TRANSFER OF OXYGEN IN THE GLUTAMINE SYNTHETASE REACTION*

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The condensation of a carboxylic acid with alcohols, amides, and mercaptans under physiological conditions usually requires an external source of energy. In most systems the external source has been shown to reside in the apparent hydrolysis of adenosine triphosphate (ATP), but the detailed way in which the energy is made available for the condensation is obscure. In the action of glutamine synthetase (3–6), for example, it is clear that the equation for the reaction (Equation 1) can be represented as

\[ \text{RC}-\text{O}^\bullet + \text{NH}_2^+ + \text{ATP}^\bullet \rightarrow \text{RC}-\text{NH}_2 + \text{ADP}^\bullet + \text{HOPO}_4^\bullet \]

a sum of two reactions, the hydrolysis of ATP to adenosine diphosphate (ADP) and inorganic phosphate (P_i) (the exergonic part of the reaction) and the condensation of glutamic acid and ammonia to form glutamine (the endergonic part of the reaction). A similar situation exists in other systems in which ATP acts as energy source for a carboxyl condensation (7–9). Since, in chemical systems, transfer of energy between such coupled reactions occurs by way of common intermediates, the first step in the elucidation of this problem involves the identification of the types of intermediates involved.

The system studied in this work was the glutamine synthetase reaction, and the approach used was the determination of the disposition of the displaced carboxyl oxygen atom after reaction with O^{18}-labeled glutamic acid.

**EXPERIMENTAL.**

*Materials—Adenosine triphosphate, disodium salt (Pabst), was chromatographed according to the method of Cohn and Carter (10) and shown to have the following composition: 1.70 mmoles of ATP, 0.12 mmole of ADP, and 0.06 mmole of P_i per gm. of material. O^{18}-labeled water (Stuart Oxygen Company) had an atom per cent excess of O^{18} of about 1.4.

* A preliminary report of this work has been published (1). Similar results have been reported by Boyer et al. (2). Research was carried out at Brookhaven National Laboratory under the auspices of the United States Atomic Energy Commission.
GLUTAMINE SYNTHETASE

Preparations—The enzyme was isolated from dried peas (6). Two preparations designated as A and B were used. Preparation A was carried through the protamine sulfate precipitation. The supernatant solution from this treatment was dialyzed and then lyophilized. Preparation B was carried through the dialysis step after the second ammonium sulfate precipitation. The dialysate was used as such. Both preparations were stored at -20° when not in use. The adenosinetriphosphatase (ATPase) activity of both preparations in the presence of hydroxylamine was investigated and found to be negligible in comparison with the rate of formation of glutamylhydroxamic acid under the same conditions.

For the preparation of O18-labeled glutamic acid, a solution of 20 mmoles of L-glutamic acid in 15 ml. of 2 N HCl in H2O18 was heated in a sealed tube at 100° for 6 to 8 hours. The solution was lyophilized, and the solid glutamic acid hydrochloride was taken up in 10 ml. of H2O16. Sodium hydroxide (3.5 M) was added to bring the pH to 3. The mixture was cooled and filtered, and the solid was washed with alcohol and ether and dried in a vacuum at 100°. The isotope content of the HCl-H2O18 mixture before addition of glutamic acid was 1.18 atom per cent excess. The final observed atom per cent excess of the medium after equilibration was 1.07. The calculated value from the known molar quantities of medium and glutamic acid assuming equilibration of all four carboxyl oxygens was 1.07, showing that HO18O18C—CH2—CH2—CHNH2—CO18O18H had been prepared.

Analytical Procedures—Analyses for inorganic phosphate were carried out by the method of Fiske and Subbarow (11) or by a modification (12, 13). Glutamylhydroxamic acid was determined by the method of Lipmann and Tuttle (14).

O18 was assayed as CO2 in the mass spectrometer. The O18 content of inorganic phosphate in the form of KH2PO4 was determined by pyrolysis to KPO3 and H2O, the resulting H2O being equilibrated with normal dry carbon dioxide according to the method of Cohn (15). The O18 content of the ADP was determined by hydrolysis to inorganic phosphate as described below. Control runs showed no exchange of oxygen between phosphate and water during hydrolysis.

The isotope content of the glutamic acid was determined by either of two methods. The first method utilized the dehydration of the acid to the lactam, 2-ketopyrrolidine-5-carboxylic acid (16), the water formed being collected and equilibrated with carbon dioxide. The glutamic acid was placed in pyrolysis tube A of Fig. 1. After evacuation and admission of a known amount of carbon dioxide in tube B, the side arm A was heated to 170° for 1 hour. The temperature was then lowered to 150° and maintained at this point for 3 hours. After completion of the reaction, the carbon dioxide and water were condensed in chamber B and equili-
brated as previously described. The second method was that of Doering and Dorfman (17), which is based on the Schütze method for the determination of oxygen (18). In this method the sample is pyrolyzed in an atmosphere of nitrogen to a mixture of water, carbon monoxide, and carbon dioxide. These gases are passed over a bed of carbon at 1120°, which converts them quantitatively to carbon monoxide. The carbon monoxide is oxidized by iodine pentoxide at 119° to carbon dioxide. Samples of glutamic acid of 10 mg. were analyzed in this manner.

Formation of Glutamylhydroxamic Acid and Glutamine from O¹⁸-Labeled Glutamic Acid—The reaction mixture (total volume, 100 ml.) consisted of 13 mmoles of tris(hydroxymethyl)aminomethane buffer, pH 7.8, 3.2 mmoles of Mg⁺⁺, 3.4 mmoles of cysteine, 3.3 mmoles of glutamic acid-O¹⁸, 1.0 mmole of ATP, 3.4 mmoles of hydroxylamine or ammonium chloride, and enough enzyme to insure complete utilization of the ATP during the course of the incubation. After incubation for 1 hour at 37°, the mixture was cooled quickly to 0°, and aliquots were removed for O¹⁸, inorganic phosphate, and hydroxamic acid analyses. Barium chloride was added in amounts just sufficient to precipitate both the inorganic phosphate and the adenine nucleotides. After centrifugation of the precipitate, glutamic acid was crystallized from the supernatant fluid and dried in a vacuum over phosphorus pentoxide.

The barium salt of the ADP together with the barium phosphate was dissolved in a minimal amount of 0.2 N HCl. The nucleotide and phosphate were separated on Norit by the method of Crane and Lipmann (19). A mixture of the Norit containing the adenosine diphosphate and 35 ml. of 1 N HCl was boiled for 15 minutes and then filtered. Potassium dihydrogen phosphate was isolated from the filtrate.

RESULTS AND DISCUSSION

The results of the reaction of O¹⁸-labeled glutamic acid with ATP and either hydroxylamine or ammonium chloride are shown in Table I.
A comparison of the values obtained for the runs of both ammonia and hydroxylamine shows a close correspondence, which indicates that the course of the reaction is similar for both ammonia and hydroxylamine.

The agreement between the isotope content of the glutamic acid used and the glutamic acid recovered indicates that no exchange of the carboxyl oxygen atoms occurred during the course of the reaction. The O\textsuperscript{18} content of the liberated ADP in all cases studied was very small or negligible. However, the inorganic phosphate contained labeled oxygen in amounts corresponding to approximately one-fourth that of the glutamic acid used. The rather large value of 0.31 in Experiment 3 probably arises from the fact that in this run the amount of inorganic phosphate recovered was very small. These results show that a stoichiometric transfer of oxygen occurs from the carboxyl group of glutamic acid to the terminal phosphate group of the ATP without the mediation of the solvent (see Equation 2, in which Ad represents the adenosine moiety).

\[
\begin{align*}
\text{O}^{18} & \quad \text{O} \quad \text{O} \\
\text{RC} & + \text{O}^{18} + \text{AdOPoPO}^{-4} + \text{NH}_4^+ \rightarrow \\
\text{O} & \quad \text{O} \quad \text{O}
\end{align*}
\]

(2)

One mechanism may be excluded immediately on the basis of these data; i.e., an indirect activation of the carboxyl group via the protein chain. Such a mechanism, shown schematically in Equations 3 and 4 with E' representing the excited form of the enzyme, would yield unlabeled phosphate and is therefore excluded by the O\textsuperscript{18} results. The same conclusion holds for any other mechanism in which the oxygen of the medium is involved in a hydrolysis of the ATP.

A second type of mechanism is also excluded; i.e., an initial rapid reaction of the enzyme with the glutamic acid to yield a glutamyl enzyme (Equation 5).

\[
\begin{align*}
\text{O} & \\
\text{Glut} & + \text{COOH} + \text{EH} \rightleftharpoons \text{Glut} \rightarrow \text{E} \rightarrow \text{E} + \text{H}_2\text{O}
\end{align*}
\]

(5)
Such a reaction should cause an exchange of oxygen between the water of the medium and the glutamic acid and is excluded by the observation that the recovered glutamic acid retained its original $^{18}O$ content.

It remains to be considered what alternatives are available. Clearly the cleavage in the ATP part of the reaction occurs between the terminal phosphorus and its bridge oxygen, as in the case of hydrolysis by muscle proteins (20) and $\alpha$-ketoglutarate activation (21). The carboxyl oxygen of the glutamic acid is transferred solely through covalently bonded compounds which undergo no ready exchange with the medium. Since carboxyl anions are known as active nucleophilic reagents in organic reactions, and since displacement mechanisms are certainly compatible with many of the observed phenomena in transfer and hydrolytic reactions (22), it is plausible to consider mechanisms which include both of these theoretical assumptions. These are a direct attack on ATP to give glutamyl phosphate (Equation 6) and a nucleophilic attack on a phosphorylated enzyme to give a glutamyl enzyme (Equation 7).

$$\text{Glut} - \text{C}-\text{O}^{18}\text{H} + \text{ATP} \rightarrow \text{Glut} - \text{C}-\text{O}^{18} \cdots \text{OPO}_{3}\text{PO}_{4}\text{Ad} \rightarrow$$

$$\text{Glut} - \text{C}-\text{O}^{18}\text{PO}_{4} + \text{ADP} \quad (6)$$

$$\text{NH}_{4}$$

$$\text{Glut} - \text{C} - \text{NH}_{2} + \text{HO}^{18}\text{PO}_{4}$$

In the latter mechanism, the initial inorganic phosphate would be unlabeled, but, since the enzyme is present only in small amounts relative to the substrates, this amount would be undetectable. Subsequent reactions would
produce labeled phosphate. (This could serve as a way of distinguishing between the mechanisms when substrate amounts of enzyme are available).

Difficulties exist with both these mechanisms in their simplest form. In relation to the first mechanism, glutamyl phosphate has not been found (4, 5). In relation to the second, a similar scheme was originally proposed by Webster and Varner on the basis of the exchange of KH₃P³²O₄ with ATP in the presence of glutamate (23). Subsequent work demonstrated that traces of ammonia were required for this exchange (24) and this, combined with other exchange and transferase studies (24–26), indicates that the

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Acceptor</th>
<th>Glutamic acid used</th>
<th>Glutamic acid isolated</th>
<th>Inorganic phosphate</th>
<th>ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>NH₂OH</td>
<td>0.97†</td>
<td>0.22</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>2†</td>
<td>“</td>
<td>1.08§</td>
<td>1.04§</td>
<td>0.24</td>
<td>0.06</td>
</tr>
<tr>
<td>3‡</td>
<td>NH₃</td>
<td>1.08§</td>
<td>1.04§</td>
<td>0.31</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Enzyme Preparation A.
† Analyzed by dehydration to 2-ketopyrrolidine-5-carboxylic acid.
‡ Enzyme Preparation B.
§ Analyzed by reduction to CO. These values are corrected for a slight blank which lowers the O¹⁸:O¹⁸ ratio.

SUMMARY

1. O¹⁸-labeled glutamic acid has been prepared and incubated with glutamine synthetase, ATP, and ammonia or hydroxylamine in an H₂O¹⁵
medium. The ADP formed contained no labeled oxygen, while the inorganic phosphate contained $^{18}O$ in amounts corresponding to a stoichiometric transfer of oxygen from the glutamic acid to the terminal phosphate of the ATP.

2. The isotopic data show that a phosphorylated intermediate is formed in which the oxygen is covalently bound, and that the medium oxygen is not involved in the energy transfer.

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