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Short Communications

The presence of soluble carbonic anhydrase in the thylakoid lumen of chloroplasts from *Arabidopsis* leaves

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ABSTRACT

Supernatant obtained after high-speed centrifugation of disrupted thylakoids that had been washed free from extrathylakoid carbonic anhydrases demonstrated carbonic anhydrase activity that was inhibited by the specific inhibitors acetazolamide and ethoxyzolamide. A distinctive feature of the effect of Triton X-100 on this activity also suggested that the source of the activity is a soluble protein. Native electrophoresis of a preparation obtained using chromatography with agarose/mafenide as an affinity sorbent revealed one protein band with carbonic anhydrase activity. The same protein was revealed in a mutant deficient in soluble stromal carbonic anhydrase β-CA1, and this indicated that the newly revealed carbonic anhydrase is not a product of the *At3g01500* gene. These data imply the presence of soluble carbonic anhydrase in the thylakoid lumen of higher plants.

Introduction

Carbonic anhydrases (CAs, EC.4.2.1.1) constitute a large group of enzymes that have been assigned to six families, which are commonly assumed to have originated in the process of convergent evolution (Hewett-Emmett and Tashian, 1996; Moroney et al., 2011). The *Arabidopsis thaliana* genome contains 19 genes encoding carbonic anhydrases (Fabre et al., 2007), but not all of their products have been identified. In chlorophyll-containing cells of plants and in algae, following the finding of soluble CAs in cytoplasm and in chloroplast stroma, CA activity of the thylakoids was revealed (Komarova et al., 1982; Vaklinova et al., 1982; Stemler, 1986, 1997; Ignatova et al., 1998). At least two sources of activity have been found in thylakoid membrane fragments enriched with photosystem II (PSII), and an additional one in membrane fragments enriched with photosystem I (PSI) (Lu and Stemler, 2002; Pronina et al., 2002; Ignatova et al., 2006). In *Chlamydomonas reinhardtii* thylakoids, a CA was found (Karlsson et al., 1998) that was identified as α-CA (Cah3) and, according to the authors' data, it is bound to the lumenal side of the thylakoid membrane near PSII. It was proposed that this CA catalyzes bicarbonate dehydration at the donor side of PSII, allowing immediate removal of the protons from the water oxidation complex, thus preventing its damage (Villarejo et al., 2002). This function was also proposed for the membrane CA bound to PSII in higher plants (Rudenko et al., 2007). Based on the other assumptions, namely, assuming the participation of CAs in the supply of CO_2 to Rubisco, the presence of a lumenal CA was postulated in algae both from indirect experiments (Pronina and Borodin, 1993) and theoretical considerations (Raven, 1997). The first indication of the presence of soluble CA in the thylakoid lumen of higher plants appeared in work with pea thylakoids (Rudenko et al., 2007). The present study conducted with *Arabidopsis* confirms the presence of a soluble CA in the thylakoid lumen of higher plants, and shows that it differs from the stromal soluble CA.

Materials and methods

Arabidopsis thaliana (L.) ecotype Columbia (WT) and the *β-CA1* knockout line (β1-mut) were grown in a chamber at 19 °C with an 8 h photoperiod under 100–120 μmol quanta m^{-2} s^{-1}. The seeds of the mutant were obtained from the Arabidopsis Biological Resource Center as a T-DNA insertion line (SALK_106570). Total RNA was extracted from the leaves of WT plants and the homozygous mutant plants containing the T-DNA insert in *At3g01500* (β1-mut) and was treated with DNase to eliminate any genomic DNA contamination. First-strand cDNA was synthesized using an Omniscript RT Kit (Qiagen) and oligo(dT) as a primer. cDNA specific for the *At3g01500* gene primer pair was used, 5′-CCT CTC CGA AAC TAG CTC TGT

Abbreviations: AZ, acetazolamide; BSA, bovine serum albumin; CA, carbonic anhydrase; Chl, chlorophyll; DTT, 1,4-dithio-DL-threitol; EZ, ethoxyzolamide; PAAG, polyacrylamide gel; PMSF, phenylmethylsulfonyl fluoride; PSI, photosystem I; PSII, photosystem II; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; WT, wild type; β1-mut, the mutant deficient in soluble stromal carbonic anhydrase.

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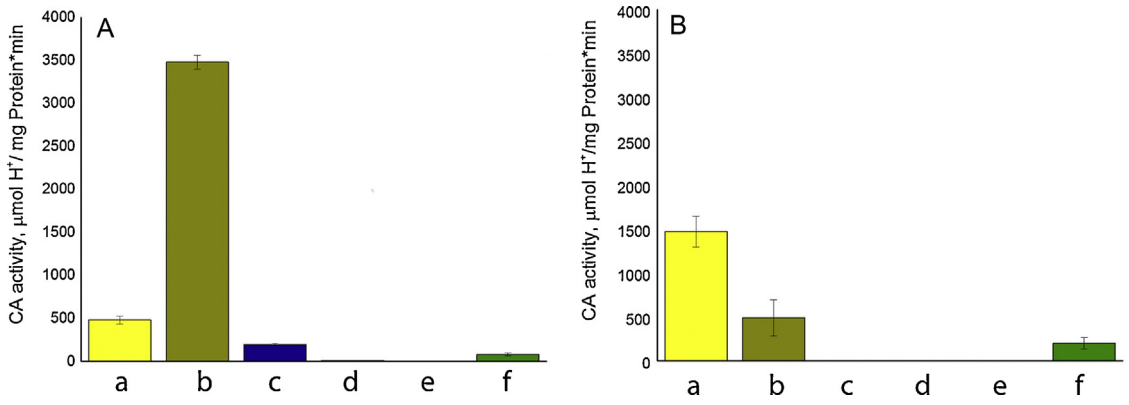

Fig. 1. Carbonic anhydrase activities of supernatants obtained in the course of isolation of the soluble proteins of thylakoid from leaves of WT (A) and β1-mut (B): 'a' – following centrifugations of homogenate; 'b' – following centrifugation of shocked chloroplasts; 'c', 'd' and 'e' – following successive washings of thylakoids; 'f' – following centrifugation of thylakoids treated with Triton X-100 (for details, see 'Materials and methods' section).

TAA-3′ and 5′-CTG TCC CCC AAG ATT TTA ATT CTG TAA A-3′, and polymerase chain reactions (PCR) were run in an IQ5 cycler (Bio-Rad). *At3g01500* gene transcripts were absent in the β1-mut leaves (Supplement 1, Fig. 1).

The thylakoids were isolated from the leaves of 1.5–2 month-old plants. The leaves were homogenized in medium containing 0.1 M Tris–H_2SO_4 (pH 8.0), 0.4 M sucrose, 2% Polyclar (w/v), 5 mM EDTA, 0.005% BSA, 1 mM benzamidine, 1 mM α-aminocaproic acid, and PMSF (Medium 1). The homogenate filtered through nylon cloth was centrifuged for 6 min at 3400 × *g*, and the supernatant was centrifuged to remove the rest of the membranes for an additional1 h at 175,000 × *g*, yielding supernatant 'a'. The pellet after the first centrifugation was suspended in Medium 1 diluted to one-tenth to break the chloroplast envelope, and the mixture was successively centrifuged twice as above, yielding finally supernatant 'b'. The thylakoids were washed three times by suspending the pellets in Medium 1 followed by centrifugation for 1 h at 175,000 × *g* yielding supernatants 'c', 'd', and 'e'. To obtain the soluble thylakoid proteins, the thylakoids, after the last washing were either incubated under stirring for 20 min at 0 °C at the required Triton X-100 concentration, or passed through a French press (Thermo electron, USA) twice, each time at 10,000 psi. Then the lysate in both the cases was centrifuged at 175,000 × *g* for 1 h to obtain supernatant 'f' and the pellet containing the thylakoid membranes. Supernatant 'f' and the pellet did not contain Rubisco, which was verified with the Western-blot assay, using antibodies to large subunits of Rubisco and Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad). This indicated that the thylakoids were not contaminated before disrupting with stromal components.

Carbonic anhydrase activity was evaluated as the difference between the rates of pH decrease, measured with pH electrode, from 8.3 to 7.8 in the course of CO_2 hydration at 2 °C in 13.6 mM Veronal buffer (pH 8.4) in the presence and in the absence of an aliquot of the preparation. The difference in the buffer capacities was taken into account to express CA activity as extent of proton release. The protein content in the supernatants and the chlorophyll content in the pellets were determined according to (Lowry et al., 1951) and (Winterman and De Mots, 1965), respectively.

Affinity chromatography was carried out by loading supernatant 'f' concentrated in a Millipore concentrator (50 kDa) and diluted with medium containing Tris–H_2SO_4 (pH 8.0), 100 mM NaCl, 5 mM EDTA, 1 mM benzamidine, and 1 mM α-aminocaproic acid (Medium 2) onto the column filled with agarose/mafenide (Sigma). After incubation for 40 min, the column was washed with Medium 2 with 0.05% Triton X-100 to remove nonspecifically bound substances. The CA was eluted from the column with Medium 2 containing 50 μM mafenide, which, being an inhibitor of the CA, was removed by centrifugation of the eluate in Millipore concentrators to restore the CA activity.

Native electrophoresis was performed according to Davis (1964) using a Mini-Protean 3 Cell (Bio-Rad). It was carried out at current strength of 10 mA for 3–4 h at 4 °C in the dark in 10% PAAG containing 20% glycerol.

The activity of CA in PAAG was visualized after incubation of the gel on ice for 20–30 min in 44 mM Veronal buffer (pH 8.1) with bromothymol blue followed by transfer into water saturated with CO_2 at 0 °C (Edwards and Patton, 1966). The blue gel became locally yellow at the place where a source of CA activity was situated. The effect of acetazolamide (AZ) was evaluated, placing the gels into the buffer with 10 mM AZ before the visualization of CA activity. The proteins in the gels were stained with Coomassie G-250 (Kashino, 2003).

Results

Fig. 1 shows CA activities of supernatants 'a'–'f' (see 'Materials and methods' section) isolated from WT (Fig. 1A) and from β1-mut (Fig. 1B) plants. Perceptible activities in supernatants 'a' obtained after breaking the cells by homogenization and in supernatant 'b' obtained after also breaking the chloroplasts in hypoosmotic media obviously reflected the presence of extrathylakoid soluble CAs from cytoplasm and chloroplast stroma: β-CA1, β-CA2, β-CA3 (Fabre et al., 2007), and α-CA1 (Villarejo et al., 2005) in the mixtures containing thylakoids both from WT and β1-mut (without β-CA1). It should be noted that CA activity of supernatant 'b' from the mutant was lower than that of the supernatant from WT. CA activities were not detectible in supernatant 'e' from WT (Fig. 1A) and already in supernatant 'c' from β1-mut (Fig. 1B). The absence of CA activity in supernatants 'e' obtained from leaves of both the WT and the mutant was confirmed by the failure to observe this activity in the gel following native electrophoresis of these supernatants, which were concentrated thirty-fold (Supplement 1, Fig. 2).

Following the detergent treatment of the thylakoids to release the soluble lumen proteins, appreciable CA activity was observed in supernatants 'f' (see 'Materials and methods' section) both from WT and β1-mut (Fig. 1A and 1B). The CA activity in the same supernatant was observed after breaking the thylakoids not only by the detergent, but also by the use of a French press (data not shown); the latter treatment does not lead to release into solution of the proteins that could adhere to the membrane surface according to Kieselbach and Schröder (1998). The CA activity of supernatant 'f', *i.e.* of the soluble lumen proteins abandoning the thylakoids during the time of Triton treatment, gradually reached a maximal value at the concentration of the detergent corresponding to Triton/Chl