Purification and Properties of an α-Dialkyl Amino Acid Transaminase*

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ABSTRACT: α-Dialkyl amino acid transaminase has been isolated 95% pure from a bacterium grown on isovaline as its sole fixed nitrogen source. The enzyme catalyzes the decarboxylation-dependent transamination of isovaline, α-aminoisobutyric acid, and cycloleucine to their respective ketones and CO_2. For this activity, the enzyme requires pyridoxal 5′-phosphate ($K_m = 5 \times 10^{-6}$ M) and pyruvate ($K_m = 2 \times 10^{-3}$ M). During the reaction pyruvate is converted to L-alanine stoichiometrically with carbon dioxide release and ketone formation. The reverse of the reaction in which CO_2 was released was not detected, whereas the exchange transamination between L-alanine and pyruvate was readily reversible.

The enzymatic decarboxylation of AIB[1] was first shown in extracts of a soil organism which had been grown on this dialkyl amino acid as its sole carbon and nitrogen source (Aaslestad and Larson, 1964). The reaction required pyruvate and yielded alanine, acetone, and CO_2 (Aaslestad and Larson, 1965). The latter two of these products were characteristic of that reaction called decarboxylation-dependent transamination by Kalyankar and Snell (1962) in their studies of the pyridoxal-catalyzed, nonenzymatic decarboxylation of AIB.

Evidently then, the over-all enzyme reaction (eq 3) was the sum of a decarboxylation-dependent transamination (eq 1) and a more common exchange transamination (eq 2).

$$\begin{array}{l} \mathrm{RCH_2C(CH_3)(NH_2)COOH + PLP\text{-}enzyme} \longrightarrow \\ \qquad \mathrm{RCH_2C(CH_3)=O + PMP\text{-}enzyme + CO_2} \end{array} \quad (1)$$

$$\mathrm{PMP\text{-}enzyme + pyruvate} \rightleftharpoons \mathrm{alanine + PLP\text{-}enzyme} \quad (2)$$

Sum

$$\begin{array}{l} \mathrm{RCH_2C(CH_3)(NH_2)COOH + pyruvate} \longrightarrow \\ \qquad \mathrm{alanine + CO_2 + RCH_2C(CH_3)=O} \end{array} \quad (3)$$

where R = H for AIB and R = CH_3 for isovaline.

We felt that the over-all reaction was of unusual interest because the enzyme involved apparently cleaved alternately, first an α-carboxyl and then an α-hydrogen in the same catalytic site. Consequently, we undertook a detailed study of this enzyme with the goal of describing the most plausible mechanism for the reaction. We report here data on the purification of a decarboxylation-dependent transaminase, the identification of the products of the reaction, the stoichiometry of the reaction, and the reversibility of the two half-reactions.

To avoid confusion we wish to make clear that decarboxylation-dependent transamination is not another example of the kind of reaction catalyzed by aspartic β-decarboxylase. This latter enzyme could β-decarboxylate continuously without any pyruvate requirement. Only when aspartate is transaminated *instead* of decarboxylated is pyruvate needed to regenerate the enzyme (Novogrodsky and Meister, 1964). A different case exists with decarboxylation-dependent transamination. Here every time a molecule of CO_2 is released, one molecule of PLP-enzyme is necessarily transaminated to PMP-enzyme. In addition, the β-decarboxylase cleaves either α-hydrogen or β bonds in the same site, whereas the decarboxylation-dependent

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[1] Abbreviations used: AIB, α-aminoisobutyric acid; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate.

transaminase cleaves either of two different α bonds in the same site.

Experimental Section

Chemicals. Commercial isovaline was crystallized from aqueous acetone for enzyme assays. PLP was purchased from the California Corp. for biochemical research or from Sigma Chemical Co.

Syntheses. [1-^{14}C]- and DL-[2-^{14}C]isovaline were synthesized from butanone, ammonia, and potassium cyanide by a Strecker reaction following the protocol of Levene and Steiger (1928) with minor modifications. K^{14}CN was used for synthesis of DL-[1-^{14}C]isovaline, whereas [2-^{14}C]butanone, prepared in this laboratory by ethyl addition to [1-^{14}C]acetyl chloride, was used for synthesis of DL-[2-^{14}C]isovaline.

Media. Nitrogen was supplied by 0.2% α-dialkyl amino acid and carbon by 0.5% glucose in a salts medium lacking ammonium sulfate but otherwise identical with that described by Dempsey (1965). The medium used for large-scale cultures was 0.15% DL-isovaline and 0.15% AIB in the above glucose salts at pH 7.5. Incubation was at 30° with sterile air introduced through a fritted-glass sparger. Foaming was suppressed with General Electric Antifoam 60. Full growth was attained in 36 hr with a 1:16 inoculum. Cells were harvested in a Sharples centrifuge and suspended in 0.01 M potassium phosphate (pH 7.5) for lyophilization. Dried cells were stored at −20°.

Enzyme Assays. Three assays were used. Assay I measured acetone from AIB by a modification of the phenylhydrazone method of Greenberg and Lester (1944). Acetone production was linear during the first 10% of the reaction and was a linear function of protein concentration when the assay contained 20 mM sodium pyruvate, 20 mM AIB, 50 mM potassium phosphate (pH 7.5), plus 0.1 mM PLP added from an acidified aqueous stock solution just prior to assay. Reaction was initiated with enzyme and was carried out at 30° with either a boiled enzyme control or a reagent blank. At various times, portions up to 0.5 ml of the reaction mixture were pipetted directly into an 18 × 150 mm screw-cap culture tube containing 0.2 ml of 5 N NaOH. Acetone was extracted by shaking for 15 min with 10 ml of carbon tetrachloride. The aqueous phase was discarded and 3.0 ml of 0.2% 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid added. The tubes were shaken another 20 min to allow formation and extraction of the neutral acetone 2,4-dinitrophenylhydrazone. The aqueous phase was again discarded and the organic phase was washed twice with 0.5 N sodium hydroxide. The absorbancy of the 2,4-dinitrophenylhydrazone in carbon tetrachloride was measured directly in the reaction tube with a Bausch and Lomb Spectronic 20 colorimeter. A standard curve was constructed by following the same isolation procedure with aliquots of the assay solution containing known amounts of acetone.

Assay II measured radioactive butanone formed from DL-[2-^{14}C]isovaline. This assay procedure was the same as assay I through the point of the termination of the reaction with base. Toluene (10 ml) was then added instead of carbon tetrachloride, and the ketone was extracted quantitatively into this solvent. The radioactivity in the toluene layer was measured directly.

Assay III measured $^{14}CO_2$ released from DL-[1-^{14}C]-isovaline by the technique devised by Cuppy and Crevasse (1963). To start the reaction, 0.2 ml of enzyme was added to 0.8 ml of the assay mixture described above. Reaction was stopped and carbon dioxide was released by injecting 0.2 ml of 2 M sulfuric acid.

Exchange between Alanine and Pyruvate. This was measured by ether extraction of [1-^{14}C]pyruvate formed from L-[1-^{14}C]alanine. In this case, up to 0.5 ml of a reaction solution, similar to that described above but containing 0.02 M [1-^{14}C]alanine instead of AIB, was pipetted after incubation into 0.2 ml of 2 M sulfuric acid. Anhydrous ether (*15 ml*) was added and the aqueous phase was extracted for 30 min. Aliquots of the ether layer were then counted in 10 ml of Bray's (1960) solution. The efficiency of the pyruvate extraction procedure was determined with reaction solutions to which known amounts of [1-^{14}C]pyruvate had been added.

Chromatographic Isolation of Amino and Keto Acids. α-[1-^{14}C]Ketobutyrate, formed by transamination of DL-α-[1-^{14}C]amino-*n*-butyrate, was separated from the labeled amino acid substrate by ion-exchange chromatography. Enzymatic reactions were stopped by heating for 3 min at 100°, and 1.0 ml of cooled mixture was placed directly on top of a 1 × 12 cm column of Dowex 50W-X4 (H^+), 50–100 mesh. The keto acids were eluted with 10 ml of water, and the eluate was counted to measure the radioactive α-keto acid formed.

To measure transamination of nonradioactive amino acids, [1-^{14}C]pyruvate was used as the α-keto acid substrate; and the [1-^{14}C]alanine produced was separated from the labeled substrate on an identical column. After the unreacted [^{14}C]pyruvate was washed from the column the bound amino acids were eluted with 2 N NH_4OH and this eluate was analyzed for radioactivity.

Miscellaneous. Radioactivity measurements were made in dioxane-based scintillation fluid (Bray, 1960) with a Model 3003 Packard Tri-Carb liquid scintillation spectrometer. Unless otherwise noted, the enzyme and fractions containing it were in 0.02 M potassium phosphate (pH 7.5) and 0.05 M in KCl. This is defined as buffer in the text. In some instances, noted in the text, this buffer was also 50 μM in PLP. Ultimately, it was determined that added PLP did not appear necessary for stabilization. All protein solutions were maintained between 0 and 12° during purification and were stored at either −20 or +5°. The enzyme remained reasonably stable for several months at either temperature when the protein concentration was greater than 2 mg/ml. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Disk electrophoresis was performed by the method

TABLE I: Purification of α-Dialkyl Amino Acid Transaminase.[a]

Purification Step	Vol. (ml)	Protein (mg)	Sp Act. (μM acetone min mg)	Yield (%)
1. Cell sonicate	1,400	36,400	0.058	100
2. 10^5g supernatant	1,350	14,300	0.14	94
3. 33–50% $(NH_4)_2SO_4$	70	3,000	0.38	56
4. Sephadex G-200	180	967	1.04	47
5. First DEAE-cellulose	150	215	4.5	46
6. Second DEAE-cellulose	10.7	23.9	8.2	10
7. 50% $(NH_4)_2SO_4$	2.6	13.5	10	6

[a] From 60 g of dry cells. Details of the procedure are described in the text.

of Clark (1964). Analytical ultracentrifugation was kindly performed by G. Norris of the Department of Pediatrics, University of Florida. Measurement of radioactive areas on thin layer chromatography plates was done by the method of Snyder (1964).

Results

The organism adopted as a source of enzyme for this study was found after screening locally available pure cultures for the ability to (a) use isovaline as a carbon or nitrogen source and (b) to release $^{14}CO_2$ from [1-^{14}C]isovaline. The organism used had the laboratory designation Ps-1. It was originally isolated as an *Escherichia coli* contaminant and appeared to be a *Pseudomonad.* Ps-1 could not use the carbon skeleton of isovaline for growth.

Purification of the Enzyme. Lyophilized cells (60 g) were suspended in 1200 ml of buffer (with PLP) and disrupted in 25-ml batches at 0–10° for 15 min with an MSE 60-w ultrasonic oscillator. More than 90% of the enzyme activity remained in the supernatant after centrifugation of the sonicate at 100,000*g* for 90 min.

Neutral ammonium sulfate was used to concentrate protein solutions for passage over gel filtration columns and also to effect a 2.5-fold purification from the 100,000*g* supernatant. The fraction soluble at 33% but insoluble at 50% ammonium sulfate saturation was dissolved in buffer (with PLP) to a final concentration of 43 mg of protein/ml and cleared of insoluble material by centrifugation. The supernatant was made 2% in sucrose, layered on the top of the 4.5 × 85 cm Sephadex G-200 column, and eluted with buffer (with PLP) at a flow rate of 20 ml/hr. The transaminase had an elution:excluded volume ratio of 1.7. Comparison of this value with data accumulated by Andrews (1965) allowed a molecular weight estimation of slightly less than 150,000 for the enzyme.

The column described here was eluted by descending buffer flow. More efficient fractionations were achieved in later preparations using presized beads of 40–60-μ diameter and ascending elution.

The most active fractions from the gel filtration step contained 970 mg of protein in 180 ml. These were combined and allowed to soak into a 2.5 × 85 cm column of Schleicher & Schuell type 40 DEAE-cellulose, which had been equilibrated with buffer. The column was then washed with 450 ml of the equilibration buffer. When protein was no longer detected in the effluent, the enzyme was eluted with a linear KCl gradient (0.05–0.5 M in 0.02 M potassium phosphate, pH 7.5) starting with 1000 ml in each reservoir. All the enzyme activity recovered from this column appeared in a single sharp peak near the center of the major protein peak. Since 95% of the added activity was recovered from the column, the exclusion of PLP from the buffer system during elution appeared to be without effect on the enzyme.

Further purification was obtained by rechromatography of the most active fractions on a smaller but identical DEAE-column. For this, the most active fractions were combined and returned to the starting salt concentration by being passed over Sephadex G-75 previously equilibrated with buffer. The protein was applied with air pressure to a 1.2 × 45 cm DEAE-cellulose column and washed with 50 ml of buffer. A KCl gradient, similar to the above one, was used for elution but with 250 ml in each reservoir to start. The most active fractions were brought to 50% saturation with solid ammonium sulfate. The fine precipitate that formed was sedimented at 27,000*g* for 30 min and dissolved in 2.6 ml of buffer.

A 170-fold purification from the sonicate had been achieved at this point. A summary of a purification is shown in Table I. The final preparation from this purification was used for purity determinations and studies of the reaction except where noted.

Enzyme Purity. Analytical ultracentrifugation and polyacrylamide gel disc electrophoresis of the final material are shown in Figure 1. Nearly all the protein sedimented in a single, apparently symmetrical peak although a slight contaminating peak could be seen running behind the major component. The observed sedimentation coefficient of the major peak was 8.3 S.

With disc electrophoresis, two fast-moving bands

TABLE II: Apparent Kinetic Constants.

Substrate	K_m (mM)	Turnover No.
Pyridoxal 5′-phosphate	0.005[a]	
Pyruvate	2[b]	
α-Aminoisobutyrate	8[c]	1550[c]
L-Alanine	33[c]	3600[c]
pH optimum for AIB, pyruvate = 8.0–8.5[d]		

[a] Pyruvate (20 mM), AIB (20 mM), or L-alanine, and potassium phosphate (50 mM) (pH 7.5); enzyme specific activity = 10.0. [b] AIB (20 mM), PLP (0.1 mM), and potassium phosphate (50 mM) (pH 7.5); enzyme specific activity = 0.14. [c] Pyruvate (20 mM), PLP (0.1 mM), and potassium phosphate (50 mM) (pH 7.5); enzyme specific activity = 5.6. Calculations were made from V_{max} values assuming a pure enzyme of molecular weight 150,000. [d] Potassium phosphate (pH 5.7–8.4) and sodium pyrophosphate (pH 7.9–9.5); enzyme specific activity = 10.0.

were evident after staining. Here again the smaller component did not appear to represent a large percentage of the total. Similar results were obtained with starch gel electrophoresis at pH 8.5, following the procedure of Smithies (1959). It was assumed that both the major ultracentrifugal peak and electrophoretic band were caused by the enzyme under study. By these analytical criteria, the protein appeared to be about 95% pure.

Certain of the apparent kinetic properties were determined for several of the substrates (Table II). Apparent K_m and V_{max} values were obtained from double-reciprocal plots with essentially saturating concentration of the second substrate and coenzyme. No reaction could be detected when PLP was excluded from assays. Although PMP appeared to serve as a coenzyme at high concentrations, chromatographic analysis of the PMP by the method of Bain and Williams (1960) showed it was 4% in PLP (W. B. Dempsey, unpublished data). Pyridoxal was ineffective.

Coenzyme Content of the Purified Enzyme. The spectrum at pH 7.5 of one preparation of the enzyme showed maxima in the near-ultraviolet region at 330 and 405 mμ with absorbances of 0.5 and 0.3, respectively, for a 1% solution in 1-cm cells against a water blank. Since these maxima are similar to maxima observed for the nonspecific binding of PLP to bovine plasma albumin (Dempsey and Christensen, 1962), we hestitate at this point to characterize them as representing coenzyme binding at the active site. The total vitamin B_6 content of the preparation used here was 1 mole/5 $\times 10^5$ g of protein as determined by the bioassay of Atkin *et al.* (1943).

Stoichiometry of the Reaction between α-Dialkyl Amino Acids and Pyruvate. From the studies of Aaslestad and Larson (1964) and Kalyankar and Snell (1962), we predicted that the enzymatic reaction between isovaline and pyruvate was

$$\text{isovaline} + \text{pyruvate} \xrightarrow[\text{enzyme}]{\text{PLP}} CO_2 + \text{butanone} + \text{alanine} \quad (4)$$

By using radioactive substrates in the reaction mixtures, we were able to identify and quantitate each product and thereby to confirm this prediction. The experiments were the following. $^{14}CO_2$ released from mixtures containing DL-[1-^{14}C]isovaline was isolated and meas-

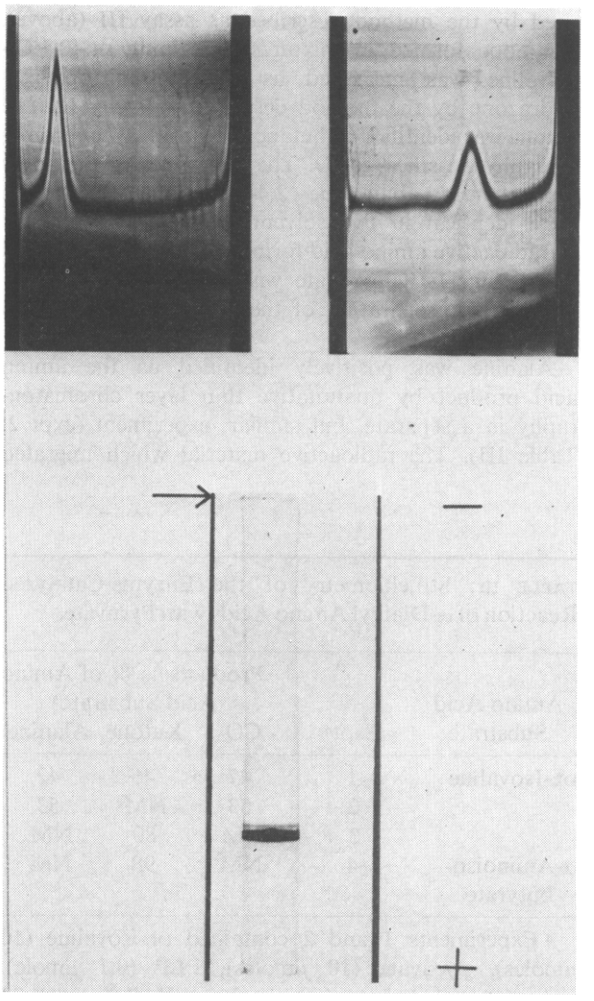

FIGURE 1: Ultracentrifugation and polyacryladmide disc electrophoresis of the 170-fold-purified enzyme. Protein concentration was 5.0 mg/ml in 0.1 M KCl–0.02 M potassium phosphate (pH 7.5). Sedimentation was at 52,640 rpm, 21°, in a Beckman-Spinco Model E. Photographs taken at 26 and 82 min after maximum speed are shown. Electrophoresis was at pH 9.0. Protein (50 μg) in 0.2 ml of 10% sucrose was applied to the top of replicate gels and subjected to a current of 5 ma/gel until the buffer front reached the anodic end of the gel.

ured by the method described as assay III (above). Butanone formed in mixtures containing DL-[2-^{14}C]-isovaline was measured as the 2,4-dinitrophenylhydrazone by the method described as assay II. The ketone was identified as butanone by thin layer chromatography on silica gel G. The R_F values of unknown and standard butanone 2,4-dinitrophenylhydrazone were identical in five solvent systems. The amount of radioactive amino acid formed in the reaction vessel containing [1-^{14}C]pyruvate was measured after chromatographic separation of the keto and amino acids (see Methods).

Alanine was positively identified as the amino acid product by quantitative thin layer chromatography in a separate, but similar, experiment (expt 2, Table III). The radioactive material which migrated during chromatography had the same R_F as alanine, and the quantity of alanine produced equalled the amount of carbon dioxide released when DL-[1-^{14}C]-isovaline replaced [1-^{14}C]pyruvate as the initially labeled substrate. Alanine and isovaline were readily distinguished by R_F values and by their respective rates of reaction with ninhydrin.

In a number of similar experiments we established: first, that the amount of butanone produced was, within experimental error, equivalent to the amount of amino group transferred to pyruvate; second, that no detectable carbon dioxide was produced in the absence of the α-keto acid substrate; and third, that in the presence of the α-keto acid, α-carboxyl release did not exceed amino group transfer or butanone production. Therefore, decarboxylation without subsequent transamination did not appear to occur to any extent. The conclusion drawn from these stoichiometry experiments (Table III) was that eq 4 correctly represented the predominant reaction catalyzed by the enzyme with isovaline.

TABLE III: Stoichiometry of the Enzyme-Catalyzed Reaction of α-Dialkyl Amino Acids with Pyruvate.

Amino Acid Substrate	Expt[a]	Products as % of Amino Acid Substrate[b] CO_2	Ketone	Alanine
DL-Isovaline	1	47	46	42
	2	53	NM[c]	53
	3	NM	80	NM
α-Aminoisobutyrate	4	NM	98	NM

[a] Experiments 1 and 2 contained DL-isovaline (10 μmoles), pyruvate (10 μmoles), PLP (0.1 μmole), potassium phosphate (50 μmoles, pH 7.5), and 21 mg of protein (specific activity = 10.0) in a total volume of 0.5 ml. Incubation was for 4 hr at 30°. Experiment 3 contained DL-isovaline (10 μmoles), pyruvate (40 μmoles), PLP (0.1 μmole), potassium phosphate (50 μmoles, pH 7.5), and 500 μg of protein (specific activity = 10.0) in a total volume of 0.5 ml. Incubation was for 6 hr at 30°. [b] Methods of identification and quantitation of products are described in the text. Average values of replicate reactions are tabulated. [c] NM = not measured.

Substrate Specificity. Decarboxylation of DL-isovaline did not appear to exceed 50% when reaction mixtures were prepared as in the above experiments; but, under similar conditions, nonoptically active AIB was transaminated up to 98%. To test whether both isomers of isovaline were acted upon, 10 μmoles of DL-[2-^{14}C]-isovaline was incubated for 6 hr with a fourfold excess of pyruvate and a 25-fold excess (500 μg) of enzyme. Under these conditions, over 80% of the radioactivity initially present as isovaline was extractable into toluene (expt 3, Table III). Possibly one isomer reacted at a slow rate relative to its mirror image, which would explain the close to 50% conversion with smaller amounts of enzyme and shorter incubation times.

The cyclic analog of diethylglycine, 1-aminocyclopentanecarboxylic acid, was also a substrate. This finding was in accord with the observation that both isomers of isovaline were acted upon.

Reversibility of the Separate Half-Reactions. Reversibility of the half-reaction involving cleavage of the α-carboxyl of α-dialkyl amino acids (eq 1) was first tested by attempting to measure exchange between isovaline, [^{14}C]bicarbonate, and butanone. Replicate reaction mixtures of 1.0 ml were prepared which contained 20 μmoles each of DL-isovaline, sodium [^{14}C]bicarbonate (1.5×10^6 cpm), and butanone; 0.5 μmole of PLP; and 50 μmoles of potassium phosphate (pH 7.5). At the end of a 6-hr incubation with 270 μg of enzyme, carbon dioxide was liberated with sulfuric acid and an aliquot of the carbon dioxide free reaction mixture was analyzed for any ^{14}C which may have become incorporated into isovaline during the incubation. None was found.

In a second experiment carbon dioxide exchange with the coenzyme-bound intermediate (*i.e.*, reversibility of the presumed aldimine-to-ketimine, α-carboxyl cleavage step) was tested analogously except that butanone was excluded from these reaction mixtures. These mixtures contained instead 0.2 μmole of pyruvate/ml to reconvert any PMP-enzyme which would be formed to PLP-enzyme. At the conclusion of this experiment also, no ^{14}C had been fixed into isovaline.

Conversely, the exchange reaction (eq 2) between L-alanine and pyruvate catalyzed by the purified transaminase was fully reversible. To test this, equimolar amounts of L-[1-^{14}C]alanine and unlabeled pyruvate were mixed with enzyme. At equilibrium, 50% of the radioactivity was found as pyruvate. A plot of the log of the loss of radioactivity from alanine against time was linear. This established that the product amino acid was of the same configuration as the substrate (Jenkins and Sizer, 1959).

Stoichiometry of the Reaction with α-Hydrogen

Amino Acids. The amino acids which participated in the exchange transamination (eq 2) were the α-hydrogen analogs of the dialkyl amino acids which participated in the decarboxylation-dependent transamination (eq 1). Since both decarboxylation-dependent and exchange transamination probably occurred at the same site, this suggested that when an α-hydrogen was present the enzyme preferentially catalyzed exchange transamination instead of decarboxylation-dependent transamination. We tested this by measuring the stoichiometry of the enzymatic reaction with the two α-hydrogen amino acids, alanine and α-amino-*n*-butyric acid. In these experiments we also paid particular attention to the detection of any $^{14}CO_2$ released from these amino acids.

After incubation under the same conditions described for Table III, but with unlabeled pyruvate and L-[1-^{14}C]alanine as substrates, the reaction was stopped with acid and carbon dioxide was collected (expt 1, Table IV). Only a small amount of radioactivity above the low control values was trapped in Hyamine. Controls in which Hyamine was suspended over solutions of [1-^{14}C]pyruvate showed the same percentage of the radioactivity trapped in the organic base.

TABLE IV: Stoichiometry of the Enzyme-Catalyzed Reaction of Alanine with Pyruvate.

Amino Acid Substrate	Expt[a]	Products of % of Amino Acid Substrate[b] CO_2	α-Keto Acid	Alanine
L-Alanine	1	<0.5	26	NM[c]
	2	NM	NM	39
D-Alanine	3	<0.3	<0.5	NM
	4	NM	NM	<2

[a] Reaction conditions were the same as for expt 1 and 2 (Table II). [b] Methods of identification and quantitation of products are described in the text. [c] NM = not measured.

Of the total radioactivity in the complete acidified reaction mixture, 26% was extractable into ether. Radioactivity could not be extracted from neutral or basic reaction mixtures. The fact that the extracted radioactive compound was acidic and contained the labeled α-carboxyl of the alanine substrate led to the conclusion that it was [1-^{14}C]pyruvate.

In similar experiments, but with [^{14}C]pyruvate as the labeled substrate, [^{14}C]alanine was formed as determined both by thin layer chromatography and by ion-exchange columns. No radioactive material other than pyruvate and alanine as detected in either experiment. Thus, the only reaction catalyzed in these experiments appeared to be transamination between L-alanine and pyruvate potentiated by loss of the α-hydrogen of L-alanine.

When D-[1-^{14}C]alanine was the amino acid substrate (expt 3, Table IV), minute amounts of acid-volatile and acid-extractable radioactivity were generated. In addition, minor amounts of [^{14}C]alanine could be detected by the ion-exchange technique when unlabeled D-alanine and [1-^{14}C]pyruvate were substrates (expt 4, Table IV). The possibility that D-alanine may have been decarboxylated or transaminated to pyruvate at a very slow rate requires further testing. On the basis of the results of these experiments, we could only conclude that D-alanine was not acted upon at a rate comparable to that with the L-enantiomer.

L-α-Amino-*n*-butyrate (but not D-α-amino-*n*-butyrate, the other α-hydrogen-bearing analog of isovaline) was transaminated to α-keto butyrate. The probable lack of reactivity of D-alanine and the low reactivity of glyoxylate used as α-keto acid substrate implied a requirement for a methyl or larger alkyl group to fit in a defined region of the active site, either for initial substrate binding, or for aldimine formation and further reaction.

Relative Reactivity of Different α-Keto Acids. Using the purified preparation and identical reaction conditions, but with DL-isovaline the substrate in one set of reactions and L-alanine the substrate in another set, we tested the ability of several α-keto acids to serve as substrates of the enzyme. The results are in Table V.

All of the α-keto acids (except pyruvate) appeared to serve less well in the transamination of L-alanine (eq 2) than in the transamination of isovaline (eq 1). This was understandable in view of the apparent irre-

TABLE V: Relative Rates of Transamination of Isovaline and L-Alanine with Several Different α-Keto Acid Substrates.[a]

α-Keto Substrate	Relative Rate DL-Isovaline[b]	L-Alanine[c]
Pyruvate	1.00	1.00
α-Keto butyrate	0.95	0.65
α-Keto valerate	0.49	0.14
Glyoxalate	0.12	0.09
α-Keto isocaproate	0.06	0.03
α-Keto phenylpyruvate	0.00	0.00
α-Keto glutarate	0.00	0.00

[a] Each reaction contained 20 mM α-keto acid, 45 mM amino acid, 0.1 mM PLP, and 50 mM potassium phosphate (pH 7.5); enzyme specific activity = 5.6. Relative rates were calculated from the mean of duplicate reactions. [b] Assayed by toluene extraction of [2-^{14}C]butanone produced from DL-[2-^{14}C]isovaline. [c] Assayed by ether extraction of [1-^{14}C]pyruvate formed from L-[1-^{14}C]alanine.

versibility of the decarboxylation-dependent transamination but ready reversibility of the L-alanine–pyruvate-exchange transamination. Thus, even though reactions in this experiment were allowed to proceed only a small extent, the fact that pyruvate was the most active α-keto acid effectively reduced the measured extent of L-[1-^{14}C]alanine transamination in all assays but the one in which unlabeled pyruvate itself was being tested. This was presumably caused by reconversion of freshly generated pyruvate to L-[1-^{14}C]-alanine.

Variable activity was observed with oxaloacetate even when freshly made solutions were used; but, as no measurable transamination of L-aspartate with [1-^{14}C]pyruvate could be detected in a separate experiment, activity measured with oxaloacetate was probably due to pyruvate arising from nonenzymatic decarboxylation of the β-keto acid. We also noted that the other most common amino acceptor, α-keto glutarate, was inactive with both amino acid substrates.

While the data in Tables III–V indicated that the same enzyme accomplished transamination of α-dialkyl amino acids by cleavage of the α-carboxyl group and transamination of the L isomers of α-hydrogen-bearing analogs by cleavage of the α-hydrogen, we felt it necessary to perform experiments to rule out the possible presence of two transaminases.

Heat Stability and Chromatographic Comparison. As a test of similarity of protein structure, the heat stability and chromatographic properties of the α-hydrogen- and α-carboxyl-labilizing activities were determined. The heat denaturation profile of decarboxylation-dependent transaminase activity was congruent with that of exchange transaminase activity. Similarly, the elution patterns of the two activities from Sephadex G-200 and DEAE-cellulose were congruent. In this latter case, a crude enzyme sample saved from early purification steps was used.

Coinduction of Enzyme. Both α-hydrogen- and α-carboxyl-labilizing activities were present in extracts of cells induced with either DL-isovaline or DL-α-amino-*n*-butyrate in the absence of other nitrogen sources, while both activities were greatly reduced in cells grown with ammonia as nitrogen source. On the basis of these data, we concluded that the purified preparation contained a single PLP-dependent transaminase which proceeded by different initial steps with α-dialkyl amino acids than with α-hydrogen-bearing analogs.

Discussion

The enzyme is induced in the same organism by DL-α-amino-*n*-butyrate, DL-isovaline, and AIB; but its physiological utility as a transaminase of naturally configured amino acids is questionable. If we assume that the amino acid analog of any α-keto acid substrate will have the same proportional substrate activity as the keto acid, then L-alanine–pyruvate exchange (eq 2) is the only rapid exchange transamination observed. In this case the apparent K_m for L-alanine is four times that for AIB! The enzyme thus seems more properly designed for decarboxylation-dependent transamination of α-dialkyl amino acids. These occur with modest abundance in nature (Miester, 1965). The failure of this organism to degrade the ketone products of the decarboxylation reactions suggests that a more "natural" amino acid substrate than those tested in this study may exist of such structure that it could be completely catabolized by sequentially or coordinately induced enzymes. A more complete analysis of the substrate specificity of the α-dialkyl amino acid transaminase is currently in progress in this laboratory in conjunction with an exploration of the variable specificity of the enzyme toward which bond is cleaved to form the presumed ketimine intermediate complex with coenzyme.

We are pursuing our study of this enzyme because an understanding of the origin of the unusual specificity of the enzyme toward which bond is cleaved promises to broaden our knowledge about the mechanism of catalysis of pyridoxal phosphate enzymes in general. In particular, we want to know if the carboxyl group of the dialkyl amino acid binds to the enzyme before CO_2 release. If it does, then the attractive model recently put forth by Dunathan (1966) suggests that either there will be two carboxyl binding sites (one for pyruvate and the other for the dialkyl amino acid) or there will be one site but the bonds cleaved will be on opposite sides of the plane of the PLP-pyridine ring. On the other hand, if it does not bind before cleaving, we have an exception to Dunathan's model which proposed that the specificity of the bond cleaved in any PLP-catalyzed reaction is determined by a specific α-carboxylate anion binding site. We are presently conducting experiments designed to answer some of these questions.

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Conformational Aspects of Polypeptide Structure. XXII. Aromatic Side-Chain Effects from Poly-L-*p*-aminophenylalanine and Derivatives*

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ABSTRACT: We synthesized poly-N^{ω}-carbobenzoxy-L-*p*-aminophenylalanine and poly-L-*p*-aminophenylalanine as analogs of poly-L-tyrosine and examined their optical rotatory dispersion (ORD) and circular dichroism (CD). In tetrahydrofuran the ORD and CD of poly-N^{ω}-carbobenzoxy-L-*p*-aminophenylalanine reveals a positive Cotton effect centered at 245 mμ which can be assigned to the $\pi-\pi^*$ electronic transition of *para*-substituted benzene rings existing in a dissymmetric environment; this band disappears in trifluoroacetic acid, a helix-breaking solvent. Poly-L-*p*-aminophenylalanine exists as a random coil between pH 1.08 and 2.56. When the pH rises from 2.56 to 2.78, a sharp change in ORD and CD is observed. Positive Cotton effects at 290 and 245 mμ corresponding to $\pi-\pi^*$ electronic transitions of aromatic side-chain amino groups appear. This can be explained by a coil–helix transition in this pH region, even though only one-half the amino groups are deprotonated, as can be seen by the molar extinction coefficient (ϵ 9000) at 250 mμ. The negative Cotton effect (trough, 230 mμ) in the ORD further suggests a right-handed helix at pH 2.78 or higher.

These results are comparable in the acidic pH range to those found for poly-L-tyrosine in the basic pH range except that soluble poly-L-*p*-aminophenylalanine of high purity and molecular weight can be prepared more easily.

Optical rotatory dispersion (ORD) (Urnes and Doty, 1960; Yang, 1961) and circular dichroism (CD) (Holzwarth and Doty, 1965) studies have been used for the conformational analysis of polypeptides with aromatic side chains (Blout, 1962). When the primary helical arrangement of the peptide bonds necessarily imposes a secondary helical arrangement on the aromatic side chains, electronic interactions can occur between side chains and main-chain chromophores, leading to unusual optical rotatory properties. Such effects have been observed for poly-L-tyrosine (Fasman *et al.*, 1964; Beychok and Fasman, 1964; Pao *et al.*, 1965) and poly-L-phenylalanine (Sage and Fasman, 1966; Auer and Doty, 1966). In both cases Cotton effects have been detected in the region of the benzene–chromophore transitions, indicating that the aromatic side-chain residues exist in dissymmetric environments.

Electronic interactions among the side-chain chromophores have been demonstrated somewhat earlier with copolymers containing poly-β-(*p*-nitrobenzyl)-L-aspartate (Goodman *et al.*, 1963) and recently with copolymers containing poly-L-*p*-(phenylazo)phenylalanine (Goodman and Kossoy, 1966). The helical porphyrin-*d*-uroblin (Moscowitz, 1964) and naturally occurring proteins (Myers and Edsall, 1965) have shown analogous effects.

In our present report we describe the synthesis and stereochemical properties of some analogs of poly-L-tyrosine. Poly-N^{ω}-carbobenzoxy-L-*p*-aminophenylalanine and poly-L-*p*-aminophenylalanine were prepared and the ORD and CD measurements of these substances were carried out.

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