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# Oxidation of *Neurospora crassa* Glutamine Synthetase

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**The glutamine synthetase of *Neurospora crassa*, either purified or in cell extracts, was inactivated by ascorbate plus $FeCl_3$ and by $H_2O_2$ plus $FeSO_4$. The inactivation reaction was oxygen dependent, inhibited by $MnCl_2$ and EDTA, and stimulated in cell extracts by sodium azide. This inactivation could also be brought about by adding NADPH to the cell extract. The α and β polypeptides of the active glutamine synthetase were modified by these inactivating reactions, giving rise to two novel acidic polypeptides. These modifications were observed with the purified enzyme, with cell extracts, and under in vivo conditions in which glutamine synthetase is degraded. The modified glutamine synthetase was more susceptible to endogenous phenylmethylsulfonyl fluoride-insensitive proteolytic activity, which was inhibited by $MnCl_2$ and stimulated by EDTA. The possible physiological relevance of enzyme oxidation is discussed.**

Our research on the regulation of nitrogen metabolism during the formation of *Neurospora crassa* aerial mycelia suggested that in the mycelium forming the aerial hyphae (1; I. Toledo, J. Aguirre, and W. Hansberg, Exp. Mycol., in press), the glutamine synthetase (GS; EC 6.3.1.2.) was regulated at the level of enzyme degradation (2). Considering that *Escherichia coli* GS is site-specifically oxidized, yielding an inactive enzyme which is more susceptible to proteolytic attack than the unmodified one (6, 8–10, 15), we set out to determine whether the GS of *N. crassa* was also inactivated by oxidation. We report that oxidation of *N. crassa* GS α and β polypeptides inactivated the enzyme, giving rise to two novel acidic polypeptides which were more susceptible to proteolysis than the unmodified ones. The data also suggest that the modification reaction can occur in vivo and is related to the physiological conditions of the fungus.

## MATERIALS AND METHODS

**Reagents and chemicals.** Reagents for enzyme determinations, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, nucleotides, and ascorbate were purchased from Sigma Chemical Co. The electrophoresis reagents were obtained from Bio-Rad Laboratories, and all other chemicals were Baker reagent grade.

**Strains and culture conditions.** Wild-type *N. crassa* strain 74A came originally from the Fungal Genetics Stock Center at the University of Kansas, Kansas City. The *gln-1bR8* strain, a revertant of the glutamine auxotrophy mutation *gln-1b*, which lacks the β polypeptide of GS (3), was kindly provided by G. Dávila from this university. This strain has completely regained the ability to grow in minimal medium.

Cultures were grown from a conidial inoculum of $10^6$ conidia per ml for 12 to 14 h at 30°C in Vogel minimal medium (22) supplemented with 1.5% sucrose. Aeration was provided by a gyratory shaker at 240 rpm. In some experiments cultures were transferred after 12 h of growth into fresh minimal medium without sucrose. Thereafter, these cultures were divided in two parts. One was agitated under the same conditions but at a high air-to-liquid volume ratio (about 20); the other was incubated without agitation at the same temperature in a closed flask filled to the top to eliminate any air chamber. At different times samples of these cultures were taken and dehydrated with acetone. Acetone powders of these and other cultures were stored at −20°C until used.

**Cell extracts and enzyme assays.** Acetone powders were ground with dry ice and suspended in 50 mM HEPES buffer (pH 7.2)–10 mM KCl for GS activity assays or in 10 mM $KH_2PO_4$ (pH 7) for catalase activity assays.

GS activity was assayed by the method of Ferguson and Sims (5). Transferase and synthetase activities are expressed as micromoles of γ-glutamyl hydroxamate per milligram of protein produced in 1 minute at 30°C. Protein was determined by the method of Lowry et al. (12).

Catalase (EC 1.11.1.6) activity was assayed with 15 mM $H_2O_2$ by the method of Munkres et al. (14) and expressed as the $\Delta A_{240}$ per minute per milligram of protein, because catalase activity units have not been defined due to abnormal kinetics. Sodium azide (10 mM) inhibited 96% of the catalase activity at 4°C.

**Inactivation of GS.** Purified GS was inactivated by addition of an ascorbate solution (neutralized with NaOH) at a final concentration of either 15 or 20 mM and an $FeCl_3$ solution at a final concentration of 0.4 mM. Both solutions were freshly prepared. Incubation was carried out at 4 or 22°C. Samples were taken at different times and assayed directly for GS activity.

The inactivation of GS in cell extracts was done with 20 mM ascorbate and 0.4 mM $FeCl_3$ at 4 or 30°C, usually in the presence of 10 mM $NaN_3$ to inhibit endogenous catalase activity (10). Freshly prepared $H_2O_2$ and $FeSO_4$ solutions (0.2 and 0.4 mM final concentration, respectively) were also used to inactivate the cell extract GS at 4°C. A third way to inactivate the cell extract GS was by adding a freshly prepared NADPH solution to a final concentration of 5 or 10 mM and incubating at 4 or 30°C.

**Susceptibility of GS to endogenous protease activity.** A cell extract in 50 mM HEPES (pH 6.1)–10 mM KCl, treated with ascorbate-$FeCl_3$ and $NaN_3$ and untreated, was incubated at 37°C for up to 4 h. Under these conditions only half of the endogenous protease activity was sensitive to 40 μM phenylmethylsulfonyl fluoride (PMSF), as measured by solubilization of radiactive proteins in 10% trichloroacetic acid (W. Hansberg, unpublished results). Following proteolysis, samples with 30 mU of initial GS activity were run for 18 h at 50 V in a quantitative immunoelectrophoresis system as

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TABLE 1. Oxidative inactivation of purified GS[a]

| Addition | Activity remaining (%) |
|---|---|
| None | 100 |
| ASC | 76.4 ± 2.8 |
| $FeCl_3$ | 98.0 ± 2.0 |
| ASC + $FeCl_3$ | 55.0 ± 4.6 |
| ASC + $FeCl_3$, $CO_2$ atm. | 85 |
| ASC + $MnCl_2$ | 90.3 ± 8.7 |
| ASC + $FeCl_3$ + EDTA | 86.0 ± 8.5 |
| ASC + EDTA | 83.3 ± 6.8 |
| ASC + $NaN_3$ | 77.0 ± 0 |

[a] Activity was assayed following incubation for 20 min at 22°C. Transferase activity without treatment was about 0.1 U/ml. Values are means for two or three determinations. Final concentrations were; ascorbate (ASC), 0.15 mM; $FeCl_3$, 0.1 mM; $MnCl_2$, 4 mM; EDTA, 1 mM; $NaN_3$, 0.5 mM.

described by Laurell (7). Goat antibody against purified GS was used (20). After the gel was stained and dried, the relative area of each precipitation peak was determined by cutting off the peaks from a photocopy and weighing them.

**Immunoprecipitation of GS.** Samples of cell extracts (0.4 mg of protein) in 50 mM HEPES (pH 7.2)–50 mM glutamate–10 mM KCl were immunoprecipitated by adding a polyclonal rabbit antibody against purified GS (20). The reaction was carried out for 12 h at 4°C in the presence of 0.4 mM PMSF, 3.5 mM β-mercaptoethanol, 3.3 mM bisulfite, 2.3 mg of bovine albumin per ml, and 4 mM $MnCl_2$ to avoid oxidation during immunoprecipitation. Immunoprecipitates were layered on top of discontinuous sucrose gradients and centrifuged at 9,000 × *g* for 30 min at 4°C (17).

**Electrophoresis and staining.** The immunoprecipitates were analyzed by two-dimensional electrophoresis in polyacrylamide gels by the method of O'Farrell (16). The final voltage pulse in the first dimension was omitted, and the second dimension was run with urea (19). The gels were stained either with Coomassie brilliant blue or by the silver stain procedure of Wray et al. (23).

## RESULTS

**Oxidative inactivation of purified GS.** Purified GS of *N. crassa* was treated with ascorbate (9) and then assayed for GS activity. Treatment with ascorbate partially inactivated the enzyme (Table 1). This inactivation was strongly enhanced by $FeCl_3$ and was dependent on molecular oxygen, since it did not occur under a $CO_2$ atmosphere (Table 1). Manganese and EDTA suppressed inactivation in the presence of air. These results suggested that the GS of *N. crassa* underwent an oxidative inactivation similar to the one described for the *E. coli* GS by Stadtman and Levine and their co-workers (6, 8–10, 15).

TABLE 2. Oxidative inactivation of GS in cell extracts[a]

| Addition | Activity remaining (%) |
|---|---|
| None | 100 |
| $NaN_3$ | 95.2 ± 2.0 |
| $NaN_3$ + ASC | 100.2 ± 4.3 |
| $NaN_3$ + $FeCl_3$ | 103.3 ± 4.7 |
| ASC + $FeCl_3$ | 79.8 ± 4.1 |
| $NaN_3$ + ASC + $FeCl_3$ | 10.3 ± 5.4 |
| $NaN_3$ + ASC + $FeCl_3$ + $MnCl_2$ | 95.9 ± 4.1 |
| $NaN_3$ + ASC + $FeCl_3$ + EDTA | 93.1 ± 2.9 |

[a] Inactivation was done at 4°C for 60 min. Sodium azide (10 mM) was used to inhibit endogenous catalase activity. Initial specific activity, 0.51 U/mg of protein. Values are means for three determinations. Concentrations: ascorbate (ASC), 20 mM; $FeCl_3$, 0.4 mM; $MnCl_2$, 4 mM; EDTA, 1 mM.

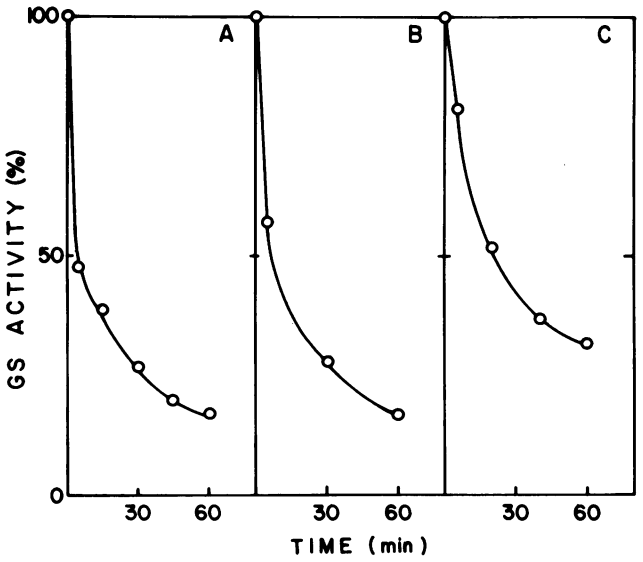


FIG. 1. Ascorbate-$FeCl_3$ inactivation of GS in cell extracts. Inactivation was done in the presence of sodium azide (10 mM) with 20 mM ascorbate and 0.4 mM $FeCl_3$ for 60 min at 4°C. (A) Transferase activity in a wild-type strain 74A cell extract after 14 h of growth. β/α ratio was about 3. (B) Synthetase activity in the same cell extract. (C) Transferase activity in a cell extract of the *gln-1b R8* mutant strain, which lacks the GS β polypeptide.

**Biochemical characterization of GS inactivation by ascorbate.** In an extract of *N. crassa* wild-type strain 74A mycelium, GS was also inactivated when treated with ascorbate plus $FeCl_3$ at 4°C (Table 2). This inactivation was stronger when sodium azide was used to inhibit the catalase activity of the extract (10). Catalase activity in this extract was 1.09 $\Delta A_{240}$ per mg per min and was 96% inhibited by the concentration of azide used. Therefore, all subsequent assays with cell extracts were done in the presence of azide. As shown for the purified GS, the inactivation reaction in the cell extracts was also supressed by $MnCl_2$ and EDTA. GS transferase and synthetase activities were inactivated by ascorbate plus $FeCl_3$ (Fig. 1A and B).

*N. crassa* has two polypeptides with GS activity, which are called α and β (3, 19). In the wild-type extract, the β/α ratio was about 3; the α polypeptide had a higher affinity for substrates but a lower turnover number than the β polypeptide (J. Guzmán and J. Mora, manuscript in preparation). The loss of activity suggested that both polypeptides had been inactivated. This result was confirmed by the fact that in the *gln-16R8* mutant strain, which has only the α polypeptide (3), the GS was also inactivated by ascorbate plus $FeCl_3$, although not as much as the wild-type GS (Fig. 1A and C).

The GS activity in the wild-type strain was to some extent protected from inactivation by ascorbate plus $FeCl_3$ when the substrates of the synthetase activity were present, particularly ATP or ATP plus glutamate (Fig. 2). Ammonium ions did not protect it, and phosphate ions and glutamine made the enzyme slightly more susceptible to inactivation (Fig. 2).

**Molecular characterization of oxidized GS.** It has been suggested that the *E. coli* GS is inactivated by oxidation of a single histidine residue, yielding a carbonylated imidazole derivative (8). When *N. crassa* wild-type mycelium extract

was immunoprecipitated with antibodies against purified GS and subjected to two-dimensional electrophoresis in a polyacrylamide slab gel, the two polypeptides, α and β, were observed. Careful observation revealed two other polypeptides with similar molecular weights, but having a slightly more acidic isoelectic point. When the extract was treated with the ascorbate-$FeCl_3$ and $H_2O_2$-$FeSO_4$ oxidizing systems prior to immunoprecipitation, the amount of α and β polypeptides in the gel diminished and the two more acidic polypeptides increased in intensity (Fig. 3). Pure GS treated with ascorbate plus $FeCl_3$ gave the same result (data not shown). These data suggested that the α and β polypeptides were partially modified by the ascorbate-$FeCl_3$ and $H_2O_2$-$FeSO_4$ oxidizing systems, giving two more acidic polypeptides. These modified α and β polypeptides are called α′ and β′ polypeptides hereafter. Beside these polypeptides, two other polypeptides of lower molecular weight than α and β were present in the oxidized cell extracts. The significance of these two polypeptides is not known and is under investigation.

Under the conditions used in the above experiment, the inactivation of GS was about 80% of the initial enzyme activity. The relative intensities of α and α′ and β and β′ were not in accord with the degree of inactivation. It was probable that oxidative modification of few monomers in the oligomeric structure, principally octamers (11), caused the loss of most of the activity. On the other hand, complete inactivation was never observed. To detect higher β′/β ratios, pure GS containing almost exclusively β polypeptide was inactivated under dissociating conditions (1 M NaCl at 4 or 20°C) and electrophoresed in two-dimensional gels (Fig. 3). The β polypeptide diminished in intensity, while the β′ polypeptide remained about the same; but at least two other more acidic polypeptides of same molecular weight were visible (Fig. 3F).

**Sensitivity of inactivated GS to endogenous protease activity.** To measure the susceptibility of the active and inactivated GS to endogenous proteolytic activity, a mycelial extract was incubated under conditions in which endogenous proteases were active and then analyzed with a quantitative immunoelectrophoresis system. The ascorbate-$FeCl_3$-inactivated GS in the presence of sodium azide was more susceptible to proteolytic attack than the GS treated with either ascorbate, $FeCl_3$, or azide alone (Table 3). It was also observed that proteolysis of the inactivated GS antigen was stimulated by EDTA (68% with 1.5 mM EDTA), was inhibited by $MnCl_2$ (65% with 4 mM $MnCl_2$), and was not affected by PMSF (16% activation with 3 mM PMSF). (The loss of antigen without any addition, about 30%, was taken as 100% proteolysis). After 4 h of proteolysis, only one-third of the active GS antigen was lost, while the inactivated GS antigen was completely lost in the same period (Fig. 4). To exclude a possible effect of ascorbate and $FeCl_3$ on the proteolytic assay, a cell extract was divided into two fractions. One was treated with ascorbate-$FeCl_3$, and then $MnCl_2$ was added; the other fraction had $MnCl_2$ added first and was then incubated with ascorbate-$FeCl_3$. Both extracts were then incubated under proteolysis conditions and run in the quantitative immunoelectrophoresis system. Manganese partially inhibited both oxidation by the ascorbate-$FeCl_3$ system and proteolytic activity. Nevertheless, loss of the GS antigen was higher in the GS inactivated with ascorbate-$FeCl_3$ before the addition of manganese, compared with the GS which was treated wtih the oxidizing system in the presence of manganese (Fig. 4). These data show that the ascorbate-$FeCl_3$-inactivated GS was preferentially degraded by an endogenous protease activity which was sensitive to divalent cations and not inhibited by PMSF.

**Inactivation of GS by NADPH.** Oxidation of *E. coli* GS is thought to be a physiological response mediated by a mixed-function oxidation enzyme which requires NADPH (10). We incubated a cell extract with NADPH in the presence of sodium azide. GS was inactivated by NADPH or NADH in

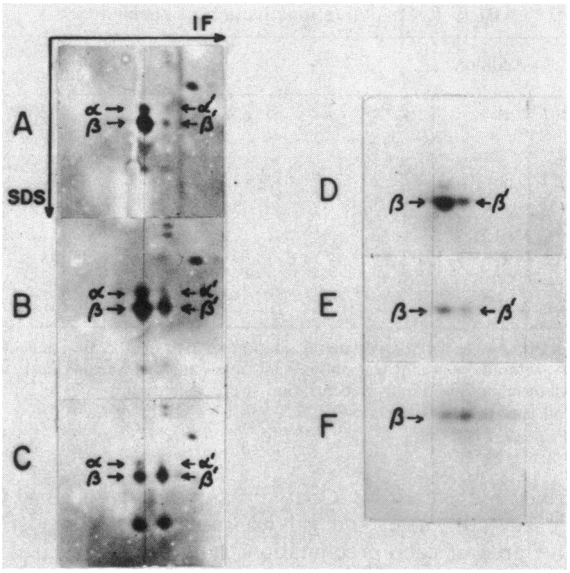


FIG. 3. Oxidative modification of α and β polypeptides. Inactivation with ascorbate-$FeCl_3$ was done as described in the legend to Fig. 1. Inactivation with $H_2O_2$ (0.2 mM) plus $FeSO_4$ (0.4 mM) was carried out for 60 min at 4°C. GS immunoprecipitates and purified GS were separated by two-dimensional electrophoresis in polyacrylamide gels. (A) Nonoxidized cell extract; (B) ascorbate-$FeCl_3$-treated cell extract; (C) $H_2O_2$-$FeSO_4$-treated cell extract; (D) purified GS from a mycelium which had almost exclusively β polypeptide; (E) the same enzyme incubated with 1 M NaCl at 4°C and then treated with ascorbate-$FeCl_3$; (F) the same enzyme incubated with 1 M NaCl at 20°C and then treated with ascorbate-$FeCl_3$. (A, B, C) Silver stained; (D, E, F) stained with Coomassie brilliant blue.

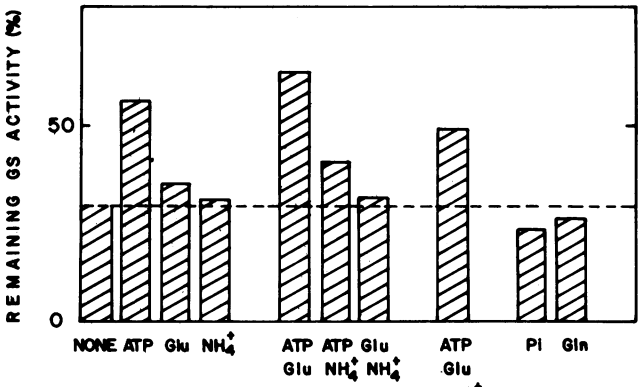


FIG. 2. Effect of substrates and products on inactivation of GS of ascorbate-$FeCl_3$. The conditions were as for Fig. 1 except that the wild-type enzyme activity was assayed after 30 min of inactivation. Values above the dashed line indicate protection from inactivation; values below indicate labilization of the enzyme to inactivation. Final concentrations: ATP, 15 mM; glutamate, 5 mM; $NH_4^+$, 10 mM; glutamine, 14 mM; $P_i$, 1 mM.

TABLE 3. Disappearance of GS antigen in extracts with proteolytic activity[a]

| Addition | % Loss of antigen |
|---|---|
| None | 0 |
| $FeCl_3$ | 8 |
| $NaN_3$ | 10 |
| Ascorbate | 22 |
| Ascorbate + $NaN_3$ | 27 |
| $FeCl_3$ + $NaN_3$ | 28 |
| Ascorbate + $FeCl_3$ | 37 |
| Ascorbate + $FeCl_3$ + $NaN_3$ | 46 |

[a] Oxidation was done at 4°C for 60 min. Incubation for proteolysis was for 4 h at 30°C with 1.5 mM EDTA. Final concentrations: $FeCl_3$, 0.4 mM; $NaN_3$, 10 mM; ascorbate, 20 mM.

the extract (Fig. 5). Manganese and also EDTA inhibited the inactivation reaction, and $FeCl_3$ stimulated it (Table 4). There was 25% more inactivation of GS at room temperature than at 4°C (Fig. 5), indicating a probable enzyme activity in the extract.

To be certain that the inactivation mediated by NADPH was equivalent to the oxidation brought about by ascorbate and $FeCl_3$, an NADPH-treated extract was immunoprecipitated and analyzed by two-dimensional electrophoresis. More α′ and β′ were observed with the NADPH-inactivated extract (Fig. 5, inset) than with the untreated active GS (Fig. 3A). This suggested that the α and β polypeptides of GS could be modified to the α′ and β′ polypeptides by an endogenous NADPH-dependent activity.

**Oxidation of GS is related to physiological conditions.** The experiments above suggested that GS was oxidized by an endogenous enzymatic activity which required NADPH. It has been reported that GS is degraded when carbon sources are limiting (13). To detect whether degradation of GS due to carbon source limitation is dependent on molecular oxygen, liquid-grown mycelium of *N. crassa* was transferred into minimal medium without a carbon source and then divided into two cultures. One was agitated as usual but with a high air-to-liquid volume ratio, and the other was kept still and incubated in a closed flask filled to the top to avoid an air

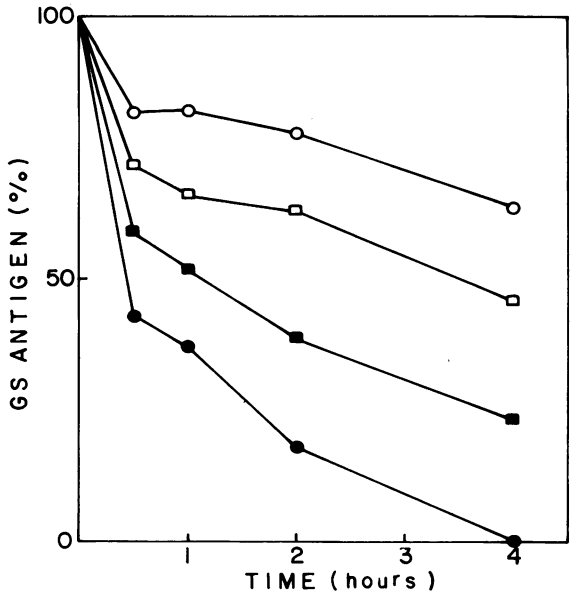


FIG. 4. GS sensitivity to endogenous proteolytic activity. A cell extract was treated with ascorbate-$FeCl_3$ and then incubated at 37°C for the times indicated. Symbols: ○, not treated; ●, treated with ascorbate-$FeCl_3$; □, treated with ascorbate-$FeCl_3$ in the presence of $MnCl_2$ (4 mM); ■, treated with ascorbate-$FeCl_3$, with $MnCl_2$ added before incubation at 37°C. Values are means for two experiments, with a maximal variation of 3%.

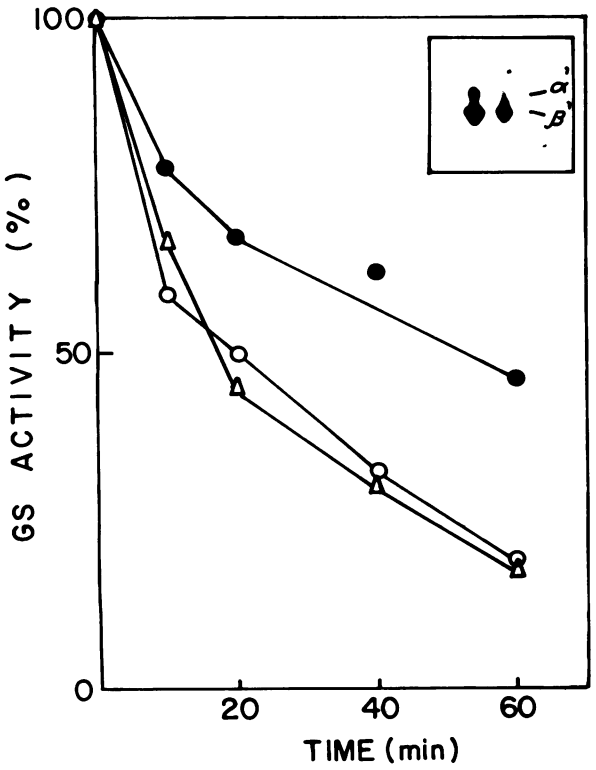


FIG. 5. GS inactivation by NADPH in cell extracts. Inactivation was done by adding either NADPH or NADH (5 mM) to the cell extract and incubating at 30 or 4°C for the times indicated. The extract contained sodium azide (10 mM) and $FeCl_3$ (0.4 mM). Sodium azide plus $FeCl_3$ alone gave 59% remaining activity after 60 min of incubation at 30°C. Addition of NADPH in the absence of sodium azide gave 66% remaining activity after 60 min at 30°C. Symbols: ○, NADPH at 30°C; ●, NADPH at 4°C; △, NADH at 30°C. (Inset) Immunoprecipitated GS from a 60-min NADPH-treated cell extract electrophoresed in a polyacrylamide gel in two dimensions and then silver stained. α′ and β′ are indicated.

TABLE 4. Inactivation of GS in cell extracts in the presence of NADPH[a]

| Addition | Activity remaining (%) |
|---|---|
| None | 100 |
| NADPH | 86.2 ± 0.8 |
| NADPH + $NaN_3$ | 88.0 ± 1.3 |
| NADPH + $NaN_3$ + $FeCl_3$ | 25.1 ± 3.8 |
| NADPH + $NaN_3$ + $FeCl_3$ (4°C) | 54.1 ± 0.6 |
| NADPH + $NaN_3$ + $FeCl_3$ + $MnCl_2$ | 103.3 ± 1.2 |
| NADPH + $NaN_3$ + $FeCl_3$ + EDTA | 89.7 ± 2.0 |

[a] Incubation was done at 22°C unless stated differently. Initial specific activity, 0.43 U/mg of protein. Values are means for three determinations. Final concentrations: NADPH, 5 mM; $NaN_3$, 10 mM; $FeCl_3$, 0.4 mM; $MnCl_2$, 4 mM; EDTA, 1 mM.

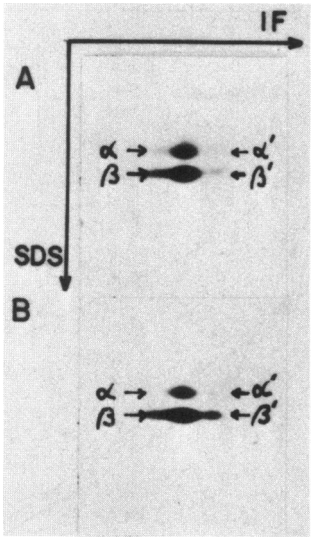


FIG. 6. Polypeptides of in vivo-modified GS. Mycelia of a 12-h culture were transferred into fresh medium without sucrose and then incubated for 3 h either in a closed flask without an air chamber or by agitating in a flask with a high air-to-liquid volume ratio. Cell extracts were immunoprecipitated with anti-GS antibodies, and the immunoprecipitates were electrophoresed in polyacrylamide gels in two dimensions and then silver stained. Panels show GS immunoprecipitated from unaerated mycelium (A) and hyperaerated mycelium (B). The directions of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoelectrofocusing (IF) are indicated.

chamber. At different times the two mycelia were harvested and dehydrated with acetone, and cell extracts were prepared. GS activity in the extracts of the nonaerated mycelia remained at nearly the pretreatment level (90% activity remaining at 3 h), whereas the enzyme activity diminished with time in the extracts of the aerated mycelia (33% activity remaining at 3 h). The immunoprecipitate of the nonaerated mycelia (3 h) had some α′ and β′ polypeptides, but the amounts of these two polypeptides were clearly higher in the extract immunoprecipitate of the aerated mycelia (Fig. 6). The immunoprecipitate of the extract before treatment is shown in Fig. 3A. These results suggest that GS polypeptides α and β are modified in vivo under strong aerating conditions.

## DISCUSSION

In this paper we have studied the oxidative inactivation of GS in *N. crassa*. Using the oxidizing system described by Stadtman, Levine, and their co-workers (6, 8–10, 15), we have been able to show that the α and β polypeptides of the active GS of *N. crassa* were modified, giving rise to two more acidic polypeptides, called α′ and β′. The modification reaction was dependent on the addition of a compound, ascorbate or NADPH in cell extracts, that can be readily oxidized in the presence of molecular oxygen, giving rise to $H_2O_2$. Endogenous catalase activity inhibited the modification of GS, and $H_2O_2$ in the presence of Fe(II) brought about the same enzyme modification. This suggests that $H_2O_2$ participates in the inactivation reaction. Hydrogen peroxide in the presence of FE(II) ions gives rise to an activated oxygen species which is thought to be responsible for the enzyme oxidation (10).

Higher proportions of α′ and β′ were detected under different conditions: ascorbate-$FeCl_3$-inactivated pure GS; NADPH-, ascorbate-$Fecl_3$-, or $H_2O_2$-$FeSO_4$-inactivated GS in cellular extracts; and in vivo conditions in which GS is degraded. In all these conditions the amount of α and β polypeptides diminished and proportionally more α′ and β′ were detected. The displacement to a more acidic isoelectric point suggested the gain of negative charges. More acidic polypeptides than α′ and β′ were observed when the modification was done under dissociating conditions. Under these conditions probably other sites for modification are exposed. The distances in the gel between a polypeptide and its more acidic form were similar and consistent with a sequential gain of probably a single negative charge.

The oxidative inactivation of GS was not quantitatively related to the appearance of the α′ and β′ polypeptides. This could mean that only a few modification sites are exposed in the oligomeric structures of *N. crassa* GS. Alternatively, it could indicate negative cooperativity in the oligomer, e.g., one or a few oxidized monomers in the oligomer bring about a conformational change that renders the enzyme less active but inaccessible to further oxidation. Both possibilities are in accord with the higher proportion of modified polypeptides that were obtained by inactivation under dissociating conditions. Another possible explanation is that the modification reaction is reversible and that equilibrium is reached after only 1 h. Quantitative correlation between the degree of activity inhibition and the amount of modified polypeptides present in the immunoprecipitates was also prevented by the fact that these polypeptides were more susceptible to proteolytic attack and proteolysis could occur during the immunoprecipitation procedure. The appearance of lower-molecular-weight polypeptides, particularly when $H_2O_2$ and Fe(II) were added to a cell extract, could be due to the presence of degradation products in these immunoprecipitates (Fig. 3).

With respect to the physiological occurrence and significance of the oxidative modification of GS, we detected the in vivo-modified α and β polypeptides in prooxidative conditions in which the GS antigen and activity disappeared. That the in vivo modification reaction could be enzymatically mediated is suggested by the temperature sensitivity of GS oxidation in a cell extract in the presence of NADPH and $O_2$ in contrast to the ascorbate-$FeCl_3$ and $H_2O_2$-$FeSO_4$ inactivation reactions. It is also significant that the GS was to some extent protected from inactivation by the substrates of its physiological synthetase activity, particularly by ATP. Inactivation of an ATP-consuming enzyme during carbon starvation makes sense. That under this condition the enzyme was not inactivated when the cells were incubated in the absence of air indicates the oxidative nature of the inactivating reaction.

Finally, it is also significant that a proteolytic activity which preferentially degraded the modified GS in the cell extracts was detected. This protease activity was insensitive to PMSF, stimulated by EDTA, and inhibited by $MnCl_2$. A protease from *E. coli* has been purified which specifically recognizes the oxidized GS at neutral pH, is not inhibited by serine protease inhibitors, and does not require ATP for its activity (J. E. Roseman and R. L. Levine, Fed. Proc. 44:1092, 1985). Also, other partially purified protease activities from mouse liver preferentially degrade the oxidatively modified form of the *E. coli* GS (18).

Oxidative inactivation (6, 21) and activation (21) of other enzymes have been reported. Whether the modified enzymes are related to specific physiological states and whether they are always more susceptible to endogenous

proteolytic activity will certainly help us to determine the physiological significance of the oxidative modification of enzymes. We are currently investigating some other enzymes which are either activated or inactivated during the course of aerial mycelium formation in *N. crassa*. Our general view is that oxidative modification of enzymes could be a very general mechanism for regulation of cellular metabolism through simultaneous activation and inactivation of different pathways together with a high turnover of the modified enzymes.

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