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Subcellular distribution of carbonic anhydrase in *Solanum tuberosum* L. leaves

Characterization of two compartment-specific isoforms

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Abstract. The intracellular compartmentation of carbonic anhydrase (CA; EC 4.2.1.1), an enzyme that catalyses the reversible hydration of CO_2 to bicarbonate, has been investigated in potato (*Solanum tuberosum* L.) leaves. Although enzyme activity was mainly located in chloroplasts (87% of total cellular activity), significant activity (13%) was also found in the cytosol. The corresponding CA isoforms were purified either from chloroplasts or crude leaf extracts, respectively. The cytosolic isoenzyme has a molecular mass of 255 000 and is composed of eight identical subunits with an estimated M_r of 30 000. The chloroplastic isoenzyme (M_r 220 000) is also an octamer composed of two different subunits with M_r estimated at 27 000 and 27 500, respectively. The N-terminal amino acid sequences of both chloroplastic CA subunits demonstrated that they were identical except that the M_r-27 000 subunit was three amino acids shorter than that of the M_r-27 500 subunit. Cytosolic and chloroplastic CA isoenzymes were found to be similarly inhibited by monovalent anions (Cl^-, I^-, N_3^- and NO_3^-) and by sulfonamides (ethoxyzolamide and acetozolamide). Both CA isoforms were found to be dependent on a reducing agent such as cysteine or dithiothreitol in order to retain the catalytic activity, but 2-mercaptoethanol was found to be a potent inhibitor. A polyclonal antibody directed against a synthetic peptide corresponding to the N-terminal amino acid sequence of the chloroplastic CA monomers also recognized the cytosolic CA isoform. This antibody was used for immunocytolocalization experiments which confirmed the intracellular compartmentation of CA: within chloroplasts, CA is restricted to the stroma and appears randomly distributed in the cytosol.

Key words: Carbonic anhydrase purification – Intracellular compartmentation – *Solanum*

Introduction

Carbonic anhydrase (CA; EC 4.2.1.1) is a zinc-containing metalloenzyme that catalyses the reversible hydration of CO_2 to bicarbonate. It is an ubiquitous enzyme among living organisms. Animal CAs have been extensively studied; however, many aspects concerning the structure, localization and physiological role of plant CAs remain to be elucidated.

In C_4 plants, CA activity appears largely confined to the cytosol of mesophyll cells. Phosphoenolpyruvate carboxylase (PEPc) which is similarly located, catalyses the initial carboxylation reaction in C_4 photosynthesis and uses HCO_3^- rather than CO_2 as a substrate. To provide substrate for this reaction, atmospheric CO_2 entering the mesophyll cells has to be rapidly converted into HCO_3^-, a process which is mediated by CA (Hatch and Burnell 1990). A cytoplasmic CA has been purified to homogeneity from maize (Burnell 1990) and antibodies reacting with a subunit of 25 kDa, were obtained. Recently, Cavallaro et al. (1994) have reported the sequence of a cDNA encoding a cytosolic isoform of CA in the leaves of *Flaveria bidentis*, a C_4 dicotyledonous plant. The *F. bidentis* cDNA sequence encodes a predicted polypeptide of 26 kDa which seems not to be processed following translation of mRNA.

In C_3 plants, CA represents one of the most abundant proteins in leaves and is mainly located in the chloroplast. However, some lines of evidence suggest the existence of a cytosolic isoform (Reed and Graham 1981). Within the chloroplast, CA activity has been suggested to be involved in photosynthetic CO_2 accumulation by facilitating CO_2 diffusion in the stroma and supplying substrate for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Badger and Price 1994). Carbonic anhydrase could also play a role in the regulation of the chloroplastic pH and protect stromal enzymes from denaturation during pH changes

Abbreviations: BSA = bovine serum albumin; CA = carbonic anhydrase; PMSF = phenylmethylsulphonyl fluoride; BAM = benzamidine; DTT = dithiothreitol; 2-ME = 2-mercaptoethanol; PVDF = polyvinylidene difluoride

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occurring in response to drastic and rapid changes in light regimes (Reed and Graham 1981). Experimental evidence for any of these roles are rather scarce. Chloroplastic CA has been purified from several angiosperms and further characterizations have led to the distinction between dicotyledonous and monocotyledonous plant CAs (Reed and Graham 1981). The dicotyledonous enzyme has been proposed to form an oligomer composed of six or eight identical subunits of M_r ranging from 24 000 to 36 000 while the M_r of the monocotyledonous monomer, partially characterized from *Tradescantia* and barley, has been estimated to 42 000 and 45 000, respectively (Reed and Graham 1981). Chloroplastic CA cDNAs have been isolated from tobacco (Majeau and Coleman 1992), spinach (Burnell et al. 1990; Fawcett et al. 1990), pea (Röeske and Ogren 1990; Majeau and Coleman 1991), *Arabidopsis thaliana* (Raines et al. 1992; Fett and Coleman 1994) and rice (Suzuki and Burnell 1995). Chloroplastic CA appears to be a nuclear-encoded protein with a transit peptide for import into the chloroplast. Chloroplastic CA synthesis is light-regulated and dependent on lead and/or chloroplast development (Fett and Coleman 1994; Majeau and Coleman 1994). A correlation between CA and Rubisco expression has been demonstrated in pea (Majeau and Coleman 1994). Although there are brief reports concerning the occurrence of cytosolic CA in non-green tissues such as etiolated leaves and roots (Reed and Graham 1981), there has been scant regard given to characterizing the cytosolic enzyme. Kachru and Anderson (1974) reported the partial purification of chloroplastic and cytosolic forms of pea. Recently, Fett and Coleman (1994) have predicted the amino acid sequence of a putative cytosolic CA isoform from a cDNA clone from *Arabidopsis thaliana*. The authors have also shown that the chloroplastic and the putative cytosolic isoforms are differentially regulated by light and suggested a role for the cytosolic CA distinct from photosynthesis.

In this paper, we have identified and purified to homogeneity two compartment-specific CA isoforms from potato leaves and characterized some biochemical parameters of both isoenzymes. The subcellular distribution of both isoenzymes has been studied using antibodies directed against the N-terminal amino acid sequence of the chloroplastic isoform.

Materials and methods

Plant material. Potato plantlets (*Solanum tuberosum* L. cv. Kennebec, supplied by Dr. Tibbits, University of Wisconsin, Madison, Wis., USA) were cultivated in vitro from stem axillary buds as described by Tourneux and Peltier (1995). After two weeks of growth, plantlets were transferred to soil in a greenhouse with temperatures at approx. 20 °C during the day and 15 °C at night. They were continuously watered with a nutrient solution (Hoagland and Arnon 1950).

Preparation of potato leaf extract. Potato leaves were quickly frozen in liquid nitrogen and ground into a fine powder with a chilled mortar and pestle. The powder was resuspended in extraction buffer ($2\,ml \cdot g^{-1}$ of plant material) containing 0.1 M sodium phosphate (pH 7.5), 1 mM Na_2EDTA, 3% (w/v) polyvinyl polypyrrolidone (PVPP), 10 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM benzamidine (BAM). The homogenate was filtered through two layers of muslin and the filtrate was centrifuged at $15\,000 \cdot g$ for 15 min. The supernatant fraction comprises the crude extract and was used for PAGE. For preparative isoelectric focusing the crude extract was fractionated with solid ammonium sulphate. The protein precipitating between 30 and 60% of saturation was resuspended in 10 mM Tris-HCl (pH 8), 10 mM DTT and desalted using a PD10 Sephadex G-25 column pre-equilibrated in the same buffer according to the instructions of the manufacturer (Pharmacia, Orsay, France).

Preparation of chloroplasts and of soluble (stroma) proteins from potato leaf chloroplasts. Intact chloroplasts were isolated and purified from potato leaves using discontinuous Percoll (Pharmacia) gradients according to a modified method of Mills and Joy (1980): Batches of freshly harvested leaves (25 g) were deribbed, cut into small pieces and homogenized for 2×8 s with 150 ml of solution A [50 mM N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycin (Tricine) pH 7.8; 33 mM Sorbitol; 2 mM Na_2EDTA; 1 mM $MgCl_2$; 2 mM ascorbate; 5 mM DTT] in a Polytron homogenizer (Poly-Labo, Strasbourg, France). The resulting slurry was filtered through two layers of muslin and two layers of nylon net (60 µm) and the filtrate was centrifuged at $3000 \cdot g$ for 5 min. Each pellet was resuspended in 5 ml of solution A and underlayered with 20 ml of Percoll medium (40%, v/v solution A) and 10 ml of Percoll medium (90% v/v solution A). The gradients were centrifuged for 15 min at $5000 \cdot g$. Intact chloroplasts were recovered between the two Percoll layers, removed by aspiration, diluted in 20 ml of solution A and pelleted by centrifugation for 5 min at $3000 \cdot g$. Chloroplasts were lysed in 60 ml of extraction buffer without PVPP. The lysate were centrifuged at $70\,000 \cdot g$ for 40 min to remove all the membranes (envelope membranes and thylakoids). The supernatant comprises the stromal extract and was used for further purification of the chloroplastic CA.

Purification of the chloroplastic CA. Step I: Ammonium sulphate fractionation. The chloroplast extract was fractionated with solid ammonium sulphate and the protein precipitating between 45 and 60% of saturation was resuspended in 20 mM Tris-HCl (pH 8.5), 1 mM DTT, 1 mM PMSF, 1 mM BAM and desalted using a PD 10 Sephadex G-25 column (Pharmacia) pre-equilibrated in the same buffer.

Step II: Anion-exchange chromatography. Anion-exchange chromatography was carried out using a high performance liquid chromatography system (Waters, Saint Quentin, France) and a Resource Q high performance column (1 ml; Pharmacia) at a flow rate of $2\,ml \cdot min^{-1}$. The column was equilibrated with 20 mM Tris-HCl (pH 8.5), 1 mM DTT. The desalted extract was loaded and unbound protein was removed by washing with equilibration buffer. Elution of proteins was performed with a linear gradient of NaCl ranging from 0 to 1 M. Fractions (1 ml) containing CA activity were pooled and crystalline ammonium sulphate was added to 1 M before loading onto the Phenyl-Superose column.

Step III: Hydrophobic interactions chromatography. The Phenyl-Superose column (HR 5/5; Pharmacia) was equilibrated with 20 mM phosphate buffer (pH 7) containing 5 mM DTT and 1 M $(NH_4)SO_4$. After loading the above protein solution, the column was washed with the equilibration buffer (6 ml) and protein was eluted with a decreasing linear gradient of ammonium sulphate (flow rate $0.5\,ml \cdot min^{-1}$). Fractions (0.5 ml) containing pure CA were pooled and used for enzyme characterization.

Purification of cytosolic CA. Step I. Ammonium sulphate fractionation. A crude extract from potato leaves was fractionated with solid ammonium sulphate. The protein that precipitated between 45 and 60% of saturation was collected by centrifugation. The resulting pellet was desalted into the Resource Q chromatography buffer using a PD 10 Sephadex G-25 column (Pharmacia).

Step II. Anion exchange chromatography. The protein sample was loaded onto a Resource Q column previously equilibrated with 20 mM phosphate buffer (pH 6.5) containing 1 mM DTT. Bound protein was eluted with a linear gradient of NaCl ranging from 0 to 1 M. Fractions containing CA were pooled and protein was concentrated by ammonium sulphate precipitation (60% of saturation).

Step III. Gel filtration chromatography. Protein was loaded onto a Superdex 200 column (26 × 600; Pharmacia) previously equilibrated with 20 mM phosphate buffer (pH 7) containing 1 mM DTT and 0.15 M NaCl. Elution was performed at a flow rate of 1 ml·min^{-1}. Fractions (1 ml) containing cytosolic CA were pooled and crystalline ammonium sulphate was added to 1 M before loading onto a Phenyl-Superose column.

Step IV. Hydrophobic interactions chromatography. The above protein sample was loaded onto a Phenyl-Superose column previously equilibrated with 20 mM phosphate buffer (pH 7) containing 1 mM DTT and 0.15 M NaCl. After washing, protein was eluted with a decreasing linear gradient of ammonium sulphate. Fractions containing pure cytosolic CA were used for further characterization.

Assay for CA activity. Carbonic anhydrase was assayed by measuring oxygen exchange from $^{13}C^{18}O^{18}O$ to water caused by the hydration and dehydration of CO_2 and HCO_3^-. The ^{18}O exchange was monitored by coupling a mass spectrometer (model MM 14–80; VG Instruments, Winsford, UK) to a cuvette via a membrane inlet system as described by Cournac et al. (1993). The assay contained 1.5 ml of 20 mM barbitone buffer (pH 7.5), 1 mM EDTA, 10 mM DTT, 50 mM Na_2SO_4 and 3 µl of 1 M $NaH^{13}C^{18}O_3$ (Peltier et al. 1995). The non-catalyzed ^{18}O exchange was followed for about 2–3 min at 25 °C. The protein extract was then injected and catalyzed exchange activity was followed for 2–3 min. One unit of CA activity was determined as a 100% stimulation of the non-catalyzed depletion of ^{18}O from $^{13}C^{18}O_2$ and calculated from $\alpha = \theta 2 - \theta 1/\theta 1$ (Peltier et al. 1995). Constants θ1 (for the non-catalyzed reaction) and θ2 (for the catalyzed reaction) was determined experimentally as the logarithmic derivatives of the curves $\log(\tau) = f(t)$, τ representing the ^{18}O isotopic abundance in CO_2.

Polyacrylamide gel electrophoresis and electrotransfer. Polyacrylamide gel electrophoresis was performed using a dual slab cell apparatus (MiniProtean II; BioRad, Ivry, France). Electrotransfer was performed using a semi-dry transfer apparatus (Milliblot Graphite Electroblotter; Millipore, Saint-Quentin, France).

Denaturing SDS-PAGE was performed as described by Laemmli (1970) using 13% (w/v) acrylamide gels. Proteins were either stained with Coomassie Brilliant Blue or electrotransferred onto nitrocellulose membrane (0.45 µm; Schleicher and Schuell, Aubervilliers, France) using the buffer system of Towbin *et al.* (1979) for 10 min at 12 V followed by 30 min at 24 V. For sequencing, peptides were transferred onto polyvinylidene difluoride membrane (Immobilon-P, 0.45 µm; Millipore). The PVDF membrane was briefly immersed in acetone, methanol and acetonitrile successively and equilibrated in transfer buffer containing 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, pH 11), 10% (v/v) methanol (Matsudaira 1987). After protein transfer the membrane was stained with Ponceau S. The peptide bands were excised from the membrane for amino acid sequencing.

Native PAGE was performed in 10% (w/v) acrylamide resolving gels using the buffer system of Aktins et al. (1972) except that DTT was used instead of 2-mercaptoethanol (2-ME). The CA activity was detected by ultraviolet fluorescence of bromcresol purple after exposure to CO_2 at low temperature (Patterson et al. 1971). Gels were photographed using Polaroid 665 film and one-dimensional densitometry scans (Hoeffer Scientific Instruments, Bioblock, Illkirch, France) were performed on the photographs.

For molecular-mass determination, non-denaturing PAGE was performed in gradient gels 4–15% (w/v; BioRad). High-molecular-weight markers from Pharmacia were used. Proteins were electrotransferred onto nitrocellulose as previously described and molecular-weight markers were detected by Ponceau S staining before CA immunodetection.

Amino acid sequence analysis. The N-terminal amino acid residues were determined by Edman degradation on a 470A Applied Biosystems protein sequencer (CNRS, Marseille, France).

Preparation of antiserum. An oligopeptide containing the 15 amino acid residues of the N-terminal end of the chloroplastic CA was synthesized and conjugated to KLH (Keyhole-limpet haemocyanin). Antisera against this conjugated protein were obtained from rabbits (Biocytex, Marseille, France). For immunocytolocalisation studies, immune and preimmune antisera were partially purified by precipitation with 50% (saturation) ammonium sulphate.

Immunodetection. Nitrocellulose filters were blocked for 1 h in blocking solution [0.1 M Tris-HCl, pH 8; 0.25% (w/v) bovine serum albumin (BSA); 0.25% (w/v) gelatin; 0.3% (v/v) Tween-20]. Filters were then probed wih 1:1000 diluted CA antiserum in blocking solution for 1 h at room temperature. Filters were washed (3 × 20 min) in 10 mM Tris-HCl (pH 8), 150 mM NaCl, 0.05% (v/v) Tween-20, probed with 5000-fold-diluted goat anti-rabbit IgG alkaline-phosphatase-conjugated secondary antibody (Jackson, Interchim, Montluçon, France) and washed as before. Filters were rinsed with 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$ and immunolabelled polypeptides were detected by incubating the filters at room temperature with 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$, 0.33% (v/v) dimethylformamide, 0.33 mg·ml^{-1} nitro blue tetrazolium, 0.17 mg·ml^{-1} 5-bromo-4-chloro-3-indoyl phosphate.

Immunocytolocalization. Potato leaf pieces of approx. 2 mm^2 were fixed in 0.1 M sodium cacodylate buffer (pH 7.1), containing 1% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde for 2 h at room temperature. Samples were rinsed with buffer alone, dehydrated with an ethanol series (10–80%) and embedded in LR-White resin according to the manufacturer's instructions (TAAB, Saint-Germain-en-laye, France). Ultrathin sections were first preincubated on drops of phosphate-buffered saline (PBS) containing 0.1 M glycine for 35 min at room temperature and then floated for 10 min at room temperature on a drop of normal goat serum (1:20 dilution) in 0.01 M PBS (pH 7.1). Next sections were incubated on the partially purified CA antiserum in PBS containing 1% (w/v) BSA, 0.05% (v/v) Tween 20. Various antiserum dilutions and periods of incubation were tested to determine the optimal labelling conditions. In the present study, sections were incubated for 2 h at 37 °C with the partially purified antiserum (1:10). The sections were washed with PBS, 1% (w/v) BSA, 0.05% (v/v) Tween 20 six times for 5 min each and placed on a drop of 20 mM Tris-HCl, 1% (w/v) BSA, 0.05% (v/v) Tween 20 for 10 min before incubation for 1 h in the same buffer containing a 50-fold dilution of goat anti-rabbit IgG conjugated to gold particles (15 nm; BioCell Research Laboratories, Cardiff, UK). After extensive washing in 20 mM Tris-HCl, 1% (w/v) BSA, 0.05% (v/v) Tween-20, and then water, the sections were post-stained with uranyl acetate and lead citrate. For controls, incubation was carried out with a preimmune rabbit serum instead of the CA antiserum.

For conventional ultrastructural observations, samples were fixed first in glutaraldehyde (2.5%, v/v) and then in osmium tetroxide (1%, w/v). After dehydration in alcohol and propylene oxide, samples were embedded in Epon 812 (TAAB, Berkshire, UK).

Sections were examined with a Jeol electron microscope operating at 80 kV (Cirad, Montpellier, France).

Protein determination. Protein was determined according to Lowry et al. (1951) using a calibration curved obtained with BSA.

Chlorophyll determination. Chlorophyll content was measured by the method of Lichtenthaler and Wellburn (1983).

Results

Characterization of CA activity in potato leaf extracts. Following potato chloroplast purification on Percoll gradients, intact chloroplasts were lysed and soluble (stroma) proteins were separated from membrane by centrifugation. Each fraction was assayed for CA activity by mass spectrometry. About 97% of the CA activity was found in the soluble protein fraction (Table 1). When

Table 1. Specific activity of CA in crude extract and in chloroplast extract from potato leaves

	Ratio mg protein · mg^{-1} chlorophyll	CA activity	
		U · mg^{-1} protein	U · mg^{-1} chlorophyll
Crude extract	20	308	6160
Chloroplasts			
Lysed chloroplasts	10	295	2950
Stroma	6.25	372	3720
Membranes	3.75	12	120

Note. Upon chloroplast lysis and centrifugation, the supernatant corresponds to stroma and the pellet to membranes. Activities were reported either on the basis of the chlorophyll present in the crude extract or the lysed chloroplast, or on the basis of protein present in each fraction.

expressed on a chlorophyll basis, CA activity was about twofold higher in total extract than in the chloroplast extract, suggesting that CA activity is also present in the extrachloroplastic compartment. Total leaf proteins and stromal proteins were assayed for CA activity using native polyacrylamide gels and the pH-indicating dye, bromcresol purple (Fig. 1). Two bands of CA activity were detected after electrophoresis of a crude extract prepared from mature potato leaves (Fig. 1, lane 1). Only a single band of CA activity was detected after separation of soluble proteins from purified chloroplasts (Fig. 1, lane 2). Both the chloroplastic band and the lower band of the total extract exhibited the same mobility. Increasing the concentration of the protein extracts up to fivefold did not reveal any additional band. These results confirm the presence of compartment-specific CA activity in potato leaf tissue. The upper band corresponding to extrachloroplastic CA activity represented about 13% of the total activity as estimated by densitometric scanning of photographs of native gels (Fig. 1, lane 1).

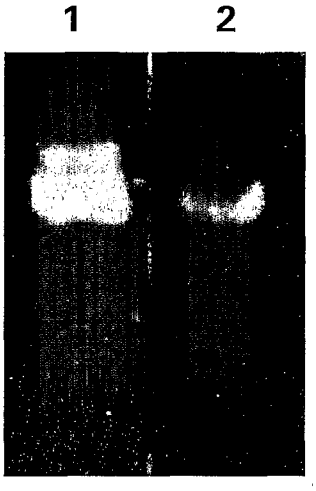

Fig. 1. Detection of potato leaf CA activity on a polyacrylamide gel. The CA activity was detected on a polyacrylamide gel (10%) with CO_2 as the substrate and bromcresol purple to indicate H^+ formation. The developed gel was fixed by freezing to −70 °C, and the enzyme bands were detected by their low-temperature fluorescence in ultraviolet light. *Lane 1*, crude extract (10 µg); *lane 2*, soluble chloroplastic (stroma) proteins (2.5 µg)

Chloroplastic CA purification. Chloroplastic CA purification was achieved from a stromal extract using anion exchange (Resource Q) and hydrophobic interactions (Phenyl-Superose) chromatography (Table 2). Anion-exchange chromatography was performed at pH 8.5. At this pH, most of the CA activity eluted as a single peak. This step resulted in a 25-fold increase in specific activity; it appeared to be the most efficient purification step. Hydrophobic interactions chromatography was used as a final step to complete the purification. A 50-fold purification was achieved by this relatively rapid procedure. The purity of the enzyme preparation was assessed by SDS-PAGE and Coomassie blue staining at various stages of purification (Fig. 2). The final product migrated as a doublet in the 13% SDS-polyacrylamide gel corresponding to molecular masses of 27 kDa and 27.5 kDa, respectively (Fig. 2, lane 3). The double faint bands observed in the 50- to 60-kDa region were attributed to staining artefacts due to the presence of 2-ME in the sample loading buffer, since the same bands were observed with the loading buffer alone (data not shown).

N-Terminal amino acid sequence of the chloroplastic CA. The 27.5- and 27-kDa polypeptides separated by SDS-PAGE and transferred onto PVDF membranes were analyzed to determine the N-terminal amino acid sequence (Fig. 3). The two potato polypeptides had identical N-terminal sequences except that the 27-kDa polypeptide was three amino acids shorter than the 27.5-kDa polypeptide. At this stage, we cannot exclude that the 27 000-M_r band could be formed by partial degradation of the 27 500-M_r polypeptide although protease inhibitors PMSF and BAM were routinely included in the extraction and chromatography buffers. Potato chloroplastic CA N-terminal sequences were aligned together with the sequences of tobacco, pea, spinach, and *Arabidopsis* chloroplastic CA (Fig. 3). Sequence similarities were observed, with the most striking homology found with CA from tobacco which also belongs to the Solanaceous plant family; among 18 aligned residues, 14 are identical and 2 are similar.

Purification of the cytosolic CA. In order to characterize the cytosolic CA, we carried out its purification from a total leaf extract. Cytosolic CA purification was achieved using a procedure very similar to that used to purify the chloroplastic CA except that an additional

Table 2. Purification of CA from potato leaf chloroplasts

Purification step	Protein (mg)	Total activity (U)	Specific activity ($U \cdot mg^{-1}$)	Purification factor	Yield (%)
Stroma	32	240	7.5		100
Ammonium sulphate	7.4	240	32	4.3	100
Resource Q	0.726	142	195	26	60
Phenyl Superose	0.061	22.7	372	49.6	10

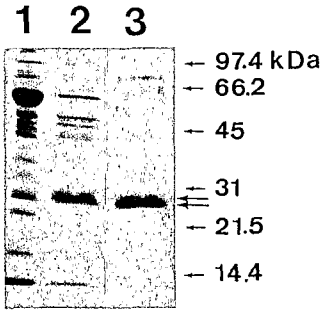

Fig. 2. Coomassie-blue-stained SDS-polyacrylamide gel of purified chloroplastic CA from potato leaves. *Lane 1*; crude leaf extract (10 µg); *lane 2*, Resource Q chromatography (10 µg); *lane 3*, Phenyl-Superose chromatography (5 µg). Protein standards were phosphorylase B (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soyabean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa)

gel-filtration step was added between the anion-exchange chromatography and the hydrophobic-interaction-chromatography steps. At each step, the cytosolic CA was detected in the fractions using non-denaturing polyacrylamide gels and bromcresol purple. In order to eliminate chloroplastic CA that persistently co-eluted with cytosolic CA, fractions enriched in cytosolic CA activity were pooled after each step and used for the subsequent purification step. For this reason, the recovery yield was low (about 0.05%) and did not reflect the relative abundance of cytoplasmic CA in potato leaf. The purity of the enzyme preparation was assessed by SDS-PAGE and Coomassie staining (Fig. 4). The cytosolic CA appeared as a single band corresponding to a molecular mass of 30 kDa. Two attempts to obtain N-terminal sequence were unsuccessful suggesting that the N-terminus was blocked.

Antiserum and immunoblot analysis. A peptide corresponding to the 15-amino-acid N-terminus (ELKSADGSKAFDPVE) of the chloroplastic CA were synthesized, conjugated to KLH and used for raising a polyclonal antibody in rabbit. The antiserum was used to probe blots of total leaf proteins and stromal proteins following separation on SDS-PAGE (Fig. 5, lanes 1, 2). The major (M_r 27 000) and the minor (M_r 27 500) peptides detected in the purified chloroplastic CA (Fig. 5, lane 3) also appeared in the crude extract (Fig. 5, lane 1) and in the stromal fraction (Fig. 5, lane 2). This indicates that the chloroplastic protein was not subjected to proteolytic attack during the different purification steps. The antibodies raised against the chloroplastic CA N-terminal sequence also cross-reacted with the cytosolic CA (Fig. 5, lane 4), suggesting that both CAs are immunologically related. The 30-kDa band was also detected in the crude extract showing that no proteolytic degradation of this peptide occurred during the purification. The antiserum was then used to probe blots of native gradient polyacrylamide gels. A lower band appeared in the total leaf extract (Fig. 6, lane 1) and in the stromal extract (Fig. 6, lane 2) at a position corresponding to that of the purified chloroplastic enzyme (Fig. 6, lane 3). The antiserum reacted with an upper band corresponding to the purified native cytosolic isoform (Fig. 6, lane 4) and present in the crude extract (Fig. 6, lane 1).

Physicochemical properties of the two CA isoforms. The full size of both CA isoenzymes was determined by probing native polyacrylamide gradient gels with the antiserum as shown in Fig. 6. Molecular-mass standards loaded onto the same gel and revealed by Ponceau S staining allowed the determination of CA molecular mass (Fig. 7). The M_r of the chloroplastic CA was estimated to be 220 000 and the extrachloroplastic CA to be 255 000. As a single band was detected in the purified chloroplastic enzyme fraction following electrophoresis on a non-denaturing gel (Fig. 6, lane 3), while two bands were detected following electrophoresis on a denaturing gel (Fig. 2, lane 3 and Fig. 5, lane 3), we concluded that the chloroplastic enzyme is an octamer composed of two different subunits. The subunit with an M_r estimated at 27 000 Da which appeared dominant on SDS-PAGE gels (Fig. 2, lane 3) represents the major component of the native chloroplastic enzyme whereas the 27 500-Da subunit represents the minor component of the enzyme. The extrachloroplastic CA is also an octamer with the 30-kDa polypeptide as the monomer.

The inhibition of CO_2-hydration activity of the two potato CAs by sulfonamides and monovalent anions was determined by mass spectrometry (Table 3). Both CA isoforms were found to be similarly sensitive to sulfonamides and monovalent anions. Ethoxyzolamide, acetazolamide and azide were found to be more potent inhibitors than nitrate and iodide. Chloride at physiological concentrations did not significantly inhibit potato CAs.

The K_m for the CO_2-hydration reaction was determined by mass spectrometry in 20 mM barbitone buffer (pH 7.5) containing 1 mM Na_2-EDTA, 10 mM DTT, 50 mM Na_2SO_4. The measured values, i.e. 9.4 mM for the chloroplastic isoform and 10 mM for the cytosolic CA, were not significantly different.

Potato CAs were found to depend on the presence of a reducing agent to maintain catalytic activity. Very low

ORIGIN	TYPE	SEQUENCE	DETERMINATION
Solanum tuberosum[a]	chloroplastic	SADGS----KA-FDPVEHMR	N-ter
Solanum tuberosum[a]	chloroplastic	ELKSADGS----KA-FDPVEHMR	N-ter
Nicotiana tabacum[b]	chloroplastic	ELQSSDGS----KP-FDPVEHMK	cDNA
Arabidopsis thaliana[c]	chloroplastic	ALQTGTSSD--KKA-FDPVETIK	cDNA
Arabidopsis thaliana[d]	cytosolic	DVQAASSSD--SKS-FDPVERIK	cDNA
Pisum sativum[e]	chloroplastic	QLGTTSSSDGIPKS--EASERIK	cDNA, N-ter
Spinacea oleacera[f]	chloroplastic	EL--ADGGT--PSASY-PVQRIK	cDNA, N-ter

[a] This work; [b] Majeau and Coleman 1992; [c] Raines et al. 1992, Fett and Coleman 1994; [d] Fett and Coleman 1994; [e] Roeske and Ogren 1990, Majeau and Coleman 1991, Johansson and Forsman 1992; [f] Burnell et al. 1990, Fawcett et al. 1990

Fig. 3. Multiple alignment of N-terminal amino acid sequences of plant CAs. Multiple alignment was performed by a software PC-GENE (Intelligenetics, Bruxelles, Belgium). The determination column refers either to the direct analysis of the N-terminal amino acid sequence of the purified protein (*N-ter*) or to the putative terminus sequence deduced from sequencing of cDNA (*cDNA*). Gaps are indicated as (–)

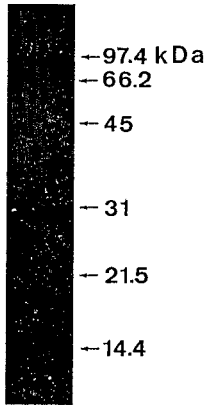

Fig. 4. Coomassie blue-stained SDS-polyacrylamide gel of purified cytosolic CA from potato leaves. Protein standards were as described in Fig. 2

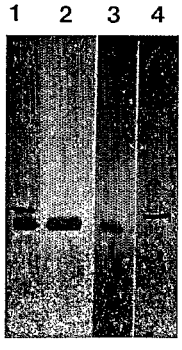

Fig. 5. Immunodetection of CA in various protein extracts from potato leaves. Proteins were separated by SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The membrane was then probed with the CA antiserum. *Lane 1*, crude extract (10 μg); *lane 2*, stromal protein extract (5 μg); *lane 3*, purified chloroplastic CA (1 μg); *lane 4*, purified cytosolic CA (1 μg)

activities were obtained when purification of both isoforms was carried out in the absence of reducing agent (data not shown), but incubation with DTT or cysteine or reduced glutathione almost fully restored the activity. In contrast, 2-ME at low concentration (1 mM) rapidly inactivated (50% of inhibition) CA isoenzymes.

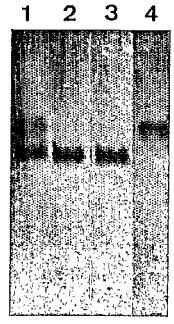

Fig. 6. Immunodetection of CA in various protein extracts from potato leaves. Proteins were separate by electrophoresis on a 5–15% polyacrylamide gradient gel in non-denaturing conditions and electrotransferred onto a nitrocellulose membrane. The membrane was then probed with the CA antiserum. *Lane* 1, crude extract (10 μg); *lane* 2, stromal protein extract (5 μg); *lane* 3, purified chloroplastic CA (1 μg); *lane* 4, purified cytosolic CA (1 μg)

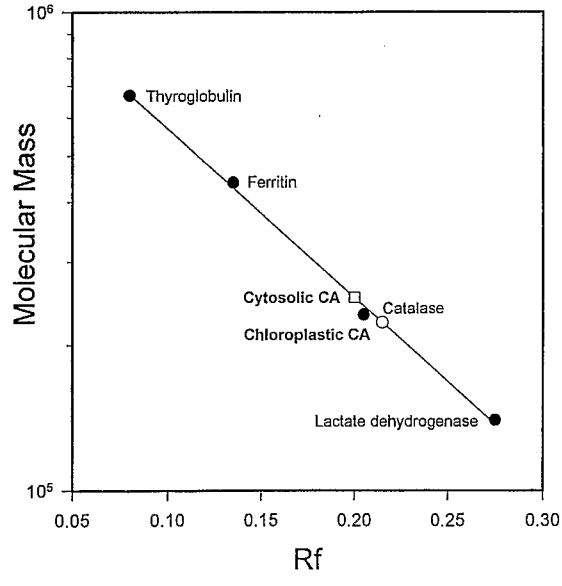

Fig. 7. Molecular-mass determination of native chloroplastic (○) and cytosolic (□) CA isoforms from potato leaves. Purified proteins were run on a non-denaturing polyacrylamide gradient gel (5–15%) and stained with Coomassie blue. High-molecular-mass standards (lactate dehydrogenase, 140 kDa; catalase, 232 kDa; ferritin, 440 kDa and thyroglobulin, 669 kDa) were run in a separate lane and used to construct a calibration curve by plotting the R_f values versus the logarithms of their corresponding molecular mass

Immunocytolocalization. In order to confirm the distribution of CA within the cell, immunocytochemical analysis was performed. Ultrathin sections of potato leaf fragments were probed with the partially purified CA antiserum followed by goat anti-rabbit IgG conjugated to colloidal gold (Figs. 8, 9). No labelling was seen over sections from tissue fixed with glutaraldehyde (2.5%), post-fixed with osmium tetroxide and embedded in Epon, although cell structures were well preserved (Fig. 8A). Because the presence of osmium and high concentrations of glutaraldehyde have been shown, in some cases, to alter the antigenicity of a target protein, the tissue was fixed with low concentrations of glutaraldehyde (1%) and without post-fixation with osmium. Under these conditions, the antigenicity of CA was preserved, although the endomembrane system was not as well defined (Fig. 8B, C and Fig. 9A–C). Numerous gold particles were observed over ultrathin sections made from leaf tissue. The chloroplasts and the cytosol of both spongy (Fig. 8B, C) and palisade (Fig. 9A, B) parenchyma cells were found to be similarly labelled. Within the plastid compartment, gold particles appeared to be preferentially located in the stromal areas surrounding the grana and thylakoids (Fig. 8B and Fig. 9B). However, some gold particles were found associated with the membranes (thylakoids and

Table 3. Inhibition of potato CAs by sulfonamides and monovalent anions. The CA activity was determined by mass spectrometry. Measurements were performed in 20 mM barbitone buffer (pH 7.2) containing 1 mM Na_2-EDTA, 10 mM DTT, 50 mM Na_2SO_4 at 25 °C. The I_{50} values correspond to the concentrations giving 50% inhibition and were determined by semi-log plot of percentage of inhibition versus log concentration of inhibitors

Inhibitor	I_{50} (mM)	
	Chloroplastic CA	Cytosolic CA
Ethoxyzolamide	0.015	0.021
Acetazolamide	0.05	0.04
Cl^-	10	12
I^-	1.2	1.5
NO_3^-	1.2	1.25
N_3^-	0.044	0.034

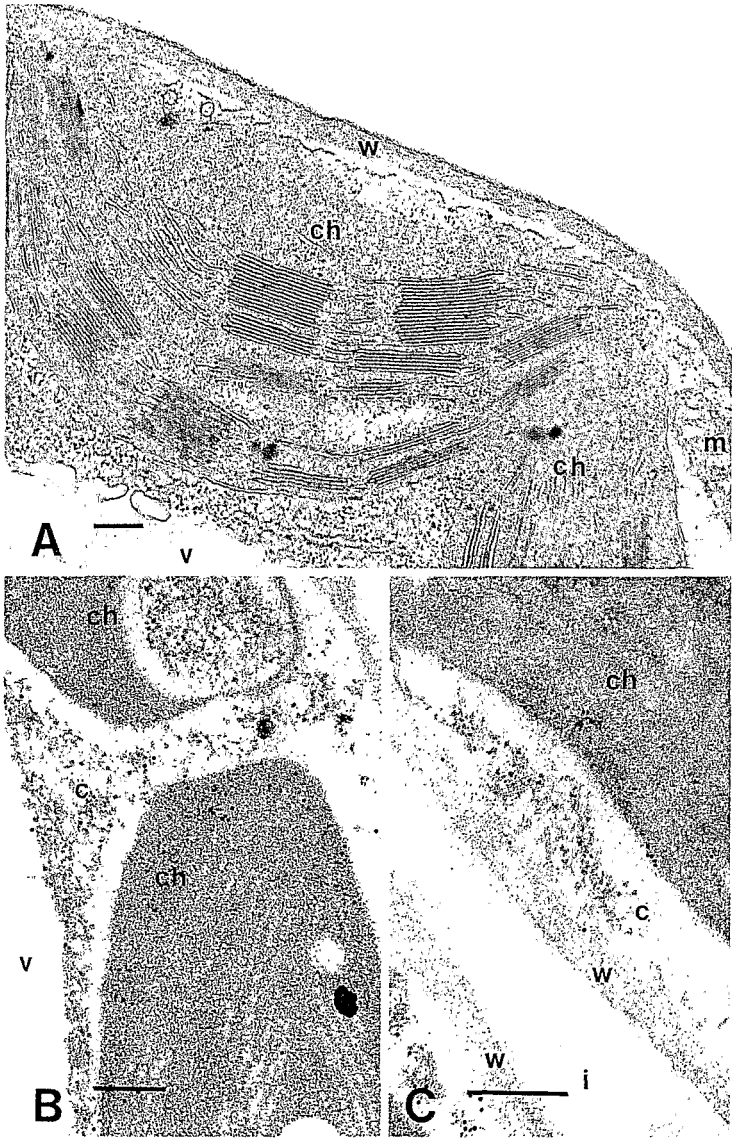

Fig. 8A–C. Transmission electron micrographs showing the localization of CA in spongy parenchyma cells of potato leaves. Tissues were fixed using either 2.5% glutaraldehyde plus post-fixation in osmium tetroxide (**A**) or 1% glutaraldehyde and 4% paraformaldehyde (**B, C**). Fixed tissues were then embedded in LR-White resin (**A–C**). **A** No gold particles are observed over cell organelles and the cell walls. **B** Numerous gold particles are present in the chloroplast and the cytosol. **C** Labelling can be seen over the chloroplast and the cytosol of two adjacent cells. Few gold particles are detected over the plant cell-walls and the intercellular space. *c*, cytosol; *ch*, chloroplast; *i*, intercellular space; *m*, mitochondrion; *v*, vacuole; *w*, cell wall. Bars = 0.2 μm

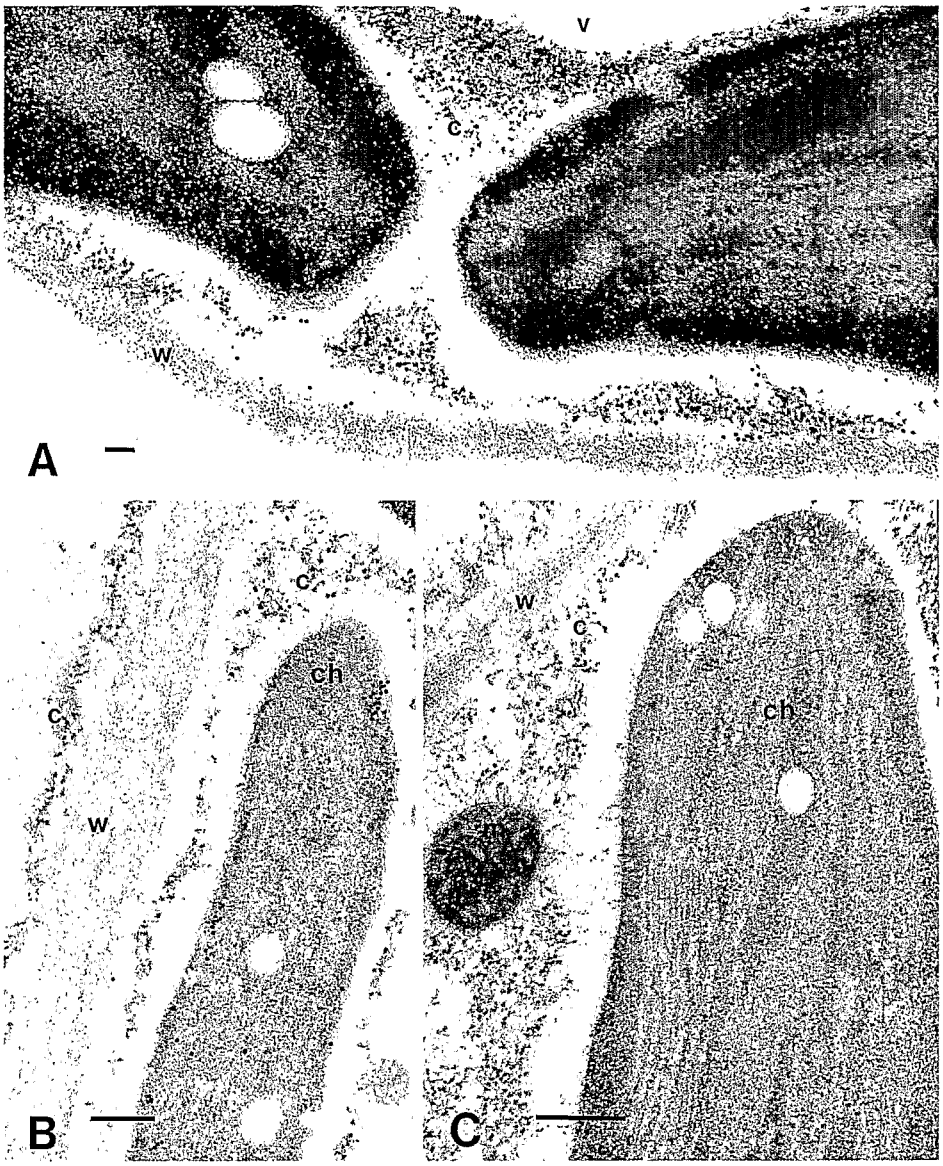

Fig. 9A–C. Transmission electron micrographs showing the localization of CA in palisade parenchyma cells of potato leaves. Tissues were fixed using 1% glutaraldehyde and 4% paraformaldehyde. **A, B** Immunolocalization of CA in ultrathin sections of LR-White-embedded tissues. **A** The gold particles are evenly distributed over two chloroplasts and in the cytosol, while the plant cell wall is devoid of any appreciable labelling. **B** In this micrograph the labelling is located at the edge of the chloroplast. No gold particles are present over the plant cell wall. **C** Control; sections were incubated with the preimmune rabbit serum, no appreciable labelling is present over the cell, including the organelles. *c*, cytosol; *ch*, chloroplast; *m*, mitochondrion; *v*, vacuole; *w*, cell wall. Bars = 0.2 μm

grana; Fig. 9A). In the cytosol, an even distribution of gold particles was observed. No appreciable labelling was seen over the cell walls, the mitochondria, the vacuoles or the nucleus. The use of preimmune rabbit serum for assessing immunocytochemical specificity did not yield any appreciable labelling over the plant cell, including the chloroplasts (Fig. 9C).

Discussion

In this study, we identified, purified and characterized two compartment-specific isoforms of potato CA. The presence of multiple isoforms of CA in leaf protein extracts was previously noticed in studies conducted with various higher-plants species (Atkins et al. 1972). Chloroplastic CA has been purified to electrophoretic homogeneity from various C_3 plants including spinach (Pocker and Ng 1973; Kandel et al. 1978; Burnell et al. 1990; Fawcett et al. 1990), pea (Kiesel and Graf 1972; Majeau and Coleman 1991; Johansson and Forsman 1992), parsley (Tobin 1970) and chick-pea (Guliev et al. 1992). In contrast cytosolic CA has been largely ignored, probably because it is thought to represent only a negligible part of total CA activity in C_3 leaf tissue. The most-convincing data on the characterization of a C_3 plant cytosolic CA are probably those published by Kachru and Anderson (1974). These authors used isoelectric focusing and characterized two isoforms of pea CA from chloroplastic and cytosolic protein extracts. However, the subcellular compartmentation of the two pI-distinct isoforms was not confirmed in this study. We have purified chloroplastic CA from a preparation of potato chloroplasts, obtaining a good yield with a high level of purity. Purification of cytosolic CA was conducted from a crude leaf extract and provided cytosolic CA at a high level of purity although the yield was fairly low. Such a low yield is probably due to the fact that cytosolic and chloroplastic CAs exhibit similar physiochemical properties and subsequently showed similar behaviour during the different steps used for

purification. The strong similarity between the two isoforms was confirmed through inhibitor studies. Indeed, no significant difference was found with regard to the inhibitory effect of monovalent anions (I^-, NO_3^-, Cl^- and N_3^-) and sulfonamides (ethoxyzolamide and acetazolamide), suggesting that both isoforms have a similar active-site conformation. As found for chloroplastic CA from spinach (Pocker and Ng 1973) and pea (Johansson and Forsman 1992), potato CA isoforms appear less sensitive to the sulfonamide group of inhibitors than the animal enzymes. Sensitivity to azide is relatively high, whereas chloride has little effect at physiological concentrations. Contradictory effects of sulphydryl-reducing agents on plant CAs have been reported (Reed and Graham 1981). Pocker and Ng (1973) found spinach CA to be stable in the absence of such agents and inactivated by 10 mM 2-ME while Cybulski et al. (1979) found that, at 100 mM, 2-ME made the spinach enzyme retain its activity. Pea CA needs to be reduced to be active, but 2-ME induces a rapid loss of activity when added to the buffer systems using in stop-flow measurements (Johansson and Forsman 1993). Oxidised, inactive potato isoenzymes could be activated by the addition of an SH-agent such as cysteine, reduced-glutathione and DTT, but 2-ME appears to be a potent inhibitor at millimolar concentration. This effect of 2-ME will need more investigation to be explained.

The oligomeric structure of CA from dicotyledonous plants is generally accepted, but different values for the native molecular weight of CA from pea and spinach have been reported and the number of monomers remains uncertain (Reed and Graham 1981). Our results from electrophoresis indicate an M_r of 255 000 for the potato cytosolic CA and 220 000 for chloroplastic CA. The cytosolic isoform is composed of eight identical subunits (M_r 30 000). The chloroplastic CA is also composed of eight subunits but two different monomers were identified by SDS-PAGE. The N-terminal amino acid sequence of both chloroplastic monomers was determined. They were found to be identical except that one polypeptide is three amino acids shorter than the other, suggesting either that an alternative processing occurred during transit peptide removal or that the peptides are encoded by two different genes. The latter hypothesis could be sustained by the identification and characterization of two potato genes encoding the corresponding polypeptides, an approach currently in progress in our laboratory. The former hypothesis was already suggested by Johansson and Forsman (1992) who found the pea CA to be three amino acids shorter than reported by Roeske and Ogren (1990). As a single chloroplastic CA gene was identified in pea by Majeau and Coleman (1994), Johansson and Forsman (1992) speculated that the cleavage occurring during the removal of the transit peptide from pea CA precursor might not be directed by a specific sequence but rather by a secondary or tertiary structure.

The recognition of cytosolic CA by polyclonal antibodies directed against the N-terminus of potato chloroplastic CA suggests that the isoenzymes are immunologically related. Fett and Coleman (1994) have isolated two *Arabidopsis thaliana* cDNA clones encoding putative cytosolic and chloroplastic CAs. Comparison of the predicted amino acid sequences indicated a 84.6% identity. Alignment of the N-terminal sequence of the *A. thaliana* chloroplastic CA with the corresponding region of the *A. thaliana* cytosolic CA shows that among 20 amino acids, 12 are identical and 4 are similar. It seems likely that in potato, also, both isoenzymes exhibit similar homology. Again, the characterization of the corresponding cDNAs will confirm the immunological similarity.

Tissue and intracellular location of the CA have been demonstrated using a histochemical technique (Hansson 1967). The staining procedure, which results in the precipitation of CoS at the site of CA, has been used to show the presence of the enzyme in the chloroplasts of both C_3 and C_4 plants (Triolo et al. 1974). However, a critical evaluation has shown that the cobalt method is not specific for CA detection in tissues although it is satisfactory for the isolated enzyme (Muther 1972). Here, we used antibodies to localize CA within potato leaf cells. As the antibodies recognize chloroplastic and cytosolic isoenzymes, both isoforms were simultaneously detected. In such experiments, the precursor of chloroplastic CA was not detected. If a precursor of the chloroplastic CA had been labelled by the antiserum, then a higher-molecular-weight polypeptide should have been immunolabelled after SDS-PAGE of the crude extract. Yet this was not the case (Fig. 5). In the cytosol, CA was found to be randomly distributed, while within the chloroplast CA was mainly located in the stroma. However, some labelling was found associated with the membranes of the chloroplast (grana and thylakoids). This result is consistent with the detection of some CA activity in the membrane fraction using mass spectrometry (Table 1). Membrane-associated CA activity has been reported in maize thylakoids by Stemler (1986) who suggested that one of the polypeptide component of the photosystem II complex could be a form of CA. Within the cells, we noticed that gold labelling did not reflect the large abundance of chloroplastic CA (87% of total activity) versus cytoplasmic CA (13%), as was estimated by scanning activity gels. Indeed, cytosol and chloroplast stroma occupy different volumes in mesophyll cells. Winter et al. (1994) estimated the subcellular volumes in leaves of spinach and found that stroma and cytosol represented 9.5% and 3.4% of the mesophyll cell volume, respectively. If we assume a similar ratio between chloroplastic and cytosolic volumes in potato, we estimate that the CA concentration in the chloroplast is about twofold higher than in the cytosol. Indeed, the first determination of each CA isoform activity from a crude extract led to an overestimation of the chloroplastic CA activity because the volume of each compartment was not taken into account. These results highlight cytosolic CA abundancy and reinforce the interest in determining the role of cytosolic CA. As suggested by Fett and Coleman (1994), it is possible that cytosolic CA is required for catalysis of HCO_3^- synthesis, the substrate for a cytosolic phosphoenolpyruvate carboxylase in a role similar to that evoked for the mesophyll cell-localized CA in C_4 plants. The anaplerotic synthesis of carbon skeletons by a C_3 phosphoenolpyruvate carboxylase in the cytoplasm is required for amino acid biosynthesis as well as replenishment of Krebs cycle intermediates.

In conclusion, from this work and others, it is clear that two biochemically-realted isoforms of CA are present in

leaves. Located in two different cell compartments, their roles remain unknown. Using the methods of characterization presented here, the study of their differential regulation is currently underway.

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