Ornithine Degradation in *Clostridium sticklandii;* Pyridoxal Phosphate and Coenzyme A Dependent Thiolytic Cleavage of 2-Amino-4-ketopentanoate to Alanine and Acetyl Coenzyme A†

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ABSTRACT: The third enzymic step in the fermentation of D-ornithine by *Clostridium sticklandii* was identified as a thiolytic cleavage of 2-amino-4-ketopentanoate by coenzyme A to form acetyl-CoA and alanine. Satisfactory radiochemical and spectrophotometric assays were developed for the 2-amino-4-ketopentanoate thiolase catalyzing this reaction. The activity of this partially purified thiolase is completely dependent on the presence of pyridoxal 5′-phosphate. The apparent K_m for this coenzyme is 2.5×10^{-7} M. The enzyme is completely inactivated by dialysis against 100 mM cysteine and can be reactivated by the addition of pyridoxal 5′-phosphate. Other pyridoxal phosphate analogs and α-keto acids cannot replace pyridoxal 5′-phosphate. The structure of 2-amino-4-ketopentanoate was verified by several chromatographic and electrophoretic procedures and by identification of the products of iodoform degradation.

Clostridium sticklandii and related organisms anaerobically ferment ornithine to alanine, acetate, carbon dioxide, and ammonia (Stadtman, 1954; Dyer and Costilow, 1968). Alanine was found to be derived from carbon atoms 1 to 3 of ornithine and acetate from carbon atoms 4 and 5, suggesting a cleavage between carbon atoms 3 and 4. The first step in the pathway of ornithine fermentation catalyzed by ornithine mutase (reaction 1) is a coenzyme B_{12} dependent migration of the amino group

$$\underset{\text{ornithine}}{CH_2CH_2CH_2CHCOOH} \rightleftharpoons \underset{\text{DAP}}{CH_3CHCH_2CHCOOH} \quad (1)$$

$$\text{DAP} + \text{DPN(TPN)} \rightleftharpoons \underset{\text{KAP}}{CH_3COCH_2CHCOOH} + NH_3 + \text{DPNH(TPNH)} \quad (2)$$

$$\text{KAP} + \text{CoASH} \longrightarrow CH_3COSCoA + CH_3CHCOOH \quad (3)$$

from carbon atom 5 to carbon atom 4 forming 2,4-diaminopentanoate (Dyer and Costilow, 1968; Tsuda and Friedmann, 1970). In the second step (reaction 2), 2,4-diaminopentanoate undergoes an oxidative deamination catalyzed by a TPN- or DPN-dependent dehydrogenase to yield 2-amino-4-ketopentanoate (AKP)[1] (Tsuda and Friedmann, 1970). Both enzymes have been purified to homogeneity and the ornithine mutase has been shown to be dependent on PLP (Somack and Costilow, 1973a,b). An early observation that the formation of alanine in the overall reaction was dependent on coenzyme A (Dyer and Costilow, 1970) suggested that coenzyme A may react with AKP to form acetyl-CoA and alanine (reaction 3).

In this paper we report the presence and some properties of a pyridoxal 5′-phosphate-dependent enzyme that catalyzes this reaction in extracts of *C. sticklandii*. The substrate of the enzyme, 2-amino-4-ketopentanoate, has also been more completely characterized.

Experimental Section

Growth of Cells and Preparation of Extracts. The procedures for growing *C. sticklandii* and obtaining cell extracts were those described by Somack *et al.* (1971). The crude extract was either dialyzed against sonication buffer (50 mM potassium phosphate, pH 7.5) prior to use or subjected to further purification as described below. All steps were carried out at 0–4°.

Enzymes. Partial purification of AKP-thiolase was done by the following procedure. Three milliliters of nondialyzed crude extract containing 130 mg of protein/ml was diluted with sonication buffer to a protein concentration of 44 mg/ml. One-third the volume of 1.5% neutralized protamine sulfate was added slowly with stirring. After an additional 20 min the solution was centrifuged at 13,000*g* and the precipitate was discarded. The supernatant solution was brought to 50% saturation by the addition of 2.91 g of ammonium sulfate/10 ml of solution. After stirring for an additional 15 min, the precipitate was removed by centrifugation and discarded. To the supernatant solution 1.25 g of ammonium sulfate was added per 10 ml of solution to bring the ammonium sulfate saturation to 70%. After 15 min the precipitate was collected by centrifugation, redissolved in 1 ml of sonication buffer, and the solution was then dialyzed in washed Visking tubing against 125 ml of sonication buffer for 24 hr. The AKP-thiolase was purified about 7.6-fold with an 88% recovery of activity. he final solution contained 5.1 mg of protein/ml; the specific activity was 0.90 unit/

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[1] The following abbreviations are used: AKP, 2-amino-4-ketopentanoate; DAH, diaminohexanoate; DAP, 2,4-diaminopentanoate; *p*-ClHgBzO, *p*-chloromercuribenzoate; PL, pyridoxal; PLP, pyridoxal 5′-phosphate; PM, pyridoxamine; PMP, pyridoxamine 5′-phosphate; PN, pyridoxine.

mg. Thiolase activity in the crude extract (22 mg/ml, specific activity 0.12 unit/mg) remained constant at −20° for at least 10 days and at 24° for at least 2 days.

Apothiolase was prepared by adding 1 ml of crude extract (130 mg; 0.12 unit/mg) to 1 ml of a solution containing 1 mM cysteine, 1 mM dithiothreitol and 100 mM potassium phosphate (pH 7.5) and dialyzing the enzyme for 22 hours at 4° against 125 ml of the same solution without cysteine. The buffer was changed three times in 24 hr.

DAP C_4-dehydrogenase was purified from *C. sticklandii* through the hydroxylapatite step by the method of Somack and Costilow (1973a). The preparation had a specific activity of 80 units/mg.

Chemicals. DAP was prepared by the method described by Somack *et al.* (1971). AKP was prepared from ornithine by the methods of Tsuda and Friedmann (1970), using an extract of *C. sticklandii* purified through the ammonium sulfate step. Unlabeled or [^{14}C]AKP was separated from unreacted ornithine and DAP by passing the neutralized reaction solution through a column of Dowex 50-X8 (H^+ form) and eluting the AKP with 0.5 N HCl. The yield of AKP from ornithine varied from 40 to 60%. AKP was estimated by the enzymic procedure described below or by the radioactivity recovered when [^{14}C]ornithine was used.

Acetoacetyl-CoA was prepared from diketene and coenzyme A (Lynen and Wieland, 1955). The Dowex 50 used in this study was AG 50-X8, 200–400 mesh (Bio-Rad). The resin was prepared for use as described by Hirs *et al.* (1952).

Assays for AKP-thiolase. Spectrophotometric and radiochemical assays were developed for AKP-thiolase. For the spectrophotometric assay the reaction solution (1.0 ml) contained 100 mM Tris-Cl (pH 8.0), 80 μM coenzyme A, 100 μM PLP, and 0.28 mM AKP. The reaction was initiated by addition of enzyme and was followed at 26° by the increase in absorbance at 232 nm attributable to the thioester bond of acetyl-CoA; $\Delta E_{232nm} = 4.5 \times 10^3\ M^{-1}\ cm^{-1}$ (Stadtman, 1955). One unit of enzyme catalyzes the formation of 1 μmol of acetyl-CoA/min under the above conditions. Activity was shown to be proportional to enzyme level up to 8 munits/ml ($\Delta A = 0.036$/min) under these conditions.

Enzyme must be incubated with PLP to obtain maximal activity, even though PLP is present in the spectrophotometric assay solution. For activation, enzyme was incubated in Tris-Cl buffer (pH 8.0) containing 100 μM PLP for 30 min at 25°. An aliquot was then assayed as described above. With crude extract, prior incubation with PLP results in a two- to three-fold increase in activity; with enzyme purified through the ammonium sulfate step, a three- to four-fold increase in activity is obtained. Unless otherwise stated, the enzyme was activated prior to the assay only in those experimets where specific activity is reported.

The radiochemical assay employed a 50-μl reaction solution containing 10 mM dithiothreitol, 32 μM PLP 1.7 mM coenzyme A, 100 mM Tris-Cl (pH 8.0), 14 mM [U-^{14}C]AKP (5.1 nCi/μmol), and enzyme. [^{14}C]AKP was added to start the reaction. The assay solution was incubated at 24° for 5 min and the reaction was terminated by addition of 0.2 ml of 40 mM HCl. The resulting solution was passed through a small Dowex 50-X8 (H^+ form) column with a bed volume of about 0.4 ml. The column was washed with two 0.4-ml aliquots of water and the effluent containing [^{14}C]acetyl-CoA was collected and counted in Bray's scintillation fluid as described below. Residual substrate and alanine, the other reaction product, are retained by the resin. The rate of [^{14}C]acetyl-CoA formation is linear with enzyme level up to 15 munits/assay.

Analytical Methods. A rapid enzymatic method using DAP-C_4-dehydrogenase was developed to estimate AKP after some of the properties of this enzyme were reexamined. These studies[2] indicated that the reaction is less favorable for the reduction of AKP than previously reported (Tsuda and Friedmann, 1970) and that the enzyme is more active with TPN than with DPN. For the estimation of AKP, 1.1 units of DAP-C_4-dehydrogenase was added to a solution (1.0 ml) containing 100 mM Tris-Cl (pH 7.4), 250 mM NH_4Cl (pH 7.4), 0.17 mM TPNH, and AKP at 25°. The decrease in A_{340} was measured after the absorbance became constant, usually within 10 min. The absorbance change was proportional to the amount of AKP added between 10 and 60 nmol per ml of assay solution. The concentration of a sample of [^{14}C]AKP estimated by this method (140 mM) agreed well with the concentration (144 mM) calculated from the conversion of [^{14}C]ornithine of known specific activity to [^{14}C]AKP in the reactions used to produce AKP.

The iodoform test was carried out as follows: 7 μmol of AKP in 100 μl was mixed with 60 μl of 10% sodium hydroxide. About 100 μl of an iodine solution containing 12.5% (w/v) iodine and 25% potassium iodide was slowly added to the alkaline AKP solution. The disappearance of the brown color with the formation of a yellow crystalline precipitate with a characteristic odor was judged to indicate the formation of iodoform. The aqueous layer was extracted three times with 0.3 ml of chloroform to remove iodoform and was then evaporated under vacuum to dryness, and taken up in 1.0 ml of water. One microliter of the resulting solution was examined for aspartic acid with an amino acid analyzer.

For the reduction of AKP, a solution of AKP (30 μl; 0.21 μmol) was added to 15 μl of 1 M $NaBH_4$ in 0.01 N NaOH. After 20 min the reaction was stopped by addition of 15 μl of acetone.

3-Ketoacyl-CoA-thiolase activity was assayed by the decrease in absorbance at 310 nm as described by Stern (1955) except that glutathione was not included in the reaction solution.

General Methods. Protein was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard. Absorbance measurements were made with a Beckman DU spectrophotometer equipped with a Gilford multiple sample absorbance recorder.

Radioactivity of ^{14}C was measured with a Packard Tri-Carb scintillation spectrophotometer, Model 3214. One milliliter of aqueous sample was added to 10 ml of Bray's (1960) solution.

Paper electrophoresis was performed with the apparatus described by Crestfield and Allen (1955) using Schleicher and Schull Grade No. 580 white ribbon paper. Vitamin B_{12} and picrate were used as reference compounds. When pH 4.75 electrophoresis buffer was used, the paper was sprayed with 1 M K_2HPO_4 before drying to reduce the loss of volatile acids.

Paper chromatography was done by the ascending method with the same paper used for paper electrophoresis.

A Beckman Model 120C amino acid analyzer was used for amino acid analyses following the methods of Benson and Patterson (1965). A 50 × 0.9 cm column containing Beckman custom spherical AA-15 cation-exchange resin was used.

Results

Identification of the Degradation Products of AKP and the Stoichiometry of the AKP-thiolase Reaction. To determine the products of the degradation of AKP and their origin in the amino acid, [^{14}C]AKP labeled either on carbon-2 or carbon-5

[2] These results will be published in a separate paper.

TABLE I: Chromatographic Behavior of the Labeled Products of the AKP-thiolase Reaction.[a]

Compound	Paper Chromatography[b]	Electrophoresis (cm/hr) pH 4.75 + Bisulfite[c]	Electrophoresis (cm/hr) pH 7.6[d]
[^{14}C]Product of [2-^{14}C]AKP	0.28	0	0
[^{14}C]Product of [5-^{14}C]AKP	0.085	2.85	18.0
Acetate		3.55	13.9
Acetyl-CoA	0.086	2.88	18.5
Alanine	0.29	0	0
AKP	0.23	1.38	0

[a] The reaction solution contained 40 mM potassium phosphate (pH 7.5), 20 μM PLP, 4.0 mg of protein of ammonium sulfate fractionated extract (specific activity 1.0 unit/mg), 1.2 mM coenzyme A, and 0.5 mM [^{14}C]AKP in a volume of 1 ml. The reaction was carried out at 24° for 30 min and terminated by cooling to 0°. The positions of radioactive compounds were detected with a Packard radiochromatogram scanner. [b] Paper chromatography (ascending) utilized butanol–acetate–H_2O (60:15:25). [c] In 100 mM sodium acetate buffer containing 100 mM sodium bisulfite (pH 4.75) (10 V/cm). [d] In 50 mM sodium phosphate buffer (pH 7.6) (30 V/cm).

was prepared from [2-^{14}C]- or [5-^{14}C]ornithine (see Methods). The position of labeling was assigned on the assumption that no rearrangement of the carbon skeleton occurred during the ornithine mutase and DAP-C_4-dehydrogenase reactions.

Reactions were carried out in the presence of ammonium sulfate treated extract and with an excess of coenzyme A to insure the complete conversion of AKP. The R_F values and the electrophoretic mobilities of the ^{14}C-labeled products generated from [2-^{14}C]- or [5-^{14}C]AKP are given in Table I. With [5-^{14}C]AKP all the radioactivity in the products migrated both in paper chromatography and electrophoresis with a compound that behaved like acetyl-CoA. No significant amount of acetate was formed. The formation of acetyl-CoA was confirmed by arsenolysis in the presence of phosphotransacetylase as described by Stadtman (1955) and by the increase in absorbance at 232 nm in the spectrophotometric assay (see Methods). All the evidence indicates that acetyl-CoA is a product of the AKP-thiolase reaction.

TABLE II: Stoichiometry of the AKP-thiolase Reaction.[a]

		mM Concentration Initial	Final	Diff
Substrates	AKP[b]	2.40	1.80	0.60
	Coenzyme A[c]	1.30	0.72	0.58
Products	Acetyl-CoA[d]	0.00	0.54	0.54
	Alanine[e]	0.00	0.62	0.62

[a] In addition to the substrates indicated, the reaction solution contained 20 mM potassium phosphate (pH 7.5), 20 μM PLP, and 20 μg of dialyzed ammonium sulfate fraction (specific activity 1.0 unit/mg) in a volume of 1 ml. After 15-min incubation at 25°, the reaction was chilled to 0° and aliquots was analyzed for *b* to *e*. [b] AKP, by the enzymatic method described under Methods. [c] Coenzyme A, by thiol group determination with Ellman's reagent (Ellman, 1959; Flavin, 1962). [d] Acetyl-CoA, by arsenolysis using phosphotransacetylase (Stadtman, 1955). [d] Alanine by an amino acid analyzer.

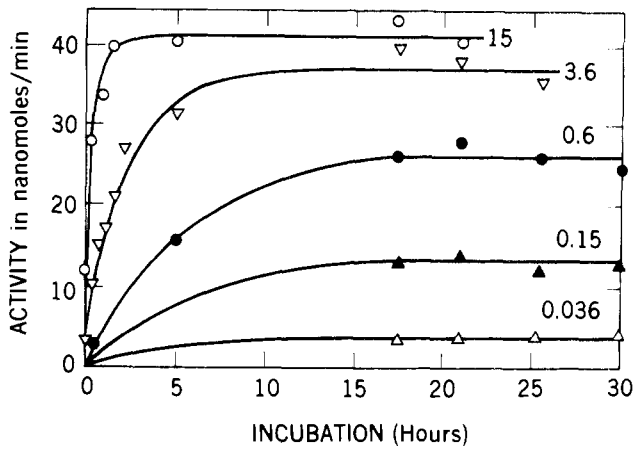

FIGURE 1: Effect of PLP concentration and incubation time on thiolase activity. Apoenzyme (5.2 mg), prepared as described under Methods, was incubated in the dark in 25 ml of 100 mM Tris-Cl (pH 8.0), 0.28 mM AKP, 1 mM dithiothreitol and the indicated concentration (μM) of PLP at 24°. Aliquots of 0.99 ml were withdrawn at the indicated times and added to cuvets. Coenzyme A (10 μl of an 8 mM solution) was added to start the reaction which was followed by the spectrophotometric method.

With [2-^{14}C]AKP as substrate, the radioactive product behaved like alanine in paper chromatography and electrophoresis systems (Table I). This observation indicates that alanine is derived from the first three carbons of AKP. Alanine was positively identified by use of the amino acid analyzer as reported in Table II.

The data in Table II show that partially purified AKP-thiolase catalyzes the formation of approximatey 1 mol each of acetyl-CoA and alanine from 1 mol each of coenzyme A and AKP. A catalytic level of coenzyme A does not allow the conversion of AKP to acetate and alanine.

Requirement for Pyridoxal 5'-Phosphate. The apoenzyme of AKP-thiolase (prepared as described under Methods) was completely inactive when assayed in the presence of all required components except PLP. The addition of PLP restored thiolase activity. Omission of cysteine from the dialysis buffer used to prepare apoenzyme, with or without prior preincubation with 5 mM hydroxylamine at 24° for 15 min, resulted in only partial dependency on PLP.

The extent of activation of apoenzyme by PLP is dependent on the concentration of PLP and incubation time. Figure 1 shows the effect of incubation time on the activation of apoenzyme by PLP. Higher concentrations of PLP shorten the time required to achieve maximal reactivation and yield higher activities. However, even 15 μM PLP restores only 70% of the original activity detected before dialysis. A double-reciprocal plot of the maximal level of activity restored by PLP *vs.* the concentration of PLP was used to estimate an apparent K_m for PLP of approximately 0.25 μM.

Compounds closely related to PLP and certain α-keto acids were tested as activators of the thiolase (Table III). of the compounds tested only PMP caused a small activation. The other compounds were either inactive, when tested alone, or inhibitory in the presence of PLP.

Components for the AKP-thiolase Spectrophotometric Assay. The spectrophotometric assay for AKP-thiolase (see Methods) requires the addition of AKP, coenzyme A and extract (Table IV). No activity was detected when any one of

TABLE III: Effect of Coenzyme B_6 Derivatives and α-Keto Acids on Thiolase Activity.[a]

Compounds Added (μM)	Act. (Acetyl-CoA Formed) (nmol/min)
None	0
PLP (20)	12.00
PMP (100)	1.20
PL (100)	0.53
PLP (20) + PMP (100)	13.90
PLP (20) + PL (100)	9.68
PLP (20) + PM (100)	9.46
PLP (20) + PN (100)	11.0
PMP (100) + α-KG (500)	0
PMP (100) + pyruvate (1000)	0.22

[a] The radiochemical assay solution contained 100 mM potassium phosphate (pH 7.5), 0.28 mM [^{14}C]AKP, 1 mM dithiothreitol, 100 μg of apoenzyme, and the indicated compound. The solution was incubated in the absence of light for 30 min and coenzyme A (10 μl of an 8 mM solution) added to start the reaction.

these compounds was omitted from the assay solution. No stimulation of the reaction was obtained by the addition of catalytic amounts of DPN, TPN, α-ketoglutarate, or pyruvate. Acetyl-CoA and either D- or L-alanine caused moderate inhibition at the concentrations used. The presence of an alanine racemase in the dialyzed crude extract precludes identification of the isomer(s) causing inhibition. D-Alanine presumably is the actual product since D-ornithine has been identified as the substrate for ornithine mutase (Somack and Costilow, 1973b) and there is no evidence for further steric rearrangements of the α-amino group during the subsequent C_4-dehydrogenase and thiolase reactions.

AKP-thiolase and 3-Ketoacyl-CoA-thiolase. Clostridia are known to be rich in 3-ketoacyl-CoA-thiolase. The possibility that this enzyme also catalyzes the thiolytic cleavage of AKP was excluded by taking advantage of the requirement of AKP-thiolase for PLP and the differential sensitivity of the two enzymes to thiol group reagents.

AKP-thiolase apoenzyme was completely inactive without PLP, whereas dialyzed crude extract of *C. sticklandii* prepared by the same procedure but without cysteine in the dialysis buffer showed a 50% dependence on PLP. The activity of 3-ketoacyl-CoA-thiolase (0.3 unit/mg) assayed with acetoacetyl-CoA as substrate was found to be identical in dialyzed crude extracts prepared with or without cysteine. PLP and PMP (15 μM) did not stimulate this activity. Since cysteine specifically inactivated only AKP-thiolase and PLP reactivated AKP-thiolase but failed to affect the activity of 3-ketoacyl-CoA-thiolase, it is concluded that these activities are associated with different enzymes.

Data on the inhibition of the two thiolases by several thiol group reagents (Table V) also support the conclusion that the enzymes are different. The 3-ketoacyl-CoA-thiolase is much more sensitive to thiol group reagents than AKP-thiolase. At the concentrations tested iodoacetamide, *N*-ethylmaleimide, and *p*-ClHgBzO completely inactivated 3-ketoacyl-CoA-thiolase whereas under the same conditions only *p*-ClHgBzO caused complete inhibition of AKP-thiolase. *N*-Ethylmaleimide partially inhibited AKP-thiolase. Arsenite did not inhibit either enzyme; these observations indicate the absence of essential vicinal thiol groups in these enzymes. It may be noted that pig heart 3-ketothiolase has been reported to be inhibited by arsenite (Lynen, 1953).

TABLE IV: Spectrophotometric Assay Requirements and Effect of Reaction Products.[a]

Omission	Addition	$\Delta A_{232\ nm}$/min	Rel Rate (%)
None	None	0.039	(100)
AKP, enzyme, or CoA	None	0.000	0
None	D-Alanine (2.5 mM)	0.026	67
None	L-Alanine (2.5 mM)	0.034	87
None	Acetyl-CoA (0.065 mM)	0.030	77

[a] Standard spectrophotometric assays were performed as outlined under Methods with 6 munits of undialyzed crude extract and the indicated omissions or additions.

As indicated earlier, acetyl-CoA inhibits the activity of AKP-thiolase. This is inconsistent with the possibility that akp accepts the coenzyme A moiety from acetyl-CoA before the thiolytic cleavage. The markedly different structures of the substrates of AKP-thiolase and 3-ketoacyl-CoA-thiolase also lend support to the conclusion that the two enzymes are distinct.

Distribution of AKP-thiolase. The specific activity of AKP-thiolase in crude extracts of *Clostridium* SB4 is about 10% of that in extracts of *C. sticklandii*. Extracts of *C. pasteurianum, C. tetanomorphum,* and *Pseudomonas* B4 had no significant activity, *i.e.*, less than 1% of that of *C. sticklandii*. These experiments were done with the radiochemical assay.

Characterization of AKP. The structure of AKP originally isolated by Tsuda and Friedmann (1970) was assigned from nuclear magnetic resonance spectrometry. Presented here are procedures developed to confirm the structure of AKP isolated by their method and to allow for practical estimation and presumptive identification of the compound.

The observation that a neutralized solution of AKP binds to

TABLE V: Effect of Thiol Group Reagents on AKP-thiolase and 3-Ketoacyl-CoA-thiolase Activity.[a]

Reagent	Concn (mM)	Residual Activity: AKP-thiolase (nmol/min per 15 μl)	Residual Activity: 3-Ketoacyl-CoA-thiolase (nmol/min per 30 μl)
None		3.8	0.68
Iodoacetamide	3.0	4.4	0
N-Ethylmaleimide	2.0	1.5	0
PCMB	0.04	0	0
Arsenite	2.0	4.4	0.64

[a] The reaction solution contained 80 mM potassium phosphate (pH 7.5), 0.2 mg/ml of protein (dialyzed ammonium sulfate fraction, specific activity of AKP-thiolase, 1.2 units/mg), and the indicated concentration of thiol group reagent. Solutions were incubated at 25° for 30 min and then the indicated aliquots were assayed. AKP-thiolase activity was determined by the spectrophotometric assay and 3-ketoacyl-CoA-thiolase activity by the method of Stern (1955).

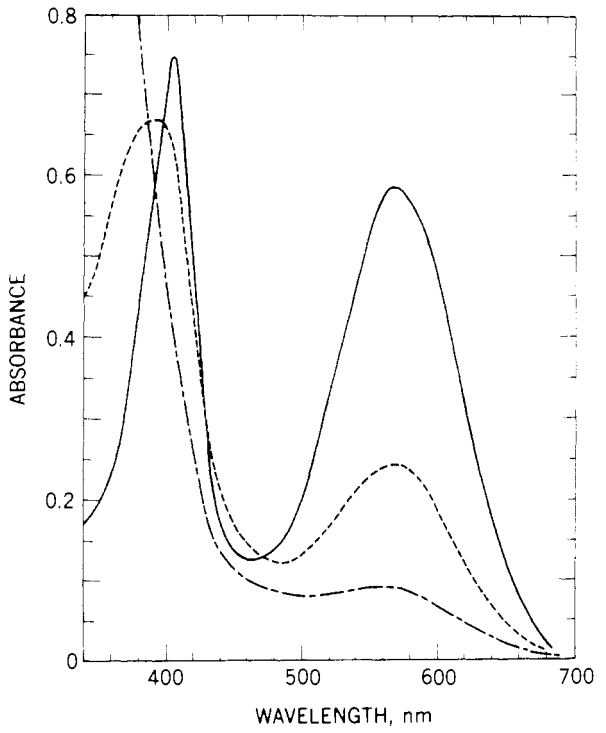

FIGURE 2: Absorption spectrum of the ninhydrin product of AKP. The ninhydrin reaction was performed by the method of Moore and Stein (1954) employing 0.32 μmol of AKP or 0.20 μmol of D-ornithine in a volume of 6 ml. Spectra were recorded against a reagent blank with a Cary spectrophotometer Model 14 employing silica cuvets with a 1-cm light path. The spectrum of the reagent blank was read against water: D-ornithine (——); AKP (- - -); reagent blank (- · - ·).

the H^+ form but not to the Na^+ form of Dowex 50 resin is consistent with the behavior of a compound with one amino and one carboxyl group. AKP behaves as a neutral compound on paper electrophoresis in 50 mM sodium phosphate (pH 7.5) or 45 mM ammonium acetate (pH 4.75). These properties indicate that the pK of the amino group is higher than 9.0 and that of the carboxyl group is lower than 3.0.

A simple paper electrophoretic method was developed for the identification of AKP. Since the addition of bisulfite to the carbonyl group of AKP should introduce an additional negative charge, paper electrophoresis was performed at pH 4.75 in the presence of 100 msmcnM sodium bisulfite. As expected, AKP moved toward the anode whereas alanine and the sodium borohydride reduced product of AKP, presumably 2-amino-4-hydroxypentanoate, remained at the origin. This observation indicated that the compound is either a methyl ketone or an aldehyde. The possibility that the compound might be an aldehyde was excluded by treating it with hypoiodite in the iodoform test (see Methods). A positive result was obtained as evidenced by the formation of hexagonal crystals typical of iodoform. As expected, the other product of hypoiodite oxidation was identified as aspartate by means of an amino acid analyzer. The yield of aspartate was 35%. No aspartate was detected in the AKP solution before treatment with hypoiodite. In short, ion-exchange chromatography, paper electrophoreses, and the iodoform test confirmed the structure of 2-amino-4-ketopentanoate proposed by Tsuda and Friedmann (1970).

AKP on paper reacts with ninhydrin to give a golden color rather than the blue characteristic of many amino acids. The spectrum of the product formed in solution by reaction with the ninhydrin reagent of Moore and Stein (1954) is compared to that of ornithine in Figure 2. It is of interest to note that the spectrum of the ninhydrin reaction product of ornithine is identical with that of DAP (not shown). The absorbance of the ninhydrin product of ornithine at 570 nm ($E_{570\,nm}$ 4.31×10^4 M^{-1} cm^{-1}) is about ten times larger than that obtained with AKP (4.96×10^3 M^{-1} cm^{-1}). Furthermore, the absorbance peak for AKP at 395 nm is shifted 15 nm relative to the position of the peak in this region for ornithine, located at 410 nm. The absorbance ratio $A_{410}/A_{570\,nm}$ for ornithine (1.16) is considerably smaller than the $A_{395}/A_{570\,nm}$ for AKP (2.67).

The behavior of AKP on the amino acid analyzer was also studied. Under the conditions outlined under Methods the retention time of AKP is 63 min. The apparent molar absorbance of the ninhydrin reaction product at 440 nm (11,100 M^{-1} cm^{-1}) is about twice that at 570 nm (5500 M^{-1} cm^{-1}). The reduction of AKP with sodium borohydride resulted in the formation of equal amounts of two ninhydrin-positive compounds with retention times of 72 and 86 min concomitant with the loss of the peak at 63 min corresponding to AKP. These compounds presumably are the two diastereomers of 2-amino-4-hydroxypentanoic acid. Both compounds give the usual blue color with ninhydrin. On the assumption that the color values of both isomers of 2-amino-4-hydroxypentanoate are equal to that of threonine, 7.6 nmol of 2-amino-4-hydroxypentanoate was estimated to be formed from 7.3 nmol of AKP.

Discussion

The third enzymic step in the degradation of ornithine by extracts of *C. sticklandii* has been shown to be a PLP-dependent thiolytic cleavage in which 2-amino-4-ketopentanoate and coenzyme A are converted to acetyl-CoA and alanine. Since [^{14}C]alanine is the main product with [2-^{14}C]AKP as substrate and [5-^{14}C]AKP yields [^{14}C]acetyl-CoA, we conclude that alanine is derived from carbon atoms 1 to 3 and the acetyl group of acetyl-CoA from carbon atoms 4 and 5 of the substrate.

Although the thiolytic activity has not been extensively purified, the relatively high yield (88%) obtained from a 7.6-fold purification suggests that it is associated with a single enzyme. The same net reaction could theoretically result from a multienzyme reaction sequence involving a transamination, or oxidative deamination, of AKP to form 2,4-diketopentanoate, a thiolytic cleavage of the latter compound to form acetyl-CoA and pyruvate, and finally amination of pyruvate to form alanine. However, this possibility appears to be eliminated by the observation that the overall reaction is not stimulated by α-keto acids, generally required for transaminations, or by DPN or TPN, usually required for oxidative aminations and deaminations. Furthermore, pyruvate could not be detected during the thiolytic cleavage of AKP. Consequently the conversion of AKP and CoA to acetyl-CoA and alanine is probably catalyzed by a single enzyme.

The PLP-dependent thiolytic cleavage of AKP is similar to the PLP-dependent hydrolytic cleavages of kynurenine to anthranilic acid and alanine, catalyzed by kynureninase (Braunstein *et al.*, 1949), and of L-aspartate to carbon dioxide and alanine, catalyzed by L-aspartate β-decarboxylase (Tate and Meister, 1971) except that coenzyme A replaces water as the nucleophilic reagent. A thiolytic cleavage is advantageous in a degradative pathway in that it conserves energy in a metabolically useful form. The only other PLP-dependent thiolytic cleavage of which we are aware is the reversible conversion of 2-amino-3-ketobutyrate and CoA to acetyl-CoA and glycine by an enzyme erroneously called aminoacetone synthetase (McGilvray and Morris, 1971) because in the reverse reaction the ketoamino acid readily decarboxylates nonenzymically to form aminoacetone. The mechanisms of the aminoacetone synthetase and the AKP-thiolase reactions must differ significant-

ly since they involve the elimination of groups α and β to the amino carbon atom, respectively [see Braunstein (1960); Snell and DiMari (1970)].

Stadtman and Tsai (1967) have shown that some clostridia degrade lysine in part by an alternate pathway that results in cleavage of the carbon chain between carbon atoms 4 and 5. The initial step in this pathway appears to be the conversion of D-lysine to 2,5-DAH (Morley and Stadtman, 1970). Although a 2,5-DAH-dehydrogenase has recently been purified to homogeneity from *C. sticklandii* extracts (Stadtman, 1973), the enzyme is 700–1000 times more active with DAP than with 2,5-diaminohexanoate. It would be attractive to propose that a thiolytic cleavage similar to that catalyzed by AKP-thiolase is operative in the D-lysine pathway: a PLP-dependent cleavage of 3-amino-5-ketohexanoate which may be derived from 2,5-diaminohexanoate by an oxidative deamination or transamination and by the migration of the amino group on C_2 to C_3. If this hypothesis is correct then it is plausible that the amino group migration precedes the formation of the 5-keto compound since 2-amino-5-ketohexanoate undergoes spontaneous ring closure to Δ^1-pyrroline-2-methyl-5-carboxylic acid (Stadtman, 1973).

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