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Inactivation of N-assimilating enzymes and proteolytic activities in wheat leaf extracts: Effect of pyridine nucleotides and of adenylates

L. Streit and U. Feller[1]

Pflanzenphysiologisches Institut der Universität Bern, Altenbergrain 21, CH-3013 Bern (Switzerland),
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Summary. Nitrate reductase was protected from inactivation in wheat leaf extracts by NADH, while NADPH was less effective. NAD, NADP or adenylates did not affect nitrate reductase inactivation in vitro. Glutamine synthetase was more stable than nitrate reductase and was protected from inactivation by ATP. ADP, AMP or pyridine nucleotides had no or only a minor effect on the decrease of glutamine synthetase activity in extracts. The caseolytic activity extracted from senescing leaves was slightly decreased by NADH and NADPH but this effect was not sufficient to explain the stabilization of nitrate reductase by NADH. Oxidized pyridine nucleotides and adenylates had no major effect on the caseolytic activity under the conditions used.

Introduction

There is evidence that nitrate reductase (NR) can be inactivated in plant extracts by endogenous peptide hydrolases[2-4]. Phenylmethylsulfonylfluoride (an inhibitor of serine peptide hydrolases) has been found to protect NR from inactivation in extracts from maize roots[5]. Leupeptin (an inhibitor of some endopeptidases) has been noted in barley leaf extracts to prevent the formation of smaller breakdown products from NR[4]. Leupeptin also improves the stability of several enzymes in extracts from castor bean endosperm[6]. Aminopeptidase has been found to be more stable under conditions of low endopeptidase activity in extracts from bean cotyledons collected at various stages of germination[7]. Treatments of the extracts causing lower endopeptidase activities delay the inactivation of aminopeptidase.

For the inactivation of enzymes by proteolysis, both the peptide hydrolase activities present and the susceptibility of the substrate proteins (e.g. enzymes) are important[8]. Substrates, coenzymes and other low molecular weight compounds have been found to change the susceptibility of various enzymes from microorganisms and animals to peptide hydrolases in vitro and in vivo[8]. ATP-stimulated peptide hydrolases have been detected in rat liver[9] and in *E. coli*[10].

Nitrate reductase which is known to be a very unstable enzyme, and glutamine synthetase (GS) are involved in the assimilation of inorganic nitrogen. While the former is needed only for the assimilation of nitrate, GS is also required for the reassimilation of ammonium liberated in the photorespiratory nitrogen cycle[11] or in other metabolic processes (e.g. by phenylalanine ammonia lyase, threonine dehydratase). NR requires for its enzymatic activity reduced pyridine nucleotides and ATP is needed for the GS reaction. In order to investigate the effect of adenylates and of pyridine nucleotides on enzyme inactivation, NR and GS are of interest because of their different cofactor requirements.

The objectives of the present work were to investigate the in vitro stability and inactivation of NR and GS in leaf extracts, and to examine the effects of adenylates and of pyridine nucleotides on the inactivation rates. Since highest endopeptidase activities had been found in wheat leaves late in senescence[12], extracts from senescing flag leaves were used as a source of proteolytic activity.

Materials and methods

Plant materials. Flag leaves of winter wheat (*Triticum aestivum* L., cv. 'Probus') were collected on a field in Zollikofen near Bern at various stages of development during summer 1981. These leaves were transported

in plastic bags on ice to the laboratory and stored frozen (−18 °C). Flag leaves collected on June 12th were used as the source of GS (extract of young leaves in experiments with GS). Extracts from senescing leaves were prepared in all experiments from senescing flag leaves collected on July 17th. The extract from senescing leaves showed very high endopeptidase activity, no NR activity and only a minor GS activity (less than 3% of the activity found in extracts from young leaves).

Since NR activity was lost upon freezing, wheat plants were also grown in a culture room in pots with coarse quartz sand. The pots with the germinated wheat were placed in a recipient containing nutrient solution with 3.5 mM nitrate as nitrogen source[13]. The plants were grown in a 14 h light/10 h dark cycle as described earlier[13]. Leaves of 2–4-week-old seedlings were used as the source of NR (extract of young leaves in experiments investigating NR inactivation). These leaves were collected immediately before extraction.

Enzyme extraction and pre-incubation of extracts. Leaf samples were extracted in 4 volumes of extraction medium (100 mM imidazole-HCl pH 7.5, 15 mM $MgSO_4$, 12 mM mercaptoethanol and 1% w/v polyvinylpolypyrrolidone) using a Polytron homogenizer (Kinematica, Luzern) for 20 sec at low speed and for 5 sec at full speed. The homogenate was passed through Miracloth (Calbiochem, San Diego) and centrifuged for 10 min at 20,000 × g in a Sorvall RC-5 centrifuge. The supernatants were then desalted by centrifugation through Sephadex G-25 columns[14] equilibrated with extraction medium minus polyvinylpolypyrrolidone. Extract from young leaves was mixed 1:1 with either buffer (control) or with extract from senescing leaves. Effectors were added to these mictures from 10-fold concentrated stock solutions (1.8 ml extract plus 0.2 ml stock solution). Buffer instead of the stock solution was added to the controls. The samples were pre-incubated in stoppered test tubes in the cold room (2 °C) or in a water bath (30 °C). In all experiments the pH was 7.5 during the pre-incubation.

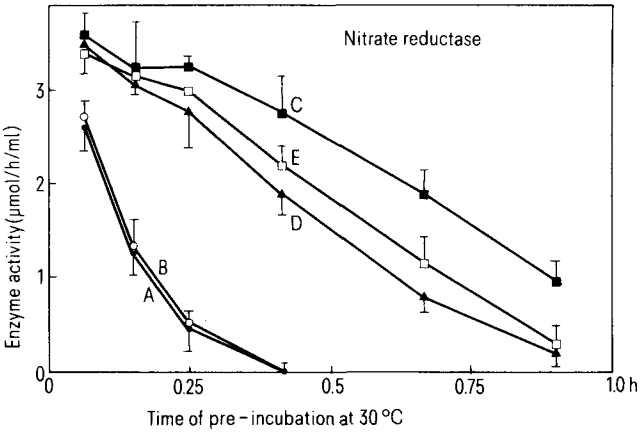

Figure 1. Stability of nitrate reductase in extracts from wheat leaves. Fresh extract from young leaves was mixed (1:1) with: fresh extract from senescing leaves (A), extract from senescing leaves precipitated with ammonium sulfate and resuspended in buffer (B), extract from senescing leaves kept for 5 min at 100 °C (C), extract from senescing leaves containing 2% casein (D) and buffer (E). The symbols represent the means ±SD of 5 separate pre-incubations.

Ammonium sulfate precipitation. Solid ammonium sulfate was added to the centrifuged extract (10 ml extract plus 4.74 g ammonium sulfate) and stirred for 30 min at 2 °C. The precipitate was collected by centrifugation (10 min at 20,000 × g), resuspended in extraction medium minus polyvinylpolypyrrolidone, and desalted on Sephadex G-25 as described above. Most of the caseolytic activity (86–97%) of the activity present in the original extract) was recovered. The resuspended and desalted precipitate was used to investigate the effect of pyridine nucleotides and of adenylates on the caseolytic activity at pH 7.5 (table 2) and for inactivation experiments as indicated (figs 1 and 2). For measurements of the caseolytic activity at pH 5.4 (table 2) the resuspended precipitate was desalted on Sephadex G-25 equilibrated with 100 mM acetate buffer pH 5.4.

Enzyme assays. Nitrate reductase was determined as described earlier[15], except that EDTA was omitted from the assay mixture. The nitrate formed was detected with the Griess-Ilosvay reagent[16]. If NADH or NADPH was present in the pre-incubation mixture, blanks were made without nitrate.

Table 1. Effect of pyridine nucleotides and ascorbic acid on the stability of nitrate reductase in extracts from wheat leaves

Compound added (final concentration during pre-incubation)	Initial activity (µmol/h/ml)	% of the initial activity min pre-incubated at 30 °C				min pre-incubated at 2 °C				
		15	24	33	54	37	60	90	120	150
Control	3.21 ± 0.08	22	4	3	< 1	44	36	32	24	12
NADP (3 mM)	3.36 ± 0.48	26	12	6	< 1	46	46	33	23	12
NADPH (1 mM)	3.22 ± 0.39	38	21	12	9	74	72	65	40	18
NADPH (3 mM)	3.48 ± 0.35	63	37	34	27	100	86	88	92	80
NADH (3 mM)	3.41 ± 0.24	96	70	60	36	108	103	103	100	105
Ascorbate (10 mM)	3.23 ± 0.52	20	5	< 1	< 1	37	33	22	17	7

The initial activity represents the mean ± SD of 5 replicates. The remaining activity at 30 °C and at 2 °C was determined for each compound in 5 separate pre-incubations and the data shown represent the means of these 5 replicates.

Glutamine synthetase was determined by measuring the ATP-dependent production of γ-glutamyl hydroxamate[17]. Blanks were made without ATP. If adenylates were added to the extracts, a 2nd set of blanks was prepared without hydroxylamine. The differences between the 2 blanks were used as indicators for the actual ATP concentration in the pre-incubated extracts. The low activities observed in blanks without ATP were compared with the γ-glutamyl hydroxamate formation when low concentrations of ATP were added to ATP-free assays.

Adenylates and pyridine nucleotides added to the pre-incubation mixtures did not affect the initial activities of NR and GS. The remaining enzyme activities in inactivation experiments are expressed in % of the initial activity.

The proteolytic activity with azocasein as substrate was determined at 37 °C as described earlier[12], except that the Tris-HCl buffer pH 7.5 was replaced by extraction medium minus polyvinylpolypyrrolidone. Acetate buffer (100 mM) was used for the measurements at pH 5.4.

In order to detect the loss of NADH during pre-incubation, 50 µl of the pre-incubation mixtures were added to 0.95 ml distilled water and the OD_{366} was read immediately after dilution. Diluted extracts without NADH were used as blanks.

Results and discussion

GS was considerably more stable than NR in extracts from wheat leaves (figs 1 and 2). This is consistent with the fact that GS is still present in young wheat leaves after freezing (6–12% loss), while essentially no NR activity was found in extracts from frozen leaves. Glutamine synthetase is present in cereal leaves in 2 forms (GS I located in the cytoplasm and GS II located in the chloroplasts)[18,19]. Since GS I contributed in young wheat leaves to only about 10% of the total activity (data not shown), the results obtained with extracts from young leaves mainly describe the properties of GS II. When extract from senescing leaves was mixed with extract from young leaves, both NR and GS were inactivated more rapidly than in extract from young leaves diluted with buffer (figs 1 and 2). In the presence of 1% casein the stability of the 2 enzymes was considerably improved in the mixed extracts and was similar to the stability observed in extract from young leaves diluted with buffer. Since the 'factor' inactivating NR and GS was

Table 2. Effect of pyridine nucleotides and of denylates on the caseolytic activity (azocaseinase) in extracts from senescing wheat leaves

Compound added (final concentration during incubation)	Azocaseinase pH 5.4 mg/h/g fresh wt*)	% **)	Azocaseinase pH 7.5 mg/h/g fresh wt*)	% **)
Control	2.66±0.42	100	2.88±0.24	100
NAD (3 mM)	2.84±0.31	107	2.90±0.17	100
NADH (3 mM)	1.63±0.28	61	2.13±0.35	74
NADP (3 mM)	2.43±0.33	91	2.73±0.32	95
NADPH (3 mM)	1.48±0.32	55	1.94±0.20	67
ATP (10 mM)	2.94±0.52	110	2.77±0.22	96
ADP (10 mM)	2.68±0.50	100	3.03±0.31	105
AMP (10 mM)	2.78±0.30	105	2.90±0.19	101

*) Mean ± SD of 5 determinations (mg azocasein hydrolyzed per h per g fresh weight); **) % of the control.

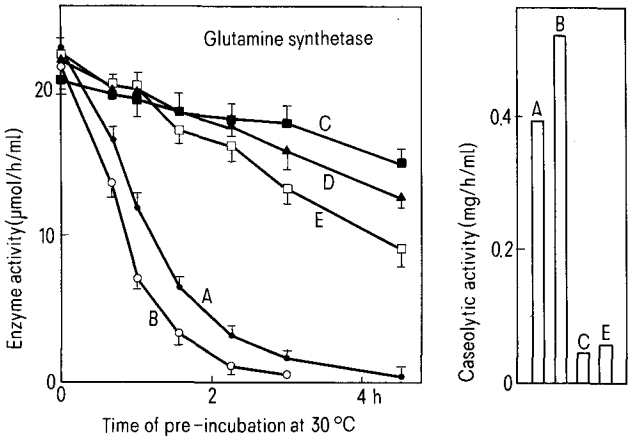

Figure 2. Proteolytic activity and stability of glutamine synthetase in extracts from wheat leaves. Fresh extract from young leaves was mixed (1:1) with: fresh extract from senescing leaves (A), extract from senescing leaves precipitated with ammonium sulfate and resuspended in buffer (B), extract from senescing leaves kept for 5 min at 100 °C (C), extract from senescing leaves containing 2% casein (D) and buffer (E). The symbols for glutamine synthetase activity represent the means ±SD of 5 separate pre-incubations. The bars for the proteolytic activity represent the means of duplicate measurements at the beginning of the pre-incubation.

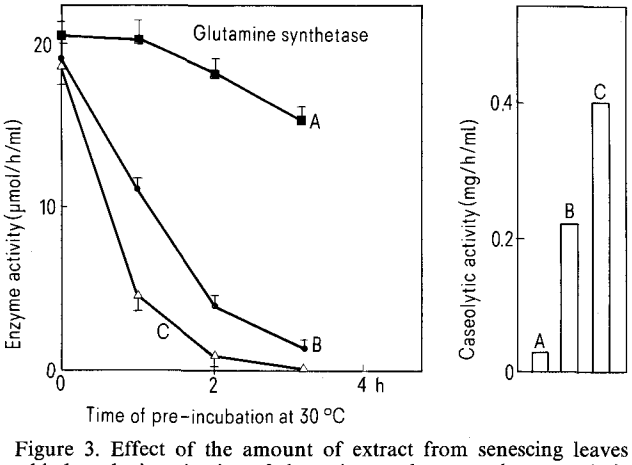

Figure 3. Effect of the amount of extract from senescing leaves added on the inactivation of glutamine synthetase and on caseolytic activity present during pre-incubation. *A* Extract from young leaves mixed with buffer (1:2). *B* Extract from young leaves mixed with buffer and with extract from senescing leaves (1:1:1). *C* Extract from young leaves mixed with extract from senescing leaves (1:2). The symbols for glutamine synthetase activity represent the means ±SD of 5 separate pre-incubations. The bars for the proteolytic activity represent the means of duplicate measurements at the beginning of the pre-incubation.

precipitable by ammonium sulfate and was ineffective after heating for 5 min at 100 °C, it appears likely that it is a protein. A good correlation was found between the caseolytic activity in the extracts and the velocity of enzyme inactivation (fig 2 and 3). Our data are consistent with the hypothesis that the rapid inactivation of NR could be due to proteolysis[2-4]. Although GS was more stable than NR, similar inactivating processes must be assumed. The endopeptidase(s) reaching highest activities during protein mobilization from senescing leaves[12] should be considered as a major factor involved in the inactivation of NR and GS.

NR was protected from inactivation when NADH (1 mM) was present in the preincubation mixture, while the addition of NAD or of adenylates had no effect (fig. 4). NADPH was less effective than NADH at both 1 mM and 3 mM concentrations (fig. 4, table 1), NADP (3 mM) was ineffective. The addition of the oxidized forms of the pyridine nucleotides (NAD, NADP) did not alter the inactivation rate of NR. With NADPH as coenzyme, NR activity was about 25% of the activity observed with NADH (results not shown). The more effective coenzyme (NADH) was also a better protectant than NADPH for NR. Since the addition of ascorbate (table 1) did not alter the inactivation rate, it appears unlikely that the protection of NR is a general effect of reducing substances.

The inactivation of GS was only slightly delayed in the presence of 3 mM NADH, and NAD was ineffective in the same concentration (fig. 5, A). In contrast to NR, GS was protected by ATP (fig. 5, B). AMP had no effect on GS inactivation and ADP often seemed to slightly protect GS during the initial time of pre-incubation. The effect of ADP is probably due to the formation of ATB by adenylate kinase activity present in the extracts. GS activity was, after a pre-incubation of 5.5 h at 30 °C with 10 mM ATP, still above 70% of the initial activity (data not shown). The metabolization of ATP during pre-incubation lowered the ATP concentration in the pre-incubation mixture as shown in figure 5, B. A better protection of GS should be possible if the ATP concentration could be maintained at 1 mM. The decrease of the NADH concentration was a problem as well. The rapid inactivation during the 1st h of pre-incubation, when the NADH level was still high

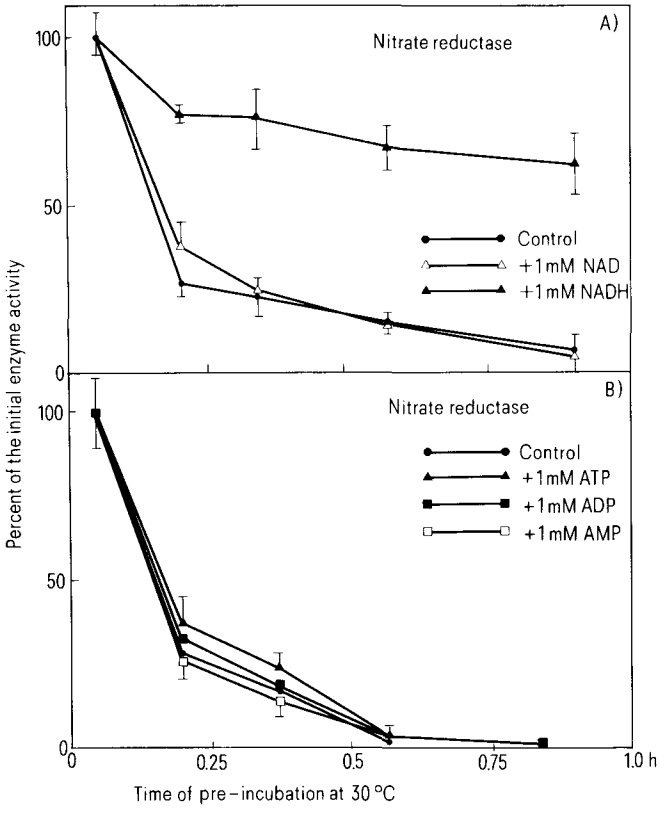

Figure 4. Effect of pyridine nucleotides (A) and of adenylates (B) on nitrate reductase inactivation. Extract from young wheat leaves was mixed 1:1 with extract from senescing flag leaves before the effectors were added from stock solutions. The initial nitrate reductase activity was 3.46 (A) and 3.61 (B) µmol/h/ml. The symbols represent the means ±SD of 5 separate pre-incubations.

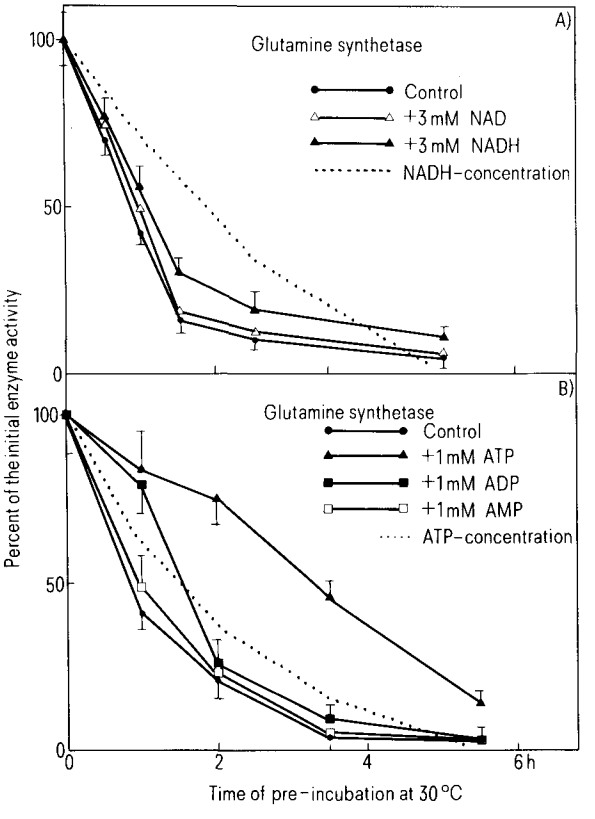

Figure 5. Effect of pyridine nucleotides (A) and of adenylates (B) on glutamine synthetase inactivation. Extract from young wheat leaves was mixed 1:1 with extract from senescing flag leaves before the effectors were added from stock solutions. The initial glutamine synthetase activity was 20.3 (A) and 23.3 (B) µmol/h/ml. The symbols represent the means ±SD of 5 separate pre-incubations.

(fig. 5, A), shows that NADH was not effectively protecting GS. In experiments with 3 mM NADPH no major stabilization of GS activity was observed. Therefore it can be concluded that pyridine nucleotides in either reduced or oxidized form had no or only a minor effect on GS inactivation. The addition of glutamate or of glutamine to the pre-incubation mixture did not alter the stability of GS (fig. 6). GS inactivation was slightly accelerated if magnesium ions were omitted from the pre-incubation mixture.

Assuming that the rapid inactivation of NR and GS after the addition of extract from senescing leaves was due to proteolysis, it remained open whether the effectors (NADH, ATP) were interacting with the substrate proteins (NR, GS) or with the proteolytic enzymes. The different behavior of NR and GS in presence of pyridine nucleotides and adenylates suggests interactions with the substrate proteins rather than with the peptide hydrolase(s). As shown in table 2, the hydrolysis of azocasein by extract from senescing leaves was not affected by adenylates. Reduced pyridine nucleotides in concentrations of 3 mM diminished the azocasein hydrolysis at pH 5.4 by 39% (NADH) and by 45% (NADPH). At pH 7.4 (pH of the pre-incubation mixtures in inactivation experiments) azocasein hydrolysis was reduced only by 26% (NADH) and by 33% (NADPH). This minor effect of reduced pyridine nucleotides on peptide hydrolase activity at pH 7.5 could be responsible for the slight stabilization of GS by NADH.

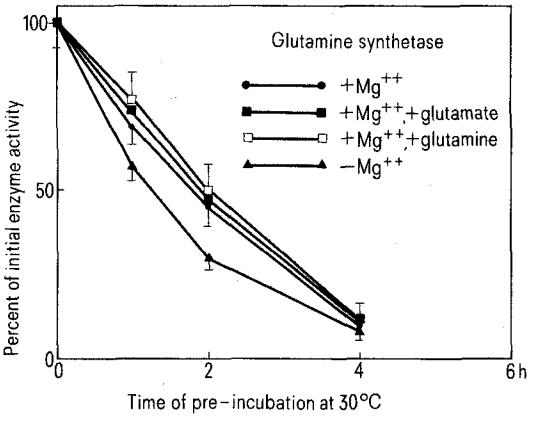

Figure 6. Influence of Mg, glutamate and glutamine on the inactivation of glutamine synthetase. Extract from young wheat leaves was mixed 1:1 with extract from senescing flag leaves. The concentration during pre-incubation was 15 mM for Mg and 20 mM for glutamate and glutamine. The initial glutamine synthetase activity was 25.7 µmol/h/ml. The symbols represent the means ±SD of 5 separate pre-incubations.

Considerable direct and indirect effects of pyridine nucleotides and of adenylates on the inactivation of NR and GS were observed in wheat leaf extracts. It remains open whether the degradation rates of these two enzymes can also be altered in vivo by adenylates or pyridine nucleotides. Interactions of low molecular weight compounds with peptide hydrolases or with substrate proteins should be considered as possible factors regulating proteolysis.

1 This work was supported by grant 3.067-0.81 from the Swiss National Science Foundation. Author for correspondence: U.F.

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