certain proteins could make the antibiological activities of SCN^- highly specific.

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Mechanism of Inactivation of Ornithine Decarboxylase by α-Methylornithine†

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ABSTRACT: Ornithine decarboxylase from *Lactobacillus* 30a is gradually inactivated by treatment with α-methylornithine, but activity is restored by treatment of the inactivated enzyme with pyridoxal phosphate. Inactivation of the enzyme is associated with formation of pyridoxamine phosphate and 5-amino-2-pentanone. α-Methylornithine is decarboxylated by the enzyme about 6000 times more slowly than is ornithine under the same conditions. These observations provide an explanation for the previously observed inhibition of ornithine decarboxylase by α-methylornithine [M. M. Adbel-Monem, N. E. Newton, and C. E. Weeks (1974), *J. Med. Chem. 17,* 447]: α-Methylornithine undergoes a decarboxylation-dependent transamination as a result of incorrect protonation of the quinoid intermediate which is formed by decarboxylation of the enzyme-bound pyridoxal phosphate–substrate Schiff base. This protonation produces inactive enzyme. Decarboxylation of ornithine by this enzyme produces a small amount of 4-aminobutanal, presumably also by decarboxylation-dependent transamination.

Ornithine decarboxylase (EC 4.1.1.7) catalyzes the decarboxylation of ornithine to form putrescine. This is the first step in polyamine synthesis, and it is ordinarily rate determining. In mammalian systems ornithine decarboxylase appears to have an important regulatory function (Russell, 1970), and the enzyme is turned over very rapidly (Russell and Snyder, 1969; Jänne and Raina, 1969). The level of ornithine decarboxylase increases during rapid cell growth (Russell, 1970), although the significance of this phenomenon is not understood. The enzymes from rat liver (Ono et al., 1972; Friedman et al., 1972; Heller et al., 1975) and from rat prostate (Jänne and Williams-Ashman, 1971) have been purified, but the levels of the enzyme in most mammalian tissues are so low that little work with the purified enzyme has been reported. The mam-

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malian enzyme contains pyridoxal phosphate, and it is clear that the coenzyme dissociates more rapidly from this enzyme than from most pyridoxal phosphate dependent enzymes (Clark and Fuller, 1976). This rapid coenzyme dissociation may have an important role in enzyme regulation (Litwack and Rosenfield, 1973).

Two different varieties of ornithine decarboxylase occur in microbial systems (Morris and Fillingame, 1974). The enzyme from *Lactobacillus* 30a[1] and the biodegradative enzyme from *Escherichia coli* (Applebaum et al., 1975) have been purified to homogeneity. Both contain covalently bound pyridoxal phosphate.

Because of the possibility that ornithine decarboxylase may play a regulatory role in cell proliferation, numerous attempts have been made to find inhibitors of this enzyme which may be useful in vivo. One of the most useful inhibitors is α-methylornithine (Abdel-Monem et al., 1974). This compound blocks proliferation of rat hepatoma cells in culture (Mamont et al., 1976), while at the same time causing an increase in the level of ornithine decarboxylase in the cells (McCann et al., 1977). In experiments with unpurified ornithine decarboxylase from rate prostate, α-methylornithine was a competitive inhibitor of ornithine decarboxylase (Abdel-Monem et al., 1974). Studies with α-[1-^{14}C]methylornithine failed to detect the formation of $^{14}CO_2$ (Abdel-Monem et al., 1975). Similar results were obtained with unpurified enzyme from other mammalian sources (Abdel-Monem et al., 1975).

α-Methyl amino acids have been studied as inhibitors of a number of pyridoxal phosphate dependent decarboxylases. α-Methyldopa and α-methyl-*m*-tyrosine are efficient inhibitors of dopa decarboxylase (Lovenberg et al., 1963; Borri Voltattorni et al., 1971; O'Leary and Baughn, 1977). These inhibitors undergo a decarboxylation-dependent transamination in which decarboxylation of the pyridoxal phosphate–substrate Schiff base is sometimes followed by transamination, leading to formation of pyridoxamine phosphate and a ketone. The fraction of decarboxylations leading to transamination is of the order of 2% (O'Leary and Baughn, 1977). The same decarboxylation-dependent transamination occurs, albeit to a lesser extent, when dopa decarboxylase acts on dopa (O'Leary and Baughn, 1975, 1977). This same decarboxylation-dependent transamination occurs when glutamate decarboxylase acts on α-methylglutamic acid (Huntley and Metzler, 1967) or glutamic acid (Sukhareva and Braunstein, 1971).

Although the inactivation of ornithine decarboxylase by α-methylornithine has been reported not to be accompanied by CO_2 evolution (Abdel-Monem et al., 1975), analogy to the studies of dopa decarboxylase and glutamate decarboxylase suggests that the rate of CO_2 evolution might be quite slow. Thus, we have investigated ornithine decarboxylase in order to determine whether decarboxylation-dependent transamination occurs when this enzyme acts on α-methylornithine or ornithine.

Experimental Section

All chemicals were the highest purity available. DL-[5-^{14}C]ornithine was obtained from New England Nuclear. Water was filtered through a Millipore Super-Q water purification system and had a resistance of greater than 18 megohms.

α-Methylornithine has been synthesized by Ellington and Honigberg (1974), by Abdel-Monem et al. (1974), and by Maehr et al. (1976). We synthesized this compound from alanine by the method of Hoppe (1975). The product thus produced was identical with an authentic sample provided by Abdel-Monem. Because of the method of synthesis, this compound should be uncontaminated by ornithine. 1-Pyrroline was prepared by hydrolysis of 4-aminobutyraldehyde diethylacetal (Aldrich) in aqueous HCl.

Biodegradative pyridoxal phosphate dependent ornithine decarboxylase was isolated from *Lactobacillus* 30a by Guirard and Snell.[1] The enzyme was homogeneous by ultracentrifugation, gel electrophoresis, and electrofocusing.

UV spectra were obtained on a Cary 118C spectrophotometer equipped with a thermostated cell compartment. The enzyme was assayed at 37 °C in 0.1 M sodium acetate buffer, pH 5.2, containing 10^{-4} M dithiothreitol using a Gilson differential respirometer.

High-voltage electrophoresis was performed with Whatman 3MM paper on a Savant flat-plate water-cooled apparatus at 2500 V. Radioactivity was measured on a Packard Tri-Carb Model 3375 scintillation counter by the automatic external standard method using an Aquasol scintillator. Thin-layer chromatography plates were counted using a Varian-Berthold radio scanner.

5-Amino-2-pentanone produced by reaction of ornithine decarboxylase with α-methylornithine was identified by the following procedure: α-Methylornithine was decarboxylated by ornithine decarboxylase at pH 5.2 in the presence of 10^{-4} M dithiothreitol and 10^{-4} M pyridoxal phosphate. After reaction was complete, the pH was adjusted to 10 and the solution was treated with $NaBH_4$. After 15 min, the remaining $NaBH_4$ was destroyed with acid, then the pH was readjusted to 10, and the solution was treated with dansyl chloride (Seiler and Wiechmann, 1969). After 1 h, the remaining dansyl chloride was destroyed by the addition of glutamic acid, after which the solution was extracted with a small amount of ethyl acetate. The extract was dried and concentrated, then samples were chromatographed on silica gel plates (Brinkmann) in 5:1 chloroform–butyl acetate, 8:3 cyclohexane–butyl acetate, 5:1 benzene–triethylamine, and 15:85:2 cyclohexane–benzene–methanol.

4-Aminobutyraldehyde produced during the decarboxylation of ornithine by ornithine decarboxylase was identified by the following procedure: DL-[δ-^{14}C]ornithine was decarboxylated by ornithine decarboxylase at 25 °C, pH 5.2, in the presence of 10^{-6} M pyridoxal phosphate. The resulting solution was concentrated by lyophilization and then chromatographed on silica gel or cellulose.

Results

Coenzyme Dissociation. Previous work on the ornithine decarboxylase from *Lactobacillus* 30a[1] indicated that dissociation of pyridoxal phosphate from this enzyme might be more facile than from other enzymes. Free pyridoxal phosphate was found to be necessary at all stages of purification. The enzyme can be readily resolved by dialysis against cysteine.

Manometric assay of ornithine decarboxylase which had been prepared and stored in the presence of pyridoxal phosphate and then desalted before use by gel filtration through Sephadex G-25 produced the same initial decarboxylation rate whether or not free pyridoxal phosphate was present. In the presence of 0.1 mM pyridoxal phosphate, a plot of CO_2 evolved vs. time was nearly linear for at least 30 min (Figure 1). However, in the absence of free pyridoxal phosphate, the rate dropped to less than 20% of its initial value within 30 min. Addition of free pyridoxal phosphate at that time restored the decarboxylation rate to its initial value. Incubation of the enzyme in dilute solution for 30 min at 37 °C in the absence of

[1] B. M. Guirard and E. E. Snell, unpublished work.

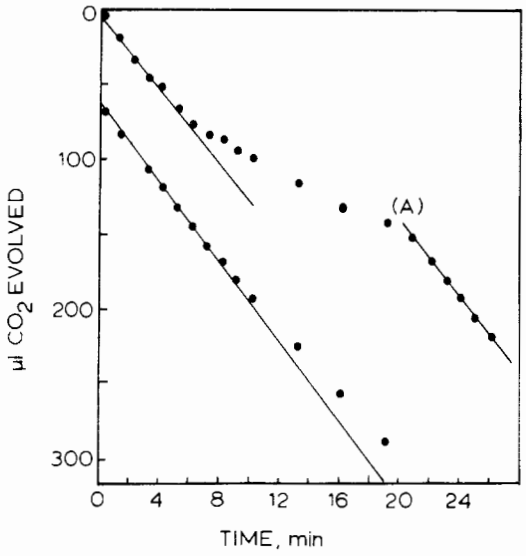


FIGURE 1: Manometric assay of ornithine decarboxylase at 37 °C, pH 5.2, in the absence of pyridoxal phosphate (upper curve) and in the presence of pyridoxal phosphate (lower curve). In the upper curve, pyridoxal phosphate was added at A.

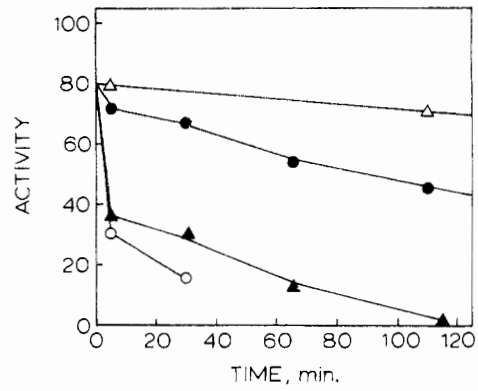


FIGURE 2: Activity of ornithine decarboxylase (in arbitrary units) vs. time of incubation with DL-α-methylornithine. The enzyme was incubated with α-methylornithine at 20 °C, pH 5.2, in the presence of 10^{-6} M pyridoxal phosphate. Samples were withdrawn at the indicated times and assayed without added pyridoxal phosphate (data shown) and with added pyridoxal phosphate (data not shown). In the latter case, activity equal to the initial activity was always observed. Concentration of α-methylornithine: (△) 0, (●) 2.5 mM, (▲) 10 mM, (○) 25 mM.

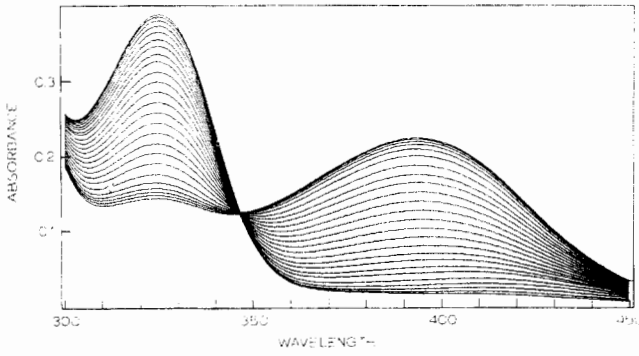
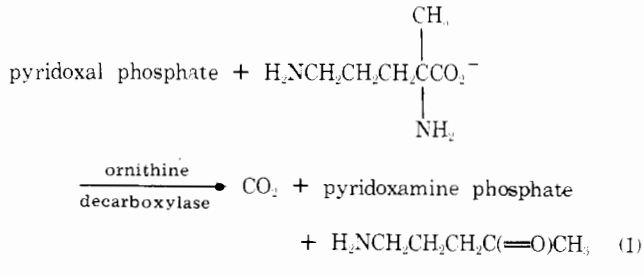


FIGURE 3: Spectra obtained during the reaction of ornithine decarboxylase with 8.3 mM α-methylornithine in the presence of 0.025 mM pyridoxal phosphate at pH 5.2, 25 °C. The spectrum was scanned at 10-min intervals.

pyridoxal phosphate led to a significant loss in activity which could be reversed by addition of pyridoxal phosphate. We found that the presence of 10^{-6} M pyridoxal phosphate was sufficient to maintain the enzyme at full activity during the assay, provided that the enzyme was fully activated prior to assay. However, the reaction of apoornithine decarboxylase with pyridoxal phosphate is slow under these conditions.

α-Methylornithine. Ornithine decarboxylase was incubated with α-methylornithine in the presence of 1 μM pyridoxal phosphate at 20 °C. Aliquots were withdrawn for assay at regular intervals. The enzyme was gradually inactivated by treatment with α-methylornithine (Figure 2), and the rate of inactivation increased with increasing concentration of α-methylornithine. Complete activity could always be restored to the inactivated enzyme by treatment with 0.1 mM pyridoxal phosphate prior to assay. At 37 °C the rate of inactivation was about tenfold faster at the same concentration of α-methylornithine.

Decarboxylation-dependent transamination of α-methylornithine would produce pyridoxamine phosphate, carbon dioxide, and 5-amino-2-pentanone (eq 1).

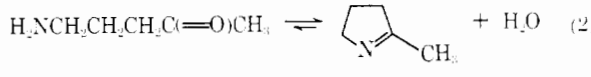


The reaction of ornithine decarboxylase with α-methylornithine was studied spectrophotometrically by use of higher concentrations of enzyme and pyridoxal phosphate (Figure 3). During inactivation of the enzyme the absorbance at 388 nm due to free pyridoxal phosphate disappears and is replaced by an absorbance at 325 nm, presumably as a result of formation of pyridoxamine phosphate.

The conversion of pyridoxal phosphate into pyridoxamine phosphate shown in Figure 3 appears to show zero-order kinetics over the course of the reaction, except that there appears to be a small initial lag before the steady-state rate is achieved. This probably occurs because the enzyme is initially not fully saturated with pyridoxal phosphate and absorbance changes due to coenzyme binding are complicating the observed spectra.

Manometric studies of the reaction of ornithine decarboxylase with α-methylornithine in the presence of 10^{-4} M pyridoxal phosphate under conditions ordinarily used to study the decarboxylation of ornithine failed to provide evidence for formation of CO_2. However, repetition of the experiment using much larger quantities of ornithine decarboxylase revealed a slow but steady formation of CO_2. In the presence of 10 mM α-methylornithine and 0.1 mM pyridoxal phosphate the rate of CO_2 evolution was roughly 6000 times slower than the corresponding rate for ornithine.

5-Amino-2-pentanone, the product expected from decarboxylation-dependent transamination of α-methylornithine, exists primarily as a cyclic imine (eq 2).



In order to establish the presence of this compound following decarboxylation, reaction mixtures were reduced with $NaBH_4$ to convert the unsaturated pyrroline (eq 2) to 2-methylpyrrolidine. This compound was then identified by dansylation and comparison with authentic dansyl 2-methylpyrrolidine by

thin-layer chromatography in several solvents.

The extent of decarboxylation-dependent transamination of α-methylornithine was measured by allowing a limited quantity of this substrate to react completely with ornithine decarboxylase in the presence of an excess of pyridoxal phosphate, as previously described by O'Leary and Baughn (1977). In such experiments, the L isomer of the substrate is assumed to be completely consumed and the change in absorbance at 388 nm can be used to determine the change in concentration of pyridoxal phosphate. A series of such experiments revealed that approximately 4% of decarboxylations of α-methylornithine lead to transamination.

Ornithine. Establishing the presence of the decarboxylation-dependent transamination for ornithine itself was significantly more difficult than for α-methylornithine. By analogy with glutamate decarboxylase[2] and dopa decarboxylase (O'Leary and Baughn, 1975, 1977), it was expected that the extent of the decarboxylation-dependent transamination would be much lower for ornithine than for α-methylornithine. In addition, problems connected with the dissociation of pyridoxal phosphate from the enzyme made it impossible to show inactivation of the enzyme during decarboxylation as a result of the decarboxylation-dependent transamination. The formation of pyridoxamine phosphate during reaction is not a useful criterion of reaction because this compound can arise from nonenzymatic transamination of free pyridoxal phosphate with ornithine.

[5-^{14}C]Ornithine was decarboxylated with ornithine decarboxylase in the presence of pyridoxal phosphate and the radioactive 1-pyrroline was identified by chromatography in several solvents. The radioactivity in the 1-pyrroline was always at least twice the background level of radioactivity obtained in the corresponding experiment in the absence of enzyme. Because of the background radioactivity, it was not possible to obtain an accurate figure for the extent of the decarboxylation-dependent transamination of ornithine. Our best estimate is that this figure is near 0.1%.

Discussion

α-Methylornithine reacts slowly with ornithine decarboxylase to produce carbon dioxide, 5-amino-2-pentanone, and pyridoxamine phosphate. In the absence of free pyridoxal phosphate, the enzyme is rapidly inactivated as a result of this reaction. In the presence of free pyridoxal phosphate decarboxylation proceeds slowly, and pyridoxal phosphate is consumed. It should be noted that we assume that 1,4-diaminopentane is also produced in this reaction, although the presence of this compound was not determined. Similarly, during the decarboxylation of ornithine itself by ornithine decarboxylase, a small amount of 4-aminopentanal (which exists primarily as the cyclic imine 1-pyrroline) is formed in addition to the normal decarboxylation products. Thus, it appears that both ornithine and α-methylornithine undergo decarboxylation-dependent transamination.

Ornithine decarboxylase is the third pyridoxal phosphate dependent amino acid decarboxylase for which this decarboxylation-dependent transamination has been demonstrated.[3] As in the case of dopa decarboxylase (O'Leary and Baughn, 1977) and glutamate decarboxylase (O'Leary and Yamada, 1977), the rate of decarboxylation of the α-methyl substrate is much lower than that of the natural substrate, but the proportion of decarboxylations leading to transamination is much higher for the α-methyl substrate.

The mechanism of this decarboxylation-dependent transamination has been discussed elsewhere (O'Leary and Baughn, 1977), and that discussion will not be repeated here. However, to date the decarboxylation-dependent transamination has been found in all three cases which have been investigated, and we know of no cases of pyridoxal phosphate dependent decarboxylations in which the reaction has been shown not to occur. The reaction may be an inevitable consequence of the decarboxylation mechanism, resulting from the inability of the enzyme to control the site of protonation of the quinoid intermediate with complete specificity.

Although we have confined our studies to the ornithine decarboxylase from *Lactobacillus* and have not studied the mammalian enzyme, it is likely that the decarboxylation-dependent transamination occurs with the mammalian enzyme as well. If so, then we can explain the previous studies of the inactivation of mammalian ornithine decarboxylase by α-methylornithine (Abdel-Monem et al., 1974; Mamont et al., 1976; McCann et al., 1977). Competitive inhibition of the enzyme by α-methylornithine has been reported (Abdel-Monem et al., 1974), and this inhibition might seem not to be consistent with the proposed mechanism. However, the reaction of ornithine decarboxylase with α-methylornithine is sufficiently slow that inhibition would appear to be competitive if inhibitor and substrate were added to the enzyme simultaneously and if assay times were kept relatively short. Dopa decarboxylase catalyzes a similar decarboxylation-dependent transamination (Borri Voltattorni et al., 1971; O'Leary and Baughn, 1977) and similar kinetic phenomena are observed; inhibition of dopa decarboxylase by α-methyldopa appears to be competitive if substrate and inhibitor are added to the enzyme simultaneously (Lovenberg et al., 1963), but if the enzyme is preincubated with α-methyldopa, the inhibition is not competitive.

It is noteworthy that Abdel-Monem et al. (1975) failed to observe decarboxylation of α-[1-^{14}C]methylornithine by the ornithine decarboxylase from rat prostate. If the relative rates of the various reactions catalyzed by that enzyme are the same as those for the enzyme for *Lactobacillus*, no measurable formation of $^{14}CO_2$ would be expected under those conditions.

Thus, the inhibition of ornithine decarboxylase in vivo is likely to be significantly more complex than has been appreciated. Clearly, the inhibition is reversible, and the extent of inhibition will depend not only on the concentration of the inhibitor but also on the time of contact between enzyme and inhibitor. Because of the reversibility of the inhibition, the observed inhibition will depend on the concentration of pyridoxal phosphate in the cell, and thus ultimately on the nutritional state of the animal with respect to vitamin B_6.

Ornithine decarboxylase is rapidly turned over during cell proliferation, and it is thought that this rapid turnover may be connected with regulation of cell growth (Russell, 1970). Degradation of ornithine decarboxylase is presumed to proceed by dissociation of the coenzyme, followed by proteolysis of the apoenzyme (Litwack and Rosenfield, 1973). Although it has been shown that coenzyme dissociation is significantly more facile than with most pyridoxal phosphate dependent enzymes (Clark and Fuller, 1976), it is not clear whether the equilibrium constant for coenzyme binding is appreciably altered compared to other enzymes. If it is not, then the extent of dissociation will

[2] H. Yamada and M. H. O'Leary, unpublished results.

[3] It should be noted that aspartate β-decarboxylase catalyzes a rather similar decarboxylation-dependent transamination, in that case, leading to the formation of pyridoxamine phosphate and pyruvate. However, the origin of that reaction is not precisely the same as the cases discussed here because of the fact that transamination is a normal part of the mechanism of action of aspartate β-decarboxylase, whereas decarboxylation is not a required part of the mechanism of action of the α-decarboxylases.

be limited by the equilibrium constant for coenzyme binding, and very little apoenzyme formation is expected.

An alternative mechanism for turnover of ornithine decarboxylase is available based on the studies reported here. Apoenzyme may be formed as a result of the decarboxylation-dependent transamination of the enzyme during the decarboxylation of ornithine. Even though the efficiency of this process is low, the decarboxylation of ornithine is sufficiently rapid that inactivation occurs within a few minutes and, as with other pyridoxal phosphate dependent decarboxylases, dissociation of pyridoxamine phosphate from the enzyme is much more facile than dissociation of pyridoxal phosphate. Thus, decarboxylation-dependent transamination may provide a key step in the control of decarboxylase levels in cells.

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