Regulation and Subunit Structure of Aspartate β-Decarboxylase. Studies on the Enzymes from *Alcaligenes faecalis* and *Pseudomonas dacunhae**

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ABSTRACT: L-Aspartate-β-decarboxylase from *Pseudomonas dacunhae* is subject to the same type of allosteric regulation involving transamination as the L-aspartate β-decarboxylase from *Alcaligenes faecalis*. The two enzymes differ in several respects, for example in the effect of treatment with *p*-mercuribenzoate on enzymatic activity. However, the two enzymes are similar with respect to K_m and V_{max} for substrate, amino acid composition, number of free sulfhydryl groups, electron microscopic appearance, and sedimentation coefficient; they both dissociate on resolution at pH 8 to give dimers, and reassociate at pH 6 or in the presence of pyridoxal 5′-phosphate. When mixtures of the two dimeric apoenzymes are induced to reassociate, seven electrophoretically separable species are formed, supporting the conclusion that these enzymes are homologous and that they are composed of twelve subunits.

Previous studies on the regulation of the activity of L-aspartate β-decarboxylase of *Alcaligenes faecalis* led to the conclusion that under conditions in which the enzyme catalyzes aspartate decarboxylation at a constant rate in the absence of α-keto acid activator, there is an equilibrium between enzyme-bound alanine–ketimine and enzyme-bound pyridoxamine 5′-phosphate and pyruvate (Tate and Meister, 1969b). It was shown that the enzyme can bind at an allosteric site about 1 mole of keto acid/minimal catalytic unit. The activity of the enzyme is increased by adding very low concentrations of pyruvate and is decreased by removal of pyruvate (*e.g.*, by adding lactate dehydrogenase and reduced diphosphopyridine nucleotide). Thus, the enzyme is subject to highly sensitive regulation effected by small changes in the concentration of keto acids (*e.g.*, pyruvate and α-ketoglutarate) by a novel allosteric mechanism involving reaction of the keto acid effector with the enzyme-bound coenzyme.

The possibility that the activity of the enzyme can be influenced by phenomena which are allosteric in a more conventional sense was also considered, especially because the enzyme is known to contain twelve subunits (Bowers *et al.*, 1968), and because earlier work on the protection of the enzyme by 4′-deoxypyridoxine 5′-phosphate (Novogrodsky and Meister, 1964) suggested the occurrence of cooperative effects. Furthermore, Chibata *et al.* (1968), who have recently reported on the aspartate β-decarboxylase of *Pseudomonas dacunhae*, concluded that the activation of the aspartate β-decarboxylase of this organism by α-keto acids is not due to a transamination mechanism but rather to an allosteric one in which α-keto acid alters the affinity for L-aspartate. In the present work, we have prepared the *Pseudomonas* enzyme by the procedure of Chibata *et al.* (1967) and Kakimoto *et al.* (1969) and have compared certain of its kinetic and other properties with those of the *Alcaligenes* enzyme. Our studies indicate that the two enzymes are similar though not identical, and that they are both subject to the same type of allosteric regulation (involving transamination). The effector α-keto acid does not affect the K_m value of the enzyme for aspartate but increases the V_{max}.

The *Alcaligenes* and *Pseudomonas* enzymes are similar with respect to amino acid composition and the number of free sulfhydryl groups. Both enzymes on resolution at pH 8 dissociate to yield apoenzyme dimers; these apoenzyme units reassociate to yield the holoenzyme structure on adjustment of pH to a value of 6 or an addition of pyridoxal 5′-phosphate. When mixtures of the dimeric apoenzymes obtained from the two holoenzymes are incubated at pH 6 or treated with pyridoxal 5′-phosphate seven electrophoretically separable species are formed; two of these correspond to the native enzymes and the remainder are hybrid forms. This result supports the conclusion that aspartate β-decarboxylase from *A. faecalis* (Bowers *et al.*, 1968, 1970) as well as the enzyme from *P. dacunhae* are composed of twelve subunits and that the two enzymes are homologous.

Experimental Section

Materials

L-Aspartate β-decarboxylase was isolated from *A. faecalis* (strain N) (ATCC 25094) as previously described (Tate and Meister, 1968). The enzyme from *P. dacunhae*[1] was isolated as described by Kakimoto *et al.* (1969). Resolution of the holoenzymes was achieved as follows: A solution containing 0.1 M phosphate buffer (pH 6), 2 mM EDTA, 40 mM L-aspartate, 1 mM DPNH, lactate dehydrogenase (15 μg/ml), and the isolated *Pseudomonas* or *Alcaligenes*

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[1] We thank Professor H. Iizuka of the Institute of Applied Microbiology, University of Tokyo, for his kindness in supplying us with a culture of *Pseudomonas dacunhae*.

enzyme (2.5 mg/ml), was incubated at 37° for 1 hr and then at 25° for a further 4 hr. The solution was dialyzed overnight at 4° against 250 volumes of 1 M sodium acetate buffer (pH 6), and subsequently against 1000 volumes of 0.02 M potassium phosphate buffer (pH 6).

L-Aspartic acid-4-^{14}C was obtained from New England Nuclear Corp. Sodium pyruvate-1-^{14}C, L-cysteinesulfinic acid, and pyridoxal 5′-phosphate were purchased from Calbiochem. Lactate dehydrogenase (rabbit muscle) and DPNH were obtained from Sigma Chemical Co. *p*-Mercuribenzoate and 5,5′-dithiobis(2-nitrobenzoic acid) were obtained from Mann Research Laboratories, Inc., and Aldrich Chemicals, respectively. *p*-Mercuribenzoic acid-carboxyl-^{14}C was obtained from Tracer Lab Radiochemicals.

Methods

Cysteinesulfinate desulfinase activity was determined by colorimetric measurement of the sulfite liberated from L-cysteinesulfinic acid (Soda *et al.*, 1964) by the procedure previously described (Tate and Meister, 1968). The decarboxylation of L-aspartate and transamination between pyruvate-1-^{14}C and L-aspartate were determined by methods which have been described previously (Tate and Meister, 1969a,b).

Amino Acid Composition. Samples of the enzyme were hydrolyzed in 6 N hydrochloric acid at 107° for 24–72 hr. The hydrolysates were analyzed with a Beckman amino acid analyzer Model 120C by the method of Spackman *et al.* (1958). Half-cystine residues were determined as cysteic acid after performic acid oxidation of the enzyme according to the procedure of Moore (1963). Tryptophan was determined by the spectrophotometric procedure of Edelhoch (1967).

Titration with pMB[2] *and DTNB.* Spectrophotometric titrations of the sulfhydryl groups of the *Pseudomonas* enzyme were carried out with a Cary Model 15 spectrophotometer using the DTNB method of Ellman (1959) and the *p*MB procedure of Boyer (1954); the experimental details were the same as previously applied to studies on the enzyme from *A. faecalis* (Tate and Meister, 1968).

Polyacrylamide Gel Electrophoresis. These studies were carried out essentially as described by Davis (1964). A continuous buffer system was used consisting of 0.05 M Tris-acetate buffer (pH 8) containing 1 mM EDTA. Electrophoresis was performed in 4 and 6% gels at 25°. In one experiment, a 4% gel at pH 6 (0.05 M potassium phosphate buffer) was used. The enzyme samples (10–20 μl) containing about 6–8% sucrose and bromophenol blue as a tracking dye were layered through the buffer on top of the gels. The protein bands were fixed in methanol–glacial acetic acid–water (60:10:30, v/v) and stained with Amido-Schwarz. After destaining in 7.5% acetic acid the gels were scanned at 520 mμ in a Joyce-Loebl Chromoscan.

Results

Effect of α-Keto Acids and of Lactate Dehydrogenase on Aspartate β-Decarboxylase. Figure 1 describes the time course of the decarboxylation of L-aspartate catalyzed by the *Pseudomonas* enzyme under conditions similar to those used earlier in studies on the aspartate β-decarboxylase from *A. faecalis* (see Figure 2 of Tate and Meister, 1969b). The behavior of the two enzymes is quite similar. Thus, a constant rate of decarboxylation was observed in the absence of added α-keto acid with both enzymes; in the presence of α-keto acid (curve 1 of Figure 1) this rate was increased almost threefold with the *Pseudomonas* enzyme, a result similar to that found with the *Alcaligenes* enzyme (Tate and Meister, 1969b). The ratio of the initial rate of decarboxylation to that of transamination (pyruvate-^{14}C plus aspartate) for the *Pseudomonas* enzyme was about 1600 compared with 2380 for the *Alcaligenes* enzyme (Tate and Meister, 1969b). Addition of lactate dehydrogenase and reduced diphosphopyridine nucleotide brought about a marked decrease in decarboxylase activity (curve 3 of Figure 1), and, as in the studies on the *Alcaligenes* enzyme, activity was restored on subsequent addition of α-ketoglutarate (curves 3A,B of Figure 1). However, in contrast to the result with the *Alcaligenes* enzyme, the initial activity was not fully restored when α-ketoglutarate was added (after 9 min) to the *Pseudomonas* enzyme (curve 3B of Figure 1), but when both pyridoxal

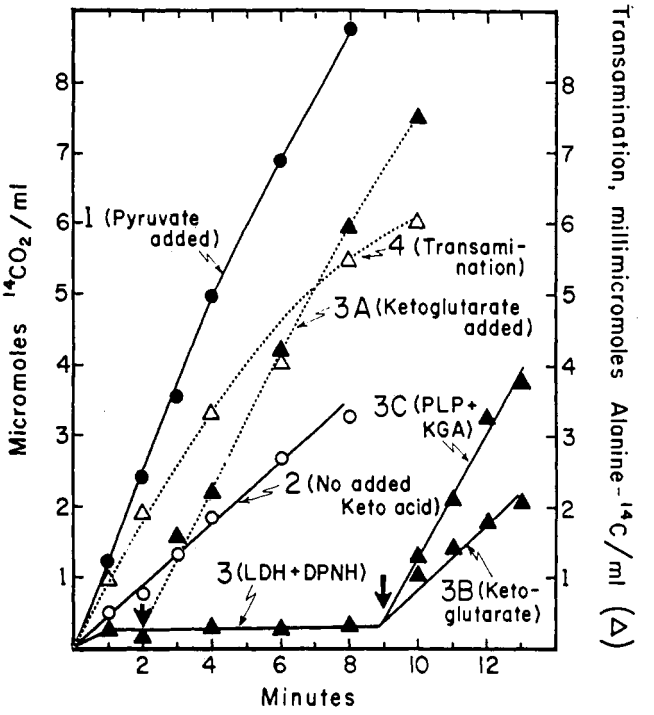

FIGURE 1: Effects of α-keto acids and of lactate dehydrogenase plus reduced diphosphopyridine nucleotide on the activity of aspartate β-decarboxylase of *Pseudomonas dacunhae*. Enzyme activity was determined at 37° in reaction mixtures containing 0.2 M sodium acetate buffer (pH 5.5), 10 mM L-aspartate-4-^{14}C, and 10 μg of aspartate β-decarboxylase/ml. Curve 1: decarboxylation of aspartate in the presence of 1 mM sodium pyruvate or 1 mM sodium α-ketoglutarate. Curve 2: decarboxylation of aspartate in the absence of added α-keto acid. Curve 3: decarboxylation of aspartate in the presence of lactate dehydrogenase (10 μg/ml) and DPNH (0.5 mM). At the points indicated by heavy arrows (curve 3A,B), sodium α-ketoglutarate was added, in separate experiments, to yield a final concentration of 1 mM. Curve 3C: sodium α-ketoglutarate and pyridoxal-5′-P were added together to yield final concentrations of 1 and 0.1 mM, respectively. Curve 4: transamination between L-aspartate (10 mM) and sodium pyruvate-1-^{14}C (1 mM).

[2] Abbreviations used are: *p*MB, *p*-mercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Gd·HCl, guanidine hydrochloride.

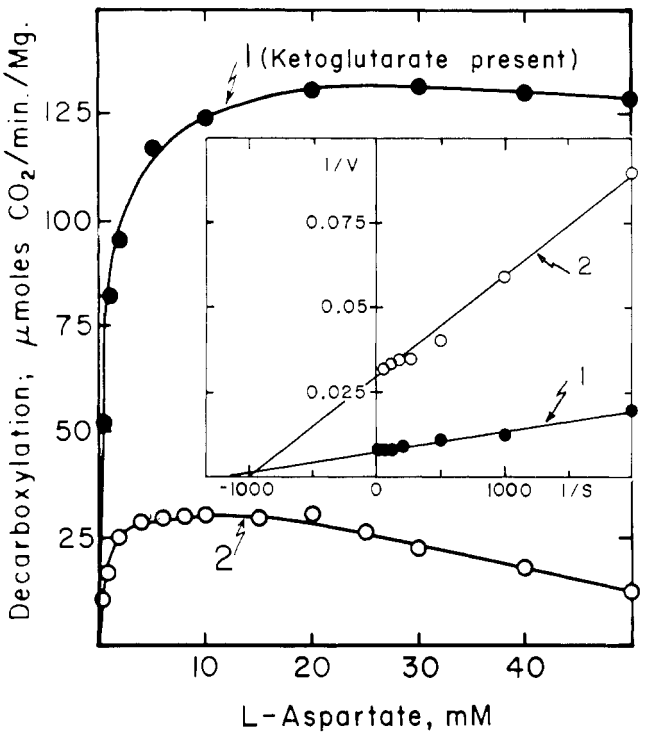

FIGURE 2: Effect of L-aspartate concentration on the activity of the *Pseudomonas* enzyme. The reaction mixtures (volume 1 ml) contained 0.2 M sodium acetate buffer (pH 5.5), L-aspartate-4-^{14}C in the indicated concentrations and enzyme (20 μg/ml); 37°. Curve 1: 1 mM sodium α-ketoglutarate present. Curve 2: no α-keto acid added. The inset shows the double-reciprocal plots of the data. The lines drawn are the least-squares fit (in curve 2, velocity data for 0.5–10 mM aspartate were used).

TABLE I: Amino Acid Composition of Aspartate β-Decarboxylase of *P. dacunhae*.

Amino Acid	Extrapolated Values (moles/60,000 g of Enzyme)	Av No. of Residues/60,000 g of Enzyme
Lysine	25.4	25 (25)[a]
Histidine	5.6	6 (6)
Arginine	35.4	35 (34)
Aspartic	57.5	58 (60)
Threonine	23.1	23 (21)
Serine	30.6	31 (36)
Glutamic	57.5	58 (57)
Proline	26.8	27 (25)
Glycine	39.6	40 (38)
Alanine	57.8	58 (55)
Half-cystine	2.4,[b] 2.3[c]	2 (2)
Valine	32.8	33 (32)
Methionine	14.6	15 (13)
Isoleucine	30.6	31 (28)
Leucine	73	73 (66)
Tyrosine	16.7	17 (23)
Phenylalanine	25	25 (26)
Tryptophan	3.9[d]	4 (4)

[a] The values in parentheses give the amino acid composition of aspartate decarboxylase of *A. faecalis*, ATCC 25094 (Tate and Meister, 1968). [b] As cysteic acid. [c] From DTNB titration of the reduced enzyme. [d] Determined spectrophotometrically (Edelhoch, 1967).

5′-phosphate and α-ketoglutarate were added (curve 3C), a rate close to the initial one (curve 1) was observed. The findings therefore indicate that there is appreciable dissociation of pyridoxamine 5′-phosphate from the *Pseudomonas* enzyme under these conditions; such dissociation was much less marked in analogous studies on the *Alcaligenes* enzyme (Tate and Meister, 1969b).

The effect of L-aspartate concentration on the decarboxylase activity of the *Pseudomonas* enzyme is described in Figure 2. The inset gives double-reciprocal plots; the K_m values are 10×10^{-4} and 8.5×10^{-4} M in the absence (curve 2) and presence (curve 1) of added α-ketoglutarate. The corresponding V_{max} values are 34 and 136, respectively. Similar kinetic parameters were obtained for the *Alcaligenes* enzyme (Tate and Meister, 1969b). It is of interest that considerable inhibition of decarboxylase activity was observed at relatively high concentrations of aspartate (25–50 mM) in the absence of added keto acid (curve 2 of Figure 2). However, such inhibition was not apparent in the presence of 1 mM α-ketoglutarate (curve 1 of Figure 2). Similar results have been obtained with the *Alcaligenes* enzyme.

Amino Acid Analysis and Sulfhydryl Group Titration of the Pseudomonas Enzyme. Table I gives the amino acid analysis of the *Pseudomonas* enzyme; the comparative values for the *Alcaligenes* enzyme are given in parentheses. In general, the amino acid compositions of the two enzymes are quite similar. Thus, the values for the basic and acidic amino acids were, within experimental error, the same, as were also the values for tryptophan, half-cystine, proline, glycine, valine, and phenylalanine. Although the values for threonine, serine, methionine, isoleucine, leucine, and tyrosine are probably outside the range of analytical error, they are not markedly different for the two enzymes.

Titration with DTNB in 5 M Gd·HCl gave values close to 2 moles of sulfhydryl/60,000 g of enzyme (Table II). On the other hand, when the holoenzyme was treated with *p*-mercuribenzoate-^{14}C about 1 mole of *p*-mercuribenzoate-^{14}C was bound per 60,000 g of enzyme. Similar treatment of the apoenzyme led to binding of about 2 moles of *p*-mercuribenzoate-^{14}C. These results contrast with those obtained in analogous studies on the *Alcaligenes* enzyme in which close to 2 moles of *p*-mercuribenzoate was bound to both the holoenzyme and the apoenzyme (Tate and Meister, 1968).

The *p*MB–holoenzyme of *Pseudomonas* was about 95% as active in catalyzing the desulfination of L-cysteinesulfinate as the untreated holoenzyme (Figure 3), while the *p*MB–apoenzyme of *Alcaligenes* exhibited about 25% more desulfinase and aspartate β-decarboxylase activities than did the untreated holoenzyme (Tate and Meister, 1968). Both the *p*MB–apoenzyme of *Pseudomonas* (Figure 3) and that of *Alcaligenes* (Tate and Meister, 1968) after reconstitution exhibited about 50% of the activities of the corresponding untreated holoenzymes.

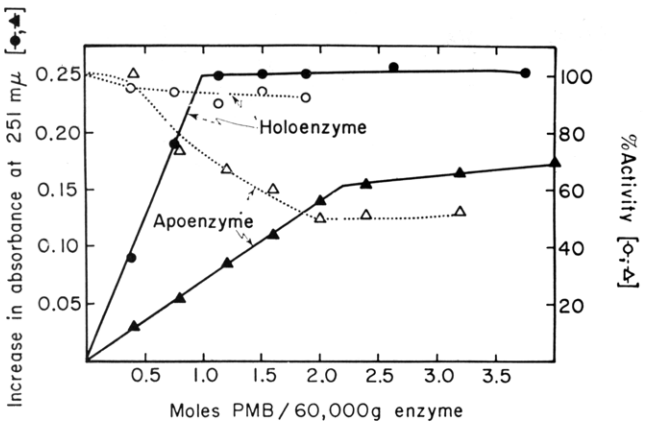

FIGURE 3: Reaction of *p*MB with the holoenzyme and apoenzyme forms of aspartate β-decarboxylase of *P. dacunhae*. The holoenzyme (1.6 mg/0.5 ml of 0.2 M sodium acetate buffer, pH 5.5) was titrated with 5-μl portions of 2 mM *p*MB-^{14}C at 25° (open and closed circles). The apoenzyme (0.63 mg/0.5 ml of 0.05 M potassium phosphate buffer, pH 6) was titrated with 2-μl portions of 2 mM *p*MB-^{14}C (open and closed triangles). The increase in absorbance was recorded 5 min after addition of the reagent. Cysteinesulfinate desulfinase activity was determined at 37° in the standard assay system; in studies with the apoenzyme, assay was carried out after reconstitution with pyridoxal-5′-P. Open symbols, enzyme activity; closed symbols, increase in absorbance at 251 mμ.

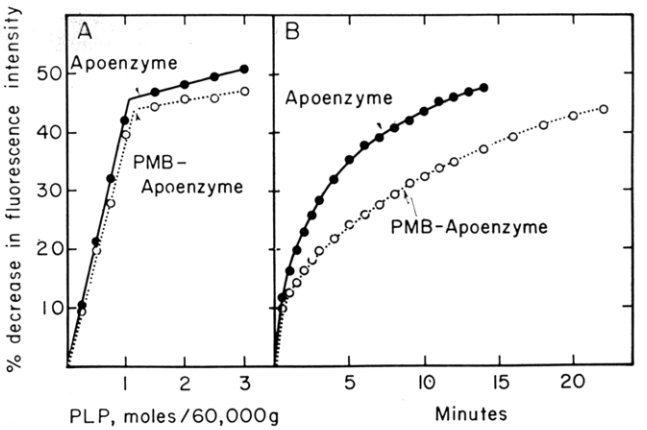

FIGURE 4: Fluorimetric studies of the interaction of pyridoxal-5′-P with the apoenzyme. (A) Titration of the apoenzyme and the *p*MB–apoenzyme with pyridoxal-5′-P. The apoenzyme (0.24 mg/ml of 0.05 M sodium acetate buffer, pH 6) and the *p*MB–apoenzyme (0.24 of the apoenzyme was incubated with 20 mμmoles of *p*MB for 10 min) were treated with 1-μl portions of 1 mM pyridoxal-5′-P. The fluorescence intensity at 350 mμ was measured at 22° after 15-min incubation. The activating wavelength was 280 mμ. (B) Time-dependence of the decrease in fluorescence intensity. The apoenzyme and the *p*MB–apoenzyme (0.24 mg/ml; as in part A) were treated with 10 μl of 1 mM pyridoxal-5′-P; the fluorescence intensity was recorded as a function of time.

Fluorimetric titration of the *Pseudomonas* apoenzyme and the corresponding *p*MB–apoenzyme with pyridoxal-5′-P indicated that both forms of enzyme bind close to 1 mole of pyridoxal-5′-P/60,000 g of enzyme (Figure 4A); it is of note, however, that reaction with pyridoxal-5′-P takes place at a substantially slower rate with the *p*MB–apoenzyme (Figure 4B).

TABLE II: Sulfhydryl Groups of Aspartate β-Decarboxylase of *P. dacunhae*.

Expt	Enzyme Form	Procedure	Thionitrobenzoate Formed or *p*MB Bound/60,000 g of Enzyme (moles)
1	Holoenzyme	DTNB[a]	2.22
2	Holoenzyme[b]	DTNB[a]	2.31
3	Holoenzyme[c]	*p*MB	1.06
4	Apoenzyme[c]	*p*MB	2.18

[a] The enzyme (2–3 mg) was treated with DTNB by the Ellman (1959) method in 5 M Gd·HCl in 0.05 M potassium phosphate buffer (pH 6.8) containing 5 mM EDTA. [b] Reduced by treatment with $NaBH_4$ (Cavallini *et al.*, 1966). [c] Treated with *p*MB-^{14}C as described in Figure 3 and then passed through a column (1 × 45 cm) of Sephadex G-25. *p*MB-holoenzyme was eluted with 0.05 M sodium acetate buffer (pH 5.5) and the *p*MB–apoenzyme was eluted with 0.05 M potassium phosphate buffer (pH 6.0).

Hybrid Formation between the Enzymes from Alcaligenes faecalis and P. dacunhae. Studies in which the two holoenzymes were subjected to electrophoresis in polyacrylamide gels at pH 8 indicated a marked difference in their migration

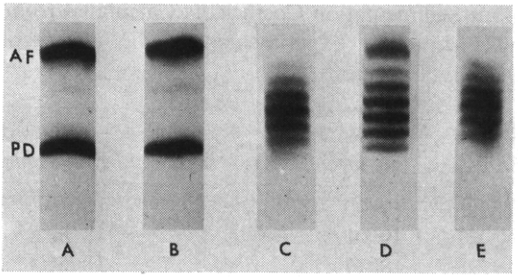

FIGURE 5: Polyacrylamide gel electrophoresis of the hybrids produced by mixing the decarboxylases from *A. faecalis* and *P. dacunhae*. In expt A–D gels (4%) were run at 25° in 0.05 M Tris-acetate buffer (pH 8) containing 1 mM EDTA. Expt A: the two holoenzymes were mixed and applied to the gel. AF, *Alcaligenes*, PD, *Pseudomonas* (16 μg of each enzyme/10 μl of 0.2 M sodium acetate buffer, pH 5.5). Expt B: the two apoenzyme forms ($s_{20,w}$ = 19 S; 16 μg each/10 μl of 0.2 M sodium acetate buffer, pH 6) were mixed and then incubated at 25° for 10 min with 50 mμmoles of pyridoxal-5′-P. Expt C: the two dimeric apoenzymes (24 μg each) were mixed and incubated at pH 8 (0.15 M Tris-acetate buffer) at 25° for 10 min. The pH was then adjusted to about 6.0 by addition of 1 M sodium acetate buffer (pH 5.5). Pyridoxal-5′-P (50 mμmoles) was added to the mixture which was then incubated for 10 min at 25°. Expt D: the two dimeric apoenzymes (24 μg each) were mixed and incubated at 25° for 10 min in 30 μl of 0.15 M Tris buffer (pH 8). Pyridoxal-5′-P (50 mμmoles) was added and incubation was continued for 10 min at 25°. Expt E: the two dimeric apoenzymes (24 μg each) were mixed and incubated at pH 8 as in expt C for 10 min. The pH was then adjusted to 6.0 by addition of 1.0 M sodium acetate buffer. The apoenzyme mixture was then subjected to electrophoresis in a 4% gel at pH 6 (0.05 M potassium phosphate buffer); 25°.

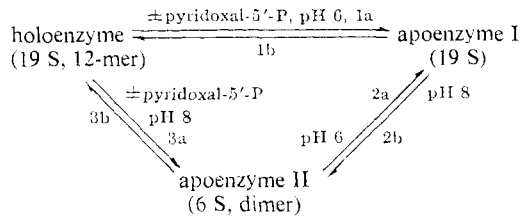

FIGURE 6: Subunit interactions of L-aspartate β-decarboxylase (see the text).

properties, the *Alcaligenes* enzyme being more anionic than the *Pseudomonas* enzyme (Figure 5A). This difference seems to be primarily associated with a difference in charge since the relative migration rates of the enzyme in 4 and 6% gels were the same. The *Alcaligenes* apoenzyme at pH 8 dissociates to dimeric units which move ahead of the holoenzyme in acrylamide gels (Tate and Meister, 1968; Bowers *et al.*, 1968). This phenomenon is represented in Figure 6 as reaction 3a. Reassociation of the dimeric units (apoenzyme II) to yield the holoenzyme occurs on addition of pyridoxal-5′-P (reaction 3b); adjustment of the pH to 6 leads to association of the dimeric apoenzyme to form the 19S apoenzyme (apoenzyme I; reaction 2a, Figure 6). The same phenomena have been observed with the enzyme from *Pseudomonas*. Table III gives the sedimentation coefficients of the two forms of the enzymes; we are indebted to Dr. R. H. Haschemeyer and Mr. W. F. Bowers for these data.

When the apoenzyme I forms of the *Pseudomonas* and *Alcaligenes* enzymes ($s_{20,w}$ = approximately 19S) were mixed and treated with pyridoxal-5′-P (to preserve association during electrophoresis at pH 8), the pattern shown in Figure 5B was obtained. No interaction between the two enzymes was evident. However, when equal amounts of the two dimeric apoenzymes (apoenzymes II) were mixed at pH 8, followed by adjustment of the pH to 6 to induce association, and addition of pyridoxal-5′-P to preserve association, the pattern shown in Figure 5C was obtained. Seven protein bands were formed, two of which correspond to the native enzyme and five to the hybrid forms. Inspection of the stained gel indicates essentially random association of the dimeric protomers, and this is confirmed by densitometric studies (Figure 7C). Hybridization was also induced by treating a mixture containing equal amounts of the two dimeric apoenzymes with pyridoxal-5′-P at pH 8. Again

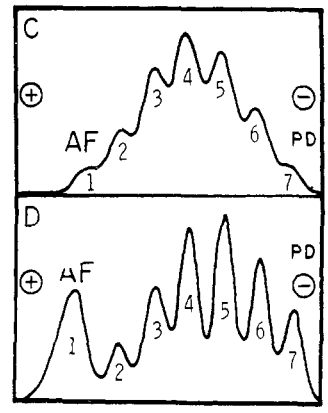

FIGURE 7: Densitometric study of the gels obtained in Figure 5C,D.

TABLE III: Sedimentation Coefficients Calculated from Simultaneous Multiplex Experiments on the *Alcaligenes* and *Pseudomonas* Enzymes.[a]

Enzyme Form	$s_{20,w}$ of *A. faecalis* (S)	$s_{20,w}$ of *P. dacunhae* (S)
Holoenzyme[b]	19.00	18.70
	19.01	18.68
	18.73	18.51
	Mean = 18.9 ± 0.2	Mean = 18.6 ± 0.1
Apoenzyme[c]	5.78	5.54
	5.83	5.48
	5.76	5.42
	Mean = 5.79 ± 0.04	Mean = 5.48 ± 0.06

[a] The $s_{20,w}$ values of corresponding pairs of the two enzymes were computed from data obtained simultaneously in the four-hole ANF rotor using the multiplex ultraviolet-scanning optical system. Sedimentation coefficients were measured and corrected as described elsewhere (Bowers *et al.*, 1970). All experiments were performed at 23.5° with an initial protein concentration of 0.5 mg/ml and a nominal speed of 52,000 rpm. [b] In 0.05 M sodium acetate, 0.1 M NaCl, and 1 mM Na_2EDTA, titrated to pH 5.5 with acetic acid. [c] In 0.05 M Tris, 0.1 M NaCl, and 1 mM Na_2EDTA, titrated to pH 8.0 with acetic acid.

TABLE IV: Activities of Reconstituted Forms of the *Alcaligenes* and *Pseudomonas* Enzymes and Their Hybrids.

Enzyme Derivative	Sp Act. (Desulfination)
Alcaligenes apoenzyme	
i. Reconstituted with pyridoxal-5′-P at pH 6	48 (53)[a]
ii. Reconstitution at pH 8	40
Pseudomonas apoenzyme	
i. Reconstituted with pyridoxal-5′-P at pH 6	34 (42)[a]
ii. Reconstitution at pH 8	32
Equimolar mixture of the reconstituted (pH 6) forms of the two enzymes	40
Hybridization of the two enzymes induced by pyridoxal-5′-P at pH 8 (pathway 3b, Figure 5)	39
Hybridization of the two enzymes induced by pH change (pathways 2b and 1b, Figure 5)	46

[a] Figures in parentheses are the specific activities of the isolated holoenzymes.

seven bands were obtained (Figures 5D and 7D); however, in this case the associations were not random. A result similar to that shown in Figure 5C was obtained when the two dimeric apoenzymes were mixed in equal amounts and hybridization was achieved by adjustment of the pH to 6; electrophoresis was carried out at pH 6 in the absence of pyridoxal-5′-P (Figure 5E).

The cysteinesulfinate desulfinase activities of mixtures of the several enzyme forms were determined (Table IV). The data indicate that there is no marked change in enzymatic activity after hybridization. Thus, the mixed hybrids (whether produced by pH change or addition of pyridoxal-5′-P) exhibit activities intermediate between the specific activities of the two enzymes.

Discussion

The results obtained in the experiments in which the dimeric apoenzymes from the *Alcaligenes* and *Pseudomonas* enzymes were hybridized can best be explained in terms of

$$(A)_2 \times (P)_2 \rightarrow \begin{cases} 6\,(A)_2 & (1) \\ 5\,(A)_2\,(P)_2 & (2) \\ 4\,(A)_2\,2\,(P)_2 & (3) \\ 3\,(A)_2\,3\,(P)_2 & (4) \\ 2\,(A_2)\,4\,(P)_2 & (5) \\ (A)_2\,5\,(P)_2 & (6) \\ 6\,(P)_2 & (7) \end{cases}$$

where A and P represent the monomeric units of the *Alcaligenes* and *Pseudomonas* enzymes, respectively, and $(A)_2$ and $(P)_2$ are the corresponding dimeric protomers. Estimates of the areas under the curves obtained in the densitometric studies (Figure 7) indicate that there is essentially a binomial distribution of the various forms in the experiment in which hybridization was induced by adjustment of pH to a value of 6 (reaction 2a, Figure 6, Figure 5C, and Figure 7C); thus, the relative amounts of forms 1–7 are (approximately) 1.2, 5.7, 11.3, 14.2, 12.6, 6.6, and 1.0, respectively. In the experiment in which hybridization was achieved by addition of pyridoxal-5′-P (reaction 3b, Figure 6, Figure 5D, and Figure 7D), the corresponding area estimates are (approximately) 3.0, 1.0, 2.2, 2.8, 3.1, 2.3, and 1.5, respectively. A definitive explanation for the differences in the relative amounts of the seven forms obtained in the two types of hybridization experiments is not yet apparent. It would seem that the properties of the two types of subunits responsible for association at pH 6 are quite similar. On the other hand, in the association induced by pyridoxal-5′-P the associating subunits must be sufficiently different as to favor or hinder certain combinations. It seems possible that addition of pyridoxal-5′-P to the dimeric subunits leads to conformationally different protomers and hence to a nonrandom association pattern.

The finding of seven species in the hybridization studies indicates that the enzymes are homologous.[3] The present findings thus confirm the elegant hydrodynamic studies of Bowers *et al.* (1970), who demonstrated that the *Alcaligenes* enzyme contains twelve subunits and that the molecular weights of the *Alcaligenes* holoenzyme, dimeric apoenzyme, and monomeric subunit are, respectively, 675,000, 114,000, and 57,000. The present findings indicate that the *Pseudomonas* enzyme also contains twelve subunits. The electron microscopic appearance of the *Pseudomonas* enzyme (Figure 8) is very similar to that of the *Alcaligenes* enzyme (Bowers *et al.*, 1970).

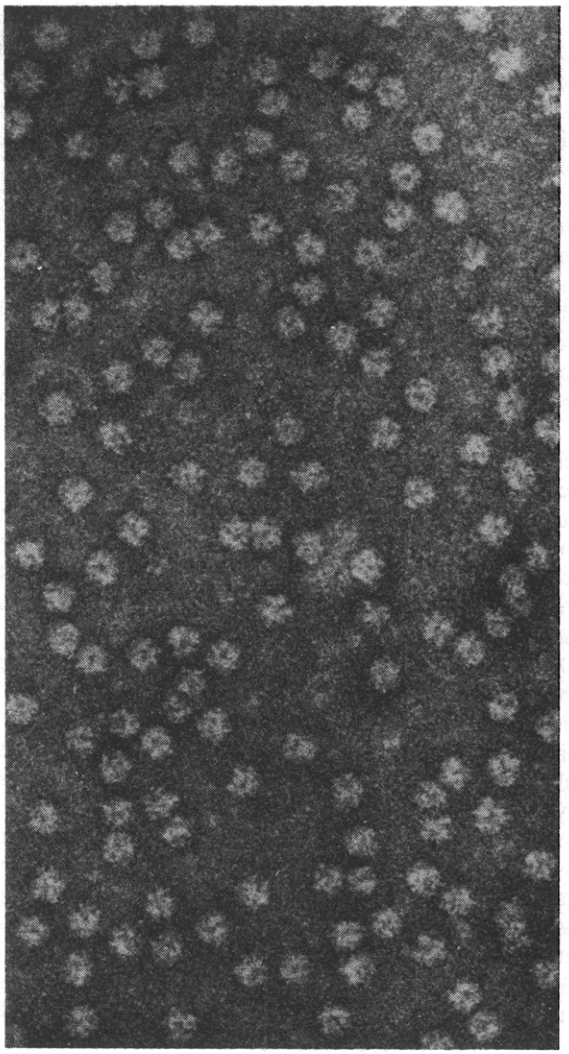

FIGURE 8: Electron micrograph of the *Pseudomonas* enzyme (courtesy of R. H. Haschemeyer and W. F. Bowers). The enzyme was fixed in 0.5% glutaraldehyde and negatively stained with uranyl oxalate at pH 6.8; mean particle diameter is 150 Å.

Although the two enzymes are quite similar with respect to subunit structure, sedimentation coefficient, amino acid composition, number of free sulfhydryl groups, and in their relative decarboxylase, desulfinase, and transaminase activities, there are some interesting differences. Thus, the two holoenzymes differ in gel electrophoretic mobility, and indeed

[3] Cook and Koshland (1969) have recently provided evidence that the intersubunit binding sites of several enzymes are "highly specific and have been selected over evolutionary time for correct assembly."

this difference has facilitated the gel electrophoretic separations of the hybrids described here. Since the relative electrophoretic mobilities of the enzymes were the same in 4 and 6% gels, it seems likely that the different migration rates exhibited by the two enzymes reflects a difference in charge. However, examination of the amino acid compositions of the two enzymes (Table I) offers no support for this. Nevertheless, it is possible that there is a difference in the number of amide groups, or conceivably that there are conformational differences between the two enzymes which result in different electrophoretic mobilities similar to the situation observed with malate dehydrogenases (Kaplan, 1968). It is notable that, in contrast to the *Alcaligenes* enzyme, which increased about 25% in activity after reaction with *p*MB, the activity of the *Pseudomonas* enzyme was not altered after such treatment. In addition, although both apoenzymes reacted with 2 moles of *p*MB/catalytic unit, the *Alcaligenes* holoenzyme reacted with 2 moles of *p*MB while the *Pseudomonas* holoenzyme reacted with only 1 mole of *p*MB. Titration of both holoenzymes with DTNB in 5 M Gd·HCl gave values for free sulfhydryl groups that are not far from 2 moles/catalytic subunit.

Our results on the *Pseudomonas* enzyme are at variance with those of Chibata *et al.* (1968). The latter workers reported a sigmoidal relationship between decarboxylase activity and aspartate concentration in the absence of added α-keto acid and they derived a K_m value of 0.1 M from this sigmoidal curve. We have been unable to find such a sigmoidal relationship even at concentrations of aspartate much lower than those used by Chibata *et al.* Furthermore, the K_m values obtained in our experiments are considerably smaller than those obtained by Chibata *et al.* As we have commented previously (Tate and Meister, 1969b), it is notable that Chibata *et al.* added large amounts of bovine serum albumin to their reaction mixtures. Bovine serum albumin has been found to bind various compounds including pyruvate and aspartate, and the presence of these compounds in commercially available samples of this protein has been reported (Hanson and Ballard, 1968). It thus seems possible that the results of Chibata *et al.* may reflect effects related to phenomena which are extraneous to the action of the decarboxylase itself.

The present data indicate that *Pseudomonas* enzyme like the *Alcaligenes* enzyme can produce the α-keto acid effector from the substrate. In studies on the *Alcaligenes* enzyme, the effector was shown to bind to a separate site at the active center and participate in a reaction with enzyme-bound cofactor to maintain a constant amount of the active form of the cofactor (Tate and Meister, 1969b). The homology between the two enzymes and the similarities in their kinetic behavior suggest a similar model for the regulation of the *Pseudomonas* enzyme. Thus the findings are consistent with the conclusion that both enzymes are controlled by the same type of allosteric transamination mechanism described previously (Tate and Meister, 1969b), and do not appear to support the view that the α-keto acid effector alters the affinity for substrate or that there is cooperativity in the binding of substrate.

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