Reactivation of Glutamine Synthetase from *Escherichia coli* after Auto-inactivation with L-Methionine-S-sulfoximine, ATP, and Mn²⁺

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*Escherichia coli* glutamine synthetase auto-inactivated with L-methionine-S-sulfoximine and ATP can be completely reactivated at pH 3.5-4.6 in 1 M KCl and 0.4 M (NH₄)₂SO₄. Both unadenylated and adenylylated magnesium and manganese enzymes can be reactivated. Reactivation of fully inactivated enzyme is first order (t½ = 2 min at pH 4.1 and 37 °C) and coincides with the stoichiometric release of 0.95 ± 0.06 eq each of L-methionine-S-sulfoximine phosphate and ADP and 2.0 ± 0.2 eq of Mn²⁺ from each subunit. The rate of reactivation increases with decreasing pH and is proportional to the 3rd to 4th power of the hydrogen ion activity; the protonation of 3-4 carboxylic acid groups/subunit therefore may be required to disrupt the enzyme complex. Reactivation rate also increases with increasing KCl concentrations and temperature, with an Arrhenius activation energy of ~26 kcal/mol, suggesting that some protein structural perturbation is required to disrupt the complex. Upon neutralization of reaction solutions, the ligands and metal ions recombine with the enzyme resulting in its complete reactivation. Thus, inactivation of glutamine synthetase at neutral pH is due to the extremely tight, but reversible, binding of L-methionine-S-sulfoximine phosphate, ADP, and Mn²⁺ (Kₐ > 10¹² M⁻¹) for ADP) to the enzyme. Under certain conditions, ATP or ADP can partially inhibit inactivation. Excess ADP inhibits reactivation by L-methionine-S-sulfoximine phosphate, and ATP has a complex effect on the rate and extent of the auto-inactivation reaction.

The glutamine synthetase of *Escherichia coli* is composed of 12 identical polypeptides arranged in 2 superimposed hexagonal rings (1). In addition to cumulative feedback inhibition, enzymatic activity of glutamine synthetase is modulated by covalent modification catalyzed by an adenylyltransferase that is part of an exquisitely regulated bicyclic cascade system (1, 2). Each subunit contains an active site (3), separate binding sites for several feedback inhibitors (1, 4), and a specific tyrosyl residue that is the acceptor for the 5'-adenylate group (5). Also, each subunit has two essential sites for divalent metal ion binding; both sites must be occupied for full expression of activity (3). The n₁ site binds Mn²⁺ or Mg²⁺ with high affinity and has a structural and possibly a catalytic role (6-8). The n₂ site has lower affinity for Mn²⁺ and binds the nucleotide-metal ion complex with higher affinity than Mn²⁺ alone (3). Metal ions play a complex role in catalysis by glutamine synthetase: unadenylated enzyme is active with magnesium but inactive with manganese in the biosynthetic reaction and is active with both metal ions in the y-glutamyltransferase reaction; adenylylated enzyme is active with manganese but inactive with magnesium in both the biosynthetic and the y-glutamyltransferase reactions (1).

Glutamine synthetases from *E. coli* and various other organisms undergo auto-inactivation in the presence of the L-glutamate analog, L-methionine-S-sulfoximine, metal ions, and ATP (9). Inactivation results from the extremely tight binding to the enzyme of the transition state analog L-methionine-S-sulfoximine phosphate and ADP, which are formed on the enzyme by transfer of the y-phosphoryl group of ATP to the imino nitrogen of L-methionine-S-sulfoximine (10, 11). This reaction is analogous to the first step of the biosynthetic reaction which involves the formation of an activated intermediate between L-glutamate and ATP, possibly y-glutamylphosphate (9, 12, 13). Glutamine synthetase, however, does not display the same discrimination for metal ions in catalyzing the phosphorylation of L-methionine-S-sulfoximine as in catalysis of the biosynthetic reaction; the reaction occurs with both the manganese and magnesium forms of the adenylylated and the unadenylated enzymes (14). Although it is known that all 12 subunits of glutamine synthetase are catalytically active (3), it is uncertain whether all 12 subunits can catalyze the auto-inactivation reaction. Using enzyme with various extents of adenylylation, Weisbrod and Meister (14) found that fully inactivated glutamine synthetase from *E. coli* contained 9-11 mol of L-methionine-S-sulfoximine phosphate/mol of dodecameric enzyme, and Rhee et al. (15) found that partially inactivated enzyme contained less than 1 eq of L-methionine-S-sulfoximine phosphate bound/eq of activity lost. However, Hunt and Ginsburg (8) found that the inactivated enzyme contains 2 eq/subunit of Mn²⁺ or Mg²⁺ which are bound with very high affinity (Kₐ > 10¹⁰ M⁻¹). Also, the stoichiometry for the reversible binding of L-methionine-S-sulfoximine to glutamine synthetase is 1 eq/subunit (16).

This paper describes conditions for the complete restoration of enzymatic activity to glutamine synthetase inactivated with ATP and L-methionine-S-sulfoximine. Reactivation experiments allowed characterization of several metal ion and ligand interactions with the enzyme and indicated the presence of inactive complex on all subunits of the dodecamer.

**EXPERIMENTAL PROCEDURES**

**Materials**—All aqueous solutions were made with distilled water that was deionized and filtered through a Millipore Millicell Q2 reagent grade system. L-Methionine-SR-sulfoximine, Hepes, Tris, and ATP

1 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GS₃, glutamine synthetase with some number (2 - 12) of 5'-adenylate groups attached/dodecameric enzyme; [Me-MSOXP-ADP-MeKGS, inactivated complex of glutamine synthetase with L-methionine-S-sulfoximine phosphate, ADP, and twometal ions (Mn²⁺ or Mg²⁺).
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were from Sigma. Polyethyleneimine cellulose was from Eastman, Chelex 100 was from Bio-Rad, and the tetrasodium salt of xylenol orange was obtained from Fisher. Separated S- and R-diastereoisomers of 1-methionine-SR-sulfoximine were a kind gift of Dr. F. C. Wedler of Pennsylvania State University. [14C]ATP and [γ-32P]ATP were obtained from New England Nuclear. All other materials were reagent grade.

Preparation of Glutamine Synthetase—Undenatured glutamine synthetase was prepared from E. coli W by the method of Woolf et al. (17) and stored as before (18). Working stock solutions were dialyzed against 20 mM Hepes/KOH or 20 mM imidazole/HCl at pH 7.2 containing 100 mM KCl and 1 mM MnCl2 and stored at 4 °C. Magnesium enzyme was prepared by treating purified manganese enzyme with magnesium-Titreplex (Merck) and dialyzing the enzyme against 3 × 1000 volumes of buffer containing 50 mM MgCl2 and 100 mM KCl. Adenylylated enzyme was the gift of R. J. Hofman and was prepared from undenatured glutamine synthetase by enzyme-catalyzed adenylylation (18).

Assays of Enzyme Activity and Protein—Enzymatic assays for glutamine synthetase and its state of denaturation were performed using the γ-glutamyltransferase assay at pH 7.5 as described (19). Concentrations of active enzyme were calculated using a specific activity for pure enzyme of 107 units/mg and protein concentrations determined after correcting UV absorbance for light scattering using absorption coefficients of A280 = 3.85 and A260 = 7.33 + (6/120)0.50 (20). Protein concentrations of inactive and other modified forms of glutamine synthetase were determined by the colorimetric assays of Bradford (21) and Lowry et al. (22) with purified glutamine synthetase as the protein standard.

Preparation of Inactive Enzyme Complex—Manganese or magnesium glutamine synthetase (1–10 mg/ml) was combined with 1.5 mM L-methionine sulfoximine (either the commercially available S- and R-diastereoisomeric mixture or the resolved S-isomer) in 20 mM Hepes or 20 mM imidazole, pH 7.2, 100 mM KCl, and 50 mM MgCl2. ATP (0.2–2 mM) was then added and the solution was incubated at 37 °C for 1–2 h and then overnight at room temperature. Unbound small molecules were removed by dialysis against 3 × 1000 volumes of buffer containing 100 mM KCl. Manganese enzyme retained its enzymatic activity nearly 100% after this treatment. Storage for up to 4 months at 3-6 mg/ml led to no significant return of activity and only a very slow release of tightly bound ligands (2-3%) as measured by release of 45Ca2+. Inactive enzyme complex containing either [14C]ADP or L-[32P]methionine-S-sulfoximine phosphate was prepared as described above with the addition of either [14C]ATP or [γ-32P]ATP. Fully inactivated, undenatured enzyme had 11–12 eq of tightly bound ligands/dodecamer; fully inactivated adenylylated enzyme contained 9–10.5 eq of tightly bound ligands/dodecamer.

Measurements of Rates of Inactivation or Reactivation—Reactivation solutions contained 50 mM acetic acid adjusted to various pH values with KOH and KCl and (NH4)2SO4 as indicated. For measurement of rates of inactivation or reactivation, incubations were performed at 25 °C with 5–20 μl aliquots to the assay solution for convenient determination of enzymatic activity. Inactivation and reactivation were terminated upon transfer as judged by the linearity of the assay determined for the intact enzyme. Concentrations of active enzyme were calculated using a specific rate constant for an unmodified glutamine synthetase subunit. At the end of each Mn2+ determination, 1 mM EDTA (pH 7.2) was added to the spectrophotometer cell confirmed the absorbance change due to the Mn2+--xylenol orange interaction. An aliquot of the dialyzed, inactive enzyme used for each reaction was added directly to a xylenol orange solution and the free Mn2+ initially present was determined to be ≤10% of the protein-bound Mn2+.

pH Measurements—Radiometer pHM Type 26C pH meter equipped with a GR 2322 electrode (Radiometer) was adjusted with standard pH buffers at the temperatures indicated for pH measurements.

Scintillation Counting—All scintillation counting was done with 10 ml of Aquasol-1 and 0.50-1.0-ml aqueous sample in a Beckman LS250 liquid scintillation counter.

Identification of ADP and L-Methionine-S-Sulfoximine Phosphate—Inactive enzyme complex containing [14C]ADP or L-[32P]methionine-S-sulfoximine phosphate was reactivated at pH 4.1 in 1 mM LiCl. Precipitated protein was removed by filtering through a 0.45 μ filter and 20 μl of the filtrate was spotted on a poly(ethylene)imine cellulose thin layer chromatogram. The spots were washed with anhydrous methanol and ascending chromatography was carried out in a 100:0:5:1 vol/vol/vol mixture of chloroform-acetone-ether. All radiolabeled compounds were: ATP, ADP, 0.18; orthophosphate, 0.49; and L-methionine-S-sulfoximine, 0.40. Compounds were detected under UV light or by autoradiography.

Fluorescence Measurements—Protein tryptophan fluorescence was measured using a Hitachi Perkin Elmer MPA 2A Spectrophotometer. Cuvettes were housed in a water jacketed cuvette holder at 25 °C. Excitation was at 300 nm and emission was at 340 nm with slits set at 6 nm.

**RESULTS**

**Stability of Inactive Complex**—The complex formed when glutamine synthetase from mammalian brain or from E. coli is inactivated with ATP and L-methionine-S-sulfoximine is extremely stable. Previous efforts to reactivate the inactive enzyme complex of sheep brain glutamine synthetase by various methods (dialysis, gel filtration, ion exchange chromatography, urea treatment, or ethanol precipitation) were unsuccessful (24). Similarly, we have observed that the inactive complex of E. coli glutamine synthetase is not rapidly disrupted at neutral pH by any of the following treatments: incubation in buffers at pH 8-11; incubation with 2 M KCl, 1
Reactivation of Inactivated Glutamine Synthetase—Complete reversal of inactivation with good recovery of the enzymatic activity of glutamine synthetase was obtained in these studies by incubating the inactive complex at 25 °C at pH 4.0 in the presence of 1 M KCl and 0.4 mM (NH₄)₂SO₄ (Table 1). Although quantitative yields of enzymatic activity can be obtained, the yields of activity vary from 60–100% primarily because of the susceptibility of glutamine synthetase to denaturation in acid and the great variability in the rate of reactivation (see below). This layer chromatograph established that L-methionine-S-sulfoximine phosphate and ADP, which are the products released by heat or acid treatment (24), were released from the enzyme during reactivation. Equally good recoveries of activity were obtained with inactive complexes of unadenylated glutamine synthetase containing either Mn⁺⁺ or Mg⁺⁺ and with adenylylated enzyme containing Mn⁺⁺ (data not shown).

Fig. 1. Release of components of inactive complex during reactivation. Unadenylated glutamine synthetase was inactivated with L-methionine-S-sulfoximine and ATP as described under “Experimental Procedures.” Four different preparations were made and in all cases the fully inactivated enzyme contained 11.2–11.7 eq of complex/dodecamer of active enzyme present initially. To measure release of radioactive ligand, samples from reactivation solutions were diluted directly into assay mixtures, and a portion of the assay solution was analyzed for free ³²P or ¹⁴C ligand by the Millipore filter assay and the remainder was analyzed for enzymatic activity. For Mn⁺⁺ release, samples were withdrawn for enzymatic assay and 20 s later samples were taken for Mn⁺⁺ assay. Correction for the differences in sample times were made by interpolation of the kinetic data for return of activity. The percent of Mn⁺⁺ released was based on the expected amount of Mn⁺⁺ present, 2 metal ions bound/subunit (8). Reactivations were performed in 1 M KCl as follows: complex with 2500 dpm of [³²P]L-methionine-S-sulfoximine phosphate/pg of manganese inactive complex at pH 4.1 and 25 °C (A); complex with 3900 dpm of [¹⁴C]ADP/pg of manganese inactive complex at pH 4.1 and 23 °C (C); complex with 4000 dpm of [¹⁴C]ADP/pg of magnesium inactive complex reactivated at pH 4.1 and 25 °C (V); complex with manganese inactive complex reactivated at pH 4.15 and 37 °C (C, inset). Inset, data showing the decrease of inactive enzyme as a function of time for the reactivation of inactive complex of manganese enzyme containing L-³²P[methionine-S-sulfoximine phosphate shown by triangles.

Effects of pH, Salt, and Temperature on the Rate of Reactivation—Fig. 2 shows that above pH 4, the first order rate constant of reactivation at 25 °C increased with the 3rd or 4th power of the hydrogen ion activity (Δ log k/Δ pH = 3.6 ± 0.5). KCl, which increased the rate of reactivation, had no effect on the slope of the pH curves. The falling off of the reactivation rates below pH 4 is most likely due to the irreversible denaturation of glutamine synthetase in acid, since the recovery of enzyme activity was lower at pH <4 than at pH >4. If the linear portions of the pH curves in Fig. 2 are extrapolated to the lower pH (which assumes that the rate-limiting step remains the same), a rate constant for reactivation at pH 7.0 of 10⁻¹¹ to 10⁻¹² min⁻¹ can be estimated. Such a remarkably slow dissociation rate is consistent with the seemingly "irreversible" nature of the inactivation carried out at neutral pH.

Reactivation of the inactive complexes of unadenylated manganese and magnesium enzymes and the adenylylated activity of reactivated enzyme was linear when assayed from 0.5 to 30 min. Thus, any protein conformational changes that may occur are rapid upon dilution into the same assay solution. The correspondence between the kinetics of reactivation and ligand release suggest that the rate-limiting step in reactivation of inactivated glutamine synthetase is dissociation of the ligands bound at the active site.
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![Graph showing the pH dependence of reactivation](image)

**Table II**

| Salt present | Concentration | Conditions | k (min⁻¹) |
|--------------|---------------|------------|-----------|
| KCl          | pH 4.2, 37 °C, 0.4 M (NH₄)₂SO₄ | 0.016      |
|              | 0.25          | 0.050      |
|              | 0.50          | 0.11       |
|              | 1.0           | 0.23       |
|              | 2.0           | 0.42       |
| KCl          | pH 4.1, 25 °C, 0.4 M (NH₄)₂SO₄ | 0.36       |
| NH₄Cl        | 1.0           | 0.43       |
| K₂SO₄        | 0.50          | 0.03       |
| (NH₄)₂SO₄    | 0.50          | 0.05       |

Increasing concentrations of KCl increased the rate of reactivation for both the manganese and the magnesium forms of the inactive complex (Figs. 2 and 3). The effect of KCl on reactivation of unadenylated manganese complex is shown in Table II. Other neutral salts such as LiCl, NaCl, and NH₄Cl also accelerated the reactivation. Ammonium and potassium sulfates however were less effective than the respective chlorides (Table II). Incubation of the inactive complex at 25 °C for 1 h in 50 mM acetic acid (pH 3) with no additional salt, resulted in no reactivation and no dissociation of the complex.

The temperature dependence of the rate of reactivation of the inactive complex is shown in Fig. 3. Below 50 °C, the Arrhenius activation energies for both the magnesium and the manganese inactive complexes and for either complex in the absence and the presence of KCl were the same (~26 kcal/mol). With 1 M KCl present, the increase in first order rate constants was ~20-fold for unadenylated [Mn-MgSO₄-ADP-Mn]GS and ~8-fold for unadenylated [Mg-MgSO₄-

**FIG. 2.** pH dependence of reactivation. Fully inactive manganese or magnesium complexes were incubated at 25 °C in 50 mM citric acid at the pH values indicated. From each incubation mixture samples were taken at times intervals and assayed. First order rate constants were calculated from semilog plots of the data. Manganese unadenylated inactive enzyme incubated with 0.4 M (NH₄)₂SO₄ only (V) and with 1 M KCl only (A); manganese unadenylated (O), magnesium unadenylated (O), or manganese fully adenylylated (O) inactive enzyme incubated with 1 M KCl and 0.4 M (NH₄)₂SO₄.

manganese enzyme showed the same pH dependence. However, the unadenylated manganese complex was more stable at acidic pH (reactivated more slowly) than the unadenylated magnesium complex or the adenylylated manganese complex. This order of stability is consistent with the previously determined binding affinities of Mn²⁺ and Mg²⁺ to glutamine synthetase (3, 25) and with the relative affinities of the unadenylated and adenylylated enzymes for ADP-Mn²⁺ (3). It is likely that the steep pH dependence of reactivation reflects a requirement to protonate several ionized carboxylate groups in order to weaken metal ion binding in the complex.

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**FIG. 3.** Temperature dependence of the reactivation at pH 4.3. Inactive complexes were incubated at pH 4.3 in 50 mM acetic acid with 0.4 M (NH₄)₂SO₄ with or without KCl at the indicated temperatures and the first order rates of reactivation (k) were determined. Manganese inactive enzyme, no KCl (O); magnesium inactive enzyme, no KCl (A); manganese inactive complex plus 1 M KCl (O); magnesium inactive complex plus 1 M KCl (- - - -).
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ADP-MgGS. Below 55 °C, KCl increased the rate of reactivation of inactive enzyme complexes equally at all temperatures. The break occurring above 55 °C in the Arrhenius curve for reactivation of unadenylated [Mn-MSOX phosphate - ADP-Mn] GS in the absence of KCl (Fig. 3) was observed whether return of enzymatic activity (two experiments) or the release of [14C] ADP from the inactive complex (one experiment) was measured. KCl (1 m) appeared to eliminate this thermal transition. A similar break in the curve for the reactivation of the magnesium complex in the absence of KCl was not seen, indicating some difference in conformation between the manganese and the magnesium forms of the complex.

Reversible Binding of the Components of the Inactive Complex—When the pH of the reactivation solution was raised above pH 6, L-methionine-S-sulfoximine phosphate, ADP, and Mn^2+ bound rapidly to the enzyme, resulting in complete reactivation of the enzyme (Fig. 4). Reactivation involved the rebinding of the free components of the inactive complex, as [14C] ADP released from the inactive complex during reactivation and remaining in the filtrate (unbound) when reactivation was carried out in the presence of 50 μM unlabeled ADP, reactivation of glutamine synthetase reactivated at pH 4.1 with 1 mM KCl and then diluted into 200 mM Hepes, pH 7.2, at different final concentrations of enzyme subunits: 2.0 μM (Δ), 1.0 μM (□), 0.4 μM (○), and 0.2 μM (○). The line to the asterisk indicates the counts of [14C] ADP released from the inactive complex during reactivation and remaining in the filtrate (unbound) when reactivation was carried out in the presence of 50 μM unlabeled ADP, Mn^2+ tightly bound to each enzyme subunit, and that reactivation of glutamine synthetase reactivated at pH 4.1 with 1 mM KCl and then diluted into 200 mM Hepes, pH 7.2, at different final concentrations of enzyme subunits: 2.0 μM (Δ), 1.0 μM (□), 0.4 μM (○), and 0.2 μM (○).

![Figure 4](http://www.jbc.org/)

**Fig. 4.** Reconstitution of inactive complex at pH 7.2 following reactivation at pH 4.1. A. 200 μg/ml of inactive complex was reactivated at pH 4.1 with 1 mM KCl. The yield was 92%. The reactivated enzyme was diluted 1:1 into 200 mM Hepes buffer, pH 7.2, and incubated at 37 °C in Hepes buffer only (Δ); plus 1 mM MnCl₂ (□); plus 1.06 mM MnCl₂ and 50 μM ADP (▼); plus 1.5 mM MnCl₂ and 500 μM ADP (○); plus 2 mM EDTA (○). The line to the asterisk indicates the counts of [14C] ADP released from the inactive complex during reactivation and remaining in the filtrate (unbound) when reactivation was carried out in the presence of 50 μM unlabeled ADP, Mn^2+ tightly bound to each enzyme subunit, and that reactivation of glutamine synthetase reactivated at pH 4.1 with 1 mM KCl and then diluted into 200 mM Hepes, pH 7.2, at different final concentrations of enzyme subunits: 2.0 μM (Δ), 1.0 μM (□), 0.4 μM (○), and 0.2 μM (○).

![Figure 5](http://www.jbc.org/)

**Fig. 5.** Auto-inactivation with limiting L-methionine-S-sulfoximine or ATP. Glutamine synthetase (100 μg/ml; 2 μl subunit) was incubated in 20 mM Hepes/KOH, 100 mM KCl, and 2 mM MnCl₂, pH 7.2, with either 1 mM ATP and varying concentrations of L-methionine-S-sulfoximine (△) or 1 mM L-methionine-S-sulfoximine and varying concentrations of ATP (○). Mixtures were incubated at 37 °C for 20 min and at 4 °C for 12 h, and aliquots were diluted directly into γ-glutamyltransferase assay solution. Longer incubation gave no further inactivation. Limiting reactant concentration is expressed as the molar ratio of reactant to enzyme subunit.
The effects of ATP on the rates of inactivation are not reflected by the enhancement of enzyme tryptophanyl fluorescence observed upon binding of ligands. Manganese unadenylated enzyme gave identical quantum yields when treated with ATP and L-methionine-S-sulfoximine whether ATP was added first or last. Similarly fluorescence of magnesium unadenylated enzyme was the same when inactivated in the presence of 10 mM MgCl₂ (~50% inactive) or 50 mM MgCl₂ (~95% inactive). Thus the fluorescence change occurs upon binding of ATP and L-methionine-S-sulfoximine and is not affected by the inactivation (phosphoryl transfer) reaction.

**Discussion**

This study shows that *E. coli* glutamine synthetase inactivated with ATP and L-methionine-S-sulfoximine in the presence of Mn²⁺ or Mg²⁺ can be reactivated in good yield under relatively mild conditions. Reactivation coincides with the stoichiometric release of 0.9–1 eq of each of L-methionine-S-sulfoximine phosphate and ADP and 2 eq of Mn²⁺/subunit from the inactive complex. On the time scale of the experiments (>0.1 min), all components of the complex appear to be released simultaneously; however, an ordered release of ligands in which the rate-limiting release of the first component is followed by rapid release of the other components cannot be ruled out from these data. Release of L-methionine-S-sulfoximine phosphate, ADP, and Mn²⁺ is a first order process. Reactivation follows exactly the same first order kinetics as dissociation of the complex, as would be expected if dissociation of the complex were the rate-limiting step in reactivation. Upon transfer of the enzyme from the reactivation mixture to assay solution there is no discernible lag in enzymatic activity (0.5–30 min). Either the enzyme is in a fully active conformation following dissociation of the complex at low pH or any conformational changes that occur are rapid compared to the rate of dissociation.

Reversibility of inactivation is clearly shown by the release of Mn²⁺ and the products of the auto-inactivation reaction from glutamine synthetase during reactivation at pH <4.6 and by the rapid re-binding of these components to the enzyme during reactivation at pH 7 (Scheme 1). With the *E. coli* enzyme, the binding of stoichiometric amounts of L-methionine-S-sulfoximine phosphate alone to the manganese enzyme (0.2 µm subunit) is sufficient to inactivate the enzyme, which indicates that this transition state analog binds with very high affinity. These results are in agreement with those of Meister et al. (9–11) who showed that, at high concentrations, chemically synthesized L-methionine-S-sulfoximine phosphate inactivates sheep brain glutamine synthetase and that the apparent rate of inactivation is increased by added ADP and Mg²⁺ (10). The ability of very low concentrations of ADP to bind to the manganese unadenylated enzyme in the presence of L-methionine-S-sulfoximine phosphate at pH 7.2 reflects enormous synergism in the binding of these compounds to the enzyme. Measurements of the exchange of [³⁰S]ADP from the inactive complex in the presence of excess unlabeled ADP gave a maximum rate constant for dissociation of ADP at pH 7.2 and 25 °C of 10⁻⁷ s⁻¹. This rate constant is 5–6 orders of magnitude lower than that for ADP dissociation from manganese unadenylated glutamine synthetase (26). Assuming no change in the rate of binding in the absence and presence of L-methionine-S-sulfoximine phosphate and using $K'_A = 3.5 \times 10^3$ M⁻¹ for ADP binding to the manganese enzyme at pH 7.2 (3) the association constant for ADP binding to the Mn₆-enzyme-L-methionine-S-sulfoximine phosphate complex is >10²² M⁻¹. Recently Hunt and Ginsburg (8) estimated $K'_A > 10^9$ M⁻¹ for Mn²⁺ binding to both n₁ and n₂ sites of the inactive unadenylated glutamine synthetase complex.

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**Figure 6.** Nucleotide-induced heterogeneity in auto-inactivation kinetics. Glutamine synthetase (100 µg/ml; 2 µm subunits) was incubated in 20 mM imidazole/HCl and 100 mM KCl, pH 7.2, with the additions noted below. Aliquots were withdrawn at the times indicated and assayed directly in γ-glutamyltransferase assay solution. A, unadenylated (circles) or adenylated (triangles) enzyme was preincubated in buffer plus 1 mM MnCl₂ and either 1 mM ATP (●, ▲) or 1 mM L-methionine-S-sulfoximine (○, □). Inactivation was initiated by the addition of the reactant omitted in the preincubation. B, unadenylated enzyme was incubated in buffer and 1 mM L-methionine-S-sulfoximine with 10 mM MgCl₂ (circles) or 100 mM MgCl₂ (triangles) and either 0.2 mM ATP (○, ▲) or 5 mM ATP (●, □). Inactivation reactions were initiated with ATP, although the order of addition had no effect when the magnesium enzyme was used.

Subunit inactivates that subunit and in addition partly inhibits adjacent subunits. This ratio is close to that observed by Rhee et al. (15), who measured 0.83 eq of complex/subunit inactivated, but appears to contradict the results obtained with excess reactants present which gave 0.95 to 1.0 eq of complex/enzyme subunit (Fig. 1).

**Nucleotide-induced Heterogeneity of Inactivation—Previous investigations have shown that the rate of auto-inactivation of glutamine synthetase (Scheme 1) decreases as the enzyme is inactivated (14, 15). The decrease in rate of inactivation is not simply a function of the extent of inactivation, since, when partially inactivated enzyme was isolated and incubated again with ATP and L-methionine-S-sulfoximine, the fast and slow phases of inactivation had similar rates when starting with 100, 50, 30, or 25% active enzyme (data not shown). Moreover, the distribution between the rapidly inactivated and the slowly inactivated enzyme populations was influenced by ATP. Fig. 6A shows that when the unadenylated enzyme was preincubated with ATP and Mn²⁺, ~50% of the enzyme was inactivated in 1 min following addition of L-methionine-S-sulfoximine but the remaining 50% was inactivated at least 50 times more slowly. This effect was not dependent on the time of preincubation with ATP from 0.5–30 min. With the manganese adenylylated enzyme, the rate and extent of inactivation was not significantly influenced by the order of addition of ATP and L-methionine-S-sulfoximine. When the magnesium unadenylated enzyme was used, preincubation with ATP was not required to induce heterogeneity in the rate of inactivation. At a low ratio of MgCl₂ to ATP (2:1) >60% of the enzyme was inactivated slowly, whereas at a high ratio of MgCl₂ to ATP (20:1) only 5–10% of the enzyme was inactivated slowly (Fig. 6B). When a fixed concentration of 10 mM MgCl₂ was used, decreasing ATP concentrations from 5 mM to 0.2 mM led to progressively faster inactivation rates and more complete inactivation (~80%) in 2 min at 0.2 mM ATP.
The fact that the rate of reactivation of inactive complex is a simple function of pH, temperature, or KCl concentration indicates that reactivation proceeds in a single well defined mechanism under a variety of conditions. The pH dependence of reactivation indicates that protonation of 3–4 acidic groups with \( \text{pK}_a \sim 3.5 \) is required to weaken binding of the components of the complex. The groups are probably carboxylate ions on the protein and may be involved in chelating one of both metal ions. Calorimetric evidence of Hunt et al. (27) suggests that carboxylic acid groups are at the \( n_1 \text{Mn}^{2+} (\text{Mg}^{2+}) \) binding sites of glutamine synthetase. Furthermore, the affinity constants for binding \( \text{Mn}^{2+} \) to \( n_1 \) and \( n_2 \) sites are strongly dependent on pH (25, 28) with 2 and 1 eq of protons released/\( \text{Mn}^{2+} \) bound to \( n_1 \) and \( n_2 \) sites at pH 7.2, respectively (28). Our results also indicate that the metal ions play a key role in stabilizing the inactive enzyme complex. Since both the manganese and magnesium inactive complexes of the unadenylated enzyme have the same pH dependence of reactivation, the protonation of equal numbers of carboxylate groups are apparently involved in disruption of these complexes. Also, both adenylylated and unadenylated enzymes are reactivated with the same pH dependence, despite the difference in the conformations of these enzyme forms induced by L-methionine-S-sulfoxime binding (26). Although the protonation of carboxylate groups within metal ion binding clusters could be the rate-limiting step in reactivation of the inactive enzyme complexes, protonation of one or more functional groups on the ligands or on the protein outside of the active site could also be involved.

It is likely that both high KCl and high temperatures accelerate reactivation by disrupting protein conformations that favor the stability of the complex. The much smaller rate enhancement produced by \( K_2 \text{SO}_4 \) (one-tenth of that of KCl) may reflect the counteracting influence of sulfate which is known to promote “ordering” of proteins in solutions (30). The break in the Arrhenius curve for reactivation of manganese inactive complex without KCl indicates the melting of a particular protein conformation that contributes to the stability of the complex. Since no thermal transition was observed in the presence of 1 mM KCl, it appears that high concentrations of KCl dominate this stabilizing conformation. The smaller effect of KCl on the rate of reactivation of magnesium inactive complex suggests the absence of a similar conformation in the magnesium inactive complex, for which no break in the Arrhenius activation curve is seen. The above considerations indicate that favorable conformational changes of the protein make important contributions to the free energy of binding of the ligands in the inactive complex. The considerable resistance to dissociation and unfolding of the protein polypeptide chains upon formation of the inactive complex, described elsewhere, show that the inactivating ligands markedly stabilize secondary, tertiary, and quaternary structures of the dodecamer.

The rate of auto-inactivation of glutamine synthetase decreases in a non-first order manner during inactivation (Fig. 6 and Refs. 14 and 18). Rhee et al. (15) have suggested that the non-first order kinetics of inactivation of magnesium unadenylated enzyme is caused by negative interactions between inactive and active subunits within the dodecamer. Spectrophotometric and fluorescence titrations (15, 29) have provided direct evidence for negative cooperativity in the reversible binding of L-methionine-S-sulfoximine to unadenylated enzyme. Our findings that nucleotide-metal ion ratios and the order of addition of reactants drastically affect the rates and extents of inactivation (Fig. 6) indicates a more complex kinetic mechanism for the auto-inactivation reaction. Also, reactivation at neutral pH was inhibited by excess Mn-ADP (Fig. 4). Those data are consistent with a model in which ATP or metal ion-ATP binds to and partially stabilizes a form of glutamine synthetase that catalyzes the phosphorylation of L-methionine-S-sulfoximine either slowly or not at all. A rate-limiting interconversion between the resistant and susceptible conformers would result in non-first order kinetics of inactivation. Likewise, excess Mn-ADP may stabilize a form of the enzyme that has decreased ability to bind L-methionine-S-sulfoximine phosphate. It is probable that homologous subunit interactions produced by L-methionine-S-sulfoximine binding and the heterogeneity induced by nucleotide binding both contribute to the complex kinetics of the formation of the inactive enzyme complex.

Different apparent stoichiometries for inactive complex formation in the completely inactive enzyme were obtained by titration with limiting amounts of reactants (10–11/dodecamer) or by radioactive labeling with excess reactants (11/12/dodecamer). These results imply that γ-glutamyltransferase activity of all subunits is inhibited when 10–11 subunits of the dodecamer are inactivated, but phosphorylation of L-methionine-S-sulfoximine may occur on the remaining 1–2 subunits at a reduced rate. This possibility is not unreasonable since phosphorylation of L-methionine-S-sulfoximine is readily catalyzed by the magnesium adenylylated enzyme which is inactive in both the biosynthetic and the γ-glutamyltransferase reactions. Despite the differences in stoichiometries obtained by the two methods, the amount of enzymatic activity is proportional to the amount of inactive complex present throughout the entire saturation range measured either by inactivation with limiting reactants (Fig. 5) or by dissociation of the inactive complex at low pH (Fig. 1). Enzyme heterogeneity and subunit interactions are often invoked to explain less than 1:1 stoichiometry of ligand binding to subunits of oligomeric enzymes. However, in this case, such effects would have to occur equally at all extents of saturations, suggesting that an alternative explanation or additional considerations are required.

In summary, the very tight, synergistic binding of 4 components containing a transition state analog to the active site of each subunit of dodecameric glutamine synthetase has been demonstrated. The conditions for reversibly binding these ligands give some insight into the role that protein structure has during catalysis. Additionally, recent studies of the stability of the inactive enzyme complex have shown that both intra- and inter-subunit bonding domains are strengthened by the presence of the inactivating ligands bound at the active site. Further studies should help characterize the physical interactions between liganded and unliganded subunits of glutamine synthetase and possibly clarify the effect of these interactions on catalysis by the enzyme.

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