Studies on Phosphoserine Aminotransferase of Sheep Brain*

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SUMMARY

Phosphoserine aminotransferase has been purified about 500-fold from sheep brain extracts by ammonium sulfate precipitation and Sephadex and diethylaminoethyl cellulose chromatography. On ultracentrifugation, the enzyme migrated in a single peak with a sedimentation constant of 4.2 S. The molecular weight determined by the Trautman modification of the Archibald method is 96,000. The spectrum of the aminotransferase shows an absorption peak at 415 mp. The enzyme could be inactivated by dialysis against cysteine, and the activity was restored upon incubation with pyridoxal phosphate. This and other evidence suggest that the latter is a coenzyme for this enzyme.

The enzyme utilizes glutamic acid preferentially for transamination. The reaction rate with alanine as amino group donor is about 10% of that of glutamate. The optimum activity is at pH 8.15. In characterizing the enzyme, the transamination reaction was followed in both directions and the stoichiometry, the equilibrium constant, and the Michaelis constants for 3-phosphohydroxypyruvate (0.25 mm) and glutamate (0.7 mm) have been determined.

This paper presents the results of a study of the purification and properties of the enzyme phosphoserine aminotransferase from sheep brain. Bridgers (1) has shown that, in brain, serine appears to be synthesized exclusively by the pathway proposed by Ichihara and Greenberg (2). In view of the more limited metabolic processes occurring in brain cells than in liver, this tissue appeared to be advantageous for the isolation and study of the properties of the enzymes of the phosphorylated pathway in the mammal. No reports have appeared of the preparation of highly purified phosphoserine aminotransferase from any source.

EXPERIMENTAL PROCEDURE

Materials Hydroxypyruvic acid phosphate dimethylketal, O-phospho-L-serine, L-serine-3-14C, crystalline lactate dehydrogenase, and glutamate dehydrogenase were obtained from Calbiochem. Alkaline phosphatase was purchased from Nutritional Biochemicals. The various resins and other chemicals employed in this work were commercial preparations purchased from reliable suppliers.

The phosphohydroxypyruvate was prepared by hydrolysis of the hydroxypyruvic acid phosphate dimethylketal according to the procedure of Ballou (3). Incubation at 40° was carried out for only 24 hours, which gives a 50% yield of the phosphohydroxypyruvate. In this time period only a trace of inorganic phosphate was found in the hydrolysate. Reaction with lactate dehydrogenase indicated that less than 1% of free hydroxypyruvate was present. O-Phospho-L-serine-3-14C was prepared from the labeled serine and purified as described by Neuhaus and Byrne (4).

Enzyme Assay—The enzyme activity was determined quantitatively by coupling the phosphoserine aminotransferase with glutamate dehydrogenase according to the following equations.

\[
P\text{-hydroxypyruvate} + \text{glutamate} \rightarrow \text{serine-P} + \alpha\text{-ketoglutarate} \quad (1)
\]

\[
\alpha\text{-Ketoglutarate} + \text{NH}_4^+ + \text{NADH} \rightarrow \text{glutamate} + \text{NAD}^+ + \text{H}_2\text{O} \quad (2)
\]

The decrease in NADH absorption at 340 mp was measured at 25° in a Gilford recording spectrophotometer. The incubations were performed in quartz cuvettes with a 1-cm light path. For standard activity tests, the assay mixture contained 1.25 micromoles of hydroxypyruvate, 20 micromoles of glutamate, 10 micromoles of NaF, 80 micromoles of ammonium acetate, 0.5 micromole of NADH, 0.2 mg of glutamate dehydrogenase, and 0.02 to 0.2 ml of the enzyme preparation in 0.05 M Tris buffer, pH 8.2, in a total volume of 2.5 ml. The temperature was maintained at 25° by means of a thermostatted cuvette holder.

Specific activity is expressed in enzyme units per mg of protein. Protein concentrations were determined by absorption at 280 and 260 mp (5).

Corrections for other enzyme activities than the phosphoglycerate dehydrogenase that utilized NADH or decomposed the substrate were made from blank runs containing no glutamate or phosphohydroxypyruvate and subtracting the decrease in the absorption at 340 mp from the total decrease. An illustrative example is shown in Fig. 1. The figure also establishes the linearity of the rate of the reaction under the condition of the assay.

In other experiments, the linearity of the response of enzyme activity to enzyme concentration was shown to hold over a 5-fold range of concentration.

Chromatographic Identification—Formation of serine-P was shown qualitatively by paper chromatography of aliquots of deproteinized incubation mixtures on Whatman No. 1 paper developed with 90% C_6H_5OH-34% NH_3 (77:33). This solvent gives a clear separation of serine-P, glutamate, and serine (R_F = 0.02, 0.15, 0.31, respectively). Increasing the incubation time

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from 10 to 30 min yielded a progressive increase in the serine-P
formed.

**ENZYME PURIFICATION**

**Homogenization**—Fresh brains (250 g) were washed with cold
distilled water and homogenized in batches of about 50 g each
in a Waring Blender at low speed for 5 min in 60 ml of an ice-cold
solution of 0.154 M KCl-0.01 M MgCl₂. The homogenate was
then centrifuged at 35,000 × g, and the supernatant liquid was
dialyzed for 5 hours against 4 liters of 0.1 M Tris-HCl buffer, pH
7.2. All operations in the purification were carried out in the
cold.

**Ammonium Sulfate Fractionation**—Solid ammonium sulfate
was added with stirring to the dialysate to 45% saturation (277 g
per liter). The mixture was then mechanically stirred for 1 hour
in the cold and then centrifuged at 35,000 × g for 20 min. The
supernatant liquid was decanted and more solid ammonium
sulfate was stirred in to give 65% saturation (430 g per liter).
This was equilibrated by continued stirring and was centrifuged
as described above.

The precipitate from this step was collected, redissolved to a
volume of 40 ml with 0.05 M Tris-HCl buffer (pH 7.2), and dia-
yzed overnight against 2 liters of the same buffer.

**DEAE-cellulose Column Chromatography**—A DEAE-cellulose
column (30 × 2.5 cm), prepared as described by Peterson and
Sobey (6), was equilibrated with 0.05 M Tris-HCl buffer, pH 7.2.
The red enzyme solution was pipetted onto the absorbent bed,
and the column was then washed with 150 ml of the 0.05 M
Tris-HCl buffer at a rate of 1 ml per min. At this ionic strength
the red pigment was not absorbed and was washed out by the
eluting fluid. The column was then successively eluted with 100
ml each of 0.1, 0.2, and 0.4 Tris-HCl buffer, pH 7.2. The 0.4 M
buffer eluate was saved and the others were discarded. Solid
ammonium sulfate was added to this solution to 70% saturation,
and the mixture was stirred for ½ hour more. The protein
precipitate was then isolated by centrifugation at 50,000 × g
for 15 min, dissolved in a small volume of the 0.05 M Tris-HCl
buffer, and dialyzed for 2 hours against 2 liters of the same buffer.

An ultracentrifugal run of this preparation in a Beckman model
E instrument yielded a schlieren pattern with three peaks: one
main peak with a sedimentation coefficient of 4.2 S, a second peak
which sedimented about twice as fast, and a third small peak of a
very high molecular weight substance. The latter gave the
protein solution a slight opacity. This was eliminated by cen-
trifuging the solution in a Spinco preparative ultracentrifuge at
100,000 × g for 1 hour.

**Sephadex Fractionation**—The enzyme solution was introduced
onto a Sephadex G-150 column (36 × 1.5 cm) that had been
equilibrated with 0.05 M Tris-HCl buffer, pH 7.2. Elution was
performed with the same buffer at a flow rate of 3 ml/10 min.
Fractions of 3 ml were collected. The enzyme activity appeared
in Fractions 12 to 22 after the void volume. The fractions of
highest activity were used for the study of the enzyme properties.
The other fractions were pooled and stored by freezing. A
summary of the purification procedure is given in Table I.

**EXPERIMENTAL RESULTS**

No significant effects on the enzyme activity were observed
with either Mg²⁺ or K⁺ (0.1 M).

**Homogeneity and Molecular Weight**—The homogeneity of the
enzym e preparation was indicated by a single symmetrical
sedimentation peak in the ultracentrifuge (Fig. 2).1

The molecular weight was estimated by the Trautman modifi-
cation (7) of the Archibald approach to equilibrium method.
Determinations were carried out with a 0.7% enzyme solution in
0.05 M Tris-HCl buffer, pH 7.2. Sedimentation was performed in
a double sector cell at 50,740, 39,460, and 29,500 rpm at 20°C.
Pictures were taken with schlieren optics as soon as the operating
speed was attained at 2- or 4-min intervals until the peak was
fully sedimented out and the concentration at the meniscus
became zero. A drawing of each picture was taken with a 10-fold
magnifying Nicon microcomparator. The height of the gradient
was obtained from the slope of a Trautman plot of ΔCm against qm (Fig. 3). The slope is represented by the following equation.

\[ M_w = \frac{(q_mV_p)\Delta t}{(1 - V_p)C_0} \]

\[ q_m \] is derived from the following equation.

\[ q_m = \frac{R.T. \cdot (dc/dx)_n}{\alpha^2 \cdot q_m} \]

1 An acrylamide gel electrophoresis determination showed the presence of two bands, the faster migrating one being much larger
than the slower one. This could represent the more negatively
charged holoenzyme and a smaller amount of apoenzyme from
which the pyridoxal-P had been disassociated. Evidence for
this is being sought.
In the above, $C_0$ equals the intercept on the $C_n$ axis, $M_n$, the weight average molecular weight, $(dc/dx)$ the concentration gradient, and $r_m$, the distance of the gradient from the rotation center. The other symbols have their usual meaning. Evaluation of the numerical data yielded a mean molecular weight of 96,000 for the serine-P aminotransferase.

**pH Activity**—The pH activity curve in Fig. 4, obtained as described in the legend, shows that serine-P aminotransferase has a relatively sharp optimum peak at pH 8.15. Optimum activity of glutamate dehydrogenase extends over the range of pH 7.8 to 8.4 (8). Thus its activity is virtually independent of pH over the range used for the study of serine-P aminotransferase.

**Michaelis Constants**—The samples for analysis were prepared as described in Fig. 4 except that the concentrations of phosphohydroxypyruvate or glutamate were varied from 0.05 to 1.2 mm. The pH was maintained at 8.2.

$K_m$ values were derived from Lineweaver-Burk plots (Figs. 5 and 6). The figures obtained were 0.25 and 0.7 mm for phosphohydroxypyruvate and glutamate, respectively.

**Stoichiometry of Reaction**—This was determined both for the forward and backward reactions. The incubation mixture for the forward reaction is described in Fig. 4. The pH was maintained at 8.2. At the end of incubation, the residual phosphohydroxypyruvate was determined on aliquots by adjusting the pH to 10.5 and incubating the sample with 1 mg of alkaline phosphatase for 20 min. The pH was then lowered to 7.5 and NADH and lactic dehydrogenase were added and allowed to react with the hydroxypyruvate. The concentration of the latter was calculated from the decrease in absorption at 340 nm.

The stoichiometry for the reverse reaction was determined with serine-P-3-14C in a manner similar to that for the forward reaction. The procedure is described in the legend to Table III.
PHOSPHOHYDROXYPYRUVATE

\[ \frac{d[\text{P-O-P}]}{dt} = \frac{d[\text{Glu}]}{dt} = \text{Rate} \]

Fig. 6. Concentration activity curve and Lineweaver-Burk plot of utilization of glutamic acid.

**TABLE II**

**Stoichiometry of forward reaction**

<table>
<thead>
<tr>
<th>System</th>
<th>Zero time</th>
<th>After incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-O-pyr</td>
<td>Glutamate</td>
</tr>
<tr>
<td></td>
<td>μmoles</td>
<td>μmoles</td>
</tr>
<tr>
<td>Complete without glutamic acid</td>
<td>1.25</td>
<td>1.15</td>
</tr>
<tr>
<td>Complete</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

**TABLE III**

**Stoichiometry of reverse reaction**

The reaction mixture, containing 24 μmoles of serine-P, 12 μmoles of α-ketoglutarate, 0.6 mg of serine-P aminotransferase, and 8 ml of Tris-HCl buffer (pH 8.2), was incubated for 20 min at 25°. After the incubate was divided into two equal parts, the residual α-ketoglutarate was determined with NADH and glutamate dehydrogenase in one part; the other half was spotted on a paper chromatogram and the amount of formed radioactive phosphohydroxypyruvate was eluted and determined by scintillation counting.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Zero time</th>
<th>After incubation</th>
<th>Net Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>counts/hr</td>
<td>μmoles</td>
<td>counts/hr</td>
</tr>
<tr>
<td>Serine-P</td>
<td>20,520</td>
<td>12</td>
<td>18,060</td>
</tr>
<tr>
<td>P-O-Pyruvate.......</td>
<td>4,500</td>
<td>2.7</td>
<td>3.15</td>
</tr>
<tr>
<td>α-Ketoglutarate....</td>
<td>6.0</td>
<td>3.15</td>
<td></td>
</tr>
</tbody>
</table>

* Solvent, 1-butanol-acetic acid-H₂O (35:10:10). Rf values for phosphohydroxypyruvate and serine-P = 0.32 and 0.06, respectively.

results in Table III show the formation of 2.7 μmoles of phosphohydroxypyruvate, and a decrease of 2.85 μmoles of α-ketoglutarate. The reversibility of the reaction is evident.

**Determination of Equilibrium Constant**—Experiments to determine the equilibrium constant of the transamination reaction were carried out both in the forward and reverse directions. The incubations for the forward reaction were carried out with reaction mixtures prepared as described for determination of the pH activity curve, but with variation of the glutamate between 3.75 to 18.75 μmoles. The pH was maintained at 8.2.

The absorbance of the incubation mixture minus glutamate was scanned until it became constant. Glutamate was then added, and the decrease in absorbance was recorded until the absorption returned to the starting point.

**TABLE IV**

**Determination of equilibrium of transamination from forward reaction**

<table>
<thead>
<tr>
<th>Ratio of P-O-pyr</th>
<th>Zero time</th>
<th>α-Ketoglutarate</th>
<th>Ketoglutarate formed</th>
<th>Kₑq</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-O-pyr to Glu</td>
<td>μmoles</td>
<td>μmoles</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>1:15</td>
<td>125</td>
<td>90</td>
<td>72</td>
<td>0.121</td>
</tr>
<tr>
<td>1:8</td>
<td>125</td>
<td>75</td>
<td>60</td>
<td>0.122</td>
</tr>
<tr>
<td>1:3</td>
<td>125</td>
<td>52</td>
<td>42</td>
<td>0.128</td>
</tr>
</tbody>
</table>

**TABLE V**

**Determination of equilibrium of transamination from reverse reaction**

The equilibrium of the reverse reaction was determined with labeled serine-P-3H. Labeled serine-P (20 μmoles) was incubated with 20 or 15 μmoles of α-ketoglutarate, 10 μmoles NaF, and 2 mg of serine-P aminotransferase in Tris-HCl buffer, pH 8.2. Incubation time was for 2 hours, temperature was 25°.

**FIG. 7.** Absorption spectrum of serine-P aminotransferase at pH 6.5 (solid line). Dotted line represents the spectrum of the NaBH₄-reduced product at same pH.
NADH was depleted. A second portion of 0.25 μmole of NADH was pipetted in, and, when this was depleted, the process was repeated until there was no further oxidation of NADH and the absorbance remained constant.

The amount of α-ketoglutarate formed was assumed to be equal to the amount of NADH oxidized. The equilibrium constant was calculated from the following equation.

\[ K_{eq} = \frac{\text{[serine-P]α-ketoglutarate}}{\text{[P-O-pyruvate]glutamate}} \]

The results obtained and the calculated values of \( K_{eq} \) are given in Table IV.

The equilibrium for the reverse reaction was measured with serine-P-3-14C as described in the legend to Table V. The P-O-hydrolysis by reducing Schiff’s base. Reduction with NaBH₄ resulted in complete loss of enzyme activity, which could almost also a slight reaction with glutamine and aspartate.

Experimental results. The spectrum of the enzyme solution shows a peak at 415 mp. This suggests the binding of pyridoxal-P enzyme was inferred from the following experimental results. The spectrum of the enzyme solution shows a peak at 415 mp and produced an increased absorption at 330 mp (Fig. 7). The enzyme activity was then lost. The absorption spectra of the active enzyme and its NaBH₄-treated product are plotted in Fig. 7.

Role of Pyridoxal-Phosphate—That the serine-P aminotransferase is a pyridoxal-P enzyme was inferred from the following experimental results. The spectrum of the enzyme solution shows a peak at 415 mp. This suggests the binding of pyridoxal-P by the enzyme.

Thorough dialysis for 5 days slightly reduced the magnitude of the 415 mp peak, but did not eliminate it, which shows that the pyridoxal-P is bound rather tightly to the enzyme. The dialyzed enzyme solution showed a 40% decrease in enzyme activity, which could be fully recovered on dialysis against a 10⁻³ M pyridoxal-P solution. Dialysis against cysteine-HCl resulted in complete loss of enzyme activity, which could almost completely be recovered by the addition of pyridoxal-P.

The absorption maximum of the pyridoxal-P enzyme complex is characteristic of a Schiff’s base bond between pyridoxal-P and enzyme, and it should be possible to form a compound stable to hydrolysis by reducing the Schiff’s base. Reduction with NaBH₄ (ratio of enzyme to NaBH₄ = 1:10) at pH 5.5 eliminated the peak at 415 mp and produced an increased absorption at 330 mp (Fig. 7). The enzyme activity was then lost. The absorption spectra of the active enzyme and its NaBH₄-treated product are plotted in Fig. 7.

Identification of the amino acid residue to which the pyridoxal-P is bound in the enzyme protein was made in the following manner. The NaBH₄-reduced product was hydrolyzed for 24 hours at 105° in 6 N HCl. The hydrolysate was vacuum-dried and chromatography was performed on Whatman No. 1 paper in the following solvent systems: (a) water-methanol-ethanol-benzene-pyridine-dioxane (25:25:10:10:10:10), and (b) 1-butanol-acetic acid-water (4:1:1). Each chromatogram showed the presence of a strongly fluorescent compound whose \( R_f \) value was in good agreement with that of synthetic ε-pyroxylysinine (9); \( R_f = 0.49 \) in Solvent A and 0.27 in Solvent B. The fluorescent compound was eluted and rechromatographed on paper. It gave a positive ninhydrin reaction.

**DISCUSSION**

The phosphorylated pathway for serine biosynthesis has been shown to be present in several bacteria (10, 11) and in vertebrate tissues (1, 12). In many instances this appears to be the predominant pathway for the formation of this amino acid. Walsh and Sallach (12) have shown serine-P aminotransferase and D-glyceraldehyde-3-P dehydrogenase activity in the liver, kidney, and brain of a considerable number of mammalian and avian species. Fallon, Hackney, and Byrne (13) have shown that the enzymes of the phosphorylated pathway are inducible in rat liver and are greatly increased on a low protein diet. The occurrence of a specific phosphatase for the hydrolysis of serine-P was discovered earlier (14, 15). We have found no previous report in the literature of the purification and study of the properties of the enzyme serine-P aminotransferase. The other primary enzyme in the pathway D-glyceraldehyde-3-P dehydrogenase has been purified 700-fold from chicken liver by Walsh and Sallach (16).

It is interesting to speculate on the reason for the occurrence of this fairly active system of serine biosynthesis in brain. One possible reason would be as a source of the phosphatidic bases, ethanolamine and choline. However, Bremer and Greenberg (17) observed only a slight degree of radioactivity phosphatidyl choline synthesis with methyl-3-C-adenosymethionine in adult rat brain. In view of the numerous synthetic functions of glycine, the activity of this enzyme system may be needed to form required glycine from serine.

**REFERENCES**

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