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Cu^{2+}-Catalyzed Peptide Bond Formation in the Reaction of 5-Deoxypyridoxal and α-Phenyl-α-aminomalonic Acid

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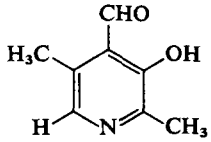
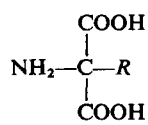
The reaction of 5-deoxypyridoxal with α-phenyl-α-aminomalonic acid in the presence of excess Cu^{2+} ions is shown to lead to the formation of *N*-5-deoxypyridoxoyl-α-phenylglycine, **III**, as the only 5-deoxypyridoxal-derived product. This reaction occurs anaerobically under very mild conditions of temperature and pH and involves the oxidative formation of a peptide bond. It represents a hitherto undescribed reaction type for vitamin B_6 and its analogs; a mechanism for the reaction is proposed.

INTRODUCTION

Previous investigations from this laboratory have demonstrated that the reactions of 5-deoxypyridoxal, **I**, a vitamin B_6 analog, with α-aminomalonic acid (**II**, $R = H$) and α-substituted α-aminomalonic acids (**II**, $R = CH_3$, C_6H_5) occur very rapidly under mild conditions of temperature and pH (*1–4*). As a continuation of these studies, we



(I)



(II)

have been investigating the effects of metal ions on these reactions (*5*) and, in the process, have discovered an unusual and hitherto unreported type of reaction for vitamin B_6.

The data reveal that the reaction of 5-dexoypyridoxal with α-phenyl-α-aminomalonic acid, in the presence of excess Cu^{2+} ions, leads to the formation of a peptide bond that incorporates the 4′-aldehyde carbon of the vitamin analog and the amino group of the decarboxylated amino acid, structure **III**.

This reaction proceeds at pH 6.2 at room temperature in a nitrogen atmosphere; **III** is the only 5-deoxypyridoxal-derived product that can be isolated from the reaction

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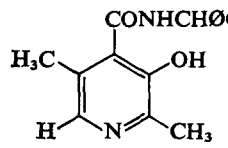
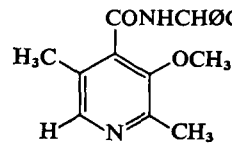


(III)



(IV)

solution. The structure of this compound has been confirmed by comparison of **IV**, the ethyl ester, *O*-methyl ether of **III**, with an authentic sample of **IV** obtained by an unambiguous synthesis.

EXPERIMENTAL

Materials. 5-Deoxypyridoxal, **I**, and the ammonium salt of α-phenyl-α-aminomalonic acid, containing an ethanol of solvation, were prepared as described previously (*1, 3, 6, 7*). Water used in these experiments was distilled, deionized water that had been boiled, gassed with a stream of nitrogen, and stored under nitrogen. All other reagents were the highest quality available from commercial sources.

Methods. Nuclear magnetic resonance (nmr) spectra were recorded on a Perkin–Elmer R-12 spectrometer; this instrument was not equipped with a signal lock device, and the reported shifts are considered to be accurate to ±0.1 ppm. Infrared spectra were recorded on a Perkin–Elmer Model 137 spectrometer. Chemical ionization and electron impact mass spectra were obtained on a Finnigan Model 6000 mass spectrometer.

Isolation of N-5-deoxypyridoxoyl-α-phenylglycine, III, from the reaction of 5-deoxypyridoxal and α-phenyl-α-aminomalonic acid in the presence of Cu^{2+} ions. 5-Deoxypyridoxal (75.5 mg, 0.5 mmole) and $CuCl_2 \cdot H_2O$ (852 mg, 5.0 mmoles) were dissolved in 400 ml of 0.3125 *M* potassium cacodylate buffer (pH 6.21 at 30°C, ionic strength 1.25 *M* with KCl). To this green solution were added 3.23 g (12.5 mmoles) of α-phenyl-α-aminomalonic acid in 100 ml of water. The resulting blue solution was kept overnight in the dark in a nitrogen atmosphere in a tightly stoppered polyethylene bottle. The pH was then adjusted from 6.08 to approximately 1 with 50 ml of 6 *N* HCl. The acidified solution was applied, under nitrogen, to a 1.9 × 58-cm column of Bio-Rad AG50W-X8 (200–400 mesh) cation-exchange resin in the hydrogen form. After application of the sample, the column was eluted with 0.5 *N* HCl (1860 ml), 1.0 *N* HCl (1300 ml), and 2.0 *N* HCl (2100 ml). The flow rate was 50–75 ml/hr, fractions of 13 ml were collected, and the ultraviolet absorbancies of the fractions were measured at 255 and 295 nm. The only fractions having maximal absorbancies at 295 nm, corresponding to 5-deoxypyridoxal-derived products, appeared in a bell-shaped elution profile in the 2 *N* HCl eluate. Subsequent elution of the column with 4 *N* HCl produced no further ultraviolet absorbing material. The 295-nm-absorbing fractions (150 in all) were combined and concentrated at 40°C on a rotary evaporator to a buff-colored solid that was further dried *in vacuo* over KOH and P_2O_5 to yield 145 mg of the product **III**. The nmr spectrum in D_2O is provided in Fig. 1. Thin-layer chromatography on Eastman 6060 fluorescent silica gel sheets in three different solvent systems showed only one ultraviolet fluorescent spot in each case. For analysis, the compound was dissolved in a small volume of water,

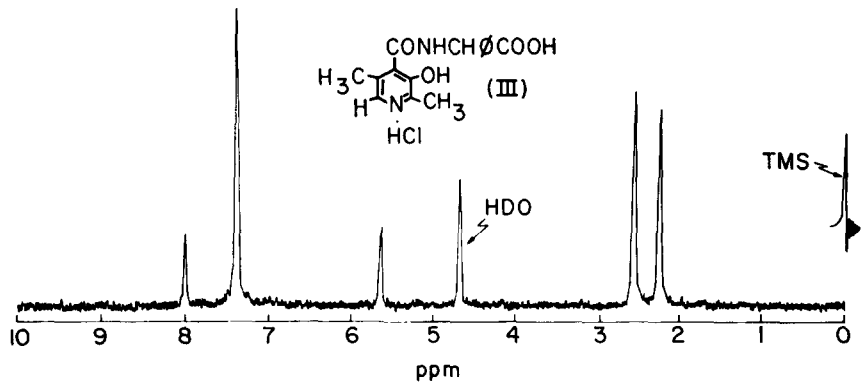


FIG. 1. Nuclear magnetic resonance spectrum of *N*-5-deoxypyridoxoyl-α-phenylglycine, **III**. Solvent, D_2O; external standard, tetramethylsilane (TMS).

and concentrated HCl was added to achieve a final concentration of 2 *N*. Upon refrigeration, granular white crystals separated out; these were washed with ice-cold 2 *N* HCl and dried *in vacuo* over KOH and P_2O_5.

Anal. Calcd for $C_{16}H_{16}N_2O_4 \cdot HCl \cdot H_2O$ (354.79): C, 54.16; H, 5.39; N, 7.89; Cl, 9.99. Found: C, 53.97; H, 5.40; N, 7.87; Cl, 10.27.

Chemical ionization and electron impact mass spectrometry of the free base gave $(M + 1)^+$ and M^+ ions at 301 and 300, respectively.

Preparation of IV by esterification and methylation of III. Fifty milligrams (0.141 mmole) of **III** were quantitatively converted to the ethyl ester by a conventional Fischer esterification procedure using absolute ethanol and dry HCl gas. After decolorization with Norite A and Darco G-60 in boiling ethanol, the ester hydrochloride was recrystallized from ethanol/ether; 47 mg of white powder were obtained. The ethyl ester was then reacted for 5 hr at 0°C and 11 hr at room temperature, in 2.5 ml of methylene chloride, with diazomethane generated from 1 mmole of Aldrich *N*-methyl-*N*-nitroso-N^1-nitroguanidine using the procedure and apparatus described by Fales *et al.* (*8*). After removal of solvent and excess diazomethane, the residual amber oil was decolorized in ethyl acetate with a mixture of Norit A and Darco G-60. The yellow oil (45 mg) that was obtained was dissolved in a small volume of ether/acetic acid (19/1) and passed through a 0.6 × 5.0-cm column of Merck No. 60 silica gel, using the same solvent as the eluant. The first 25 ml of eluant were collected and the solvents were removed to yield 42 mg of a very pale yellow oil (95% yield from ester). This was converted to the hydrochloride by passing dry HCl gas over an ice-cooled ether solution with stirring. The material that was obtained after evaporation of the solvent was recrystallized from ethanol/ether, yielding a fluffy snow-white solid, **IV**, mp 162.5–163.5°C (dec).

Anal. Calcd for $C_{19}H_{22}N_2O_4 \cdot HCl$ (378.86): C, 60.23; H, 6.12; N, 7.40; Cl, 9.36. Found: C, 60.28; H, 6.11; N, 7.47; Cl, 9.13.

This compound was found to be identical in all of its properties to a product obtained by an independent and unambiguous synthesis, as described below. Criteria used were mixed melting point, nmr spectra, electron impact and chemical ionization mass spectra,

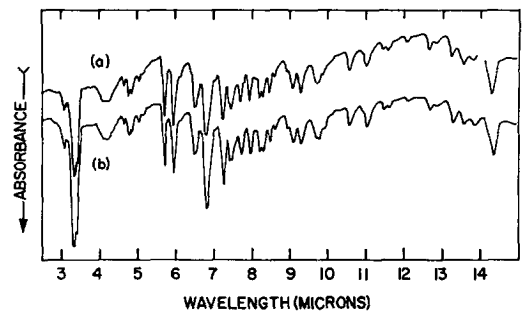


FIG. 2. Infrared spectra (nujol mulls) of *N*-5-deoxypyridoxoyl-α-phenylglycine ethyl ester *O*-methyl ether, IV, obtained (a), by esterification and methylation of III, and (b), by the synthetic route shown in Scheme I.

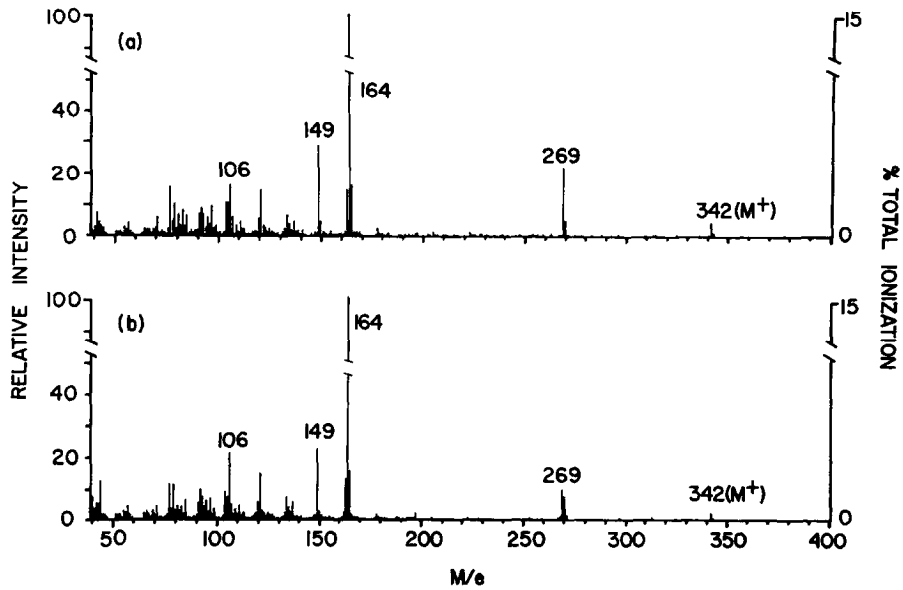


FIG. 3. Electron impact mass spectra of *N*-5-deoxypyridoxoyl-α-phenylglycine ethyl ester *O*-methyl ether, IV, obtained (a), by esterification and methylation of III, and (b), by the synthetic route shown in Scheme II.

and infrared spectra. The infrared and electron impact mass spectra are compared in Figs. 2 and 3, respectively.

5-Deoxypyridoxal oxime, V. 5-Deoxypyridoxal (453 mg, 3.0 mmoles) was dissolved in 30 ml of hot water containing 2.92 g of sodium acetate trihydrate. To this solution were added, with stirring, 417 mg (6 mmoles) of hydroxylamine hydrochloride. The reaction mixture was chilled in ice and the white precipitate was filtered, washed with water, and dried over KOH and P_2O_5. Yield, 472 mg (95%); mp 226–228°C. Recrystallization from ethanol did not change the melting point.

2,5-Dimethyl-3-acetoxy-4-cyanopyridine, VI. The procedure of Heyl (*9*) was adapted for this and the subsequent synthesis. The oxime, **V** (472 mg), was refluxed under nitrogen for 2.5 hr with 5 ml of redistilled acetic anhydride. Concentration and evaporation from ethanol yielded a brown solid that was dissolved in ether and extracted with saturated sodium bicarbonate and water. The ether solution was dried over magnesium sulfate and decolorized with Darco G-60. The yellow product obtained after solvent removal was recrystallized from carbon tetrachloride/petroleum ether; large yellow crystals were obtained, 380 mg (67%); mp 74–75°C. A second crop of 102 mg (18%) could be obtained from the filtrate and wash.

Anal. Calcd for $C_{10}H_{10}N_2O_2$ (190.20); C, 63.14; H, 5.29; N, 14.73. Found: C, 63.14 H, 5.43; N, 14.52.

2,5-Dimethyl-3-hydroxypyridine-4-carboxylic acid, (5-deoxypyridoxic acid), VII. Compound **VI** (846 mg, 4.45 mmoles) was refluxed for 7.5 hr under a slow stream of nitrogen with 20 ml of 3 *N* KOH. After dilution with 2 vol of water, the solution was extracted twice with carbon tetrachloride. The aqueous layer was then acidified to a pH value between 3 and 4. The light-orange precipitate that was obtained was filtered, washed with water, and dried over KOH and P_2O_5 to yield 410 mg (55%) of the product, which does not melt below 300°C. This compound is insoluble in most solvents and for analysis was recrystallized from hot dimethylsulfoxide/water.

Anal. Calcd for $C_8H_9NO_3$ (167.16): C, 57.47; H, 5.43; N, 8.38. Found: C, 57.47; H, 5.63; N, 8.55.

Chemical ionization and electron impact mass spectra gave $(M + 1)^+$ and M^+ signals at 168 and 167, respectively.

2,5-Dimethyl-3-methoxypyridine-4-carboxylic acid methyl ester, VIII. Compound **VII** (501 mg, 3.0 mmoles) was converted to the methyl ester, methyl ether with an excess of diazomethane, in an alcoholic ether solution, generated from 4.52 g of Aldrich Diazald. The diazomethane solution (approx 50 ml) was slowly and carefully added with ice-cooling to a stirred suspension of **VII** in 15 ml of ethanol. The resulting solution was stirred overnight and then concentrated to a dark amber oil. This was decolorized by passing a chloroform solution of the oil through a 1.1 × 12.5-cm silica gel column using chloroform as the eluant. Concentration of the eluate gave 490 mg (85%) of a pale yellow oil. For analysis, the product was converted to the hydrochloride by passing HCl gas over a stirred ice-cold ether solution. The hydrochloride was recrystallized from ethyl acetate/ether; large white rods were obtained, mp 130–132°C.

Anal. Calcd for $C_{10}H_{13}NO_3 \cdot HCl$ (231.68): C, 51.84; H, 6.09; N, 6.05; Cl, 15.31. Found: C, 51.58; H, 5.94; N, 6.03; Cl, 15.27.

Chemical ionization and electron impact mass spectrometry of the free base gave $(M + 1)^+$ and M^+ signals at 196 and 195, respectively. The nmr spectrum of the free base in carbon tetrachloride gave the expected signals.

Coupling of 2,5-dimethyl-3-methoxypyridine-4-carboxylate with D,L-*α-phenylglycine ethyl ester: Proof of structure of IV.* The potassium salt of 2,5-dimethyl-3-methoxypyridine-4-carboxylic acid, **IX**, was obtained by reacting **VIII** with 1 equiv of potassium hydroxide in methanol and concentrating the resulting solution to dryness. To 98 mg (0.45 mmole) of this salt, suspended in 1 ml of benzene, were added 5 μl of distilled dimethylformamide and 99 mg (0.83 mmole) of distilled thionyl chloride (*10*). After the reaction mixture had been stirred for 10 hr at room temperature, the solvents

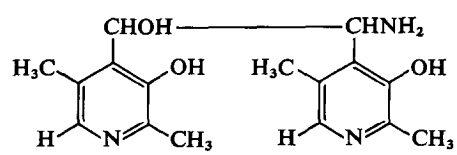
were removed, and the residual solid, containing KCl and **X**, the acyl halide of **IX**, was dried *in vacuo* over P_2O_5. Dry tetrahydrofuran (1 ml) was added to the acyl halide. To this stirred suspension were added 50 mg (0.5 mmole) of triethylamine and a suspension, in 1 ml of methylene chloride, containing 0.5 mmole each of D, L-α-phenylglycine ethyl ester hydrochloride and triethylamine. The reaction mixture was stirred for 1 hr at room temperature and 1 hr in a 60°C bath. The solids were filtered, and the filtrate was concentrated to 140 mg of an amber oil. This was dissolved in chloroform and extracted with water, 3% sodium bicarbonate, and water. The chloroform solution was dried over magnesium sulfate, decolorized with Norit A and Darco G-60, and concentrated to 108 mg of yellow oil. An nmr spectrum showed this oil to be a mixture of the desired product and unreacted D,L-α-phenylglycine ethyl ester. After treatment of an ether solution of the oil with HCl gas, the hydrochloride of DL-α-phenylglycine ethyl ester could be removed by crystallization from an acetone solution; the hydrochloride of the desired product **IV** is soluble in acetone. Purification of the product to homogeneity was achieved by silica gel chromatography of the free base (yield after chromatography, 80 mg; 52%), and crystallization of the hydrochloride salt from ethanol/ether, as described above in the preparation of **IV** from **III**; mp 164–164.5°C (dec.) The mixed mp of this preparation with that from **IV** obtained above is 163–164°C (dec.)

Anal. Calcd for $C_{19}H_{22}N_2O_4 \cdot HCl$ (378.86): C, 60.23; H, 6.12; N, 7.40; Cl, 9.36. Found: C, 60.03; H, 6.17; N, 7.13; Cl, 9.10.

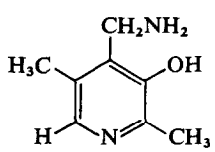
The nmr spectrum of the hydrochloride in $CDCl_3$, with tetramethylsilane as an internal reference, gave signals at 9.1 (*d*, 1), 8.0 (*s*, 1), 7.5 (quintet, 5), 5.8 (*d*, 1), 4.2 (*q*, 2), 3.9 (*s*, 3), 2.6 (*s*, 3), 2.4 (*s*, 3), and 1.2 (*t*, 3) ppm downfield from the standard. The infrared and electron impact mass spectra are provided in Figs. 2 and 3. Chemical ionization mass spectrometry of both this product and **IV**, obtained above, gave base peaks at 343 $(M + 1)^+$.

RESULTS

It was shown previously that the reaction of 5-deoxypyridoxal with α-phenyl-α-aminomalonic acid in the absence of Cu^{2+} ions leads to the formation of **XI** (two diastereoisomeric pairs) and **XII** as the 5-deoxypyridoxal-derived products (*3*).



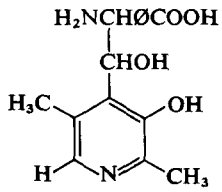
(XI)



(XII)

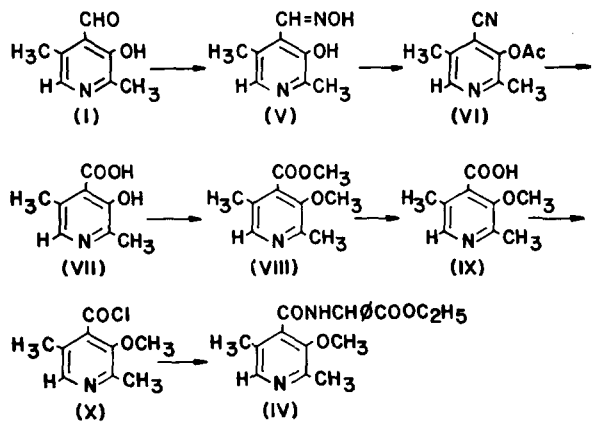
However, after cation-exchange chromatography of a reaction solution that was initially 0.025 *M* in α-phenyl-α-aminomalonic acid, 0.001 *M* in 5-deoxypyridoxal, and 0.01 *M* in Cu^{2+} ions, only one 5-deoxypyridoxal-derived product, **III**, was isolated in high yield. Its proton magnetic resonance spectrum is shown in Fig. 1. It is apparent from the figure that the benzene ring of the amino acid has been incorporated into a structure that also contains the components of the 5-deoxypyridoxal ring. This com-

pound formed a monohydrochloride, a monoethyl ester, and monoacetate and monobenzoate ethyl esters. Mass spectrometry indicated a molecular weight of 300 for the parent compound **III**. These data ruled out a structure such as **XIII**, which can arise from carbon–carbon condensations of 5-deoxypyridoxal and aminomalonates (*1*, *2*).



(XIII)

The available data, however, did fit the unexpected peptide structure **III**. Because of the unusual nature of the reaction, we decided to establish the structure of **III** by unambiguous synthesis. This was done according to Scheme I; for the purposes of comparison, **III** was converted to its ethyl ester, *O*-methyl ether, **IV**. The structures of the intermediates in Scheme I were confirmed by elemental analyses, nmr and infrared spectra, and chemical ionization and electron impact mass spectra (see Experimental).



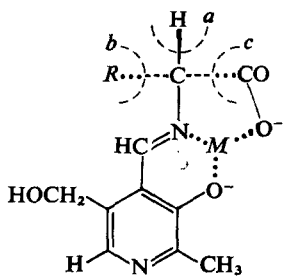
SCHEME I

In Figs. 2 and 3 are provided the infrared and mass spectra of **IV** obtained by esterification and methylation of **III**, and of **IV** obtained by the synthetic route shown in Scheme I. It can be seen that these are identical, providing unequivocal confirmation of the peptide structure **III**.

DISCUSSION

The classical studies of Snell and his colleagues (*11*, *12*) and of Braunstein (*13*) established the mechanistic principles behind vitamin B_6-catalyzed reactions. Subse-

quent investigations in model systems, such as those of Auld and Bruice (*14*), have confirmed and expanded our understanding of the mechanistic basis for vitamin B_6 action. Although metal ions do not appear to play a role in the large majority of B_6-dependent enzymes, the effects of metal ions on B_6-catalyzed reactions in model systems are well established, and there have been a number of investigations dealing with complexes of Schiff bases of pyridoxal and amino acids (*15–17*). These have confirmed that structures such as **XIV** are involved as intermediates in reactions catalyzed by vitamin B_6 and its analogs in chemical systems; in **XIV**, *M* is any of a number of di- and trivalent metal ions.



(XIV)

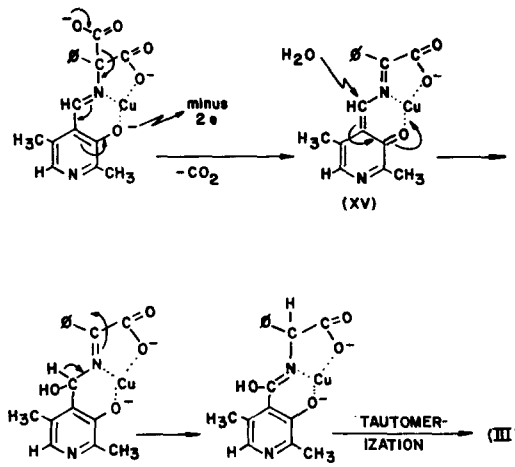
These reactions include, among others, oxidative deamination (*18*) and transamination (*19*), carbon–carbon bond cleavage and condensation (*20*), and decarboxylation (*21*), resulting from bond-making and bond-breaking processes occurring at bonds *a*, *b*, and *c*, respectively, in **XIV**. The role of the metal ion is to promote a planar conformation of the Schiff base and to provide electrophilic catalysis by favoring electron withdrawal from the α-carbon of the amino acid (*11*).

More recently, Dunathan has provided arguments that the reactive position in the Schiff base is the one occupied by bond *a* in **XIV**, and that reaction specificity in vitamin B_6-dependent enzymes can be achieved by rotation about the C_α–N bond of the amino acid so as to fix the bond undergoing scission in this position (*22*, *23*).

We have shown that α-aminomalonates and α-substituted-α-aminomalonates are extremely reactive towards vitamin B_6 analogs in chemical systems and uniquely reactive towards decarboxylation (*1–4*). Pyridoxal-catalyzed decarboxylation of amino acids is not usually observed because of other competing reactions that occur more readily (*21*). Presumably, decarboxylation is enhanced in aminomalonates because one of the carboxyl groups can occupy the reactive position *a* while the other is in the unreactive position *c*. In addition, we have established that addition of metal ions to these systems can alter reaction pathways and impart specificity to potential multipath reaction pathways (*5*). An example of such specificity is provided in this paper where it is shown that the presence of excess Cu^{2+} ions causes the formation of only one 5-deoxypyridoxal-derived product which is different from any of the 5-deoxypyridoxal derived products formed in the absence of the metal ion. Furthermore, this reaction is unusual among pyridoxal reactions in that it leads to the oxidative formation of a peptide bond incorporating the 4′-aldehyde carbon of the vitamin analog and the amino nitrogen atom of an amino acid under extremely mild conditions of temperature and pH.

Oxidative pyridoxal-catalyzed reactions of a variety of amino acids in the presence of metal ions have been observed (*18*, *24*). In addition, there are several pyridoxal phosphate-dependent amine oxidases that apparently require Cu^{2+} (*25–27*), and model systems for this type of reaction have been reported (*28*). The presence of a metal ion in these particular enzymes is in contrast to most vitamin B_6-requiring enzymes which generally appear to have no metal ion dependency.

Mechanisms of electron transfer in oxidative reactions catalyzed by metalloenzymes, including copper and B_6-dependent amine oxidases, have been discussed by Hamilton (*29*). In the presence of oxygen, Hamilton proposes that electron transfer in the copper and B_6-dependent amine oxidases involves a nonradical two-electron transfer to recipient molecular oxygen to form hydrogen peroxide, with no change in the valence state of the metal (*28*, *29*). The formation of **III**, to our knowledge, has no precedent in pyridoxal chemistry. Considering that the reaction is run in a nitrogen atmosphere, it seems that the reaction may involve the reduction of Cu^{2+} to Cu^{1+}, presumably by way of two one-electron transfers that occur via a copper-chelated Schiff base of the amino acid and 5-deoxypyridoxal. This reaction is being investigated further in an attempt to define the mechanism and the valence state(s) of the metal. A proposed



SCHEME II

reaction sequence leading to the formation of **III** is provided in Scheme II. This scheme incorporates some of the features of the oxidative mechanism of Hamilton (*29*) for copper- and vitamin B_6-dependent amine oxidases, as well as the generally accepted mechanistic principles of pyridoxal-catalyzed reactions.

The key intermediate in Scheme II is **XV**, an *o*-quinone methide, which undergoes nucleophilic addition of water at C_4'. A very recent report by Frater-Schröder and Mahrer-Busato (*30*) provides support for the proposed reaction pathway. These authors have demonstrated nucleophilic addition of a number of reagents to C_4' of an *o*-quinone methide derived from pyridoxine.

Whatever the details of the mechanism of the reaction, it is clear that excess Cu^{2+} ions exert a profound effect on the 5-deoxypyridoxal-catalyzed reactions of α-phenyl-α-aminomalonic acid. The alteration of pathway and imposition of reaction specificity

seen in this system must be, at least in part, a reflection of the steric constraints introduced into the system by the metal ion. Such constraints are no doubt an important aspect of the absolute specificity achieved by vitamin B_6-dependent enzymes (*23*, *31*).

ACKNOWLEDGMENTS

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