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Identification and Levels of 2′-Carboxyarabinitol in Leaves[1]

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ABSTRACT

2′-Carboxyarabinitol 1-phosphate (CA1P) is a naturally occurring inhibitor of ribulose-1,5 bisphosphate carboxylase/oxygenase activity. A chloroplast phosphatase has previously been identified that degrades CA1P *in vitro* to carboxyarabinitol (CA) plus phosphate, but CA has not yet been detected in plants. Here, we detail procedures to isolate and assay CA from leaves and utilize mass spectrometry to demonstrate for the first time that CA is present in plants. CA was present in leaves of all 13 species examined, including those of C_3, C_4, and Crassulacean acid metabolism photosynthetic subgroups. CA was present both in species with high levels of CA1P (*e.g. Phaseolus vulgaris, Lycopersicon esculentum, Beta vulgaris*) as well as in species with low levels of CA1P (*e.g. Spinacea oleracea, Triticum aestivum*). CA levels in the light were sometimes greater than those in the dark. Bean leaves had the most CA of any species tested, with levels in the light approaching 1 micromole per milligram of chlorophyll. In illuminated bean leaves, about 63% of the CA is located outside the chloroplast. CA is one of only a few branched chain sugar acids to be identified from plants.

CA1P[2] (also known as 2-C-hydroxymethyl-D-ribonic acid 2′-P and hamamelonic acid 2′-P; ref. 2) is a naturally occurring, transition state analog of the Rubisco carboxylation reaction (3, 6). CA1P inhibits *in vitro* activity of both cyanobacterial and higher plant Rubisco (20) and occurs in leaves of all plant species thus far examined (11). CA1P accumulates to varying levels in many species under low light or dark conditions, with the highest amounts reported in leaves of *Phaseolus vulgaris* (1.5 mol CA1P mol^{-1} Rubisco catalytic site; ref. 11).

The biochemical pathway for CA1P metabolism remains largely unknown. CA1P can be degraded *in vitro* by a specific chloroplast phosphatase to yield CA (5, 9), a compound that has not yet been identified in plants. Schramm *et al.* (15) have demonstrated that 2-C-hydroxymethyl-pentonic acid does occur in leaves of *P. vulgaris*, but apparently it has not been determined whether this acid has the *ribo* or *arabino* configuration. There is one report that describes the occurrence of CA in a strain of *Pseudomonas* grown on hamamelose plus citrate, in which CA was an oxidative product that was not further metabolized (22).

CA can be synthesized *in vitro* in a carboxyl-labeled ^{14}C form (6). Thus, we were able to develop procedures to detect nmol amounts of CA based on the UV absorbance of its carbonyl group, assay CA by using isotope dilution principles, and purify CA from leaf extracts by liquid chromatography using anion exchange and ion exclusion separation techniques. In this study, we demonstrate that CA occurs in leaves of 13 higher plant species, quantitate the levels of CA under both light and dark conditions, and determine the intracellular distribution of CA within leaves of *P. vulgaris*.

MATERIALS AND METHODS

Plant Material

Plants were grown as described in ref. 11. CAM expression in *Mesembryanthemum crystallinum* was induced by irrigating the plants 6 weeks with 0.5 M NaCl, and was monitored by the increase in leaf activity of PEP carboxylase (23).

Synthesis and Purification of [2-^{14}C]CA

[2-^{14}C]CABP (specific radioactivity, approximately 1.5 Ci/mol) was synthesized and purified as described elsewhere (7, 11, 14). [2-^{14}C]CA was prepared by treating the anionic form of [^{14}C]CABP with alkaline phosphatase (Type VII-NL, Sigma Chemical Co.) in a 5-mL solution containing 50 mM Na_2CO_3 (pH 10), [^{14}C]CABP, and 5 units enzyme/μmol CABP. After 8 h at 25°C, the reaction was stopped by passing the solution through a 5-mL column of Dowex 50 (H^+). The CA anion was then loaded on a 12.5-mL column of QAE-Sephadex (formate form) and eluted with a 300-mL gradient of 0 to 250 mM HCO_2H. CA eluted after about 60 mL, and P_i remained bound to the column. HCO_2H was removed by rotary evaporation. CA was lactonized by drying under acidic conditions (pH 1.5, HCl) and was finally resuspended in 5 mM H_2SO_4 (4–5 μCi/mL).

Leaf CA Measurements by Isotope Dilution

Leaves were collected into liquid N_2, the principal veins were removed, and 10 g of fresh weight were added to boiling 80% (v/v) ethanol that contained 0.2 μmol of [2-^{14}C] CA. As previously described for CA1P (11), [^{14}C]CA was extracted and the solution passed through a 35-mL column of Dowex 50 (H^+). The eluate was concentrated, and the CA anion was chromatographed on QAE-Sephadex as described

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[2] Abbreviations: CA1P, 2′-carboxyarabinitol-1-P; CA, 2′-carboxyarabinitol; CABP, 2′-carboxyarabinitol-bisP; PEP, phospho*enol*pyruvate; 2D-TLE/TLC, two-dimensional thin-layer electrophoresis/thin-layer chromatography.

above. Appropriate fractions were pooled and then dried by rotary evaporation. The CA anion was then loaded onto a 12.5-mL column of Dowex 1 (formate form) and eluted with the HCO_2H gradient described above. CA eluted after about 80 mL, and at this stage did not contain any visible pigments. The sample was dried, converted to the lactone form, and finally resuspended in 0.25 mL of 5 mM H_2SO_4. Samples were stable for at least 1 year at 4°C.

CA samples were fractionated by HPLC (Spectra Physics SP 8750) using an Aminex HPX-87H organic acid column (Bio-Rad). The mobile phase was 5 mM H_2SO_4 and flow rate was maintained at 0.4 mL/min. The eluate was monitored for $A_{190\ nm}$ (Kratos SF 757 detector) and ^{14}C content (Radiomatic Beta One flow-through scintillation detector). A [^{14}C]CA standard was tested daily to determine the peak height ratio of ^{14}C to $A_{190\ nm}$; the daily relative SD of this ratio was about 1.1%. Sample peak height ratios were determined from two or three injections, and sample specific radioactivity (Ci/mol) was calculated as the proportion of the sample to the standard peak height ratios multiplied by the standard's specific radioactivity. The leaf CA content was then calculated as follows:

nmol endogenous CA/extract =

$$(\text{nmol}[^{14}\text{C}]\text{CA standard added}) \times \frac{(C_o - C)}{C},$$

where C_o equals the specific radioactivity of the standard and C equals the specific radioactivity of the sample. CA amounts were expressed per g fresh weight or per mg Chl (25).

Alternate CA Sample Preparation

In one experiment, about 20,000 dpm of bean leaf extract were largely purified by anion exchange chromatography (see above) and then subjected to 2D-TLE/TLC (12, 16). [^{14}C]CA was located by autoradiography and extracted in H_2O, the lactone was prepared, and the specific radioactivity was measured.

MS

The CA standard (1.46 Ci/mol) or bean CA sample (0.0373 Ci/mol) was collected after HPLC, and the mobile phase was removed by chromatography on QAE-Sephadex (see above). The ^{14}C-containing fractions were dried twice by rotary evaporation. Electron impact mass spectra of the CA standard and plant sample were obtained with a Finnigan 4023 gas chromatograph mass spectrometer used in a 70 eV EI mode. For pyrolysis studies, samples were resuspended in 25% ethanol and introduced by direct probe at a source temperature of 270°C.

CA Intracellular Localization

Protoplasts and chloroplasts were isolated from leaves of *Phaseolus vulgaris* (10). These were illuminated (0.8 mg Chl/sample) at 1100 μmol quanta m^{-2} s^{-1} for 10 min, frozen in liquid N_2, and then assayed for CA content using the isotope dilution assay.

Nonaqueous fractionation of leaves of *P. vulgaris* was done by a modified procedure of Gerhardt and Heldt (4) as described previously (19). Leaf material was collected into liquid N_2 after treatment for 12 h dark or 12 h dark followed by 2 h light. After gradient centrifugation, four 2-mL fractions were collected. One-third of each fraction was used for marker enzyme assays, and two-thirds of each fraction were used for measuring CA content by the isotope dilution assay. The subcellular distribution of CA was calculated as described by Heineke *et al.* (8). In this method, each possible distribution (in 1% increments for each of the stroma, cytosol, and vacuole) was used to predict the distribution of CA through the gradient. The distribution that gave the closest fit to the observed data was used.

RESULTS

Elution of the [^{14}C]CA standard from an Aminex HPLC organic acids column was examined by monitoring the effluent for $A_{190\ nm}$ and ^{14}C content using a flow-through system. When the standard was injected in its lactone form, a single $A_{190\ nm}$ and ^{14}C peak was observed in the eluate (Fig. 1, A and B), with a retention factor of 0.594. Absorbance was linear with concentration, and the apparent extinction coefficient was 15.9 M^{-1} cm^{-1} (data not shown).

To perform the isotope dilution assay, exogenous [^{14}C]CA was added at the initial point of leaf extraction, and the [^{14}C]CA then purified. The plant samples had enough [^{14}C]CA added initially to allow an easily measured $A_{190\ nm}$ signal after purification (≥15X the detection limit), even if there was no additional CA within the leaf extract. A representative sample elution profile (Fig. 1, C and D) indicates both that the CA samples were highly purified prior to HPLC and that the amount of ^{14}C per unit $A_{190\ nm}$ was reduced relative to the CA standard (*i.e.* the exogenous [^{14}C]CA was diluted with [^{12}C]CA from the leaves; compare with Fig. 1, A and B). That the Aminex column was effective in separating a mixture of different acids was verified by adding known standards to a purified leaf sample (data not shown).

Two additional separation analyses were employed to validate the conclusion that the plant samples did contain endogenous CA. First, partially purified sample CA was subjected to 2D-TLE/TLC, a procedure that separates CA from its epimer carboxyribitol and from other metabolites (12). The specific radioactivity of the recovered [^{14}C]CA was equivalent to that of the sample prior to the TLE/TLC treatment (data not shown), thus indicating that the measured CA sample was pure. Second, the CA standard and plant CA sample were each collected after HPLC and analyzed by MS. Sample identity was confirmed as CA, and its purity established by obtaining a fragmentation pattern after pyrolysis identical to that of the CA standard (Fig. 2). Additionally, the mass spectrum of the trimethylsilyl-derivatized plant sample was equivalent to that previously published for CA lactone (data not shown; see figure 2 in ref. 2). It should be noted that the relative abundance of standard present in the plant sample was only 2.5%.

After validating the identity and purity of the plant CA sample, we assayed for the presence and amount of CA in leaves of 13 species (Table I). All species examined (C_3, C_4, CAM) were found to contain CA, with the highest amounts

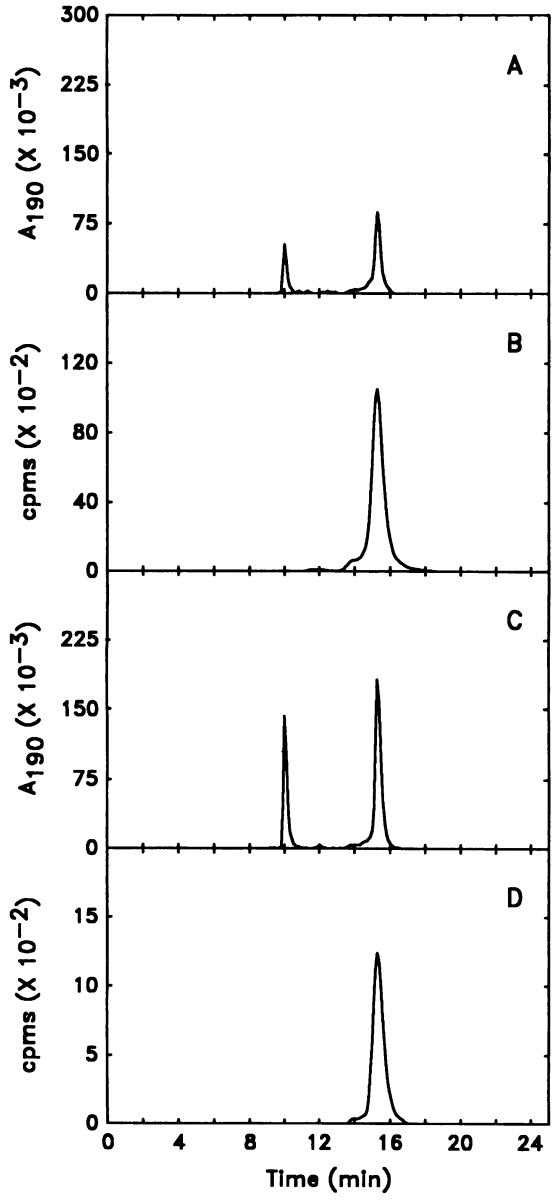

Figure 1. HPLC chromatograms of [2-^{14}C]CA standard (A, B) and CA purified from bean leaves (C, D) after injection onto an Aminex HPX-87H HPLC column; A and C, $A_{190\ nm}$; B and D, ^{14}C content. The plot of UV absorbance was delayed 60 s; actual CA retention time was 14.3 min. The CA detection limit was 1.1 nmol. The initial UV peak (t_r = 8.9 min) was always present after injecting the standard or plant sample and corresponds to the column void volume. The identity of this material is unknown. The specific radioactivity of the standard was 1.46 Ci/mol, and that of the plant sample was 0.81 Ci/mol (due to having added ^{14}C-labeled standard during initial extraction).

in bean, wheat, sunflower, and ice plant, and the lowest in maize, sugar beet, and spinach. In five of the species, CA levels in the light were 45% or more higher than in the dark.

To determine, in part, the intracellular location of CA in bean leaves, protoplasts and chloroplasts were isolated and illuminated and their CA contents then measured. Chloroplasts were found to contain only about 30% of the CA within the protoplast when corrected for their 87% intactness (data not shown). Given this result, we then carried out nonaqueous fractionation of illuminated or darkened bean leaves and measured the CA contents of the isolated fractions (Table II). In the illuminated leaf, 37% of the CA was in the chloroplast and the remainder in the vacuole. However, in the darkened leaf, very little, if any, of the CA was found in the chloroplast, with most occurring in the cytosol and the vacuole.

DISCUSSION

We conclude that CA occurs in plant leaves because a CA standard and putative plant CA copurify and have equivalent mass spectra (Fig. 2). In both pyrolysis spectra, the detected

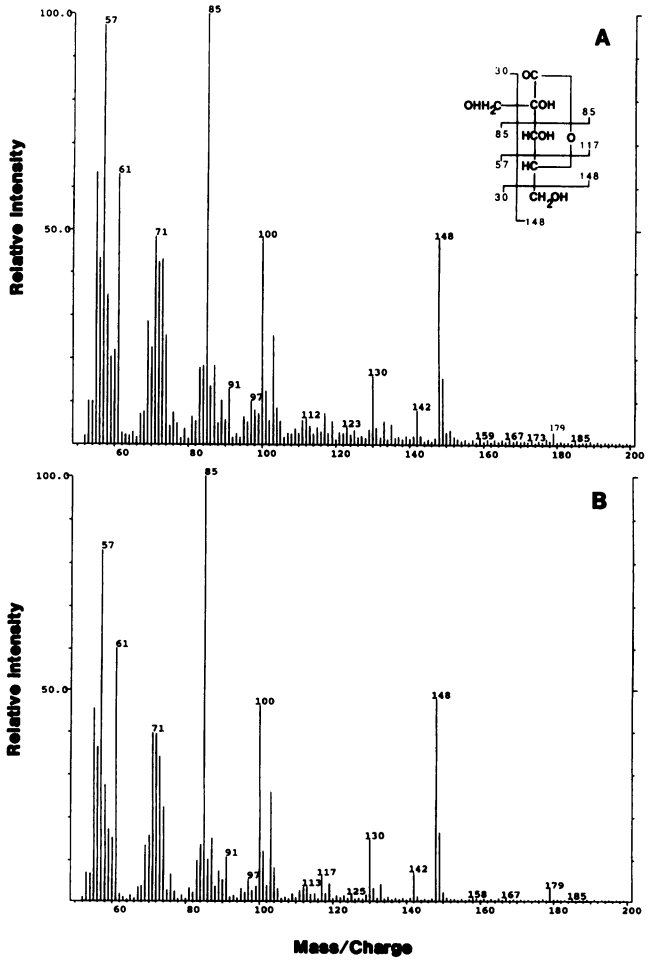

Figure 2. Mass spectra of pyrolyzed CA: A, authentic standard; B, purified plant sample.

Table I. *CA Contents of Leaves Isolated in Light or Dark from Selected Species*

Leaves were collected at midday or after 16 h of dark. Amounts are the means of two to three measurements, generally from a single extraction.

Species	Irradiance	Amount CA	
	$\mu mol\ m^{-2}\ s^{-1}$	*nmol/g fresh weight*	*nmol/mg Chl*
C_3			
Arabidopsis thaliana	1300	4.0	6.6
	0	8.4	10.6
Alocasia macrorrhiza	550	19.0	6.0
	0	15.1	4.8
Beta vulgaris (sugar beet)	1300	4.2[a]	3.2[a]
	0	4.3[a]	3.7[a]
Glycine max (soybean)	1300	17.6	10.1
	0	8.3	4.8
Helianthus annus	1300	303	129
(sunflower)	0	312	154
Lycopersicon esculentum	1300	115	78.1
(tomato)	0	66.7	47.9
Petunia hybrida (petunia)	1300	22.3	20.9
	0	9.5	9.7
Phaseolus vulgaris (bean)	1300	1632[a]	953[a]
	0	939[a]	657[a]
Spinacea oleracea	550	4.5	2.3
(spinach)	0	3.2	1.7[b]
Triticum aestivum (wheat)	1300	425	229
	0	367	247
C_4			
Panicum maximum	1300	20.9	9.5
	0	28.2	13.0
Zea mays (maize)	1300	12.0	6.3
	0	6.6	3.4
CAM			
Mesembryanthemum	1300	76.4	235
crystallinum (ice plant)	0	91.2	299

[a] Average of two extractions. [b] Sample specific radioactivity differed from that of the standard at P = 0.05. In all other cases, specific radioactivities of the samples differed from those of the standards at P = 0.01 (*t* test).

parent ion *m/e* ratio was 179, a value greater by 1 than that predicted from the formula mass ($C_6H_{10}O_6$, *e.g.* see ref. 3). This slight difference is likely due to self-protonation during sample pyrolysis. Both spectra further indicate the presence of a lactone due to the detected, large pseudomolecular ion with a *m/e* of 148. The presence of CA in plant leaves supports the hypothesis that CA1P is metabolized *in vivo* by CA1P phosphatase (5, 9).

The level of CA in illuminated leaves of some species was shown to be quite high (Table I), with the amounts being comparable to or greater than levels of most photosynthetic metabolites (18). CA levels in the light were 2 to 4 times the corresponding dark CA1P pool size in most of the 13 species examined (see ref. 11). The amounts of CA in bean, wheat, and sunflower were in particularly large excess of the amounts of CA1P, with respective light/dark molar ratios (Chl basis) of 953:67, 229:2.5, and 129:8.7. These high ratios

Table II. *Intracellular Distribution of CA in Leaves of Phaseolus vulgaris*

Leaf samples were isolated using nonaqueous fractionation procedures following treatments of 12 h of dark or 12 h of dark/2 h of light. Values represent the mean ± 1 SD of CA contents calculated from four separate extractions.

Fraction	Distribution of CA		Amount of CA	
	Light	Dark	Light	Dark
	%		nmol mg Chl^{-1}	
Chloroplast	37 ± 17	5 ± 9	149	16
Cytosol	2 ± 3	54 ± 2	6	176
Vacuole	62 ± 11	42 ± 9	250	137
Whole leaf	100	100	404	326

indicate either that there is a source for CA other than CA1P or that there is relatively slow turnover of the CA pool. In sugar beet, the pool size of CA1P in the dark is much greater than the pool size of CA in the light, indicating that in this species, CA may be further metabolized in the light. Furthermore, the fact that the light minus dark CA levels in most species were seldom equivalent to the dark CA1P pool (see ref. 11) indicates additional complexity in CA metabolism.

Although CA1P is thought to occur only within the chloroplast (17), the intracellular distribution of CA is more complicated. In an illuminated bean leaf, CA occurs in the chloroplast and the vacuole (Table II). However, in the dark there is less CA in the leaf (Tables I and II), and the CA present is about equally distributed between the cytosol and vacuole. Much of the chloroplast CA in the light is likely utilized for dark CA1P synthesis (13), but not all of the depletion of chloroplast CA can be attributed to this factor. Possibly some of the chloroplast CA is utilized in synthesis of other minor, unknown products (13) and/or is a source for some of the dark pool of cytosolic CA. The presence of substantial pools of extrachloroplastic CA does indicate that CA may also function in other aspects of plant metabolism unrelated to the regulation of Rubisco activity.

In conclusion, CA is one of a very few branched chain sugar acids identified from plants, two others being 2-C methyl-tetronic acid (15) and 3-C-carboxyl-5-deoxy-L-xylose (aceric acid, ref. 21). Branched chain monosaccharides have been identified only from plants and microorganisms, with the branch group being generally a hydroxymethyl or a methyl group (24). There are now at least nine branched chain monosaccharides, or derivatives, identified from plants (1, 15), but the functions of most of these are not well defined. Sugars that branch at C-2 have enhanced stability in both acid and alkali conditions (24). This fact indirectly supports the suggestion that hamamelose and hamamelitol (which do branch at C-2) may function as storage and/or cryoprotectant compounds (1). It remains to be established whether CA may have any such functions and whether CA and CA1P are metabolically related to the structurally similar compounds hamamelose, hamamelitol, and hamamelose 2',5-bisP.

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