THE TEMPERATURE-SENSITIVITY OF DARK-INACTIVATION AND LIGHT-ACTIVATION OF THE RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE IN SPINACH CHLOROPLASTS

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1. Introduction

The isolated RuBP-Case (EC 4.1.1.39) can be activated by CO_2 and Mg^{2+} at pH > 7.0 [1]. Oxygen is another important factor in the activation/inactivation process [2]. The actual rate of the activated enzyme is modulated by a number of stromal metabolites [3]. In isolated chloroplasts the RuBP-Case was found to be in a relative inactive state in the dark and was activated by illumination [4,5]. The light-dependent increase in the stromal pH and $[Mg^{2+}]$ may play a crucial role in light activation. However, in [6] a complete lack of dark inactivation of the enzyme in isolated protoplasts was observed; they assumed that the dark inactivation observed with isolated chloroplasts was an artefact due to the isolation procedure.

Part of this discrepancy can be explained by the result shown here that the activation/inactivation of the RuBP-Case in isolated chloroplasts is strictly temperature-dependent: around 5°C the enzyme is in an activated state, even in the dark; around 25°C the enzyme is partly inactivated in the dark but can be fully reactivated by saturating light conditions. Pretreatment at >25°C does not cause further inhibition of the dark-adapted enzyme but it inhibits the reactivation by light. This inhibition is accompanied by a decrease in the overall photosynthetic CO_2-fixation. The results give rise to the suggestion that high temperatures interfere with a stromal or membrane-bound factor involved in the activation/inactivation process rather than with the enzyme protein itself.

Abbreviations: chl, chlorophyll; FBPase, fructose-1,6-bisphosphatase; GAPDH, glyceraldehydephosphate dehydrogenase; MDH, malate dehydrogenase; PRK, ribose-5-phosphate kinase; RuBP, ribulose-1,5-bisphosphate; RuBP-Case, ribulose-1,5-bisphosphate carboxylase

I conclude that the light activation of the RuBP-Case is one of the most heat-sensitive functions of the photosynthetic apparatus.

2. Materials and methods

Intact chloroplasts were isolated from spinach (*Spinacia oleracea* L.) as in [7]. The chloroplasts (100 μg chl/ml) were preincubated for 12 min at different temperatures in 0.25 ml of a medium containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.5 mM P_i, 40 mM Hepes–KOH (pH 7.5), 10 mM NaCl and air-levels of CO_2 and O_2. After preincubation the chloroplast suspension was diluted with 0.75 ml of the same medium reaching the measuring temperature (20–25°C) within 30 s and was then incubated in presence of 2 mM HCO_3^- and catalase for 5 min in the dark or in the light (RG 630 filter, Schott; 400 W/m^2). During illumination the photosynthetic O_2-evolution was recorded with a Clark-type oxygen electrode. After 5 min 100 μl of the suspension were rapidly transferred to 0.9 ml a medium containing 30 mM bicine–NaOH (pH 7.8), 2 mM $H^{14}CO_3^-$ (37 GBq/mol) and 0.4 mM RuBP. Samples (100 μl) were withdrawn every 30 s, mixed with HCl, dried and measured for radioactivity.

Light activation of the FBPase, PRK, GAPDH and NADP-dependent MDH has been measured as in [8].

3. Results

Fig.1(a–c) shows the activity of the RuBP-Case immediately following the osmotic rupture of the chloroplasts. In the absence of RuBP the carboxyla-

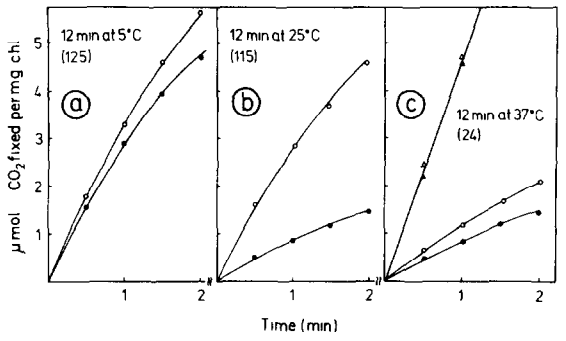

Fig.1. RuBP-Case activity from chloroplasts which were preincubated at different temperatures in the dark (•) or in the light (○) measured immediately after chloroplast rupture. To obtain full activation of the RuBP-Case the predarkened (▲) or preilluminated (△) chloroplasts were lysed and then incubated for 10 min in a medium containing 60 mM bicine–NaOH (pH 8.0), 4 mM HCO_3^- and 20 mM $MgCl_2$ before measuring. The numbers in brackets indicate the photosynthetic CO_2-fixation of the intact chloroplasts in µmol . mg chl^{-1} . h^{-1}. All measurements were done at 20°C.

tion rate was <3% of the standard test. The fixation of CO_2 was almost linear for 60 s and then gradually declined as in [4]. The relative linearity of the RuBP-dependent activity after chloroplast rupture indicates that the enzyme did not undergo significant change during the assay. Thus, in the following experiments the initial activity of the RuBP-Case was used to indicate the relative degree of activation of the stromal enzyme before lysis. The figure shows that the degree of activation depends on both preillumination and preincubation temperature. In chloroplasts stored at 0–5°C the RuBP-Case was always in an active state; 5 min illumination did not increase the activity significantly (fig.1a). Preincubation at 25°C in the dark at air level of CO_2 caused a strong decrease in enzyme activation (fig.1b). The time necessary for inactivation (10–15 min at 25°C) decreased with increasing temperatures (e.g., 3 min at 38°C). Illumination caused reactivation to the high-activity state (light activation).

In [6] a complete lack of dark inactivation of the enzyme in isolated protoplasts of wheat or barley was observed. However, under appropriate conditions such as air levels of CO_2 and extended dark-incubation at higher temperatures (20–30 min at 25°C) one can also show dark-inactivation and subsequent light activation of the enzyme from isolated spinach protoplasts, similar to that shown in fig.1b (not shown).

The photosynthetic CO_2-fixation was not or only little affected by preincubation at 25°C (see values in fig.1(a–b)). Preincubation at >25°C did not cause further inactivation of the dark-adapted enzyme which was almost insensitive to preincubation temperatures between 25°C and 45°C. Reactivation by light, however, was inhibited at >25°C, accompanied by an inhibition of the photosynthetic CO_2-fixation (fig.1c), i.e., only the light-dependent enzyme activity was sensitive to mild heating (30–40°C). As shown in fig.2 the decrease of the photosynthetic CO_2-fixation is clearly related to the light-dependent RuBP-Case activity. In [9] it has been demonstrated with patterns of photosynthetic metabolites that, indeed, a blockage of the carboxylation reaction very probably is supposed to be the primary cause for the inhibition of photosynthesis by mild heating. Photosynthetic electron transport from H_2O to NADP as well as photophosphorylation were not inhibited.

A RuBP-dependent oxygenase activity of illuminated chloroplasts in the order of 6–10 µmol O_2 . mg chl^{-1} . h^{-1}, measured with an oxygen electrode immediately following osmotic rupture of chloroplasts, was also inhibited by a mild heat treatment. In most cases the degree of inhibition was similar to that observed with the RuBP-Case activity; in some cases, however, the inhibition of the oxygenase was somewhat smaller than that of the carboxylase activity (not shown).

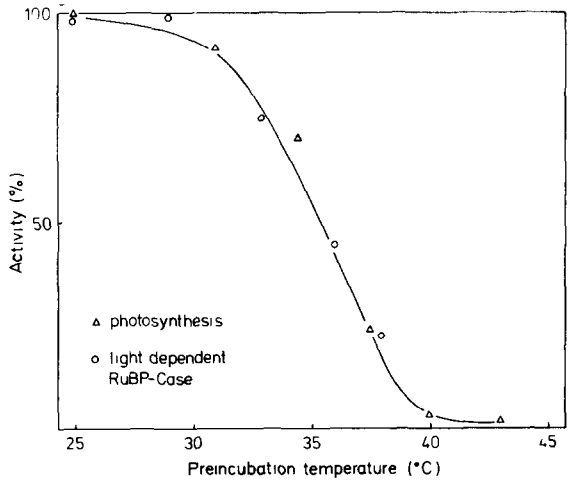

Fig.2. Photosynthetic CO_2-fixation and light-dependent RuBP-Case activity of isolated chloroplasts as a function of the preincubation temperature. Light-dependent activity of the RuBP-Case: total activity of the enzyme from light-adapted chloroplasts minus activity from dark-adapted chloroplasts.

Table 1
The activities (μmol . mg chl^{-1} . h^{-1}) of photosynthetic CO_2-fixation and light activation of some stromal enzymes after preincubation of the intact chloroplasts for 12 min at 20°C and 38°C in the dark and in the light (red light, 200 W/m²)

	20°C		38°C	
	Dark	Light	Dark	Light
CO_2-fixation	–	120	–	30
RuBP-Case	60	185	45	60
GAPDH	61	150	100	230
FBPase	10	115	12	96
PRK	131	362	128	350
NADPH–MDH	2	23	2	25

The ferredoxin-mediated reductive light-activation of the FBPase, PRK, GAPDH and NADP-dependent MDH [10] remained unaffected by mild heating (table 1). The same is valid for the light inactivation of the stromal glucose-6-phosphate dehydrogenase (not shown).

The maximum rate of photosynthetic CO_2-fixation was decreased by preincubation at 35°C without affecting the apparent affinity to HCO_3^- (fig.3). The

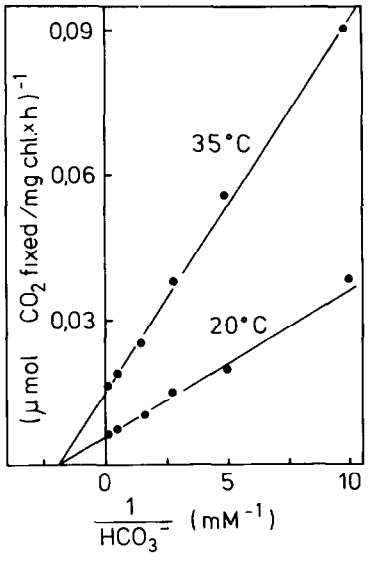

Fig.3. Photosynthetic CO_2-fixation of intact chloroplasts pretreated at 20°C and 35°C as a function of [HCO_3^-] (Lineweaver-Burk plot). The measuring medium (section 2) contained 20 mM Na-acetate to obtain pH-equilibration between stroma and medium [11]. CO_2-free measuring medium was prepared by evacuating the solution at pH 4.0 before adjusting the pH to 8.0 with freshly prepared KOH.

apparent K_m for HCO_3^- at pH 8.0 is 0.56 mM (K_m for CO_2 = 10 μM) and may reflect the affinity of the stromal RuBP-Case to CO_2. Therefore, it is suggested that mild heating inhibits the activation of the RuBP-Case without affecting the affinity for its substrate, CO_2.

The inhibition of photosynthetic CO_2-fixation by mild heating was partially prevented when chloroplasts were illuminated during heat-pretreatment with red light (not shown). Similar inhibition of photosynthetic CO_2-fixation by mild heating (e.g., 60% inhibition after 20 min at 32°C) has also been observed with intact leaf tissue; in this case the inhibition was completely reversible with recovery $t_{1/2} \sim 15$ min [9,12].

4. Discussion

It has been questioned whether dark inactivation of the RuBP-Case [6] has physiological significance. However, the physiological advantage of light regulation may not be derived from a complete switching-off in the dark, as is suggested for the light regulation of the FBPase and other stromal enzymes. Rather, in the case of the RuBP-Case there may be a close linkage between light and temperature regulation, which will modulate the carboxylation efficiency during short-term adaptation of plants to environmental temperature and light intensity. It seems that there are two sites of the action of temperature: low temperatures (0–25°C) affect the activation state of the dark-adapted enzyme, whereas mild heating (30–40°C) affects the mechanism of light activation. Prevention of dark inactivation by low temperatures (see fig.1a) has also been reported in [4]. The physiological significance of this result seems to be clear: at extremely low temperatures the carboxylation efficiency is high, even in the dark and consequently at very low light intensities. Dark inactivation followed by complete light activation only occurs in chloroplasts preincubated at relatively moderate temperatures (~25°C) whereas after mild heating the reactivation by light and consequently the overall photosynthetic CO_2-fixation is inhibited, before the heat-sensitive water-splitting reaction and photophosphorylation are damaged (see also [9]). The results in fig.1c confirm the well-known phenomenon that the RuBP-Case itself as well as other stromal enzymes are relatively heat stabil (e.g., [13,14]). Rather, the mild heating

interferes with the activation of RuBP-Case occurring in the light. Light-induced alkalisation of the stroma, however, suggested to be an important event in the activation of RuBP-Case [3], was not inhibited after mild heating [9]. The results in fig.3 suggest that the stromal [Mg^{2+}] was not a limiting factor after mild heating; a decrease in [Mg^{2+}] would lower the affinity of the enzyme to its substrate, CO_2. From patterns of photosynthetic intermediates [9] it seems to be unlikely that inhibition is caused by changes in the stromal levels of intermediates such as sugar phosphates.

Alternatively it is conceivable that heat-sensitive membrane-bound factors participate in the enzyme activation. For example, reactions occurring at the thylakoid membrane and forming reactive oxygen intermediates (review [15]) could interfere with the activation reaction. Rather indirect evidence for the involvement of the thylakoid membrane in heat-sensitivity of CO_2-fixation has been shown in [9,12].

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