Purification and Properties of Tyrosine-activating Enzyme of Hog Pancreas*  

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A reaction between ATP and amino acids, which may be the first stage in protein synthesis, has been called amino acid activation by Hoagland (1). The proposed mechanism (2) of this reaction is shown in Equation 1.

\[
\text{ATP} + \text{amino acid} + \text{enzyme} \rightarrow [\text{enzyme-amino acyl-AMP}] + \text{P-P}^{1}  
\]

Equation 1

Activation reactions of this type are catalyzed by enzymes from various sources (2-6) and specific activating enzymes may be required for each amino acid (2). The first highly purified, activating enzyme was a tryptophan-activating enzyme from beef pancreas, studied by Davie et al. (7). The isolation and properties of a tyrosine-activating enzyme from hog pancreas (8) are described in this report.

EXPERIMENTAL

The hydroxamic acid assay was similar to that described previously (5). The reaction mixture contained 5 μmoles of 1-tyrosine; 30 μmoles of dipotassium ATP (Pabst Laboratories), adjusted to pH 7.5 with potassium hydroxide; 30 μmoles of magnesium chloride; 100 μmoles of Tris buffer, pH 7.5; 3000 μmoles of hydroxylamine hydrochloride, adjusted to pH 7.0 with potassium hydroxide just before use; approximately 60 μg. of purified enzyme; and water to a final volume of 3.0 ml. The mixture was shaken in a Dubnoff metabolic shaker for 1 hour at 37°.

The reaction mixture for the P-P exchange assay contained 1 μmole of tyrosine; 10 μmoles of dipotassium ATP (crystalline, Pabst Laboratories), adjusted to pH 7.5 with potassium hydroxide; 10 μmoles of magnesium chloride; 100 μmoles of Tris buffer, pH 7.5; 3000 μmoles of hydroxylamine hydrochloride adjusted to pH 7.0 with potassium hydroxide just before use; approximately 60 μg. of purified enzyme; and water to a final volume of 1.0 ml. The mixture was shaken in a Dubnoff metabolic shaker for 1 hour at 37°.

The reaction mixture for the P-P exchange assay contained 1 μmole of tyrosine; 10 μmoles of dipotassium ATP (crystalline, Pabst Laboratories), adjusted to pH 7.5 with potassium hydroxide; 10 μmoles of magnesium chloride; 100 μmoles of Tris buffer, pH 8.5 with potassium hydroxide; 5 μmoles of radioactive potassium P-P containing approximately 200,000 c.p.m.; approximately 30 μg. of purified enzyme; and water to make a final volume of 1.0 ml. The mixture was incubated at 37° for 10 minutes. The radioactivity in ATP was determined as before (5). The percentage of exchange and conversion to 37° for 10 minutes. The radioactivity in ATP was determined as before (5). The percentage of exchange and conversion to

1 The abbreviations used are: P-P, pyrophosphate; Tris, tris-(hydroxymethyl)aminomethane.

2 The amount of enzyme activity varies somewhat with the acetone powder used. Therefore, in some cases, as much as 40 gm. of acetone powder were treated as described to obtain a fraction I containing 4 units per ml.

3 The Tris buffer was adjusted to pH 7.5 at 20°.
Fraction II—To the clear supernatant (220 ml.) was added 40 ml. of calcium phosphate gel. The gel had previously been centrifuged and the supernatant discarded to minimize dilution of the enzyme extract. The slurry was adjusted to pH 7.5 and stirred gently for 30 minutes. It was then centrifuged at 5600 X g for 15 minutes. The supernatant solution was discarded. The gel was suspended in 110 ml. of 0.2 m potassium phosphate buffer, pH 8.1, and homogenized in a Potter-Elvehjem homogenizer if any lumps were apparent. The mixture was stirred for 30 minutes and then centrifuged for 60 minutes at 70,000 X g. The slurry was centrifuged as before and the supernatant discarded. The gel was then mixed with 110 ml. of 2.0 m potassium phosphate buffer, pH 8.1, and homogenized in a Potter-Elvehjem homogenizer if any lumps were apparent. The mixture was stirred for 30 minutes and then centrifuged for 60 minutes at 70,000 X g. The gel was discarded and the supernatant dialyzed with stirring for 18 hours against two changes of 21. of 0.02 M Tris buffer, pH 7.5.

Fraction III—To the dialyzed supernatant (200 ml.) were added 120 gm. of solid ammonium sulfate. The pH was maintained at 8.0 (measured on a 1:5 dilution) by the addition of cold N potassium hydroxide. After standing for 2 to 3 hours, the mixture was centrifuged and the supernatant discarded. The precipitate was dissolved in 10 ml. of 0.1 M Tris buffer, pH 7.5, and dialyzed overnight against 1 l. of 0.02 M Tris buffer, pH 7.5.

Fraction IV—The clear solution, after dialysis, was adjusted slowly to pH 5.3 with ice-cold N acetic acid. The solution was centrifuged at once and the mud-colored precipitate discarded. The supernatant was adjusted immediately to pH 7.5 by addition of cold N potassium hydroxide. Sufficient M Tris buffer, pH 7.5, was added so that the final buffer concentration was 0.12 M. To the solution (60 per cent saturation) was added powdered ammonium sulfate (5.4 gm. to 15 ml.). The mixture was centrifuged after 10 minutes. The supernatant was taken to 70 per cent saturation by the further addition of 1.12 gm. of ammonium sulfate. After standing for 2 hours, the mixture was centrifuged and the precipitate dissolved in 4 ml. of 0.1 M Tris buffer, pH 7.5, and dialyzed overnight against 500 ml. of 0.02 M Tris buffer, pH 7.5.

Fraction V—Usually two batches of enzyme Fraction IV were combined for the following ethanol fractionation. To 6.2 ml. of enzyme Fraction IV was added 0.7 ml. of 0.4 M magnesium chloride. The solution was placed in an ice-salt bath and 1.5 ml. of absolute ethanol at −15° were added slowly, with stirring. The mixture was centrifuged at −10° for 10 minutes at 10,000 X g. The precipitate was dissolved in 2 ml. of 0.02 M Tris buffer (Fraction Vₐ). An additional 1.45 ml. of ethanol were added to the supernatant, and this precipitate was collected and taken up in the same way (Fraction Vₐ). The last fraction contained the most highly purified enzyme. The supernatant was discarded, and the two active fractions were dialyzed as usual. Fraction Vₐ was saved, recombined with other batches, and fractionated with ethanol again for a further recovery of highly purified enzyme. The various enzyme fractions were stored at −15°.

**RESULTS**

**Enzyme Purification**—As shown in Table I, an approximately 400-fold purification was obtained with a yield of 21 per cent. This is actually a low estimate of the yield, since refractionation of Fraction Vₐ yields more enzyme of high specific activity. A key step is the retention of the enzyme on the gel during the 0.2

**TABLE I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein content</th>
<th>Total activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml.</td>
<td>mg/ml.</td>
<td>units</td>
<td>Yield Hydroxamid</td>
</tr>
<tr>
<td>I. Acetone powder extract</td>
<td>220</td>
<td>52.0</td>
<td>1255</td>
<td>100</td>
</tr>
<tr>
<td>II. Calcium phosphate gel eluate</td>
<td>180</td>
<td>0.84</td>
<td>613</td>
<td>67</td>
</tr>
<tr>
<td>III. 1st ammonium sulfate precipitate</td>
<td>14.5</td>
<td>8.6</td>
<td>520</td>
<td>56</td>
</tr>
<tr>
<td>IV. 2nd ammonium sulfate precipitate</td>
<td>5.8</td>
<td>6.1</td>
<td>435</td>
<td>47</td>
</tr>
<tr>
<td>Vₐ Ethanol precipitate</td>
<td>1.5</td>
<td>4.4</td>
<td>198</td>
<td>21</td>
</tr>
</tbody>
</table>

* In the hydroxamid assay, no enzyme activity was observed without added tyrosine, except with Fraction I. The data for this fraction were obtained by removing residual tyrosine as described previously (3). In the P-P exchange assay, the enzyme activity without added tyrosine was less than 5 per cent of the complete system with Fractions IV and V and varied from 10 to 15 per cent with Fraction II. These blanks have not been subtracted from the values of the least column.

**FIG. 1. Ultracentrifuge patterns of tyrosine-activating enzyme.** The photographs show enzyme of specific activity = 30 at the times indicated up to 160 minutes, moving from right to left. The conditions were: temperature, 2.7°; enzyme concentration, 4 mg. per ml. in 0.2 M Tris buffer, pH 7.5; rotor speed, 50,760 r.p.m.
Fig. 2. Effect of enzyme concentration on activity. Standard assay conditions were used (see “Experimental”) and the data are given in micromoles per reaction tube. The P-P exchange results (A) were calculated from the usual 10-minute assay with the use of the amounts of enzyme shown. Enzyme of specific activity = 11.0 was used in both assays.

Fig. 3. Time course of tyrosine activation. Standard assay conditions were used. For the P-P exchange, 40 µg. of enzyme of specific activity = 15.2 were used; for hydroxamide formation, 155 µg. of the same preparation.

Fig. 4. Effect of tyrosine concentration on the rate of P-P exchange. Standard assay conditions were used with 15 µg. of enzyme of specific activity = 28.0.

although the tyrosine-activating enzyme from this tissue is also more stable to storage than the other activating enzymes.5

Enzyme Purity—Studies in the Spinco analytical ultracentrifuge6 showed a single peak (Fig. 1), which moved with a sedimentation constant (s20, w) of 4.2 S. Enzyme recovered from the cell at the end of a 3-hour centrifugation at 2.7° was fully active.

The enzyme also has been studied with the use of paper electrophoresis. Only one component was observed with the most highly purified material when used in 0.02 M phosphate buffer, pH 7.5, for 12 hours at 5 volts per cm. The enzyme does not move under these conditions, probably because of adsorption to the paper. Electrophoresis at pH 9 gave a single component which spread rapidly during the experiment, and moved toward the anode.


6 The authors are indebted to Dr. Jerome Vinograd and Dr. Paul Ts'io, California Institute of Technology, for these analytical ultracentrifuge studies.

No P-P exchange in the absence of amino acids, or with amino acids other than tyrosine, was detected. Adenylic kinase, ATP-hydrolyzing enzymes, and pyrophosphatases were not detected at enzyme concentrations used in the assay. However, ribonuclease was present even in the best preparations. The 260:280 absorbance ratio was 1.7, suggesting that no nucleotide-containing material was present.

Enzyme Characteristics—The enzymatic activity was proportional to protein concentration and linear with time under the standard assay conditions (Figs. 2 and 3). In the hydroxamide assay, the rate decrease after 1 hour is the result of inhibition by P-P, a product of the reaction.

Figs. 4-6 show curves of reaction rate with increasing concentrations of each of the three substrates in the P-P exchange reaction. Straight line plots were obtained by the Lineweaver-Burk method (14) and the following Kₘ values calculated: tyrosine, 6 × 10⁻⁵ M; P-P, 3 × 10⁻⁵ M; ATP, 5 × 10⁻³ M. The optimal Mg ion concentration was equimolar with the ATP concentration at all ATP levels. The Mg ion concentrations were the same as the ATP concentrations in the experiments shown in Fig. 5.

The insensitivity of the hydroxamide assay does not permit accurate determination of Michaelis constants. However, for ATP and tyrosine it seems that the results would be similar to the P-P exchange. For example, the rate of hydroxamide formation decreased at ATP concentrations below 0.01 M. The affinity for tyrosine was much higher, since an amount of enzyme which formed 2 µmoles of hydroxamide per hour in the standard assay with a saturating concentration of tyrosine, formed 1.83 µmoles of hydroxamide in 1 hour, when the initial amount of tyrosine was only 2 µmoles. The rate of the hydroxamide reaction increased linearly with hydroxylamine concentration up to about 1.0 M. This concentration has been chosen for the standard assay, although higher levels gave somewhat greater activity. Hydroxamide formation was inhibited by P-P, as previously reported (5, 7). The addition of 1.0 µmole of P-P per ml., initially, inhibited hydroxamide formation 28 per cent. The P-P exchange...
alanine, proline, serine, threonine, tryptophan, and valine. 

Inhibition here is considerably less than with the tryptophan-activating enzyme studied by Davie et al. (7).

The pH optimum for maximal hydroxamide formation is at 7.3, whereas the maximal P-P exchange rate was obtained at pH 8.5 (Fig. 7). Glycine buffer was employed in the standard P-P assay because 0.1 m Tris gave 10 to 15 per cent lower P-P exchange (Fig. 7).

**Substrate Specificity**—Tyrosine analogues have been tested for activity with the purified enzyme and the following compounds were inactive in both assays when tested at the same concentration as tyrosine: 15 other common amino acids, n-tyrosine, 2,5-dihydroxyphenylalanine, 3,4-dihydroxyphenylalanine, 3-amino tyrosine, 3-nitrotyrosine, 3-nitrotyrosine, p-fluorophenylalanine, o-tyrosine, m-tyrosine, N-chloroacetyltyrosine, 3,5-diiodotyrosine, and tyrosine amide. The only compound tested which acted as a substrate was L-3-fluorotyrosine. Even this compound, which resembles tyrosine so closely, was not as effective as tyrosine. In the hydroxamide assay (Table II), fluorotyrosine approached the maximal rate obtained with tyrosine, but only at higher concentrations. However, in the P-P exchange assay, fluorotyrosine gave only 50 per cent of the maximal rate with tyrosine. It seems from these results that almost any substitution on the benzene ring yields an inactive compound. Further, the free amino group in the L-configuration seems necessary for activity. Further, the free amino group in the L-configuration seems necessary for activity. However, it would be of interest to test p-hydroxyphenyllactic acid and p-hydroxyphenylserine in connection with side-chain specificity.

**Effects of Inhibitors**—None of the compounds listed above showed any appreciable inhibition at the same concentration as tyrosine, except tyrosine amide. This compound inhibited hydroxamide formation by 30 per cent. Tyramine, however, which cannot be a substrate, was the best inhibitor of the compounds tested. Tyramine inhibited P-P exchange 80 per cent at a concentration of 2 × 10⁻⁴ M and hydroxamide formation 50 per cent at 6 × 10⁻⁴ M. If the tyrosine concentration of the standard assay was doubled, the inhibition decreased to 50 and 35 per cent, respectively. Phenylethylamine did not inhibit either reaction. The structural requirements for inhibitors which are substrate analogues, also seem to show a high degree of specificity.

In contrast to P-P, phosphate at 0.01 M final concentration was not inhibitory in either assay, and at 0.1 M, inhibited hydroxamide formation 30 to 35 per cent and P-P exchange 20 per cent. Chloramphenicol did not inhibit at 0.01 M. The P-P exchange reaction was inhibited 28 per cent by 5 × 10⁻⁴ M tested.

### Table II

**Comparison of tyrosine and fluorotyrosine as enzyme substrates**

<table>
<thead>
<tr>
<th>Concentration of substrate</th>
<th>Hydroxamide formed</th>
<th>P-P exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tyrosine</td>
<td>Fluoro-tyrosine</td>
</tr>
<tr>
<td>µmoles/ml</td>
<td>µmoles/br.</td>
<td>µmoles/br.</td>
</tr>
<tr>
<td>0.5</td>
<td>---</td>
<td>1.5</td>
</tr>
<tr>
<td>1.0</td>
<td>2.1</td>
<td>1.5</td>
</tr>
<tr>
<td>1.6</td>
<td>2.1</td>
<td>1.8</td>
</tr>
<tr>
<td>2.0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3.0</td>
<td>2.1</td>
<td>2.0</td>
</tr>
<tr>
<td>3.3</td>
<td>2.1</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* The ratio is the activity with fluorotyrosine divided by the activity with tyrosine at the same concentration. The dashes indicate that experiments with these concentrations were not done.

![FIG. 7. Effect of pH on the rate of enzyme activity. Standard assay conditions were used and the data are given as micromoles per hour. For the hydroxamide assay (---), 0.9 µg. of enzyme of specific activity = 25.0 were used. The P-P exchange results (---) were calculated from the standard 10-minute assay with 9.0 µg. of the same enzyme.](http://www.jbc.org/)

![FIG. 8. Effect of cation concentration on the rate of P-P exchange. The ATP, P-P, and tyrosine were used as the Tris salt (see "Experimental"), and the cation was added as the chloride in the indicated amount. Other conditions were standard, with the use of 15 µg. of enzyme of specific activity = 25.0.](http://www.jbc.org/)

![FIG. 9. Effect of extra salt on the rate of P-P exchange. The reaction mixture was the same as given in Fig. 8 with 0.05 M KCl added in all tubes. An additional amount of the appropriate chloride was added as shown by the bars. Other conditions were standard, with the use of 40 µg. of enzyme of specific activity = 20.0.](http://www.jbc.org/)
The tyrosine-activating enzyme described here, because of its purity and stability, should provide an excellent tool for the more detailed study of the general mechanism of carboxyl-group activation. The nature of the active groups at the enzyme site, the chemistry of the amino acid-AMP-enzyme complex, and the equilibrium constant, are problems which can be studied with the purified enzyme. A comparison of the differences among the various amino acid-activating enzymes would also be of interest. For example, this enzyme contains little or no nucleotide-containing material, in contrast to the tryptophan-activating enzyme (7). This finding also indicates that the reactions studied here do not require the presence of ribonucleic acid, in contrast to the recent report of the participation of ribonucleic acid in alanine activation (17). An estimate of the turnover number of the P-P exchange reaction can be made from the sedimentation constant. This value is very approximately 400 moles per mole of enzyme per minute, which is lower than that for many enzymatic reactions.

SUMMARY

Methods for the isolation of a tyrosine-activating enzyme are described. The physical and enzymatic criteria cited indicate that the best preparations are nearly pure. The only compound which served as a substrate, in addition to tyrosine, was 3-fluorotyrosine. Tyramine was the best inhibitor among the compounds tested. Two cations, K and Mg, were required for enzymatic activity. Differences in the formation of tyrosine hydroxylamine and pyrophosphate exchange are discussed in relation to the mechanism of the reaction.

REFERENCES


p-chloromercuribenzoate. Tryptophan-activating enzyme (7) and activating enzymes from guinea pig liver are more sensitive to inhibition by this reagent. The relative insensitivity of the tyrosine enzyme from pancreas may be related to its stability to storage, since the most stable activating enzymes from guinea pig liver (tyrosine and threonine) are also inhibited the least by SH inhibitors.

Requirements for Cations—No enzyme activity was observed in the absence of added Mg ion. Of more interest is the requirement for K ion shown in Fig. 8 for the P-P exchange reaction. The K ion requirement for the hydroxylamine reaction was observed also when salt-free hydroxylamine (see "Experimental") was used (6). No such requirement has been found for other activating enzymes (5). Although Rb ion (Fig. 8) is about 65 per cent as active as K ion at low concentrations, the superiority of K ion is shown at higher salt concentrations. When 0.2 M salt is added to a system containing a suboptimal amount of K various amino acid-activating enzymes would also be of interest.

Recently, it has been shown that P-P exchange can be made from the sedimentation constant.

The K ion requirement for the hydroxylamine reaction was observed only when added extra K ion (0.05 M), only extra K ion stimulates (Fig. 9). Other monovalent cations either have no effect or are inhibitory.

Relationship of P-P Exchange and Hydroxylamine Formation—The two activities, P-P exchange and hydroxylamine formation, are probably catalyzed by the same enzyme. This is indicated by the substrate specificity, salt requirement and other properties of both reactions, and by the constant activity ratio of 10: 1 in favor of P-P exchange shown at various stages of purification (Table 1). This ratio varies widely for different enzymes and seems to be a characteristic of the particular amino acid-activating enzyme studied (2, 3, 5, 7). The two reactions, however, differ quantitatively in many ways. In addition to the much smaller amount of P-P than of hydroxylamine needed for obtaining maximal reaction rates, the two reactions differ in their pH optima, the effectiveness of 3-fluorotyrosine as substrate and tyramine as inhibitor, and in their sensitivity to excess salt (5). The available evidence suggests that enzyme-bound, amino acid-AMP (see Equation 1) is the intermediate in both reactions (2, 15, 16) and, therefore, the difference in the two reactions may reside in the reaction of the enzyme-amino acyl-AMP intermediate with P-P vescus hydroxylamine. It is possible that hydroxylamine formation occurs as a result of a nonenzymatic reaction between hydroxylamine and a small amount of free amino acid-AMP dissociated from the enzyme by high concentrations of hydroxylamine. Differences in the ratio of P-P exchange to hydroxylamine formation which have been reported for different activating enzymes (2-5) might then be due to differences in dissociation of amino acid-AMP from the various enzymes.

SUMMARY

The tyrosine-activating enzyme described here, because of its purity and stability, should provide an excellent tool for the more detailed study of the general mechanism of carboxyl-group activation. The nature of the active groups at the enzyme site, the chemistry of the amino acid-AMP-enzyme complex, and the equilibrium constant, are problems which can be studied with the purified enzyme. A comparison of the differences among the various amino acid-activating enzymes would also be of interest. For example, this enzyme contains little or no nucleotide-containing material, in contrast to the tryptophan-activating enzyme (7). This finding also indicates that the reactions studied here do not require the presence of ribonucleic acid, in contrast to the recent report of the participation of ribonucleic acid in alanine activation (17). An estimate of the turnover number of the P-P exchange reaction can be made from the sedimentation constant. This value is very approximately 400 moles per mole of enzyme per minute, which is lower than that for many enzymatic reactions.
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