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Evolutionary Relationships Among γ-Carboxymuconolactone Decarboxylases

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γ-Carboxymuconolactone decarboxylase (EC 4.1.1.44) from *Azotobacter vinelandii* resembled the isofunctional enzymes from *Acinetobacter calcoaceticus* and *Pseudomonas putida*. All three decarboxylases appeared to be hexamers formed by association of identical subunits of about 13,300 daltons. The *A. vinelandii* and *P. putida* decarboxylases cross-reacted immunologically with each other, and the NH_2-terminal amino acid sequences of the enzymes differed in no more than 7 of the first 36 residues. In contrast, the *A. calcoaceticus* decarboxylase did not cross-react with the decarboxylase from *A. vinelandii* or *P. putida*; the NH_2-terminal amino acid sequences of these enzymes diverged about 50% from the NH_2-terminal amino acid sequence of the *A. calcoaceticus* decarboxylase.

Immunological comparisons conducted with protocatechuate oxygenase (EC 1.13.11.3) (2) and γ-carboxymuconolactone decarboxylase (EC 4.1.1.44) (1), enzymes of the protocatechuate branch of the β-ketoadipate pathway, indicated that the *Azotobacter* species enzymes are evolutionarily homologous to isofunctional enzymes formed by fluorescent *Pseudomonas* species. As described here and elsewhere (1, 10, 13), isofunctional enzymes formed by members of other bacterial genera appear to be immunologically distant, and these organisms govern expression of the enzymes with induction patterns unlike those shared by *Azotobacter* and *Pseudomonas* species (12). The conclusions drawn from immunological evidence are supported by chemical and physical data presented here. The results show that the γ-carboxymuconolactone decarboxylases of *Azotobacter* and *Pseudomonas* species are closely related to each other and more distantly related to the evolutionarily homologous γ-carboxymuconolactone decarboxylase of *Acinetobacter* species.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Azotobacter vinelandii* OP was obtained from Paul E. Bishop, North Carolina State University, Raleigh, N.C. Large-scale growth of *A. vinelandii* was accomplished at 30°C in a New Brunswick Fermacell CF-130 fermentor containing 100 liters of modified Burk medium (14). *Pseudomonas putida* PRS 2260 (4) and *Acinetobacter calcoaceticus* ADP 152 (17) were described recently. For immunodiffusion studies, the appropriate strains of *A. vinelandii*, *P. putida*, and *A. calcoaceticus* were grown in 500-ml Erlenmeyer flasks containing 150 ml of mineral medium (5, 14) supplemented with 10 mM p-hydroxybenzoate.

Purification of γ-carboxymuconolactone decarboxylase from *A. vinelandii*. All purification procedures were performed between 0 to 4°C. Buffer A was 20 mM Tris-hydrochloride (pH 7.5) containing 25 μM dithiothreitol; buffer B was 10 mM Na_2HPO_4-KH_2PO_4 (pH 7.0); and buffer C was 20 mM Tris-hydrochloride containing 0.1 M NaCl. Cell pastes with a wet weight of 200 g were suspended in three volumes of buffer A and disrupted by passage through an American Instrument continuous-flow French pressure cell at 12,000 lb/in^2. Unbroken cells and debris were removed by centrifugation at 40,000 × *g* for 20 min. The resultant crude extract (Table 1 step 1) was brought to 30% saturation by the addition of ammonium sulfate followed by centrifugation at 40,000 × *g* for 20 min. Ammonium sulfate treatment was repeated on the supernatant fraction until 75% saturation was reached. After centrifugation, the protein pellet was dissolved in buffer A (Table 1, step 2) and dialyzed against three changes of this buffer over 48 h. The dialysis was applied onto a DEAE-cellulose column (5 by 30 cm) previously equilibrated with buffer A. The column was washed with three volumes of the same buffer, after which a continuous linear gradient, constructed from 0 to 0.3 M NaCl in buffer A in a total volume of 6 liters, was applied. Fractions of 15 ml were collected at a flow rate of 60 ml/h, and those containing γ-carboxymuconolactone decarboxylase activity, which eluted between 0.16 to 0.19 M NaCl, were pooled (Table 1, step 3). The DEAE-celluose eluate was treated with ammonium sulfate, and the protein pellet that precipitated between 45 to 75% saturation was dissolved in buffer B (Table 1, step 4) and dialyzed thoroughly against the same buffer. The dialysate was

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TABLE 1. *Purification of γ-carboxymuconolactone decarboxylase from A. vinelandii*

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Yield (%)	Purification (fold)
1. Crude extract	610	34,000	17,670	1.92	100	1.0
2. First 30 to 75% saturated ammonium sulfate fraction	310	30,130	8,290	3.63	89	1.9
3. First DEAE-cellulose eluate	500	32,000	1,575	20.3	94	10.6
4. Second 45 to 75% saturated ammonium sulfate fraction	33	31,350	726	43.2	92	22.5
5. Second DEAE-cellulose eluate	120	26,400	356	74.2	78	38.6
6. Third 40 to 65% saturated ammonium sulfate fraction	7	23,920	248	96.5	70	50.3
7. Sephadex G-200 eluate	32	19,840	30.4	653	58	340
8. Sephadex G-100 eluate	35	12,600	10.0	1,260	37	656
9. Quaternary aminoethyl-Sephadex eluate	12	6,040	4.8	1,258	18	655

applied onto a second DEAE-cellulose column (1.6 by 20 cm) previously equilibrated with buffer B. The column was washed with 2 volumes each of 0.01, 0.05, and 0.1 M sodium potassium phosphate buffer, and then a continuous linear gradient, constructed from 0.1 M sodium potassium phosphate buffer to the same buffer containing 0.3 M NaCl in a total volume of 300 ml, was applied. Fractions of 3 ml were collected at a flow rate of 20 ml/h, and those containing the decarboxylase activity were pooled (Table 1, step 5) and fractionated with ammonium sulfate. The protein pellet that precipitated between 40 to 65% saturation was dissolved in buffer A (Table 1, step 6) and applied onto a Sephadex G-200 column (2.5 by 100 cm) previously equilibrated with buffer A. Fractions of 3 ml were collected at a flow rate of 14 ml/h, and those containing a specific activity of the decarboxylase greater than 600 U/mg were combined (Table 1, step 7) and concentrated with ammonium sulfate (0 to 70% saturation). The resultant pellet was dissolved in buffer A and loaded onto a Sephadex G-100 column (2.5 by 40 cm) previously equilibrated with buffer A. The eluate containing the decarboxylase activity (Table 1, step 8) was concentrated with ammonium sulfate (0 to 70% saturation), dialyzed thoroughly against buffer C, and applied onto a quaternary aminoethyl-Sephadex column (0.9 by 8 cm) previously equilibrated with buffer C. The column was washed with 10 volumes of the same buffer, and then a continuous linear gradient, constructed from 0.1 to 0.4 M NaCl in 20 mM Tris-hydrochloride (pH 7.2) in a total volume of 50 ml, was applied. Fractions of 2 ml were collected at a flow rate of 10 ml/h, and those containing the decarboxylase activity were pooled (Table 1, step 9). The quaternary aminoethyl-Sephadex eluate was stored at 4°C in the presence of ammonium sulfate at 70% saturation.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed with 10% disc gels (9). The subunit size of *A. vinelandii* decarboxylase was estimated by sodium dodecyl sufate-gel electrophoresis (15) with previously described standard proteins (17).

Amino acid analysis. The amino acid composition of *A. vinelandii* decarboxylase was determined by a previously described procedure (4) and analyzed with a computer program (8) for the minimum molecular weight, which is the subunit size, of the enzyme.

NH_2-terminal amino acid sequence determination. Previously published procedures (16) were used for determination of the NH_2-terminal amino acid sequence of *A. vinelandii* decarboxylase.

Serological studies. Antisera against *A. vinelandii*, *P. putida*, and *A. calcoaceticus* decarboxylases were prepared (2), and the method of Stanier et al. (13) was used to examine serological cross-reaction on Ouchterlony double-diffusion plates (7).

Chemicals. Chemicals were described previously (2, 17). Quaternary aminoethyl-Sephadex was obtained from Pharmacia Fine Chemicals.

RESULTS

Purity of *A. vinelandii* decarboxylase. The most purified preparation of *A. vinelandii* decarboxylase (Table 1, step 9) possessed a specific activity of 1,260 U/mg. When subjected to electrophoresis on 10% polyacrylamide gels, the decarboxylase preparation migrated as a major band and a slight minor band (Fig. 1). The minor band may have been an electrophoretically different form of the decarboxylase because, as described below, the preparation was immunologically homogeneous, and the NH_2-terminal amino acid sequence of the enzyme was determined without detectable contamination.

Molecular weight and subunit size determinations. The molecular weight of the *A. vinelandii* decarboxylase, close to the molecular weights of the *A. calcoaceticus* and *P. putida* decarboxylases, was estimated to be 85,500 by gel filtration (Fig. 1). The size of the *A. vinelandii* decarboxylase subunit was determined as 13,300 by sodium dodecyl sulfate-gel electrophoresis and as 13,460 by computer-aided analysis of the amino acid composition of the enzyme (8).

Serological properties. Antisera prepared against the purified *A. vinelandii* decarboxylase formed a single precipitin band when diffused against a crude extract of *A. vinelandii* cells in which the enzyme had been induced (Fig. 2). This band formed a spur with a precipitin band formed by a *P. putida* extract containing the

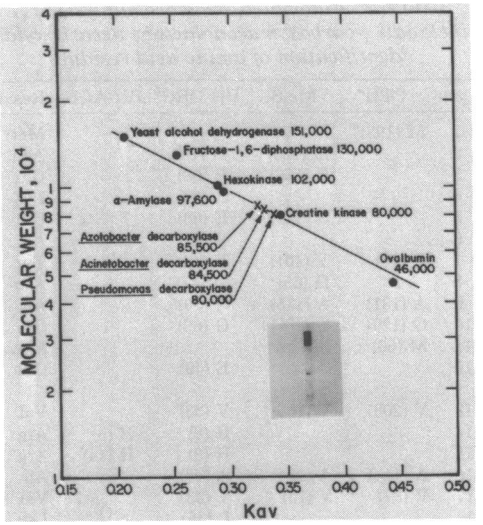

FIG. 1. *Insert depicts a stained 10% polyacrylamide gel on which 50 μg of A. vinelandii decarboxylase (Table 1, step 9) had migrated electrophoretically. The graph shows data indicating the molecular weights of decarboxylases determined by filtration on a standardized Bio-Gel agarose A 1.5m column (2.6 by 100 cm).*

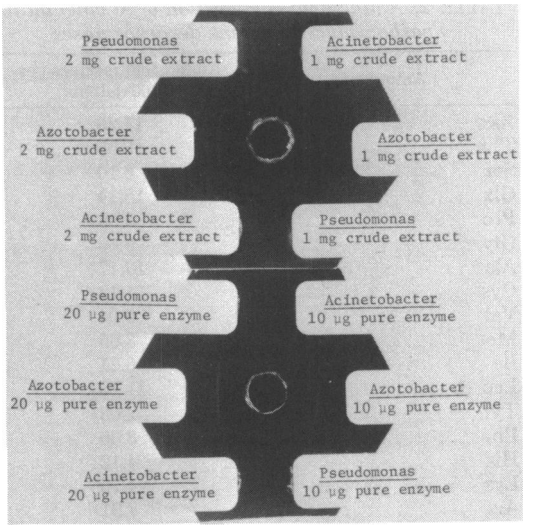

FIG. 2. *Double-diffusion plates showing the immunological cross-reactions of enzymes with A. vinelandii decarboxylase. The center wells received 0.2 ml (containing approximately 3 mg of protein) of antiserum against the A. vinelandii decarboxylase. The outer wells of the top plate received crude extracts of cells in which the decarboxylase had been induced by growth with p-hydroxybenzoate. The outer wells of the bottom plate received the indicated amounts of purified decarboxylases.*

decarboxylase (Fig. 2). No precipitin band was formed with extracts of uninduced *A. vinelandii* and *P. putida* cells (not shown) or with *A. calcoaceticus* extract containing the enzyme (Fig. 2). Similar precipitin patterns were formed when purified decarboxylase was substituted for crude extract in the outer wells (Fig. 2). Thus, the *A. vinelandii* decarboxylase appears to be an immunologically homogeneous preparation that cross-reacts strongly with the *P. putida* decarboxylase and not at all with the *A. calcoaceticus* decarboxylase.

The conclusion that the *A. vinelandii* and *P. putida* decarboxylases resemble each other more closely than they resemble the *A. calcoaceticus* decarboxylase is fortified by the immunodiffusion patterns shown in Fig. 3. Antisera prepared against the *P. putida* decarboxylase form a precipitin band with the *P. putida* and *A. vinelandii* decarboxylases, but not with the *A. calcoaceticus* decarboxylase (Fig. 3). Antisera prepared against the *A. calcoaceticus* decarboxylase formed a precipitin band with this enzyme, but not with the corresponding decarboxylase from *A. vinelandii* or *P. putida* (Fig. 3).

Amino acid composition. The amino acid composition of the *A. vinelandii* decarboxylase is shown in Table 2. Quantitative comparison of amino acid compositions may be achieved by measurement of SΔQ, the sum of the square of the difference in mole fraction of each amino acid that can be readily quantitated in a protein

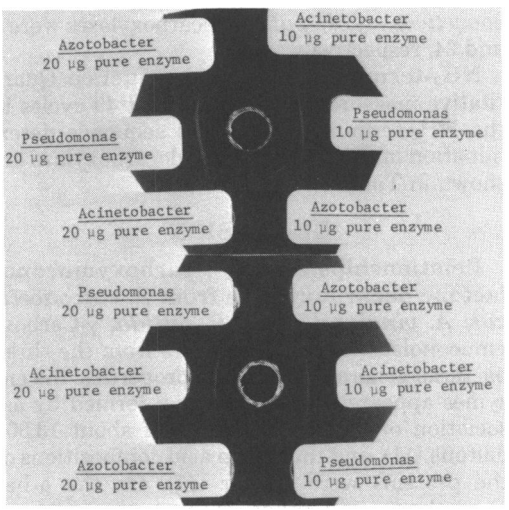

FIG. 3. *Double-diffusion plates on which purified decarboxylases from different bacterial genera were diffused against antiserum prepared against P. putida decarboxylase (center well, top) and against antiserum prepared against A. calcoaceticus decarboxylase (center well, bottom).*

hydrolysate. In over 5,000 pairwise comparisons, Marchalonis and Weltman (3) found that an SΔQ of less than 50 invariably reflected a structural similarity that was revealed by comparison

TABLE 2. *Amino acid composition of A. vinelandii γ-carboxymuconolactone decarboxylase*

Amino acid	Amino acid residues per 13,460 daltons[a]
Asx	11.26
Thr	5.95
Ser	6.10
Glx	15.14
Pro	4.95
Gly	9.86
Ala	13.17
Cys[b]	1.14
Val	9.04
Met	3.05
Ile	6.21
Leu	11.70
Tyr	2.63
Phe	3.66
His	4.12
Lys	4.76
Arg	7.91
Trp[c]	1.18

[a] From computer-aided analysis of amino acid composition (8).

[b] Estimated as cysteic acid after performic acid oxidation (4).

[c] Determined after hydrolysis of a protein sample with 3 N mercaptoethanesulfonic acid (4).

of amino acid sequences. With the *A. vinelandii* decarboxylase as reference we found that SΔQs for the amino acid compositions of the *A. calcoaceticus* and *P. putida* decarboxylases were 9 and 24, respectively.

NH_2-terminal amino acid sequence. Quantitative measurements for the first 49 cycles in the NH_2-terminal amino acid sequence determination of the *A. vinelandii* decarboxylase are shown in Table 3.

DISCUSSION

Relationships among γ-carboxymuconolactone decarboxylases from *A. calcoaceticus*, *A. vinelandii*, and *P. putida*. γ-Carboxymuconolactone decarboxylases from the three bacterial genera shared some properties: the enzymes appeared to be hexamers formed by association of identical subunits of about 13,300 daltons (11), and the amino acid compositions of the proteins were similar (17). On the other hand, the specific activity of the *A. calcoaceticus* decarboxylase (140 U/mg) was substantially lower than the specific activities of the *A. vinelandii* and *P. putida* decarboxylases (1,260 and 1,310 U/mg, respectively), and the latter two decarboxylases cross-reacted immunologically with each other, but not with the *A. calcoaceticus* decarboxylase. Thus, it appears that all three decarboxylases were derived from a com-

TABLE 3. *Automated sequence analysis of A. vinelandii γ-carboxymuconolactone decarboxylase: identification of amino acid residues*

Cycle	PTH[a]	Me_3Si	BH-ORG[b]	BH-AQU[c]	Residue
1	M (193)[d]	M (181)			Met
2		D (125)	D (73)		Asp
3		E (166)	E (68)		Glu
4			K (37)		Lys
5		E (125)	E (68)		Glu
6			R (4)	R (17)	Arg
7	Y (18)	Y (101)	Y (46)		Tyr
8		D (65)	D (47)		Asp
9	A (131)	A (115)	A (53)		Ala
10	G (129)	G (106)	G (66)		Gly
11	M (66)	M (82)			Met
12		Q (20)	E (49)		Gln
		E (36)			
13	V (208)	V (153)	V (33)		Val
14			R (3)	R (8)	Arg
15			R (3)	R (14)	Arg
16	A (114)	A (104)	A (47)		Ala
17	V (97)	V (75)	V (33)		Val
18			L (35)		Leu
19	G (38)	G (33)	G (26)		Gly
20		D (25)	D (31)		Asp
21	A (72)	A (85)	A (43)		Ala
22				H (7)	His
23	V (230)	V (145)	V (26)		Val
24		D (48)	D (25)		Asp
25			R (2)	R (7)	Arg
26					(Cys or Ser)[e]
27	LI[f] (181)	LI (119)	L (26)		Leu
28			K (10)		Lys
29		D (11)	D (22)		Asn[g]
30	LI (163)	LI (117)	L (25)		Leu
31			T (14)		Thr
32	P (39)		P (11)		Pro
33	F (32)	F (34)	F (14)		Phe
34		D (7)	D (20)		Asn[g]
35		E (46)	E (23)		Glu
36		E (42)	E (25)		Glu
37	F (32)	F (31)	F (11)		Phe
38		Q (5)	E (22)		Gln
		E (26)			
39		E (30)	E (24)		Glu
40	M (9)	M (14)			Met
41	LI (97)	LI (59)	I (11)		Ile
42			T (12)		Thr
43			R (1)	R (3)	Arg
44				H (3)	His
45		A (45)	A (19)		(Ala)
46					(Cys or Ser)[e]
47		G (16)	G (12)		Gly
48		D (10)	D (11)		Asp
49	LI (138)	LI (67)	I (8)		Ile

[a] PTH, Phenylthiohydantoin.

[b] BH-ORG, Back hydrolysis-extracted organic phase.

[c] BH-AQU, Back hydrolysis-remaining aqueous phase.

[d] Results indicate the single-letter amino acid designation and (within parentheses) the number of nanomoles recovered.

[e] The only two amino acid residues that could not be identified by the procedures used in this sequence determination are cysteine and serine.

[f] PTH-leucine and PTH-isoleucine were coeluted by the gas chromatographic technique.

[g] PTH-asparagine was distinguished from PTH-asparatic acid by high-pressure liquid chromatography.

mon ancestor; the structural genes for the *A. vinelandii* and *P. putida* decarboxylases seem to have diverged recently relative to their divergence from the *A. calcoaceticus* decarboxylase. These conclusions are strengthened by comparison of the NH_2-terminal amino acid sequences of the decarboxylases. The data allow a three-way comparison of 34 of the first 36 residues. The *A. vinelandii* and *P. putida* sequences were identical in 83% of the compared positions (Fig. 4), whereas comparison of the *A. vinelandii* sequence with the *A. calcoaceticus* sequence reveals an identity of 47% (Fig. 5).

Early events in the evolution of γ-carboxymuconolactone decarboxylases and muconolactone isomerases. γ-Carboxymuconolactone decarboxylases and muconolactone isomerases, enzymes that mediate analogous biochemical reactions, appear to share a common ancestral gene. Alignment of the NH_2-terminal

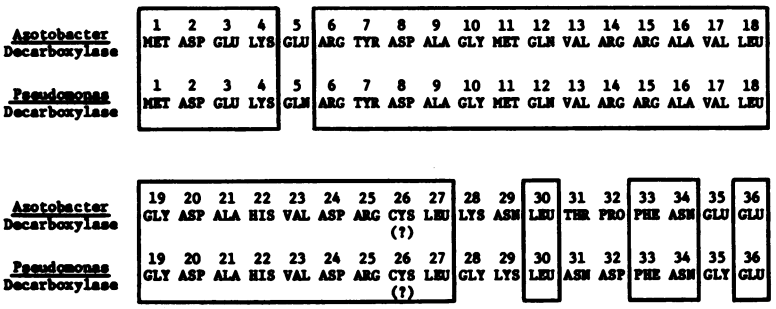

FIG. 4. *Comparison of the NH_2-terminal amino acid sequences of A. vinelandii and P. putida γ-carboxymuconolactone decarboxylases. Identical residues are enclosed in boxes.*

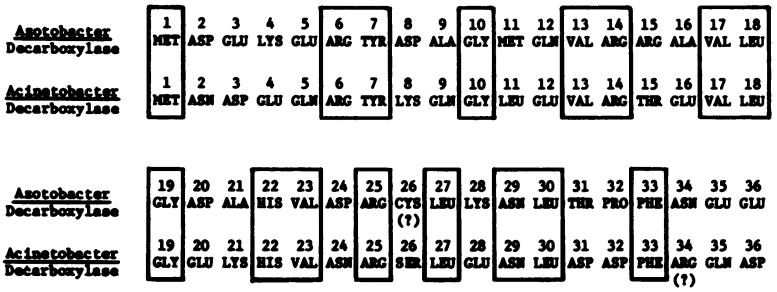

FIG. 5. *Comparison of the NH_2-terminal amino acid sequences of A. vinelandii and A. calcoaceticus γ-carboxymuconolactone decarboxylases. Identical residues are enclosed in boxes.*

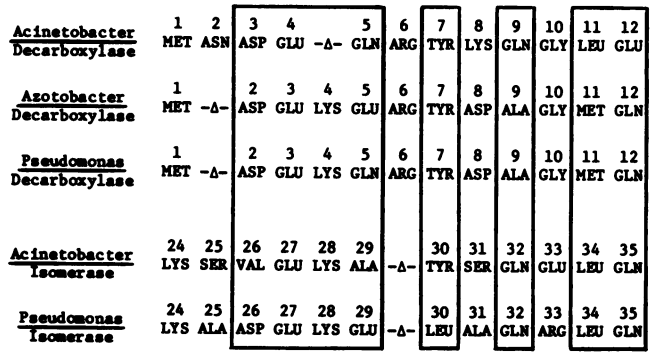

FIG. 6. *A portion of the NH_2-terminal amino acid sequence of γ-carboxymuconolactone decarboxylase appears to be conserved within the primary structure of muconolactone isomerase. Numbers indicate the positions of residues in the primary sequences of the proteins. Boxes enclose residues at positions where identical residues are found in the decarboxylase-isomerase comparison.*

amino acids of the enzymes reveals sequence similarity suggesting a low overall homology (17). In addition, the NH_2-terminal amino acid sequence of the decarboxylase appears to be conserved within the primary structure of the muconolactone isomerase: the tetrapeptide extending from residues 2 through 5 in the *A. vinelandii* decarboxylase is represented at residues 26 through 29 in the amino acid sequence of the *P. putida* muconolactone isomerase (Fig. 6). This is consistent with the proposal that, as genes for the β-ketoadipate pathway became established, oligonucleotide substitution mutations placed sequences coding for peptides in novel structural contexts (6, 18).

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