STUDIES ON THE MECHANISM OF ACTION OF GLUTATHIONE SYNTHETASE*

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It has been established that the synthesis of GSH from glutamylcysteine and glycine is accompanied by the splitting of ATP to ADP and Pi, but the detailed mechanism of this process has not been elucidated. It is reasonable to assume that the formation of the peptide bond which is catalyzed by glutathione synthetase occurs in several steps. Nevertheless, present evidence indicates that the reaction is catalyzed by a single enzyme (3, 4). Also, it has not been possible so far to accumulate intermediates or to ascertain their chemical nature. It is likely therefore that the intermediates that are formed in the course of the reaction are bound to the enzyme, possibly by covalent linkages. In an attempt to elucidate the mechanism of GSH synthesis, various isotope exchange experiments have now been carried out with glutathione synthetase. Some other properties and the purification of this enzyme are described in the accompanying paper (3).

EXPERIMENTAL

Materials—P32-ATP was generously supplied by Dr. Eugene Kennedy. P32-ADP was prepared from AMP and radioactive ATP by the action of muscle adenylic kinase (5). Both nucleotides were purified on a Dowex 1 column according to the method of Cohn and Carter (6). The enzyme used, unless stated otherwise, was the preparation which had been purified 5500-fold, as described in the preceding paper (3). The other materials were the same as those used previously (4).

Methods—Hydroxamic acid formation was measured according to the

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1 The following abbreviations are used: AMP, ADP, ATP, adenosinemoono-, adenosinedi-, and adenosinetriphosphate, respectively; GSH, glutathione; Pi, orthophosphate; Tris, tris(hydroxymethyl)aminomethane.

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method of Lipmann and Tuttle (7). Whenever it was necessary to separate the adenosine nucleotides, Siekevitz and Potter's modification (8) of the Dowex 1 chromatographic technique (6) was employed. For the determination of P\textsuperscript{32}-ATP in the presence of radioactive phosphate, separations were carried out by means of charcoal as described by Crane and Lipmann (9).

**Results**

It had been observed earlier in this laboratory (10) that ADP is a competitive inhibitor of ATP in GSH synthesis. Since ADP is also a product of the over-all reaction, the inhibitory action of this nucleotide could be taken to indicate a reversal of the process by which ADP is liberated from ATP. Results which are in support of this interpretation have been obtained by incubation of P\textsuperscript{32}-ADP and normal ATP in the presence of low concentrations of GSH synthetase. Incorporation of isotope into ATP took place readily and the exchange of P\textsuperscript{32} between ADP and ATP was essentially complete after 60 minutes (Table I). The exchange of phosphate between ADP and ATP requires magnesium but is not affected in any way by the addition of either glutamylcysteine or glycine. AMP was not formed in this reaction, and, since the concentrations of ADP and ATP remained constant under the conditions of the experiment, the observed exchange could not have been due to adenylic kinase. The participation of the latter enzyme was unequivocally ruled out by isolation and degradation of the radioactive ATP (16,000 c.p.m. per \(\mu\)mole) that had been formed in the exchange reaction with P\textsuperscript{32}-ADP. This ATP was added to the GSH synthetase system which splits the nucleotide in accordance with

### Table I

*Exchange between ATP and P\textsuperscript{32}-ADP*

<table>
<thead>
<tr>
<th></th>
<th>ADP</th>
<th>ATP</th>
<th>Exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>535</td>
<td>405</td>
<td>91</td>
</tr>
<tr>
<td>Omit enzyme</td>
<td>2730</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>&quot; magnesiu</td>
<td>2720</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Add 0.06 m glycine</td>
<td>524</td>
<td>421</td>
<td>92</td>
</tr>
<tr>
<td>&quot; 0.06 &quot; glutamylcysteine</td>
<td>515</td>
<td>494</td>
<td>99</td>
</tr>
</tbody>
</table>

The reaction mixture, 3.0 ml. in volume, contained 0.003 m ATP, 0.00057 m P\textsuperscript{32}-ADP, 0.01 m Tris buffer, 0.01 m KCl, 0.005 m KCN, 0.004 m MgSO\textsubscript{4}, 3.0 mg. of serum albumin, and 9 \(\times\) 10\textsuperscript{-4} mg. of enzyme protein per ml. The incubation was for 60 minutes at 37\(^\circ\), pH 8.3. The nucleotides were separated by Dowex 1 chromatography (8).
The equation

Glutamylcysteine + glycine + ATP → GSH + ADP + Pi

The inorganic phosphate which represents the terminal phosphate of ATP was separated from the nucleotides by charcoal and found to contain only 45 c.p.m. On the other hand, if the formation of P\textsuperscript{32}-ATP had resulted from the action of adenylic kinase, the P\textsuperscript{32} concentration in the terminal phosphate of ATP should have accounted for one-half of the total isotope

\[ 2\text{AMP} \sim P\text{32} \rightarrow \text{AMP} \sim P\text{32} \rightarrow \text{AMP} \sim P\text{32} \sim P\text{32} \]  

(1)

in ATP. Since the data in the preceding paper (3) indicate the absence of nucleoside diphosphokinase (11) and in view of the highly purified state of GSH synthetase, it appears probable that the phosphorus exchange between ADP and ATP is indeed catalyzed by the same enzyme which is responsible for GSH synthesis. These findings have previously been accepted as evidence for a reversible phosphorylation of the enzyme by ATP (1, 2), an interpretation which is consistent with the powerful inhibition of GSH synthesis by ADP. Accordingly, it has been postulated that the first step in GSH synthesis from glutamylcysteine is represented by the equation

\[ \text{Enzyme} + \text{ATP} \rightleftharpoons \text{ADP} + \text{enzyme-P} \]  

(2)

If it is correct that GSH synthetase catalyzes the splitting of ATP into ADP and a phosphorylated enzyme, one would not expect an exchange reaction to occur if radioactive orthophosphate instead of ADP\textsuperscript{32} is added to the same system. This had, indeed, proved to be the case. When enzyme and normal ATP were incubated with Pi\textsuperscript{32} alone or in combination with glutamylcysteine, glycine, or both, no significant incorporation of P\textsuperscript{32} into ATP took place.\textsuperscript{2} The negative outcome of this experiment confirms earlier observations with the corresponding enzyme from pigeon liver (4).

Since relatively large amounts of the purified enzyme were available, an attempt has been made to test the hypothesis that GSH synthesis involves the formation of a phosphorylated enzyme. 50 mg. of the purified enzyme were incubated, in the presence of magnesium ions, with 20 \(\mu\)moles of P\textsuperscript{32}-ATP which contained approximately \(1 \times 10^6\) c.p.m. per \(\mu\)mole in the terminal phosphate group. In order to remove the radioactive ATP remaining at the close of the experiment, the protein was precipitated with 3 volumes of saturated ammonium sulfate, which had been adjusted to

\textsuperscript{2} Since, as will be shown later, the synthesis of GSH is a reversible reaction, one would expect P\textsuperscript{32}-orthophosphate to be incorporated into ATP in the presence of glutamylcysteine and glycine. However, under the conditions of the experiment, the concentrations of the products ADP, Pi, and GSH were too small to permit any appreciable synthesis of ATP.
The protein was redissolved and reprecipitated five more times with ammonium sulfate. After the last precipitation, the protein was found to contain only 200 c.p.m. per mg. If a molecular weight of 100,000 is assumed for the enzyme, the protein would be expected to contain 10,000 c.p.m. per mg. if it contained 1 phosphoric acid residue per molecule of enzyme. This calculation is based on the assumption that the enzyme preparation is pure.

**Glycine Exchange**—It had been previously concluded that GSH synthetase from pigeon liver was incapable of catalyzing an exchange of the glycine moiety of GSH with free glycine (4). This problem has now been reinvestigated with the yeast enzyme. Incubation of GSH, C\(^4\)-glycine, and GSH synthetase does, indeed, result in the formation of C\(^4\)-GSH, i.e., a replacement of the glycine moiety of the tripeptide. As shown in Table II, the exchange reaction requires the presence of magnesium ions and is also dependent upon either ADP or ATP. This requirement for nucleotides cannot be met by AMP. Arsenate is a further component that is necessary, although it can be replaced to some extent by phosphate. As Fig. 1 shows, in the exchange of glycine the nucleotides are effective in extremely low concentrations, indicating that their rôle is a catalytic one.

That the exchange of glycine into GSH is due to the same enzyme that catalyzes GSH synthesis is indicated by the data in Table III. Throughout the purification of the enzyme, the ratio of synthetic to exchange activities has remained constant. Moreover, both reactions proceed op-
timally at pH 8.3. The exchange reaction is only one-tenth as rapid as the synthetic reaction, even under optimal conditions, a finding which may explain why previous results obtained with pigeon liver enzyme were negative.

In Fig. 2 is shown the effect of magnesium and manganese ions on the exchange of free glycine with the glycine moiety of GSH. At low concentrations manganese appears to be the more effective of the 2 ions for the exchange reaction. However, at $1 \times 10^{-2} \text{ M}$, which is the optimal concentration for magnesium, the relative effectiveness of the 2 ions is reversed. This is probably attributable to the formation of an insoluble manganese

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**Table III**

*Synthetic and Exchange Activities of Glutathione Synthetase at Different Stages of Purification*

<table>
<thead>
<tr>
<th></th>
<th>Specific activity*</th>
<th>Synthesis Exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSH synthesis</td>
<td>Glycine exchange</td>
</tr>
<tr>
<td>Original autolysate</td>
<td>0.212</td>
<td>0.0186</td>
</tr>
<tr>
<td>Fraction after heat denaturation</td>
<td>13.60</td>
<td>1.28</td>
</tr>
<tr>
<td>Nucleic acid fraction</td>
<td>324.00</td>
<td>28.6</td>
</tr>
</tbody>
</table>

* Expressed either as micromoles of GSH synthesized or as micromoles of glycine exchanged in 60 minutes per mg. of protein.
mercaptide of GSH. In the synthetic reaction, manganese is far less effective than magnesium. Over a concentration range of manganese from $1 \times 10^{-4}$ to $2 \times 10^{-2} \text{ M}$, the amount of GSH synthesized is never greater than 2 per cent of that observed with the corresponding concentrations of magnesium.

The ability of GSH synthetase to catalyze the exchange of the glycine moiety of the tripeptide recalls the analogous property of glutamine synthetase to catalyze the replacement of the amide of glutamine by free ammonia (13). Since the glutamine-synthesizing enzyme is also capable of exchanging the amide moiety of glutamine for hydroxylamine (13), it became of interest to determine whether GSH synthetase could catalyze a similar reaction. That hydroxylamine can indeed serve as a replacement agent for the glycine moiety of GSH is shown by the data in Table IV. As in the exchange reaction with glycine, the replacement of glycine residues by hydroxylamine requires arsenate or phosphate. When phosphate is present, the formation of hydroxamic acid is dependent upon the addition of ADP. However, in the presence of arsenate, ADP, although it stimulates the exchange with hydroxylamine, is not essential. The possibility exists that the hydroxamic acid formation which is apparently ADP-independent is actually promoted by trace amounts of ADP in the enzyme preparation. This, however, is not likely, since the exchange of glycine into GSH in the presence of arsenate did not occur at all unless nucleotide was added even when the enzyme concentration was increased 10-fold over that used in the experiments in Fig. 1. It is evident, there-

![Fig. 2](http://www.jbc.org/)

**Fig. 2.** Effect of magnesium ions (●) and manganese (▲) ions on glycine exchange. The experimental conditions were the same as those given in Fig. 1, except that the incubation mixtures contained 0.001 M ADP. In the experiments in which the manganese concentrations were $2 \times 10^{-2}$ and $1 \times 10^{-2} \text{ M}$, a precipitate appeared in the reaction mixture.
fore, that the glycine exchange and the hydroxamic acid formation are not strictly comparable reactions.

The ability of GSH synthetase to promote an exchange of the glycine moiety of GSH suggests that under certain conditions the synthesis of GSH may be reversed. The reversibility of glutamine synthesis has recently been demonstrated by Levintow and Meister (14), who have shown that ATP may be synthesized from glutamine, ADP, and Pi. That the over-all reaction glutamylcysteine + glycine + ATP → GSH + ADP + Pi is also a reversible process has been established in the following manner.

### Table IV

**Formation of Hydroxamic Acid from GSH**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Additions</th>
<th>Hydroxamic acid formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmoles</td>
<td>µmoles</td>
<td>µmoles</td>
</tr>
<tr>
<td>0.05</td>
<td>10.0</td>
<td>20.0</td>
</tr>
<tr>
<td>0.05</td>
<td>10.0</td>
<td>20.0</td>
</tr>
<tr>
<td>0.05</td>
<td>10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>0.05</td>
<td>10.0</td>
<td>50.0</td>
</tr>
<tr>
<td>0.05</td>
<td>10.0</td>
<td>20.0</td>
</tr>
</tbody>
</table>

The reaction mixtures, 1.0 ml. in volume, contained 0.1 M Tris buffer, 0.1 M KCl, 0.015 M KCN, 0.015 M MgSO₄, 0.013 M GSH, and 0.40 M neutralized hydroxylamine. The incubation was for 60 minutes at 37°, pH 8.3. The hydroxamic acid values are expressed as succinohydroxamic acid equivalents.

### Table V

**Synthesis of ATP from ADP, Pi, and GSH**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ADP</th>
<th>GSH</th>
<th>Acid-labile P</th>
<th>ATP synthesized</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmoles</td>
<td>µmoles</td>
<td>c.p.m. per µmole</td>
<td>µmole</td>
<td></td>
</tr>
<tr>
<td>0.032</td>
<td>10.0</td>
<td>13.0</td>
<td>511</td>
<td>0.68</td>
</tr>
<tr>
<td>0.008</td>
<td>10.0</td>
<td>13.0</td>
<td>296</td>
<td>0.38</td>
</tr>
<tr>
<td>0.008</td>
<td>10.0</td>
<td>13.0</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>0.008</td>
<td>10.0</td>
<td>13.0</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

The reaction mixtures, 1.0 ml. in volume, contained 0.1 M Tris buffer, 0.1 M KCl, 0.015 M KCN, 0.015 M MgSO₄, 1.0 mg. of serum albumin, and 50 µmoles of P³²-orthophosphate (8060 c.p.m. per µmole). The incubation was for 60 minutes at 37°, pH 8.3. The nucleotides were separated from orthophosphate by adsorption on charcoal (9).
then subjected to hydrolysis in 1 N HCl at 100° for 7 minutes. The acid-labile phosphate was determined and its radioactivity measured. The amount of ATP synthesized was calculated from the equation specific activity of labile phosphate = (specific activity of orthophosphate added × ATP synthesized)/(ADP added + ATP synthesized). The identification of the radioactive nucleotide as ATP was established by adding carrier ATP and separating the nucleotides on a Dowex 1 column. The radioactive component was found to travel with the ATP peak. Furthermore, with the specific activity of labile phosphate as a measure of the amount of ATP formed, the specific activity of the eluted nucleotide, after correction for dilution, differed only by 8 per cent from that of the P³²-orthophosphate added to the incubation mixture.

Assuming that equilibrium has been reached in the experiment with the larger amount of enzyme, an equilibrium constant of 1.8 × 10⁴ and a difference of -6040 calories between the standard free energies of hydrolysis of the cysteinyl-glycine bond in GSH and of the terminal pyrophosphate bond of ATP can be calculated. Since it is not certain that in the experiment listed in Table V equilibrium has been attained, the calculated values provide only an approximate measurement of the magnitude of the energy change involved.

**DISCUSSION**

For the purpose of evaluating the present results in terms of the mechanism of action of GSH synthetase it will be useful to enumerate the various reactions which are promoted by catalytic amounts of the enzyme.

Glutamylcysteine + glycine + ATP ⇌ GSH + ADP + Pi (3)

ATP + ADP³² ⇌ ATP³² + ADP (4)

³ To insure the removal of P³²-orthophosphate, the charcoal was washed three times with water, two times with 0.02 M normal phosphate buffer, and finally again five times with water.

⁴ In a previous attempt (2) to demonstrate the formation of ATP from ADP, Pi, and GSH, the concentration of Pi used was only 5 × 10⁻⁴ M. The amount of ATP that could have been formed was too small to detect.

⁵ The standard free energy of hydrolysis of a peptide bond in a dipeptide is generally assumed to be -3500 calories (15). There are indications that this value becomes smaller as the length of the peptide chain increases. If the same value is assigned to the cysteinyl-glycine bond of GSH as has been calculated for the hydrolysis of the glycyglycine bond of benzoylglycylglycine, namely -2400 calories (15), then the value for the hydrolysis of ATP would be estimated to be about -8500 calories. This is substantially smaller than the generally accepted value of 10,500 calories, but of the same magnitude as that obtained for ATP hydrolysis from a study of the glutamine system by Levintow and Meister (14).
AsO₄ or PO₄
GSH + C¹⁴-glycine $\xrightarrow{\text{ADP or ATP}}$ C¹⁴-GSH

(5)

GSH + NH₃OH $\xrightarrow{\text{AsO₄ or PO₄ (ADP)}}$ hydroxamic acid

(6)

Glutamylcysteine + hydroxylamine + ATP $\rightarrow$

hydroxamic acid + ADP + Pi

(7)

Because of the present finding that GSH synthetase catalyzes the synthesis of ATP from ADP and orthophosphate in the presence of GSH, it has now become necessary to postulate that every one of the partial reactions of the over-all process (Reaction 3) can also proceed in the reverse reaction. On the basis of various observations two alternative mechanisms for GSH synthesis have been considered (2). In both of the suggested schemes the initial reaction was assumed to be the reversible phosphorylation of the enzyme by ATP (Reaction 2). For the subsequent steps, Schemes A (Reactions 8 and 9) and B (Reactions 10 and 11) were presented as alternatives.

Enzyme-P + glutamylcysteine $\rightleftharpoons$ enzyme-glutamylcysteine + Pi

(8)

Enzyme-glutamylcysteine + glycine $\rightleftharpoons$ enzyme + GSH

(9)

Enzyme-P + glutamylcysteine $\rightleftharpoons$ enzyme + glutamylcysteine-P

(10)

Glutamylcysteine-P + glycine $\rightleftharpoons$ Pi + GSH

(11)

Although the isotope data indicate that the enzyme labilizes the terminal linkage of ATP in such a manner as to permit the free exchange of the ADP moiety, it has not been possible to prove the hypothesis that phosphate becomes concomitantly linked to the enzyme. The present data do not favor the hypothesis (Scheme A) of an enzyme-glutamylcysteine compound as an intermediate. If the formation of this intermediate (Reaction 8) were reversible, Pi should be incorporated into ATP in the presence of glutamylcysteine. This was, however, not the case. On the other hand, the failure of glutamylcysteine to inhibit the ATP-ADP exchange can be taken as evidence against the formation of an enzyme-glutamylcysteine by an irreversible reaction. Since glycine neither effects the ADP-ATP exchange nor promotes the incorporation of Pi into ATP, the formation of an enzyme-glycine intermediate can be ruled out on the same grounds. On the other hand, the formation of a phosphorylated glutamylcysteine (Reaction 10) is still a possibility, though attempts to isolate this intermediate have met with failure. The formation of this intermediate was previously considered unlikely, since it was believed that the phosphorylation of glutamylcysteine as in Reaction 11 would be ener-
getically improbable. However, the present finding that ATP can be synthesized from ADP, Pi, and GSH removes this objection. To postulate a phosphorylated glutamylcysteine as an intermediate is an attractive hypothesis, since a hydroxamic acid, presumably of glutamylcysteine, can be formed from glutamylcysteine, hydroxylamine, and ATP as well as from GSH and hydroxylamine.

If Scheme B were valid, it should follow that glycine can exchange with GSH, and that a hydroxamic acid is capable of formation from GSH in the absence of ADP. Although the data presented suggest that ADP is not essential for the hydroxamic acid formation, ADP nevertheless stimulates this process markedly, and in the case of the glycine exchange the requirement for either ADP or ATP is absolute. In order to explain the effect of the nucleotides it might be argued that Reaction 11 does occur with rupture of the cysteinyl-glycine bond of GSH, but that the glycine is bound tightly to the enzyme through linkages that are not covalent and that glycine is free to exchange only after complete reversal of the process.

The effect of arsenate in the glycine exchange, as well as in the synthesis of hydroxamic acid from GSH, is interesting in that it implies the formation of arseno compounds of glutamylcysteine and perhaps of ADP. Since both the glycine exchange and hydroxamic acid formation occur more rapidly in the presence of arsenate than of phosphate, it would appear that these arseno compounds are formed more rapidly than the corresponding phosphates, and also that they have considerable stability.

Although the present data are not incompatible with Scheme B, it should be pointed out that the formation of a free phosphorylated glutamylcysteine is difficult to accept if GSH synthetase is indeed a single catalytic entity as the present evidence would seem to indicate.

SUMMARY

Various experiments bearing on the mechanism of the enzymic synthesis of GSH from glutamylcysteine, glycine, and ATP have been carried out with purified GSH synthetase. The enzyme catalyzes an exchange of phosphate between ATP and ADP in the presence of magnesium ions. An exchange of the glycine moiety of GSH for either free glycine or for hydroxylamine is catalyzed by the same enzyme. The synthesis of GSH has been demonstrated to be a reversible reaction.

BIBLIOGRAPHY

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