A Relationship between Carbonic Anhydrase and Rubisco in Response to Moderate Cadmium Stress during Light Activation of Photosynthesis

Anna Siedlecka[a,*], Per Gardeström[b], Göran Samuelsson[b], Leszek A. Kleczkowski[b] and Zbigniew Krupa[a]

[a] Department of Plant Physiology, Maria Curie-Skłodowska University, Akademicka 19, 20–033 Lublin, Poland. Fax: (+48-81) 5375102. E-mail: asiedlec@biotop.umcs.lublin.pl
[b] Department of Plant Physiology, Umeå University, S-90187 Umeå, Sweden

* Author for correspondence and reprint requests

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In our previous research, we showed that low Cd concentration increases the effectiveness of the processes leading to activation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). This stimulation was dependent on carbonic anhydrase (CA) activity and resulted in protecting Rubisco activity against Cd toxicity. The aim of the present paper was to test whether this mechanism has any influence on light activation of photosynthesis during the first 2 h of illumination. Both the "activation mechanism" of plant response to Cd-stress conditions and its full efficiency at low Cd concentration were confirmed. The CA-dependent light activation of Rubisco at low Cd level was correlated with accelerated attaining of the maximum Rubisco activity by these plants. The amount of Rubisco was also Cd- and time-dependent and varied from continuous accumulation in control plants till reaching the maximum level within 30 minutes for the high Cd concentration. An increase in CA activity that was found to be parallel to the decrease of the amount of CA suggested activation of the enzyme by low Cd concentration.

Introduction

The Calvin cycle plays a very important role in the environmental stress susceptibility and in the stress adaptation of higher plants. Weigel (1985a and b) claimed this cycle to be the main target of cadmium toxicity. The "effect of multiplication", defined by Krupa and Baszyński (1995), defines heavy metal toxicity as the result of a number of direct and indirect effects – from functional to structural disorders. Individual changes can be small, but when they reach the photosynthetic apparatus they cause severe disturbances in photosynthesis, even at low heavy metal content in chloroplasts. It seems to be evident that plant response to heavy metal stress includes various adaptation mechanisms (Krupa and Baszyński, 1995). These mechanisms are expected to differ in their efficiency, depending on the individual heavy metal and its concentration, as well as on plant tolerance.

The undisturbed functioning of Rubisco, the carboxylating enzyme in C_3 plants, is regulated by a sophisticated activation system, even under optimal physiological conditions (Portis, 1990, 1992; Sültemeyer *et al.,* 1993; Salvucci and Ogren, 1996). A specific enzyme – Rubisco activase, as well as a set of chloroplast constituents such as: RuBP level, Mg^{2+} ions, ATP/ADP ratio, concentration of some carbohydrates, natural nocturnal inhibitor (CA1-P), the CO_2 concentration and CO_2/O_2 ratio belong to the most important factors affecting Rubisco carboxylating activity. Availability of CO_2, which is an activator of the enzyme, as well as its substrate and one of the main limiting factors for photosynthesis, must play an important role in plant adaptation to stress conditions. Moreover, mechanisms of Rubisco activity regulation upon heavy metal stress may differ from those operating under optimal physiological conditions. CA is a good example of these differences. This Zn-containing enzyme, comprising as much as 1–2% of the total leaf protein

Abbreviations: CA, carbonic anhydrase (EC 4.2.1.1); CA1P, 2-carboxyarabinitol 1-phosphate; DTT, dithiothreitol; ME, β-mecaptoethanol; PPFD, photosynthetic photon flux density; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.11.39); RuBP, ribulose-1,5-bisphosphate.

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content (the "third place" after Rubisco – 60% and Rubisco activase – 5%) and responsible for $HCO_3^- \leftrightarrow CO_2$ conversion with a very high maximum turnover rate of 10^6 had been, even a few years ago, considered almost as an "evolutionary ballast" in C_3 plant chloroplasts (Johanson and Forsman, 1992, 1993; Price *et al.*, 1994). In experiments with transgenic tobacco plants Price *et al.* (1994) showed that transformants with a CA level as low as 2% of the wild type maintained 95.6% assimilation rate. From that moment, CA was thought not to play any important role in C_3 photosynthesis. On the other hand, synthesis of 2% of the total protein only as a "ballast" seems to be an effort for cell metabolism which is too important to be maintained during evolution. Some elucidation came from stress physiology experiments: an increase in CA activity was observed for cyanobacteria and marine algae in the presence of Cd in their growth environment (Price and Morel, 1990; Pawlik *et al.*, 1993; Morel *et al.*, 1994). Moreover, for the latter organisms it was documented that Zn substitution by Cd was the reason of growth promotion for Zn-deficient organisms (Price and Morel, 1990; Morel *et al.*, 1994). An increased CA activity was reported for C_3 plant chloroplasts during adaptation to high light and drought (Fridlyand, 1995; Williams *et al.*, 1996). For bean plants treated with Cd, an interesting phenomenon was observed: Rubisco activation mechanisms responded to Cd treatment by increasing ATP level, ATP/ADP ratio and the enzyme activation state, but the activity of Rubisco itself was decreased (Siedlecka *et al.*, 1997). Only at a low Cd concentration, full reactivation took place, and it was strictly correlated with increase in CA activity (Siedlecka *et al.*, 1997). This "activation response" seemed to be at least as complicated as "multiplying effect" of heavy metals toxicity to photosynthesis (Krupa and Baszyński, 1995). The aim of the present paper was to elucidate the CA effects on the light activation of Rubisco as an element of the "activation response" of plant adaptation to Cd toxicity.

Materials and Methods

Etiolated, 7-days-old seedlings of bush bean (*Phaseolus vulgaris* L. cv. Słowianka) were grown for 6 days on full, modified Hoagland nutrient solution containing 0.25 mM Fe in the form of ferric citrate (Siedlecka and Krupa, 1996). The day/night regime was 16/8h and 22/18 °C with PPFD of 150 μmol $m^{-2}s^{-1}$. Plants were then transferred into fresh nutrient solution containing 0, 10 or 50 μM Cd^{2+} ions (in form of cadmium sulfate). On the 7^{th} day of growth under the conditions described above, primary leaves from 3 independent series of plants were collected for analyses.

Whole primary leaves were taken with regard to the onset of illumination in the climate chamber: in darkness (5 min before light was turned on) and 2, 5, 30, 60 and 120 min after turning the light on in the climate chamber. The leaves were immediately frozen in liquid nitrogen and stored in a Dewar pot till the next step of preparation. Leaves were ground to a very fine powder in liquid nitrogen. The powder was packed into frozen Eppendorf tubes and kept in liquid nitrogen (for immediate measurements of Rubisco activity and homogenization in extraction buffer for protein SDS-PAGE) or stored at −80 °C (for CA activity measurements). This procedure has allowed us to obtain homogeneous material for all subsequent analyses.

Protein content measurements were carried out using the Bio-Rad Protein Assay, according to the manufacturer's instruction.

Rubisco activity was measured according to Hurry *et al.* (1995) and expressed as micromoles of RuBP converted to PGA per mg of total soluble leaf protein. The data shown in the present paper as "Rubisco activity" refer to the enzyme activity measured as the so-called initial activity. Total activity of Rubisco was measured after 5 min incubation in activating medium, according to Hurry *et al.* (1995). Rubisco activation state – the% of protein active *in vivo* – was calculated as initial activity expressed in % of total activity of the enzyme (Hurry *et al.* 1995).

CA activity was measured following the electrochemical method of Karlsson *et al.* (1995). Both protocols were described in detail by Siedlecka *et al.* (1997).

Western-blot: leaf powder stored in liquid nitrogen was ground on ice with extraction buffer (0.2 M Tris-HCl [tris(hydroxymethyl)aminomethane], pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT) and centrifuged for 20 s. From the supernatant a suitable volume was taken for protein assay, then 250 μl of supernatant was mixed 1:1 (v/v) with

electrophoretical sample loading buffer (4% w/v SDS, 4% v/v ME, 20% v/v glycerol, 1% w/v bromophenol blue) and stored at −20 °C until used (after Kleczkowski *et al.*, 1993, modified). SDS-PAGE (12% acrylamide) gels were run according to Laemmli (1970). Western-blot was run as described before (Towbin *et al.*, 1979; Salinovich and Montelaro, 1986) but modified as in Siedlecka *et al.* (1997). Antiserum against pea chloroplastic CA and antibodies against spinach native Rubisco were used on separate membrane transfers, followed by Amersham horseradish peroxidase linked secondary antibodies and ECL (Amersham) detection of antibody-antigen conjugation (Larsson *et al.*, 1997, modified).

Results and Discussion

The early steps in Rubisco activity development have been measured so far mainly by isotope methods. The enzyme measurements at this stage are thought to be difficult and not reliable due to rapid changes in stroma pH and redox state as well as due to "jumping" release and re-bounding of CA1P. Therefore, activity measurements are usually performed 30 min after the exposure of plants to light (Sage *et al.*, 1993). In our experiments, this initial phase was finished within the first 5 min of measurements, and at least from that time-point a clear picture could be observed not only for Rubisco but also for CA activity (Fig. 1a and b, Fig. 3).

From the fifth minute after onset of the light the initial activity of Rubisco in control and 10 μM Cd-treated plants showed the same scheme of increase and the enzyme activity in low Cd-treated plants was maintained at the control level. At 50 μM Cd Rubisco activity was markedly decreased in comparison with control (Fig. 1a). The only difference between control and 10 μM Cd-treated plants was the time required for reaching the maximum rate of Rubisco activity: for control plants, the activity showed constant increase, while for low Cd-treated ones it reached its maximum after 60 min of light exposure (Fig. 1a). For the high Cd stress conditions, Rubisco activity was low and it is therefore irrelevant to discuss its light-activation during the experiment (Fig. 1a). The changes in Rubisco content showed similar pattern as its activity. For control plants, a continuous accumulation of Rubisco was observed (Fig. 2a, lanes 1–4).

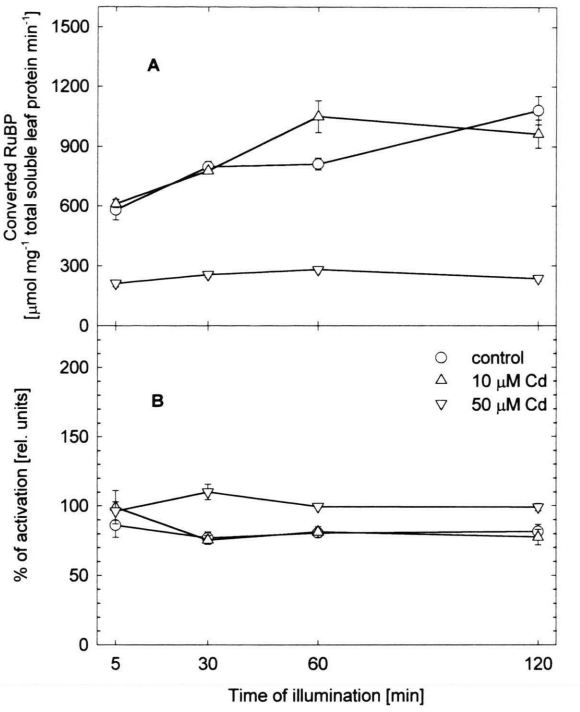

Fig. 1. Rubisco activity (A) and Rubisco activation state – the% of enzyme protein active *in vivo* – (B) in control plants and upon Cd-stress conditions. Results represent mean values of three replicates ± SE.

At 10 μM Cd, the maximum was reached after 60 min exposure, but in general the amount of the enzyme was lower than in control (Fig. 2a, lanes 5–8). For 50 μM Cd, the enzyme content was also lower than in control and remained stable after reaching its maximum after half an hour of light exposure (Fig. 2a, lanes 9–12). The increase in the enzyme activation state (Fig. 1b) at the high Cd concentration confirmed the maximum effort of the "activation response" to overcome the Rubisco activity depletion. At the low Cd concentration, the Rubisco activation state was the same as for control plants, so these plants still have some unused capacity for stress adaptation (Fig. 1b). These differences in plant response to various Cd concentrations, resulting in maintaining Rubisco activity at control (low Cd) or highest possible level (high Cd), confirmed the importance and effectiveness of the plant "activation response" to Cd-stress.

An important aspect to be addressed in the present paper was the expected role of CA in the acti-

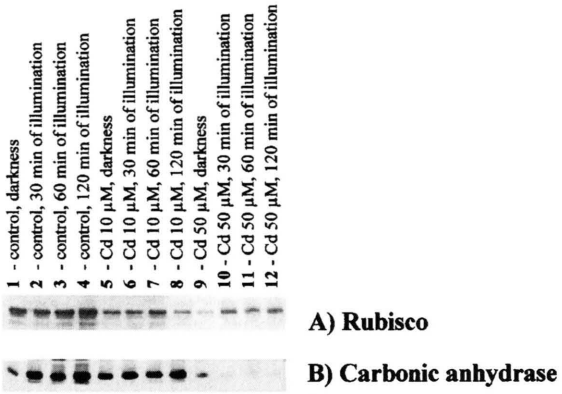

Fig. 2. Western-blot with Rubisco (A) and carbonic anhydrase (B) antibodies (see Methods for details).
Lanes: 1 – control, darkness; 2 – control, 30 min of illumination; 3 – control, 60 min of illumination; 4 – control, 120 min of illumination; 5–10 μM Cd, darkness; 6–10 μM Cd, 30 min of illumination; 7–10 μM Cd, 60 min of illumination; 8–10 μM Cd, 120 min of illumination; 9–50 μM Cd, darkness; 10–50 μM Cd, 30 min of illumination; 11–50 μM Cd, 60 min of illumination; 12–50 μM Cd, 120 min of illumination.

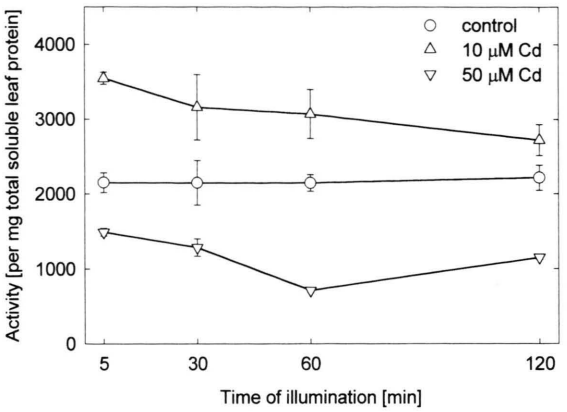

Fig. 3. Activity of carbonic anhydrase in control and Cd-treated plants. Results represent mean values of three replicates ± SE.

vation. For control plants, CA activity is light-independent, with only a slight increase during the experiment, while the amount of CA protein increases (Fig. 2b, lanes 1–4 and Fig. 3). There is an excess of CA in the chloroplasts (Johanson and Forsman, 1992, 1993). The observed changes can be explained as maintaining the necessary activity of the enzyme with its continuous synthesis during the first 2 h of the day rhythm. At high Cd concentration, both CA activity and its amount in chloroplasts markedly decreased (Fig. 2b, lanes 9–12 and Fig. 3). The treatment inhibited the CA-dependent mechanism of Rubisco activation (Fig. 1a and b, Siedlecka *et al.* 1997). At the low Cd concentration, the CA activity increased, despite a slight decrease in its protein amount in comparison with values obtained for control plants (Fig. 2b, lanes 5–8 and Fig. 3). Moreover, at this Cd concentration CA activity was highest at the beginning of lightening, during development of Rubisco activity (Figs. 1a and 3). All these correlations suggested a Cd-stimulated increase in CA activity, resulting in a very efficient mechanism of Rubisco activation and maintaining of full activity of this main enzyme of the Calvin cycle. The increase in CA activity in parallel with the CA protein content decrease suggested activation of CA in the presence of Cd. A possible explanation is the Zn/Cd replacement described before by Price and Morel (1990) and Morel *et al.* (1994).

The conclusion from the presented results is the operation of different mechanisms of plant adaptation to Cd-stress conditions, depending on the Cd concentration. The presence of Cd resulted in faster stabilization of Rubisco activity at the maximal level: continuous increase for control plants, 60th minute at low Cd and 30th minute at high Cd concentration (Fig. 1a). At the high Cd-stress, plant adaptation mechanisms yielded an increased Rubisco activation state, but this strategy was insufficient and Rubisco activity remained decreased. Moreover, no light-activation of the enzyme occurred at the high Cd stress. At the low Cd concentration plants fully adapted to stress by operation of the "activation response", based on the Cd-stimulated increase in the CA activity. Light-activation of Rubisco at low Cd-treated plants was fully effective and prompted to compare with control.

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