Studies on the Reaction Mechanism of Adenosine Triphosphate: Glutamine Synthetase Adenylyltransferase from Escherichia coli B

CONFORMATIONAL CHANGES ELICITED BY EFFECTORS AND SUBSTRATES: REACTIVITY OF SULFHYDRYL GROUPS

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SUMMARY

Glutamine establishes feedback control of glutamine synthetase (EC 6.3.1.2) in Escherichia coli over a novel loop, viz. by its effect on an enzymatic adenylylation of glutamine synthetase, whose activity is thus modulated. Positive effectors of the adenylyl transfer reaction, magnesium ion and glutamine, increase the number of —SH groups in adenylyltransferase (EC 2.7.7.-) titratable by 5,5'-dithio-bis(2-nitrobenzoic acid); inhibitors of the transfer reaction, α-ketoglutarate and 3-phosphoglycerate, decrease the number of titratable —SH groups. It is concluded that effector control of the adenylylation reaction is exerted at the level of adenylyltransferase.

The substrates of the adenylyltransfer reaction, MgATP and nonadenylylated glutamine synthetase also expose additional —SH groups of adenylyltransferase to titration. A substrate of the reverse reaction (pyrophosphorolysis of AMP-glutamine synthetase) MgPPi·−-buries —SH groups. The relationship between the number of —SH groups modified and residual enzyme activity is established.

EXPERIMENTAL PROCEDURES

Materials—ATP, UTP, α-ketoglutarate, and 3-phosphoglycerate were products of Boehringer (Mannheim, Germany). L-Glutamine and tetrasodium pyrophosphate were obtained from Merck (Darmstadt, Germany). 5,5'-Dithiobis(2-nitrobenzoic acid) and sodium dodecyl sulfate were obtained from Serva (Heidelberg, Germany).

Adenylyltransferase—The enzyme was purified as described by Ebner et al. (14). Its activity was measured as inactivation of glutamine synthetase under standard conditions (14). Glutamine Synthetase—Glutamine synthetase was purified by

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the procedure of Woolfolk et al. (16), except that their first two steps were replaced by the two initial steps used for purifying adenylyltransferase. This variation allows both enzymes to be purified from the same batch of bacteria. Glutamine synthetase activity was measured as magnesium-dependent, glutamylhydroxamate synthesis as described by Kohlhaw et al. (17).

Protein Determination—Protein was determined by the method of Lowry et al. (18) using bovine serum albumin as standard. Values thus obtained related to values obtained using the biuret method (19): Lowry protein x 1.02 = biuret protein.

Reaction of Protein Sulfhydryl with DTNB—This reaction was carried out in 0.1 M Tris-HCl, pH 7.6, containing 0.1 mM EDTA in a total volume of 0.6 ml in 10-mm cuvettes thermostated at 25°. Adenylyltransferase was present at concentrations ranging from 1.7 to 2.3 \( \mu \)M, based on a molecular weight of 115,000 daltons (20). The reaction was started by addition of 0.5 mM DTNB. Changes in absorbance at 412 nm were followed with a Zeiss PM4 spectrophotometer. The molar extinction coefficient of the TNB-ester of sulfhydryl was determined from the reaction of DTNB with cysteine, the rate of the reaction being proportional to the concentration of DTNB, and \( X = \text{concentration of DTNB at time } t. \)

Change in adenylyltransferase activity during reaction with DTNB was measured by removing 50 \( \mu l \) samples at the times indicated in the tables and assaying them for 30 s under standard assay conditions.

RESULTS

Reactivity of Adenylyltransferase Sulfhydryl Groups as a Function of pH—Disulfide exchange is a second order reaction involving nucleophilic attack by the mercaptide ion on the disulfide (22). The apparent rate constant of the over-all reaction, \( k' \), is thus a function of the pK of the sulfhydryl group. For protein-bound sulfhydryl, influence of local environment on pK and steric barriers to the approach of the sulfhydryl may both be velocity determinants.

Reaction of adenylyltransferase with DTNB at increasing pH in the range 7.0 to 8.0 showed (a) an increase in velocity of the disulfide exchange; and (b) an increase in the total number of reactive -SH groups. The latter process was presumably the consequence of a conformation shift exposing more sulfhydryl groups to the solvent, and was complete at pH 7.6. Subsequent sulfhydryl titrations were carried out at this pH, which corresponds also to the pH optimum of the adenylyltransferase (14).

Titrations of Native and Denatured Adenylyltransferase—Fig. 1 is representative of titration curves for native and sodium dodecyl sulfate-denatured enzyme. In a molecule of native enzyme five to six —SH groups were accessible to DTNB, two of which reacted rapidly (\( k' \) on the order of \( 3 \times 10^4 \text{ M}^{-1} \text{ min}^{-1} \)). Upon denaturation, two additional —SH groups appeared, both rapidly reacting. In native enzyme these presumably lie in the inaccessible interior.

Influence of Substrates and Effectors—Fig. 2 presents a typical titration of native adenylyltransferase in the presence of various combinations of magnesium, substrate ATP, and inhibitor UTP. Addition of 25 mM Mg\(^{2+}\), the concentration for optimal enzymatic activity (14), caused the appearance of 0.5 to 1 additional

\[ \	ext{FIG. 1.} \text{ Reaction of adenylyltransferase with DTNB in the presence and absence of sodium dodecyl sulfate. The reactions were carried out at 25° in 0.1 M Tris-HCl, pH 7.6, 0.1 mM EDTA, and 0.5 mM DTNB as described under } \text{ "Experimental Procedures."} \]  

Protein concentration was 0.233 mg per ml. Reaction in absence of sodium dodecyl sulfate (○); in the presence of 0.5% sodium dodecyl sulfate (○).

\[ \	ext{FIG. 2.} \text{ Reaction of adenylyltransferase with DTNB in the presence of Mg\(^{2+}\), ATP, and UTP. Reaction mixtures were as described in the legend to Fig. 1, with the following additions: 25 mM MgCl\(_2\) (O), 25 mM MgCl\(_2\) plus 5 mM ATP (△), 25 mM MgCl\(_2\) plus 20 mM ATP (▲), 25 mM MgCl\(_2\) plus 10 mM UTP (○), reference reaction without additions (●). Protein concentrations were 0.216 mg per ml in all cases.} \]
TABLE I
Change in the number of titratable thiol groups of adenylyltransferase by effectors of the adenylylation reaction

The reaction mixtures contained 0.1 M Tris-HCl, pH 7.0, 0.1 mM EDTA, 1.7 to 2.3 units adenylyltransferase, and 0.5 mM DTNB. Effectors were added at 20 mM. Values of ΔSH are given per molecule enzyme and were taken after 60 min when the reaction was completed. The range summarizes the results from 4 experiments.

<table>
<thead>
<tr>
<th>Effector added</th>
<th>Reference conditions</th>
<th>ΔSH with respect to reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Glutamine</td>
<td>Without effector</td>
<td>0</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>Without effector</td>
<td>-0.05</td>
</tr>
<tr>
<td>3-Phosphoglycerate</td>
<td>Without effector</td>
<td>-0.25</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>25 mM Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>25 mM Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>-0.3 to -0.5</td>
</tr>
<tr>
<td>3-Phosphoglycerate</td>
<td>25 mM Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>-0.5 to -1.0</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>25 mM Mg&lt;sup&gt;2+&lt;/sup&gt; + 1 mM ATP</td>
<td>+0.2 to +0.3</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>25 mM Mg&lt;sup&gt;2+&lt;/sup&gt; + 20 mM α-ketoglutarate</td>
<td>+0.3 to +0.5</td>
</tr>
</tbody>
</table>

TABLE II
Sensitivity of adenylyltransferase to activators and inhibitors before and after sulfhydryl modification

Enzyme, 56 µg, was incubated at 25°C for 2 hours with 25 mM MgCl<sub>2</sub>, 0.1 M Tris/HCl, pH 7.0, 0.1 mM EDTA with and without DTNB, 0.5 mM. Subsequently effectors were added as indicated and the enzymatic reaction was started by addition of adenylyltransferase. For each column 100% activity is that without addition of effectors.

<table>
<thead>
<tr>
<th>Activator or inhibitor added</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>% control</td>
<td></td>
</tr>
<tr>
<td>L-Glutamine, 20 mM</td>
<td>1750</td>
</tr>
<tr>
<td>α-Ketoglutarate, 20 mM</td>
<td>86</td>
</tr>
<tr>
<td>3-Phosphoglycerate, 20 mM</td>
<td>34</td>
</tr>
<tr>
<td>ATP, 20 mM</td>
<td>26</td>
</tr>
<tr>
<td>UTP, 10 mM</td>
<td>92</td>
</tr>
<tr>
<td>Pyrophosphate, 2 mM</td>
<td>72</td>
</tr>
</tbody>
</table>

UTP (48% inhibition, see Table II) was similar to that of 20 mM ATP. These effects of ATP' and UTP' are not to be laid to a sequestering of Mg<sup>2+</sup>, since compensating increments of Mg<sup>2+</sup> (50 mM) did not alter the picture. ATP in the absence of magnesium was without effect.

Table I summarizes the results of several thiol titrations of adenylyltransferase in the presence of various combinations of positive and negative effectors. Inhibitors of enzymatic activity, α-ketoglutarate and 3-phosphoglycerate, alone or in the presence of magnesium, rendered a portion of the protein —SH groups inaccessible for reaction with DTNB. The stronger inhibitor, 3-phosphoglycerate, had the stronger effect. Activator glutamine per se produced no measurable changes in titration behavior. It did, however, augment the increase in number of titratable —SH groups seen with ATP and compensate the decrease seen with α-ketoglutarate. Under these conditions, at least, a conformational change in adenylyltransferase may be attributed to glutamine.

Inorganic pyrophosphate is a product of the adenylyltransferase reaction (11), and as such inhibits this reaction (14). Pyrophosphate forms two types of complex with magnesium, MgPP<sub>2</sub> and Mg<sub>2</sub>PP<sub>2</sub>, and it appears likely from studies of the adenylylation reaction equilibrium that the form Mg<sub>2</sub>PP<sub>2</sub> is the actual reactant (13). We have attempted to assess the interaction of the various complex forms of pyrophosphate with adenylyltransferase. From the known total concentrations of magnesium and pyrophosphate, the concentrations of MgPP<sub>2</sub> and Mg<sub>2</sub>PP<sub>2</sub> were calculated as described in Reference 13. In the absence of magnesium, pyrophosphate (4.17 mM) had no noticeable effect on sulfhydryl titration. The presence of magnesium pyrophosphate caused a decrease in the number of titratable sulfhydryl groups, in this case measured 15 min after start of reaction, to avoid interference from a pyrophosphate precipitate noticeable at 60 min. With 4.17 mM total PP<sub>1</sub> and 4.17 mM MgCl<sub>2</sub>, the calculated concentration of the Mg<sub>2</sub>PP<sub>2</sub> complex is 2.8 mM, that for MgPP<sub>2</sub> 0.4 mM. Under these conditions decreases of 0.3 to 0.4 —SH groups per molecule adenylyltransferase were observed. With 1.67 mM total PP<sub>1</sub> and 20 mM MgCl<sub>2</sub>, the calculated concentrations of MgPP<sub>2</sub> and Mg<sub>2</sub>PP<sub>2</sub> are 0.35 and 1.10 mM, respectively. Under these conditions a decrease of 0.15 —SH group was measured.

Those substances which bury —SH groups (20 mM ATP, UTP, α-ketoglutarate, 3-phosphoglycerate and pyrophosphate) might do so either by inducing conformational shifts in the protein or by blocking access to —SH groups which lie at their binding sites. We sought to distinguish between these possibilities with the rationale that if an —SH group lay at the binding site, prior modification of it with DTNB would prevent binding of inhibitor and thus inhibition. Table II shows that this is not the case, that adenylyltransferase was as sensitive to inhibition after modification as before. Thus, the disappearance of titratable sulfhydryl groups in the presence of inhibitors may be ascribed also to shifts of protein conformation.

Interaction between Glutamine Synthetase and Adenylyltransferase—Glutamine synthetase itself has no titratable sulfhydryl
groups (23). The interaction between glutamine synthetase and adenylyltransferase results, however, in the appearance of additional sulfhydryls. The course of a titration with adenylyltransferase in the presence of adenylylated and nonadenylylated glutamine synthetases is shown in Fig. 3. Control titrations with both forms of glutamine synthetase confirmed the absence of reactive sulfhydryl groups. In the absence of effectors glutamine synthetase did not alter the total number of titratable —SH groups, but accelerated the rates of reaction. The over-all kinetic changes correspond to an increased apparent rate constant for 0.55 —SH group in the case of nonadenylylated glutamine synthetase and for 0.45 —SH group in the case of adenylylated glutamine synthetase.

In the presence of concentrations of magnesium (25 mM) and glutamine (20 mM) optimal for transferase activity, however, glutamine synthetase (8.6 x 10^-6 m) caused the appearance of additional reactive —SH groups. The effect was more pronounced with nonadenylylated glutamine synthetase (+1 —SH group) than with adenylylated (+0.4 —SH group).

These experiments indicate that the conformational changes elicited by glutamine synthetase in the absence of effectors of the transferase reaction are independent of the state of adenylylation of glutamine synthetase. However, in the presence of effectors nonadenylylated glutamine synthetase has a larger effect than an equal concentration of adenylylated enzyme.

The simplest interpretation of these data is that glutamine synthetase, like the other substrates, elicits a conformational shift in adenylyltransferase which exposes additional —SH groups. The data, however, do not exclude the possibility that the conformational shift takes place in glutamine synthetase. Whichever protein may be the source of the new titratable —SH groups, the conclusion holds that the interaction between the proteins is influenced both by the effectors and state of adenylylation.

**Thiol Groups and Enzymatic Activity**—Kinetic analysis of the reaction of the thiol groups of adenylyltransferase with DTNB allows classification of these according to apparent velocity constants, k' (see Table III). Thiols of Class I react essentially to completion within 1/2 min; those of Class II within 23 min. A third class reacts within 20 min, a fourth class within 60 min.

The additional thiol (0.5 to 1 per protein molecule) uncovered in the presence of magnesium fall into Class I. The rate constant of this class, furthermore, was doubled in the presence of magnesium ion; k' of Class II was increased 20%. With or without magnesium, the reaction of Group I thiols was so rapid as to prohibit accurate determination of k'.

The following experiment (Fig. 4) examined the relation of enzymatic activity to chemical modification of thiols belonging to these various kinetic classes. In the absence of magnesium, reaction of Class I and II thiols left enzymatic activity unaltered. Reaction of the single thiol of Class III resulted in a 20% loss of activity: reaction of the Class IV thiol diminished activity by a further 40%. The remaining 40% activity was unaltered by prolonged incubation with DTNB.

Reaction in the presence of 25 mM MgCl2 presented a different picture. Reaction of thiols of Class I led to the loss of about 5% of enzymatic activity; a similar loss occurred when Class II thiols reacted. Modification of thiols III and thiol IV reduced activity by 45 and 75%, respectively. The 20% residual activity survived prolonged incubation with DTNB. A similar residual activity was observed when the titration was carried out in the presence of glutamine synthetase under conditions where the adenylylation reaction was in process. As was shown in Table II, this residual activity is responsive to activation by glutamine.

**DISCUSSION**

The catalytic efficiency of an enzyme depends largely upon the conformation it assumes during the transformation of substrate into product. The catalytic potential of a large number of enzymes is modulated by effectors bound at sites distinct from the substrate-binding site.

Glutamine synthetase is subject to feedback inhibition by a number of end products of glutamine metabolism (24). Glutamine synthetase itself establishes feedback control over a novel loop, namely via its effect on an enzymatic reaction which inactivates glutamine synthetase by covalent modification (5). The target of glutamine action might be the inactivating enzyme, ATP: glutamine synthetase adenylyltransferase, whose activity might be enhanced by binding glutamine, or it might be glutamine synthetase itself, whose vulnerability to attack from adenylyltransferase might be heightened by binding glutamine. Both glutamine and Mg2+, another activator of the adenylyltransferase reaction, bind to glutamine synthetase. As has been shown here,
both also bind to adenylytransferase as gauged by their abilities to cause conformational changes in this enzyme. That glutamine affects the state of adenylytransferase has also been demonstrated by fluorescence measurements (7, 25).

Crucial to our interpretation that regulatory significance lies in the binding to adenylytransferase is the fact that the effects of several molecules on the titratability of transferase thiols correlate to their effects on the transferase reaction. For example, concentrations of Mg$^{2+}$ which support less than 10% of maximal enzymatic activity cause no perceptible change in titratability, while optimal concentrations expose 0.5 to 1 additional —SH group to DTNB titration. Activator glutamine, in the presence of substrate ATP, also increases the number of titratable thiol groups, and counteracts the decrease caused by inhibitor α-ketoglutarate. UTP, α-ketoglutarate and 3-phosphoglycerate, in contrast, cause a reduction in the number of titratable thiols and inhibit enzymatic activity. The magnitudes of both effects are correlated.

It seems to us, therefore, most likely that adenylytransferase is target for effector control of the inactivation of glutamine synthetase. That effector binding directly to glutamine synthetase is completed without influence on its activation, of course, is not established.

We would like to stretch this conclusion into a hypothesis that adenylytransferase is also the regulated member in the glutamine synthetase deadenylylating (reactivating) complex. This complex consists of adenylytransferase and a second protein factor (8, 9). In a way not yet fully understood, this protein factor determines whether adenylytransferase operates as such (GS + ATP → GS-AMP + PP$_i$) or as a phosphorylase, deadenylylating glutamine synthetase (GS-AMP + P$_i$ → GS + ADP). In fact, those metabolites investigated here which alter the state of adenylytransferase deadenylylating (reactivating) complex. This complex consists of adenylytransferase and a second protein factor (8, 9). In a way not yet fully understood, this protein factor determines whether adenylytransferase operates as such (GS + ATP → GS-AMP + PP$_i$) or as a phosphorylase, deadenylylating glutamine synthetase (GS-AMP + P$_i$ → GS + ADP). In fact, those metabolites investigated here which alter the state of adenylytransferase and inhibit the transferase reaction, α-ketoglutarate, UTP, and 3-phosphoglycerate, activate the phosphorylolytic reaction of the complex; the reverse is true for glutamine (26, and unpublished observations). The hypothesis could neatly accommodate this reciprocity.

Thiol titrations of adenylytransferase in the presence of effectors suggest that there exist two behaving conformation, open and closed. We propose that the open conformation is catalytically most efficient for the transfer of the adenylyl group to glutamine synthetase; the closed conformation is more suited for deadenylylation, pyrophosphorolytic in the case of adenylyltransferase: protein factor complex mentioned above. Conformational response to magnesium supports this idea. Optimal Mg$^{2+}$ concentrations for both deadenylylating reactions are considerably lower, 10 mm (20) and 3.3 mm (12), respectively, than for the adenylytransferase reaction. At these lower concentrations of Mg$^{2+}$, adenylytransferase exists in a closed conformation; the optimal Mg$^{2+}$ concentration for the adenylytransferase reaction, 25 mm, promotes an open conformation. The effect of pyrophosphate is also consistent with this picture: magnesium pyrophosphate, substrate for the pyrophosphorolytic deadenylylation closes the conformation. Furthermore, inhibition studies indicate that nonadenylylated glutamine synthetase has a roughly 4 fold greater affinity for adenylytransferase under conditions supporting an open conformation (14) than does adenylylated glutamine synthetase. As seen in the present study, under these conditions nonadenylylated enzyme enhances —SH turnover as compared to adenylylated glutamine synthetase. Under conditions supporting a closed conformation (— Mg$^{2+}$, — glutamine), however, only slight kinetic changes are observed in titration behavior, and these are essentially indifferent to adenylylation state. Thus, it is reasonable to include in our picture of open and closed conformations a heightened affinity of the open conformation for nonadenylylated glutamine synthetase. Direct binding studies between these two proteins have been put off out of technical considerations.

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