Two Ways of Hydrogen Peroxide Formation in the Oxidative Inactivation of Cyanobacterial Glutamine Synthetase

Gottfried Martin* and Peter Böger

Lehrstuhl für Physiologie und Biochemie der Pflanzen, Universität Konstanz, P. O. Box 5560, D-78434 Konstanz, Germany

Z. Naturforsch. **52c,** 812–816 (1997); received August 12, 1997

Glutamine Synthetase, Protein Oxidation, Ammonia Effect, Photosynthetic Hydrogen Peroxide Production, Cyanobacterium, *Anabaena variabilis*

Using crude extracts from the cyanobacterium *Anabaena variabilis* glutamine synthetase (GS) activity was rapidly irreversibly reduced to about 60% during dark incubation („spontaneous GS inactivation"). An additional decrease was observed by the addition of ammonia in the light („ammonia-mediated inactivation"). Both effects were prevented by EDTA, $MnCl_2$ or catalase indicative of the involvement of H_2O_2. This is a key intermediate in oxidative enzyme inactivation. In both spontaneous and ammonia-mediated GS inactivation H_2O_2 is produced in different ways. Spontaneous inactivation is prevented by depletion of reduced pyridine nucleotides which apparently donate electrons to produce H_2O_2. Fractionation of the crude extract showed that the light-enhanced GS inactivation by ammonia required the presence of thylakoid membranes. The photosynthesis inhibitor DCMU decreased GS inactivation by ammonia. For the inactivation in the light apparently H_2O_2 is produced from superoxide during photosynthetic electron transport.

Introduction

Glutamine synthetase (GS) is a key enzyme in nitrogen assimilation and subject to different kinds of regulation. Long-term regulation is caused by repression of the *glnA* gene (encoding GS) if ammonia is present in the culture medium. This is the main type of GS regulation in cyanobacteria (Rowell *et al.,* 1979; Flores and Herrero, 1994). Furthermore, in many prokaryotes the activity is down-regulated in a short-term effect following the addition of ammonia which is the result of a covalent GS modification by adenylylation (Stadtman, 1990b; Engelhardt and Klemme, 1982). Such a down-regulation of GS activity was also expected in cyanobacteria, but it was found not to exist. Only in *Synechocystis* PCC 6803 a short-term GS inactivation was observed (Merida *et al.,* 1991).

Recently, the influence of ammonia on GS activity in cyanobacteria was reinvestigated. In crude extracts incubated in the light a marked reduction of GS activity was found, and in *Synechocystis* PCC 6803 this effect was interpreted as ADP ribosylation (Silman *et al.,* 1995). On the other hand GS in crude extracts from *Anabaena variabilis* was shown to be oxidatively inactivated upon the addition of NADH (Martin *et al.,* 1997). The purified inactive GS did not exhibit any property indicative of ADP ribosylation or adenylylation. In this report we show that GS inactivation in crude extracts is caused by oxidation of GS and that hydrogen peroxide is involved in GS inactivation. Two different modes of H_2O_2 generation are discussed.

Materials and Methods

Anabaena variabilis ATCC 29413 was grown in Arnon medium without nitrate (Arnon *et al.,* 1974). Cultures were started by inoculating 200 ml of medium with a cell density equivalent to 1.5 µg per ml of chlorophyll. The tubes were gassed with CO_2-enriched air (1.6% CO_2, v/v) and illuminated with 150 µE m^{-2} s^{-1} fluorescent light at 30 °C.

Crude extracts were prepared from cells at the end of the log phase (1–2 days old cultures). Cells were collected by centrifugation (5 min at 6500 × *g*)

* Present address: Lehrstuhl für Entwicklungsgenetik, Universität Tübingen, Spemannstraße 37–39, D-72076 Tübingen, Germany.

Reprint requests to Prof. Dr. Peter Böger.
Telefax: +49-7531-883042.

0939–5075/97/1100–0812 $ 06.00

and washed once in 50 mM Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, pH 7.0. The sediment was 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. It was incubated in a water bath at 25 °C with an illumination of 200 μE m^{-2} s^{-1}. At the times indicated samples were assayed for GS activity.

GS was generally assayed by the transferase reaction according to (Orr *et al.*, 1981). The reaction mixture (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 and 20 mM Na_2HAsO_4 and an appropriate amount of GS. After a 10-min incubation at 37 °C the reaction was stopped by the addition of 660 μl stop mix (3.3% $FeCl_3$, 2.0% trichloroacetic acid and 0.25 N HCl). Absorption was measured at 540 nm. Biosynthetic activity was determined as described in (Martin *et al.*, 1997).

Chlorophyll was determined in methanol extracts using the extinction coefficient from (Mackinney, 1941). Protein was measured by the bicinchoninic acid (BCA) method (Smith *et al.*, 1985).

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%.

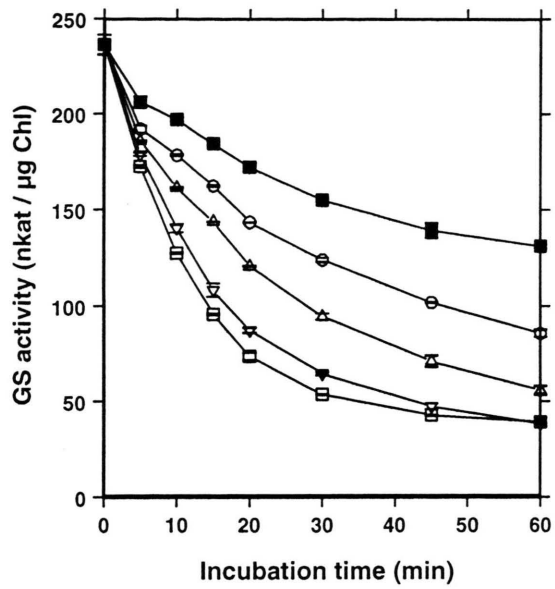

Fig. 1. Inactivation of GS by ammonia in crude extracts of *Anabaena variabilis*.
The experiment was started by adding ammonium chloride to 200 μl of crude extract and incubating it at 25 °C with illumination at 200 μE m^{-2} s^{-1}. Incubations were stopped by starting the GS transferase assay. Standard deviations are derived from double assays. Incubations contained: ■, 0 mM ammonia; ○, 0.16 mM ammonia; △, 0.63 mM ammonia; ▽, 2.5 mM ammonia; □, 10 mM ammonia.

Results and Discussion

During incubation of crude extracts prepared with Hepes buffer GS activity was reduced to about 60% of the initial activity (Figs. 1, 2 and 3). This effect was called „spontaneous GS inactivation" and was completed within 30 to 60 min. GS inactivation was enhanced by the addition of ammonia (Fig. 1). At least 100 μM ammonia was necessary and saturation was reached at 4 mM; the reaction was completed within 30 min. Both effects are seen using either the assay based on transferase or biosynthetic activity. pH did not change during incubation. GS inactivation was not prevented by protease inhibitors like PMSF (phenylmethylsulfonyl fluoride), leupeptin or pepstatin. Only little GS degradation was detected in immuno blots of crude extracts using a polyclonal antiserum raised against GS indicating that proteolytic degradation was not the reason for GS inactivation. The ammonia effect was absent when ammonia was substituted by glutamine or glutamate. This finding is not compatible with the view that GS is down-regulated by products of nitrogen assimilation as observed in *E. coli* or purple bacteria. Furthermore, no GS inactivation was observed with partially purified GS. Obviously, the crude extract contains substances mediating GS inactivation.

Both kinds of GS inactivation (spontaneous and ammonia-mediated) are impaired by EDTA, catalase and $MnCl_2$ (Table I). These substances are known to inhibit oxidative GS inactivation in *E. coli* (Levine *et al.*, 1981; Fucci *et al.*, 1983) and cyanobacteria (Martin *et al.*, 1997). Obviously, the observed GS inactivation in crude extracts is caused by oxidation. We have reported previously that in crude extracts GS oxidation requires NAD(P)H (Martin *et al.*, 1997), therefore a depletion of reduced pyridine dinucleotides should diminish GS

Table I. Effect of inhibitors on glutamine synthetase inactivation.

Inhibitor	GS activity (% of initial activity)	
	(-) NH_4Cl	(+) NH_4Cl, 4 mM
EDTA, 1 mM	100	99
$MnCl_2$, 400 µM	97	97
Catalase, 8 mU / µl	101	98
Control (water added)	72	51

100 µl of crude extract were incubated in the absence or presence of ammonia for 30 min in the light at 25 °C and then assayed for GS activity. Catalase was from bovine liver (Boehringer Mannheim). Spontaneous GS inactivation is documented for the incubations without ammonia (1st col.), while the incubations containing ammonia show a combination of spontaneous and ammonia-enhanced GS inactivation (2nd col.).

Table II. Effect of depletion of reduced pyridine nucleotides on glutamine synthetase inactivation.

Additions	Incubation with:	GS activity (nkat/µg Chl)	
		30 min	60 min
– Alcohol dehydrogenase	0 mM ammonia	53	46
	4 mM ammonia	38	37
	Ratio	0.73	0.82
+ Alcohol dehydrogenase	0 mM ammonia	90	82
	4 mM ammonia	63	62
	Ratio	0.70	0.76

100 µl of crude extracts were incubated in the absence or presence of ammonia for 30 min or 60 min in the light at 25 °C and then assayed for GS activity. Incubations with lowered content of reduced pyridine nucleotide contained 240 U / ml alcohol dehydrogenase (ADH) from yeast (Boehringer Mannheim) and 50 mM acetaldehyde to form ethanol with consumption of NADH. Starting GS activity was 110 nkat / µg Chl. Spontaneous GS inactivation is given in the incubations without ammonia. The incubations containing ammonia show a combination of spontaneous and ammonia-enhanced GS inactivation. To get an estimate for the latter the ratio of the activity with added ammonia to that without ammonia is shown. Chl, chlorophyll.

inactivation. Table II shows that removal of reduced pyridine nucleotides by alcohol dehydrogenase only abolishes the spontaneous GS inactivation while the ammonia mediated GS inactivation is not affected.

While the spontaneous GS inactivation is independent of light the enhancement of GS inactivation by ammonia is only observed in the light (Fig. 2). To investigate the effect of light the influence of photosynthesis was tested. After a short lag phase presence of the electron transport inhibitor DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] inhibited the ammonium-enhanced GS inactivation (Fig. 3) giving a result very similar to that obtained with ammonia in the dark (Fig. 2). The spontaneous GS inactivation was not influenced. To corroborate this finding thylakoid membranes were removed from the crude extract by ultracentrifugation which resulted in reduced GS inactivation by ammonia (Table III) while the spontaneous GS inactivation was even enhanced.

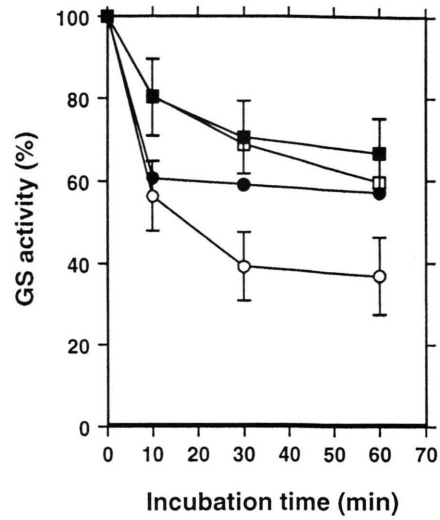

Fig. 2. Effect of light on glutamine synthetase inactivation.
Crude extracts were incubated in the light (200 µE m^{-2} s^{-1}) or in darkness with or without the addition of ammonium chloride. Then GS activity was assayed. The values are normalized to 100% since they are derived from 23 (light) or 6 (dark) independent experiments. The initial GS activity was in the range of 180 to 240 nkat / µg chlorophyll. Incubations were: ■, 0 mM ammonia, dark; ●, 4 mM ammonia, dark; □, 0 mM ammonia, light; ○, 4 mM ammonia, light.

The enhancement of GS inactivation by ammonia is probably not caused by uncoupling of photophosphorylation which would result in increased NADP reduction and subsequent GS inactivation. In *Anabaena variabilis*, the half maximum concentration of uncoupling is 10 mM ammonia (Duane *et al.*, 1965) while GS inactivation by ammonia is saturated at 4 mM (Fig. 1). Moreover, the ammonia-mediated GS inactivation is not influenced by depletion of the NAD(P)H pool by alcohol dehydrogenase and acetaldehyde (Table II).

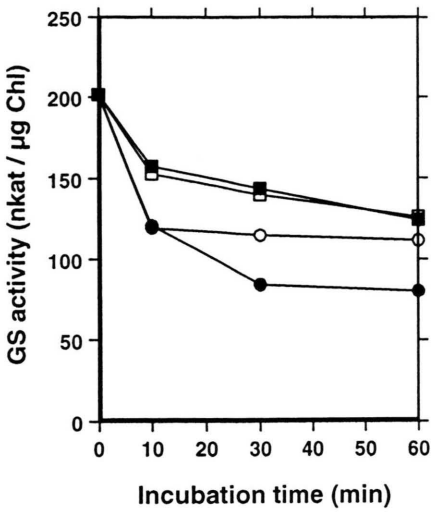

Fig. 3. Effect of the photosynthesis inhibitor DCMU on GS inactivation in crude extracts.
Crude extracts were incubated in the light (200 µE m^{-2} s^{-1}) or in darkness with or without the addition of ammonium chloride. Then GS activity was assayed. A typical experiment is shown. Incubations were: ■, 0 mM DCMU, 0 mM ammonia; ●, 0 mM DCMU, 4 mM ammonia; □ 100 µM DCMU, 0 mM ammonia; ○, 100 µM DCMU, 4 mM ammonia.

GS inactivation by ammonia is only observed when photosynthetic electron transport is operating, and it is assumed that H_2O_2 is produced during photosynthesis from superoxide (Takahashi and Asada, 1988) generated mainly by reduction of O_2 through photosystem I. O_2 is much more soluble in the thylakoid membranes than in the cytosol. Accordingly, most of superoxide is generated in the membranes. To release this superoxide to the cytosol it must be protonated. Protonation is enhanced by ammonium penetrating the membrane which results in disproportionation of superoxide to O_2 and H_2O_2 according to the following reaction (Takahashi and Asada, 1988):

$$2\ O_2^- + 2\ NH_4^+ \longrightarrow O_2 + H_2O_2 + 2\ NH_3$$

Oxidative GS inactivation was first detected in *E. coli* (Levine *et al.*, 1981). In this organism inactivated GS was more rapidly degraded than the active enzyme. It was assumed that oxidative GS inactivation modifies the protein for subsequent proteolytic degradation (Stadtman 1990a). We described a similar system in *Anabaena variabilis* (Martin *et al.*, 1997); recently ubiquitin was detected in *Anabaena* which is also involved in protein degradation (Durner and Böger, 1995). Irreversible oxidative inactivation was reported for cyanobacterial nitrogenase (Bagchi *et al.*, 1991, Durner *et al.*, 1996) and protein degradation was detected on SDS gels. These examples indicate that protein oxidation is an important step to start cyanobacterial protein degradation.

Table III. Inactivation of glutamine synthetase in fractions prepared by ultracentrifugation.

No.	Treatment	Incubation with:	GS activity (% of initial activity) 30 min	60 min	Activity at start (nkat / µg protein)
1	Supernatant	0 mM ammonia	54	33	3.3
2		4 mM ammonia	44	28	3.3
3		Ratio	0.83	0.84	
4	Pellet, containing	0 mM ammonia	79	68	2.1
5	the membranes	4 mM ammonia	58	39	2.1
6		Ratio	0.74	0.58	
7	Control without	0 mM ammonia	68	56	4.0
8	ultracentrifugation	4 mM ammonia	47	39	4.0
9		Ratio	0.69	0.70	

7.5 ml of crude extract were centrifuged at 170,000 × *g* for 1 h at 4 °C. The sediment was resuspended in 1.5 ml of 50 mM Hepes, pH 7.0. 100 µl of the extract were incubated in the light at 25 °C in the absence or presence of ammonia for 30 min or 60 min, respectively. Then a 5-µl sample was used to assay GS activity. Spontaneous GS inactivation is seen by the lines 1, 4, 7 (incubations without ammonia) . The incubations containing ammonia (lines 2, 5, 8) show a combination of spontaneous and ammonia-enhanced GS inactivation. To estimate the latter inactivation the activity ratio with added ammonia to that without ammonia is shown (lines 3, 6, 9).

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft through its Sonderforschungsbereich 248 "Stoffhaushalt des Bodensees" and by the Fonds der Chemischen Industrie.

Arnon, D. I., McSwain, B. D., Tsujimoto, H. Y. and Wada, K. (1974), Photochemical activity and components of membrane preparations from blue-green algae. Biochim. Biophys. Acta **357**, 231–245.

Bagchi, S. N., Ernst, A. and Böger, P. (1991), The effect of activated oxygen species on nitrogensase of *Anabaena variabilis*. Z. Naturforsch. **46c**, 407–415.

Duane, W. C., Hohl, M. C. and Krogmann, D. W. (1965), Photophosphorylation activity in cell-free preparations of a blue-green alga. Biochim. Biophys. Acta **109**, 108–116.

Durner, J. and Böger, P. (1995), Ubiquitin in the prokaryote *Anabaena variabilis*. J. Biol. Chem. **270**, 3720–3725.

Durner, J., Böhm, I., Knörzer, O. C. and Böger, P. (1996), Proteolytic degradation of dinitrogenase reductase from *Anabaena variabilis* (ATCC 29413) as a consequence of ATP depletion and impact of oxygen. J. Bacteriol. **178**, 606–610.

Engelhardt, H. and Klemme, J.-H. (1982), Purification and structural properties of adenylylated and deadenylylated glutamine synthetase from *Rhodopseudomonas sphaeroides*. Arch. Microbiol. **133**, 202–205.

Flores, E., and Herrero, A. (1994), Assimilatory nitrogen metabolism and its regulation. In: D. A. Bryant (ed.): The Molecular Biology of Cyanobacteria, pp. 487. Kluwer Academic Publishers.

Fucci, L., Oliver, C. N., Coon, M. J. and Stadtman, E. R. (1983), Inactivation of key metabolic enzymes by mixed-function oxidation reactions: Possible implication in protein turnover and ageing. Proc. Natl. Acad. Sci. USA **80**, 1521–1525.

Levine, R. L., Oliver, C. N., Fulks, R. M. and Stadtman, E. R. (1981), Turnover of bacterial glutamine synthetase: Oxidative inactivation precedes proteolysis. Proc. Natl. Acad. Sci. USA **78**, 2120–2124.

Mackinney, G. (1941), Absorption of light by chlorophyll solutions. J. Biol. Chem. **140**, 315–322.

Martin, G., Haehnel, W. and Böger, P. (1997), Oxidative inactivation of glutamine synthetase from the cyanobacterium *Anabaena variabilis*. J. Bacteriol. **179**, 730–734.

Merida, A., Candau, P. and Florencio, F. J. (1991), Regulation of glutamine synthetase activity in the unicellular cyanobacterium *Synechocystis sp.* strain PCC 6803 by the nitrogen source: Effect of ammonium. J. Bacteriol. **173**, 4095–4100.

Orr, J., Keefer, L. M., Keim, P., Nguyen, T. D., Wellems, T., Heinrikson, R. L. and Haselkorn, R. (1981), Purification, physical characterization, and NH_2-terminal sequence of glutamine synthetase from the cyanobacterium *Anabaena* 7120. J. Biol. Chem. **256**, 13091–13098.

Rowell, P., Sampaio, M. J. A. M., Ladha, J. K. and Stewart, W. D. P. (1979), Alteration of cyanobacterial glutamine synthetase activity in vivo in response to light and NH_4. Arch. Microbiol. **120**, 195–200.

Silman, N. J., Carr, N. G. and Mann, N. H. (1995), ADP-ribosylation of glutamine synthetase in the cyanobacterium *Synechocystis sp.* strain PCC 6803. J. Bacteriol. **177**, 3527–3533.

Smith, P. K., Krohn, R. J., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985), Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**, 76.

Stadtman, E. R. (1990a), Covalent modification reactions are marking steps in protein turnover. Biochemistry **29**, 6323–6331.

Stadtman, E. R. (1990b), Discovery of glutamine synthetase cascade. Methods Enzymol. **182**, 793–809.

Takahashi, M. and Asada, K. (1988), Superoxide production in aprotic interior of chloroplast thylakoids. Arch. Biochem. Biophys. **267**, 714–722.