0.45 H, vinyl proton), 5.63-5.51 (m, 0.55 H, vinyl proton), 4.99-4.89 (m, 2 H, terminal vinyl protons), 4.22-4.18 (m, 1 H, acetal methine proton), 3.40, 3.33, 3.26, 3.25 (four s, 6 H), 2.52-2.46 (m, 0.5 H, methine proton at C_2), 2.21-2.09 (m, 0.5 H, methine proton at C_2), 1.8-0.93 (m, 7 H), 0.90, 0.88, 0.86, 0.85 (four s, 6 H); ^{13}C NMR ($CDCl_3$/75 MHz) δ 143.7, 141.7, 113.9, 113.8, 108.0, 105.2, 57.0, 55.8, 53.5, 52.4, 46.5, 45.5, 43.5, 41.2, 40.1, 39.7, 38.4, 37.2, 32.9, 30.4, 30.3, 30.1, 29.9, 24.5, 21.0, 19.2; IR (neat/NaCl) 2950, 2899, 2840, 1637, 1457, 1384, 1375, 1364, 1209, 1190, 1165, 1128, 1072, 1059, 995, 961, 911, 858, 840 cm^{-1}; GC/MS for isomer one (PCI) *m/e* (rel intensity) 212 (M^+, 0.6), 182 (24), 181 ($M^+ - CH_3O$, 100), 165 ($M^+ - C_2H_7O$, 13), 149 ($M^+ - C_2H_7O_2$, 90), 133 (20), 109 (8), 75 (64); GC/MS for isomer two (PCI) *m/e* (rel intensity) 212 (M^+, 0.4), 181 ($M^+ - CH_3O$, 100), 165 ($M^+ - C_2H_7O$, 10), 149 ($M^+ - C_2H_7O$, 34), 133 (12), 109 (5), 75 (22); HRMS (EI) *m/e* calcd for $C_{12}H_{20}O$ ($M^+ - CH_4O$) 180.1514, found 180.1514. Anal. Calcd for $C_{13}H_{24}O_2$: C, 73.58; H, 11.32. Found: C, 73.47; H, 11.48. The spectral data for compound **32** (obtained as a mixture of diastereomers) are as follows: ^1H NMR ($CDCl_3$/300 MHz) δ 4.53 (d, 0.23 H, J = 8.1 Hz, acetal methine proton), 4.46 (d, 0.46 H, J = 6.4 Hz, acetal methine proton), 4.35 (d, 0.31 H, J = 2.0 Hz, acetal methine proton), 3.37-3.19 (m, 10 H, methoxy protons and the methine proton α to the OCH_3), 2.11 (m, 0.5 H), 1.92-0.95 (m, 0.5 H), 0.90, 0.88, 0.87, 0.85 (four s, 6 H), 0.01 and 0.00 (two s, 9 H); ^{13}C NMR ($CDCl_3$/75 MHz) δ 107.9, 105.0, 103.8, 80.9, 80.4, 80.3, 56.0, 55.9, 55.2, 54.3, 52.8, 51.4, 42.1, 40.7, 40.2, 39.0, 38.7, 37.8, 37.5, 36.5, 36.2, 35.5, 35.0, 34.7, 33.3, 33.1, 30.5, 25.2, 24.7, 22.9, 20.2, 19.2, 18.7, 17.0, -0.53, -0.78, -1.1; IR (neat/NaCl) 2950, 2824, 1456, 1364, 1248, 1080, 1096, 1117, 960, 946, 861, 837 cm^{-1}; GC/MS (PCI) *m/e* (rel intensity) 316 (M^+, 1), 301 ($M^+ - CH_3$, 2.6), 285 ($M^+ - CH_3O$, 4), 269 ($M^+ - C_2H_7O$, 19), 253 ($M^+ - C_2H_7O_2$, 35), 237 (17), 181 (22), 149 (96), 131 (22), 89 (11), 75 (100), 73 (12); HRMS (EI) *m/e* calcd for $C_{16}H_{33}O_2Si$ ($M^+ - CH_4O$) 284.2171, found 284.2164.

Conversion of Compound 32 to 31. Compound **32** (0.037 g/0.12 mmol) was taken up in 4 mL of methanol and 1 mL of acetic acid. The reaction was stirred at room temperature for 16 h. After this period, there was still evidence of starting material by TLC so the reaction was refluxed for an additional 3 h. The reaction was then cooled to room temperature, diluted with ether (10 mL), and washed with saturated sodium bicarbonate (2 × 50 mL). The organic layer was dried over potassium carbonate, concentrated in vacuo, and chromatographed through 10 g of silica gel that had been packed with a 2% ether/hexane solution containing 1% triethylamine. The column was eluted with 2% ether/hexane to afford 0.018 g (74%) of the desired compound **31**.

Acknowledgment. This work was supported by Washington University, the donors of the Petroleum Research Fund, administered by the American Chemical Society, and the Biomedical Research Support Program, Division of Research Resources, National Institutes of Health. We also gratefully acknowledge the Washington University High-Resolution NMR Facility, partially supported by NIH 1S10R02004, and the Washington University Mass Spectrometry Resource Center, partially supported by NIHRR00954, for their assistance.

Stereochemistry and Mechanism of Aldol Reactions Catalyzed by Kynureninase

Robert S. Phillips*,† and Rajesh K. Dua‡

Contribution from the Departments of Chemistry and Biochemistry, School of Chemical Sciences, and Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Georgia, Athens, Georgia 30602. *Received January 24, 1991*

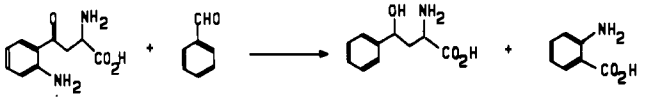
Abstract: Kynureninase from *Pseudomonas* has been reported to catalyze aldol and retro-aldol reactions, in addition to the physiological hydrolytic cleavage of L-kynurenine to anthranilic acid and L-alanine. However, the stereochemistry of these novel aldol reactions has not been previously determined. We have determined that the reaction of L-kynurenine and benzaldehyde catalyzed by kynureninase results in (2*S*,4*R*)-2-amino-4-hydroxy-4-phenylbutanoic acid. Similarly, the 4*R* isomer of dihydro-L-kynurenine readily undergoes retro-aldol cleavage, while the 4*S* isomer is unreactive as a substrate. Both isomers of dihydro-L-kynurenine are competitive inhibitors of kynureninase from *Pseudomonas*. However, the 4*S* isomer of dihydro-L-kynurenine is the most potent inhibitor, with a K_i of 0.3 μM. These results provide additional support for a general base mechanism for kynureninase, and suggest that the hydration occurs on the *re* face of the carbonyl group of kynurenine to give an (*S*)-*gem*-diolate intermediate.

Introduction

Kynureninase (L-kynurenine hydrolase, EC 3.7.1.3) is a pyridoxal-5'-phosphate-dependent enzyme that catalyzes the hydrolytic cleavage of L-kynurenine to give L-alanine and anthranilic acid (eq 1). This enzyme plays a central role in the catabolism

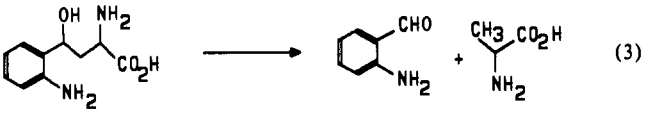
(1)

of L-tryptophan in some bacteria, including *Pseudomonas fluorescens*.[1] A similar enzyme, 3-hydroxykynureninase, is involved in L-tryptophan metabolism to NAD in animals and plants.[1] Kynureninase has also been found to catalyze an aldol-type condensation of benzaldehyde with incipient L-alanine formed from L-kynurenine to give 2-amino-4-hydroxy-4-phenylbutanoic acid[2] (eq 2). However, the stereochemistry of the aldol product at the



(2)

4-position was not determined, although Bild and Morris believed that only a single isomer was formed.[2] Furthermore, Tanizawa and Soda have reported that dihydro-L-kynurenine (2-amino-4-hydroxy-4-(2'-aminophenyl)butanoic acid) is a substrate for kynureninase, yielding 2-aminobenzaldehyde and L-alanine[3] in a retro-aldol cleavage reaction (eq 3). However, the diastereo-



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† School of Chemical Sciences.
‡ College of Pharmacy.

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(2) Bild, G. S.; Morris, J. C. *Arch. Biochem. Biophys.* **1984**, *235*, 41-47.
(3) Tanizawa, K.; Soda, K. *J. Biochem. (Tokyo)* **1979**, *86*, 1199-1209.

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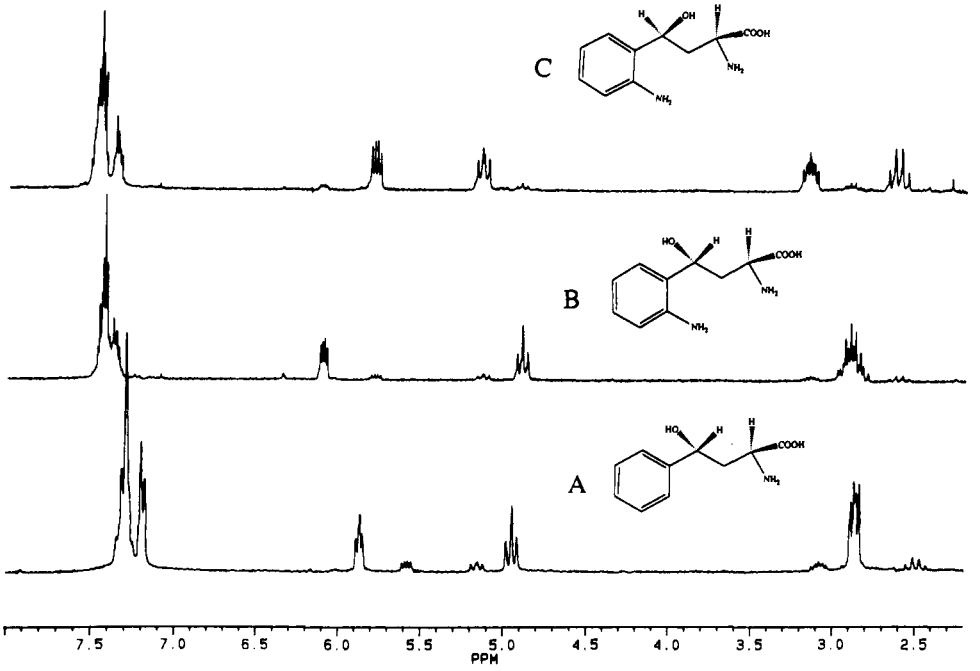


Figure 1. NMR spectra of 2-amino-4-hydroxy-4-phenylbutanoic acid and dihydrokynurenines. (A) Spectrum of the product obtained from the reaction of L-kynurenine and benzaldehyde catalyzed by kynureninase. The major signals arise from the 2*S*,4*R* diastereomer. (B) Spectrum of the 4*R* diastereomer of dihydro-L-kynurenine. (C) Spectrum of the 4*S* diastereomer of dihydro-L-kynurenine.

meric composition of the dihydro-L-kynurenine used in these studies was not known, and the stereospecificity of this retro-aldol reaction has not been examined. We have now determined the stereochemical course of these kynureninase-catalyzed aldol and retro-aldol reactions. The implications of these results for the mechanism of kynureninase are discussed.

Results

Aldol Condensation with Benzaldehyde. Incubation of L-kynurenine and excess benzaldehyde with kynureninase from *P. fluorescens* (ATCC 11250)[4], under the conditions described by Bild and Morris,[2] resulted in 2-amino-4-hydroxy-4-phenylbutanoic acid (eq 2), which was purified by preparative HPLC in nearly quantitative yield based on L-kynurenine. This product exhibited a circular dichroism (CD) spectrum with a negative extremum at 260 nm, exhibiting vibronic splitting characteristic of a chirally substituted benzyl alcohol chromophore. Comparison of this CD spectrum with those of (*R*)- and (*S*)-mandelic acids demonstrated that the major enzymatic aldol product exhibits the same absolute configuration as (*S*)-mandelic acid, and thus has the 4*R* configuration.[5] However, the 300-MHz ^{1}H NMR spectrum of this product demonstrates that it is a 4:1 mixture of diastereomers, with the major signals assigned to the 2*S*,4*R* isomer of 2-amino-4-hydroxy-4-phenylbutanoic acid (Figure 1A).[6] Although the reaction of benzaldehyde with L-alanine could also lead to the aldol product, the incubation of L-alanine and benzaldehyde in the presence of kynureninase does not result in any significant aldol reaction, in agreement with Bild and Morris.[2]

Retro-Aldol Cleavage Reaction of Dihydrokynurenine. Tanizawa and Soda reported preparation of dihydro-L-kynurenine by electrochemical reduction of L-kynurenine.[3] In our hands, L-kynurenine was conveniently reduced with $NaBH_4$ in aqueous solution, and the resultant 2:3 mixture of diastereomers was separated by preparative HPLC. The 300-MHz ^{1}H NMR spectra of the separated diastereomers of dihydro-L-kynurenine are shown in Figure 1. The second isomer to emerge from the HPLC column exhibits a proton spectrum very similar to that of the major product of the aldol condensation with benzaldehyde, and thus has the 4*R* configuration (Figure 1B). In contrast, the first peak exhibits an NMR spectrum which closely matches that of the minor isomer formed in the reaction with benzaldehyde, and thus is assigned the 4*S* configuration (Figure 1C). The differences in the β-hydrogen region of the NMR spectra of these compounds are especially striking. If the configuration at C-4 is *R*, the diastereotopic β-hydrogens overlap at 2.85 ppm, whereas the 4*S* isomer shows well-resolved multiplets at about 2.50 and 3.05 ppm (Figure 1). These assignments were confirmed by the CD spectra of the dihydro-L-kynurenines, which show the expected negative extremum at 290 nm for the 4*R* isomer and a similar positive extremum for the 4*S* isomer.

The reaction of kynureninase with the two dihydro-L-kynurenine diastereomers was then examined. The 4*R* isomer readily forms 2-aminobenzaldehyde, as determined spectrophotometrically by the increase in absorbance at 360 nm[3]. This reaction obeyed Michaelis–Menten kinetics, with a K_m of 1.5 μM and a V_{max} about 9% that for L-kynurenine.[7] In contrast, no significant absorbance increase at 360 nm was observed for the (4*S*)-dihydro-L-kynurenine in the presence of kynureninase. This is the stereospecificity anticipated from the stereoselectivity of the aldol condensation with benzaldehyde; however, this stereospecificity seems much higher than expected from the 4:1 mixture of diastereomers observed in the reaction of benzaldehyde (Figure 1A). Perhaps the presence of the aromatic amino group assists in stereochemical discrimination.

Both isomers of dihydro-L-kynurenine were found to act as competitive inhibitors; however, the 4*S* isomer is a more potent inhibitor, with an apparent K_i of 0.3 μM, than is the 4*R* isomer,

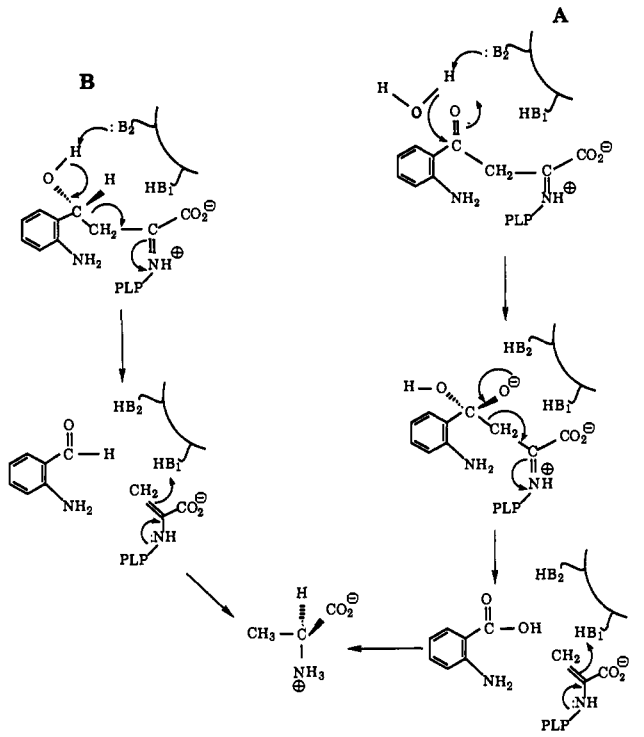
(4) Hayaishi, O.; Stanier, R. Y. *J. Biol. Chem.* **1952**, *195*, 735–740.

(5) Although the spatial arrangement of the substituents around the benzylic position is identical in the benzaldehyde condensation product and in (*S*)-mandelic acid, as shown by the similarity in the CD curves, the enzymatic product is assigned the *R* configuration due to the change in the substituent priorities according to the Cahn–Ingold–Prelog rules.

(6) We assume that the stereospecificity at the α-carbon is retained in this product. This assumption is supported by the reaction stereospecificity for kynurenine, since D-kynurenine exhibits no measurable substrate activity.[3] Since the stereochemical arguments in this paper are only concerned with C-4, the configuration at C-2 will be assumed to be L (*S*) for all compounds.

(7) Although Tanizawa and Soda[3] reported that dihydro-L-kynurenine reacts with a V_{max} about 65% of that of L-kynurenine, we find that the 2*S*,4*R* diastereomer of dihydro-L-kynurenine reacts at about 9% of the rate of L-kynurenine. In addition, the K_m for dihydro-L-kynurenine was reported to be 65 μM, but our K_m is much lower (1.5 μM). These differences may be due to differences in the substrate specificities of the kynureninases from *P. fluorescens* (this work) and *P. marginalis* (Tanizawa and Soda), or to some differences in reaction conditions. However, our K_m value for L-kynurenine is in good agreement with Tanizawa and Soda.

Scheme I



with K_i of 1.4 μM (in good agreement with the K_m for the retro-aldol reaction). The K_m for the reaction of L-kynurenine under these conditions was found to be 25 μM, in good agreement with the K_m of 35 μM reported by Tanizawa and Soda.[3]

Discussion

The mechanism of kynureninase has been the subject of considerable interest, due to the unique nature of this pyridoxal-5'-phosphate-dependent reaction, which occurs with electrophilic rather than nucleophilic substitution at the β-carbon. Early mechanisms were proposed based on implausible redox reactions[8] or transamination;[9] however, the recent consensus suggests either a nucleophilic mechanism, with an "acyl–enzyme" intermediate,[10] or a general-base-catalyzed hydrolytic mechanism.[3] Our results, taken together with the studies of Bild and Morris[2] and Tanizawa and Soda,[3] provide strong additional support for the general-base mechanism (Scheme I). Kishore reported that a carboxylate group is modified by suicide substrate inhibitors.[11] Although Kishore proposed that this carboxylate is responsible for α-proton abstraction,[11] stereochemical studies by Palcic et al. found that the α-proton of L-kynurenine is scrambled between the α- and β-positions of the L-alanine product, and thus the proton abstraction at the α-C is probably due to a polyprotic base, most likely a lysine ϵ-amino group.[12] Thus, there are apparently two catalytic bases in the active site of kynureninase. One of these bases removes the α-proton, forming the iminium ion to serve as an electron sink in the subsequent C–C bond cleavage. In the hydrolysis of L-kynurenine, the second base would be required to assist in hydration of the ketone, by abstraction of a proton from a water molecule (Scheme I, mechanism A). The stereospecificity for cleavage of the (4*R*)-carbinol of the dihydro-L-kynurenines is likely a reflection of favorable orientation for this basic group to initiate the retro-aldol cleavage by proton abstraction from the OH (Scheme I, mechanism B). The observed stereochemistry of the aldol reactions suggests that the water attacks on the *re* face of the carbonyl group of kynurenine, giving the (*S*)-*gem*-diolate anion.[13] In contrast, the 4*S* isomer of dihydro-L-kynurenine would have the OH oriented projecting away from the catalytic base, and is not cleaved, since proton abstraction cannot occur. However, since both dihydro-L-kynurenines are similar in structure to the *gem*-diol intermediate (Scheme I, mechanism A), the potent competitive inhibition exhibited by these compounds suggests that they are "transition-state analogue" inhibitors.[14] The K_m/K_i for L-kynurenine and (4*S*)-dihydro-L-kynurenine is 83, similar to what we observed for inhibition of tryptophan synthase and tryptophan indole-lyase by the diastereomers of 2,3-dihydro-L-tryptophan, a transition-state analogue for these latter PLP-dependent enzymes.[15] Subsequent rapid collapse of the tetrahedral *gem*-diol intermediate in Scheme IA is likely and would generate the enzyme-bound PLP–enamine of L-alanine and anthranilic acid. This enamine can be intercepted by electrophiles such as benzaldehyde, resulting in the aldol product. Protonation of this enamine at the β- and α-carbons and subsequent transaldimination produce L-alanine.

Experimental Methods

Instrumentation. UV and visible spectra and steady-state kinetic measurements were performed on a Gilford Response II spectrophotometer, equipped with a Peltier-type thermoelectric cell block for temperature control. ^{1}H and ^{13}C NMR spectra were obtained in CF_3CO_2D on a Bruker AC-300 at 300.13 and 75.4 MHz, respectively. Circular dichroism spectra were obtained on a JASCO J-500C spectropolarimeter with a DP-501 data collection module. Typically, eight scans were collected per CO spectrum and averaged to improve signal to noise ratios.

Preparation of Kynureninase. The cells of *P. fluorescens* (ATCC 11250) were grown as described by Hayaishi and Stanier,[4] in a minimal medium containing 0.1% L-tryptophan as the sole carbon and nitrogen source. From 100 L of medium, grown for 18 h at 30 °C, was obtained 230 g of wet cell paste. The cells were suspended in 1 L of 0.01 M potassium phosphate, pH 7.0, and disrupted by 2 passages through a Manton–Gaulin homogenizer. After centrifugation of the cell extract for 1 h at 10000*g*, the enzyme was partially purified by ion-exchange chromatography on DEAE–cellulose and ammonium sulfate precipitation.[16] The preparation used in these studies exhibited a specific activity of 0.2 μmol min^{-1} mg^{-1}.

Assay of Kynureninase. Kynureninase activity was measured from the decrease in absorbance at 360 nm (ϵ = −4500 M^{-1} cm^{-1})[11] upon conversion to anthranilic acid. The reaction mixtures contained 0.4 mM L-kynurenine in 0.04 M potassium phosphate, pH 7.8, containing 40 μM pyridoxal-5'-phosphate, at 25 °C. The reactions of the dihydro-L-kynurenines were followed from the increase in absorbance at 360 nm (ϵ = 3900 M^{-1} cm^{-1}),[3] due to the formation of *o*-aminobenzaldehyde. Determination of inhibition of kynureninase by the dihydro-L-kynurenines was performed by variation of L-kynurenine concentration at several fixed values of dihydro-L-kynurenine concentration. K_m and V_{max} values were calculated by fitting of initial rate data to the Michaelis–Menten equation with ENZFITTER (Elsevier) on a Zenith Z-286 personal computer. K_i values were determined from eq 4.[17]

$$v = V_{max}[S]/(K_m(1 + [I]/K_i) + [S]) \quad (4)$$

Aldol Condensation with Benzaldehyde. The reaction mixture contained 52 mg of L-kynurenine and 200 μL (208.8 mg) of benzaldehyde in 10 mL of 65 mM Tris·HCl, pH 8.0, and 65 μM pyridoxal-5'-phosphate. A 1-mL portion of *Pseudomonas* kynureninase (17.7 units; specific activity 0.2 units/mg) was added, and the reaction mixture was stirred at room temperature for 24 h. The reaction mixture was then acidified with glacial acetic acid, and the precipitated protein was removed by centrifugation. The clear supernatant was extracted with ether

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(2 × 5 mL) to remove excess benzaldehyde. The aldol product was purified by preparative HPLC on a Dynamax 20 × 250 mm C18 column, eluting with 0.1% acetic acid/20% methanol at 5 mL/min. The product elution was followed at 254 nm; lyophilization of the product gave 46 mg (94.5%) of 2-amino-4-hydroxy-4-phenylbutanoic acid. The 300-MHz ^{1}H NMR of this product is shown in Figure 1A.

Preparation of Dihydrokynurenines. To a solution of 21 mg of L-kynurenine (Sigma) in 5 mL of H_2O was added 10 mg of $NaBH_4$. The reaction mixture was stirred overnight at room temperature, and the initially pale yellow solution became colorless. The UV spectrum of the solution showed formation of a new peak at 283 nm and disappearance of the peak at 360 nm due to kynurenine. The reaction was then lyophilized, and the solid was dissolved in 5 mL of 0.1% acetic acid. The diastereomers of dihydro-L-kynurenine were separated by preparative HPLC on a Rainin HPLC system, using a 20 × 250 nm C18 column (Dynamax, Rainin) in 0.1% acetic acid at 5 mL/min. The elution of the dihydro-L-kynurenines was detected by following the UV absorbance at 283 nm on an LDC Spectromonitor 3000. The separation of these diastereomers is difficult, and less than 0.5 mg could be separated during each run. The fractions containing the separated diastereomers were pooled and lyophilized to give 7.2 mg of the first peak (4*S* isomer) and 12.4 mg of the second peak (4*R* isomer). The 300-MHz ^{1}H NMR spectra of the diastereomers are shown in Figure 1B and C.

Acknowledgment. This research was supported by a grant from the National Institutes of Health to RSP (GM-42588).

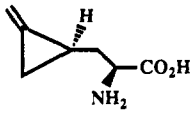
Mechanistic Study on the Inactivation of General Acyl-CoA Dehydrogenase by a Metabolite of Hypoglycin A

Ming-tain Lai, Li-da Liu, and Hung-wen Liu*

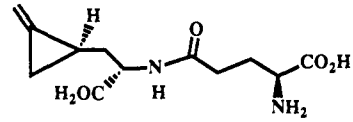
Contribution from the Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received March 28, 1991*

Abstract: General acyl-CoA dehydrogenase (GAD) is a flavin-dependent (FAD) enzyme that catalyzes the oxidation of a fatty acyl-CoA to the corresponding α,β-enolyl-CoA. When GAD is exposed to (methylenecyclopropyl)acetyl-CoA (MCPA-CoA), a metabolite of hypoglycin A that is the causative agent of Jamaican vomiting sickness, time-dependent inhibition occurs with concomitant bleaching of the active-site FAD. The inactivation mechanism is generally believed to be initiated by C_α anion formation followed by ring fragmentation and the covalent modification of FAD. However, formation of a cyclopropyl radical intermediate through one-electron oxidation followed by ring opening and then radical recombination to yield a modified FAD is an appealing alternative. As described herein, studies of the inactivation of GAD by (1*S*)- and (1*R*)-MCPA-CoA bearing a stereospecific tritium label at C_α have provided direct evidence suggesting that C_α proton abstraction occurs during inactivation and the two diastereomers of MCPA-CoA bind to the same locus in the active site of GAD. Despite the fact that the inactivations mediated by (1*R*)- and (1*S*)-MCPA-CoA proceed at different rates, the observed partition ratios are almost identical. Using $[\alpha,\alpha\text{-}^2H_2]$MCPA-CoA as inhibitors, we have found that the sluggish inactivation observed for (1*S*)-MCPA-CoA is not due to mechanistic rerouting, but is instead a result of the retardation of the initial deprotonation step. Thus, the equivalent partition ratios found in these studies clearly indicate that inactivation by either (1*R*)- or (1*S*)-MCPA-CoA follows the same chemical course. Such a lack of stereospecificity for the bond rupture at C_β of MCPA-CoA in the enzyme active site suggests that the ring-opening step leading to inactivation is likely a spontaneous event. Since the rearrangement of α-cyclopropyl radicals to ring-opened alkyl radicals is extremely rapid, the ring cleavage induced by an α-cyclopropyl radical may bypass the chiral discrimination normally imposed by the enzyme. Thus, the mechanistic insights deduced from this study support our early notion that inactivation of GAD by MCPA-CoA is likely to proceed through a radical mechanism.

Hypoglycin A (**1**), a methylenecyclopropane-containing amino acid, has been isolated together with its γ-glutamyl conjugate hypoglycin B (**2**) from the arillus and seeds of unripe ackee



HYPOGLYCIN A (**1**)



HYPOGLYCIN B (**2**)

(*Blighia sapida*).[1] While ripe ackee fruit serves as a dietary staple in Jamaica, ingestion of hypoglycin from unripe fruit has been recognized as the cause of Jamaican vomiting sickness.[2] The ingested hypoglycin is metabolized in vivo to methylenecyclopropaneacetic acid (MCPA) in two steps, and the subsequent coenzyme A derivative (MCPA-CoA, **3**) is the actual causative agent of Jamaican vomiting sickness.[3] The site at which hypoglycin toxicity occurs has been shown to be flavin-containing acyl-CoA dehydrogenases.[4] Since acyl-CoA dehydrogenase catalyzes the first step of β-oxidation, converting a fatty acyl thioester substrate to the corresponding α,β-enolyl-CoA product,[5]

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