Studies on the Tyrosine Aminotransferase Mechanism*

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ABSTRACT: Further studies have been made on the mechanism of the tyrosine aminotransferase (EC 2.6.1.5) system isolated from rat liver. The strength of binding of coenzyme to apoenzyme has been compared to aspartate aminotransferase (EC 2.6.1.1) using gel filtration, aging, and dialysis, and the results show that under the conditions used, the coenzyme of tyrosine aminotransferase is more dissociable. The catalytic reaction is not inhibited under conditions where essentially all of the pyridoxal phosphate added is combined as Schiff base with tyrosine, necessitating a reinterpretation of previous kinetic studies which by not taking Schiff base formation into account had been interpreted to indicate a sequential mechanism. Isotopic exchange between tyrosine-U-^{14}C and *p*-hydroxyphenylpyruvate in the absence of α-ketoglutarate, the equivalent activation of this exchange by pyridoxal phosphate and pyridoxamine phosphate, and the direct demonstration of a half-reaction producing net *p*-hydroxyphenylpyruvate-U-^{14}C from tyrosine at rates comparable with those for isotopic transfer in the complete three-reactant system all indicate also that a Ping-Pong mechanism occurs. A rate equation which predicts most of the experimental findings has been derived on the assumption that the mechanism is Ping-Pong, the coenzymes are freely dissociable from the enzyme, and that either Schiff base, or coenzyme followed by substrate, may combine with the free enzyme to give a catalytically active complex.

In a previous report (Diamondstone and Litwack, 1963) on the kinetics of tyrosine aminotransferase (EC 2.6.1.5), evidence was presented to show that the reaction catalyzed by this enzyme was substantially different from the mechanisms suggested for aspartate aminotransferase (EC 2.6.1.1) from pig heart (Velick and Vavra, 1962; Henson and Cleland, 1964), or for alanine aminotransferase (EC 2.6.1.2) as studied by Bulos and Handler (1965). For the latter two enzymes, it is now clear that the coenzyme does not dissociate, and transamination involves conversion of enzyme-bound pyridoxal phosphate into pyridoxamine phosphate by reaction with an amino acid, and subsequent reconversion by reaction of the pyridoxamine phosphate with a keto acid. The coenzyme is thus a "shuttle" for the amino group (Snell and Jenkins, 1959). The tyrosine aminotransferase reaction, however, appeared to involve free pyridoxal phosphate as a substrate and to require the presence of all three reactants (including pyridoxal phosphate) at the enzymatically active site before detectable product could be released.

Kinetic studies showed that when tyrosine was varied at different pyridoxal phosphate levels (with α-ketoglutarate concentration held high and constant) an intersecting initial velocity pattern was observed. Likewise, α-ketoglutarate and pyridoxal phosphate gave an intersecting pattern. However, when α-ketoglutarate and tyrosine were varied at a high fixed level of pyridoxal phosphate, a parallel initial velocity pattern was observed (which became intersecting when a constant level of glutamate was present). These experiments were interpreted at the time as indicating an ordered addition of the three reactants, particularly since the pattern when α-ketoglutarate and tyrosine were varied appeared to become intersecting at low pyridoxal phosphate concentrations. However, the concentrations plotted on these graphs were the total concentrations added, and no recognition was made of the fact that a varying amount of the pyridoxal phosphate present was tied up as Schiff base, depending on the level of tyrosine added.

The experiments in the present paper show that the coenzymes are not tightly bound to the enzyme, Schiff bases form between coenzymes and substrates and may act as substrates, and either pyridoxal phosphate or pyridoxamine phosphate may function as coenzyme. In addition, isotopic exchange has been observed between tyrosine and *p*-hydroxyphenylpyruvate, dependent only on enzyme and coenzyme, and not on α-ketoglutarate or glutamate. This exchange has the same initial rate as does the chemical reaction catalyzed by the enzyme, and further, net chemical synthesis of *p*-hydroxyphenylpyruvate from tyrosine in the absence of α-ketoglutarate, but presence of pyridoxal phosphate and enzyme, has been observed. These data all indicate a Ping-Pong mechanism similar to that found for the other transaminases, but with the difference that the coenzyme can dissociate freely from the enzyme. A partial report of this work has been made (Litwack and Cleland, 1967).

Materials and Methods

Chemicals. Reagent chemicals of the best quality were obtained commercially. L-Tyrosine-U-^{14}C obtained

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from New England Nuclear Corporation (Boston, Mass.) had a specific activity of 393 mCi/mmole and was purified routinely by unidimensional paper chromatography on Whatman No. 1 paper developed with 1-butanol–acetic acid–water (5:1:4). *p*-Hydroxyphenylpyruvate-U-^{14}C was prepared from L-tyrosine-U-^{14}C following transamination catalyzed by tyrosine transaminase and purified on columns of Dowex 50W-8X (Weinstein *et al.*, 1967). The product was taken to dryness in a stream of nitrogen. Bio-Gel P-10 and Bio-Gel P-300 molecular sieve gels were obtained from Calbiochem and were swelled and washed extensively in the indicated buffer. DEAE-Sephadex A-50 was obtained from Pharmacia. Dowex 50W-8X was obtained from J. T. Baker and prepared as previously described (Weinstein *et al.*, 1967). The neutral surface-active agent, Triton X-100, was obtained from Rohm and Haas Corp. (Philadelphia).

Enzyme Preparation. Purified rat liver tyrosine aminotransferase was used in all the studies reported. The enzyme was purified through four fractionation steps: ammonium sulfate, adsorption and elution on calcium phosphate gel, chromatography on Bio-Gel P-300, and chromatography on DEAE-Sephadex A-50. The livers of 20–50 adrenalectomized male Fisher CD rats (Charles River Breeding Laboratories) weighing 150–250 g each were used for purification 4 hr after an inducing dose of cortisol and RNA was injected (Litwack and Diamondstone, 1962). The chilled and blotted livers were homogenized with a Teflon homogenizer in three volumes of cold 0.14 M KCl. After adjustment to pH 7.9 the homogenate was centrifuged at 35,000*g* at 0° for 30 min. The supernatant was brought to 70% saturation of ammonium sulfate at pH 7.8–7.9 at 1° by slow addition of the solid salt. The precipitate was centrifuged in the cold. The precipitate was washed successively for 10 min each time with 90-ml portions of ammonium sulfate solutions starting with 60, then 50, 45, 35, 30, and 25% ammonium sulfate saturation at pH 7.9. Most of the enzymatic activity was distributed in the 45 and 25% ammonium sulfate saturation washes. The fractions of 45–25% saturation were pooled and yielded 75–100% of the starting activity with a two- to threefold purification. The protein was concentrated by precipitation at 70% saturation of ammonium sulfate and dissolved in a minimal volume of 4 mM Tris-HCl buffer (pH 8.0). This preparation was adsorbed and eluted on calcium phosphate gel according to Jacoby and LaDu (1964) giving a three- to tenfold purification step. The most active fractions were pooled, concentrated by ammonium sulfate precipitation, and dissolved in a minimal volume of Tris-HCl buffer (pH 8.0). This preparation was heated in a 60° water bath for 3–6 min until coagulation just started and centrifuged in the cold to remove precipitated protein. The supernatant was applied to a Bio-Gel P-300 column which had been swelled and washed in 0.2 M potassium phosphate buffer containing 1 mM EDTA at pH 6.5. The bed measured 2.8 × 20 cm. A top layer of Bio-Gel P-60 was added to 1 cm over the bed. Potassium phosphate buffer (0.2 M) (pH 6.5) containing 1 mM EDTA and 0.5 mM dithiothreitol was the eluent. The major portion of the eluted enzyme was concentrated by ultrafiltration at 4° and was applied to a column of DEAE-Sephadex A-50 (4.8 × 23 cm) and eluted stepwise with 250 ml of 0.05 M potassium phosphate buffer (pH 6.5) containing 1 mM EDTA and 0.5 mM dithiothreitol, 250 ml of the same buffer plus 0.2 M NaCl, 300 ml of the same buffer except with 0.3 M NaCl, and 500 ml of the same buffer except with 0.5 M NaCl. The most active fractions of the peak of enzymatic activity were pooled and used as the enzyme preparation which was 500- to 1000-fold purified over the starting homogenate (Litwack *et al.*, 1966) and essentially homogeneous by ultracentrifugation. Hayashi *et al.* have crystallized the enzyme after fractionation to 500-fold over the crude extract (Hayashi *et al.*, 1967). Aspartate aminotransferase prepared from pig heart (Jenkins *et al.*, 1959) was obtained from Boehringer-Mannheim (New York) and did not require additional pyridoxal phosphate for full activity. Further information regarding specific treatments of enzyme preparations is given in footnotes to Table I.

Enzymatic Assays. Tyrosine aminotransferase activity was assayed by the conversion of *p*-hydroxyphenylpyruvate into its corresponding aldehyde (Diamondstone, 1966) after incubation in a system described previously (Litwack, 1962), or the enzyme was assayed using the Briggs' reaction to detect *p*-hydroxyphenylpyruvate (Briggs, 1922; Canellakis and Cohen, 1956) where indicated. Aspartate aminotransferase was assayed at 280 mμ in the direction of oxaloacetate formation by the continuous spectrophotometric procedure of Cammarata and Cohen (1951). Spectrophotometric measurements were made with Zeiss PMQII or Gilford instruments.

Schiff Base Measurements. The Schiff base of tyrosine and pyridoxal phosphate was estimated by a procedure developed in this laboratory[1] using the ratio of absorbancy at 400 mμ to that at 390 mμ. This ratio is approximately linear with increasing concentrations of the Schiff base of tyrosine and pyridoxal phosphate and the method is very similar to that recently reported by Zenker (1966). A fixed concentration of pyridoxal phosphate (from 1 to 38 μM) was combined with a given amount of L-tyrosine (from 0.5 to 7 mM) in 0.2 M phosphate buffer (pH 7.6). The final volume was 2.9 ml. After equilibration of the solution for 20 min at room temperature for maximal formation of Schiff base, the absorbancy was measured at 280, 330, 390, and 400 mμ against a blank of phosphate buffer in a Beckman DU spectrophotometer. The concentration of Schiff base was expressed as the ratio of absorbancies at 400–390 mμ. In order to determine the effect of various amounts of preformed Schiff base on enzymatic activity, the system was brought to 37° and the enzymatic reaction was initiated by addition of 0.1 ml of purified tyrosine aminotransferase and α-ketoglutarate to a final concentration of 10 mM. The initial velocity of the enzymatic reaction was measured after 10-min incubation by the Briggs' reaction (Canellakis and Cohen, 1956).

Isotopic Exchange Studies. Study of the exchange be-

[1] Plost, C., and Litwack, G., unpublished experiments.

TABLE I: Comparison of the Dissociability of the Coenzymes of Tyrosine Aminotransferase and Aspartate Aminotransferase.

Treatment	Per Cent Dissociated by Treatment[a]			
	Tyrosine Aminotransferase		Aspartate Aminotransferase[d]	
	0.2 M Phosphate (pH 6.5)	0.1 M Bicarbonate (pH 6.5)	0.05 M Phosphate (pH 7.4)	0.1 M Bicarbonate (pH 6.5)
Heat[b]	45 ± 6[e] (6)[f]	18 (13–25)[g] (3)[f]	28 (1)[f]	
Gel filtration on Bio-Gel P-10[c]	60 ± 6 (7)	48 (41–52) (3)	3 (1)	
Storage, 24 hr at 4° after gel filtration[h]	60 ± 12 (6)	35 (26–40) (3)	5 (3–6)[g] (4)	5 (0.9)[g] (2)[f]
Dialysis at 4° against 200 volumes of H_2O for 2 hr	77 ± 4 (4)	41 (29–53) (2)	16 (14–19) (5)	5 (0–9) (2)

[a] Dissociation of coenzyme was determined by assay of the treated fraction in the presence or absence of 38 μM pyridoxal phosphate and

$$\text{per cent dissociated} = 100 - \left[\frac{\text{activity without pyridoxal phosphate}}{\text{activity with pyridoxal phosphate}} \times 100\right]$$

[b] Purified tyrosine aminotransferase (20–30 mg) in a 3.03-ml system with the indicated buffer containing 1 mM ethylenediaminetetraacetic acid and 90 μM pyridoxal phosphate was heated to 60° for 2–3 min until a precipitate began to form. This treatment was used to simulate the heat step in the purification of aspartate aminotransferase after which the enzyme remains virtually completely associated with pyridoxal phosphate. The heat treatment of aspartate aminotransferase indicated in the table refers to a *second* treatment identical with the one used for tyrosine aminotransferase. [c] Gel filtration was performed on the heat-treated tyrosine aminotransferase (first treatment) on Bio-Gel P-10 (1 × 26 cm bed) at 4° in order to remove pyridoxal phosphate not associated with the enzyme. The entry for aspartate aminotransferase refers to enzyme that was not heat treated a second time, and all entries for this enzyme except the one for heat treatment are made for the enzyme preparation *per se* without further heating. Heating tyrosine aminotransferase was done in all cases and was followed by gel filtration in all cases. The other treatments in the table were done with enzyme that was already treated by heat and gel filtration. The only sequential treatments in the case of tyrosine aminotransferase are heat treatment, gel filtration, and other treatment. In the case of aspartate aminotransferase gel filtration, only, precedes a specific other treatment except for the single entry showing a second heat treatment. [d] Aspartate aminotransferase from pig heart was essentially fully active without addition of pyridoxal phosphate in the assay. [e] Standard error of the mean = $\sqrt{\Sigma d^2/n - 1}/\sqrt{n}$. [f] Number of experiments. This value for aspartate aminotransferase shows that an additional heat step causes some dissociation of the holoenzyme. Other values in the table for aspartate aminotransferase are given for preparations which were not heated a second time (the first heat step was used in the preparation of the enzyme). [g] Range of values. [h] Similar effects were noted when storage was for 48 or 72 hr.

tween L-tyrosine-U-^{14}C and *p*-hydroxyphenylpyruvate was made possible by the method of complete separation of these reactants on Dowex 50W-8X (Weinstein *et al.*, 1967). The incubation was stopped by addition of 100% (w/v) trichloroacetic acid to give a final concentration of 5%. The supernatant was applied to the ion-exchange resin; the *p*-hydroxyphenylpyruvate was eluted with water and labeled tyrosine was eluted with 3 N HCl (Weinstein *et al.*, 1967).

Radioactivity Measurements. A dioxane–Cab-O-Sil–fluor system was used as described previously (Weinstein *et al.*, 1967) and radioactivity was determined in a Nuclear-Chicago Model 723 liquid scintillation spectrometer. All reported measurements were corrected for background and converted into disintegrations per minute by the channels ratio procedure. Efficiency was approximately 70% for ^{14}C.

Results

Dissociability of Coenzyme. One of the most important differences between tyrosine aminotransferase and aspartate or alanine aminotransferase appears to be the degree of association of the coenzyme. To study this difference, tyrosine aminotransferase was compared with aspartate aminotransferase by using treatments which would be expected to cause dissociation of a loosely held coenzyme. This study is presented in Table I. Partially purified tyrosine aminotransferase was heat treated in the presence of excess pyridoxal phosphate in an at-

tempt to simulate the heating step in the preparation of aspartate aminotransferase (Jenkins *et al.*, 1959). Heated preparations were chromatographed on columns of Bio-Gel P-10 at 4° to remove unattached coenzyme. It is obvious from the first two entries in the table that gel filtration results in some dissociation of the holoenzyme, as is expected if the coenzyme has a measurable dissociation constant.

Aspartate aminotransferase was also subjected to this procedure prior to other treatments, but the gel treatment did not appreciably affect the stability of the holoenzyme. Storage of tyrosine aminotransferase at 4° resulted in only a slight increase in the amount of dissociation of the holoenzyme. Dialysis for 2 or 4 hr resulted in further increases in dissociation of the holoenzyme. The extent of dissociation of this enzyme under these conditions appears to be significantly greater in 0.2 M phosphate at pH 6.5 than in 0.1 M bicarbonate at the same pH, confirming similar results obtained by Scardi *et al.* (1963) with aspartate aminotransferase. It has been shown that phosphate ion or arsenate ion can partially protect tyrosine aminotransferase from inhibition by urea, which may produce a structural change in the enzyme (Litwack *et al.*, 1966). Since pyridoxal phosphate produces a similar but more extensive protection of the enzyme from urea inactivation, it seems possible that phosphate ion might bind to some of the same sites as pyridoxal phosphate. If this were so, use of phosphate buffer could result in a decreased ability of the enzyme to bind the coenzyme and bring about a greater dissociation of holoenzyme under the conditions of these experiments. In contrast to the effects of these treatments upon the stability of the tyrosine aminotransferase holoenzyme, there are only comparatively small effects upon holoaspartate aminotransferase. Thus, after gel filtration and storage, there is 7–12 times more dissociation of tyrosine aminotransferase, and after dialysis against water for 2 hr, there is 2–15 times more dissociation than with holoaspartate aminotransferase.

Activity of Schiff Bases as Substrates. Schiff bases of pyridoxal phosphate and amino acids form very rapidly in the absence of enzyme or metals (Matsuo, 1957), and this occurs with tyrosine and pyridoxal phosphate (Diamondstone and Litwack, 1963). It is therefore of great interest to determine whether such a Schiff base will act as a substrate in the enzymatic reaction, will act as an inhibitor, or will have no apparent effect on the kinetics. These possibilities were tested by increasing the concentration of tyrosine at a constant level of pyridoxal phosphate to a point where all of the coenzyme would be in the form of Schiff base. Various levels of tyrosine were mixed with pyridoxal phosphate in buffer and equilibrated for 20 min at room temperature (Matsuo, 1957), and the amount of Schiff base formed was determined spectrophotometrically. The enzymatic reaction was then started by the addition of enzyme and α-ketoglutarate at 37°. When 38 μM total pyridoxal phosphate was present, the velocity increased with increasing tyrosine concentration up to about 5 mM tyrosine and then levelled off. The concentration of Schiff base also increased with tyrosine concentration, reaching 38 μM tyrosine. In other experiments at lower pyridoxal phosphate concentrations (down to 1 μM, approximately the Michaelis constant), the results were similar, and in no case was any substrate inhibition by tyrosine seen at levels where pyridoxal phosphate was completely tied up as Schiff base. These data show that the Schiff base of tyrosine and pyridoxal phosphate is not an inhibitor of the reaction. If the rate constants for dissociation of tyrosine and pyridoxal phosphate from the enzyme greatly exceed the maximum velocity of the reaction (that is, the portion of the reaction sequence involving addition of these molecules is at thermodynamic equilibrium), one cannot tell whether the enzyme–pyridoxal phosphate–tyrosine complex, whose further reaction is one of the rate-limiting steps, forms by obligatory addition of pyridoxal phosphate followed by tyrosine (a rapid equilibrium ordered mechanism), or whether preformed Schiff base can also react with free enzyme. As noted later (see Discussion) formation of an enzyme–tyrosine complex would lead to substrate inhibition, and can be ruled out. However, if the rapid equilibrium assumption is not valid

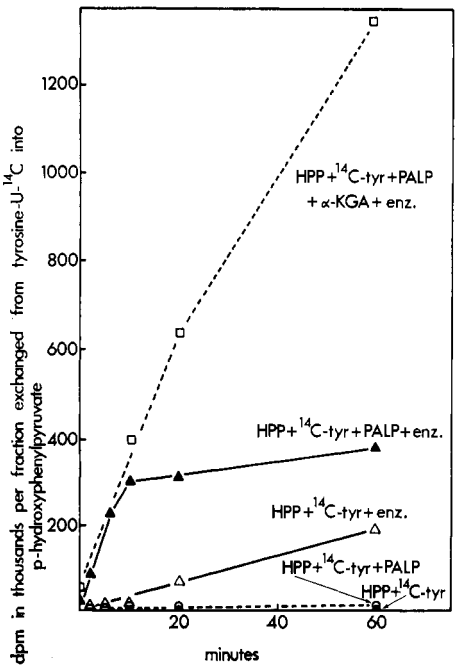

FIGURE 1: Isotopic exchange between L-tyrosine-U-^{14}C and *p*-hydroxyphenylpyruvate with various components of the tyrosine aminotransferase system as a function of time. Depicted is one of three similar experiments. Each reaction mixture contained in 3.2-ml total volume: L-tyrosine containing 10 μCi of L-tyrosine-U-^{14}C (Tyr-^{14}C), 3.75 mM; *p*-hydroxyphenylpyruvate (HPP), 1.25 mM; and potassium phosphate (pH 7.6), 0.1 M. The concentrations of the other components, when added, were: pyridoxal phosphate (PALP), 38 μM; α-ketoglutarate (α-KGA), 31 mM; and partially purified tyrosine aminotransferase (Enz), 5.6 mg. Systems were incubated at 37°, and 0.5-ml portions were removed at the indicated times and combined with 1.4 ml of 0.2 M potassium phosphate buffer (pH 7.6) and 0.1 ml of 100% trichloroacetic acid in a glass centrifuge tube (to precipitate enzyme when it was present in the incubation). The mixture was centrifuged and the supernatant was added to the Dowex 50W-8X ion-exchange column (see Materials and Methods section). *p*-Hydroxyphenylpyruvate was eluted with water and the radioactivity measured as described previously (Weinstein *et al.*, 1967).

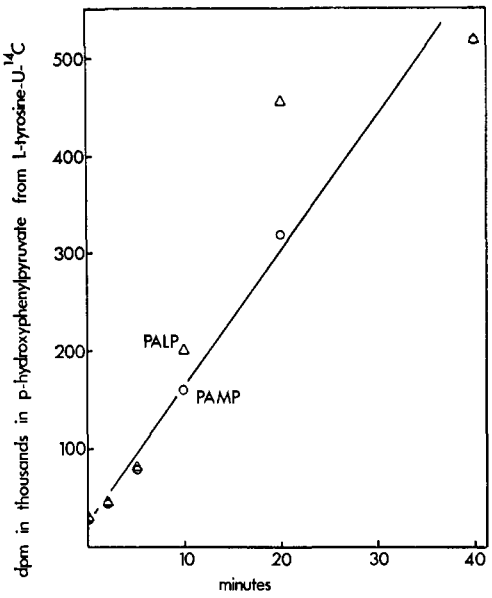

FIGURE 2: Comparison of pyridoxal phosphate and pyridoxamine phosphate as activators of the exchange reaction between tyrosine-U-^{14}C and *p*-hydroxyphenylpyruvate in the absence of α-ketoglutarate. Depicted is one of two similar experiments. The reaction mixtures contained in 3.2-ml total volume: 3.75 mM L-tyrosine together with 10 μCi of tyrosine-U-^{14}C; 38 μM pyridoxal phosphate or pyridoxamine phosphate; 2 mg of purified tyrosine aminotransferase; and 0.2 M potassium phosphate (pH 7.6). The exchange reaction was initiated by the addition of 4 μmoles of *p*-hydroxyphenylpyruvate (final concentration, 1.25 mM), and exchange rates were measured as described in Figure 1.

(and some of the data given above for the degree of dissociation of the coenzyme under different conditions suggest that it may not be valid for coenzyme), substrate inhibition by tyrosine should be observed, unless preformed Schiff base can react with free enzyme. Until comparison can be made between the rate constant for dissociation of pyridoxal phosphate and the maximum velocity for the reaction, the question of whether Schiff bases may act as substrates must remain open.

Isotopic Exchange Studies. If tyrosine aminotransferase has a Ping-Pong mechanism like other transaminases, it should catalyze isotopic exchange between tyrosine and *p*-hydroxyphenylpyruvate in the presence of pyridoxal phosphate or pyridoxamine phosphate, but in the absence of α-ketoglutarate or glutamate. If the mechanism is sequential as postulated earlier (Diamondstone and Litwack, 1963), all components of the reaction would have to be present in order to see exchange, and pyridoxamine phosphate might not serve as coenzyme with the same efficiency as pyridoxal phosphate, or at all. To test these possibilities, isotopic exchange experiments were carried out as shown in Figure 1. Negligible exchange occurred when *p*-hydroxyphenylpyruvate and radioactive tyrosine were incubated with or without pyridoxal phosphate, showing that isotopic exchange was dependent on the presence of enzyme.

In the presence of enzyme and a saturating level of pyridoxal phosphate, however, rapid isotopic exchange was observed with a half-life for approach to isotopic equilibrium of about 10 min. Slow exchange with an initial velocity about 8% of this value was observed in the absence of added pyridoxal phosphate; this rate reflects the inability to remove bound coenzymes completely from the enzyme during purification. Of particular importance is the equality of initial velocities of isotopic exchange in the presence of saturating pyridoxal phosphate and of the chemical reaction catalyzed by the enzyme under similar conditions when α-ketoglutarate is added in excess. The half-life of approach to chemical equilibrium when α-ketoglutarate is present is nearly 1 hr, although the amount of label in *p*-hydroxyphenylpyruvate is much greater in this case than when only isotopic exchange is measured, since the concentration of *p*-hydroxyphenylpyruvate is increasing during the experiment and should reach nearly 5 mM at chemical equilibrium, as opposed to the constant level of 1.25 mM during the exchange experiment. These data are strong evidence that the partial reaction shown by the isotopic exchange is an integral part of the mechanism for chemical reaction, and that the mechanism is thus Ping-Pong, rather than sequential.

The Role of Pyridoxamine Phosphate. Pyridoxamine phosphate appears to be just as efficient an activator for the rat liver enzyme as pyridoxal phosphate. In six experiments the K_m ± standard error of the mean for pyridoxamine phosphate was found to be 1.2 ± 0.1 × 10^{-6}

TABLE II: Direct Demonstration of Partial Reaction between Tyrosine and Pyridoxal Phosphate.[d]

Enzyme Added (mg/ml)	*p*-Hydroxyphenylpyruvate[a] Formed (μM)	Tyrosine[b] Remaining (μM)	Protein-Bound[c] Substrate (μM)
0.004	4.4	26.7	0.2
0.026	6.5, 7.5	23.7	0.5, 0.6
0.052	10.3	19.9	1.1
0.103	11.1, 13.1	17.1	2.0, 2.2
0.258	16.7	10.7	3.9
0.517	16.4	9.5	5.4

[a] Includes total free and combined as Schiff base. [b] Determined by difference between initial 31.3 μM tyrosine added and the values shown in the second and fourth columns. Includes total free and combined as Schiff base. [c] Measured by radioactivity precipitated with protein by trichloroacetic acid. [d] The incubation system contained in a total volume of 3.0 ml: purified enzyme in the amounts shown; L-tyrosine-U-^{14}C, 99,000–160,000 dpm, 0.094 μmole; pyridoxal phosphate, 0.114 μmole; and 0.2 M potassium phosphate (pH 7.6). The reactions were stopped after 30 min, and the mixtures were analyzed for *p*-hydroxyphenylpyruvate as described in Figure 1. The amount of radioactivity associated with material precipitable by trichloroacetic acid is also shown. The radioactivity associated with the precipitated enzyme was not removed by treatment with 5% Triton X-100.

M which agrees very well with the K_m value of about 0.7×10^{-6} M for pyridoxal phosphate in this reaction (Kenney, 1959; Diamondstone and Litwack, 1963; Jacoby and LaDu, 1964). The tyrosine aminotransferase from chicken liver also appears to be activated by pyridoxamine phosphate (Constantsas and Knox, 1967) and the rat liver enzyme is protected from urea inhibition by pyridoxamine phosphate as well as by pyridoxal phosphate (Litwack *et al.*, 1966). Figure 2 depicts an experiment to test whether pyridoxamine phosphate would activate isotopic exchange between tyrosine-U-^{14}C and *p*-hydroxyphenylpyruvate in the absence of α-ketoglutarate. It can be seen that pyridoxamine phosphate supports the same exchange rate as pyridoxal phosphate. These data indicate that the enzyme catalyzes reaction between free pyridoxamine phosphate and either *p*-hydroxyphenylpyruvate or α-ketoglutarate, and suggest that in the steady state both forms of the coenzyme are present and that the distribution between the two should not depend on which one was added initially (assuming that the other substrates are always added in considerable excess over the coenzyme, which is normally true in either an initial velocity or isotopic exchange experiment). Such a situation is consistent with a Ping-Pong mechanism, but is more difficult to reconcile with a sequential one.

Direct Demonstration of Half-Reaction. Reaction between tyrosine and pyridoxal phosphate to give *p*-hydroxyphenylpyruvate is demonstrated by the data in Table II. In these experiments 31.3 μM tyrosine-U-^{14}C was incubated with 38 μM pyridoxal phosphate in the presence of various levels of enzyme for 30 min, and the amount of labeled *p*-hydroxyphenylpyruvate was determined. In addition, the amount of label bound to the enzyme was measured. With the larger amounts of enzyme, the reaction reached equilibrium during the incubation, while with the lower levels, it did not. The initial velocities calculated for the lowest enzyme levels are somewhat higher, when corrected for enzyme concentration and degree of saturation with tyrosine, than those shown in Figure 1 for the over-all chemical reaction catalyzed by the enzyme and for isotopic exchange between tyrosine and *p*-hydroxyphenylpyruvate. This is to be expected, since no *p*-hydroxyphenylpyruvate was present initially to cause product inhibition, and these data serve as additional proof that the mechanism of the over-all chemical reaction catalyzed by this enzyme is Ping-Pong.

At the concentrations used here, the enzyme should be saturated with pyridoxal phosphate and/or pyridoxamine phosphate, while the reaction should be first order with respect to tyrosine, which is present at only several per cent of its Michaelis constant level. As much as one-third of the tyrosine might be initially in the form of a Schiff base with pyridoxal phosphate, depending on the value assumed for the dissociation constant of this complex. The reaction catalyzed is L-tyrosine + pyridoxal phosphate $\rightleftharpoons$ *p*-hydroxyphenylpyruvate + pyridoxamine phosphate, but because the amount of the coenzyme bound to the enzyme and tied up as Schiff bases cannot be estimated accurate'y, it is not possible to calculate an equilibrium constant for this reaction from the available data. The value would appear to be slightly more than unity.

The amount of radioactivity precipitated with the enzyme by trichloroacetic acid was essentially constant at the lower enzyme levels, representing a combining weight for the enzyme of about 50,000, but dropped off at the two highest levels, which gave combining weights of 66,000 and 96,000. Since the tyrosine concentration was so far below its Michaelis constant, this binding probably represents nonspecific interaction between Schiff bases present in solution and the newly exposed hydrophobic regions of the denatured enzyme, rather than the true level of substrates bound at the active site as Schiff bases in central complexes with active enzyme.

Discussion

The data presented above show that tyrosine aminotransferase has a Ping-Pong mechanism like other transaminases, but that the coenzymes can dissociate from and recombine with the enzyme. In addition, it is clear that Schiff bases of the coenzymes with the substrates are not inhibitors, and may be able to combine with the enzyme to give catalytically active complexes. The previous kinetic data obtained for this enzyme by varying the total concentrations present of tyrosine, α-ketoglutarate, and pyridoxal phosphate must therefore be reinterpreted. The derivation which follows yields a rate equation expressed in terms of total concentrations of reactants, which is consistent with most of the kinetic data obtained previously (Diamondstone and Litwack, 1963).

Schiff base formation and dissociation are assumed to be rapid and to occur for all components: A = tyrosine, B = α-ketoglutarate, C = pyridoxal phosphate, D = pyridoxamine phosphate, P = *p*-hydroxyphenylpyruvate, Q = glutamate, A–C = Schiff base of A + C, B–C = Schiff base of B + C, P–D = Schiff base of P + D, and C–Q = Schiff base of C + Q.

$$A + C \rightleftharpoons A\text{–}C \qquad A\text{–}C = \frac{AC}{K_{iac}}$$

$$B + D \rightleftharpoons B\text{–}D \qquad B\text{–}D = \frac{BD}{K_{ibd}}$$

The same is true for P–D or C–Q. It is assumed that: (a) the concentrations of A and B exceed those of C and D sufficiently so that they are not altered by Schiff base formation; (b) as appears to be the case experimentally, tyrosine, or pyridoxal phosphate, or tyrosine–Schiff base can act as substrates; (c) only pyridoxal phosphate, pyridoxamine phosphate, or Schiff base may add to free enzyme;[2] (d) Schiff base formation may take place be-

[2] If tyrosine combines with free enzyme, the resulting rate equation predicts substrate inhibition by tyrosine; since this is not observed experimentally, it is assumed that a tyrosine-free enzyme complex does not exist. If it is assumed that Schiff bases cannot react directly with free enzyme, the form of the equation is not altered, but K_{ac} and K_{bd} do not exist, and are replaced in all equations by K_aK_{ic}/K_{iac} and K_bK_{id}/K_{ibd}.

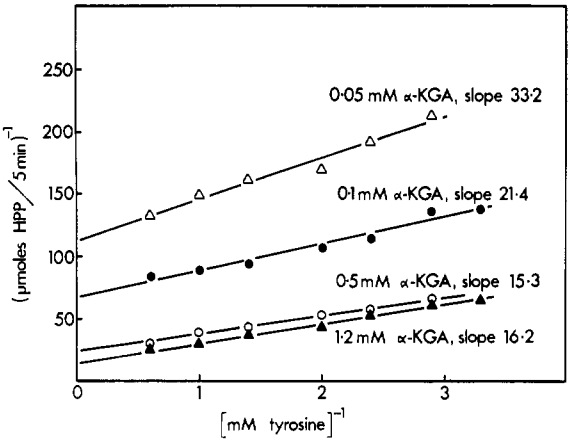

FIGURE 3: Apparent convergence of double-reciprocal plots of initial velocity as a function of tyrosine concentration when the fixed concentration of α-ketoglutarate is lowered below 0.1 mM. All lines are drawn by the least-squares procedure. Initial velocity is expressed as micromoles of *p*-hydroxyphenylpyruvate formed per 5 min at 37°. The concentration of pyridoxal phosphate was fixed at 40 μM.

tween bound coenzyme and free substrates. Further, it is assumed that all of these steps are rapid compared with the catalytic interconversion of Schiff bases A–C $\rightleftharpoons$ P–D or B–D $\rightleftharpoons$ C–Q. Therefore

$$\mathrm{E} + \mathrm{C} \rightleftharpoons \mathrm{EC}^3 \qquad EC = E\left(\frac{C}{K_{ic}}\right)$$

$$\mathrm{E} + \mathrm{D} \rightleftharpoons \mathrm{ED} \qquad ED = E\left(\frac{D}{K_{id}}\right)$$

$$\mathrm{E} + \mathrm{A\text{–}C} \rightleftharpoons \mathrm{EAC} \qquad EAC = E\frac{A\text{–}C}{K_{ac}} = E\left(\frac{A}{K_{ac}}\right)\left(\frac{C}{K_{iac}}\right)$$

$$\mathrm{EC} + \mathrm{A} \rightleftharpoons \mathrm{EAC} \qquad EAC = EC\frac{A}{K_a} = E\left(\frac{A}{K_a}\right)\left(\frac{C}{K_{ic}}\right)$$

Thus, $K_{ac}K_{iac} = K_aK_{ic}$.

$$\mathrm{E} + \mathrm{B\text{–}D} \rightleftharpoons \mathrm{EBD} \qquad EBD = E\frac{B\text{–}D}{K_{bd}} = E\left(\frac{B}{K_{bd}}\right)\left(\frac{D}{K_{ibd}}\right)$$

$$\mathrm{ED} + \mathrm{B} \rightleftharpoons \mathrm{EBD} \qquad EBD = ED\frac{B}{K_b} = E\left(\frac{B}{K_b}\right)\left(\frac{D}{K_{id}}\right)$$

Thus, $K_{bd}K_{ibd} = K_bK_{id}$. Similar equilibria may be written for P + Q. Since $dP/dt = k_1EAC$ (for conversion of EAC into EPD) and $dQ/dt = k_3EBD$ (for conversion of EBD into ECQ) and, in the steady state $dP/dt = dQ/dt$, we have $k_1EAC = k_3EBD$ (in the absence of products) or $k_1E(AC/K_aK_{ic}) = k_3E(BD/K_bK_{id})$, from which it follows that $D/C = AK_bK_{id}k_1/K_aBK_{ic}k_3$. This equation describes the ratio of pyridoxamine phosphate to pyridoxal phosphate in the steady state in the absence of products.

We now consider the conservation equation for coenzyme. As long as C_t (total coenzyme concentration) exceeds E_t (enzyme concentration), we can write $C + D + (\mathrm{A\text{–}C}) + (\mathrm{B\text{–}D}) = C_t$. This is

$$C + D + \frac{AC}{K_{iac}} + \frac{BD}{K_{ibd}} = C_t$$

or eliminating D by using the equation for D/C derived above

$$C = \frac{C_t}{1 + \frac{A}{K_{iac}} + \frac{AK_bK_{id}k_1}{K_aBK_{ic}k_3} + \frac{AK_{bd}k_1}{K_aK_{ic}k_3}}$$

The conservation equation for enzyme is

$$E + EC + EAC + ED + EBD = E_t$$

or

$$E + E\frac{C}{K_{ic}} + E\frac{AC}{K_aK_{ic}} + E\frac{D}{K_{id}} + E\frac{BD}{K_bK_d} = E_t$$

from which

$$E = \frac{E_t}{1 + \frac{C}{K_{ic}} + \frac{AC}{K_aK_{ic}} + \frac{D}{K_{id}} + \frac{BD}{K_bK_{id}}}$$

Since $v = k_1EAC = k_1E(AC/K_aK_{ic})$

$$v = \frac{(k_1E_t)}{\frac{K_aK_{ic}}{AC} + \frac{K_a}{A} + 1 + \frac{K_aK_{ic}D}{K_{id}AC} + \frac{K_aK_{ic}BD}{K_bK_{id}AC}}$$

Substituting from our expressions for D/C and C, we get an expression containing only A, B, and C_t as variables, which can be written as eq 1, where

$$V = \frac{k_1k_3E_t}{k_1 + k_3}$$

[3] *E*, *EC*, *ED*, *EAC*, *EBD*, and *ECQ* mean the concentration of an enzyme form as such and are different from expressions where E is not on the same line with, or separated by brackets from, reactants or products. The latter expression indicates the multiplication of an enzyme form times the concentration of reactants or products indicated.

$$v = \frac{V}{1 + \left(\frac{k_3}{k_1 + k_3}\right)\frac{K_a}{A} + \left(\frac{k_1}{k_1 + k_3}\right)\frac{K_b}{B} + \left(\frac{k_3}{k_1 + k_3}\right)\frac{K_a K_{ic}}{AC_t} + \left(\frac{k_1}{k_1 + k_3}\right)\frac{K_b K_{id}}{BC_t} + \frac{1}{C_t}\left(\frac{k_3 K_{ae} + k_1 K_{bd}}{k_1 + k_3}\right)} \quad (1)$$

This equation predicts the kinetic patterns reported previously by Diamondstone and Litwack (1963), namely, intersecting patterns when A and C_t were varied at constant B, or when B and C_t were varied at constant A, and a parallel pattern when A and B were varied at constant C_t. In addition, this mechanism explains why the latter pattern became intersecting when glutamate was present.

If the rate equation is derived with the presence of glutamate allowed for, there are $Q/(AB)$ and $Q(ABC_t)$ terms present in the denominator, and these cause an intersecting rather than parallel pattern when A and B are varied at constant C_t and Q.

This equation is derived on the assumption that A and B are considerably higher than C_t, the total coenzyme concentration. Under conditions where this is not the case, a conservation equation for the substrate whose concentration does not exceed C_t must be considered, and the final rate equation becomes much more complex than the one derived here. In addition, it is no longer possible to assume that a steady-state distribution between C and D is established without appreciable change in the level of the other substrates, and as a result the steady-state approach must be abandoned. No attempt will be made here to derive equations for such situations, but the expected deviations from the equation derived here can be clearly seen from the data in Figure 3, where the parallel pattern seen at normal α-ketoglutarate concentrations is altered at concentrations which are low and equivalent to that of the pyridoxal phosphate added.

The assumption made in deriving this rate equation that either of the coenzymes may combine with free enzyme is clearly supported by the experimental data. However, the assumption that the rate-limiting steps are solely the interconversion of central complexes may not be valid, and in fact, the coenzymes may normally dissociate from the enzyme only once in a large number of catalytic cycles. If this is true, however, Schiff bases must be capable of combining with free enzyme, since no inhibition was observed at tyrosine levels sufficient to reduce the level of free pyridoxal phosphate far below its Michaelis constant. The use of the rapid equilibrium assumption in deriving the equation can be justified by the fact that simulation studies in the laboratory of one of the authors (W. W. C.), on mechanisms with alternate reaction sequences such as are present in the mechanism considered here, have shown that the observed kinetics are nearly the same whether such an assumption is valid or not. It is clear from the data, in any case, that the major difference between this enzyme and other transaminases previously studied lies in the degree of dissociation of the coenzymes, and that in the presence of saturating amounts of coenzyme, the kinetics of the other substrates show the Ping-Pong initial velocity pattern characteristic of other transaminases.

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