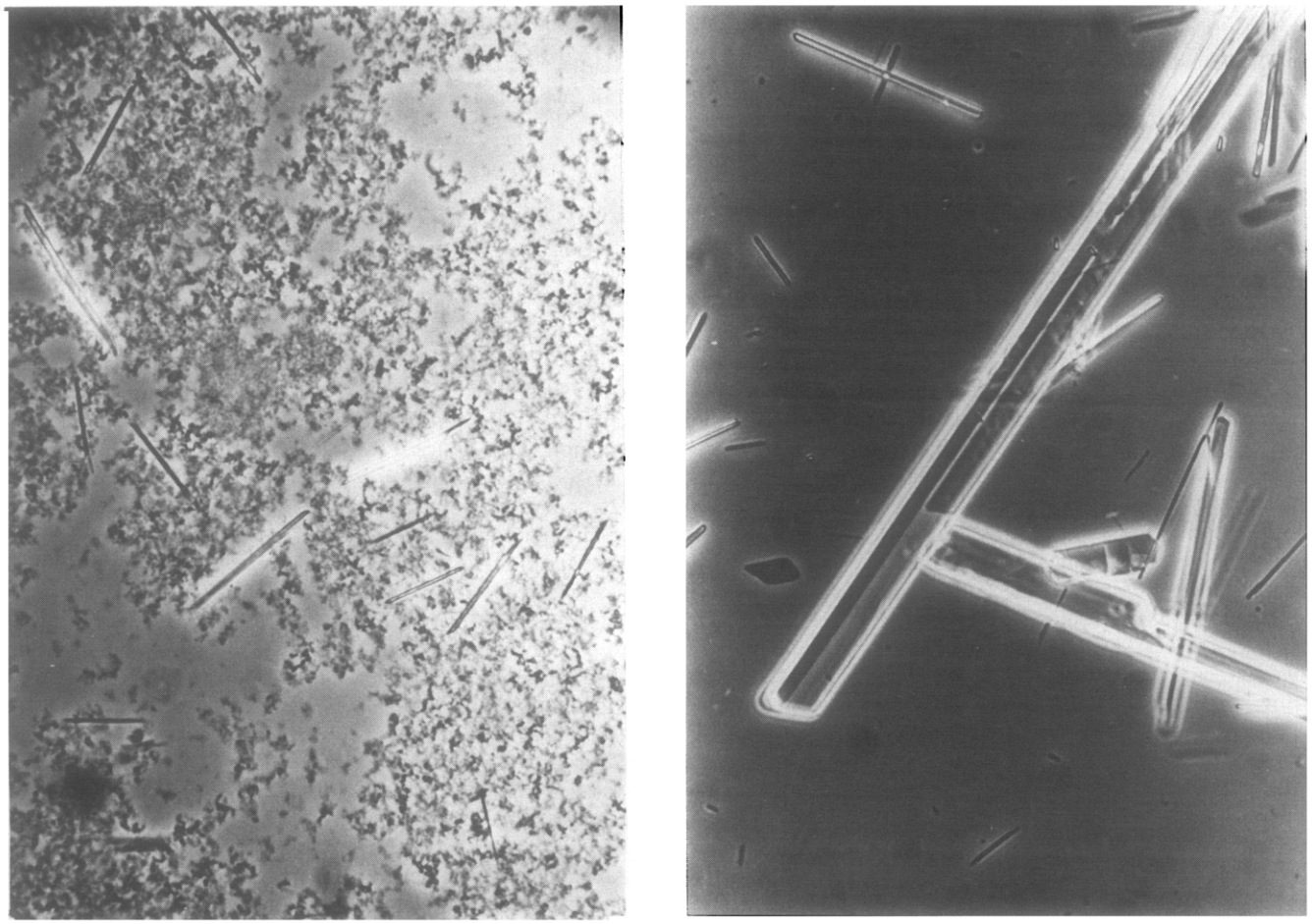

FIGURE 3: Crystals of γ-carboxymuconolactone decarboxylase (left) and of muconolactone isomerase (right) formed in ammonium sulfate. The magnification is 1500× (CMD) and 500× (MI).

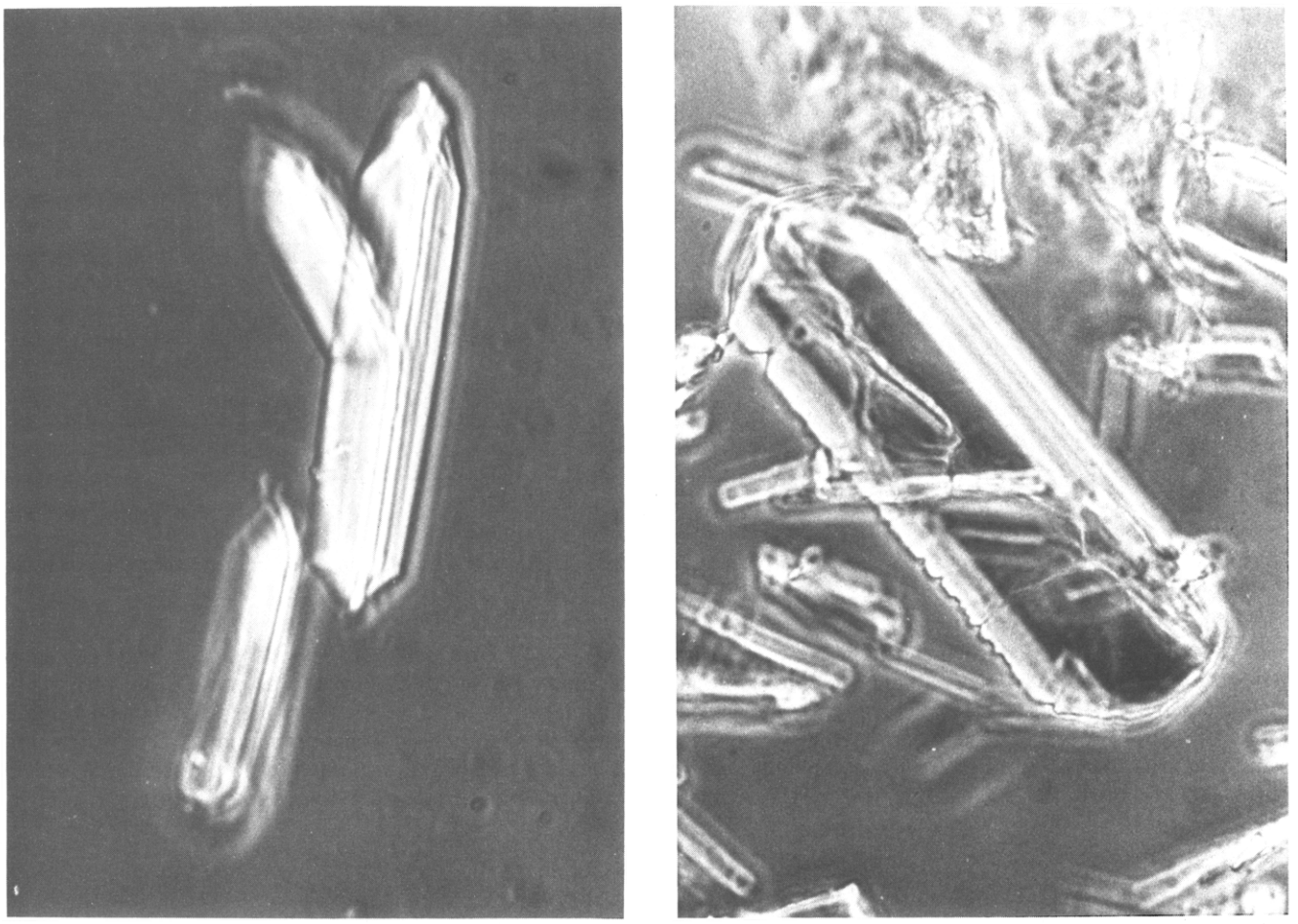

FIGURE 4: Crystals of γ-carboxymuconolactone decarboxylase (left) and of muconolactone isomerase (right) obtained by dialysis against distilled water. The magnification is 1200× (CMD) and 1000× (MI).

ing 0.3 M NaCl. Fractions of 3.3 ml were collected; tubes containing γ-carboxymuconolactone decarboxylase were pooled (step 7, Table I) and were used as a source of crystalline enzyme.

Solid ammonium sulfate was added to bring the QAE-Sephadex eluate to 70% of saturation with respect to ammonium sulfate. After centrifugation of the preparation, the pellet was dissolved in buffer B at a protein concentation of 14 mg/ml (step 8, Table I). Microcrystals of the decarboxylase were formed by slowly adding buffer B that was saturated with ammonium sulfate to bring the solution to 55% of saturation. The crystals were collected by centrifugation, washed with buffer B that was 55% saturated with respect to ammonium sulfate and dissolved at 23° in buffer B that was 7.5% saturated with respect to ammonium sulfate (step 9, Table I). Denatured protein was removed by centrifugation, and the enzyme was twice recrystallized by the same procedure. The microcrystals were stored in buffer B that contained ammonium sulfate at 30% of saturation. This material was used for characterization of the enzyme. The enzyme also could be crystallized by dialysis against distilled water.

Immunological Techniques. Antisera against the decarboxylase were prepared by previously described methods (Meagher and Ornston, 1973), and serological cross reactivity was tested on Ouchterlony double-diffusion plates by the method of Stanier *et al.* (1970).

Acrylamide Gel Electrophoresis. Disc gel electrophoresis (Davis, 1964) was performed at 5° with 7% gels as described previously (Meagher and Ornston, 1973); the reservoir buffer was 25 mM Tris–25 mM glycine, pH 9.0, containing 1 mM mercaptoethanol.

A discontinuous system was used for sodium dodecyl sulfate electrophoresis (Weber and Osborn, 1969); the reservoir buffer was 25 mM Tris–25 mM glycine, pH 9.0, containing 0.1% sodium dodecyl sulfate and 0.1% mercaptoethanol. The 15% gel was composed of 15% acrylamide, 0.36% bisacrylamide, 0.1% sodium dodecyl sulfate, 0.005% ammonium persulfate, 0.08% *N,N,N′,N′*-tetramethylethylenediamine, and 25 mM Tris–25 mM glycine titrated to pH 7.0 with HCl. Gels were stained and destained by previously described procedures (Meagher and Ornston, 1973).

Amino Acid Analysis. Amino acid analyses were conducted by previously described methods (Meagher and Ornston, 1973; Patel *et al.*, 1973). The half-cystine content was measured as cysteic acid on a Beckman 120B amino acid analyzer after a 24-hr hydrolysis in the presence of 2% dimethyl sulfoxide (Spencer and Wold, 1969). Tryptophan content was determined in 24-, 48-, and 72-hr hydrolysates that had been prepared in the presence of 3 N *p*-toluenesulfonic acid in 0.02% 3-(2-aminoethyl)indole (Liu and Chang, 1971). An independent determination of tryptophan was made by the method of Goodwin and Morton (1946).

Amino-Terminal Residues. The amino-terminal residue of γ-carboxymuconolactone decarboxylase was identified as its dansyl derivative (Wiener *et al.*, 1972).

TABLE II: Amino Acid Composition of γ-Carboxymuconolactone Decarboxylase.[a]

Amino Acid	Calcd No. of nmoles	No. of Residues/13,000 Daltons
Lysine	12.8[b]	4.2
Histidine	14.6[b]	4.8
Arginine	29.0[b]	9.5
Aspartic acid	40.0	13.2
Threonine	14.2	4.7
Serine	11.7	3.9
Glutamic acid	43.0	14.2
Proline	8.4	2.8
Glycine	23.5	7.7
Alanine	34.1	11.2
½-Cystine	5.6[c]	1.8
Valine	18.3	6.0
Methionine	16.4	5.4
Isoleucine	19.9	6.6
Leucine	31.4	10.3
Tyrosine	6.2	2.0
Phenylalanine	7.7	2.5
Tryptophan	8.5	2.9[d]

[a] Hydrolysate derived from 40 μg of protein was applied to each column of the amino acid analyzer. [b] Values for the basic amino acids were derived from hydrolysis in the presence of toluenesulfonic acid. [c] Hydrolysis in the presence of dimethyl sulfoxide. [d] Average of values from timed hydrolysis in the presence of thioglycolic acid (2.8 residues) and from a determination of material absorbing at 280 nm (3.1 residues).

Results

Crystalline Shape. When prepared in ammonium sulfate, crystals of γ-carboxymuconolactone decarboxylase and of muconolactone isomerase possess similar slender, elongated shapes (Figure 3). Crystallization of the two enzymes in distilled water produces shorter, thicker forms (Figure 4).

Serological Studies. Antisera against thrice crystallized γ-carboxymuconolactone decarboxylase formed a single sharp precipitin band when tested on Ouchterlony double diffusion plates against either the crystalline enzyme or against crude extracts of induced cells. Muconolactone isomerase did not cross react with antisera against γ-carboxymuconolactone decarboxylase.

Disc Gel Electrophoresis. A single protein band was observed when crystalline γ-carboxymuconolactone decarboxylase was subjected to disc gel electrophoresis; as shown in Figure 5, the decarboxylase migrates slightly more rapidly than muconolactone isomerase under these conditions.

Subunit Size. Three independent runs of sodium dodecyl sulfate electrophoresis yielded subunit molecular weights ranging from 12,300 to 13,400 daltons for γ-carboxymuconolactone decarboxylase. The average value was taken to be 13,000 daltons.

Amino Acid Composition. The amino acid composition of γ-carboxymuconolactone decarboxylase hydrolysates is shown in Table II.

Amino Terminus. Dansyl-methionine, slightly contaminated with dansyl-glycine, was the only α-amino-substituted amino acid recovered after dansylated γ-carboxymuconolactone decarboxylase was hydrolyzed. Therefore the amino terminus of the decarboxylase, like that of muconolactone isomerase, appears to be methionine.

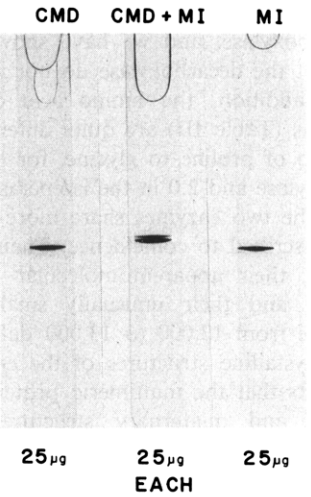

FIGURE 5: Disc gel electrophoresis of γ-carboxymuconolactone decarboxylase (CMD) and of muconolactone isomerase (MI). The middle gel contained both CMD and MI.

Discussion

Homogeneity of Crystalline γ-Carboxymuconolactone Decarboxylase. After three crystallizations, γ-carboxymuconolactone decarboxylase behaves as a single protein in disc gel electrophoresis, in sodium dodecyl sulfate electrophoresis and on Ouchterlony double-diffusion plates. It thus appears that unlike former preparations of the decarboxylase (Ornston, 1966a) the crystalline enzyme is homogeneous.

Comparison of γ-Carboxymuconolactone Decarboxylase and Muconolactone Isomerase. γ-Carboxymuconolactone decarboxylase and muconolactone isomerase are immunologically remote. Stanier *et al.* (1970) demonstrated that

TABLE III: Mole Fractions of Amino Acids in γ-Carboxymuconolactone Decarboxylase, Muconolactone Isomerase, and Crude Extract of *P. putida*.

Amino Acid	Mole Fraction (%) in: γ-Carboxymuconolactone Decarboxylase	Muconolactone Isomerase	Crude Extract
Lysine	3.9	5.6	6.8
Histidine	4.4	6.3	2.3
Arginine	8.7	6.4	5.6
Aspartic acid	12.1	10.5	8.2
Threonine	4.3	4.0	4.6
Serine	3.6	4.9	4.9
Glutamic acid	13.0	9.7	10.0
Proline	2.6	6.6	4.9
Glycine	7.1	3.3	11.1
Alanine	10.3	7.7	11.5
Valine	5.5	7.3	7.3
Methionine	5.0	5.3	2.2
Isoleucine	6.1	3.0	5.4
Leucine	9.5	12.9	9.3
Tyrosine	1.8	3.4	2.3
Phenylalanine	2.3	3.2	3.8

antisera prepared against the isomerase do not cross-react with the decarboxylase, and we have shown that antisera prepared against the decarboxylase do not interact with the isomerase. In addition, the amino acid compositions of the two proteins (Table III) are quite different in some respects: the ratio of proline to glycine, for example, is 0.36 in the decarboxylase and 2.0 in the isomerase. Despite these dissimilarities, the two enzymes share more properties than can be readily ascribed to coincidence. Their amino termini are methionine, their apparent molecular sizes are about 93,000 daltons, and their unusually small subunit sizes are in the range from 12,000 to 13,000 daltons. The similarity of the crystalline structures of the enzymes (Figures 4 and 5) suggests that the multimeric proteins may possess similar tertiary and quaternary structures. It therefore appears likely that the two enzymes are the products of homologous structural genes. The validity of this proposal can be tested only by direct comparison of the primary structures of the proteins.

Acknowledgments

We thank May K. Ornston for her valuable contributions to the preparation of this manuscript.

References

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.
Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.
Liu, T.-Y., and Chang, Y. H. (1971), *J. Biol. Chem. 246*, 2842.
Lowry, O. H., Rosebrough, N. J., Farr, A. C., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
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., and Stanier, R. Y. (1966), *J. Biol. Chem. 241*, 3776.
Patel, R. N., Meagher, R. B., and Ornston, L. N. (1973), *Biochemistry 12*, 3531.
Spencer, R. L., and Wold, F. (1969), *Anal. Biochem. 32*, 185.
Stanier, R. Y., Wachter, D., Gasser, C., and Wilson, A. C. (1970), *J. Bacteriol. 102*, 351.
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.

Elevated Levels of Acceptor Activity of Hepatoma Transfer Ribonucleic Acid†

Andrew J. Ouellette‡ and Milton W. Taylor*

ABSTRACT: It was originally observed that phenylalanyl-tRNA of hepatoma 5123 accepted 3- to 4-fold the amount of phenylalanine than isogenic liver tRNA. A comparison of the extents of aminoacylation of liver and hepatoma tRNAs for 15 other amino acids were compared. In 7 cases, the extent of hepatoma aminoacyl-tRNA formation was significantly greater than that for liver. This difference was less striking for another four amino acids, and the remainder gave essentially identical extents of acylation. No instances were found in which greater amounts of amino acid were incorporated into liver tRNA. Control experiments effectively eliminated several sources of artifact which might have accounted for these differences. Periodate oxidation experiments revealed that the ratio of periodate protected:potentially acylatable tRNA was the same in both tissues. It appears, therefore, that hepatoma 5123D contains, per microgram of tRNA, a greater number of tRNA molecules capable of amino acid acceptance than liver, and it is suggested that this may represent differential rates of tRNA processing and maturation in these tissues.

The observation of tumor-specific phenylalanyl-tRNAs in Morris hepatomas 5123C (Gonano *et al.*, 1971) and 5123D (Volkers and Taylor, 1971), and the suggestion from several laboratories (Strehler *et al.*, 1967; Ilan *et al.*, 1970; Kanabus and Cherry, 1971) that processes of differentiation might be accompanied by changes in aminoacyl-tRNA synthetases, led us to investigate whether the phenylalanyl-tRNA synthetases of hepatoma 5123D and rat liver had different properties. No differences were found between the liver and hepatoma enzymes (Ouellette, 1972); however, it was noted that equivalent amounts of tRNA from these tissues differed in their extents of phenylalanine acceptance. Regardless of whether the liver or hepatoma enzyme was used, the aminoacylation of hepatoma tRNA consistently yielded twice the amount of phenylalanyl-tRNA as did liver tRNA. This paper describes a study determining the general nature of this phenomenon, and reveals significant differences between the tRNA populations of these two tissues.

Materials and Methods

The source and maintenance of tissues and the preparation of total tRNA have previously been described (Ouellette, 1972).

† From the Department of Microbiology, Indiana University, Bloomington, Indiana 47401. *Received March 6, 1973.* Supported by U. S. Public Health Service Grant No. CA-11496 and CA-10417. A. J. O. was a predoctoral fellow on Public Health Service Grant No. 5T1-GM503.

‡ Present address: Department of Surgery, Massachusetts General Hospital, Boston, Mass.