



# Influence of the activation status and of ATP on phosphoribulokinase degradation

Lea Kamber and Urs Feller<sup>1</sup>

Institute of Plant Physiology, University of Bern, Altenbergrain 21, CH-3013 Bern, Switzerland

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## Abstract

The light-regulated chloroplast enzyme phosphoribulokinase (EC 2.7.1. 19) exists in two forms. In darkness this enzyme is present in an oxidized form, which is inactive. It is activated in the light by a thioredoxin-mediated reduction. In extracts from young wheat leaves (*Triticum aestivum* L.) phosphoribulokinase as well as some other thioredoxin-modulated enzymes can be activated by the artificial reductant dithiothreitol (DTT). The influence of the activation status and of the substrate ATP on phosphoribulokinase stability was investigated in the presence of endogenous endopeptidases from senescing wheat leaves. Similar experiments were performed with purified phosphoribulokinase from spinach in the presence of exogenous, purified endopeptidases (chymotrypsin and trypsin). Phosphoribulokinase stability was analysed by immunoblotting and activity measurements. Both systems led to similar conclusions. DTT (reductant) and ATP (substrate) stabilized phosphoribulokinase in wheat leaf extracts as well as partially purified phosphoribulokinase from spinach. The combination of both effectors was far more protective than either effector alone. DTT had hardly any effect on the degradation of thioredoxin-independent chloroplast enzymes such as glutamate synthase and glutamine synthetase. These results suggest that the activation status and substrate concentrations are not only important for the activity of phosphoribulokinase, but are also relevant for the susceptibility of this enzyme to proteolysis.

Key words: ATP, enzyme inactivation, phosphoribulokinase, proteolysis, redox modulation.

## Introduction

The hydrolysis of peptide-bonds in higher plants is important for the modification of newly synthesized

polypeptides, for the degradation of damaged proteins, for the mobilization of storage proteins in germinating seeds, and for the remobilization of proteins in senescing cells (Huffaker, 1990). Cellular protein degradation must be well regulated. Possible mechanisms for regulation are the quality and quantity of proteolytic enzymes, the compartmentation, and the susceptibility of the substrate proteins. Vacuoles are organelles of high proteolytic activity (Boller and Kende, 1979; Lin and Wittenbach, 1981; Peoples and Dalling, 1979), while the ubiquitin system is found in the cytosol, where degradable proteins are tagged with ubiquitin and selectively degraded by the proteasome (Bachmair *et al.*, 1986). Peptide hydrolases have also been detected in chloroplasts (Dalling, 1986; Bushnell *et al.*, 1993; Shanklin *et al.*, 1995). The susceptibility of substrate proteins to degradation might be influenced by conformational changes as a consequence of interactions with, for example, substrate or other low molecular weight substances. Previous work has shown that phosphoenolpyruvate carboxylase was protected by phosphoenolpyruvate and by magnesium ions (Wedding and Black, 1987), and glucose-6-phosphate-dehydrogenase was protected from the proteolytic activity by NADP and phosphate (Fischer *et al.*, 1992). These ligands improved the stability of this enzyme also against heat and denaturing reagents (Kurlandsky *et al.*, 1988). As another example, glutamine synthetase was protected by ATP from attack by endopeptidases (Streit and Feller, 1982). Low molecular weight substances may also have an effect on the activity of endopeptidases. For example, an endopeptidase from *Medicago sativa* has been reported to be inhibited by polyamines (Ballestreri *et al.*, 1987). On the other hand the activity of endopeptidases of germinating cotyledons from bush beans was stimulated by the addition of  $\beta$ -mercaptoethanol (Feller, 1979).

An important mechanism for the regulation of chloroplast enzymes by light is the ferredoxin/thioredoxin system

<sup>1</sup> To whom correspondence should be addressed. Fax: +41 331 332 20 59. E-mail: feller@pfp.unibe.ch

which allows a covalent redox-modification driven by photosynthetic electron flow in the light and deactivation in darkness (Latzko *et al.*, 1970; Buchanan, 1991). Such redox modifications allow an efficient control of the metabolism in chloroplasts. Oxidative inactivation of the Calvin cycle enzymes phosphoribulokinase, glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphatase, and sedoheptulose-1,7-bisphosphatase prevents a simultaneous degradation and assimilation of carbohydrates in the dark (Wolosiuk *et al.*, 1993; Scheibe, 1990; Cséke and Buchanan, 1990). Phosphoribulokinase can be activated by the artificial reductant DTT (Farr *et al.*, 1994). The regulatory cysteines of phosphoribulokinase in spinach leaves were identified as cys-16 and cys-55. Current work identified the sequence around the cys-16 as the ATP-binding site (Porter *et al.*, 1988). Furthermore, the activation of phosphoribulokinase can be influenced by the concentrations of metabolites (Faske *et al.*, 1995). The aim of the work presented here was to compare the susceptibility of the oxidized (inactive) and reduced (active) forms of phosphoribulokinase from wheat leaves to proteolysis.

## Materials and methods

### Plant material

Grains from winter wheat (*Triticum aestivum* L., variety Arina) were germinated for 2 d on wet tissue paper and then for 2 d on quartz sand in the dark. The seedlings were transferred to nutrient solution and cultivated hydroponically (Hildbrand *et al.*, 1994) during 10–15 d in a culture room with 14 h light ( $105\text{--}150\ \mu\text{E m}^{-2}\ \text{s}^{-1}$  photosynthetic active radiation,  $25^\circ\text{C}$ ) and 10 h darkness ( $21^\circ\text{C}$ ) per day. Senescing wheat leaves were collected in a field and stored at  $-18^\circ\text{C}$ .

### Extraction

The extraction medium contained 100 mM TRIS-HCl pH 7.8, 1% (w/v) polyvinyl-pyrrolidone and 0.1% (w/v)  $\beta$ -mercaptoethanol. Fresh leaves (1 g), collected at the end of the dark phase were extracted in 4 ml extraction buffer with a Polytron mixer (Kinematica, Littau) for 20 s at medium and 5 s at full speed. Extracts were passed through Miracloth (Calbiochem, La Jolla) and centrifuged for 10 min at 4000 g. The supernatants were desalted by centrifugation through Sephadex G-25 (Feller *et al.*, 1977) equilibrated with 10 mM TRIS-HCl pH 7.8 containing 0.1%  $\beta$ -mercaptoethanol. All steps were performed at  $0\text{--}4^\circ\text{C}$  and the extracts were kept in the dark. Phosphoribulokinase was very stable in extracts from young leaves kept at  $0\text{--}4^\circ\text{C}$ . Senescing leaves were extracted as described above, except that 6 ml buffer per leaf were used.

### Preincubations

Extracts from young and senescing leaves were mixed 1:1. Controls were mixed with buffer instead of an extract from senescing leaves. Mixed extracts were treated with either 10 mM DTT, 10 mM ATP (neutralized with sodium bicarbonate) or a combination of 10 mM DTT and 10 mM ATP. The stoppered tubes were incubated in darkness in a water bath at  $25^\circ\text{C}$ . Samples were taken during incubation for the detection of

enzyme activity (photometric assay) and enzyme abundance (immunoblotting). Experiments with purified phosphoribulokinase were performed with partially purified powder containing approximately 50% protein (Sigma). Instead of senescing leaf extracts, 0.01% purified endopeptidases chymotrypsin and trypsin (Sigma) were used.

### Measurement of phosphoribulokinase activity

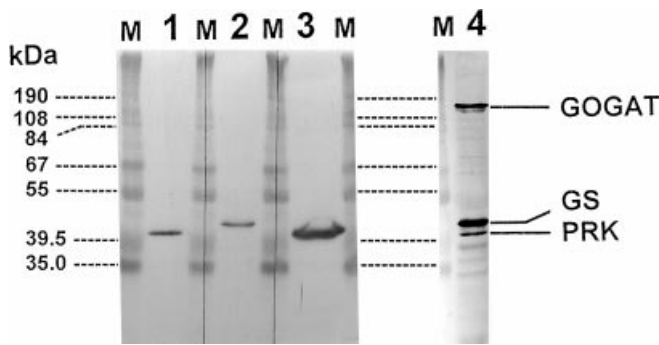
Phosphoribulokinase activity was measured by monitoring the NADH oxidation at 334 nm in a coupled assay (Surek *et al.*, 1985) with a Multiskan Photometer (DYNATEC). The incubation medium contained: 100 mM TRIS-HCl pH 7.8, 10 mM  $\text{MgCl}_2$ , 0.25 mM NADH, 20 mM KCl, 20 mM DTT, 2 units  $\text{ml}^{-1}$  lactate dehydrogenase, 2 units  $\text{ml}^{-1}$  pyruvate kinase, 1.5 units  $\text{ml}^{-1}$  ribose-5-phosphate isomerase, 1 mM ATP (neutralized with sodium bicarbonate), 0.6 mM phosphoenolpyruvate, and  $5\ \mu\text{l}$  desalted extract in a total volume of  $200\ \mu\text{l}$  per well. The reaction was started by addition of 2.5 mM ribose-5-phosphate. The assays were incubated at room temperature.

### SDS-PAGE and immunoblotting

Samples taken during preincubation were mixed with sample buffer and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini Protean II Dual Slab Cell (Biorad, Richmond) according to Laemmli (1970). Immunoblotting was performed basically according to Tijssen (1985) as described previously by Mitsunashi and Feller (1992). Polyclonal antibodies raised in rabbits against phosphoribulokinase (SJ Crafts-Brandner, Western Cotton Research Laboratory, USDA/ARS, Phoenix), ferredoxin-dependent glutamate synthase (RM Wallsgrove, IACR-Rothamsted) and the plastidial form of glutamine synthetase (G Ochs and A Wild, Johannes-Gutenberg-Universität, Mainz) were used to identify the enzymes under investigation. The blots were then treated with goat-anti-rabbit-IgG for bridging and peroxidase-anti-peroxidase soluble complex as described previously (Mitsunashi and Feller, 1992). The peroxidase activity was visualized with 4-chloro-1-naphthol as substrate. The appropriate enzyme proteins were recognized specifically by the antibodies used (Fig. 1).

## Results

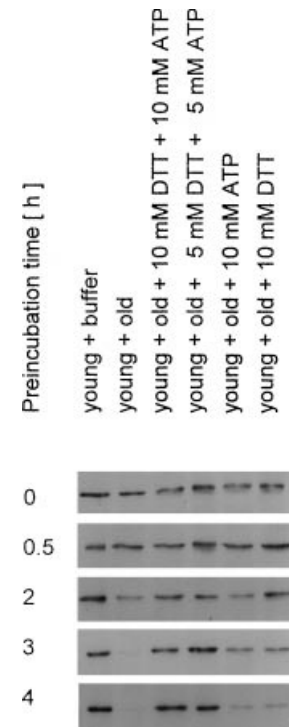
Rapid inactivation of phosphoribulokinase in leaf extracts from 10–14-d-old wheat plants was initiated by the addition of extract from senescing wheat leaves (Table 1). The activity of phosphoribulokinase in extract of young wheat leaves in the absence of extract from senescing leaves declined more slowly (Fig. 1). DTT and ATP caused no major effects on this slow inactivation (data not shown). DTT, an artificial reductant replacing the natural activator thioredoxin, protected this enzyme in the presence of extract from senescing leaves. The substrate ATP also delayed phosphoribulokinase inactivation under these conditions. The protective effects mentioned above were observed consistently in two similar experiments, although the inactivation rates differed considerably (Table 1, experiments 1 and 2). DTT and ATP in combination stabilized the activity far better than either effector alone. The decline in enzyme activity may be due to modifications of the enzyme protein or to its degradation.



**Fig. 1.** Specificity of the antibodies. Extract of young wheat leaves (lanes 1, 2, 4), partially purified phosphoribulokinase (lane 3) and prestained markers (M) were loaded on 12% gels. After electrophoresis the proteins were transferred to nitrocellulose and further processed according to the standard protocol for immunoblotting. The membrane was cut in the marker lanes, decorated with primary antibodies against phosphoribulokinase (lanes 1, 3) or glutamine synthetase (lane 2) and arranged after colour development in the original sequence for the photograph. Lane 4 was treated initially with antibodies against ferredoxin-dependent glutamate synthase (GOGAT), then with those against plastidial glutamine synthetase (GS) and finally with those against phosphoribulokinase (PRK). Only one band at the appropriate position was detected after decorating immunoblots with primary antibodies against GOGAT (data not shown). The increased background in lane 4 was due to the repeated probing with the various antibodies.

Immunological studies allowed us to distinguish between these two possibilities (Fig. 2). The band intensity for the intact subunit of phosphoribulokinase on immunoblots (Fig. 2) reflected the activity measurements (Table 1). The enzyme protein was protected by DTT, ATP and, most efficiently, by a combination of the two solutes. It should be noted that a combination of 5 mM each or 10 mM each protected the enzyme in a similar manner. The immunoblots indicated that the enzyme activity and the enzyme were lost simultaneously and suggested that the inactivation was caused by proteolytic attack.

Solutes may affect proteolysis in a general manner by interacting with peptide hydrolases or, more specifically,



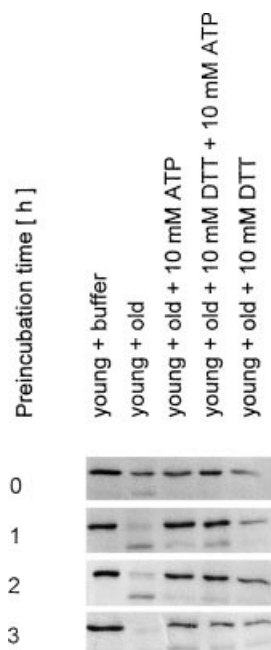
**Fig. 2.** Protection of phosphoribulokinase in extracts from wheat leaves by ATP and DTT. Extract from young wheat leaves (young) was mixed 1:1 with either buffer or extract from senescing wheat leaves (old). Samples were collected throughout the preincubation period of 4 h and analysed by SDS-PAGE and immunoblotting.

by interacting with substrate proteins and altering their susceptibility. Glutamine synthetase (Fig. 3) also depends on ATP and the observed protection by this ligand (as judged by the loss of the intact subunit and the transient accumulation of a fragment) is consistent with previously reported results (Fröhlich *et al.*, 1994). DTT added simultaneously did not increase the protection caused by ATP further. Glutamate synthase was not protected by

**Table 1.** Protection of phosphoribulokinase activity in wheat leaf extracts by ATP and DTT

Extract from young leaves (young) was mixed 1:1 with extract from senescing leaves (old). Extract from young leaves was mixed 1:1 with buffer for the control. ATP and DTT were added to a final concentration of 10 mM when indicated. Means  $\pm$  SD of four separate preincubations are shown.

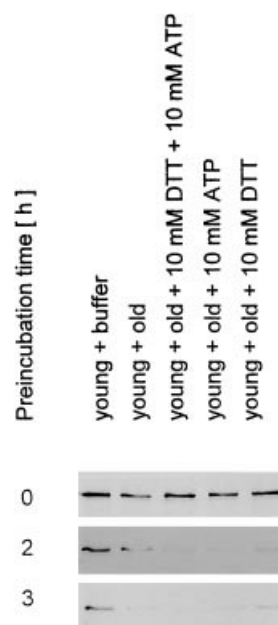
Time (h)	Remaining phosphoribulokinase activity (nkat ml <sup>-1</sup> )				
	Young (control)	Young + old	Young + old + ATP	Young + old + DTT	Young + old + ATP + DTT
Experiment 1 (plant age: 10 d)					
0	406 $\pm$ 22	376 $\pm$ 6	460 $\pm$ 60	382 $\pm$ 18	454 $\pm$ 24
0.5	385 $\pm$ 13	320 $\pm$ 3	430 $\pm$ 98	299 $\pm$ 76	357 $\pm$ 56
2	270 $\pm$ 9	152 $\pm$ 6	302 $\pm$ 15	308 $\pm$ 14	350 $\pm$ 8
3	228 $\pm$ 12	110 $\pm$ 4	301 $\pm$ 8	304 $\pm$ 9	460 $\pm$ 18
4	214 $\pm$ 15	112 $\pm$ 16	278 $\pm$ 48	288 $\pm$ 98	360 $\pm$ 99
Experiment 2 (plant age: 13 d)					
0	232 $\pm$ 14	194 $\pm$ 9	244 $\pm$ 15	270 $\pm$ 8	302 $\pm$ 14
0.5	200 $\pm$ 9	178 $\pm$ 5	246 $\pm$ 12	234 $\pm$ 16	286 $\pm$ 16
2	176 $\pm$ 2	124 $\pm$ 2	218 $\pm$ 4	193 $\pm$ 14	264 $\pm$ 8
3	154 $\pm$ 4	74 $\pm$ 5	199 $\pm$ 6	193 $\pm$ 9	258 $\pm$ 18
4	132 $\pm$ 4	42 $\pm$ 4	138 $\pm$ 4	148 $\pm$ 8	259 $\pm$ 9



**Fig. 3.** Protection of glutamine synthetase in extracts from wheat leaves by ATP and DTT. Extract from young wheat leaves (young) was mixed 1:1 with either buffer or extract from senescing wheat leaves (old). Samples were collected throughout the preincubation period of 3 h and analysed by SDS-PAGE and immunoblotting. A fragment of glutamine synthetase (below the intact subunit) became visible during incubation in the presence of extract from senescing wheat leaves.

ATP or DTT (Fig. 4), indicating that the stabilization by the solutes was enzyme specific and not a general effect on proteolysis. These results suggest that the protective effect of DTT on phosphoribulokinase was due to the reduction of the activity-regulating disulphide bond.

Experiments were also performed with partially purified phosphoribulokinase from spinach to avoid interactions with other proteins present in crude extracts. Two exogenous endopeptidases of the serine type (chymotrypsin and trypsin) caused a rapid decrease in phosphoribulokinase activity (Tables 2, 3). Lower values at the beginning of the experiment (0 h) in the presence of the bovine endopeptidases can be explained by proteolysis during the few minutes between the preparation of the tubes and the initial sampling. Partially purified phosphoribulokinase was stabilized in the presence of high endopeptidase activity by DTT or ATP. DTT was a better protectant than ATP, but the highest stability was observed when a combination of the two effectors was added. Similar results were obtained with trypsin (Table 2) and chymotrypsin (Table 3), although these endopeptidases differ in their substrate specificities. Activity measurements were confirmed by immunoblots in a separate experiment (Fig. 5). From the results mentioned above, it became evident that DTT and ATP protected phosphoribulokinase from proteolysis regardless of the properties of the endopeptidases added. These findings suggest that the



**Fig. 4.** Protection of glutamate synthase in extracts from wheat leaves by ATP and DTT. Extract from young wheat leaves (young) was mixed 1:1 with either buffer or extract from senescing wheat leaves (old). Samples were collected throughout the preincubation period of 3 h and analysed by SDS-PAGE and immunoblotting.

altered susceptibility of phosphoribulokinase to proteolysis in the presence of the reductant DTT and the substrate ATP was due to changes in its three-dimensional structure.

## Discussion

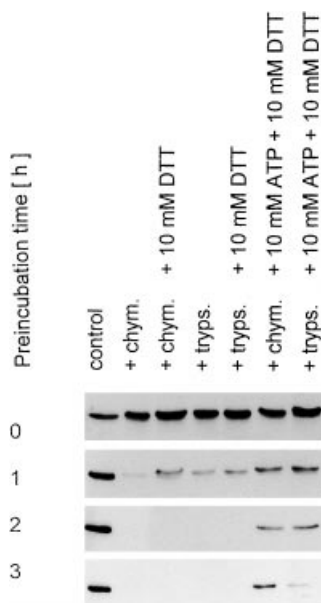
The inactivation of phosphoribulokinase in extracts from young wheat leaves was accelerated by the addition of extracts from senescing wheat leaves. Partially purified phosphoribulokinase from spinach leaves was rapidly inactivated after the addition of purified endopeptidases. The decrease of the intact phosphoribulokinase subunit always correlated well with the loss of enzyme activity. These results suggest that the irreversible phosphoribulokinase inactivation was caused by proteolysis in this system. The artificial reductant DTT protected this enzyme *in vitro* as judged by the enzyme activity and the subunit abundance. The reduction of the disulphide bond in the oxidized enzyme (present in darkness, inactive) probably decreased the susceptibility to proteolytic attack. *In vivo*, the thioredoxin-dependent activation may also influence the stability of the enzyme protein. On the other hand, no such protection by DTT *in vitro* was observed for the thioredoxin-independent enzymes glutamine synthetase and glutamate synthase. Since phosphoribulokinase was protected by DTT in the presence of various endopeptidases and other enzymes were not protected by DTT, it appears likely that the effect was due to a

**Table 2.** Protection of partially purified phosphoribulokinase from spinach by 10 mM ATP and 10 mM DTT in the presence of 0.01% trypsin: means  $\pm$  SD of four replicates are shown

Time (h)	Remaining phosphoribulokinase activity (nkat ml <sup>-1</sup> )				
	Control (– trypsin)	+ Trypsin	+ Trypsin + ATP	+ Trypsin + DTT	+ Trypsin + ATP + DTT
0	769 $\pm$ 61	301 $\pm$ 16	378 $\pm$ 18	576 $\pm$ 45	591 $\pm$ 6
0.25	720 $\pm$ 51	117 $\pm$ 23	253 $\pm$ 19	384 $\pm$ 24	637 $\pm$ 12
0.5	547 $\pm$ 58	62 $\pm$ 34	211 $\pm$ 42	224 $\pm$ 23	525 $\pm$ 44
1	250 $\pm$ 32	53 $\pm$ 19	205 $\pm$ 24	136 $\pm$ 19	397 $\pm$ 11
2	218 $\pm$ 21	34 $\pm$ 32	203 $\pm$ 23	123 $\pm$ 12	291 $\pm$ 99

**Table 3.** Protection of partially purified phosphoribulokinase from spinach by 10 mM ATP and 10 mM DTT in the presence of 0.01% chymotrypsin (chymotr.): means  $\pm$  SD of four replicates are shown

Time (h)	Remaining phosphoribulokinase activity (nkat ml <sup>-1</sup> )				
	Control (– chymotrypsin)	+ Chymotrypsin	+ Chymotrypsin + ATP	+ Chymotrypsin + DTT	+ Chymotrypsin + ATP + DTT
0	769 $\pm$ 61	380 $\pm$ 19	332 $\pm$ 20	776 $\pm$ 25	721 $\pm$ 38
0.25	720 $\pm$ 51	103 $\pm$ 15	346 $\pm$ 14	664 $\pm$ 42	999 $\pm$ 46
0.5	547 $\pm$ 58	78 $\pm$ 10	178 $\pm$ 48	387 $\pm$ 16	921 $\pm$ 99
1	250 $\pm$ 32	52 $\pm$ 12	163 $\pm$ 61	201 $\pm$ 32	848 $\pm$ 36
2	218 $\pm$ 21	40 $\pm$ 34	132 $\pm$ 21	180 $\pm$ 12	721 $\pm$ 97

**Fig. 5.** Protection of partially purified phosphoribulokinase from spinach by ATP and DTT in the presence of 0.01% trypsin (tryps.) or 0.01% chymotrypsin (chym.). Samples were collected throughout the preincubation period of 3 h and analysed by SDS-PAGE and immunoblotting.

conformational change of phosphoribulokinase caused by its reduction.

Fragments were detected on immunoblots of partially purified phosphoribulokinase incubated for several hours in the presence of low concentrations of trypsin or chymotrypsin (data not shown). Therefore partially degraded phosphoribulokinase was still recognized by the

antibodies used. In contrast, no fragments of phosphoribulokinase were observed on immunoblots after the incubation of a mixture of extracts from young and old wheat leaves. These facts suggest that the first cleavage was rate-limiting in wheat leaf extracts and that the fragments produced were rapidly degraded later to free amino acids or small peptides which are no longer detectable on a blot. Several endo- and exopeptidases are present in wheat leaf extracts and may contribute to the complete hydrolysis of proteins (Barrett, 1994; Dalling, 1986). This is consistent with the finding that enzyme fragments do not, in general, accumulate in intact cells. In contrast, typical fragments (depending on the endopeptidase added (trypsin or chymotrypsin) were visible on immunoblots of purified phosphoribulokinase incubated with purified endopeptidases (data not shown). Under these conditions fragments may not be rapidly degraded and may thus accumulate.

ATP, one of the substrates for phosphoribulokinase protected the enzyme *in vitro*. The interaction with ligands may stabilize a protein in the presence of endopeptidases by modifying its shape and, as a consequence, its accessibility. Protection by ligands has been observed previously for a series of other enzymes (Fischer *et al.*, 1992; Streit and Feller, 1982). Very effective protection of phosphoribulokinase was observed when both DTT and ATP were added to the incubation medium. Since phosphoribulokinase was stabilized by DTT and ATP in the presence of various peptide hydrolases (proteolytic activities from senescing wheat leaves, trypsin or chymotrypsin), this protective effect was a general phenomenon and not

restricted to a particular endopeptidase. It must be considered that such interactions may also be relevant for the regulation of phosphoribulokinase degradation in intact chloroplasts by proteolytic activities present in these organelles. These results suggest that, *in vivo*, the activation status and the availability of substrates may influence the degradation of phosphoribulokinase. The thioredoxin-mediated reduction of this enzyme is not only relevant for its activity but may also affect its susceptibility to proteolysis.

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