On the Mechanism of Glutamine-dependent Reductive Amination of \( \alpha \)-Ketoglutarate Catalyzed by Glutamate Synthase

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Highly purified preparations of glutamate synthase catalyze the TPNH-dependent reductive amination of \( \alpha \)-ketoglutarate to form L-glutamate in the presence of either L-glutamine or NH\(_3\). Preparations of the enzyme that lack flavins (FAD and FMN) or the flavins and iron sulfide, catalyzed NH\(_3\)-mediated glutamate synthesis, but not glutamine-mediated glutamate synthesis. Participation of enzyme-flavin in the reductive amination reaction with glutamine as the nitrogen donor, but not with NH\(_3\), as the nitrogen donor, was also indicated by studies in which the enzyme was reduced with dithionite. Thus, incubation of the enzyme with Na\(_2\)S\(_2\)O\(_4\), \( \alpha \)-keto\(^{14} \)C\textit{glutarate}, and glutamine led to substantial formation of \(^{14} \)C\textit{glutamate}, which was shown to be of the l-configuration; however, only trace amounts of \(^{14} \)C\textit{glutamate} were formed when glutamine was replaced by NH\(_3\). Studies with stereospecifically labeled \(^{1} \)H\textit{TPNH} showed that glutamate synthase, like glutamate dehydrogenase, uses the hydrogen atom at the B side of C\(_4\) of the nicotinamide ring of TPNH. In studies with \(^{1} \)H\textit{TPNH} (B) it was found that the products formed in the reductive amination of \( \alpha \)-ketoglutarate with glutamine and NH\(_3\) are different. Thus, in the reaction with glutamine, \(^{3} \)H is transferred from \(^{1} \)H\textit{TPNH} (B) to water; \( H_2O \) and glutamate are formed stoichiometrically. On the other hand, in the reaction with NH\(_3\), \(^{1} \)H\textit{glutamate} is formed. The enzyme also catalyzes rapid exchange of \(^{3} \)H from \(^{1} \)H\textit{TPNH} (B) with \( H_2O \), and a much slower exchange of \(^{1} \)H from [\(^{1} \)H\textit{glutamate} with water. The findings show that reductive amination with glutamine is flavin-mediated, whereas reductive amination with NH\(_3\) is not and therefore closely resembles the reaction catalyzed by glutamate dehydrogenase in which \(^{3} \)H is transferred from \(^{1} \)H\textit{TPNH} (B) to \(^{1} \)H\textit{glutamate}. The findings are consistent with the presence in highly purified glutamate synthase preparations (from \textit{Escherichia coli} and \textit{Aerobacter aerogenes}) of two different catalytic entities. The pH dependence of the glutaminase activity of glutamate synthase was determined; this activity, like the glutaminase activity of carbamyl phosphate synthetase, is enhanced by storage of the enzyme at pH 9.

Glutamate synthase is one of thirteen presently known enzymes (glutamine amidotransferases) (1-4) that catalyze the utilization of the amide nitrogen atom of glutamine in various biosynthetic reactions. Glutamate synthase catalyzes the reductive amination of \( \alpha \)-ketoglutarate according to the following equation (5-7).

\[
\text{L-Glutamine + TPNH + H}^+ + \alpha\text{-ketoglutarate} \rightarrow \text{2 L-glutamate + TPN}^+
\]

This enzyme has been obtained in highly purified form from \textit{Escherichia coli} (8, 9) and from \textit{Aerobacter aerogenes} (10); both the \textit{E. coli} enzyme and that isolated from \textit{A. aerogenes} are composed of two unequal subunits. In glutamine-dependent carbamyl phosphate synthetase (4, 11-18), which is also composed of two unequal subunits, one of the subunits (the light subunit) functions to bind glutamine and to facilitate transfer of its amide nitrogen to the heavy subunit, which can synthesize carbamyl phosphate from ammonia in the absence of the light subunit. Similar results have been obtained in studies on anthranilate (19-22) and p-aminobenzoate (23, 24) synthetases. However, recent work in this laboratory showed that the subunits of glutamate synthase do not function in this way. Thus, studies on glutamate synthase from \textit{A. aerogenes} (10) revealed that the glutamine binding site is located on the heavy subunit \( (M, -175,000) \), which also contains the iron sulfide and flavins (FAD and FMN) known to be associated with the holoenzyme. A number of the glutamine amidotransferases have been found to use ammonia in place of glutamine (1-4). However, \textit{E. coli} glutamate synthase was reported to be inactive when glutamine is replaced by ammonia (8, 9); on the other hand, it was also reported that the enzyme from \textit{A. aerogenes} (10) and that from \textit{E. coli} (25, 26) are significantly active with ammonia. The reaction in which ammonia is substituted for glutamine is identical to that catalyzed by glutamate dehydrogenase:

\[
\text{NH}_3 + \text{TPNH} + \text{H}^+ + \alpha\text{-ketoglutarate} \rightarrow \text{L-glutamate + TPN}^+
\]

There is evidence that glutamate dehydrogenase cannot use glutamine in place of ammonia in the reductive amination of \( \alpha \)-ketoglutarate (27). However, the possibility that glutamate synthase can utilize ammonia as well as glutamine is suggested by published data and would be consistent with the behavior of other glutamine amidotransferases.

In the present work, we have obtained new data on glut-
Glutamate Synthase Mechanism

We have used TPNH specifically labeled with tritium on the B side of C, of the nicotinamide ring, and preparations of the enzyme that lack flavins (deflavoenzyme) or both flavins and iron sulfide reductive amination (Reaction 1) is fundamentally different from that of the ammonia-mediated reductive amination (Reaction 2).

**Experimental Procedures**

**Materials**

*Escherichia coli* and *Aerobacter aerogenes* were grown (25 through log phase) at the New England Enzyme Center, Tufts University School of Medicine, Boston, Mass., on minimal salt medium (28). L-glutamate decarboxylase (E. coli), glucose-6-phosphate dehydrogenase, and bovine liver catalase were obtained from Sigma. a-Ketoglutaric acid was prepared as described (29). Thio-TPN was purchased from Calbiochem. L-2-Amino-4-oxo-5-chloropentanoic acid was prepared as described (28). The TPNH was purchased from P-L Biochemicals. [3H]Glucose was obtained from Schwarz/Mann. a-Keto[14C]glutarate and L-[14C]glutamate were obtained from New England Nuclear Corp.

[3H]TPNH labeled on the B side of C, of the nicotinamide ring ([1H]TPNH (B)) was prepared by incubating 125 μmol of TPN with 140 nmol of a-[1H]-glucose, 0.5 mM ATP, 2 mM MgCl₂, 4 mM diithiothreitol, 0.5% unit of hexokinase, and 0.95 unit of glucose-6-phosphate dehydrogenase in a final volume of 1 ml of 100 mM Tris/HCl (pH 7.8). Glucose-6-phosphate dehydrogenase exhibits B-side specificity for C, of the nicotinamide ring of TPNH (20). [1-14C]Glucose-6-phosphate, produced in the hexokinase reaction, was oxidized by glucose-6-phosphate dehydrogenase to yield 6-phosphogluconate and [3H]TPNH. The products were separated by chromatography on a column (1.2 x 12 cm) of Whatman DE52 cellulose (31), which was eluted with a linear gradient established between 200 mM of 10 mM glycylglycine (pH 7.5) and 200 mM of 0.03 mM NaCl in 10 mM glycylglycine (pH 7.5). L-[1-14C]Glutamate was prepared by incubating a mixture (final volume, 0.5 ml) containing 0.5 mM [3H]TPNH (specific activity, 1.7 x 10⁶ cpm/mmol), 100 mM NH₄Cl, 10 mM sodium a-ketoglutarate, 200 μg of bovine liver glutamate dehydrogenase and 0.1 mM Tris/HCl (final pH 7.8). After the absorbance at 340 nm had decreased from about 3.5 to 1.0, the products were separated by chromatography on DE-52 cellulose as described above.

**Methods**

**FMN and FAD** were quantitatively determined from their fluorescence at 527 nm (32) with an Amino-Bowman spectrophotofluorometer. The iron was determined by a modification of the colorimetric method of Langenbeck et al. (33); prior to assay, iron was converted to the Fe³⁺ form (34). The sample was added to 1 ml of 0.27 M HCl and then heated at 80° for 10 min. The solution was cooled to 4°, 0.1 ml of freshly prepared 0.12 M Na₂S₂O₇ was added, followed by 0.5 ml of 0.2 M pol of Medicine, Boston, Mass. 0.14 ml of a-α-aminophenylalanine. After standing at 26° for 5 min, the mixture was centrifuged at 45,000 x g to remove the small precipitate that formed; the absorbance at 512 nm was then measured.

Radioactivity was measured in a Beckman LS-100 liquid scintillation counter. The sample (1 ml) was counted in 10 ml of scintillation mixture (35). Protein was determined from the absorbance at 280 nm or by the method of Lowry et al. (36). During purification, glutamate synthase activity was assayed at 25° in a reaction mixture (final volume, 0.5 ml) containing 0.35 mM TPNH, 5 mM sodium a-ketoglutarate, 100 mM Tris/HCl (pH 7.8), and either 10 mM a-α-aminophenylalanine or 100 mM NH₄Cl. Initial velocities were obtained from the decrease in absorbance at 340 nm, and are reported as units (micromoles per h).

**Assays** were performed in which [3H]glutamate was determined (19). Glutaminase activity was determined as described (17). Separation of [3H]- and [14C]-labeled components present in the glutamate- and NH₄⁺-dependent glutamate synthase reactions (glutamate, TPNH, α-ketoglutarate) was carried out by chromatography on DE52 cellulose as described in the miniprint.

**Purification of Glutamate Synthase from A. aerogenes**

The enzyme was isolated by a modification of earlier procedures developed in this laboratory (16, 26). All steps were carried out at 4° and the centrifugations were done at 16,000 x g for 30 min, unless otherwise stated.

**Step 1: Sonication** — The frozen cell paste (400 g) was suspended in 1500 ml of 0.2 M potassium phosphate buffer (pH 7.8) containing 0.5 mM Na₂EDTA for 18 h. The cells were ruptured in batches of 350 ml each by sonication with a Branson sonifier for 10 min and the mixture was then centrifuged.

**Step 2: Heat Denaturation of Impurities** — Solid L-glutamine was added to the supernatant solution obtained in Step 1 to yield a final concentration of 50 mM. The solution was then heated with continuous stirring in a water bath at 90°. The temperature of the solution increased to 53° within 5 min and this temperature was maintained for an additional 5 min. The solution was then cooled and the precipitated protein was removed by centrifugation.

**Step 3: Proline Sulfate Precipitation** — The supernatant obtained in Step 2 was treated with 0.25 volume of proline sulfate solution (2%; pH 5.5). After stirring for 10 min, it was centrifuged.

**Step 4: Ammonium Sulfate Precipitation** — The supernatant obtained in Step 3 was treated with 350 g of solid ammonium sulfate and 0.2 g of Na₂EDTA per liter. The suspension was stirred for 10 min and then centrifuged. The pellet was dissolved in 150 ml of 0.2 M potassium phosphate (pH 7.6) containing 0.5 mM Na₂EDTA.

**Step 5: Gel Filtration on Sephadex G-50** — The solution from Step 4 was applied to a column (5 x 75 cm) of Sephadex G-50 equilibrated with 0.14 M potassium phosphate (pH 6.8) containing 0.5 mM Na₂EDTA. Equiluent corresponding to the void volume of the column (about 500 ml) was collected. The protein was collected in the next 500 ml, and this solution was immediately processed as described in Step 6.

**Step 6: Chromatography on DEAE-Sephadex** — The solution from Step 4 was applied to a column (5 x 25 cm) of DEAE-Sephadex (A-50) equilibrated with 0.14 M potassium phosphate buffer (pH 6.8) containing 0.5 mM Na₂EDTA. Equiluent corresponding to the void volume of the column (about 500 ml) was collected. The protein was collected in the next 500 ml, and this solution was immediately processed as described in Step 7.

**Step 7: Gel Filtration on Sephadex G-200** — The solution obtained in Step 6 was applied to a column (3 x 100 cm) of Sephadex G-200 equilibrated with 0.2 M potassium phosphate buffer (pH 7.6) containing 0.5 mM Na₂EDTA, and 0.3 g of ammonium sulfate (35/100 ml) was added. The fraction containing activity were pooled and solid ammonium sulfate (35 g/100 ml) was added. The fraction was centrifuged and dissolved in 0.2 M potassium phosphate buffer (pH 7.6) containing 0.5 mM Na₂EDTA. Elution was carried out with this buffer at a flow rate of 15 ml/h; fractions of 5 ml were collected. Glutamate synthase (Fig. 1A) was eluted just after the void volume of the
Column (250 ml) was collected. The elution profile of ammonia-dependent activity exhibited a shoulder under the peak of glutamine-dependent activity, and a maximum which appeared several fractions later. The fractions containing glutamine-dependent glutamate synthase of the highest specific activities were pooled and concentrated in an Amicon cell fitted with an XM-50 membrane.

Step 8: Gel Filtration on Sepharose 6B – The concentrate obtained in Step 7 was applied (in two batches) to a column (2 x 65 cm) of Sepharose 6B equilibrated with 0.2 M potassium phosphate buffer (pH 7.6) containing 0.5 mM Na₂EDTA. Elution was carried out with this buffer at a flow rate of 10 ml/min and fractions of 1.2 ml were collected. The fractions containing enzyme with the highest glutamine-dependent activity were pooled (Fig. 1B, Fractions 54 to 65) and concentrated in an Amicon cell. In this chromatography, the elution of glutamine-dependent activity closely corresponds to that of protein, but the elution profile for ammonia-dependent activity shows two peaks (Fig. 1B), the first of which corresponds to the major glutamate synthase peak. The second peak appears to be glutamate synthase peak. The second peak appears to be glutamate synthase peak.

Preparation of Other Enzymes

Chloroketone-inhibited glutamate synthase was prepared essentially as described previously (10). This form of the enzyme is inhibited with respect to its glutamine-dependent functions, but exhibits unaltered ammonia-dependent activity. A. aerogenes glutamate synthase (500 μg) was added to a solution (final volume, 1 ml) containing 0.2 M potassium phosphate (pH 7.6), 0.5 mM Na₂EDTA, and 55 μM chloroketone. Glutamine- and ammonia-dependent TPNH oxidation, measured as the decrease in absorbance at 340 nm, were determined at 10-min intervals, until the glutamine-dependent activity decreased to about 1% of the initial activity. The chloroketone concentration was then doubled and the activities were monitored for 15 min, after which there was no further decrease in absorbance of TPNH at 340 nm. The inhibited enzyme was dialyzed at 4° against 0.2 M potassium phosphate buffer (pH 7.6) containing 0.5 mM Na₂EDTA for 6 h.

Preparations of the enzyme that lack flavin and iron sulfide (apo-enzyme) were obtained by precipitation at pH 4.6 as described by Mantsilii and Zalkin (25). Enzyme preparations from which only the flavins were removed (deflavoenzyme) were obtained by application of the procedure of Rajagopalan and Handler (34). A solution of the enzyme (approximately 1 mg/ml) in 0.2 M potassium phosphate (pH 7.6) containing 0.5 mM Na₂EDTA, 5 mM dithiothreitol, 10 mM L-glutamine, and 5 mM sodium α-ketoglutarate was treated with 4 volumes of ice cold methanol and allowed to stand at 0° for 10 min. The sample was centrifuged at 1000 x g. The supernatant was evaporated to dryness in the dark with a stream of air and the residue was analyzed for its content of flavin. During this procedure, virtually all of the flavin is extracted into methanol (see miniprint, Table I). The pellet obtained after centrifugation is red-brown. Analysis for iron in the pellet and in the supernatant showed that 90% of the iron remains enzyme-bound and that 10% is extracted by the methanol. The precipitate was triturated with 0.2 M potassium phosphate (pH 7.6) containing 0.5 mM Na₂EDTA, 2 mM urea, and 10 mM dithiothreitol. The insoluble material was removed by centrifugation and the extraction was repeated. The urea and dithiothreitol present in this solution were removed by dialysis against 0.2 M potassium phosphate buffer containing 0.5 mM Na₂EDTA.

Glutamate dehydrogenase from A. aerogenes was obtained in the gel filtration of glutamate synthase preparations on Sephadex G-200 (i.e. Step 7 was repeated), the glutamine- and ammonia-dependent activities coincided closely (Fig. 1C). Table I gives a summary of a typical purification procedure. The isolated enzyme exhibited glutamine-dependent specific activity of 1250 μmol/mg/h and ammonia-dependent activity of 105 μmol/mg/h.

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exhibit glutamine-dependent synthase activity; however, preparations of the deflavoenzyme were active in catalyzing ammonia-dependent synthesis of glutamate (see miniprint, Table II). Addition of FAD and FMN (10^{-4} \text{ M}) (separately or together) did not restore glutamine-dependent activity.

Substantially the same results were obtained with the apoenzyme, which lacks flavins and iron sulfide. Thus, the apoenzyme was inactive in catalyzing the glutamine-dependent reaction and fully active in catalyzing the ammonia-dependent reaction as judged by TPNH oxidation and glutamate formation. Addition of FAD (10^{-4} \text{ M}), FMN (10^{-4} \text{ M}), Na_2S (10^{-4} \text{ M}), 2-mercaptoethanol (10^{-4} \text{ M}), and FeSO_4 (10^{-4} \text{ M}) did not restore glutamine-dependent activity.

Utilization of Glutamine for Reductive Amination of \alpha-Ketoglutarate in the Presence of Dithionite - The results summarized above suggest that enzyme flavin is not significantly involved in the ammonia-dependent reaction, and that the glutamine-dependent reaction requires flavin for the synthesis of glutamate from \alpha-ketoglutarate. Further studies have supported this conclusion. Thus, in one approach, sodium dithionite was substituted for TPNH and the formation of glutamate was determined. The results of these studies (Table II) indicate that significant glutamate formation occurred only in the glutamine-dependent glutamate synthase reaction. Only slight formation of glutamate from ammonia and \alpha-ketoglutarate was observed with glutamate synthase preparations from Aerobacter aerogenes and Escherichia coli and with bovine liver glutamate dehydrogenase. These findings are in accord with spectral studies which showed that glutamine, but not ammonia, reoxidized chemically reduced E. coli glutamate synthase in the presence of \alpha-ketoglutarate (26). The [14C]glutamate formed by glutamate synthase in the presence of dithionite and glutamate was incubated with L-glutamate decarboxylase. About 95% of the glutamate radioactivity was converted to \gamma-amino[14C]pyruvate, indicating that the glutamate formed in this reaction is predominantly of the L-configuration and is thus produced on the enzyme. The reduction of \alpha-amino
glutamate in solution would be expected to yield racemic glutamate.

**Table II**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>With [14C]Glutamate formol</th>
<th>With glutamate</th>
<th>With ammonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>No enzyme</td>
<td>5,820</td>
<td>4,640</td>
<td></td>
</tr>
<tr>
<td>A. aerogenes, glutamate synthase</td>
<td>346,000</td>
<td>6,680</td>
<td></td>
</tr>
<tr>
<td>E. coli, glutamate synthase</td>
<td>22,400</td>
<td>6,640</td>
<td></td>
</tr>
<tr>
<td>Glutamate dehydrogenase (bovine liver)</td>
<td>7,520</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

other substrates including [14C]-labeled \alpha-keto
glutarate; the oxidation of TPNH was followed spectrophotometrically and the formation of [\text{H}^+]- and [14C]-labeled glutamate and of H_2O was also determined; the results are summarized in Table III.

In Experiment 1, bovine liver glutamate dehydrogenase was incubated with [3H]TPNH (B), ammonia, and \alpha-keto
gluturate. The oxidation of [3H]TPNH was virtually equivalent to the formation of [3H]glutamate; no tritium appeared in water. These findings verify that the tritium in the reduced pyridine nucleotide is on the B side. The specificity of this enzyme for the hydrogen atom on the B side was first demonstrated by Nakamoto and Vennesland (41), who showed that glutamate dehydrogenase transfers this hydrogen atom to the \alpha-carbon of glutamate. Experiment 2 gives the results obtained in two experiments in which glutamate synthase was incubated with \alpha-keto[14C]glutarate, [3H]TPNH (B), and glutamine. Tritium was transferred to water in amounts equivalent to both the amount of [3H]TPNH oxidized and to the [14C]glutamate formed. No tritium was found in glutamate. In Experiment 3, in which glutamate synthase was incubated with [3H]TPNH (B) in the absence of other substrates, slight TPNH oxidation was observed, but the incorporation of tritium into water was far greater than the oxidation of TPNH.

The findings indicate that the enzyme catalyzes relatively rapid exchange of hydrogen between the reduced pyridine nucleotide hydrogen and water; a reaction probably mediated by the enzyme flavin. Although the rate of such exchange (375 \mu mol/mg/h) is greater than the rate of the TPNH oxidase reaction (2.1 \mu mol/mg/h), it is considerably slower than the rate of the glutamine-dependent glutamate synthase reaction (1250 \mu mol/mg/h).

In Experiment 4, glutamate synthase was incubated with [3H]TPNH (B), \alpha-keto[14C]glutarate, and ammonia. The amount of TPNH oxidized was about equivalent to the formation of [14C]glutamate, but more than half of the [H] removed from [3H]TPNH was transferred to water and only about 20% was incorporated into glutamate. These results seem to reflect a typical glutamate dehydrogenase-catalyzed reaction (as seen in Experiment 1) and in addition the rapid proton exchange between TPNH and water observed in Experiment 3. That such exchange is mediated by enzyme-bound flavin is consistent with the results of Experiment 5 in which apoglutamate synthase was incubated with [3H]TPNH (B), \alpha-keto[14C]glutarate and ammonia; here, no tritium appeared in the water and the disappearance of reduced pyridine nucleotide was equivalent to the formation of glutamate, which contained about equimolar amounts of [14C] and \text{H}. When this experiment was carried out with the chloroketone-inhibited enzyme (Experiment 6) results similar to those found in Experiment 4 were obtained; thus, tritium appeared in both water and glutamate. In comparing the results obtained in Experiments 4 and 6, it should be noted that in Experiment 4, all of the [3H]TPNH was oxidized, so that the amount of [3H]TPNH oxidized is equivalent to the sum of the amounts of tritium transferred to water and to glutamate. In Experiment 6, about half of the [3H]TPNH was oxidized and since the exchange of tritium with water is more rapid than oxidation of [3H]TPNH (Experiment 3), it would be expected (and was observed) that the amount of tritium present in glutamate plus water (147 nmol) would be greater than the amount of [3H]TPNH oxidized (100 nmol).

In Experiment 7, glutamate dehydrogenase isolated from A. aerogenes was incubated with [3H]TPNH (B), \alpha-keto
gluturate, and ammonia; there was substantial incorporation of
The reaction mixtures contained (final volume, 0.5 ml) enzyme, potassium phosphate buffer (0.2 M; pH 7.6), Na<sub>2</sub>EDTA (0.5 mM), [14C]TPNH (0.35 mM; 170,000 cpm/µmol), NH<sub>4</sub>Cl (100 mM), L-glutamate (10 mM), α-ketoglutarate (1 mM; α-keto[14C]glutarate, 500,000 cpm/µmol) and Thio-TPN (0.35 mM) as indicated. The amounts of enzymes and incubation periods (25°) were as follows: Experiment 1, 10 µg, 20 min; Experiment 2a, 1 µg, 20 min; Experiment 2b, 1 µg, 10 min; Experiment 3, 2.4 µg, 10 min; Experiment 4, 9 µg, 40 min; Experiment 5, 2 µg, 40 min; Experiment 6, 11 µg, 9 min; Experiment 7, 1 µg, 60 min; Experiment 8, 10 µg, 40 min; Experiment 9, 5 µg, 16 min; Experiment 10, 10 µg, 25 min. The oxidation of TPNH was determined from the decrease in absorbance at 340 nm. The formation of Thio-TPN was determined from the increase in absorbance at 398 nm. The other products were separated by chromatography on DE52 cellulose and their radioactivity was determined as described under "Methods."

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Reaction compounds</th>
<th>Enzyme*</th>
<th>TPNH oxidized</th>
<th>H&lt;sub&gt;2&lt;/sub&gt;O formed</th>
<th>[14C]glutamate formed</th>
<th>[14C]glutamate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TPNH, NH&lt;sub&gt;4&lt;/sub&gt;, α-ketoglutarate</td>
<td>Bovine liver GDH</td>
<td>148</td>
<td>0</td>
<td>139</td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>TPNH, glutamine, α-keto[14C]glutarate</td>
<td>Glu syn (A.a.)</td>
<td>22</td>
<td>22</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>2b</td>
<td>TPNH, glutamine, α-keto[14C]glutarate</td>
<td>Glu syn (A.a.)</td>
<td>10</td>
<td>14</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>[14C]TPNH</td>
<td>Glu syn (A.a.)</td>
<td>0.84</td>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>[14C]TPNH, NH&lt;sub&gt;4&lt;/sub&gt;, α-keto[14C]glutarate</td>
<td>Glu syn (A.a.)</td>
<td>208</td>
<td>168</td>
<td>40</td>
<td>195</td>
</tr>
<tr>
<td>5</td>
<td>[14C]TPNH, NH&lt;sub&gt;4&lt;/sub&gt;, α-keto[14C]glutarate</td>
<td>Apo-glu syn (A.a.)</td>
<td>53</td>
<td>0</td>
<td>55</td>
<td>59</td>
</tr>
<tr>
<td>7</td>
<td>[14C]TPNH, NH&lt;sub&gt;4&lt;/sub&gt;, α-keto[14C]glutarate</td>
<td>GDH (A.a.)</td>
<td>133</td>
<td>8</td>
<td>195</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>[14C]TPNH, glutamine, α-keto[14C]glutarate</td>
<td>Glu syn (E.c.)</td>
<td>122</td>
<td>110</td>
<td>0</td>
<td>112</td>
</tr>
<tr>
<td>9</td>
<td>[14C]TPNH, NH&lt;sub&gt;4&lt;/sub&gt;, α-keto[14C]glutarate</td>
<td>Glu syn (E.c.)</td>
<td>44</td>
<td>156</td>
<td>0.4</td>
<td>56</td>
</tr>
<tr>
<td>10</td>
<td>[14C]TPNH, Thio-TPN</td>
<td>Glu syn (A.a.)</td>
<td>63</td>
<td>142</td>
<td>[57]</td>
<td></td>
</tr>
</tbody>
</table>

* Enzymes and sources: GDH, glutamate dehydrogenase; Glu syn, glutamate synthase; A.a., Aerobacter aerogenes; E.c., Escherichia coli; CK, chloroketone-treated.

** Nanomoles of Thio-TPN formed.

The findings summarized in Table III indicate that the B-hydrogen atom of TPNH is specifically utilized in the glutamine-dependent glutamate synthase reaction catalyzed by the enzymes from A. aerogenes and E. coli. The B-hydrogen atom of TPNH is also specifically utilized in the TPNH-water exchange and in the ammonia-dependent reductive amination of α-ketoglutarate catalyzed by both glutamate synthase preparations and by A. aerogenes glutamate dehydrogenase. The findings also show that the utilization of the B-hydrogen atom of TPNH takes place by a flavin-independent mechanism when ammonia is the nitrogen donor. The mechanisms of reductive amination with glutamine and ammonia are clearly different; when glutamine is used the B-hydrogen atom of TPNH is transferred to water, whereas when ammonia is the substrate the hydrogen atom released by TPNH is incorporated into glutamate.

**Inhibition of Glutamate Synthase by TPNH** In the course of these studies, it was found that preincubation of glutamate synthase with concentrations of TPNH ordinarily used for the assay of enzyme activity (0.35 mM) led to relatively rapid loss of glutamate-dependent glutamate synthase activity. On the other hand, the ammonia-dependent activity was not affected by incubation of the enzyme with TPNH. Since incubation of the enzyme with TPNH would be expected to lead to reduction of enzyme-bound flavin, and since the enzyme is known to catalyze TPNH oxidation in the absence of the other substrates, we considered the possibility that hydrogen peroxide might be formed in the oxidation of reduced flavin by molecular oxygen. This hypothesis was examined by determining the effect of catalase on the inactivation of the enzyme produced by TPNH. As shown in Table IV, when catalase was present during incubation of glutamate synthase with TPNH, virtually no glutamine-dependent activity was lost; under these conditions, most of the activity disappeared in the absence of...
Glutamate Synthase Mechanism

The findings, which are in accord with the hypothesis expressed above, also indicate a significant difference between the ammonia- and glutamine-dependent reactions. It is possible that hydrogen peroxide oxidizes a group (or groups) at the active site of the enzyme required for the binding or reaction (or both) of glutamine. The findings are reminiscent of earlier studies on glutamine-dependent carbamyl phosphate synthetase, in which it was found that hydrogen peroxide markedly inhibits the glutamine-dependent functions of this enzyme without affecting its ability to utilize ammonia (16).

Glutaminase Activity of Glutamate Synthase—Glutamate synthase, like a number of other glutamine amidotransferases, exhibits glutaminase activity. The glutaminase activity of A. aerogenes glutamate synthase is about 2% of the glutamine-dependent glutamate synthase activity. Studies on the pH dependence of the glutaminase activity of glutamate synthase revealed substantial activity between pH 6 and 9.5 with small peaks of activity at pH values of about 3, 6, 7.5, and 9.2 (Fig. 2, lower two curves). It seems notable that glutamine-dependent carbamyl phosphate synthetase also exhibits more than a single pH optimum (17); studies on the pH dependence of the glutaminase activities of the other amidotransferases have apparently not yet been carried out. When glutamine-dependent carbamyl phosphate synthetase is stored at pH 5, the glutaminase activity of the enzyme increases significantly (with concomitant loss of synthetase activity); the enhanced glutaminase activity exhibits an optimum at about pH 9 (17, 18). After glutamate synthase was stored at pH 9 for 7 days at pH 9, its glutaminase activity was found to be increased at all values of pH, and there was a distinct optimum at about pH 9 (Fig. 2, upper curve). Under these conditions of storage, the glutaminase activity at pH 9.2 increased from 29 pmol/mg/h to 65 pmol/mg/h, and there was a marked decrease in the glutamate-dependent synthetase activity.

Studies on the deflavoenzyme showed that removal of flavin does not destroy the glutaminase activity (miniprint, Table III); the 40% loss in activity observed may result from denaturation by methanol during preparation of this form of the enzyme. The deflavoenzyme exhibited a lower glutaminase activity in the presence of $10^{-5}$ to $10^{-4}$ M flavin. Miller and Stadtman (8) have observed partial inactivation of glutamine-dependent glutamate synthase activity in the presence of $10^{-5}$ to $10^{-4}$ M flavin. We have obtained partial (about 50%) protection against inactivation by $10^{-4}$ M flavin by adding $10$ mM l-glutamine and $5$ mM sodium α-ketoglutarate.

**Table IV**

<table>
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<tr>
<th>Experiment</th>
<th>Conditions</th>
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<tr>
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<tr>
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<td>Enzyme + TPNH + catalase</td>
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**FIG. 2.** pH dependence of the glutaminase activity of *Aerobacter aerogenes* glutamate synthase. Lower two curves (2 separate experiments): the enzyme (15.6 μg) was incubated at 27°C for 20 min in a final volume of 0.4 ml of buffer containing 1-14C glutamine (10 μM, 7000 cpm/μmol). The reaction was stopped by adding 0.1 ml of 1 M HCl and the sample was then placed at 0°C. After 10 min, 0.1 ml of 1 M Tris was added, and the 1-14C glutamate was quantitatively recovered by chromatography on Dowex 1-acetate (17). The following buffers were used: A, 0.1 M potassium phosphate, 0.1 M acetic acid (1); B, 0.1 M Tris/acetate (2); C, 0.1 M Veronal, 0.1 M potassium phosphate (1); D, 0.1 M sodium carbonate, 0.1 M potassium phosphate (2). Upper curve: pH dependence of the glutaminase activity of *A. aerogenes* glutamate synthase after storage at pH 9. The enzyme (160 μg) in 0.2 ml potassium phosphate (pH 7.6) containing 0.5 mM Na₂EDTA was adjusted to pH 9 by addition of 0.2 ml Tris and brought to a final volume of 250 μl. The solution was stored at 0°C for 6 days, and then assayed as described above using Buffer A, pH 2 to 6, and Buffer B, pH 6 to 10.2. Abscissa, final pH; ordinate, micromoles of glutamate formed per 20 min.
hydrogen is transferred from TPNH to water during the ammonia-mediated reaction may be ascribed to the simultaneous hydrogen was transferred from TPNH to water in the ammonia-mediated reaction catalyzed by apoglutamate synthase (Table III). On the basis of these considerations it seems unlikely that the reductive amination reaction with ammonia represents a partial reaction of glutamate synthase. It thus appears that the ammonia-mediated reaction is catalyzed by a separate catalytic entity. In this connection it may be relevant to note that the specific activity of the preparation of A. aerogenes glutamate synthase used here in the ammonia-dependent reaction is about 8% of that of the glutamine-dependent reaction; values of 2 to 10% have been observed for other preparations isolated in this laboratory. A value of 4% was obtained for A. aerogenes glutamate synthase in the earlier work in this laboratory (10). Our preparation of E. coli B glutamate synthase used ammonia at about 1.4% of the rate found with glutamine, and Mantsalä and Zalkin found values of 5 to 7% for their preparation from E. coli K-12. Although considerable effort was made to purify glutamate synthase from contaminating glutamate dehydrogenase, one cannot exclude the possibility that a trace of the latter enzyme may be present in the glutamate synthase preparation. Such an explanation would seem to be consistent with all of the present findings and would not be inconsistent with the findings of other investigators. Mantsalä and Zalkin (25, 26, 42) have made several observations, published while the present paper was in preparation, which led them to conclude that the ammonia-dependent activity is not due to the presence of glutamate dehydrogenase. Thus, they were unable to detect structural relatedness between E. coli glutamate synthase and glutamate dehydrogenase by micromethod fixation, double antibody precipitation, immunodiffusion, and sodium dodecyl sulfate-gel electrophoresis; they estimated that the double antibody precipitation procedure should detect a contamination of 0.1 to 1%. These workers also reported that the ammonia-dependent activity of E. coli K-12 glutamate synthase was increased 5-fold after removal of flavin and nonheme iron. We did not observe this effect in similar studies on the A. aerogenes enzyme. Mantsalä and Zalkin (42) also reported that the isolated light subunit of E. coli glutamate synthase (separated from the heavy subunit by treatment with sodium dodecyl sulfate) exhibited ammonia-dependent TPNH oxidase activity. However, the magnitude of this activity was less than 1% of that of the native enzyme, and glutamate formation was not reported. Although their conclusion that the light subunit of glutamate synthase is responsible for catalysis of the ammonia-mediated reaction may be correct, other interpretations of the presently available data cannot be excluded.

Both our studies and those of Mantsalä and Zalkin (25, 26, 42) suggest that the heavy subunit can use glutamine, but not ammonia, in the reductive amination of α-ketoglutarate. This raises the question as to the nature of such a reaction in which an intermediate equivalent to iminoglutarate can be formed on the enzyme by reaction with glutamine but not with ammonia. The possibility must be considered that the intermediate formed in this reaction is actually a γ-glutamyl dehydropeptide which undergoes reduction followed by hydrolysis. On the other hand, if iminoglutarate is the intermediate, the binding site for α-ketoglutarate must be so situated as to prevent interaction with ammonia.

Further work is needed to establish the functional role of the light subunit. It will be of particular importance to achieve separation of the subunits without destruction of catalytic activity as has been accomplished with certain glutamine amidotransferases (3, 4). Previous work has shown that the glutamine-binding site as well as the flavins and iron sulfide of glutamate synthase are located on the heavy subunit (10), but the locations of the sites for binding of TPNH and the α-ketoglutarate utilized in the glutamine-dependent reaction require further study.

It has been suggested that glutamine amidotransferases may have arisen in the course of evolution as a combination of a glutaminase with an ammonia-utilizing synthetase. However, this idea would seem to be inconsistent with the data now available about glutamate synthase, since it is clear that two basically different mechanisms of reductive amination are involved. Nevertheless it may be significant from the standpoint of evolution that both glutamate synthase and glutamate dehydrogenase specifically use the B-side of TPNH.

Acknowledgment—We are indebted to Dr. Owen Griffith for many stimulating discussions of this work, and for his help in preparing the labeled TPNH.

REFERENCES

**Table I**

<table>
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<th>Phosphorylase</th>
<th>Phosphatase</th>
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**Table II**

| Enzyme | Molar Equivalent of Enzyme Activity | Molar Equivalent of Enzyme Activity
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<td>Glucose 6-phosphate dehydrogenase</td>
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**Table III**

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<td>3</td>
<td>NADPH</td>
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*All values are in millimolar, unless stated otherwise. Values were obtained in a final volume of 0.02 ml containing 0.1 M Tris-HCl (pH 7.4), 5 mM EDTA, 0.1 mM 2-mercaptoethanol, and 0.1 mM NADH (5.0 mg/ml). The reaction mixture contained 0.005 M of the substrates for 1 min at 37°C.*
On the mechanism of glutamine-dependent reductive amination of alpha-ketoglutarate catalyzed by glutamate synthase.

L E Geary and A Meister


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