Studies on the Sulfhydryl Groups of L-Aspartate β-Decarboxylase*

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ABSTRACT: L-Aspartate β-decarboxylase, which contains 16 moles of pyridoxal 5′-phosphate/mole of enzyme, has 2 cysteine residues/50,000 g (minimal catalytic unit) as determined by amino acid analysis and by studies in which the enzyme was reacted with *p*-mercuribenzoate, iodoacetate, and 5,5′-dithiobis(2-nitrobenzoate). Treatment of the holoenzyme with *p*-mercuribenzoate gave a *p*-mercuribenzoate derivative (*p*-mercuribenzoate–holoenzyme) which exhibited about 25% more desulfinase and decarboxylase activity than the holoenzyme. On the other hand, the *p*-mercuribenozate–apoenzyme (after reconstitution with pyridoxal 5′-phosphate) was only 55% as active as the holoenzyme. The transaminase activity of these forms of the enzyme was not altered. Removal of the *p*-mercuribenzoate moieties of the pyridoxal 5′-phosphate reconstituted *p*-mercuribenzoate enzyme and the *p*-mercuribenzoate–holoenzyme restored enzyme activity levels to that of the holoenzyme. Similar studies were carried out with pyridoxamine 5′-phosphate. It is concluded that enzyme sulfhydryl groups are not required for the several catalytic activities exhibited by the enzyme or for linkage of pyridoxal 5′-phosphate or pyridoxamine 5′-phosphate to the enzyme, but that introduction of the *p*-mercuribenzoate moiety can affect the conformation of the enzyme in such a manner as to increase or decrease activity. In the course of these studies it was found that resolution of the holoenzyme or the *p*-mercuribenzoate–holoenzyme ($s_{20,w}$ = 19 S) at pH 8.0 leads to dissociation to an apoenzyme of much lower molecular weight ($s_{20,w}$ = 6 S); when this enzyme form is treated with pyridoxal 5′-phosphate, the 19S holoenzyme is restored.

Aspartate β-decarboxylase has been isolated from *Alcaligenes faecalis* (Novogrodsky *et al.*, 1963; Novogrodsky and Meister, 1964; Soda *et al.*, 1964; Wilson and Meister, 1966) and from *Achromobacter* (Wilson, 1963). In addition to the β decarboxylation of L-aspartate, the enzyme catalyzes the desulfination of L-cysteine sulfinate (Soda *et al.*, 1964) and acts as a relatively nonspecific L-amino acid transaminase (Novogrodsky *et al.*, 1963; Novogrodsky and Meister, 1964; Soda *et al.*, 1964). The enzyme has been shown to bind 1 mole of pyridoxal 5′-phosphate/50,000 g of enzyme (Wilson and Meister, 1966) and about 16 moles of pyridoxal 5′-phosphate/mole of enzyme. The enzyme is thus unusually large when compared with other vitamin B_6 enzymes that have been isolated. The enzyme from *Achromobacter* has a molecular weight of 760,000 (Lloyd, 1963).

The present communication, which deals with the function of the sulfhydryl groups of this enzyme, represents part of a study of the relationship between the structure and function of aspartate β-decarboxylase. Data on the amino acid composition of the enzyme are reported; these and other findings indicate that the minimal catalytic unit contains two sulfhydryl groups. These are not necessary for activity, but undergo reaction with *p*-mercuribenzoate and other reagents which have made possible preparation and study of several new derivatives of the enzyme. In the course of this work it has been observed that resolution of the holoenzyme at pH 8.0 leads to reversible formation of an apoenzyme of much lower molecular weight.

Experimental Section

Materials. L-Cysteinesulfinic acid, pyridoxal 5′-phosphate, pyridoxamine 5′-phosphate, and dithiothreitol were purchased from California Corp. for Biochemical Research. Protamine sulfate, enzyme grade ammonium sulfate, *p*-mercuribenzoate, and 5,5′-dithiobis(2-nitrobenzoic acid) were obtained from Nutritional Biochemical Corp., Mann Research Laboratories, Inc., and Aldrich Chemicals, respectively. Sodium pyruvate-1-^{14}C was a product of New England Nuclear Corp., and *p*-mercuribenzoic acid-carboxyl-^{14}C was obtained from Tracerlab Radiochemicals. DEAE-cellulose, preswollen microgranular grade DE52, a product of Whatman, was purchased from Reeve Angel, N. J.

Methods. Enzyme activity was measured by colorimetric determination of the sulfite liberated from L-cysteinesulfinic acid (Soda *et al.*, 1964). The standard assay contained 0.1 M sodium acetate buffer (pH 5.5), 1 mM sodium α-ketoglutarate, and 10 mM cysteinesulfinate; pyridoxal 5′-phosphate, when present, was 0.5 mM. After equilibration at 37°, the reactions were initiated by addition of enzyme. Portions (50–100 μl) of the reaction mixture were pipetted into 200 μl of 1 N acetic acid in order to terminate the reaction and the sulfite formed was determined by the *p*-rosaniline method of West and Gaeke (1956). The colors were

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compared at 560 mμ. A unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmole of sulfite/min under the above conditions.

Transamination between pyruvate-1-^{14}C and L-aspartate was determined by adding the enzyme to a reaction mixture containing 0.4 M sodium acetate buffer (pH 5.5), 5 mM sodium L-aspartate, and 1 mM pyruvate-1-^{14}C. Portions (200 μl) were withdrawn after incubation for 30 and 60 min at 37° and placed on small columns of Dowex 1 (acetate) prepared in Pasteur pipets. Labeled alanine was eluted with 2 ml of water and a sample (0.5 ml) of the eluate was taken for determination of radioactivity in 15 ml of liquid scintillation medium (Bray, 1960). Transaminase activity is expressed in terms of millimicromoles of alanine-^{14}C formed per minute under these conditions.

Protein concentration was determined by the biuret procedure of Gornall *et al.* (1949) and by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

Spectrophotometric titrations of the sulfhydryl groups of the enzyme were carried out with a Cary Model 15 spectrophotometer using the DTNB[1] method of Ellman (1959) and the PMB procedure of Boyer (1954).

Resolution of the enzyme was achieved as described by Wilson and Meister (1966); the apoenzyme thus obtained was dialyzed against 0.05 M sodium acetate buffer (pH 6.0).

Amino Acid Composition. Samples of the enzyme were hydrolyzed in 6 N HCl at 110° for 24–72 hr. The hydrolysates were analyzed with a Beckman amino acid analyzer Model 120 C by the method of Spackman *et al.* (1958).[2] Half-cystine residues were determined (a) as cysteic acid after performic acid oxidation of the enzyme according to the procedure of Moore (1963), and (b) as *S*-carboxymethylcysteine after carboxymethylation of reduced enzyme with iodoacetic acid according to the method of Craven *et al.* (1965). Tryptophan was determined by the spectrophotometric procedure of Edelhoch (1967).

Polyacrylamide Gel Electrophoresis. These were performed essentially as described by Davis (1964). A continuous buffer system was used consisting of 0.05 M Tris and 2 mM Na_2EDTA, pH adjusted to 8.1 by addition of acetic acid. Electrophoresis was carried out in 4 and 6% gels at 25°. 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 electrophoresis was terminated when the bromophenol blue band reached the bottom of the gel.

Purification of the Enzyme. Satisfactory methods for the isolation of the enzyme have been developed (Novogrodsky and Meister, 1964; Soda *et al.*, 1964; Wilson and Meister, 1966). The improved method given here incorporates some features of the earlier procedures and a number of modifications; it reproducibly gives an enzyme preparation which is homogeneous in the analytical ultracentrifuge and on acrylamide gel electrophoresis. The streptomycin precipitation step, used earlier, has been eliminated since, in general, higher yields of enzyme are obtained when this procedure is omitted.

A. faecalis (strain N) was grown in batches of 500 l. on the medium previously described (Novogrodsky and Meister, 1964; Wilson and Meister, 1966). The fermentations were carried out at the New England Enzyme Center, Tufts University School of Medicine, Boston, Mass.[3] Cells (1 kg of frozen paste) were suspended in 1 l. of 0.05 M sodium acetate buffer containing 1 mM EDTA (pH 5.5) (referred to hereafter as acetate buffer) and sonicated in batches of 350 ml using a Branson Sonifier cell disrupter (Heat Systems Co., N. Y.). The extract was centrifuged at 20,000*g* for 45 min. The supernatant (step 1, Table I) was decanted and treated with 300 ml of 3% protamine sulfate solution (2 g of protamine sulfate/10 g of protein). Stirring was continued for 20 min after the addition and then the extract was adjusted to pH 4.6 by dropwise addition of 2 N acetic acid. After standing at 4° for 30 min, the extract was centrifuged at 20,000*g* for 30 min.

Solid ammonium sulfate was added to the pH 4.6 supernatant (step 2, Table I) to achieve 65% saturation, and the precipitate obtained after centrifugation at 20,000*g* was dissolved in about 80 ml of acetate buffer. The resulting suspension was clarified by centrifugation at 9000*g* for 20 min. The supernatant (step 3) was layered on top of a Sephadex G-200 column (5 × 90 cm) and eluted with acetate buffer. The fractions containing the enzyme were combined (step 4) and run into a DEAE-cellulose column (2 × 22 cm) prepared in acetate buffer. Air pressure (4 psi) was used in preparing the column and applying the sample. The column was washed with about 200 ml of acetate buffer to remove unadsorbed protein and then developed with a linear gradient between 1800 ml of acetate buffer and 1800 ml of 0.3 M acetate buffer containing 1 mM EDTA (pH 5.5). The enzyme was eluted when about 700 ml of buffer had emerged from the column. The active enzyme fractions were combined and treated with 2 μmoles of PLP and placed at 25° for 1 hr. The enzyme solution was then brought to 75% $(NH_4)_2SO_4$ saturation by addition of solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and then dissolved in acetate buffer. This solution was passed through a coarse grade Sephadex G-25 column (2 × 65 cm) prepared in acetate buffer (without EDTA). The active fractions were combined (step 5). This preparation, which exhibited a specific activity of 57 units/mg, contained one minor component as determined by acrylamide gel electrophoreis. This impurity was removed in the final step.

Portions of fraction 5 (containing about 25 mg of protein) were brought to 60% $(NH_4)_2SO_4$ saturation and the resulting precipitate was successively extracted with

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PMB, *p*-mercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoate); PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate.

[2] We are indebted to Dr. Shigetaka Suzuki and Dr. Daniel Wellner for the amino acid analyses.

[3] We thank Dr. Stanley E. Charm and his associates for their valuable help in this work.

TABLE I: Purification of Aspartate β-Decarboxylase.

Fraction	Vol (ml)	Protein		Activity		Yield (%)
		Concn (mg/ml)	Total (mg)	Sp (units/mg)[a]	Total (units)	
1. Cell extract	1665	27	45,000	0.7	31,500	100
2. Supernatant from protamine and pH 4.6 treatment	1685	5.8	9,690	2.2	21,300	68
3. $(NH_4)_2SO_4$ fraction (0–65%)	95	57.5	5,460	3.3	18,000	57
4. Eluate from Sephadex G-200	195	5.6	1,100	12.7	14,000	44
5. DEAE-cellulose eluate after $(NH_4)_2SO_4$ precipitation and gel filtration (Sephadex G-25)	25	7.9	197	57	11,200	37
6. Final preparation[b]	41	3.4	140	60	8,400	27

[a] Micromoles of product formed per minute (unit) per milligram of protein under the conditions described in the text. [b] Batches (25 mg) of fraction 5 were extracted with solutions containing decreasing concentrations of $(NH_4)_2SO_4$ as described in the text.

TABLE II: Amino Acid Composition of Aspartate β-Decarboxylase.

Amino Acid	Extrapolated Values from 2 Independent Analyses (moles/50,000 g of enzyme)		Av No. of Residues/50,000 g of Enzyme
Lysine	20.1	20.9	21
Histidine	5.2	5	5
Arginine	28.2	27.9	28
Aspartic	52.0	47.8	50
Threonine	17.3	18.4	18
Serine	28.8	30.9	30
Glutamic	49.5	46.1	48
Proline	21.5	20.7	21
Glycine	30.5	32.4	31
Alanine	45.1	46.7	46
Half-cystine	2.2,[a] 2.5[b]	1.95[c]	2
Valine	26.3	26.6	26
Methionine	10.7	10.9	11
Isoleucine	22.8	22.3	23
Leucine	53.7	57.1	55
Tyrosine	18.8	19.0	19
Phenylalanine	22.5	20.3	21
Tryptophan	3.7[d]		4
PLP			1[e]

[a] As cysteic acid. [b] As *S*-carboxymethylcysteine. [c] From DTNB titration of reduced enzyme (see Table III). [d] Determined spectrophotometrically (Edelhoch, 1967). [e] Wilson and Meister (1966).

4-ml volumes of acetate buffer solutions which were made 50, 42, 38, and 30% with respect to $(NH_4)_2SO_4$ saturation by trituration with a glass rod and then centrifuged at 27,000*g* for 20 min. The supernatant was decanted and the residue was resuspended in a solution of lower salt concentration; the process was then repeated.

The contaminants present in fraction 5 were extracted along with some enzyme in solutions which were 50 and 42% $(NH_4)_2SO_4$ saturation, while the major portion of the enzyme was solubilized at 38 and 30% $(NH_4)_2SO_4$ saturation. The extracts were pooled and brought to 60% $(NH_4)_2SO_4$ saturation by adding sat-

TABLE III: Determination of the Sulfhydryl Groups of Aspartate β-Decarboxylase by Reaction with 5,5′-Dithiobis(2-nitrobenzoate).

Reaction Conditions	Moles of Thionitrobenzoate Produced/50,000 g of Enzyme (values from separate determination)	Av No. of Sulfhydryl Groups/50,000 g of Enzyme
Holoenzyme in 0.1 M phosphate buffer containing 1 mM EDTA (pH 6.8)	0, 0	0
Apoenzyme in 0.1 M phosphate buffer containing 1 mM EDTA (pH 6.8)	0, 0	0
Apoenzyme in 6.4 M urea, 0.05 M phosphate buffer containing 1 mM EDTA (pH 6.5)[a]	1.55, 1.73	1.64
Apoenzyme in 5.5 M guanidine hydrochloride, 0.05 M phosphate, and 1 mM EDTA (pH 6.5)[a]	1.63, 1.80	1.72
Apoenzyme in 5 M guanidine hydrochloride, 0.1 M phosphate, and 10 mM EDTA (pH 6.2); after reduction with dithiothreitol[b]	1.93, 1.97	1.95

[a] A determination of this type on the holoenzyme was attempted; however, PLP dissociates in the presence of high concentrations of guanidine hydrochloride or urea and the absorbance of the resulting free PLP at 412 mμ interferes significantly with the determination of thionitrobenzoate. [b] Apoenzyme (2.4 mg) was incubated at 37° for 2 hr in 0.5 ml of a solution containing 0.2 M $NaHCO_3$, 15 mM EDTA, 40 mM dithiothreitol, and 5 M guanidine hydrochloride. The mixture was then applied to the top of a coarse Sephadex G-25 column and eluted with 0.1 M potassium phosphate buffer containing 10 mM EDTA and 5 M guanidine hydrochloride (pH 6.2). The protein was determined from the absorbance at 280 mμ. The reaction with DTNB was carried out immediately after elution of the protein from the column.

urated $(NH_4)_2SO_4$ solution. The precipitate obtained by centrifugation was dissolved in acetate buffer and passed through a Sephadex G-25 column as described above. The enzyme thus obtained was homogeneous when examined in the analytical ultracentrifuge and by the acrylamide gel technique. The specific activity of this preparation was about 60 units/mg. A representative purification is summarized in Table I.

Results

Amino Acid Composition of Aspartate β-Decarboxylase. The results of amino acid analyses on two preparations of the enzyme are given in Table II. Since the enzyme contains 1 mole of PLP/50,000 g (Wilson and Meister, 1966), we have taken this value as the molecular weight of the minimal catalytic unit, and have thus expressed the amino acid composition as the number of residues/50,000 g of enzyme. Study of the products of tryptic digestion of aspartate β-decarboxylase indicate that about 43 peptides are formed (M. Jensen, unpublished data); this result is in accord with the view that the enzyme consists of identical units having a molecular weight of about 50,000. The least frequent amino acid residue is cysteine; the value (2.2) obtained for cysteic acid in hydrolysates of the performic acid oxidized enzyme is not far from that obtained for *S*-carboxymethylcysteine in hydrolysates of the enzyme which had been reduced and then carboxymethylated in 8 M urea. Titration of the apoenzyme with DTNB in 6 M urea or in 5.5 M guanidine hydrochloride (Table III) gave values approaching 2 for the number of sulfhydryl groups/50,000 g of enzyme; these data are therefore in accord with the amino acid analyses (Table II). Titration of the enzyme immediately after reduction with dithiothreitol (Table III) also gave values very close to 2 sulfhydryl groups/50,000 g of enzyme. It may therefore be concluded that the enzyme does not contain disulfide linkages.

Reaction of the Enzyme with PMB. Titration of the apoenzyme and holoenzyme with PMB-^{14}C was carried out and the increase in absorbancy at 251 mμ was determined (Figure 1, left-hand ordinate). These studies indicate that both forms of the enzyme react with close to 2 moles of PMB/50,000 g of enzyme. After titration was complete, the PMB-^{14}C-treated enzyme preparations were subjected to gel filtration as described in Table IV. These data indicate that both the PMB–holoenzyme and the PMB–apoenzyme contain close to 2 moles of bound PMB-^{14}C. It is of interest, however, that the PMB–holoenzyme exhibited about 25% more desulfinase activity than did the untreated holoenzyme (Figure 1). On the other hand, the PMB–apoenzyme, after incubation with PLP, was only about 55% as active as the untreated holoenzyme (or the untreated apoenzyme after incubation with PLP). The effects of PMB

TABLE IV: Reaction of Holoenzyme and Apoenzyme with PMB-^{14}C.[a]

Enzyme Derivative	Fraction from Sephadex G-25 Column	Protein Concn (mg/ml)	PMB-^{14}C (mμmoles/fraction)	moles of PMB-^{14}C Bound/50,000 g of Enzyme
PMB–holoenzyme	9	0.58	25.0	2.14
	10	0.24	10.8	2.25
PMB–apoenzyme	10	0.16	6.24	2.01
	11	0.18	6.74	1.90

[a] Holoenzyme and apoenzyme were treated with PMB-^{14}C (Figure 1) and then passed through a coarse Sephadex G-25 column (1 × 25 cm). Elution was carried out with 0.05 M sodium acetate buffer (pH 5.5, PMB–holoenzyme; pH 6.0, PMB–apoenzyme). The eluates were analyzed for protein and radioactivity as described in the text.

treatment of the enzyme on decarboxylase were similar to those on desulfinase activity. However, it is notable that the aspartate–pyruvate transaminase activity was essentially unaffected by treatment of the enzyme with PMB. Thus, values for the specific activity of transaminase of 11.7, 12.6, and 11.0 units/mg were obtained for the untreated holoenzyme, PMB–holoenzyme, and PLP-reconstituted PMB–apoenzyme, respectively.

The finding that the PMB–holoenzyme and PLP-reconstituted PMB–enzyme exhibit different desulfinase activities suggests that these forms of the enzyme differ structurally. The observation that the ultraviolet absorption curves of these enzyme forms are significantly different (Figure 2) is consistent with this view. The observed spectral differences are apparently not related to differences in the amount of PLP bound; thus, determinations of bound PLP by the phenylhydrazone method of Wada and Snell (1961) gave values that were the same, within experimental error, for the PMB–holoenzyme and the PLP-reconstituted PMB–apoenzyme.

Titration of the pyridoxamine 5′-phosphate–enzyme with PMB indicated that this form of the enzyme also reacts with 2 moles of PMB (Figure 3). The desulfinase activity of this form of PMB–enzyme was determined (after incubation with pyruvate); like the PLP-reconstituted PMB–apoenzyme, it exhibited substantially reduced desulfinase activity as compared to the holoenzyme (Figure 3).

Removal of PMB from the Enzyme. The bound PMB in the several forms of aspartate β-decarboxylase can be removed by dialysis against 0.05–0.1 M sodium acetate buffer (pH 5–6) containing 5–10 mM L-cysteine (Table V). Similar results were obtained when either dithiothreitol or 2-mercaptoethanol was substituted for L-cysteine. When the PMB–apoenzyme (IV) was treated in this manner and then reconstituted by incubation with PLP, a preparation exhibiting about 73% desulfinase activity of the holoenzyme was obtained. This compares with a recovery of 94% desulfinase

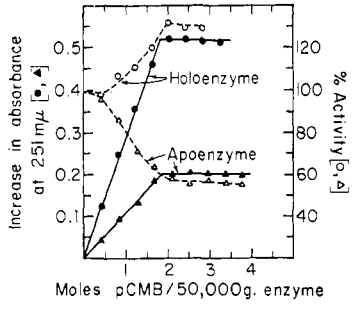

FIGURE 1: Reaction of PMB with the holoenzyme and apoenzyme. Left-hand ordinate: increase in absorbance at 251 mμ after addition of PMB to apoenzyme (▲) and holoenzyme (●). The apoenzyme (0.6 mg/0.5 ml of 0.1 M sodium acetate buffer, pH 6) was titrated with 10-μl portions of 0.5 mM PMB-^{14}C at 25°. The holoenzyme (1.57 mg/0.5 ml of 0.1 M acetate buffer, pH 5.5) was titrated with 5-μl portions of 2.53 mM PMB-^{14}C. The increase in absorbancy at 251 mμ was recorded 5 min after addition of the reagent. Enzyme activity (determined in the standard assay system with cysteinesulfinate as substrate at 25°): of holoenzyme (○); of apoenzyme (△) (after reconstitution with PLP).

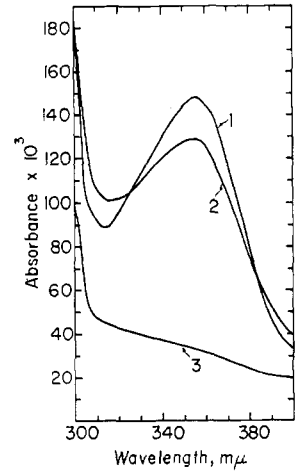

FIGURE 2: Ultraviolet absorption curves of PLP-treated apoenzyme (curve 1) and PLP-reconstituted PMB–enzyme (curve 2). Curve 3 is apoenzyme. Protein concentration, 1 mg/ml in 0.05 M sodium acetate buffer (pH 5.5). Reconstitution with PLP was carried out by incubating the apoenzyme forms with 1 mM PLP in 0.05 M acetate buffer (pH 5.5) for 1 hr at 25°, followed by removal of free PLP by gel filtration on a Sephadex G-25 column (1 × 25 cm); elution was carried out with 0.05 M acetate buffer (pH 5.5). The curves for the isolated holoenzyme and PMB–holoenzyme were identical with that for the PLP-treated apoenzyme (curve 1).

TABLE V: Desulfinase and Transaminase Activities of the Various Forms of L-Aspartate β-Decarboxylase.

Derivation of Enyzme Form[a]	Moles of PMB Bound/50,000 g of Enzyme	Desulfinase Act. Units/mg	Transaminase Act. Units/mg
Holoenzyme (I; isolated)	0	55.0 (103)[b]	11.7
PMB–apoenzyme (IV) → apoenzyme (III) → holoenzyme (I)	0.03	40.0	
PMB–holoenzyme (II) → holoenzyme (I)	0.03	51.6	
Holoenzyme (I) → PMB–holoenzyme (II)	2	72.2 (120)	12.6
Apoenzyme (III) → PMB–apoenzyme (IV) → PLP-reconstituted PMB–enzyme (V)	2	26.8 (59.1)	11.8
PMB–holoenzyme (II) → PMB–apoenzyme (IV) → PLP-reconstituted PMB–enzyme (V)	1.6	22.8	
PLP-reconstituted PMB–enzyme (V) → holoenzyme (I)	0.03	48.1	

[a] The Roman numerals given in parentheses indicate the pathway by which the enzyme form was obtained (see also Figure 4). Reconstitution was achieved by incubating 1.5 mg of apoenzyme or PMB–apoenzyme with 0.5 μmole of PLP in 0.5 ml of 0.05 M sodium acetate buffer (pH 6) at 25° for 1 hr. The excess PLP was removed by passing the enzyme solution through a Sephadex G-25 column (1 × 25 cm). PMB was removed by dialyzing against 200 volumes of 0.05 M acetate buffer (pH 6) containing 0.01 M L-cysteine for 15 hr; some loss of PLP occurred during dialysis and the enzyme preparations were therefore incubated with PLP as described above after dialysis. [b] Aspartate β-decarboxylase activities are given in parentheses.

activity when PMB holoenzyme (II) was converted into the holoenzyme (I). When the PMB–apoenzyme (IV) (prepared from the apoenzyme (III)) was incubated with PLP, a PLP-reconstituted PMB enzyme was obtained (V), which was far from identical with the PMB holoenzyme (II) with respect to desulfinase activity. Resolution of the PMB–holoenzyme (II) to yield the PMB–apoenzyme (IV) followed by treatment with PLP gave a PLP-reconstituted PMB–enzyme (V), which had about the same activity as the form obtained from PMB–apoenzyme (IV) derived from the apoenzyme (III). Thus, it appears that this form of the enzyme (V) may be obtained by two routes (Figure 4). When the PLP-reconstituted PMB–enzyme (V) was dialyzed against L-cysteine, a preparation was obtained which exhibited about 87% of the activity of the holoenzyme.

Evidence for Dissociation of the Enzyme. The migration patterns obtained on polyacrylamide gel electrophoresis in 4% gels at pH 8.0 were similar for holoenzyme (I), PMB–holoenzyme (II), and PLP-reconstituted PMB–enzyme (V). However, the apoenzyme (III) (and PMB–apoenzyme (IV)) moved considerably more rapidly than the holoenzyme forms of the enzyme (Figure 5). Under these conditions of gel electrophoresis the primary separation effect is due to molecular sieving (Hjersten *et al.*, 1965). The R_F values, expressed as the ratio of the distance moved by the protein to the length of the gel column, are 0.41 and 0.26 for the apoenzymes and the holoenzymes, respectively, in 4% gels. In 6% gels, the corresponding values are 0.32 and 0.08, respectively. The ratios of the R_F values (apoenzyme:holoenzyme) are 1.6 and 4.0 in 4 and 6% gels, respectively; this supports the view that the difference in migration between the apoenzyme and holoenzyme forms reflects a difference in size rather than in charge. This interpretation has been substantiated by ultracentrifugal studies which show that the apoenzyme (at pH 8.0) exhibits a sedimentation coefficient ($s_{20,w}$) of 6 S; the sedimentation coefficient of the holoenzyme was found to be 19 S, in agreement with earlier findings (Novogrodsky and

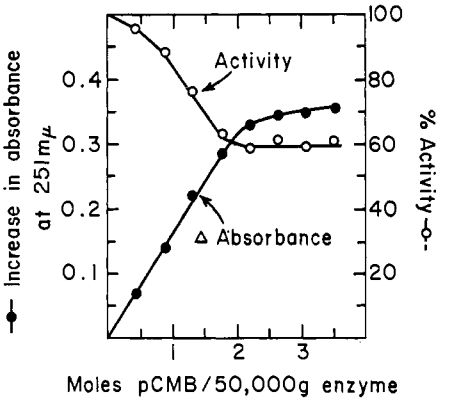

FIGURE 3: Titration of the PMP–enzyme with PMB. The apoenzyme (0.57 mg) was incubated in 0.5 ml containing 0.1 M sodium acetate buffer (pH 6) and 0.01 M PMP for 15 min at 26°. Titration was carried out with 5-μl portions of 1 mM PMB. Samples (5 μl) were withdrawn at intervals and added to a standard assay mixture containing 2 mM sodium pyruvate, but no cysteinesulfinate. After incubation at 25° for 30 min, the assay reactions were initiated by addition of cysteinesulfinate.

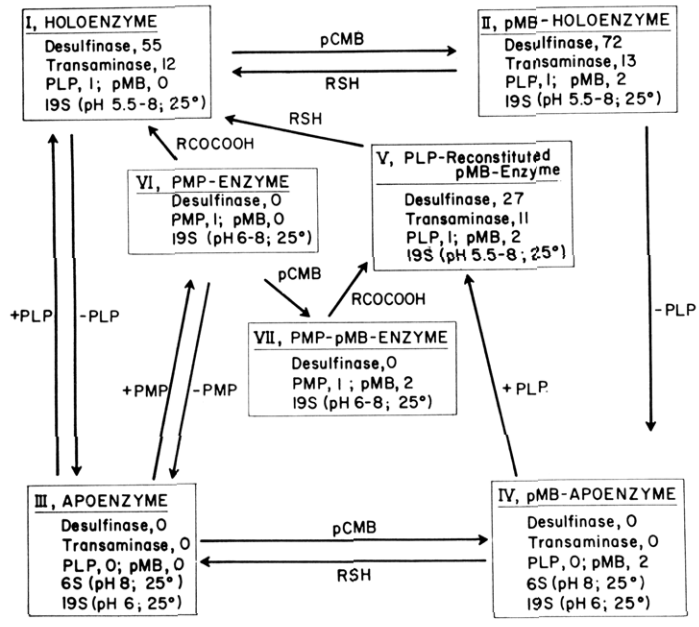

FIGURE 4: Summary of the interrelationships and properties of the several forms of the enzyme. The values for PLP and PMB are given as moles/50,000 g of enzyme. Specific transaminase and desulfinase activities were determined and are expressed as described in the Methods section.

Meister, 1964).[4] When the apoenzyme was treated with PLP, there was prompt conversion of the 6S apoenzyme into the 19S holoenzyme; this was established by the gel electrophoresis procedure (Figure 5). Similar studies on the 6S PMB–apoenzyme indicated that this form of the enzyme also undergoes association to form the 19S PMB–holoenzyme in the presence of PLP.

Discussion

The amino acid analyses of aspartate β-decarboxylase show that (a) the least frequent residue is cysteine, (b) there are 2 residues of cysteine/50,000 g of enzyme (the minimal catalytic unit), and (c) the enzyme does not have disulfide bonds. The sulfhydryl groups of the enzyme appear to be masked since 5,5′-dithiobis(2-nitrobenzoate) fails to react with them unless the reaction is carried out in relatively high concentrations of urea or guanidine hydrochloride. However, PMB reacts readily in the absence of these reagents, both the apoenzyme and the holoenzyme bind 2 moles of PMB/50,000 g of enzyme. The PMP–enzyme also binds 2 moles of PMB. It is evident that the same sulfhydryl groups in the several forms of the enzyme react with PMB. The effect of such treatment on desulfinase (and decarboxylase) activity is strikingly different for the two forms of the enzyme. Thus, the activity of the holoenzyme increases by about 25% and this increase in activity is closely parallel to the uptake of PMB by the enzyme. In contrast, treatment of the apoenzyme with PMB followed by reconstitution with PLP gives a form

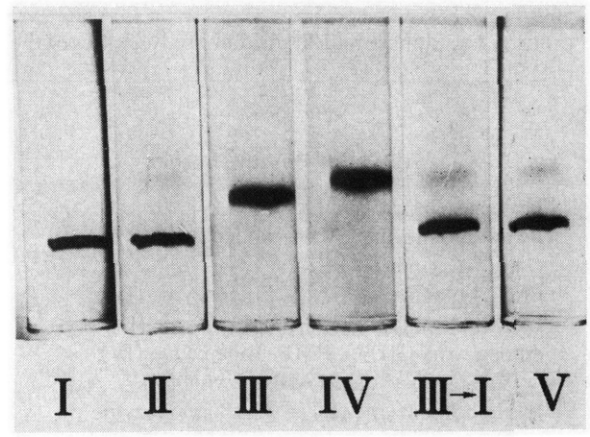

FIGURE 5: Polyacrylamide gel electrophoresis patterns of various forms of the enzyme. The Roman numerals correspond to those given in Figure 4 (see also Table V).

of the enzyme which exhibits only about 50% of the activity of the reconstituted holoenzyme. This difference in enzymatic activity is apparently not related to the PLP content of the two preparations of the enzyme. Furthermore, it is of interest that the aspartate–pyruvate transaminase activities of the holoenzyme (I), PMB–holoenzyme (II), and the PLP-reconstituted PMB enzyme (V) are virtually the same. The failure of PMB to affect aspartate–pyruvate transaminase activity is of interest and suggests that there is a fundamental difference in the manner in which the enzyme participates in this reaction and in the decarboxylation of aspartate or the desulfination of cysteine sulfinate. Data to be published later on the effect of various PLP analogs also indicate that the desulfinase (or decarboxylase) and transaminase activities can be separately affected.

Both the activation and the inhibition phenomena associated with attachment of PMB to the enzyme are reversed by treatment of the enzyme with mercaptan. It is notable that the activated form (PMB–holoenzyme, II), when resolved (II → IV) and then treated with PLP to effect reconstitution (IV → V), exhibits only about half of the activity of the holoenzyme. It seems probable that the alterations in desulfinase and decarboxylase activities related to attachment of PMB to the enzyme are associated with conformational changes in the enzyme apparently brought about by reaction of the sulfhydryl groups with the mercurial reagent. The differences between the ultraviolet absorption spectrum of the holoenzyme and that of the PLP-reconstituted PMB–enzyme (Figure 2) are consistent with this interpretation. It seems pertinent that treatment of the holoenzyme with PMB leads to activation of the desulfinase and decarboxylase activities, while PMB treatment of the apoenzyme or of the PMP enzyme (VI) results in a less active form which is convertible (by removal of PMB) into a form of the enzyme that is closely equivalent to the holoenzyme. It is probable that the type or extent of conformational change associated with binding of PMB to the PLP–enzyme differs from that which occurs on PMB binding to the apoenzyme or an enzyme form containing a vitamin B_6 derivative which does not form a Schiff base linkage with the enzyme.

[4] The authors are indebted to Dr. Rudy H. Haschemeyer and Mr. William F. Bowers for the ultracentrifugation studies.

The present data indicate that desulfinase, decarboxylase, and transaminase activities of aspartate β-decarboxylase are not dependent upon the presence of enzyme sulfhydryl groups. The findings also indicate that the sulfhydryl groups are not directly involved in the binding of PLP to the enzyme. However, it is evident that attachment of a bulky substituent to the sulfhydryl groups can alter, *i.e.*, activate or inhibit, enzymatic activity. It is conceivable that this type of reaction, which involves combination of the enzyme sulfhydryl groups with a small molecule, functions in the physiological mechanism for the control of enzymatic activity.

A by-product of these studies on the function of the sulfhydryl groups of aspartate β-decarboxylase is the interesting finding that a striking structural change results upon resolution of the enzyme at pH 8 as demonstrated by the acrylamide gel electrophoresis and ultracentrifuge studies. Thus, the apoenzyme exists, at pH 8 and 25°, entirely as a dissociated form of the enzyme, with an $s_{20,w}$ value of 6 S. This compares with a value of 19 S for the holoenzyme. Under these conditions conversion of the 6S apoenzyme into the 19S component is brought by PLP. It was found previously in this laboratory (Novogrodsky and Meister, 1964) that resolution of the holoenzyme at pH 5 yields an apoenzyme possessing a sedimentation coefficient ($s_{20,w}$) of 19 S. This finding has been confirmed; it thus appears that the tendency of the enzyme to associate and dissociate is affected by pH as well as by the presence of PLP. A detailed study of the subunit structure of aspartate β-decarboxylase by means of electron microscopy and analytical ultracentrifugation by W. Bowers and Dr. R. H. Haschemeyer is now in progress. It is of considerable interest that conversion of the 6S apoenzyme into the 19S holoenzyme occurs at pH 8 in the presence of vitamin B_6 analogs such as PMP and 4′-deoxypyridoxine 5′-phosphate. This indicates that a covalent linkage between coenzyme and the apoenzyme is not required for the processes that bring about aggregation of the 6S units. It is notable, however, that the phosphate moiety of PLP is required; thus, pyridoxal was ineffective. These findings are of particular interest in relation to those of Morino and Snell (1967), who have shown that apotryptophanase of *Escherichia coli* exists as a tetramer (mol wt 220,000; $s_{20,w}$ = 9.5 S) at room temperature and pH 8, but dissociates reversibly at low temperature and protein concentration to a dimer (mol wt 110,000; $s_{20,w}$ = 6 S) when K^+ or Na^+ is present. The holotryptophanase, however, does not dissociate under these conditions. Addition of PLP or some of its analogs to the dimeric apotryptophanase causes the formation of the tetrameric structure and this is accompanied by a large conformational change in the enzyme. It would be of interest to determine whether this effect of PLP on the subunit interactions of these two enzymes is, in fact, a general phenomenon exhibited by vitamin B_6 enzymes.

References

Boyer, P. D. (1954), *J. Am. Chem. Soc. 76*, 4331.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Craven, G. R., Steers, E., and Anfinsen, C. B. (1965), *J. Biol. Chem. 240*, 2468.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Edelhoch, H. (1967), *Biochemistry 6*, 1948.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Hjersten, S., Jerstedt, S., and Tiselius, A. (1965), *Anal. Biochem. 11*, 211.

Lloyd, P. H. (1963), *Biochem. J. 88*, 587.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Moore, S. (1963), *J. Biol. Chem. 238*, 235.

Morino, Y., and Snell, E. E. (1967), *J. Biol. Chem. 242*, 5591.

Novogrdosky, A., and Meister, A. (1964), *J. Biol. Chem. 239*, 879.

Novogrodsky, A., Nishimura, J. S., and Meister, A. (1963), *J. Biol. Chem. 238*, PC1903.

Soda, K., Novogrodsky, A., and Meister, A. (1964), *Biochemistry 3*, 1450.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Wada, H., and Snell, E. E. (1961), *J. Biol. Chem. 236*, 2089.

West, P. W., and Gaeke, G. C. (1956), *Anal. Chem. 28*, 1816.

Wilson, E. M. (1963), *Biochim. Biophys. Acta 67*, 345.

Wilson, E. M., and Meister, A. (1966), *Biochemistry 5*, 1166.