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Inhibition of Aromatic L-Amino Acid Decarboxylase by Coenzyme–Amino Acid Adducts†

Edwin A. Rudd‡ and John W. Thanassi*

ABSTRACT: The coenzyme–amino acid adducts *N*-(5′-phosphopyridoxyl)-L-3,4-dihydroxyphenylalanine and *N*-(5′-phosphopyridoxyl)-L-*m*-NH_2-tyrosine inhibit hog kidney aromatic L-amino acid decarboxylase (Dopa decarboxylase). Kinetic studies on the nature of the inhibition caused by these adducts appeared to distinguish two distinct decarboxylase activities in purified enzyme preparations. It was determined that the appearance of two activities in purified enzyme preparations is an artifact of the system resulting from the following properties of Dopa decarboxylase: (1) the enzyme has a high affinity for pyridoxal 5′-phosphate, (2) Dopa decarboxylase can follow a decarboxylation-dependent transamination pathway forming apoenzyme as one of the products of this pathway, and (3) the phosphorylated adducts investigated readily bind to apo-Dopa decarboxylase but do not readily displace pyridoxal phosphate from holoenzyme. Incubation of holo-Dopa decarboxylase with *N*-(5′-deoxypyridoxyl)-DL-Dopa, in the absence of added coenzyme, causes a rapid inactivation of enzyme ($t_{1/2}$ = 5 min) which is associated with a decrease in the coenzyme content of the enzyme. However, incubation of holoenzyme with the phosphorylated adduct, PPxy-L-*m*-NH_2-Tyr, causes a much slower inactivation of enzyme ($t_{1/2}$ = 30 min), while a short incubation (10 min or less) with either of the phosphorylated adducts increases the activity of holoenzyme. Calculations indicate that the extent of reactivation of apoenzyme, formed via the decarboxylation-dependent transamination pathway, by excess exogenous coenzyme cannot be accounted for solely by reconstitution of holoenzyme. It is proposed that Dopa decarboxylase has either a second active site which has a low affinity for pyridoxal phosphate or a site(s) which when occupied by pyridoxal phosphate leads to an increase in the activity of the enzyme.

Coenzyme–amino acid adducts are inhibitors of many pyridoxal phosphate (PLP)[1] dependent enzymes (Ayling & Snell, 1968; Bayon et al., 1977; Borri-Voltattorni et al., 1975; Heller et al., 1975; Orlacchio et al., 1980; Raso & Stollar, 1975; Tunnicliff et al., 1977). Since these adducts combine both substrate and coenzyme into a single molecule, they might be expected to be potent enzyme inhibitors (Byers, 1978). In addition, these adducts can be useful in exploring mechanistic aspects of PLP-dependent enzymes. In a previous publication (Rudd et al., 1979), we reported that *N*-(5′-phosphopyridoxyl)-L-Dopa (PPxy-L-Dopa) and *N*-(5′-deoxypyridoxyl)-DL-Dopa were inhibitors of mouse liver Dopa decarboxylase (aromatic L-amino acid decarboxylase, EC 4.1.1.28). We report in this paper a detailed characterization of the interactions between coenzyme–amino acid adducts and purified hog kidney Dopa decarboxylase.

Dopa decarboxylase has been purified from porcine kidney (Christenson et al., 1970; Lancaster & Sourkes, 1972; Borri-Voltattorni et al., 1979). It has a very high affinity for its coenzyme and can exist as holoenzyme in the absence of ex-

† From the Department of Biochemistry, University of Vermont College of Medicine, Burlington, Vermont 05405. *Received July 28, 1981.* This research was supported by Grant AM 25490 from the National Institutes of Health, U.S. Public Health Service.
‡ Present address: The Hormel Institute, Austin, MN 55912.

[1] Abbreviations used: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; Dopa, 3,4-dihydroxyphenylalanine; Tyr, tyrosine; PPxy, 5′-phosphopyridoxyl; BSA, bovine serum albumin; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

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ogenous PLP (Christenson et al., 1970; Borri-Voltattorni et al., 1979). The molecular weight of the native enzyme is reported to be $M_r \sim 110000$, most likely being a dimer of two equal subunits, and there is 1 mol of PLP per mol of enzyme (Christenson et al., 1970; Borri-Voltattorni et al., 1979; Maycock et al., 1980). However, preparations of purified porcine kidney Dopa decarboxylase are not the same from every laboratory, in particular, with respect to specific activity and subunit size and composition. The question of possible multiple enzyme forms is still apparently unsettled (Maycock et al., 1980).

Holo-Dopa decarboxylase can be stimulated 2–5-fold by the addition of exogenous coenzyme, and Christenson et al. (1970) proposed that other coenzyme binding sites were responsible for the activation of the enzyme by exogenous PLP. In 1977, O'Leary and Baughn showed that Dopa decarboxylase also catalyzes a decarboxylation-dependent transamination. This reaction simultaneously converts Dopa into carbon dioxide and 3,4-dihydroxyphenylacetaldehyde and enzyme-bound pyridoxal phosphate into pyridoxamine phosphate, PMP. In the absence of exogenous PLP, holoenzyme is rapidly inactivated as the active coenzyme is converted to coenzymatically inactive PMP via the decarboxylation-dependent transamination pathway. In the presence of exogenous coenzyme, holoenzyme is reconstituted and much of the apparent inactivation can be reversed. The decarboxylation-dependent transamination pathway could conceivably account for the stimulation of Dopa decarboxylase caused by exogenous PLP as reported by Christenson et al. (1970).

The adducts employed in this study appear to indicate that there are two kinetically distinct Dopa decarboxylase activities which differ in their requirements for PLP and in their susceptibility to inhibition by the adducts. However, it was determined that these two activities are in fact associated with a single enzyme.

Studies concerned with the effects of exogenous PLP on enzyme activity suggest that in addition to a high-affinity coenzyme-binding active site, Dopa decarboxylase has at least one additional low-affinity PLP binding site which might be either a second active site or an allosteric site which causes an increase in enzyme activity when occupied by coenzyme. The decarboxylation-dependent transamination pathway which causes loss of active coenzyme and the proposed second site are suggested to be responsible for the stimulation of enzyme activity by exogenous PLP.

Materials and Methods

Materials. L-Dopa, L-5-hydroxytryptophan, L-tyrosine, L-*o*-tyrosine, L-*m*-nitrotyrosine, acrylamide, *N*,*N*′-methylenebis(acrylamide), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. Pyridoxal 5′-phosphate was obtained from Aldrich Chemical Co. DEAE-Bio-Gel A, Bio-Gel A-15 M, and Bio-Gel P-2 were supplied by Bio-Rad Laboratories. PCS liquid scintillation fluid was purchased from Amersham/Searle Corp. Econofluor, Protosol, and [1-^{14}C]-DL-Dopa were obtained from New England Nuclear. All other reagents and chemicals used in these studies were reagent grade or the highest quality available. PPxy-L-Dopa and *N*-(5′-deoxypyridoxyl)-DL-Dopa were prepared as described previously (Rudd et al., 1979). *N*-5′-(Phosphopyridoxyl)-*m*-NH_2-Tyr was synthesized according to Raso & Stollar (1973). *n*-Butylagarose was prepared from cyanogen bromide activated Bio-Gel A-15 M as described by Shaltiel (1974).

Dopa Decarboxylase Assay. Dopa decarboxylase activity in hog kidney preparations was measured by a procedure adapted from one used to assay tyrosine decarboxylase (Meisler & Thanassi, 1980). The assays were performed in 15-mL Corex centrifuge tubes capped with rubber serum stoppers; a plastic well was suspended from the stopper. The serum stoppers and plastic wells were obtained from Kontes Glass Co.

For a typical assay, 50 μL of enzyme solution was added to 0.70 mL of potassium phosphate buffer (pH 6.8) containing 10 mM 2-mercaptoethanol, inhibitor, and/or PLP at the desired concentrations. After a 5-min preincubation at 37 °C, the reaction was initiated by the addition of 0.25 mL of 4 mM L-Dopa containing 0.4 μCi of [1-^{14}C]-DL-Dopa. The tube was then quickly capped with a serum stopper, and incubation at 37 °C was continued. After the desired incubation period, a syringe was used to inject 0.4 mL of 2 N H_2SO_4 through the septum stopper into the tube, stopping the reaction and causing the release of $^{14}CO_2$ which was trapped in the Protosol-containing plastic well over a 90-min period (37 °C). The well and its contents were then transferred to a scintillation vial and 5 mL of Econofluor containing 10% methanol was added to the vial. The radioactivity was measured with a Beckman LS-150 liquid scintillation counter.

The spectrophotometric assay of O'Leary & Baughn (1977) was used to identify enzymatically active fractions during the purification of hog kidney Dopa decarboxylase.

Enzyme Purification. Dopa decarboxylase was purified from kidneys obtained from freshly killed hogs by a method adapted from the procedures used by Borri-Voltattorni et al. (1979) and Srinivasan & Awapara (1978). The extraction and ammonium sulfate fractionation procedures of Borri-Voltattorni et al. (1979) were performed with the following changes: an ultracentrifugation step (1 h at 100000*g*) was added at the end of the extraction procedure, and the cellulose centrifugation step at the end of the ammonium sulfate fractionation was replaced with an ultracentrifuge step (1 h at 100000*g*). The supernate was applied to a 5 × 14 cm column of DEAE-Bio-Gel and washed in with 600 mL of buffer A (10 mM potassium phosphate, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, and 10 μM PLP, pH 7.2). The column was eluted with a linear gradient of potassium phosphate (10–100 mM) in buffer A (total volume 2 L). The active fractions were pooled, and the protein was precipitated by the addition of ammonium sulfate to 55% saturation. The precipitate was collected by centrifugation (20 min at 15000*g*) and dissolved in 30 mL of 10 mM potassium phosphate buffer (pH 7.1) containing 0.1 mM EDTA, 1 mM 2-mercaptoethanol, and 10 μM PLP and 20% saturated with ammonium sulfate. This solution was applied to a 2 × 12 cm column of *n*-butylagarose and eluted with the 20% ammonium sulfate buffer solution. Under these conditions, the enzyme was not absorbed to the column. The active fractions were pooled and made 25% saturated with ammonium sulfate. This solution was applied to a 2.5 × 24 cm column of *n*-butylagarose, and the column was eluted with a decreasing linear gradient of ammonium sulfate (25–0% saturation in buffer A, total volume 1.0 L). The active fractions were pooled, and the enzyme was precipitated by the addition of ammonium sulfate to 55% saturation. The precipitate was dissolved in 2 mL of 100 mM potassium phosphate buffer (pH 6.8) containing 0.1 mM EDTA and 1 mM 2-mercaptoethanol and dialyzed against several changes of this buffer. The dialyzed enzyme solution was divided into aliquots and stored at −20 °C. One unit of enzyme activity is defined as that amount of enzyme which produces 1 nmol of CO_2 per min (37 °C) using 100 mM potassium phosphate buffer (pH 6.8) containing 1 mM Dopa, 10 mM 2-mercaptoethanol, and 10 μM PLP. Enzyme ob-

tained in this fashion had a specific activity of ~4600 units/mg of protein and was stable for at least 3 months when stored at −20 °C.

The molecular weight of the purified enzyme as determined by gel filtration on a calibrated column of Sephacryl S-300 superfine was M_r 91 000–95 000. Electrophoresis on 7–25% gradient polyacrylamide gels in the presence of sodium dodecyl sulfate was performed as described by Poduslo & Rodbard (1980). There was one major band (75% of the total protein) having a mobility corresponding to a protein whose molecular weight was 47 000–49 000. The PLP content of purified enzyme was determined to be 1.27 μg/mg of protein, equal to 0.49 mol of PLP/mol of enzyme, assuming a molecular weight of 95 000 for holoenzyme.

Dilution of purified enzyme caused the specific activity to decrease. This decrease could be prevented by the addition of methylated BSA (50 μg/mL) to all buffers used to dilute the enzyme. The BSA was exhaustively methylated by the procedure of Jentoft & Dearborn (1951). Exhaustive methylation eliminated the possibility of PLP binding to BSA (Dempsey & Christensen, 1962).

Protein was determined by the procedure of Lowry et al. (1951) or by the fluorescamine procedure of Bohlen et al. (1973). PLP was determined by a procedure routinely employed in this laboratory (Meisler & Thanassi, 1980).

Results

Purification of Dopa Decarboxylase. The purification of hog liver Dopa decarboxylase yielded an enzyme preparation having a specific activity of 4600 units/mg of protein when assayed at 37 °C. This compares with previously reported specific activities of purified enzyme of ~2000 (Lancaster & Sourkes, 1972), 7500–9500 (Christenson et al., 1970), and 4700–11 400 units/mg of protein (Maycock et al., 1980). Specific activities of 1400 (O'Leary & Baughn, 1977) and 2000–3000 units/mg of protein (Borri-Voltattorni et al., 1979) have been reported for purified preparations of porcine kidney Dopa decarboxylase when the activities were measured at 25 °C, with the difference in activity between 37 and 25 °C having been determined to be a factor of 2.8 (O'Leary & Baughn, 1977).

Molecular sieve chromatography and polyacrylamide gel electrophoresis under denaturing conditions indicate that the holoenzyme has a molecular weight of approximately M_r 95 000 and is composed of two equal subunits. Although the molecular and subunit weights are somewhat smaller than the values determined by others (Christenson et al., 1970; Borri-Voltattorni et al., 1979; Maycock et al., 1980), the agreement is reasonably close and most nearly approximates the results of Lancaster & Sourkes (1972). The PLP content was determined to be 0.49 mol of coenzyme per mol of enzyme compared to literature values of 0.2–0.4 (Lancaster & Sourkes, 1972), 0.7–1.1 (Christenson et al., 1970), 0.86–1.02 (Borri-Voltattorni et al., 1979), and ~1 mol of coenzyme per mol of enzyme (Maycock et al., 1980). Taken together, the specific activity, PLP content, and densitometric analysis of protein patterns obtained after gradient polyacrylamide gel electrophoresis under denaturing conditions indicate that the enzyme preparation obtained in the present studies was 50–75% pure.

Interaction of Coenzyme–Amino Acid Adducts with Dopa Decarboxylase. The adduct, *N*-(5′-phosphopyridoxyl)-*m*-NH_2-Tyr (PPxy-*m*-NH_2-Tyr), appeared to distinguish between two Dopa decarboxylase activities since, as shown in Figure 1, a plot of velocity^{-1} vs. the concentration of adduct at various PLP concentrations resulted in curves which appear to asymptotically approach a line parallel to the abscissa. One

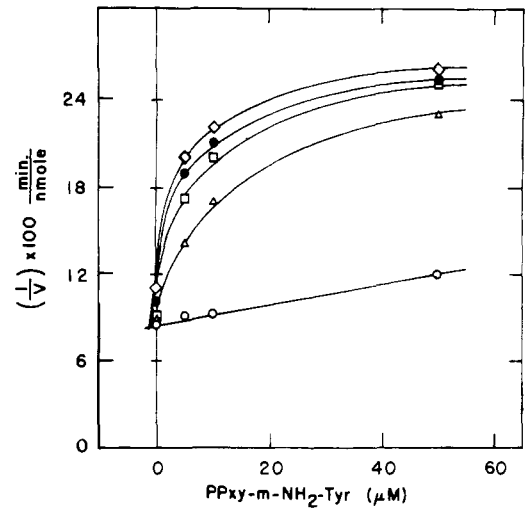

FIGURE 1: Dixon plot of enzyme activity as a function of PPxy-*m*-NH_2-Tyr concentration in the presence of various coenzyme concentrations. Purified hog kidney Dopa decarboxylase (11 units) was assayed in 1.0 mL of 50 mM potassium phosphate buffer (pH 6.8) containing 1.0 mM Dopa and the indicated concentrations of PPxy-*m*-NH_2-Tyr (0–50 μM). The assay was performed as described under Materials and Methods; an assay time of 6 min was used. The following coenzyme concentrations were used: 0.10 μM (diamonds); 0.13 μM (filled circles); 0.2 μM (squares); 0.4 μM (triangles); 10.0 μM (open circles).

activity did not appear to require exogenous PLP and was not inhibited by PPxy-*m*-NH_2-Tyr (linear portions of plots in Figure 1). The other activity required PLP and was inhibited by PPxy-*m*-NH_2-Tyr (curved portion of plots of Figure 1). Similar results were obtained when the coenzyme–amino acid adduct, *N*-(5′-phosphopyridoxyl)-L-Dopa (PPxy-Dopa), was used in place of PPxy-*m*-NH_2-Tyr (data not shown). Because of the rapid and spontaneous Pictet–Spengler reaction between PLP and substrate, leading to the formation of a tetrahydroisoquinoline derivative (Schott & Clark, 1952), the coenzyme concentration decreased markedly during the assay period. When the rate constants determined by O'Leary & Baughn (1977) are used, it can be calculated that as much as 60% of the PLP is consumed during the assay period. Thus, the coenzyme concentration was constant only in the relative sense that the same amount of coenzyme was consumed at corresponding points along the lines. Because of the large change in coenzyme concentration, the Dixon plot shown in Figure 1 cannot be used to determine a dissociation constant for the adduct.

Since the large decrease in the concentration of PLP which occurred during the 6-min assay period was objectionable, the assay time was shortened to a still technically feasible 2 min. The Pictet–Spengler reaction under these conditions was calculated to consume less than 10% of the coenzyme during the assay. The effect of PPxy-*m*-NH_2-Tyr on enzyme activity when assayed for 2 min in the presence of varying concentrations of PLP is shown in Figure 2 in the form of a double-reciprocal plot. Within the limits of experimental error, the two lines intersect at a common point on the ordinate axis, indicating that there is competitive inhibition between PPxy-*m*-NH_2-Tyr and PLP, at least at high concentrations of coenzyme.

In the absence of exogenous PLP, holoenzyme is rapidly inactivated, as expected, via the decarboxylation-dependent transamination pathway (Figure 3). The production of CO_2 by enzyme ceases after about 30 min, indicating loss of enzymatic activity under conditions where more than 95% of substrate remains. The presence of either PPxy-Dopa or

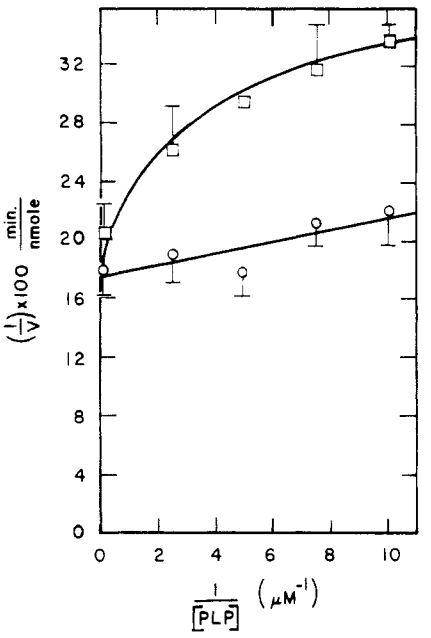

FIGURE 2: Double-reciprocal plot of enzyme activity as a function of coenzyme concentration in the presence of PPxy-*m*-NH_2-Tyr. Purified hog kidney enzyme (6 units) was assayed in 1.0 mL of 50 mM potassium phosphate buffer (pH 6.8) containing 0.25 mM Dopa and the indicated concentration of coenzyme (0.1–10.0 μM). The following inhibitor concentrations were used: none, (circles); 50 μM (squares). The assay time was 2 min.

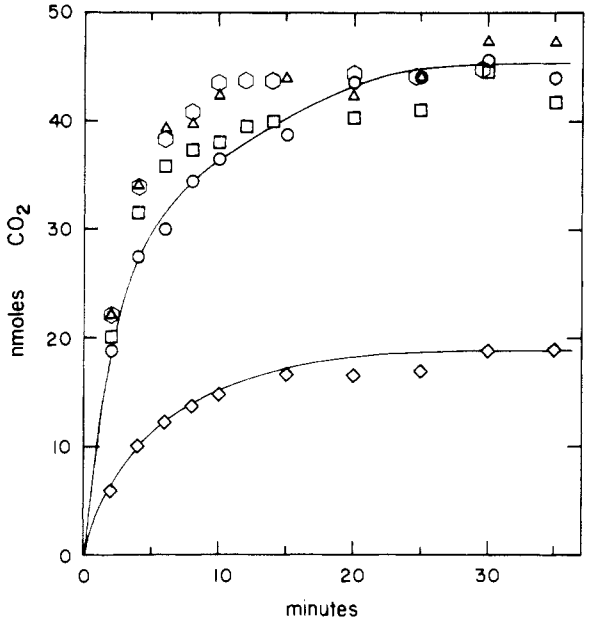

FIGURE 3: Effect of coenzyme and/or adducts on inactivation of enzyme via the decarboxylation-dependent transamination pathway. Purified hog kidney enzyme (20 units) was assayed in 1.0 mL of 200 mM potassium phosphate buffer (pH 6.8) containing 1.0 mM Dopa and the following additions for the indicated time periods: 50 μM PPxy-Dopa (squares); 50 μM PPxy-Dopa + 0.1 μM PLP (triangles); 50 μM *N*-(5′-deoxypyridoxyl)-Dopa (diamonds); 50 μM PPxy-*m*-NH_2-Tyr (hexagons); control, no additions (circles).

PPxy-*m*-NH_2-Tyr in the assay solution caused no signficant change in the plateau value for CO_2 evolution. When both PLP (0.1 μM) and PPxy-Dopa (50 μM) were present, the plateau value was the same, indicating that under these conditions, added coenzyme cannot reconstitute holoenzyme because if it could the plateau value would be higher than the control value. Significantly, the nonphosphorylated adduct, *N*-(5′-deoxypyridoxyl)-Dopa, caused a decrease in the plateau value by a factor of 2 (Figure 3).

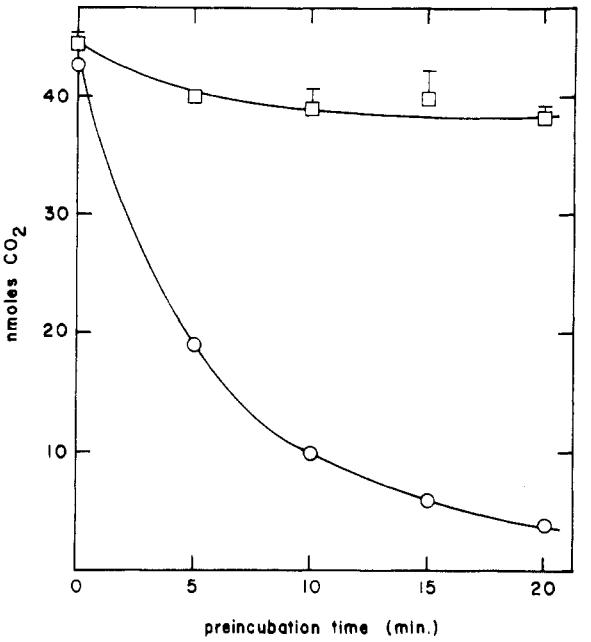

FIGURE 4: Time-dependent inactivation of Dopa decarboxylase caused by preincubating it with *N*-(5′-deoxypyridoxyl)-Dopa. Purified hog kidney enzyme (18 units) was preincubated at 37 °C for the indicated time periods in 0.75 mL of 200 mM potassium phosphate buffer (pH 6.8) containing no inhibitor (squares) or 7.14×10^{-5} M inhibitor (circles). Then 0.25 mL of the above buffer containing 4.0 mM Dopa was added, and the enzyme was assayed for 10 min. Each point was determined in triplicate, and the bars represent the standard error of the experimental values.

In the experiments described immediately above, enzyme and adducts were incubated at 37 °C for 5 min before the substrate was added. Experiments were then conducted to determine if the length of this incubation affected the activity of holoenzyme. Incubation with *N*-(5′-deoxypyridoxyl)-Dopa caused a rapid time-dependent inactivation of holoenzyme (Figure 4) which could be prevented by the presence of 10 μM PLP in the incubation medium. Incubation with PPxy-*m*-NH_2-Tyr caused a much slower inactivation of holoenzyme (half the activity was lost during a 30-min incubation). Since these adducts were interacting with holoenzyme, their mode of action could be to decrease the coenzyme content of the enzyme. The following experiment was conducted to determine if incubation with *N*-(5′-deoxypyridoxyl)-Dopa caused a change in the coenzyme content of holoenzyme. Enzyme was incubated with and without 0.1 mM *N*-(5′-deoxypyridoxyl)-Dopa. The enzyme was then separated from free coenzyme by gel filtration on a 1.6 × 4.5 cm column of Bio-Gel P-2. Determination of the PLP content of the enzyme-containing fraction revealed that more than 50% of the coenzyme had been lost when compared to the control which had not been exposed to the adduct. Therefore, incubation of holoenzyme with the adduct *N*-(5′-deoxypyridoxyl)-Dopa caused a marked decrease in the coenzyme content.

In contrast to *N*-(5′-deoxypyridoxyl)-Dopa, short incubations (less than 10 min) with PPxy-Dopa and PPxy-*m*-NH_2-Tyr actually stimulated the Dopa decarboxylase activity above the control. This case can be seen in Figure 3 where, during the earlier time points, the values in the presence of PPxy-Dopa and PPxy-*m*-NH_2-Tyr are higher than the control values.

The decarboxylation-dependent transamination pathway provides a convenient way to generate apoenzyme. Incubation of enzyme with substrate in the absence of PLP for 30 min completely inactivates the enzyme (Figure 3). The addition of PLP to inactivated enzyme restores activity to about 30%

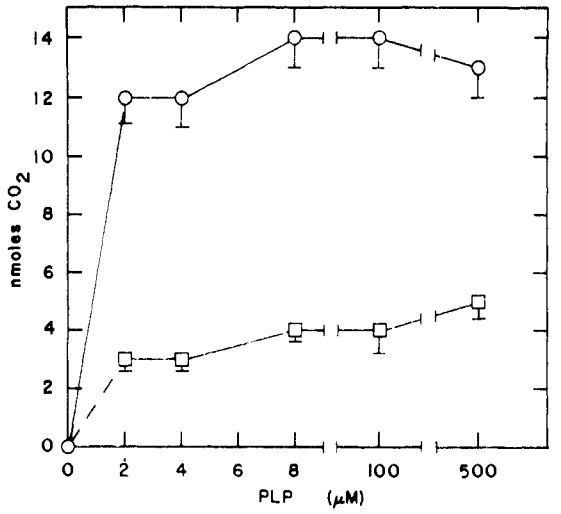

FIGURE 5: Effect of PPxy-*m*-NH_2-Tyr on the reactivation of apoenzyme by coenzyme. Hog kidney enzyme (20 units) was incubated with unlabeled 1 mM Dopa for 30 min at 37 °C. The enzyme was completely converted to apoenzyme by this step; 3% of the Dopa had been consumed. Then [1-^{14}C]Dopa with 50 μM PPxy-*m*-NH_2-Tyr (squares) or without (circles) was added, and the solutions were incubated for 5 min. Then the indicated concentrations of coenzyme were added, and the enzyme was assayed for 5 min. Final assay volume was 1.0 mL. The assay buffer was 200 mM potassium phosphate (pH 6.8); all concentrations given are the final assay concentrations.

of the original value.[2] Apoenzyme generated by the above method was incubated with the inhibitor PPxy-*m*-NH_2-Tyr and then assayed for activity in the presence of various coenzyme concentrations (Figure 5). The inhibition caused by this adduct remained constant over a wide range of PLP concentrations, indicating that prior exposure of apoenzyme to PPxy-*m*-NH_2-Tyr renders PLP ineffective in the reactivation of apoenzyme to holoenzyme.

Since the decarboxylation-dependent transamination is an intrinsic property of the enzyme, the inactivation of enzyme by this pathway should be a first-order process provided the substrate concentration remains constant during the inactivation. Under these conditions, the velocity of the enzyme-catalyzed reaction can be expressed as

$$v = k_e E_a \quad (1)$$

In eq 1, k_e is the apparent rate constant for the enzyme-catalyzed reaction and E_a is the concentration of active enzyme. For Dopa decarboxylase, the amount of active enzyme, in the presence of excess substrate, is decreasing in a first-order fashion owing to the decarboxylation-dependent transamination reaction which causes the loss of coenzymatically active PLP. Thus, at any given time, $E_a = (E_{initial} - E_{inactive})$, and the decrease in E_a is expressed by the first-order equation

$$-dE_a/dt = k_i E_a \quad (2)$$

where k_i is the apparent first-order rate constant for the inactivation process.

This system is kinetically identical with one studied by Momsen & Brockman (1976). Using their mathematics, one can derive the following equation which can be used to calculate the initial velocity of the enzyme-catalyzed reaction:

$$P_\infty k_i = k_e E_{initial} \quad (3)$$

where P_∞ is the amount of product, CO_2, formed at $t = \infty$, i.e., the plateau value in Figure 3. The values for k_i can be calculated by plotting the data shown in Figure 3 in a semilog form. If the inactivation is first order, then a plot of ln ($P_\infty - P$) vs. time will yield a straight line with a slope equal to k_i. Such plots in the absence and presence of 50 μM PPxy-*m*-NH_2-Tyr were linear, with the slopes of the lines calculated to be 0.13 (control) and 0.31 min^{-1} (plus 50 μM adduct) at 37 °C. The $t_{1/2}$ for inactivation of the control is therefore 5.8 min which compares favorably with the value of 6.6 min in the presence of 1 mM Dopa reported by O'Leary & Baughn (1975). Minelli et al. (1979) reported that the rate constant for the inactivation of Dopa decarboxylase in the presence of 28 mM Dopa was 5.4×10^{-3} s^{-1} at 37 °C, corresponding to a $t_{1/2}$ of 2.1 min. Multiplying k_i by the plateau value of 45 nmol (from Figure 3) provides $k_e E_{initial}$, the calculated value for the initial, maximal velocity if there were no decarboxylation-dependent transamination pathway, under conditions where substrate is constant and there is no other PLP except that which is bound to the holoenzyme. For the control, $k_e E_{initial}$ is calculated to be 5.9 nmol/min; in the presence of 50 μM PPxy-*m*-NH_2-Tyr, $k_e E_{initial}$ equals 14.0 nmol/min. Thus the coenzyme–substrate adduct increases both the rate of inactivation of holoenzyme and the activity of holoenzyme (Figure 3).

If one assumes that PLP binds only to the active site of Dopa decarboxylase and exerts no other effects, then one would expect that in the presence of excess, PLP, the maximum obtainable velocity would be 5.9 nmol/min. However, when the data in Figure 1 are normalized to those in Figure 3 to take into account the differences in enzyme concentration, then an observed, experimental velocity of 22.1 nmol/min is obtained when PLP is present in excess, a velocity almost 4 times greater than the calculated maximal velocity. These findings indicate that holoenzyme activity can be increased by both coenzyme–substrate adducts and excess PLP and strongly suggest that a second site(s) may be involved in positively regulating the activity of holo-Dopa decarboxylase.

Srinivasan & Awapara (1978) reported that the decarboxylation of 5-hydroxytryptophan by Dopa decarboxylase is not stimulated by the addition of PLP. This finding suggests that the decarboxylation-dependent transamination pathway may not be followed when 5-hydroxytryptophan is the substrate. A recent report by Barboni et al. (1981) also indicates that 5-hydroxytryptophan does not undergo a conventional decarboxylation-dependent transamination in the presence of Dopa decarboxylase. The following experiment was performed to investigate this possibility. Holoenzyme was preincubated with the following nonradioactive substrates for various time periods and then assayed for activity toward Dopa: 5-hydroxytryptophan, *o*-tyrosine, and Dopa (as a positive control). Relative to an untreated control, a 40-min preincubation with 5-hydroxytryptophan caused only a slight (9%) decrease in enzyme activity toward Dopa. Preincubation with *o*-tyrosine caused a significant time-dependent decrease in enzyme activity (35% after 40 min), and as expected, incubation with Dopa caused a rapid (60% after 10 min) decrease in activity. These results indicate that 5-hydroxytryptophan does not follow the decarboxylation-dependent transamination pathway to any significant extent under the experimental conditions.

Discussion

Curved Dixon plots (Figure 1) are not unusual for multisubstrate enzymes (Segal, 1975). In one sense, Dopa decarboxylase is not a multisubstrate enzyme because PLP is a cofactor and not a substrate in the decarboxylation reaction leading to CO_2 and dopamine. However, when the decarb-

[2] O'Leary & Baughn (1975), in similar studies, reported that there was a 30–50% reactivation of apoenzyme, generated in this fashion, by the addition of PLP, with the loss in activity presumably resulting from instability of apoenzyme.

oxylation-dependent transamination pathway is considered, PLP is indeed a substrate for the enzyme. The Dixon plot in Figure 1 not only is curved but also appears to be reaching a plateau value. Data obtained in the presence of either PPxy-Dopa or PPxy-*m*-NH_2-Tyr seemed to indicate that there were two distinct Dopa decarboxylase activities which differed in their requirements for coenzyme and in their susceptibility to inhibition by these adducts.

These two apparent activities result from the following properties of Dopa decarboxylase: (1) Dopa decarboxylase has a very high affinity for PLP and has significant activity in the absence of added coenzyme; (2) holoenzyme is not inhibited by these adducts; (3) the adducts cause inhibition by binding to apoenzyme; (4) the decarboxylation-dependent transamination pathway is continually generating apoenzyme during the assay. The activity that appeared not to require exogenous PLP and was not inhibited by the adducts was due to the intrinsic holoenzyme present even in the absence of free PLP. The activity that required exogenous PLP and was inhibited by the adducts resulted from the reconstitution of holoenzyme from PLP and apoenzyme, with the latter generated via the decarboxylation-dependent transamination pathway. In addition, as discussed below, excess exogenous PLP positively affects holoenzyme activity.

The adducts and coenzyme compete for binding to apoenzyme since the inhibition is kinetically competitive with respect to coenzyme (Figure 2). Borri-Voltattorni et al. (1972) reported that similar coenzyme–amino acid adducts inhibited the formation of holo-Dopa decarboxylase from apoenzyme and PLP. Bayon et al. (1978) observed similar curved Lineweaver–Burk plots while studying the inhibition of mammalian glutamate decarboxylase by coenzyme–amino acid adducts. Mammalian glutamate decarboxylase also has a very high affinity for coenzyme and appears to have two activities that differ in their requirement for coenzyme (Bayon et al., 1977; Tapia & Sandoval, 1971). Other amino acid decarboxylases including ornithine decarboxylase (O'Leary & Herreid, 1978) and tyrosine decarboxylase (Vederas et al., 1979) have been reported to possess decarboxylation-dependent transamination pathways. The decarboxylation-dependent transamination pathway may turn out to be a general property of all PLP-dependent amino acid decarboxylases, and this pathway should be considered in all kinetic mechanisms for these enzymes.

Since the coenzyme–amino acid adducts rapidly bind to apoenzyme, they are dependent on the decarboxylation-dependent transamination pathway for the expression of their inhibitory properties. This means that the maximum inhibition caused by these adducts will be affected by the length of the assay. During a short assay, a small fraction of enzyme molecules will follow the decarboxylation-dependent transamination pathway, and therefore the adduct will cause little inhibition. However, if a long assay period were used, a large fraction of enzyme molecules would follow the decarboxylation-dependent transamination pathway, and the adduct would cause significant inhibition.

In contrast to the phosphorylated adducts discussed thus far, incubation of holoenzyme with *N*-(5′-deoxypyridoxyl)-Dopa caused a rapid inactivation of the enzyme ($t_{1/2}$ = 5 min). This rapid inactivation could be prevented by the addition of coenzyme. Furthermore, incubation with *N*-(5′-deoxypyridoxyl)-Dopa caused a decrease in the coenzyme content of holoenzyme (see Results). These findings suggest that this particular adduct was acting at the active site of the holoenzyme. Binding of substrate to *S. faecalis* tyrosine decarboxylase which also decarboxylates L-Dopa has been shown to cause the opposite face of the coenzyme to become exposed to solvent, and it has been proposed that there are two conformation-dependent binding modes for the coenzyme, one for the holoenzyme form and one for the substrate–holoenzyme form (Vederas et al., 1979). If similar conformation-dependent coenzyme-binding modes exist for hog kidney Dopa decarboxylase, then *N*-(5′-deoxypyridoxyl)-Dopa may bind to the substrate–holoenzyme conformer and cause a displacement of the coenzyme. An adduct possessing a phosphate group at the 5′ position (PPxy-*m*-NH_2-Tyr) caused a much slower inactivation of holoenzyme ($t_{1/2}$ = 30 min). Thus, the phosphate group is an important factor in determining the nature of the interaction of these adducts with holoenzyme.

When PPxy-Dopa or PPxy-*m*-NH_2-Tyr was incubated with holoenzyme for short periods (10 min or less), these adducts acted as activators in that the enzyme activity was greater than the control. We propose that these adducts are binding to a site other than the active site and that binding to this site causes an increase in catalysis at the active site. Minelli et al. (1979) reported that the coenzyme in holo-Dopa decarboxylase binds in two distinct modes and that one mode forms a complex with the substrate, Dopa, 5 times faster than the other mode. It may be that binding of the adduct to a proposed second site causes all of the coenzyme to become bound in the fast reactive mode. Thus, both the rate of product formation and the rate of inactivation would be increased when the second binding site is occupied.

Under the experimental conditions used in the present study, the rate constant for the inactivation of holo-Dopa decarboxylase in the absence of exogenous PLP was determined to be 0.13 min^{-1} (Figure 3). This rate constant was used to calculate the initial velocity for holoenzyme before there had been any inactivation via the decarboxylation-dependent transamination pathway (eq 3). The value calculated in this manner, 5.9 nmol/min, is only 27% of an experimentally determined velocity, 22.1 nmol/min, for the same amount of enzyme in the presence of exogenous PLP (see Results). It is evident, therefore, that the stimulation of holo-Dopa decarboxylase by exogenous PLP is due not only to the stoichiometric reconstitution of apoenzyme but also by the influence of PLP at another site(s). The fact that holoenzyme is stimulated by added PLP over the range 0.1–10.0 μM (Figure 1) suggests that the proposed second site(s) has a much lower affinity for the coenzyme than the active site.

There are at least two explanations for these observations. One involves the existence of a second active site which has a much lower affinity for PLP than the first active site. Along these lines, pig brain γ-aminobutyrate transaminase, a PLP-dependent enzyme, has been reported to have two active sites that differ by a factor of 3×10^3 in their affinities for coenzyme (Churchich & Moses, 1981). Interestingly, like Dopa-decarboxylase, pig brain γ-aminobutyrate transaminase is a dimeric enzyme with a subunit molecular weight of 50 000; the native enzyme contains 1 mol of tightly bound PLP per dimer. It may be that Dopa decarboxylase resembles γ-aminobutyrate transaminase in having two classes of PLP-binding active sites.

A second explanation is that there is a PLP binding site(s) other than the active site which acts in a regulatory fashion, most likely leading to conformational changes in the enzyme which cause an increase in activity at the catalytic site. Proposed conformational changes of PLP-dependent enzymes (Braunstein et al., 1968) involving reorientation of the coenzyme at the active site are now well established (Ford et

al., 1980). Thus, the effects of both coenzyme–amino acid adducts and exogenous PLP on the activity of holo-Dopa decarboxylase can be interpreted to indicate the existence of a second site(s) which can alter enzyme activity.

When apoenzyme, generated in situ via the decarboxylation-dependent transamination pathway, was first incubated with PPxy-*m*-NH_2-Tyr, increasing PLP concentrations caused little change in the inhibition. This contrasts with the Dixon plot results (Figure 1) which indicated that increasing PLP concentrations nearly eliminated the inhibition caused by low concentrations of PPxy-*m*-NH_2-Tyr when one starts out with holoenzyme. It appears that coenzyme cannot cause a rapid displacement of the adduct from enzyme once the inhibitor–enzyme complex is formed. Conversely, this adduct does not cause a rapid inactivation of holoenzyme, indicating that neither coenzyme nor PPxy-*m*-NH_2-Tyr can readily displace each other once bound to enzyme. Conformational changes in the enzyme most likely accompany binding of either coenzyme or adducts. The inhibition of aspartate and tyrosine aminotransaminases by analogous coenzyme–amino acid adducts has also been reported to be irreversible (Relimpio et al., 1975; Turano et al., 1970).

The determination of dissociation constants for inhibitors by kinetic methods assumes that the binding of the inhibitors is readily reversible. The present study indicates that kinetic methods are not suitable for determining dissociation constants for PLP–amino acid adducts. Thus, the competition observed in the Dixon and Lineweaver–Burk plots is under kinetic control, and the apparent affinities of coenzyme and adduct do not reflect their true affinities for enzyme. In other words, the formation of a complex between apoenzyme and either coenzyme or adduct is determined by which compound successfully encounters apoenzyme first and not by which compound has the greater affinity for apoenzyme. Coenzyme–amino acid adducts similar to those used in the present study are being used to study other PLP-dependent enzymes, and one must determine if the binding of such adducts is readily reversible before conventional kinetic methods can be used to determine the dissociation constant for the adducts.

When holoenzyme is incubated with the alternate substrates 5-hydroxytryptophan and *o*-tyrosine, only *o*-tyrosine caused a significant time-dependent inactivation of holoenzyme. Therefore, 5-hydroxytryptophan which is decarboxylated at approximately one-third the rate of Dopa by Dopa decarboxylase (Srinivasan & Awapara, 1978) appears not to follow the decarboxylation-dependent transamination pathway to any significant degree under these conditions and is probably the substrate of choice if one wishes to minimize the effect of the decarboxylation-dependent transamination pathway.

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