Light-induced stimulation of carbonic anhydrase activity in pea thylakoids

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Abstract Stimulation of the bicarbonate dehydration reaction in thylakoid suspension under conditions of saturating light at pH 7.6–8.0 was discovered. This effect was inhibited by nigericin or the lipophilic carbonic anhydrase (CA) inhibitor ethoxyzolamide (EZ), but not by the hydrophilic CA inhibitor, acetazolamide. It was shown that the action of EZ is not caused by an uncoupling effect. It was concluded that thylakoid CA is the enzyme utilizing the light-generated proton gradient across the thylakoid membrane thus facilitating the production of CO_2 from HCO_3^- and that this enzyme is covered from the stroma side of thylakoids by a lipid barrier.

Key words: Thylakoid membrane; Proton transfer; Energy conversion; Bicarbonate; CO_2 concentration

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1. Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) is known to catalyze the interconversion between the neutral lipophilic CO_2 molecule and the bicarbonate ion thus facilitating the inorganic carbon penetration from the environment to the chloroplast [1]. CA activity of thylakoid membranes has been found in a number of algal cells [2–4] and higher terrestrial plants [5,6]. Discovered functional differences between CA activities of thylakoids and of well-known soluble CA in both C_4 [7] and C_3 plants [8,9] imply the existence of a specific enzyme, thylakoid CA (tCA), discernible from the soluble CA. Recently, genetic evidence for the existence of tCA localized near photosystem II (PSII) of *Chlamydomonas reinhardtii* was obtained [10]. Our recent electrophoretic studies have shown the presence of a specific protein with CA activity in pea thylakoids.

The role of tCA has still not been clarified (see [11] for review). Some connection between tCA and the energy-transforming machinery of the thylakoid is suggested by the existence of different, both inhibitory and stimulatory, effects of flash and weak light on the CA activity of thylakoids under specific conditions [6,12,13]. The possibility of using tCA of intrathylakoid protons to facilitate the production of CO_2 to be used by Rubisco was first hypothesized earlier [14] and was theoretically supported recently [15]. It was shown at the organism level that tCA of *C. reinhardtii* is necessary to maintain the proper rates of photosynthetic inorganic carbon utilization by this species [16]. While the authors discuss a possibility of use of intrathylakoid protons by this enzyme, there were no direct data proving the existence of such a process. Previously we demonstrated that tCA but not soluble CA of a higher plant possesses a specific pH dependence of the enzyme activity, which may indicate the presence of an intramolecular proton shuttle in the tCA [9]. In the present work we demonstrate the involvement of pea tCA in the consumption of protons accumulated in the thylakoid in light in the process of producing CO_2 from the bicarbonate ion.

2. Materials and methods

2.1. Plant material

Pea (*Pisum sativum* L.) plants were grown in a greenhouse at a temperature of 22/19°C (day/night) and 12/12 h light/dark regime. Fresh leaves from two upper tiers of 10–14-day-old plants were used. Experiments were performed with three different pea types ('Rannij 301', 'Rannij Gribovskij' and 'Bogatir''). The main result of the study, stimulation of the CA activity of thylakoids by light, was found with all types used.

2.2. Thylakoid isolation

Thylakoid isolation was performed as described previously [8], but the isolation medium contained 0.33 M sucrose, 35 mM K_2HPO_4, 15 mM NaH_2PO_4, 2 mM Na-EDTA, 3 mM $MgSO_4$, 10 mM KCl, 20 mM sodium ascorbate and 10 mM potassium bicarbonate. The suspension medium differed from the isolation medium by 100 mM sucrose, 0.5 mM Na-EDTA, 2 mM ascorbate and 1 mM bicarbonate. All procedure steps were performed at near-freezing temperature, necessary for the preservation of the tCA activity [8]. This was ensured by the presence of an ice slush in all media used.

2.3. Carbonic anhydrase activity

Carbonic anhydrase activity measurements were performed by monitoring the pH change after the addition of sodium bicarbonate to the sample at 19°C, using a fast-response glass electrode. The experimental device was described earlier [8], but instead of a line recorder, the 'L-154' ('Lcard', Russia) A/D converter with a PC was used for data recording. The sensitivity and time resolution of the equipment was adequate for competent measurement of bicarbonate dehydration kinetics even at high pH in the medium. The reaction mixture contained 100 mM sucrose, 50 mM HEPES, 20 mM KCl, 5 mM $MgCl_2$; pH was adjusted with NaOH up to 7.8. The reaction kinetics after the addition of 10 mM of bicarbonate were recorded during 20 s. The observed pH change was an indicator of carbonic anhydrase activity only, since proton uptake by thylakoids under the conditions used was two orders of magnitude lower and may be ignored. Results obtained just after bicarbonate addition, during the first seconds of the reaction, are not shown in Fig. 1 due to the high dispersion of the obtained values caused by the inhomogeneous state of the reaction mixture at that time [8]. The initial rate of the reaction obtained during the first 2.5 s after bicarbonate addition was used in the CA activity calculations presented in Table 1.

2.4. Electron transport rate

The electron transport rate was measured either as ferricyanide reduction monitored as pH change in the reaction medium or as oxygen consumption in the presence of methylviologen (MV) with a

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Clark-type oxygen electrode. 1 mM ferricyanide or 0.1 mM MV was used, respectively. The reaction medium was of the same composition as for CA measurement, pH 7.6.

2.5. Light-induced proton uptake by thylakoids

The light-induced proton uptake by thylakoids was measured in the presence of 50 µM MV. The reaction medium was the same as in the case of electron transport measurement but the HEPES concentration was lowered to 5 mM.

2.6. The generation of proton gradient on thylakoid membranes

The generation of proton gradient on thylakoid membranes was observed using measurement of light-dependent quenching of 9-aminoacridine fluorescence. While this method cannot serve as a direct measure of the transthylakoid proton gradient, it allows observation of the relative changes in the ΔpH. The reaction mixture contained 5 µM 9-aminoacridine, 0.1 mM MV and a medium identical to the one used for CA measurement, pH 7.6. The fluorescence was excited by a mercury lamp shielded with UFS-8 (Russia) and IF-365 (Carl Zeiss Jena, Germany) filters and recorded by a photomultiplier equipped with SZS-22, ZhS-17 (Russia) and IF-461 (Carl Zeiss Jena, Germany) filters. Actinic light was supplied by means of a slide projector with a red cut-off filter KS-17 (Russia).

2.7. Light intensity

The light intensity was measured using a Li-Cor quantum meter (Licor, Lincoln, NE, USA).

2.8. Chlorophyll concentration

The chlorophyll concentration was 100 µg/ml in the case of CA measurement and 30 µg/ml in other cases.

3. Results

In continuous light of high intensity at pH 7.6–8.0 in the presence of sucrose, i.e. under conditions close to those in illuminated chloroplasts in vivo, the CA activity of thylakoids increased (Fig. 1). The effect was not observed at a lower pH in the medium (7.0–7.4, not shown). The presence of nigericin (Nig) in the reaction medium removed the light stimulation and sometimes led to the development of an inhibitory effect of light. The inhibitory effect was described earlier [6] but was not obligatory in our conditions, since in most cases the illumination of thylakoids in the presence of Nig caused no effect on tCA activity during the entire measurement period, up to 20 s (not shown). The stimulating effect of light was observed during the first seconds of the reaction and then disappeared. This might be the reason why the stimulation was not observed earlier using conventional CA measurement devices. The transient manifestation of the effect arises from approaching the HCO_3^-–CO_2 equilibrium in the artificial system used, without continuous HCO_3^- input and CO_2 removal. Nevertheless, the light-induced CA activity is expected to contribute to a steady rate of bicarbonate dehydration in the illuminated chloroplast, where both HCO_3^- input and CO_2 fixation take place. The value of the stimulating effect (measured 2.5 s after bicarbonate addition) was commensurate with the electron transport rate when expressed in µmol of protons per mg Chl per hour (Table 1, rows 1, 4 and 5). We studied the stimulating effect during all seasons and with three different pea types (see Section 2). Thylakoids isolated from 'Rannij Gribovskij' pea type demonstrated the high (corresponding to 1000–2000 µmol of protons per mg Chl per hour) and therefore quickly disappearing effect of light on tCA activity (Fig. 1). The CA inhibitor acetazolamide (AA), which is known to be non-penetrative through the membrane, did not affect the light-induced part of thylakoid CA activity (Table 1) even at

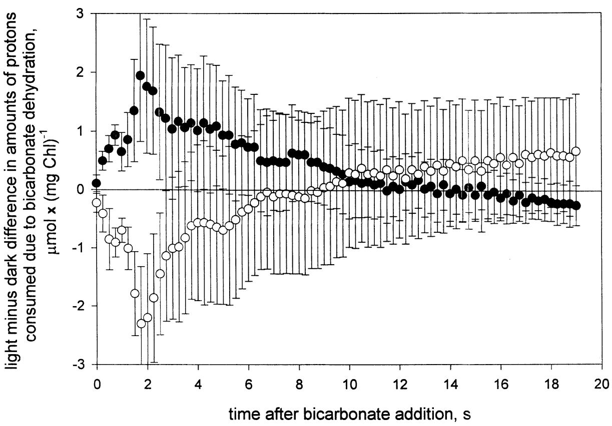

Fig. 1. The influence of 50 nM nigericin on light-stimulated bicarbonate dehydration in thylakoid suspension. The difference in amounts of protons used in the dehydration reaction in light and dark is shown for each time point. Red light (620 µmol quanta m^{-2} s^{-1}) was switched on 1 min before bicarbonate addition. 'Rannij Gribovskij' pea variation. The means ± S.E.M. calculated for four samples of a typical thylakoid preparation are shown.

Table 1

Effects of nigericin (Nig), acetazolamide (AA) and ethoxyzolamide (EZ) on the light-dependent CA activity of thylakoids, electron transport rate, proton uptake by thylakoids and generation of ΔpH on thylakoid membrane of 'Rannij 301' pea type

Parameter measured	Additions into the reaction medium				
	–	Nig (50 nM)	AA (0.5 mM)	EZ (0.5 mM)	Nig+EZ
Illumination-dependent gain in CA activity (µmol H^+ (mg Chl)$^{-1}$ h^{-1})	257 ± 72	−21 ± 76	261 ± 93	−37 ± 61	–
Proton uptake by thylakoids (nmol H^+ (mg Chl)$^{-1}$)	276 ± 1 (100%)	46 ± 6	240 ± 13 (87%)	236 ± 3 (86%)	–
Proton efflux from thylakoids after switching off the light (nmol H^+ (mg Chl)$^{-1}$ s^{-1})	0.028 (100%)	0.070 (250%)	0.028 (100%)	0.026 (93%)	–
Ferricyanide reduction (µmol (mg Chl)$^{-1}$ h^{-1})	120 ± 5 (100%)	291 ± 6 (100%*)	125 ± 5 (104%)	91 ± 4 (76%)	274 ± 13 (94%*)
O_2 consumption in presence of MV (µmol O_2 (mg Chl)$^{-1}$ h^{-1})	17.1 ± 0.5 (100%)	37.2 ± 1.1 (100%*)	16.7 ± 0.7 (98%)	12.5 ± 0.3 (73%)	33.8 ± 0.5 (91%*)
Light-dependent quenching of 9-aminoacridine (%)	15 ± 1	7 ± 1	15 ± 2	16 ± 2	–

*The rate in the presence of nigericin was taken as 100%.

The data for CA activity are the means ± S.E.M. calculated from measurements on five thylakoid preparations; data for 9-aminoacridine fluorescence from two and for the other parameters from three preparations. See Section 2 for the measurement conditions.

high concentrations (0.5 mM). On the contrary, the lipophilic CA inhibitor, ethoxyzolamide (EZ), did not allow the light-dependent CA activity to appear (Table 1).

The possibility of 'non-enzymatic' light-dependent gain in the bicarbonate dehydration rate in the thylakoid suspension could also be considered. Indeed, a low pH of the lumen generated in light may facilitate spontaneous bicarbonate dehydration taking into account the relatively high permeability of the thylakoid membrane to bicarbonate ions [17]. The suppression of the effect of light by EZ might be accounted for by a possible occasional uncoupling effect of EZ. However, the presence of both AA (which does not influence the light-dependent bicarbonate dehydration) and EZ (which eliminates it completely) reduce the light-dependent proton consumption by thylakoids slightly and equally, by 13–14% (Table 1), thus proving that the mechanism of EZ action is not of an uncoupling nature. Neither substance affected the ΔpH value estimated as light-dependent quenching of 9-aminoacridine fluorescence (Table 1, row 6). While AA did not affect the electron transport rate measured as either ferricyanide reduction (Table 1, row 4) or O_2 consumption in the presence of MV (Table 1, row 5), EZ inhibited it by 24–27%. The inhibitory action of EZ on electron transport was reduced to 6–9% in the presence of 50 nM Nig, regardless of the measurement method used (Table 1, rows 4 and 5).

4. Discussion

The main result of the present paper is the first direct demonstration of the involvement of intrathylakoid protons accumulated in light in the facilitation of the bicarbonate dehydration reaction with participation of tCA. This discovery contributes to revealing the paths of utilization of the transmembrane proton gradient and to elucidating the nature of high rates of photosynthesis measured in vivo regardless of the limited concentration of CO_2, the immediate substrate for Rubisco, in chloroplast stroma.

Our data on electron transport measurements additionally support the idea of tCA operating as a sink of intrathylakoid protons. EZ negligibly affected the rate of electron transport in the presence of Nig which implies the absence of substantial direct damage of the electron transport chain components by this inhibitor. At the same time, the inhibition of electron transport by EZ in the presence of ΔpH in thylakoids was high (Table 1). This may be interpreted as a role of tCA in maintaining the electron transport via partial removal of the photosynthetic control. This assumption appears to be in direct accordance with the CA measurement data (Table 1, row 1) demonstrating utilization of light-generated ΔpH by tCA.

Elimination of the light-dependent CA activity of thylakoids by EZ, but not AA, suggests that the active center of pea tCA is at least covered from the stroma by a hydrophobic layer and possibly directed to the lumen. The lumen-directed CA has been discovered recently in *C. reinhardtii* [10]. Our previous results demonstrating another stimulating effect of light on tCA, i.e. by weak light in the presence of DCMU, suggested that intramembrane protons located near PSII rather than bulk lumen protons are responsible for the regulation of tCA activity [13].

The phenomenon of proton removal from the lumen by tCA may be considered as a second process (after ATP synthesis) of constructive utilization of the intrathylakoid protons accumulated in light. It is noteworthy that the data, showing the obligatory Q-cycle operation in photosynthetic electron transport chain [18] and the contribution of cyclic [19] and pseudocyclic [20] electron flows in proton pumping in chloroplasts of higher plants, imply 'excessive' (related to the demand for the Calvin cycle) proton accumulation inside thylakoids. Taking into account the recent data about tCA operation in algae [16], it seems that tCA is a characteristic of a wide range of photosynthetic organisms and is involved in maintaining a previously unknown link between energy transduction in thylakoid membranes and carbon metabolism.

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