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Oxidative Inactivation of Glutamine Synthetase from the Cyanobacterium *Anabaena variabilis*

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In crude extracts of the cyanobacterium *Anabaena variabilis*, glutamine synthetase (GS) could be effectively inactivated by the addition of NADH. GS inactivation was completed within 30 min. Both the inactivated GS and the active enzyme were isolated. No difference between the two enzyme forms was seen in sodium dodecyl sulfate-gels, and only minor differences were detectable by UV spectra, which excludes modification by a nucleotide. Mass spectrometry revealed that the molecular masses of active and inactive GS are equal. While the K_m values of the substrates were unchanged, the V_{max} values of the inactive GS were lower, reflecting the inactivation factor in the crude extract. This result indicates that the active site was affected. From the crude extract, a fraction mediating GS inactivation could be enriched by ammonium sulfate precipitation and gel filtration. GS inactivation by this fraction required the presence of NAD(P)H, Fe^{3+}, and oxygen. In the absence of the GS-inactivating fraction, GS could be inactivated by Fe^{2+} and H_2O_2. The GS-inactivating fraction produced Fe^{2+} and H_2O_2, using NADPH, Fe^{3+}, and oxygen. Accordingly, the inactivating fraction was inhibited by catalase and EDTA. This GS-inactivating system of *Anabaena* is similar to that described for oxidative GS inactivation in *Escherichia coli*. We conclude that GS inactivation by NAD(P)H is caused by irreversible oxidative damage and is not due to a regulatory mechanism of nitrogen assimilation.

The regulation of glutamine synthetase (GS), the key enzyme in nitrogen assimilation, has been intensively investigated in prokaryotes. In *Escherichia coli*, a bicyclic regulation cascade involving adenylylation of GS was found (28). This system was used as a model for GS regulation in cyanobacteria, but adenylylation was never demonstrated. Moreover, it was shown that no phosphate label could be attached to GS, excluding also the possibility of GS being covalently modified by a phosphate or ADP-ribose group (18). In *Synechocystis* sp., a short-term GS inactivation was found (18). The lower specific GS activity of cyanobacteria grown on media containing ammonium can be attributed to repression of the GS gene, and it was suggested that the cyanobacterial GS is regulated by feedback inhibitors and other effectors like divalent cations (20).

GS oxidation is another kind of GS inactivation found in *E. coli*. This effect was investigated by using artificial oxidation systems, including cytochrome *c* oxidase, xanthine oxidase, cytochrome P_{450}, or a nonenzymatic system with ascorbate and iron (10, 13, 29). These systems produce Fe^{2+} and H_2O_2, which then form activated oxygen. The latter reacts with amino acid residues near the metal binding site in the catalytic center of GS, resulting in irreversible inactivation (6). GS inactivation was prevented by catalase or by iron-chelating agents like EDTA.

In cyanobacteria, GS inactivation by oxidation has not been reported. GS regulation always was considered in terms of nitrogen metabolism control but not as an effect of oxidation. This study demonstrates an oxidative inactivation of GS in *Anabaena variabilis.*

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MATERIALS AND METHODS

Cultivation. *A. variabilis* ATCC 29413 was grown in Arnon medium (1) without nitrate. Tubes containing a 200-ml culture volume were inoculated with cells equivalent to 1.5 μg of chlorophyll per ml, incubated at 30°C, bubbled with air enriched with 1.6% (vol/vol) CO_2, and illuminated with fluorescent light at 150 $\mu E\ m^{-2}\ s^{-1}$.

Crude extracts. Crude extracts were prepared from cultures at the end of the log phase (1 to 2 days old). Cells were collected by centrifugation (5 min at 6,500 × *g*), washed once in buffer (buffer A [50 mM HEPES {pH 7.0} for inactive GS and GS-inactivating enzyme and buffer B [50 mM HEPES {pH 7.0}, 5 mM $MgCl_2$, 0.5 mM EDTA] for active GS), and resuspended in a threefold volume of the same buffer. Then the cells were passed twice through a French press at 65 MPa and centrifuged at 10,000 × *g* for 5 min. The supernatant is the crude extract.

Purification of GS. All steps were carried out at 4°C. Buffered ammonium sulfate (3.8 M in buffer A or B) was added to the crude extract to give a concentration of 1.4 M. After centrifugation (10 min at 10,000 × *g*), the ammonium sulfate concentration of the supernatant was raised to 2.4 M. The pellet of a second centrifugation was dissolved in twofold volume of buffer C (buffer B complemented with 2 M NaCl) and applied to a Blue Sepharose column (5 by 0.75 cm or 20 by 2.5 cm; Pharmacia) equilibrated with buffer C. After washes with buffer C followed by buffer B, GS was eluted with buffer B including 300 μM ADP. GS was precipitated by addition of cold acetone (50%). After centrifugation at 10,000 × *g* for 15 min, the sedimented GS was dissolved in buffer A.

MS. The molecular mass was determined with a tandem quadrupole (mass spectrometry [MS]/MS) model TSQ700 mass spectrometer (Finnigan MAT, San Jose, Calif.) equipped with an interface for electrospray ionization (9) and controlled by a DEC 5000 computer. The protein solution was applied with a sample loop and a syringe pump at a flow rate of 5 $\mu l\ min^{-1}$. The molecular mass (M) is calculated from the average over a series of peaks observed at increasing values of m/z for the protein molecule associated with a decreasing number of z protons: $M = (m/z - 1)z$. The spectra were deconvoluted with the program BIOMASS and analyzed by the centroid mode.

Partial purification of the GS-inactivating enzyme. First, an ammonium sulfate precipitation step was applied as described for the purification of GS, using buffer A. The dissolved pellet was placed onto a Sephacryl S-300 gel filtration column (56 by 2.5 cm; Pharmacia) equilibrated with buffer A. The GS-inactivating activity was eluted at the end of the main protein peak. Fractions containing high GS-inactivating activity and little protein as judged by the intensity of the blue color of the phycobiliproteins were used for characterizing the GS-inactivating fraction.

Analytical assays. GS was assayed by the transferase assay as described previously (21). The reaction mix (340 μl) contained 40 mM HEPES (pH 7.0), 30 mM L-glutamine, 0.3 mM $MnCl_2$, 60 mM NH_2OH, 0.4 mM ADP, 20 mM Na_2HAsO_4, and an appropriate amount of GS. After 10 min of incubation at 37°C, the reaction was stopped by the addition of 660 μl of stop mix (3.3% $FeCl_3$,

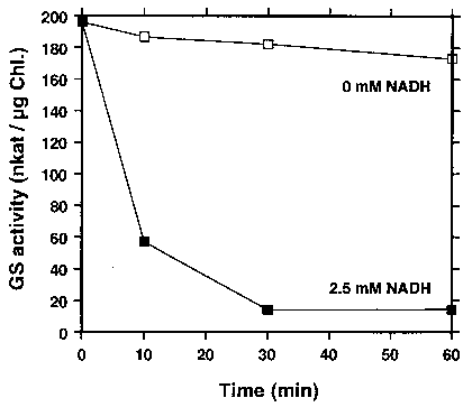

FIG. 1. GS inactivation in crude extracts. Crude extracts were incubated at 30°C. At the times indicated, samples were assayed for GS transferase activity. Chl., chlorophyll.

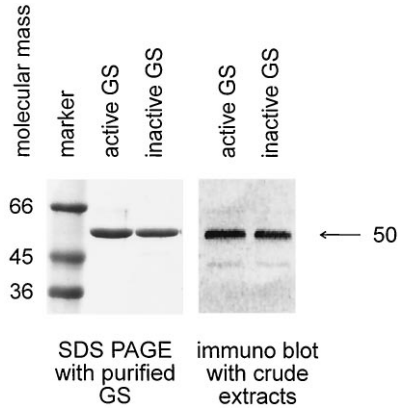

FIG. 2. SDS-PAGE of active and inactive GS from *A. variabilis*. Seven-tenths microgram of isolated GS was separated on a 12% polyacrylamide gel. For immunoblot analysis, crude extracts were used. Molecular masses are indicated in kilodaltons.

2.0% trichloroacetic acid, 250 mN HCl). Absorption was measured at 540 nm. For kinetic parameters, a modified biosynthetic assay was used (31). The reaction mix (340 µl) contained 100 mM HEPES (pH 7.6), 100 mM L-glutamate, 90 mM $MgCl_2$, 60 mM NH_2OH, 15 mM ATP, and 1.5 mM EDTA. Incubation was performed for 20 min at 37°C.

Chlorophyll was determined in methanol, using the extinction coefficient from reference 16. Protein was measured by the bicinchoninic acid method (26).

H_2O_2 was determined by the method of Avrameas and Guilbert (2). Three hundred microliters of sample, 15 µl of 6.6 mM *o*-dianisidine in 10 mM HCl, and 15 µl of horseradish peroxidase (30 U/ml; Sigma) were mixed. Absorption was measured at 440 nm.

Fe^{2+} was assayed by the ferrozin method of Gadia and Mehra (11). A 900-µl sample at neutral pH was mixed with 100 µl of 10 mM ferrozin, and the absorption was measured at 562 nm.

Statistics. Data of typical experiments are shown unless the standard error is given. All experiments were repeated three or more times. The deviation from the mean did not exceed ±10%.

RESULTS

GS inactivation by NADH in crude extracts. In crude extracts, GS was effectively inactivated after addition of 2 mM NADH. The kinetics of this inactivation is shown in Fig. 1. Inactivation is completed within 30 min. Both NADH and NADPH gave a strong inactivation, and we decided to use the cheaper one in this step.

No inhibition of GS was observed when the NADH was added to the GS assay, indicating that NADH did not interfere with the GS assay. Similarly purified GS was not inactivated by NADH. From these experiments, we concluded that the crude extract contained a component necessary for GS inactivation.

Purification of inactivated GS. GS inactivated by NADH to a residual activity of about 10% was isolated by the same procedure as the active enzyme. GS was detected by activity. Ammonium sulfate was added to the crude extract to give 1.4 M. After centrifugation by which all green material was removed, the ammonium sulfate concentration of the blue supernatant was raised to 2.4 M and the extract was centrifuged again. Then the pellet was dissolved in buffer and applied to a Blue Sepharose column (for details, see Materials and Methods). After intensive washing, GS was eluted with buffer containing ADP. Fractions containing GS were combined, and GS was concentrated by acetone precipitation as mentioned above.

Characterization of the inactivated GS. No differences between active and inactive GS were detected upon sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Fig. 2). GS was detectable a single band at 50 kDa, as expected for a prokaryotic GS. The inactivated GS did not migrate more slowly than the active GS, indicating that the inactivated GS is not ADP-ribosylated. An immunoblot of crude extracts developed with an GS antiserum again did not show a difference between active and inactive GS.

The molecular masses of active and inactive GS were determined by mass spectrometry. The value for the active GS, 52,985 ± 3.5 Da, is within the error range of the inactive GS, 53,003 ± 14 Da. These data clearly demonstrate that neither ADP-ribose, AMP, nor phosphate is covalently attached to the inactive GS.

For both the active and inactive GS, the kinetic parameters were determined (Table 1). The K_m values showed no differences between the two forms of GS. In contrast, the V_{max} values of the inactive GS were only about one-third of the values of the active GS, corresponding to the degree of inactivation of GS in the crude extract. This result demonstrates that the inactive GS remained inactive during the purification process.

Furthermore, the UV spectra of active and inactive GS were compared (Fig. 3). In both cases, the absorption maxima were at 280 nm, like that of a typical protein. There are only small absorbance differences over the spectrum, which again could not be related to an attached nucleotide. The difference spectrum is similar to that reported for GS from *E. coli* inactivated by oxidation (14). It is concluded that NADH inactivates GS by oxidation rather than by nucleotide modification.

Partial purification of the inactivating enzyme. To investigate the hypothesis that GS is inactivated by oxidation, the GS-inactivating activity was partially purified. The crude extract was fractionated by ammonium sulfate precipitations at 1.4 and 2.4 M. The inactivating activity was found in the same fraction as the GS.

TABLE 1. Kinetic data for active and inactive GS isolated from *A. variabilis*[a]

Substance	K_m (mM)		V_{max} (nkat/mg of protein)	
	Active GS	Inactive GS	Active GS	Inactive GS
Glutamate	1.2	1.0	35	12
ATP	1.5	1.3	34	11

[a] GS activity was determined by a modified biosynthetic assay, and the kinetic data were calculated by regression analysis based on Hanes plots. Before isolation, GS was inactivated to about one-third of the initial activity.

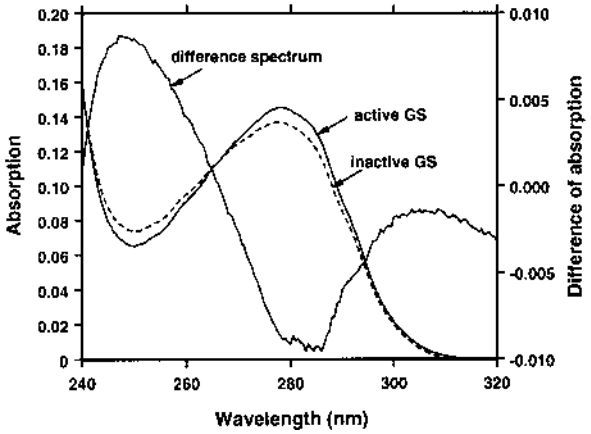

FIG. 3. UV spectra of active and inactive GS. A 100-μg/ml aliquot of isolated GS was dissolved in 100 mM ammonium acetate at pH 7.0.

For further purification, the ammonium sulfate precipitate was fractionated by gel filtration. Here the inactivating activity could be separated from GS (Fig. 4), showing that GS was not self-inactivated. It was eluted after the major bulk of protein, yielding a high purification.

Characterization of the inactivating enzyme. After gel filtration, the GS-inactivating fraction still included some contaminating proteins, as judged by SDS-PAGE. We could not yet determine the molecular weight of the GS-inactivating enzyme and could not clarify whether more than one polypeptide is involved.

GS inactivation was strongest with NADPH, but NADH also gave a strong reaction (Table 2). In contrast, the oxidized dinucleotides NAD^+ and $NADP^+$ did not promote GS inactivation, indicating that a reducing agent is needed. Again, this finding cannot be explained by GS being ADP-ribosylated.

Another substrate for GS inactivation is Fe^{3+}. Table 3 (assay A) shows that GS is inactivated only in the presence of NADPH, Fe^{3+}, and the GS-inactivating fraction. Therefore, we concluded that Fe^{3+} is reduced by NADPH, resulting in Fe^{2+}. Indeed, Fe^{2+} showed a strong GS inactivation without the GS-inactivating fraction (Table 3, assay B). The GS-inactivating activity was abolished by 30 μM EDTA, a chelator of divalent cations, or by 6 μM $MnCl_2$. Furthermore, the GS-inactivating fraction produced Fe^{2+} from Fe^{3+} (220 pkat/mg of protein with 250 μM NADPH at 30°C).

On the other hand, the GS-inactivating fraction produced H_2O_2 (3 pkat/mg of protein with 63 μM NADPH at 30°C). Consequently, GS inactivation is inhibited by catalase (1 U/ml), and GS inactivation by Fe^{2+} is enhanced by the addition of H_2O_2 (Table 3, assay B). From these results, we conclude that the GS-inactivating fraction produces H_2O_2 by reducing O_2 with NADPH. Indeed, no inactivating activity was detected when degassed buffers were used. Obviously the main characteristics described for oxidative GS inactivation in *E. coli* have been found in cyanobacteria.

DISCUSSION

In *E. coli*, GS is regulated by adenylylation in a bicyclic reaction cascade (28). This kind of regulation has often been discussed as a model for the GS regulation in cyanobacteria. Moreover, ADP-ribosylation of GS was reported. In one case, GS from *E. coli* was in vitro ADP-ribosylated by an NAD: arginine ADP-ribosyltransferase from turkey erythrocytes (19). In *Streptomyces griseus*, GS inactivated by the addition of ammonia was labeled by radioactive NAD, and a second band belonging to modified GS appeared upon SDS-PAGE (22). In another study, GS was labeled by radioactive NAD in crude extracts from *Rhodospirillum rubrum* (30). However, none of these effects were detected in cyanobacteria. Moreover, no label could be attached to GS when cells were incubated with radioactive phosphate (18). Therefore, it was concluded that GS is not covalently modified by any phosphate-containing compound.

Recently, ADP-ribosylation was reported for *Synechocystis* sp. (25). In high-speed supernatants, GS was inactivated. After dialysis, GS inactivation was abolished unless NADH or NADPH was added. NAD addition resulted in only a small loss of GS activity. These effects are very similar to the GS inactivation described in this study (Table 2), and we present data indicating that this GS inactivation is not an ADP-ribosylation but GS oxidation. The GS inactivation promoted by ammonia in crude extracts (25) probably is also caused by GS oxidation as described for *A. variabilis* in reference 17.

In the partially purified GS-inactivating system, only the reduced dinucleotides are capable of inactivating GS (Table 2) whereas ADP-ribosylation uses only NAD (15). GS inactivated by NADH in crude extracts was isolated by the same method as used for the active enzyme. The UV spectrum of inactive GS shows only minor differences from that of the active form. These differences are not caused by covalent modification of GS by a nucleotide, since in this case the difference spectrum has a maximum at 260 nm (24). Furthermore, no difference between active and inactive GS is seen upon SDS-PAGE. In references 4 and 22, different mobilities of active and adenylylated or ADP-ribosylated GS in SDS-PAGE were demonstrated. Therefore, an ADP-ribosylation should be detectable by SDS-PAGE. Finally, the molecular masses of active and inactive GS determined by mass spectrometry are almost equal. Inactive GS does not show an increased mass indicative of covalently attached ADP-ribose. In summary, our data are not compatible with ADP-ribosylation or adenylylation of GS in *A. variabilis.*

Our findings give strong support for inactive GS being oxidatively modified. In the UV region, the difference absorption spectrum of active and inactive GS is very similar to that reported for oxidatively inactivated GS from *E. coli* (14). This

TABLE 2. Effects of dinucleotides on GS inactivation[a]

Addition	GS activity (% of initial activity)				
	Without nucleotide	NAD	NADP	NADH	NADPH
Without inactivating fraction	79	75	75	82	79
With inactivating fraction	95	98	95	28	17

[a] Samples were incubated at 30°C with 1 mM dinucleotide and 50 μM $FeCl_3$. After 10 min, GS activity was assayed. The values are related to the control without incubation (rate at start, 205 nkat/μg of GS protein).

TABLE 3. Inactivation of purified GS by the inactivating fraction and various additions[a]

Assay	Addition	GS activity (% of initial activity)		0 μM H_2O_2	230 μM H_2O_2
		0 μM NADPH	500 μM NADPH		
A	0 μM $FeCl_3$				
	Without inactivating fraction	102	89		
	With inactivating fraction	112	100		
	25 μM $FeCl_3$				
	Without inactivating fraction	94	82		
	With inactivating fraction	95	9		
B	None			102	105
	50 μM $FeSO_4$			30	3
	50 μM $FeCl_3$			88	

[a] Samples (20 μl) were incubated for 10 min at 30°C. Initial activity was 276 nkat/μg of GS protein. The samples in assay B did not contain the inactivating fraction.

observation is corroborated by the enrichment of an enzyme fraction which promotes GS inactivation. This enzyme uses the reduced forms of the dinucleotides (Table 2) and Fe^{3+} for GS inactivation (Table 3, assay A), reducing Fe^{3+} to Fe^{2+} in the presence of NADPH. Moreover, the GS-inactivating fraction produced H_2O_2 apparently from NADPH and O_2. Fe^{2+} and H_2O_2 caused a strong GS inactivation, even if applied without the GS-inactivating fraction (Table 3, assay B). Consequently, GS inactivation is inhibited by EDTA, Mn^{2+}, and catalase. Since GS inactivation is reported to occur at the catalytic center of the enzyme (6, 23), Mn^{2+} bound to the catalytic site may protect histidines from oxidation or may exclude Fe^{2+} from the catalytic site and thus prevent GS oxidation. At the moment, it is not clear if only a single enzyme, several enzymes reacting one after another, or an enzyme complex is involved in GS inactivation.

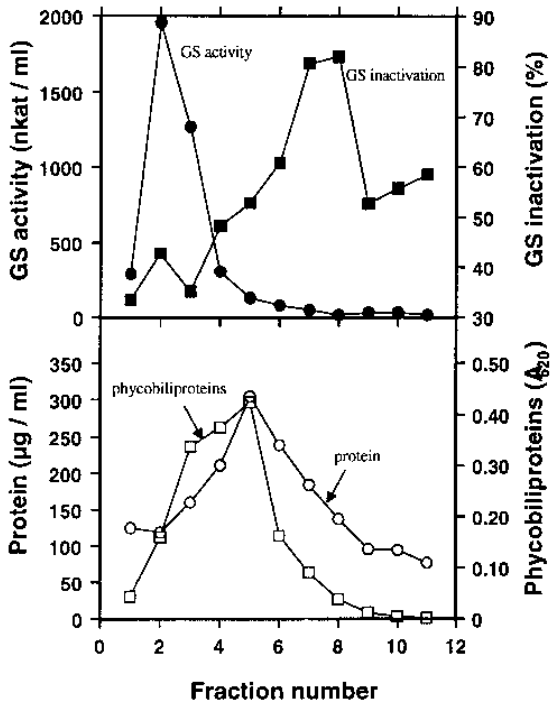

FIG. 4. Gel filtration of the ammonium sulfate precipitate. The extract was chromatographed on a Superose 12 column (Pharmacia).

The same oxidative principle of GS inactivation was reported for *E. coli*. GS is inactivated by a variety of enzyme systems, including cytochrome P_{450}/cytochrome P_{450} reductase, NADH oxidase, putidaredoxin/putidaredoxin reductase, and ferredoxin/xanthine oxidase (10, 29). Most of these systems use NADPH, O_2, and Fe^{3+} and produce Fe^{2+} and H_2O_2, which react to form reactive oxygen. They are inhibited by Mn^{2+}, EDTA, or catalase. Even the nonenzymatic systems ascorbate/Fe^{3+} and Fe^{2+} are effective in GS inactivation (10, 13). The following reactions are thought to occur (10):

$$NAD(P)H + H^+ + O_2 \rightarrow H_2O_2 + NAD(P)^+$$

$$NAD(P)H + 2Fe^{3+} \rightarrow 2\,Fe^{2+}NAD(P)^+ + H^+$$

$$Fe^{2+} + H_2O_2 \rightarrow \text{activated oxygen} + Fe^{3+}$$

$$\text{activated oxygen} + GS \rightarrow \text{oxidized GS}$$

The first two reactions are catalyzed by the GS-inactivating enzyme, while the last two reactions are spontaneous. Probably the reactive oxygen species is the hydroxyl radical reacting with amino acid residues in the catalytic center of GS.

Recently GS oxidation was also reported for the green alga *Monoraphidium braunii* (12). All of these systems inactivating GS have major characteristics in common with the GS inactivation described in this report.

The physiological role of oxidative GS inactivation is still speculative and will be further investigated. In *E. coli*, GS inactivated by oxidation was shown to be more readily degraded than active GS (14). However, in our immunoblot analysis investigating the first hour of GS inactivation, very little GS breakdown was detected. GS oxidation may serve as a signal for proteases to degrade the inactive protein and to recycle defective proteins (27). A similar function is ascribed to ubiquitin, recently detected in cyanobacteria (7). In eukaryotes, ubiquitin is attached to proteins, which then are degraded by proteases, as was also proposed for cyanobacterial nitrogenase. No hint for GS ubiquitinylation was obtained in our study. Nitrogenase inactivation by O_2 observed in cyanobacteria is discussed in terms of oxidative damage (5, 8). It is inactivated by endogenous H_2O_2 and more effectively by a xanthine/xanthine oxidase system and free iron (3). Simultaneously, protein degradation products appear on SDS-gels. Thus, there is increasing knowledge about oxidative damage of proteins initiating proteolysis, including GS.

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