Cell Biology International 32 (2008) 646–653

Cell Biology International

www.elsevier.com/locate/cellbi

# A 160 kDa protein with carbonic anhydrase activity is complexed with rubisco on the outer surface of thylakoids

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Received 19 August 2007; revised 28 September 2007; accepted 16 January 2008

**Abstract**

This study provides evidence that, in the soluble fraction from buffer-washed pea thylakoids, one form of soluble carbonic anhydrase (CA) is associated with rubisco in a stromal protein complex. On native-PAGE gels, it is present as a protein band with MW approximately 160 kDa. On SDS-PAGE gels, it is resolved as a single 25-kDa polypeptide. Analysis of Western blots developed with polyclonal antibodies to barley rubisco and to soluble pea CA shows that a 160-kDa protein with CA activity is associated with rubisco in a protein complex localized on the outer surface of thylakoid membranes.
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*Keywords:* Carbonic anhydrase; Pea (*Pisum sativum L.*) cv. Ran 1; Stromal protein complex; Rubisco

## 1. Introduction

Carbonic anhydrase (CA; EC 4.2.1.1) speeds the equilibrium between $CO_2$ and $HCO_3^-$ at physiological pH values and is believed to exist in all living organisms. The intracellular compartmentation of CA in plants remains a question even today. There are brief reports that, in $C_3$ plants, 87–92% of the total activity for soluble enzymes is located in the chloroplasts, while 7–13% resides in the cytoplasm (Popova et al., 1991; Rumeau et al., 1996). For $C_4$ plants such as *Zea mays L.*, it was established that the enzyme activity is localized in the cytosol of mesophyll cells (Atkins et al., 1972; Burnell, 2000).

It is established that soluble chloroplastic CA from higher plants belongs to the β-CA family (Kimber and Pai, 2000). In native-PAGE gels the chloroplast enzyme has MW approximately 220–230 kDa (Johansson and Forsman, 1993; Rumeau et al., 1996; Lazova and Stemler, 2005) but the results obtained for MW of the polypeptides to which CA dissociated in SDS conditions are different and range from 25 to 27 kDa. It has also been reported that stromal sCA migrates as a doublet in SDS-PAGE corresponding to molecular masses of 27 kDa and 27.5 kDa (Rumeau et al., 1996) or as an abundant band, 25.5 kDa polypeptide, and a minor band, 27.5 kDa (Majeau and Coleman, 1991). The authors explained the formation of a smaller, more abundant polypeptide as the result of proteolysis, despite the presence of protease inhibitors during the isolation procedure, or as a dissociated cytosolic isoenzyme.

Soluble carbonic anhydrase localized in the chloroplast stroma has been proposed to play an important role in the photosynthetic process by providing $CO_2$ for ribulose-bisphosphate carboxylase (rubisco). It facilitates the diffusion of inorganic carbon into the chloroplasts by catalyzing the rapid hydration of dissolved $CO_2$ as it passes across the chloroplast envelope and into the alkaline stroma (Badger and Price, 1994).

Awareness that several Calvin cycle enzymes in higher plants appear to be assembled into stable multienzyme complexes has provoked significant interest in recent years (Süss

*Abbreviations:* BN, blue native; BPB, bromophenol blue; CA, carbonic anhydrase; CN, colorless native; DTT, 1,4-dithiothreitol; LDS, laurylmaltoside; MSH, β-mercaptoethanol; MW, molecular weight; native-PAGE, native polyacrylamide gel electrophoresis; rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose bisphosphate; sCA, soluble carbonic anhydrase; SDS, sodium dodecyl sulfate.

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doi:10.1016/j.cellbi.2008.01.010

and Sainis, 1997; Anderson et al., 1996: Jebanathirajah and Coleman, 1998; Babadzhanova et al., 2002; Anderson and Carol, 2004,). The association of specific enzymes in protein complexes is presumed to facilitate channeling of pathway intermediates from one enzyme active site to another (Jebanathirajah and Coleman, 1998). These complexes are not simple artifacts resulting from fortuitous protein associations *in vitro*, but rather represent functioning complexes that facilitate photosynthetic $CO_2$ fixation in the chloroplast (Anderson and Carol, 2004). They are also certainly responsible, in part, for the very short initial lag period in photosynthetic $CO_2$ fixation by allowing the Calvin cycle to operate with low levels of intermediates.

Much earlier, Kachru and Anderson (1974) offered a similar idea. They hypothesized that rubisco and CA might associate in chloroplasts. Eventually, immunocytochemical evidence did show similar stromal localization patterns of CA and rubisco in peas (Anderson et al., 1996) and potatoes (Rumeau et al., 1996). Later, Jebanathirajah and Coleman (1998) showed that a portion of the chloroplast CA is associated with the periphery of the Calvin cycle complex, possibly in some configuration with rubisco. Anderson and Carol (2004) established that rubisco forms stable complexes with several other enzymes, including the enzymes that provide its substrates: CA and phosphoribulokinase. They presented evidence showing that rubisco activase, CA, phosphoribulokinase, and 3-P-glycerate kinase, which catalyzes the next reaction in the Calvin cycle, are co-localized with rubisco *in situ*. It was discussed also that soluble Calvin cycle enzymes not only have a tendency to associate with one another, but some of them may be organized on the surface of thylakoid membranes (Süss and Sainis, 1997; Sainis et al., 2003). In recent years great interest was provoked by the findings that free and membrane-bound stable multienzyme complexes with Calvin cycle activities were present in the chloroplast stroma (Babadzhanova et al., 2002). This was consistent with immunocytochemical evidence provided earlier (Anderson et al., 1996) that while CA is apparently located in the stroma of pea chloroplasts, a full 51% of it is in contact with thylakoid membranes.

The findings discussed above did not answer an important question: does the rubisco-containing protein aggregate associated with thylakoid membranes contain a form of carbonic anhydrase? If so, is it the 230 kDa protein, known as 'soluble carbonic anhydrase', or is it another CA isoform that is situated exclusively with rubisco? The aim of the work described here was to answer this question. The results indicate that in a soluble fraction of buffer-washed thylakoids, a 160 kDa protein with CA activity is present that can dissociate to a polypeptide with MW approximately 25 kDa. The 160 kDa protein is indeed associated with rubisco in a large protein complex localized on the outer surface of the thylakoids.

## 2. Materials and methods

### 2.1. Plant material

Seeds of peas (*Pisum sativum L.*) cv. Ran 1 were germinated for 2 days in two layers of damp filter paper in moist vermiculite at 25 °C in the dark. Then they were transferred to containers holding 2 L of tap water. The water was changed every 24 h. During the growth period the seedlings were kept in a growth chamber under conditions as follows: constant temperature of 25 °C, light by white fluorescent lamps (150 μmol photons $m^{-2}s^{-1}$), in periods of 12 h/12 h light/dark and relative humidity of about 50%. The pea leaves were collected from 10 day-old plants.

### 2.2. Preparation of pea leaf extract for isolation of a stromal aggregate

Leaves (200 g) were blended twice for 5–10 s in 330 mM sorbitol, 50 mM Hepes–KOH (pH 7.8), 10 mM KCl, 4 mM sodium ascorbate, and 7 mM L-cysteine (Kieselbach et al., 1998, with modification). The resulting homogenate was filtered through nylon mesh (20 μm) and centrifuged for 2 min at 2000 × *g*. The resulting soft pellet was resuspended in 10 mL of a solution that contained 330 mM sorbitol, 50 mM Hepes–KOH (pH 7.8), and 10 mM KCl. The yield was 60–70% intact chloroplasts that contained 40–45 mg chlorophyll. The chloroplasts were resuspended in 10 mM sodium pyrophosphate (pH 7.8) for 5 min at 4 °C and spun for 5 min at 7500 × *g*. The supernatant that contained soluble stromal components was removed. The pellet, which contained thylakoids and extrinsic thylakoid proteins, was then subjected to a wash procedure to affect separation of the two. This included washing the thylakoids twice for 5 min with 10 mM sodium pyrophosphate (pH 7.8) followed each time by centrifugation for 15 min at 12 000 × *g*. The combined supernatants were spun again at 18 000 × *g* for 20 min to remove residual membrane and the resulting supernatant was used in our experiments. The protein yield was 4.3 mg $mL^{-1}$ Protein concentration was measured according to Bradford (1976) with bovine serum albumin as the standard.

### 2.3. Electrophoretic characterization of CA protein in a stromal aggregate

The proteins in the buffer-washed supernatant were separated by:

(a) Colorless native-PAGE (CN-PAGE) in two dimensions as in Schägger et al. (1994). For both the first and second dimensions, the gradient separating gel was 6–22%, the stacking gel 3–4%, with Coomassie staining. We removed the section of the first gel that contained the protein aggregate for use in the second dimension.
(b) Blue native-PAGE (BN-PAGE) in the first dimension was performed as described in Schägger and von Jagow (1991). The gradient separating gel was 6–22%, the stacking gel was 3% with Coomassie staining. Denaturation with 10% Tris–Tricine SDS-PAGE in the second dimension was carried out as in Schägger and von Jagow (1991). The separating gel was 15% and the stacking gel was 10% with Coomassie staining. The sample buffered

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