Use of the Sodium Borohydride Reduction Technique to Identify a γ-Glutamyl Phosphate Intermediary in the *Escherichia coli* Glutamine Synthetase Reaction*

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Incubation of unadenylylated *Escherichia coli* glutamine synthetase with ATP, L-[14C]glutamate and metal ion results in the formation of γ-glutamyl-P which can under appropriate conditions be reduced by sodium borohydride. The acyl-P compound is formed catalytically as judged by the quantity of radioactive α-amino-β-hydroxyvalerate produced compared to the concentration of enzyme subunits. Formation of the glutamyl-P compound occurs in the presence of magnesium or manganous ions, and the relation of this apparent lack of metal ion specificity with regard to the highly specific Mg2+-supported biosynthetic activity of the unadenylylated form is discussed.

During the past 2 decades considerable attention has been given to studies of covalent compounds that are transiently formed during enzymatic catalysis, and there are now some 60 cases in which the participation of such intermediates has been intimated (1). One enzyme, glutamine synthetase, has been the subject of numerous chemical and kinetic studies in an effort to detect and characterize the possible involvement of a γ-glutamyl-P intermediate which would be noncovalently held at the active center in each catalytic cycle. The very early work of Kowalsky *et al.* (2) and Boyer *et al.* (3) rather clearly demonstrated that the enzymic synthesis of glutamine is attended by the transfer of an oxygen atom from the glutamate carboxyl to inorganic phosphate; however, it has been recognized for some time that this observation is compatible with the activation of that carboxyl group at the expense of ATP hydrolysis prior to or in concert with the formation of the γ-amide. Nonetheless, the mechanism involving acyl phosphate formation has gained the favor of Meister and his associates who have provided a series of elegant studies that can best be interpreted in such terms (4). Briefly, these chiefly include the observed catalytic formation of 5-oxopropyl- from ATP and glutamate in the absence of ammonium ions (5, 6), synthesis of ATP from ADP and β-aminoglutaryl-P (7), and the phosphorylation of the irreversible inhibitor, methionine sulfoximine, by ATP and divalent metal ions (8, 9). More recently, the finding that cis-1-amino-1,3-dicarboxycyclohexane acts as an effective alternative substrate with the sheep brain enzyme and can be phosphorylated has been taken as direct evidence of formation of an acyl phosphate (10).

On the other hand, isotopic equilibrium kinetic measurements by Wedler and Boyer (11) have been interpreted in a contrary fashion, and these investigators incline toward a concerted mechanism lacking an acyl-P intermediary. Although these studies were with the adenylylated form of the *Escherichia coli* enzyme, Wedler (12) has carried out more recent studies on the ovine brain, pea seed, and adenylylated as well as unadenylylated forms of the glutamine synthetase. Without exception, the relative rates of exchange are (glutamate = glutamine) > (NH2 = glutamine) > (P = ATP) = (ADP = ATP), which he interprets to indicate that the rate of net turnover at saturating substrate levels is limited more strongly by nucleotide release than by the rate of covalent interconversion. In addition, recent unpublished experiments by Huang and Purich on the γ-glutamyl transfer reaction kinetics of the unadenylylated bacterial enzyme are also in accord with the view that an acyl-P intermediate, if formed, is not kinetically discernible. As recognized by kineticists for some time (13) and discussed with regard to glutamine synthetase by Stadtman and Ginsburg (14), the initial rate and equilibrium kinetic studies do not exclude acyl-P formation in the presence of all substrates on the enzyme's surface.

Most recently, Timmons *et al.* (15) have cited indirect fluorimetric evidence for what they speculate to be the reactive intermediate. These investigators correctly state that their approach cannot distinguish between the two possibilities of complete phosphate transfer to make an acyl-P as opposed to partial bond formation between L-glutamate and MgATP2−. In fact, although fluorescence changes are particularly sensitive probes of enzyme conformational changes, assignment to acyl-P formation is no more justified than the formation of a γ-thiol ester between enzyme and glutamate. In this respect, it is rather clear that data of Timmons *et al.* (15) only indicate...
that certain conformational isomerizations attend the binding of the glutamate and nucleotide, and are in agreement with the observed synergism of binding of these compounds (12).

In view of the possibly conflicting notions regarding this reaction mechanism, we have attempted to employ yet another chemical method to the problem of detecting the glutamyl-P directly. Our approach has been to utilize the newly introduced method of Degani and Boyer (16) involving use of borohydride reduction to trap acyl-P compounds and form their respective alcohol derivatives. This method rests upon the known reactivity of acyl phosphates toward negatively charged nucleophiles such as hydroxyl ion (17, 18), and already has been advantageously employed to identify the site of phosphorylation of several ATP-phosphohydrolases (16, 19). In addition, we recently described how this method could be used to identify a glutamyl residue of E. coli acetyl kinase as the site of covalently bound phosphate in the catalysis of that reaction (20).

We report here upon similar studies with unadenylated E. coli glutamine synthetase that indicate acyl-P formation when ATP, glutamate, and a divalent metal ion are incubated with the enzyme in the absence of ammonium ions. Acyl-P formation is catalyzed by the enzyme, and Mn" or Mg" will support the process. Weisbrod and Meister (6) have made a similar observation with 5-oxoproline formation. The significance of this finding with respect to the over-all biosynthetic reaction catalyzed by the unadenylated enzyme is discussed.

EXPERIMENTAL PROCEDURE

Materials—The preparations of glutamine synthetase E. coli, where the abbreviation indicates the average state of adenylylation of the enzyme determined spectrophotometrically (21) as well as by activity assays of the γ-glutamyl transfer reaction (22), were isolated using the protocol developed by Woolfolk et al. (23). With the dimethylglutarate buffer system a specific activity of about 80 was determined for these enzyme samples, and protein homogeneity was assessed electrophoretically by the procedure described elsewhere (24). Prior to use, all enzymes were subjected to extensive dialysis in a continuous rocking dialyzer against the respective divalent metal ion, and all samples and buffers were treated to remove traces of ammonium ions (3, 4).

ATP, ADP, glutamine, glutamate, Hepes, and potassium arsenate were Sigma products. L-[U-14C]Glutamate (specific radioactivity 265 Ci/mmol), purchased from Amersham/Searle, was purified by ascending paper chromatography (3, 4). 5-Oxoproline standards were synthesized as described elsewhere (3, 4), and δ-hydroxy-α-aminovalerate was obtained from Cyclochemical Co. Silica gel chromatogram 13181 thin layer sheets were purchased from Eastman Organic Chemicals, Inc.

Methods—Sodium borohydride reduction reactions were carried out essentially as described by Degani and Boyer (16) except that the borohydride concentration was approximately 30 mM. Use of alcohol solvents was avoided because they lead to additional reduction products presumably by containing some aldehydes. The reduced samples were generally acidified with formic acid to destroy a large fraction of the remaining borohydride, and borate ion was removed by passage over Sephadex G-25 to which it is complexed at pH 8.5. To be assured that no trace borate might remain to complicate subsequent analysis, a sensitive fluorimetric spot test using alizarin was developed. A thin layer chromatogram of deproteinized samples was carried out at room temperature using a solvent of the following composition: 2,6-lutidine-collidine-water (1:1:1) with 2% diethylamine (25). Amino acids were located by ninhydrin spray, and similar samples which were not sprayed were used for counting in a Packard liquid scintillation counter. With the thin layers, the adsorbent was carefully scraped from the plastic backing prior to counting.

RESULTS

Central to a more complete understanding of the catalysis of the glutamine synthetase reaction is the determination of the bonds between substrates that are transiently formed and broken during each catalytic round. In the case of glutamyl-P formation, it was reckoned that to suitably identify the intermediate a chemical-trapping agent was needed that reacts rapidly and specifically with acyl-P compounds. The finding of Degani and Boyer (16) that sodium borohydride could be utilized to relatively rapidly reduce acyl-P compounds was an important lead, and it was felt that, since this reductant would not reductively cleave glutamine or 5-oxoproline, the reagent could be advantageously employed for our purpose. Preliminary experiments were designed to uncover favorable conditions for trapping the postulated intermediary by using l-[U-14C]glutamate and an excess of the borohydride. While this latter condition is not particularly useful in the [3H]borohydride reduction of phosphoryl enzyme compounds because of high nonspecific labeling of the protein (16, 19, 20), the use of radioactive glutamate and nonradioactive sodium borohydride eliminates a number of complicating side reactions. In our first experiments, ethanol was utilized to precipitate the synthetase after a period of incubation with Mg++, [3H]glutamine, and ATP, and sodium borohydride reduction of the deproteinized supernatant fluid followed. No radioactive δ-hydroxy-α-aminovaleate was detected by either of the separation methods described above; however, the reason for these apparent negative results became apparent when the ethanol was found to contain trace levels of acetaldehyde. In comparison to the estimated levels of glutamyl-P one would expect that the borohydride would react completely with the preponderating levels of aldehyde. Fortunately, it was found that the addition of 2 volumes of dimethylsulfoxide would essentially quantitatively precipitate the synthetase at 0° with little or no precipitation of noncovalently bound compounds. In these preliminary experiments, it was found that carbonyl compounds were unsuitable for the determination of any borohydride remaining after acyl-P reduction because of the possible formation and reduction of Schiff bases with the α-amino group. Acid was found to be quite useful for this purpose.

That a phosphorylated glutamyl intermediary is formed when the unadenylated enzyme is incubated with ATP, glutamate, and metal ions in the absence of NH₃ is suggested by the results presented in Fig. 1. In this experiment the synthetase was present at a concentration of 20 μM assuming a monomer molecular weight of approximately 50,000, and the amount of δ-hydroxy-α-aminovaleate detected was estimated to be approximately 14 μM. Suitable controls that lacked ATP or enzyme gave no δ-hydroxy-α-aminovaleate as discerned by our separation methods, and confirmed that the free unactivated amino acid was inert to the borohydride reduction conditions. Essentially all the acyl-P was trapped as δ-hydroxy-α-aminovaleate because the level of 5-oxoproline was negligibly small. Similar results were obtained when ammonium ion was substituted for magnesium ion, and this finding suggests that acyl-P formation may be relatively nonspecific with regard to metal ions, and this observation correlates with the formation of 5-oxoproline by the same enzyme form (6).

In the most rigorous sense, the findings outlined above only...
suggest that a carboxyl group has been sufficiently activated to allow attack of the negatively charged borohydride and subsequent reduction. Since the enzyme was not removed from that reaction mixture prior to reduction, it is also possible that a covalently bound glutamyl enzyme was formed in the initial incubation and the borohydride reduction product arose from other than a glutamyl-P intermediary. Indeed, Solomon and Jencks (26) were able to use borohydride reduction to identify an enzyme-γ-glutamyl-coenzyme A intermediary in the succinyl-CoA:acetoacetate coenzyme A transferase reaction. Clearly, the possibility of an enzyme glutamate thiol ester was not excluded by the data presented in Fig. 1. However, by use of dimethylsulfoxide to precipitate and separate glutamine synthetase from the putative glutamyl-P prior to borohydride reduction, it was possible to show that essentially no radioactivity coprecipitated with the enzyme. On the other hand, the supernatant fluid when treated with sodium borohydride produced a significant quantity of the δ-hydroxy-α-aminovalerate. As shown in Figs. 2 and 3, the δ-hydroxy-α-aminovalerate and a minor quantity of 5-oxoproline are well separated from the glutamate peak by electrophoresis and thin layer chromatography, respectively. It is noteworthy that the quality of resolution is drastically affected by the presence of borate ions which must be removed by Sephadex chromatography prior to chromatography. In addition, the thin layer chromatogram produced a significant quantity of the δ-hydroxy-α-aminovalerate.

FIG. 2. High voltage electropherograms of sodium borohydride-treated glutamine synthetase reaction mixtures containing enzyme, ATP, glutamate, and metal ion. Conditions were similar to those described in the legend to Fig. 1, but prior to borohydride reduction the samples were deproteinized by addition of 1.5 volumes of cold dimethylsulfoxide and subsequent centrifugation at 8000 × g for 5 min. Of each sample, approximately 14 nCi of radioactivity were spotted and electrophoresis was carried out as described above. A, radioactivity distribution from the electrophoretic origin for the Mg²⁺-supported system; B, the radioactivity distribution as a function of distance from the electrophoretic origin. As in Fig. 1, the size of the glutamate peak is depressed as a result of the solvent used in spotting samples. AHV, δ-hydroxy-α-aminovalerate.

FIG. 3. Plot of the radioactivity distribution of glutamate, 5-oxoproline (OxPro), and δ-hydroxy-α-aminovalerate (AHV) as a function of distance migrated from the origin of the thin layer chromatogram. Reaction conditions are those described in the legend to Fig. 2. Aliquots of the Mg²⁺- and Mn²⁺-supported reactions were spotted, and the chromatograms were developed for 8 hours in an ascending system containing a collidine-lutidine-water solvent. Strips of the plastic-backed thin layer sheets were cut and sprayed with ninhydrin to localize the position of authentic standards. Radioactivity was determined by carefully scraping the thin layer away from the plastic backing with a razor blade and counting in a toluene-based scintillation system. Counts have been converted to nanomoles of glutamate, 5-oxoproline, and δ-hydroxy-α-aminovalerate formed under the reaction conditions described. Represented in the figure are the magnesium ion (A)- and manganous ion (B)-supported systems.
graphs were far better resolved when the borate-free deproteinized samples were dissolved into 60% ethanol such that only a small fraction of the glutamate dissolves. In the absence of these measures, the δ-hydroxy-α-aminovalerate and 5-oxoproline peaks appear as shoulders of the glutamate peak and quantitation is impossible.

In the experiments described above, the production of δ-hydroxy-α-aminovalerate is relatively similar for both Mg2+ and Mn2+, and the formation of 5-oxoproline was found to also follow the same pattern. Indeed, the latter was first observed by Weisbrod and Meister (6). As shown in Table I, the amount of δ-hydroxy-α-aminovalerate formed under appropriate conditions exceeds the concentration of enzyme subunits and these data also accord with the observation with ovine brain (5) and unadenylylated bacterial enzyme (6) that 5-oxoproline is catalytically produced by the enzyme. The data presented here are rather clear evidence for the stability of the glutamyl-P intermediate during the reduction reaction. In the absence of ammonium ion, which is to be compared with the rate of glutamine synthesis in the presence of either Mg2+ or Mn2+ ions. Similarly, Weisbrod and Meister (6) found the same enzyme form to catalyze the formation of 5-oxoproline at a Mg2+-supported rate that was approximately 2.5-fold greater than the rate with manganous ion.

**Table I**

<table>
<thead>
<tr>
<th>Additions or conditions of assay</th>
<th>Sample No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (0.5 mM)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Magnesium ion (1 mM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Manganese ion (1 mM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-[14C]Glutamate (0.1 mM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Deproteinized before NaBH4 treatment?</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>AHV produced (nmol)</td>
<td>14.5</td>
<td>14.7</td>
<td>15.4</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>5-Oxoproline formed (nmol)</td>
<td>1.2</td>
<td>1.0</td>
<td>1.1</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

* AHV, δ-hydroxy-α-aminovalerate.

In this mechanism, coordination of the carbonyl of the γ-carboxyl group to the divalent metal ion promotes nucleophilic attack on the nucleotide and facilitates acyl-P formation. This mechanism would agree with the equilibrium exchange data (11, 12) if it is assumed that product desorption is rate-limiting.

Of considerable interest is the observation that glutamyl-P formation may take place with the unadenylylated enzyme in the presence of either Mg2+ or Mn2+ ions. Similarly, Weisbrod and Meister (6) found the same enzyme form to catalyze the formation of 5-oxoproline at a Mg2+-supported rate that was approximately 2.5-fold greater than the rate with manganous ion. This is to be compared with the rate of glutamine synthesis in the presence of Mg2+ and ammonium ions that is 20-fold higher than with Mn2+. In fact, the biosynthetic reaction rate with manganous ion can be almost completely accounted for by the presence of approximately 5% adenylylated subunits in their unadenylylated enzyme. This view is supported by the much earlier findings of Stadtman and colleagues (23, 28-30) who have shown that the unadenylylated enzyme is almost entirely Mg2+-dependent while the adenylylated form is Mn2+-dependent. Indeed, this reciprocal dependence upon divalent cations was responsible in part for the original discovery of adenylylation by the Bethesda group. The fact that there is an apparent lack of specificity with regard to acyl-P formation while the biosynthetic reaction shows high specificity suggests that some later step such as attack of ammonia on the acyl-P or product release is rate-limiting in the forward reaction. Thus, it may be possible to dissect the biosynthetic reaction into early and late phases in order to better understand the origin of this metal ion specificity in the over-all reaction. Another possibility is that the enzyme’s active center is less specific in the absence of ammonium ion, which from the kinetic studies (11, 12) is required to form the enzyme-ATP-glutamate-NH4 complex prior to product release. To date, however, there is no available evidence to exclude the possibility of partial disruption of the active site region in the absence of this nucleophile and further work is clearly necessary.

An additional word regarding the suitability of the borohydride reduction method as a specific probe of the formation of activated carboxyl groups may be helpful. Reduction of amides such as L-glutamine and lactams like 5-oxoproline do not occur with sodium, potassium, or lithium borohydrides (31). Indeed, sodium borohydride has been used in the reduction of other functional groups in amide containing compounds such as N-[2-hydroxy-1-(2-thienyl)ethyl]acetamide (32). Furthermore, the nonreduction of amides with lithium borohydride has been utilized in the reduction of peptide esters (31).
Finally, the reduction of lactams including a variety of pyrrolidones requires the use of lithiumaluminumhydride and is analogous to the reduction of open chain amides by this reductant (33). Thus it is rather clear that the δ-hydroxy-α-aminovalerate produced by sodium borohydride reduction can only arise from the reduction of a dissociable activated γ-glutamyl carboxylate such as the glutamyl-P or, probably less likely, the unsymmetrical pyrophosphate, P(\(\text{adeno-}
line{5'}\)-P\(\text{\(\gamma\)-L-glutamyl}\) \text{diphosphate}. The latter would be analogous to the postulated formation of a β-aspartyladenylate in the bacterial asparagine synthetase reaction (34).

Finally, it should be noted that the observations of Timmons et al. (19), if shown to be a bona fide reflection of acyl-P formation that is not obfuscated by other interactions on the enzyme's surface, may provide the most convenient means of following the reactions of the acyl-P intermediary. It will be of particular interest to learn whether their observed fluorescence changes upon incubation of the unadenylylated enzyme with ATP, L-glutamate, and Mg\(\text{II}\) are also observed when manganous ions are substituted. Clearly, the one drawback of the borohydride method is that it requires disruption of the active site, with the subsequent prompt reduction of liberated acyl-P. On the other hand, the chemical method is probably least ambiguous, and favorable reaction conditions utilizing a rapid multimixing device to effect rapid reduction of acyl-P in the presence of ammonium ions are now being sought.

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Use of the sodium borohydride reduction technique to identify a gamma-glutamyl phosphate intermediary in the Escherichia coli glutamine synthetase reaction.  
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