Liver Cytosol Tyrosine Aminotransferase

PRODUCT INHIBITION AND INTERACTION WITH SUBSTRATES*

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JOSEPH S. ROSENBERG AND GERALD LITWACK

From the Fels Research Institute and Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

SUMMARY

The reaction catalyzed by purified rat liver cytosol tyrosine aminotransferase (L-tyrosine + 2 oxoglutarate = p-hydroxyphenylpyruvate + L-glutamate, EC 2.6.1.5) has been analyzed with regard to product inhibition and combination with each of its rate-limiting substrates by the Hill equation. Glutamate is a linear competitive inhibitor with respect to tyrosine, a linear noncompetitive inhibitor with respect to coenzyme or a-ketoglutarate. p-Hydroxyphenylpyruvate is a linear noncompetitive inhibitor with respect to tyrosine or pyridoxal-P but is a linear competitive inhibitor with respect to a-ketoglutarate. These data, considered with replots of slopes and intercepts, indicate a “ping-pong” mechanism random in the sense that either coenzyme or Schiff’s base of coenzyme and amino acid can add to the apoenzyme.

A study of the half-reaction with apoenzyme, pyridoxamine-P, and a-ketoglutarate showed that 90% or more of the complete chemical reaction rate was achieved when freshly isolated apoenzyme was used. The pH dependence of the half-reaction paralleled that of the complete chemical reaction and supports the conclusion that the same protein catalyzes the complete and half-reaction. Furthermore, the magnitude of the half-reaction compared to the complete transamination would dictate that the mechanism does not switch between ping-pong and ordered depending upon the level of substrates available.

By use of the Hill equation, the reaction between enzyme and substrates is first order with respect to tyrosine, pyridoxal-P, and a-ketoglutarate. Reduction of the concentration of a-ketoglutarate to a rate-limiting value does not change the order with respect to tyrosine or to pyridoxal-P. Reduction of the concentration of pyridoxal-P, or of tyrosine, or of both of these reactants to rate-limiting levels when the concentration of a-ketoglutarate is varied does not affect the order of the reaction with respect to a-ketoglutarate. Thus, homotropic effects are not manifested by this system.

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† Career Development Awardee, K2 AM 16568 from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service. To whom correspondence should be addressed.

The tyrosine aminotransferase reaction catalyzed by the enzyme from rat liver cytosol has been shown to differ in certain aspects from the reaction mechanisms of other well studied transaminases. The enzyme appears to be resolved easily of coenzyme so that it behaves like a true reactant in the system (1, 2). A straightforward kinetic approach led to behavior which first was thought to support a mechanism of an ordered reaction. However, the additional approach of isotopic exchange made it clear that a binary mechanism was at least part of the reaction sequence and still was consistent with the kinetic data (2).

Values of $K_I$ with respect to each reactant in the system for the competitive inhibition by L-triiodothyronine were very similar (1) as were the values of $K_I$ with respect to each reactant in the system for the competitive inhibition by L-3-iodotyrosine (3). These observations led to the conclusion that all three reactants combined at one active site on the enzyme.

The purified enzyme appears to consist of four subunits, and four pyridoxal-P groups, suggesting the possibility of four active subunits (4). In addition, previous reports have emphasized the ability of the coenzyme, pyridoxal-P, to protect the enzyme from the effects of agents which are known to alter the conformation of certain proteins (5, 6) as well as other studies suggestive that, under certain circumstances, structural changes could take place (7). Thus, it appeared useful to determine whether there were cooperative effects involved in the interaction of enzyme and substrates. In this communication we report such studies as well as the results of product inhibition and their relationship to the mechanism of the enzymatic reaction.

MATERIALS AND METHODS

Chemicals—All chemicals were obtained from commercial sources: L-glutamic acid and L-tyrosine from Mann; a-ketoglutarate, pyridoxal-P, pyridoxamine-P, and ammonium sulfate from Nutritional Biochemicals; diethylthiocarbamic acid (sodium salt) from Eastman; p-hydroxyphenylpyruvic acid from Sigma; yeast ribonucleic acid from Schwarz BioResearch; disodium ethylenediaminetetraacetic acid (EDTA), trichloroacetic acid, and ammonium molybdate from Fisher; toluene from Matheson, Coleman and Bell; 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) and 2,5-diphenyloxazole (PPO) from Nuclear-Chicago; and diithiothreitol (Cleland’s Reagent) from Calbiochem. Sodium a-ketoglutarate-5-14C (specific activity, 17.1
mCi per mmole and 14C-L-glutamate (specific activity, 5 mCi per mmole) were purchased from Nuclear-Chicago. DE-52 anion exchange cellulose and SA-2 ion exchange resin-loaded paper were obtained from Reeve Angel Company.

All fluor reagents used were scintillation grade. All nonfluor reagents were prepared in deionized, distilled water.

Enzyme Assays—Tyrosine aminotransferase activity was determined by measuring formation of either p-hydroxyphenylpyruvate or glutamic acid. All assays were performed at 37°. For measuring p-hydroxyphenylpyruvate formation, the components of the assay system were as follows: 10 mM a-ketoglutarate, 4 mM L-tyrosine, 40 µM pyridoxal-P, 4 mM diethyl-dithiocarbamate, and enough 0.2 M potassium phosphate, pH 7.6, to give a final volume of 3.0 ml. When the purified enzyme was being assayed, diethyldithiocarbamate was omitted. Assay time was 10 min and the reaction was stopped with the addition of 0.2 ml of 100% (w/v) trichloracetic acid. To 2.0 ml of the trichloroacetic acid soluble supernatant was added 1.0 ml of 1% trichloracetic acid. The optical density of this solution was read at 850 nm 1 hour later with the Zeiss spectrophotometer. Enzyme activity has been expressed as micromoles of p-hydroxyphenylpyruvate formed per 10 min. The reaction rate was linear with respect to time and enzyme concentration.

For determining glutamic acid formation, the assay system included 30 µM pyridoxal-P, 2.5 µM a-ketoglutarate-14C, and 3 mM L-tyrosine. As in the previous assay, the enzyme was initially incubated 3 min with pyridoxal-P and a-ketoglutarate before the reaction was started with the addition of tyrosine. The reaction was stopped, after 40 min, with the addition of 0.1 ml of 2 N sulfuric acid, because the addition of trichloroacetic molybdate in 5 N hydrochloric acid (8, 9). The identity of the compounds at the origin and solvent front was determined by chromatographing samples of unreacted a-ketoglutarate and 14C-glutamate. The 14C-glutamate, positively charged because of the addition of sulfuric acid, remained at the origin, whereas the 14C-a-ketoglutarate moved with the solvent front. In each reaction mixture, the percentage of 14C-a-ketoglutarate metabolized to 14C-glutamate was determined. Unless otherwise noted, activity has been expressed as micromoles of glutamate formed during the entire assay period. Enzyme activity, as determined by this isotopic assay, was linear with respect to time and enzyme concentration. Michaelis-Menten constants determined by this method agreed with those obtained with nonisotopic procedures.

During some of the kinetic experiments it was necessary to reduce considerably the amount of 14C-a-ketoglutarate in the assay system. When the counts in the reaction mixture were low, two or more strips of ion exchange paper were spotted with 0.07 ml of the same reaction mixture and these strips were chromatographed simultaneously. After the strips were dried and sectioned, corresponding sections from each strip were combined and counted in the same scintillation vial. After the chromatography of a given reaction mixture, the percentage of radioactivity remaining at the origin (glutamate) was the same whether the strips were cut into sections. The sections were then counted individually in a 5-ml 2,5-diphenyloxazole-1,4-bis[2-(5-phenyloxazolyl)]benzene toluene scintillation system (4 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter of toluene) with a Nuclear-Chicago scintillation spectrometer model 723. After any given reaction mixture was chromatographed, 96% of the activity counted on the strip was found in either or both of two sections; one section extending from 5 mm below to 15 mm above the origin and the other section extending from 35 mm below to 5 mm above the solvent front. The identity of the compounds at the origin and solvent front were determined by chromatographing samples of unreacted 14C-a-ketoglutarate and 14C-L-glutamate. The 14C-glutamate, positively charged because of the addition of sulfuric acid, remained at the origin, whereas the 14C-a-ketoglutarate moved with the solvent front. In each reaction mixture, the percentage of 14C-a-ketoglutarate metabolized to 14C-glutamate was determined.
Fig. 2. Reciprocal plots with pyridoxal-P as the varied component and glutamate as the inhibitor. Conditions were the same as in Fig. 1, except that 4 mM tyrosine was added to each assay and the range of pyridoxal-P was 3.75 to 30 μM. Each tube contained highly purified apoenzyme. glu, glutamate.

Enzyme activity was eluted from the column as a single peak at the end of the intermediate salt concentration step. The peak of enzyme activity corresponded well with a protein peak and was purified about 1,700-fold over the 35,000 X g supernatant. Even without the addition of excess pyridoxal-P, the highly purified enzyme was quite stable in the presence of the sodium chloride with which it eluted. Table I gives a summary of the purification process. This procedure is simpler than others available and results in a good yield.

Apoenzyme was prepared by adding to the purified enzyme preparation sufficient tyrosine to give a final concentration of 5 mM (12). The enzyme was then dialyzed for 4 hours against 0.05 M potassium phosphate, pH 6.6. Enzyme activity was not detectable after dialysis. Addition of coenzyme, either pyridoxal-P or pyridoxamine-P, resulted in 100% recovery of the original activity.

Mathematical Considerations—$V_{\text{max}}$ was determined from the value of the ordinate intercept of a double reciprocal plot. All lines were drawn by the least squares procedure. The order of an enzymatic reaction defined in terms of the value of the Hill coefficient, $n$, is given by a form of the Hill equation.

\[
\log \left[ \frac{v}{V_{\text{max}} - v} \right] = n \log (S) - \log K
\]

which has been derived straightforwardly, considering Michaelis theory, by Atkinson, Hathaway, and Smith (13). In the ease of the determination of the noncompetitive interaction of inhibitor with enzyme, the form of the Hill equation used is

\[
\log \left[ \frac{v}{v_i - 1} \right] = n \log (I) + \log 1/K_i
\]

and this form of the equation has been considered by Wilson (14). This form of the Hill equation is a limiting form, valid only if a...
saturating concentration of the inhibitor gives 100% inhibition, of the more general equation.

$$\log \left( \frac{v_0 - v_i}{v_i - v_{sat}} \right) = n \log (I) + 1/K_i$$

In this case, the tyrosine aminotransferase is completely inhibited by saturation levels (10 μM) of p-hydroxymercuribenzoate, the compound used in the present studies. In these equations: $v_0$ or $v_i$ = initial velocity; $v_{sat}$ = initial velocity in the presence of a noncompetitive inhibitor; $V_{max}$ = maximal velocity; $(S)$ = concentration of substrates; $(I)$ = concentration of inhibitor; and $n$ refers to the order of the interaction of substrate or of inhibitor, as the case may be, with the enzyme.

**RESULTS**

**Product Inhibition**—Results of product inhibition aligned perfectly with the expected behavior of a binary system. As shown in Fig. 1, glutamate was a linear competitive inhibitor, when tyrosine was the varied substrate. Fig 2 depicts linear noncompetitive inhibition by glutamate when pyridoxal-P was the varied component of the reaction system. Similar results were obtained with equimolar amounts of pyridoxamine-P, if the apoenzyme was freshly prepared. As the apoenzyme aged, its activity with pyridoxamine-P as the coenzyme decreased quite rapidly. A corresponding decrease was not observed when the coenzyme of the “aged” apoenzyme was pyridoxal-P. Fig. 3 shows linear noncompetitive inhibition by glutamate when α-ketoglutarate was the varied substrate. Replots of the reciprocal velocity versus glutamate concentration at fixed concentration of the varied substrate were all linear. Replots of the slopes and intercepts of the graphs shown in Figs. 1 through 3 were also linear.

When tyrosine was the varied substrate, p-hydroxyphenylpyruvate was a linear noncompetitive inhibitor (Fig. 4). p-Hydroxyphenylpyruvate was also a linear noncompetitive inhibitor when pyridoxal-P was the varied component (Fig. 5). Fig. 6 shows linear competitive inhibition by p-hydroxyphenylpyruvate when α-ketoglutarate was the varied substrate. Replots of the slopes and intercepts of the graphs in Figs. 4 through 6 were also linear. Replots of the reciprocal velocity versus p-hydroxyphenylpyruvate concentration at fixed concentration of the varied substrate were all linear. However, at low p-hydroxyphenylpyruvate concentrations the replot for tyrosine was parabolic. Table II summarizes the modes of product inhibition and the results of various replots.

The $K_a$ values determined for tyrosine and α-ketoglutarate with the isotopic assay were $0.6 \times 10^{-4}$ and $1 \times 10^{-4}$, respectively. The $K_a$ value for pyridoxal-P was $2.9 \times 10^{-7}$. These values were in the range of values obtained by other methods (1). With the isotopic assay, the half-reaction with apoenzyme, pyridoxamine-P, and α-ketoglutarate was studied. The ability of the apoenzyme to catalyze this half-reaction decreased quite rapidly upon aging. The apoenzyme’s ability to catalyze the
Reciprocal plots with \( \alpha \)-ketoglutarate as the varied substrate and \( p \)-hydroxyphenylpyruvate as the inhibitor. Conditions were the same as in Fig. 4, except that each tube contained 3 mM tyrosine. \( \alpha \)-Ketoglutarate concentration ranged from 0.5 to 5 \( \mu \)M. \( p \)-Hydroxyphenylpyruvate concentration was held constant at 0.027 and 0.081 mM. HPP, \( p \)-hydroxyphenylpyruvate.

**TABLE II**

Summary of modes of inhibition and replots of slopes versus intercepts and replots of reciprocal velocity versus concentration of product inhibitor at fixed levels of varied substrate

Symbols are as follows: \( T \), tyrosine; \( \alpha \), \( \alpha \)-ketoglutarate; \( P \), pyridoxal-P; \( P' \), pyridoxamine-P; \( H \), \( p \)-hydroxyphenylpyruvate; \( G \), glutamate; NC, noncompetitive inhibition; C, competitive inhibition; L, linear plot; \( p \), parabolic plot.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>( H )</th>
<th>( G )</th>
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<tbody>
<tr>
<td>( T )</td>
<td>( \alpha )-ketoglutarate</td>
<td>Inhibition</td>
<td>Slope vs intercept</td>
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<tr>
<td>( \alpha )</td>
<td>( \alpha )-ketoglutarate</td>
<td>C</td>
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<td>( P )</td>
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<td>NC</td>
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<td>( P' )</td>
<td>( p )-hydroxyphenylpyruvate; G, glutamate</td>
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The other half-reaction, involving tyrosine and pyridoxal-P, decreased much more slowly in comparison.

Table III shows the activity of the apoenzyme at various pH values in the range from 5.5 to 8.4. Two reactions were studied: the half-reaction involving \( \alpha \)-ketoglutarate and pyridoxamine-P, and the complete reaction with tyrosine, \( \alpha \)-ketoglutarate, and pyridoxamine-P. The apoenzyme was freshly prepared when used in these experiments. The rate of the half-reaction at each pH was at least 90% of the rate of the complete reaction at the same pH. With aged apoenzyme, the pH profile was similar, however the rate of the half-reaction at each pH was 25% (or less) that of the complete reaction at the same pH.

**Interaction of Substrates**—The case for tyrosine is shown in Fig. 7. Under these conditions the concentrations of both pyridoxal-P and \( \alpha \)-ketoglutarate were saturating with respect to enzymatic activity. The average value of the slope from eight similar experiments is 1.00 ± 0.004 (standard error of the mean). Thus, the reaction is first order with respect to tyrosine. If the concentration of \( \alpha \)-ketoglutarate is lowered to one-half the \( K_m \) value, there is no change in the value of the Hill coefficient for interaction of tyrosine and enzyme although the \( V_{max} \) has been decreased by 50% or more.

A typical experiment is shown in Fig. 8 where the concentration of pyridoxal-P regulates the rate of reaction. In this case, the same pH.
Fig. 8. Application of the Hill equation in the case where the rate of the reaction is regulated by the concentration of pyridoxal-P. The concentration of coenzyme is expressed in micromolar and has a range between 0.12 and 4.0 μM in this particular experiment. The concentration of L-tyrosine is at a saturation level (4 × 10⁻⁴ M) as is the concentration of α-ketoglutarate (10⁻⁴ M). The slope in this experiment is 0.93. The V_max in this case was 47.2 nmoles of p-hydroxyphenylpyruvate formed per system in 5 min.

Fig. 9. Application of the Hill equation in the case where the rate of the reaction is regulated by the concentration of α-ketoglutarate which has a range between 0.5 and 10.0 × 10⁻⁴ M. The concentration of pyridoxal-P is at a saturation level (4 × 10⁻⁴ M) as is the concentration of L-tyrosine (4 × 10⁻⁴ M). The slope in this experiment has a value of 1.0. The V_max is 52.6 nmoles of p-hydroxyphenylpyruvate formed per 5 min.

Concentrations of L-tyrosine and of α-ketoglutarate are saturating. An average of the slopes of 12 similar experiments provides a value of 1.02 ± 0.4. Therefore, the reaction is also first order with respect to the coenzyme. When the same system is used except that the concentration of α-ketoglutarate is dropped to one-half the K_m concentration there is a marked reduction in the V_max and the double reciprocal plot becomes two-phase, one straight line at a lower concentration range of pyridoxal-P breaking off to another straight line at the higher concentration range. The line at the higher concentration levels of coenzyme intersects the abscissa giving a K_m value for the coenzyme in good agreement with experiments wherein the levels of the coenzyme alone are varied with saturating levels of tyrosine and α-ketoglutarate. Concentrations of both pyridoxal-P and α-ketoglutarate are at saturation levels.

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The average value for the slopes in 18 similar experiments is 1.03 ± 0.4. When the concentration of tyrosine is reduced to a level of one-half the K_m value, in this experiment, while the pyridoxal-P concentration is held at the same saturating level and the concentration of α-ketoglutarate is varied, the double reciprocal plot still follows Michaelis-Menten kinetics and the value of the slope of the Hill plot remains at 1.0 (1.04 ± 0.12) in four experiments. When the concentration of pyridoxal-P is reduced to one-half the K_m value, while the concentration of tyrosine is at a saturation level and the concentration of α-ketoglutarate is varied, the slope of the Hill plot remains at 1.0 (1.09 ± 0.07) in four experiments. When the concentration of both pyridoxal-P and tyrosine are reduced to values equivalent to one-half their K_m values and the concentration of α-ketoglutarate is varied, Michaelis-Menten kinetics are adhered to in double reciprocal plots and the value of the slope of the Hill plot remains at 1 (1.11 ± 0.05) in four experiments. Thus, the analysis with the Hill equation shows that the enzymatic reaction is first order with respect to
tyrosine, pyridoxal-P, or \( \alpha \)-ketoglutarate. These data and previous observations (1, 3) lead to a concept of the reaction wherein the combination of enzyme with each substrate is first order and cooperativity is not observed. This is particularly interesting in view of the probable tetrameric structure of the enzyme and data (4) suggesting conformational changes (5-7).

Further application of the Hill equation afforded a measure of the order of interaction of \( p \)-hydroxymercuribenzoate and \(-SH\) groups required for enzymatic activity. The use of the Hill equation for this measurement was based upon the noncompetitive inhibition of tyrosine aminotransferase activity by \( p \)-hydroxymercuribenzoate depicted in Fig. 10. The result obtained after analysis of the inhibition by the Hill equation is shown in Fig. 11. In seven similar experiments the average value of the Hill coefficient was 1.13 \( \pm \) 0.02. Attempts were made to learn if additional \(-SH\) groups that could react with \( p \)-hydroxymercuribenzoate might appear after prior incubation of the enzyme with 1 \( \mu \)M mercaptoethanol or if cooperative effects were involved. Following this treatment atypical Hill plots were obtained after incubation with \( p \)-hydroxymercuribenzoate and these consisted of a major portion of maximal negative slope with minor appendages of much lesser slope at either end of the curve. This atypical behavior was caused by a change in mode of inhibition by \( p \)-hydroxymercuribenzoate from the noncompetitive to a mixed type. The average slope of the major portion of the curve was 2.8 \( \pm \) 0.3 while those of the minor appendages were always less than one in nine experiments. These data could suggest that prior incubation with mercaptoethanol exposes additional \(-SH\) groups that are susceptible to the action of \( p \)-hydroxymercuribenzoate and that may participate in the activity of the enzyme. However, because of the mixed mode of inhibition in this case, such a conclusion must be viewed with caution.

A previous derivation of the rate law for the over-all reaction

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EP \rightleftharpoons P^\prime + G
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P^\prime \rightleftharpoons P^\alpha
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PT, PG, P'H, P^\alpha
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was based in part upon the assumption that the coenzyme is freely dissociable from the enzyme, the interconversions of central complexes are the sole rate-limiting steps, and hence the dissociation of coenzyme from holoenzyme was not a rate-limiting step in the consideration of initial velocity (2). We attempted to measure the rate of this dissociation as follows. Freshly isolated holoenzyme was stripped of coenzyme as described under "Materials and Methods." The purified apoenzyme was treated with a slight excess of $^3$H-pyridoxamine-P, on the basis of 4 moles of coenzyme per mole of apoenzyme, incubated 2 hours at 4°C, and the enzyme mixture was passed over Sephadex G-25 to remove unbound coenzyme. Unfortunately, the holoenzyme was inactivated under these conditions. The rate of dissociation would have been measured by equilibrium dialysis. In control experiments, equilibrium dialysis of $^3$H-pyridoxamine-P showed that only 36% of the labeled coenzyme permeated the membrane. It appeared that the anionically charged cofactor was avidly bound by the membrane and several treatments of the membrane failed to reduce this problem. The results also were uniformly disappointing regardless of the concentration of phosphate, temperature, or compartment size of the equilibrium dialysis cell. Thus, it was not possible, for technical reasons, to evaluate the rate of dissociation of cofactor by this experimental approach.

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