Oxidative inactivation of glutamine synthetase subunits
(mixed-function oxidase/glutamine synthetase/subunit interaction/protein modification)

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Contributed by E. R. Stadtman, December 20, 1983

ABSTRACT Escherichia coli glutamine synthetase (GS) was inactivated by a nonenzymic mixed-function oxidation system composed of ascorbate, O₂, and Fe(III). Partial inactivation of GS by this system leads to the formation of hybrid GS molecules (dodecamers) composed of both active and inactive subunits. Subunit interactions in these hybrid molecules are weaker than in the native enzyme, as indicated by the kinetics of subunit dissociation in the presence of 4 M urea. Heterologous subunit interactions in these hybrid molecules do not affect the affinity of active subunits for glutamate. Incubation of partially adenylylated GS preparations (n = 6.7) with the ascorbate system in the absence of substrates leads to preferential oxidative inactivation of unadenylylated subunits, whereas incubation in the presence of ATP and glutamate leads to preferential inactivation of adenylylated subunits.

The activity of glutamine synthetase (GS) in Escherichia coli is regulated by a cascade system that mediates the cyclic adenylylation and deadenylylation of the enzyme (1). Theoretical (2) and experimental (3) studies have shown that for each metabolic condition a steady state is reached in which the rates of adenylylation and deadenylylation are equal and that the specific activity of GS in that metabolic condition is determined by the number, n, of adenylylated subunits per GS dodecamer. Results of other studies have shown that GS preparations containing intermediate numbers of adenylylated subunits (n = 1–11) consist of molecular species (heterododecamers, hybrid molecules) comprised of both adenylylated and unadenylylated subunits (4, 5) and that heterologous interactions between the adenylylated and unadenylylated subunits affect kinetic parameters of each kind of subunit (5, 6) as well as stability of the dodecameric aggregate (4). The existence of another kind of GS heterododecamer is suggested by the recent demonstration that any one of several mixed-function oxidation (MFO) systems (7, 8) including a nonenzymic system composed of ascorbate, Fe(III), and O₂ (9) is capable of catalyzing the O₂-dependent oxidation of a single histidine residue in a GS subunit and concomitantly the inactivation of that subunit (8–10).

The inactivation reaction is regulated by the concentration of ATP and glutamate and by the state of adenylylation, n, of the enzyme. In the absence of substrate, unadenylylated GS is more susceptible to MFO-catalyzed inactivation than is adenylylated GS whereas, in the presence of ATP and glutamate, inactivation of unadenylylated enzyme is suppressed and inactivation of the adenylylated enzyme is greatly accelerated. The present study was undertaken to determine whether (i) partial inactivation of a GS preparation by the ascorbate mixed-function oxidation system leads to a mixture of fully inactivated and fully activated GS molecules or to a population of hybrid molecules containing both active and inactive subunits, (ii) partial inactivation of either fully adenylylated or unadenylylated GS affects subunit interactions of the dodecameric structure, and (iii) substrates direct the inactivation of adenylylated vs. unadenylylated subunits in GS molecules containing six subunits of each type.

MATERIALS AND METHODS

Chemicals. Adenine nucleotides (ATP, ADP, AMP), l-glutamine, monosodium l-glutamate, and pyruvate kinase were obtained from Sigma. Phosphoenolpyruvate, NADH, and lactate dehydrogenase were obtained from Boehringer Mannheim. Imidazole, 2-methylimidazole, 2,4-dimethylimidazole, triethanolamine, and 3,3'-dimethylglutaric acid were obtained from Aldrich. Ascorbic acid was from Nutritional Biochemicals. Urea was from Fisher and was recrystallized from 95% ethanol.

Purification of GS. GS was purified from E. coli by using the zinc-induced aggregation method (11) followed by ammonium sulfate and acetone treatment (12). The concentration of pure enzyme was calculated from absorbancy at 290 nm using A₂₉₀ (1 cm) = 0.387 (13).

Adenylylated and Unadenylylated Subunit Activities. The γ-glutamyltransferase activity of GS in the presence of 0.4 mM MnCl₂ and also in the presence of 0.4 mM MnCl₂/60 mM MgCl₂ was measured in the triethanolamine/3,3'-dimethylglutarate buffer (pH 7.57) assay mixture or in the mixed imidazole buffer (pH 7.15) assay mixture, as described (14). Under these conditions, activity in the presence of Mn(II) alone is a measure of both adenylylated and unadenylylated subunits (total subunit activity) whereas activity in the presence of Mn(II)/Mg(II) is a measure of unadenylylated subunit activity only. The difference between these two measurements is therefore a measure of the adenylylated subunits. The state of adenylylation, n, of a GS preparation (i.e., the average number of adenylylated subunits per GS molecule) can be calculated from the ratio of activities observed under the two sets of conditions (14).

Inactivation of GS by the Ascorbate-MFO System. Purified GS (10 units/ml) was incubated at 37°C in 50 mM Hepes buffer/100 mM KCl/10 mM MgCl₂/15 mM ascorbate, pH 7.2. The reaction was initiated by the addition of 5 μl of 450 mM ascorbate (neutralized with 1 M NaOH) to 0.145 μl of enzyme solution (8). At the desired time, 20-μl samples were added directly into the pH 7.57 γ-glutamyltransferase assay mixture described above. Where indicated, adenine nucleotides (ATP, ADP, AMP) or glutamate were added to the mixture before the reaction was initiated by addition of ascorbate.

The Kinetics of GS Inactivation in Urea. The stability of GS preparations in urea was determined by incubating GS (0.2–
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0.25 μg/ml) at 37°C in 187 mM mixed imidazole buffer, pH 7.15/0.5 mM ADP/25 mM L-glutamine/25 mM potassium arsenate, pH 7.15/4 M urea (4). After various periods of incubation time, 20-μl samples were added directly to the γ-glutamyltransferase assay mixture (mixed imidazole buffer, pH 7.15). The stability in urea of GS preparations that had been partially inactivated by prior exposure to the ascorbate inactivation system was determined as follows: purified GS (1 mg/ml) was incubated at 37°C in 0.15 ml of the ascorbate inactivation mixture described above. At various times, the inactivation reaction was stopped by addition of 1 mM MnCl₂ and the mixture was added to 3 times the volume of urea inactivation mixture. Then, 20-μl samples were added to the γ-glutamyltransferase assay mixture (mixed imidazole buffer, pH 7.15) at desired intervals.

**Determination of the Kinetic Parameters of Partially Inactivated GS by Ascorbate.** The Kᵢₐ values of GS for ATP and L-glutamate were determined by the initial velocity method using the coupled assay procedure in which the production of ADP associated with the synthesis of glutamine by GS is linked to the oxidation of NADH in the presence of saturating levels of phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase (15). The change in absorbance at 340 nm, due to oxidation of NADH, was measured (7).

**Polyacrylamide Gel Electrophoresis.** NaDodSO₄ gel electrophoresis in 10% acrylamide gel was carried out according to Laemmli's method (16).

Gel electrophoresis in the absence of NaDodSO₄ was carried out according to the method of Ornstein and Davis (17) using a 5% acrylamide gel.

**RESULTS**

**Differential Inactivation of Adenylylated and Unadenylated Subunits in Hybrid GS Molecules.** The rate of inactivation of GS by MFO systems is a function of both the state of adenylylation, n, and the concentrations of ATP and glutamate (8). In the absence of substrates, the rate of inactivation decreases as the value of n increases from 0 to 12 whereas, in the presence of ATP and glutamate, the rate of inactivation increases with increasing values of n. Therefore, the effect of n on the rate of GS inactivation by MFO systems could be due to preferential inactivation of either the adenylylated or unadenylated subunits in the presence or absence of substrates or alternatively to changes in the susceptibility of both kinds of subunits to inactivation as a consequence of heterologous subunit interactions, which have been shown (5, 6) to vary with the state of adenylylation. To examine these possibilities, three GS preparations (GS₇₃, GS₈₅, and GS₈₀) were incubated with the ascorbate-MFO system, and the loss of total GS activity as well as the losses attributable to adenylylated and to unadenylated subunits were measured over a 30-min period.

In agreement with earlier results (8), the rate of loss of total γ-glutamyltransferase activity increased as the state of adenylylation was increased in the presence of substrates (Fig. 1a) whereas, in the absence of substrates, the rate of inactivation decreased as the value of n increased (Fig. 1b). As shown in Fig. 2, these differences reflect the differential rates of inactivation of adenylylated and unadenylated subunits. In the presence of ATP and glutamate, the adenylylated subunits are preferentially inactivated by the MFO system (Fig. 2a). With the GS₇₅ and GS₈₀ preparations, 70% and 80% of the adenylylated subunits, respectively, were inactivated within 10 min, whereas there was no detectable loss (in fact a slight increase) in the unadenylated subunit activity during this same period. Under these conditions, the GS₇₃ preparation was relatively stable; even so, there was small but significant preferential inactivation of the adenylylated subunits in this preparation during the first 10 min of incubation. In contrast, the unadenylated subunits of all three GS preparations were preferentially inactivated in the absence of substrates (Fig. 2b). However, under these conditions, both kinds of subunits were inactivated at appreciable rates.

**Effect of Heterologous Interactions Between Normal and Oxidized Subunits in GS Molecules on the Stability of the Enzyme in 4 M Urea.** Ciardi et al. (4) have shown that the kinetics of inactivation (dissociation) of partially adenylylated GS preparations in 4 M urea can be used to distinguish between enzyme preparations consisting of artificial mixtures of GS₅ and GS₇₃ and naturally occurring enzyme preparations, which are comprised of hybrid molecules (i.e., molecules containing both adenylylated and unadenylated subunits). For both kinds of enzyme preparations, the kinetics and the extent of urea-induced inactivation are functions of the average of the number of adenylylated subunits initially present. It was concluded from these studies that heterologous interactions between adenylylated and unadenylated subunits in hybrid molecules affect the stability of the dodecameric structure in 4 M urea.

An analogous situation could exist when GS preparations are only partly inactivated by limited exposure to the ascor-
b-MFO system. With an all-or-none attack on any single GS molecule, partially inactivated enzyme preparations would contain mixtures of fully inactive and fully active GS molecules. But if the attack on GS subunits is random, then partially inactivated enzyme preparations would consist of hybrid GS molecules containing both active and inactive subunits.

To test these possibilities, a relatively unadenylylated preparation of GS (\( \bar{r} = 1.5 \)) was incubated with the ascorbate-MFO system until in one case 35% and in another case 15% of its initial activity was lost. The stability of the residual activity of these preparations in 4 M urea at 37°C was compared with that of untreated enzyme. As shown in Fig. 3a, both of the ascorbate-treated preparations were much less stable than an untreated sample. Moreover, the residual activity of the 35% MFO-inactivated preparation was considerably less stable (\( t_{1/2} = 7 \) min) than that of the 15% inactivated sample (\( t_{1/2} = 12 \) min). The untreated GS was very stable in the absence of urea but was rapidly inactivated in the presence of 4 M urea when 1 mM EDTA was also added. The latter condition promotes dissociation of the subunits (2). Similar results were obtained with adenylylated GS (\( \bar{r} = 11.5 \)); however, in this case, the untreated enzyme is also very unstable in the presence of 4 M urea (Fig. 3b). The half-

![Fig. 3](image-url)
lives in 4 M urea of untreated GSIII and preparations that had been 30% and 39% inactivated by the ascorbate-MFO system were approximately 2.8, 2.0, and 2.1 min, respectively.

**Effect of Partial Inactivation of GS on Subunit Dissociation During Electrophoresis.** As shown in Fig. 4, when GSIII is subjected to polyacrylamide gel electrophoresis in the presence of 10 mM MgCl₂, it migrates as a single protein band corresponding to a Mr of 600,000 (18). However, after complete inactivation of the enzyme in the ascorbate-MFO system, electrophoresis yields one major protein band, corresponding to the undissociated dodecamer, but also several other bands of higher molecular weight aggregates. In contrast, no difference was observed between the native and MFO-inactivated enzyme when the electrophoresis was carried out in the presence of NaDodSO₄; both preparations yielded a single protein band corresponding to the Mr of 50,000 subunit (data not shown).

**Effect of Partial Inactivation of GS on the Kₘ Values for L-Glutamate and ATP.** Heterologous interactions between adenylylated and unadenylylated subunits in hybrid GS molecules (i.e., in partially adenylylated GS preparations) have a significant effect on the Kₘ of unadenylylated subunits for L-glutamate (6). To the contrary, subunit interaction in hybrids composed of catalytically active and oxidized (inactive) unadenylylated subunits have little or no effect on the Kₘ for L-glutamate. Thus, as shown in Table 1, the Kₘ for L-glutamate (4.1 mM) of an untreated (fully active) GSIII preparation is about the same as that (3.6 mM) determined for a preparation in which 45% of the subunits were inactivated by the ascorbate-MFO system. As noted above (Fig. 1), treatment of partially adenylylated GS preparations with the ascorbate system leads to preferential inactivation of unadenylylated subunits in the absence of substrates but to preferential inactivation of adenylylated subunits in the presence of ATP and glutamate. It follows that partial inactivation of a GSIII preparation by the ascorbate system in the presence and absence of substrates will lead to the formation of hybrid forms in which in addition to unadenylylated and adenylylated subunits will contain variable amounts of the oxidized (inactive) forms of both of these kinds of subunits. Nevertheless, as shown in Table 1, such hybrid formation has little if any effect on the Kₘ of unadenylylated subunits for L-glutamate. There does, however, appear to be a small but significant effect of such hybrid formation on the apparent Kₘ of unadenylylated subunits for ATP; i.e., the Kₘ of both GSIII and GSIV for ATP is 4 × 10⁻³ M whereas values of 6 × 10⁻³ M are determined for the ascorbate-MFO-oxidized enzymes.

**DISCUSSION**

It is evident from the data presented here that partial oxidation of either adenylylated or unadenylylated forms of GS by limited incubation with the ascorbate-MFO system leads to the generation of heterododecamers—i.e., to enzyme molecules containing both catalytically active and inactive subunits. Like heterododecamers consisting of both adenylylated and unadenylylated subunits (4, 5), the GS molecules containing both "oxidized" and unoxidized subunits are more susceptible to dissociation by 4 M urea, showing that heterologous interactions between modified and unmodified subunits in the hybrid molecules are weaker than those in the homododecamers from which they are derived. In contrast to results obtained with heteromolecules generated by partial adenylylation of GS (6), heterologous subunit interactions between "oxidized" and unmodified subunits do not affect the catalytic parameters of the unmodified subunits in the ascorbate-treated GS preparations.

As noted earlier (8), substrates protect unadenylylated GS preparations from MFO-mediated oxidation but enhance the inactivation of adenylylated GS molecules. As reported here, these unique effects of substrates also apply to partially adenylylated forms of GS. Thus, exposure of a GSIII preparation to the ascorbate-MFO system in the absence of substrates leads to preferential oxidation (inactivation) of the unadenylylated subunits whereas, in the presence of substrates, the adenylylated subunits are preferentially oxidized.

These substrate effects could be a source of error in the standard enzyme method used for the determination of the state of adenylylation, X, of GS. In these methods (14), the value of X is calculated from measurements of the γ-glutamyltransferase activity under a condition in which adenylyl-
ated and unadenylated subunits are both active (total activity) and again under a condition in which only the unadenylated subunits are active. In view of the present results, it is evident that this procedure could give misleading results if the GS preparation contained a significant amount of MFO-oxidized subunits. For example, if a preparation of GS$_2$ is exposed to a MFO system in the presence of ATP and glutamate, the adenylylated subunits would be preferentially inactivated. Accordingly, the fractions of total γ-glutamyltransferase activity attributable to unadenylated subunits would be increased; the calculated value of $n$ would therefore be less than 6 and, depending on the extent of the oxidation reaction, the value of $n$ and could approach 0. To the contrary, exposure of a GS$_2$ preparation to a MFO system in the absence of substrates would lead to preferential inactivation of the unadenylated subunit and consequently to an increase in the measured value of $n$, up to a value of 12.

K.N. is a visiting fellow supported by the Fogarty International Center.