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A high affinity pyruvate decarboxylase is present in cottonwood leaf veins and petioles: A second source of leaf acetaldehyde emission?

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ABSTRACT

Considerable evidence indicates that acetaldehyde is released from the leaves of a variety of plants. The conventional explanation for this is that ethanol formed in the roots is transported to the leaves where it is converted to acetaldehyde by the alcohol dehydrogenase (ADH) found in the leaves. It is possible that acetaldehyde could also be formed in leaves by action of pyruvate decarboxylase (PDC), an enzyme with an uncertain metabolic role, which has been detected, but not characterized, in cottonwood leaves. We have found that leaf PDC is present in leaf veins and petioles, as well as in non-vein tissues. Veins and petioles contained measurable pyruvate concentrations in the range of 2 mM. The leaf vein form of the enzyme was purified approximately 143-fold, and, at the optimum pH of 5.6, the K_m value for pyruvate was 42 μM. This K_m is lower than the typical millimolar range seen for PDCs from other sources. The purified leaf PDC also decarboxylates 2-ketobutyric acid (K_m = 2.2 mM). We conclude that there are several possible sources of acetaldehyde production in cottonwood leaves: the well-characterized root-derived ethanol oxidation by ADH in leaves, and the decarboxylation of pyruvate by PDC in leaf veins, petioles, and other leaf tissues. Significantly, the leaf vein form of PDC with its high affinity for pyruvate, could function to shunt pyruvate carbon to the pyruvate dehydrogenase by-pass and thus protect the metabolically active vascular bundle cells from the effects of oxygen deprivation.

1. Introduction

Acetaldehyde is abundant and reactive in the atmosphere, and it has been suggested that there are large unknown biogenic sources of this aldehyde (Singh et al., 2001). Acetaldehyde emission is known to occur in leaves in response to flooding (reviewed in Kreuzwieser et al. (1999b) and Tadege et al. (1999)) and during light–dark transitions (Karl et al., 2002; Graus et al., 2004). In addition, the leaves of many higher plants under imposed anaerobic conditions produce acetaldehyde (Kimmerer and Macdonald, 1987). Two metabolic models have been proposed to explain leaf acetaldehyde emission. In the most studied model, acetaldehyde emission during flooding is primarily due to anoxic production of ethanol in roots. During flooding, roots utilize glycolysis as the main source of ATP, resulting in the formation of pyruvate. The pyruvate is then decarboxylated by the root's pyruvate decarboxylase (PDC) to form acetaldehyde, which is converted to ethanol by alcohol dehydrogenase (ADH) to regenerate NAD^+. The transpiration stream transports ethanol from root to leaves to be oxidized by ADH and reform acetaldehyde, an intermediate in acetate formation (Harry and Kimmerer, 1991; Kreuzwieser et al., 1999a). Some ethanol and acetaldehyde are lost to the air by evaporation during their transport (Kreuzwieser et al., 1999b; Tadege et al., 1999).

More recently, a second model supports the importance of PDC activity in leaves, in addition to its role in metabolism of root-derived ethanol. Our lab found that acetaldehyde is released from cottonwood and sycamore leaves following light–dark transitions (Karl et al., 2002). Graus et al. (2004) confirmed this observation in poplar leaves. This release suggests a biochemical model relating leaf acetaldehyde emission to the imbalance of cytosolic and mitochondrial pyruvate concentrations during light–dark transitions. According to this model, following a sunfleck, photosynthesis and mitochondrial pyruvate dehydrogenase (PDH), a light activated enzyme, are shut down in the shaded leaf. This could then lead to cytosolic pyruvate accumulation, which can be detrimental to cellular biological functions due to a concomitant drop in cytosolic pH. PDC, in this situation, converts pyruvate to acetaldehyde and CO_2 and re-establishes the normal cellular pH. Therefore, elevation of leaf cytosolic pyruvate concentration could lead to acetaldehyde emission in a pathway independent of ethanol oxidation. Such a

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pathway could be the PDH by-pass, which has been reviewed in work by Gass et al. (2005) in their work on PDC activity in aerobic pollen tissue.

However, Graus et al. (2004) do not completely agree with the sunfleck model. While they also showed increased acetaldehyde emissions during light–dark transitions, when PDH was inhibited or anaerobic metabolism was imposed (presumably increasing cytosolic pyruvate concentrations), they did not observe increased emissions during light–dark transitions when aldehyde dehydrogenase was inhibited. Aldehyde dehydrogenase is a plant mitochondrial enzyme that oxidizes acetaldehyde for energy metabolism as part of the PDH by-pass (Nakazono et al., 2000; Gass et al., 2005).

PDC has been studied from many plant sources including: wheat germ, maize kernels, rice, tobacco and pea, primarily from roots or seeds (reviewed in Mucke et al. (1995). It has also been studied in petunia and pollen tube tissues (Tadege and Kuhlemeier, 1997; Gass et al., 2005), where the enzyme functions in an aerobic environment. Notably, Kimmerer (1987) found PDC activity in cottonwood leaves, determined that this enzyme is constitutive, but concluded that its role in aerobic leaves is uncertain. There are multiple PDC genes in some plants – for example, *Arabidopsis* has four PDC genes (Kursteiner et al., 2003), and in *Arabidopsis* PDC1 is the most abundant form in seeds and their siliques, but the forms in leaves and stems are not known. In petunia, analysis of the PDC gene expression showed that *Pdc2* transcripts dominated *Pdc1* transcripts in leaves, similar to the pattern in roots (Gass et al., 2005). A search of the annotated *P. trichocarpa* genome (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) suggests that numerous PDC genes may be present in poplar species.

The goal of this work was to increase our knowledge of leaf PDC, one of the key enzymes involved in the biochemical scenarios proposed for acetaldehyde emissions from vegetation. We focused on characterizing this enzyme from leaves of a woody plant, cottonwood, that are known to contain constitutive levels of PDC as mentioned above (Kimmerer, 1987). The detection of PDC activity in leave tissue and petioles was analyzed, and the enzyme was partially purified from leaf veins. The kinetic behavior of the highly purified enzyme was notable and these results are discussed in relation to the possible role this enzyme plays in vascular bundle metabolism.

2. Results

2.1. Purification of pyruvate decarboxylase

PDC was partially purified from crude extracts obtained from the leaf veins of flooded cottonwood saplings. Flooded saplings were used because in some experiments there was moderate induction of leaf PDC activity. A 143-fold increase was seen in specific activity using the steps summarized in Table 1. It is notable that preparations done during the summer showed higher, but variable, purification factors, compared to preparations obtained during other seasons. The reason for this variability was not fully established. SDS–PAGE analysis of different preparations of the purified enzyme showed two major bands in the range of 67 kDa (not shown). The PDC obtained could be stored in extract buffer for 2 months at 4 °C with no loss of activity.

2.2. The pH optimum of PDC

The effect of pH on kinetic behavior of leaf PDC was also investigated. Prior to these experiments, PDC was saturated with cofactors by incubating in a buffer containing 2 mM TPP and 2 mM $MgCl_2$ to ensure co-factor saturation. It was then assayed in MES buffers of varying pH, and the velocity of the reaction was plotted. The enzyme had a pH optimum of 5.6 (Fig. 1).

2.3. The kinetic parameters of PDC

At the optimal pH, the K_m value for pyruvic acid was measured. The resultant initial velocity data was analyzed using Prism 3.0 (Fig. 2A). A K_m value for pyruvic acid of 42 μM was obtained. The pyruvate analog, 2-ketobutyric acid, was also tested. PDC activity at varying concentrations of 2-ketobutyric acid gave a K_m of 2.2 mM (Fig. 2B).

2.4. PDC activity: leaf vein vs. non-vein vs. petiole tissues

It is not known where PDC is located in leaf tissue. To determine this, vein tissue was dissected from non-vein tissues. Petioles were also removed from the leaves. Each tissue was extracted and assayed for PDC activity. In these experiments, as shown in Fig. 3, PDC activity is 5–6× higher in vein compared to non-vein tissue. In some experiments, extracts of the petioles were also made and the activity determined. Fig. 3 shows the specific activity in petiole extracts is 2.5× that in vein extracts.

2.5. Pyruvate concentration in veins and petioles

The pyruvate concentration in the vascular bundle is not known. To see if pyruvate could be detected, extracts from leaf vein and petioles were boiled and centrifuged to remove protein. The supernatant was assayed using lactate dehydrogenase and NADH. The protein concentration of the original unboiled extract was determined, and the amount of pyruvate detected per mg protein was calculated. The amount of pyruvate detected was similar for petiole and vein extracts, as shown in Table 2. Assuming 50% water content in these tissues, concentrations of pyruvate were calculated to be 1.7–3.1 mM.

3. Discussion

The work presented here is to our knowledge one of the first characterizations of leaf tissue PDC, an enzyme that converts pyruvate to acetaldehyde and CO_2. PDC has been studied extensively in plant roots, especially during anoxia induced by flooding (Tadege et al., 1998). Our interest in leaf PDC has been driven by our work

Table 1
Partial purification of leaf vein PDC from cottonwood.[a]

Step	Activity (nkatal/ml)	Protein (mg/ml)	Specific activity (nkatal/mg)	Purification factor	Yield (%)
1. Crude extract[b]	10.4	0.615	1.67	1	100
2. Ammonium sulfate precipitation supernatant (0–30%)	8.28	0.382	2.17	1.3	57.9
3. Ammonium sulfate precipitation supernatant (30–60%)	8.33	0.189	4.34	2.6	26.6
4. Ammonium sulfate precipitation supernatant (60–90%)	13.3	0.157	8.52	5.1	20.4
5. Phenyl Sepharose	4.16	0.0174	239	143	9.43

[a] Flooded cottonwood saplings were used, as explained in Section 2.1. One gram of vein tissue ground in 10 ml of extract buffer was used.
[b] Crude extract included the Sephadex G-25 and protamine sulfate pretreatment as described in the text.

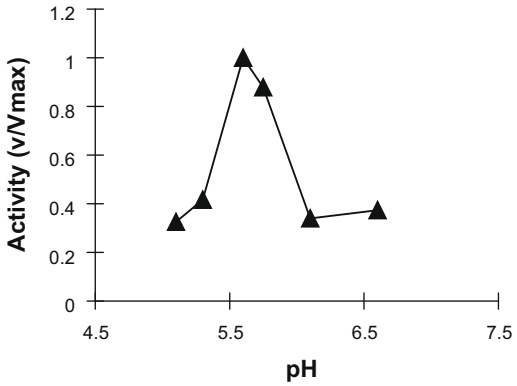

Fig. 1. Kinetic plot of PDC initial velocity vs. pH. The assay conditions are described in the Section 5. Purified PDC was used for these assays, and although statistical analysis was not carried out, each point represents the mean of triplicate determinations.

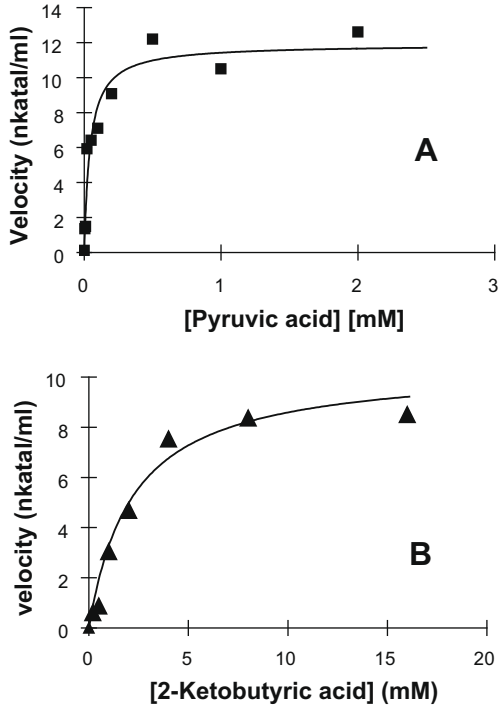

Fig. 2. Kinetic analysis of purified leaf vein PDC. Activity was measured at various concentrations of pyruvic or 2-ketobutyric acid. (A) Velocity vs. pyruvic acid concentration. (B) Velocity versus 2-ketobutyric acid concentration. Plot A summarizes the mean values of 10 experiments (each in duplicate), and K_m and V_{max} values were computed from a Prism 3.0© program; here the R^2 value of the curve fit was >0.95. For plot B results from only a single experiment are shown, again mean values were used and K_m and V_{max} values were computed from a Prism 3.0© program; R^2 value of the curve fit was also >0.95.

to understand acetaldehyde emissions from leaves and forest vegetation (Karl et al., 2002, 2003). The sources of this reactive aldehyde are of interest to atmospheric chemists. For example, Singh et al. (2001) estimated that global sources of atmospheric acetaldehyde are 80–160 Tg yr^{-1}, and also proposed that large biogenic sources are missing from the global acetaldehyde inventory. Our interest in characterizing leaf PDC is part of a larger effort to determine if enzymatic pathways utilizing PDC contribute to these missing biogenic acetaldehyde sources.

We focused on cottonwood leaves as they are a significant source of acetaldehyde emissions (Karl et al., 2002; Graus et al., 2004), but also because their leaves have large leaf veins that are easy to dissect from the surrounding mesophyll cells. We discovered that the specific activity of PDC in poplar vascular tissues, such petioles and veins, is significantly higher than in the bulk tissue of the leaf (Fig. 3); notably the non-vein tissue has low PDC specific activity, but contains the bulk of assayable soluble PDC. This suggests that PDC is enriched in the extractable protein fraction of vascular tissues. Consistent with this idea, Kimmerer and Stringer (1988) have also found PDC activity in the vascular cambium of cottonwood stems.

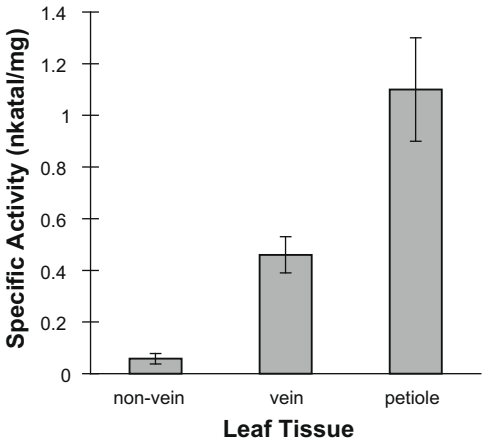

Fig. 3. PDC activity of vein, non-vein, and petiole tissue extracts from cottonwood leaves. PDC specific activity was measured in crude extracts of the three different leaf tissues. The graph includes the average and 95% confidence limits of determinations from 28-different extracts of vein, 20-different extracts of petioles and eight-different extracts of non-vein tissue. Each extract was assayed in duplicate.

Table 2
Pyruvic acid determination from crude extracts of vein and petiole tissue.

Extract type	μmol pyruvate/mg protein	Standard error of the mean
Vein[a]	0.38	0.04
Petiole[b]	0.44	0.03

[a] Six different vein extracts were assayed in duplicate.
[b] Two different petiole extracts were assayed in duplicate.

The ability to easily isolate leaf veins greatly facilitated the purification of the enzyme. Although we did not achieve homogeneity, we did purify leaf vein PDC approximately 143-fold, and determined that the enzyme had an apparent molecular mass of 67 kDa, similar to other plant PDCs (Mucke et al., 1995). This level of purification allowed further characterization of the enzyme. Like root PDCs, the leaf enzyme has a low pH optimum (pH 5.6). Unlike root PDCs that have been studied, the K_m for pyruvate is quite low (42 μM); this compares to the higher K_ms for PDC from maize seeds (0.9 mM) (Lee and Langstonunkefer, 1985), wheat germ (3 mM) (Kuo et al., 1986) and yeast (1 mM) (Boiteux and Hess, 1970). The low K_m for pyruvate in leaf PDC suggests that levels of pyruvate in vascular bundle tissues could be relatively low. In other studies by Tcherkez et al. (2005) the pyruvate concentration has been estimated to be less than 2 mM in leaf cells of the French bean (*Phaseolus vulgaris*). In this work, we measured similar levels of pyruvate in both vein and petiole extracts, but the level of pyruvate in the cellular compartment containing PDC is unknown. In addition, the cellular complexity of vascular bundles needs to be considered in future work on the metabolic role of the enzyme in this tissue (Hibberd and Quick, 2002).

Kimmerer and MacDonald (1987) suggested that the presence of PDC in highly aerobic leaves is a paradox because this enzyme

is thought be part of a fermentative pathway (Kreuzwieser et al., 1999a). Recently however, it has been shown that oxygen concentrations can fall to levels as low as 5–6% in phloem tissue (Geigenberger, 2003). Phloem tissue has a high metabolic rate, but its cells are compact and it has few intracellular air spaces. It is therefore possible, that the presence of PDC represents an adaptation to low oxygen concentrations in vascular bundle tissue. In low oxygen environments an induction in PDC activity would be a response of the plant to adopt energetically more efficient pathways that also conserve the available oxygen (van Dongen et al., 2003), such as the PDH by-pass discussed above. It is also possible that such a hypoxic response could explain in part the light-to-dark releases we observed in these leaves (Karl et al., 2002), as discussed in the Introduction.

Aerobic PDC activity has also been observed in metabolically active pollen tissue. Investigators working on developing pollen tissue from petunia, have shown the presence of an aerobic PDC (Tadege and Kuhlemeier, 1997; Gass et al., 2005). They suggested that pollen has elevated glycolysis resulting in high pyruvate levels and flux through PDC. This leads to acetyl-CoA formation independent of the mitochondrial pyruvate dehydrogenase (PDH) reaction. This PDH by-pass pathway can be used for energy and biosynthetic purposes, in these metabolically active aerobic tissues.

4. Concluding remarks

For cottonwood leaves, the localization of a high affinity PDC in the leaf veins and petioles is of interest, and it is possible that the PDH by-pass could be present in these tissues. Future work should establish if cottonwood vein cells contain the other enzymes of this by-pass, and clarify the role of these cells in leaf acetaldehyde emission. In addition, as discussed in the Introduction there is growing evidence that there are multiple PDC genes in plants. Perhaps the PDC purified from leaf veins, with its high affinity for pyruvate, represents a unique form of the enzyme, and could be a significant additional source of leaf acetaldehyde emission. It will be of interest to see which PDC gene encodes this enzyme, if its expression is tissue specific, to completely purify the enzyme, and to characterize its metabolic role.

5. Experimental

5.1. Plant materials and growth conditions

Cottonwood leaves were cut from trees of *Populus deltoides* Barr. (S7c8 East Texas day-neutral clone) growing in glasshouses at University of Colorado, Boulder as previously described in Rosenstiel et al. (2003). Trees were watered twice per day in the early morning (8 am) and early afternoon (2 pm). They were also fertilized 3× per week; 2× per week they were fertilized with Scott's General Purpose fertilizer, and one day per week they were fertilized with Scott's Acid Special fertilizer to enhance growth. The trees were also sprayed with pesticides on bimonthly intervals.

To flood plants to be used in the purification of PDC, greenhouse grown trees (in 5 gal pots) were carefully moved into 50 l containers filled with water (taking care to insure that no air bubbles were introduced). Plants were flooded for 3 days prior to leaf removal and enzyme extraction.

5.2. Enzyme extraction

The veins or mesophyll cells of the leaves were removed with a razor blade. The total tissue mass was quantified and then frozen in liq. N_2. Subsequently, they were then ground using a mortar and pestle at 4 °C in an extract buffer at a ratio of 10 ml buffer per gram of fresh plant tissue based on the method of Kimmerer (1987). Extract buffer contained 100 mM Hepes (pH 7.55), 2 mM $MgCl_2$, 1 mM NAD, 2 mM thiamine pyrophosphate (TPP) and 2 mM dithiothreitol (DTT). One gram of poly(vinylpolypyrrolidone) (PVPP) per gram of tissue was added. The enzyme extract was centrifuged at 10,000*g* for 20–30 min at 4 °C. The supernatant was isolated and passed through a G-25 Sephadex column (5 ml) to remove low molecular weight components. The protein fraction was collected and kept at 4 °C.

In some experiments a similar extraction method, except for the Sephadex G-25 step, was used to assay PDC activity in petioles.

5.3. PDC purification

PDC purification is a multi-step process involving gel filtration, salting out, and hydrophobic interaction. All purification steps, except for Phenyl Sepharose, were done at 4 °C. The first step in PDC purification was to perform gel filtration using a G-25 Sephadex column to remove low molecular weight substances; the bed volume was 5 ml per 0.5 ml of extract. The desalted pool was treated with protamine sulfate (2% w/v stock solution) at a ratio of 1 ml protamine sulfate for every 20 ml of protein extract. After stirring for 10 min and centrifugation at 10,000*g* for 20 min, the pellet was discarded. The supernatant was adjusted to 30% saturation with solid ammonium sulfate $(NH_4)_2SO_4$), stirred for 60 min, and centrifuged (10,000*g*, 20 min). The supernatant was adjusted to 60% saturation, stirred for another 60 min, and centrifuged (10,000*g*, 20 min). The supernatant of this step was then adjusted to 90% salt saturation, stirred for 60 min, and centrifuged; PDC remained in the supernatant. Aliquots of the 90% $(NH_4)_2SO_4$ supernantant were applied to a Phenyl Sepharose 4LB column in a ratio of 1 ml supernatant to 20 ml bed volume (in different experiments); in each case, the column was equilibrated with extract buffer. Bound PDC was eluted by 50% (v/v) ethylene glycol. At each stage, the fractions were tested for PDC activity. The purified enzyme was stored at 4 °C.

5.4. PDC enzyme assay

PDC activity was determined by a coupled assay (Kimmerer, 1987). In this assay, pyruvate is first decarboxylated by PDC to form acetaldehyde and carbon dioxide. Acetaldehyde is then reduced by alcohol dehydrogenase (ADH) using NADH to form ethanol. The assay buffer consisted of 100 mM MES (pH 5.6), 5 mM $MgCl_2$, 1 mM DTT, and 1 mM TPP. To perform the assay, 10 μl of 17.85 mM NADH stock solution was added to 500 μl of assay buffer, followed by 310 μl of H_2O, 100 μl of protein extract, 30 μl of equine liver ADH stock solution (2 units) purchased from Sigma–Aldrich and 10 μl of 200 mM pyruvate stock solution. NADH oxidation activity was measured by a spectrophotometer at 340 nm at 25 °C for 3 min before the introduction of pyruvate. Decarboxylation of 2-ketobutyric acid was assayed, using the same method by replacing pyruvate as the substrate. Kinetic determinations for K_m values were analyzed using Prism 3.0 software (GraphPad.com).

The relative recovery of PDC activity from different leaf tissue extracts is difficult to assess; for example, there may be different levels of polyphenols that may inactivate the enzyme. This was partly addressed by mixing extracts to determine if a soluble inhibitor was present. No inhibition was detected when extracts from vein and non-vein tissue were mixed. Inactivation during extraction is more difficult to determine. Thus we report the amount of extractable enzyme recovered only.

5.5. Pyruvate determination

The concentration of pyruvate in some of the leaf vein and petiole extracts was determined using rabbit muscle lactate dehydrogenase (LDH; Sigma–Aldrich) was diluted 1/100 in 0.2 M HEPES pH 7.3. The assay was performed in 0.2 M HEPES pH 7.3. To determine K_m and V_{max} of the purchased enzyme under the prevailing conditions, 1 ml buffer plus varying concentrations of pyruvate were added to a cuvette, then 10 μl of 17.85 mM NADH was added plus 4 μl (0.5 units) of the diluted LDH to initiate the reaction. The decrease in absorbance at 340 nm was determined. K_m for lactate was found to be 1.5 mM.

The assay was used to determine pyruvate concentrations in vein and petiole extracts. The extracts were made, leaving out NAD^+ from the extraction buffer and were not run over a Sephadex G-25 column. After centrifugation, they were placed in boiling water for 3 min. and then centrifuged at 10,000*g* for 20 min. Levels of 100 or 200 μl of the supernatant were used in the assay in place of pyruvate. The background was determined before adding LDH. Since the pyruvate concentration was far below its apparent K_m, the concentration of pyruvate was determined using K_m (v/V_{max}) = [*S*]. The protein concentrations of the extracts before boiling were determined as descried below.

5.6. Protein concentration

Protein was routinely determined by the method of Bradford (Bradford and Williams, 1976), except in samples with high ammonium sulfate concentration a precipitation method was used (Bensadoun and Weinstein, 1975). The reagents were purchased from Sigma–Aldrich, and bovine serum albumin was used as a standard.

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