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Activity of the mitochondrial pyruvate dehydrogenase complex in plants is stimulated in the presence of malate

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ABSTRACT

The effect of malate on the steady-state activity of the pea *(Pisum sativum* L.*)* and barley (*Hordeum vulgare* L.) leaf pyruvate dehydrogenase complex (PDC) has been studied in isolated mitochondria. The addition of malate was found to be stimulatory for the mitochondrial PDC, however there was no stimulation of chloroplast PDC. The stimulation was saturated below 1 mM malate and was apparently related to a partially activated complex, which activity increased in the presence of malate by about twofold. Malate also reversed the reduction of PDC activity in the presence of glycine. Based on the obtained kinetic data, we suggest that the effect of malate is rather not a direct activation of PDC but involves the establishment of NAD-malate dehydrogenase equilibrium, decreasing concentration of NADH and relieving its inhibitory effect of PDC.

1. Introduction

The pyruvate dehydrogenase complex (PDC) catalyzes the conversion of pyruvate into acetyl-CoA, NADH, and CO_2. In plants, the complex exists as two isoenzymes, one (as in animal cells) in the mitochondrial matrix, and one in the chloroplast stroma. The mitochondrial complex provides acetyl carbon units for the TCA-cycle for production of its intermediates, NADH, and ATP. The stromal PDC function is largely biosynthetic (Camp and Randall, 1985); it interacts with PEP carboxylase for channeling carbon from PEP to acetyl-CoA (Uhrig et al., 2008). The content of PDC decreases with leaf age (Luethy et al., 2001), and the distribution of the isoforms varies between plant species (Lernmark and Gardeström, 1994).

PDC is comprised of multiple copies of three primary components: the heterotetramer ($\alpha_2\beta_2$) pyruvate dehydrogenase, E1 (EC 1.2.4.1), the monomer dihydrolipoyl transacetylase, E2 (EC 2.3.1.12), and the homodimer dihydrolipoyl dehydrogenase, E3 (EC 1.8.1.4). In the mammalian systems, the complex core is made up of 60 copies of E2, to which multiple El and E3 units are attached (Patel and Roche, 1990). The components of PDC differ immunologically between plant isozymes (Camp and Randall, 1985; Rapp et al., 1987) and between animals and plants. E1 seems to be most diverged and does not cross-react with antibodies available for human E1, but cross-reacts with the yeast ditto (Taylor et al., 1992). Human antibodies raised against E2 and E3 have affinities for their plant mitochondrial counterparts, but the plant enzymes show differences in molecular weights (Taylor et al., 1992). On a molecular basis, the plant components show relatively high homology to those of other eukaryotic cells: E1 (Grof et al., 1995); E2 (Guan et al., 1995); E3 (Bourguignon et al., 1988).

The mtPDC is present at relatively high concentration in mitochondria, e.g. the 20–100-fold purification from mitochondria (depending on plant species and age) is sufficient to obtain preparations of PDC with low contamination of other proteins (Miernyk and Randall, 1987a; Millar et al., 1998; Randall et al., 1977; Thelen et al., 1998). This indicates concentration of PDC in mitochondria of 1–5% of mitochondrial protein and corresponds to a submillimolar level, which is about one order of magnitude lower than of glycine decarboxylase (GDC) in photorespiring tissues, but still quite high. On the other side, the operation of PDC is slow, the purified preparations exerted specific activities below 1 μmol (NADH formed) min^{-1} mg^{-1} protein which corresponds to the catalytic constant of ~5 s^{-1} calculated per 400 kDa enzyme (a functional unit that assembles into much larger functional units of about 20–25 nm in diameter) having four subunits of E1, monomer of E2, homodimer of E3 plus X-component of mainly structural function (Reid et al., 1977). This rate is similar to the catalytic constant of GDC and falls behind the value of

Abbreviations: GDC, glycine decarboxylase; mtPDC, mitochondrial PDC; MDH, malate dehydrogenase; NAD-ME, NAD-malic enzyme; PDC, pyruvate dehydrogenase complex; P-PDH-phosphatase, phospho-PDH-phosphatase; PDH, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; PVP, polyvinyl pyrrolidone (soluble); PVPP, polyvinyl polypyrrolidone (insoluble); TCA, tricarboxylic acid; TPP, thiamine pyrophosphate chloride.

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catalytic constant of malate dehydrogenase (MDH) (~3,000 s^{-1}) by three orders of magnitude (Bykova et al., 2014).

The mtPDC in both plants and mammals has been shown to have several levels of regulation. One is a direct regulation by substrate and product levels (Rubin et al., 1978). The other, which in plants is unique for mtPDC, is a multi-site reversible protein phosphorylation of the E1α subunit (Linn et al., 1969). The activation state, i.e. the ratio between the amount of non-phosphorylated/active complex and the total amount of enzyme, is determined by the equilibrium between PDH-kinase (EC 2.7.1.99) and phospho-PDH-phosphatase (P-PDH-phosphatase) (EC 3.1.3.43). Several mitochondrial metabolites inhibit pea PDH-kinase activity (Miernyk and Randall, 1987a). Among these, pyruvate in combination with thiamine pyrophosphate (TPP) decreases mtPDC phosphorylation most significantly (Schuller and Randall, 1990). NH_4^+ and K^+ stimulate PDH kinase, decreasing mtPDC activity (Schuller and Randall, 1989; Schuller et al., 1993). The only compound of metabolic significance that has been reported to regulate pea P-PDH-phosphatase is Pi, which inhibits the dephosphorylation (Miernyk and Randall, 1987b).

The mtPDC has been reported to be down-regulated in a light-dependent manner in leaf tissue from several species (Budde and Randall, 1990; Gemel and Randall, 1992) whereas the effect was small in barley (Krömer et al., 1994). This deactivation has been suggested to be coupled to photorespiration. It has been proposed that photorespiratory NH_4^+ released in the matrix by GDC might be involved in this deactivation (Schuller and Randall, 1989). The extent of the glycine-dependent deactivation was dependent on the amount of ATP produced by oxidative phosphorylation (Moore et al., 1993). NH_4^+, as in mammalian systems, may act by lowering the affinity of PDH-kinase for ATP (Schuller et al., 1993). The importance of substrate channelling for operation of PDC has been emphasized (Mooney et al., 2002) and several levels of regulation of the complex have been described (Tovar-Méndez et al., 2003).

In the present study, the modulation of mtPDC activity in isolated respiring mitochondria was analyzed. In contrast to the studies with the purified PDC (Miernyk and Randall, 1987c; Thelen et al., 1998) showing insensitivity of PDC to the TCA cycle intermediates, we observed stimulation of PDC by malate in mitochondria but not in chloroplasts. Based on the kinetic data and similarity of the observed phenomenon to the stimulation of glycine oxidation by malate in plant mitochondria (Bykova et al., 2014; Wiskich et al., 1990), we suggest that the stimulation of PDC by malate is attributed to the role of the mitochondrial malate dehydrogenase establishing fast equilibrium between NADH and NAD^+ and thus relieving inhibition of PDC by NADH. The results are discussed in the context of how the stimulation of mtPDC in the presence of malate optimizes its operation during pyruvate oxidation in vivo in respiring and photorespiring mitochondria.

2. Materials and methods

2.1. Plant material

Peas (*Pisum sativum* L., cv. Kelvendon wonder, Hammenhög, Sweden) and barley (*Hordeum vulgare* L., cv. Gunilla, Weibull, Sweden) were grown in a mixture of potting soil and fertilized turf for 14–16 days (pea) and 7 days (barley) before harvest. The plants were cultivated in a greenhouse at 20°C with cycles of 12 h darkness, 12 h artificial light of approximately 200 μmol quanta m^{-2} s^{-1}. The isolation was performed in the morning within 1 h after light was turned on to reduce starch content and to keep PDC in same initial conditions. Potato (*Solanum tuberosum* L. cv. Bintje) tubers were purchased at a local store.

2.2. Organelle isolation

2.2.1. Mitochondria

Leaf mitochondria were purified from fully expanded leaves. Approximately 200 g of leaf tissue or 1 kg of peeled potatoes was homogenized in 700 mL of Grinding medium (0.3 M sucrose, 25 mM MOPS-KOH (pH 7.8), 5 mM $MgCl_2$, 4 mM cysteine, 0.6% (w/v) PVPP, 0.2% (w/v) defatted BSA) using a Polytron blender (Kinematica GmbH, Switzerland) for 3 plus 3 s (maximum speed). The homogenate was filtered through 4 layers of cheesecloth and centrifuged for 10 min at 5 000 g. The supernatant was recentrifuged at 20 000 g for 10 min. The resulting pellet was carefully dissolved in minimal amounts of Percoll medium (0.25 M sucrose, 10 mM MOPS-KOH (pH 7.2), 1 mM EDTA, 0.1% (w/v) BSA (defatted), 0.5% (w/v) PVP (mol. weight 40 000), 32% (v/v) Percoll (Pharmacia, Sweden)) and layered on top of the gradient in SS34 tubes (Sorvall, DuPont, France). After centrifugation at 40 000 g for 45 min the mitochondria appeared at the lower part of the tube. This band was carefully collected and diluted in 10 times its own volume in Wash medium (0.3 M sucrose, 5 mM MOPS-KOH, pH 7.5, 1 mM EDTA, 0.1% (w/v) BSA) and pelleted by centrifugation at 20 000 g for 15 min. This final mitochondrial pellet was resuspended in approximately 0.5 mL of Wash medium to yield a protein concentration of 5–15 mg mL^{-1}. The purified mitochondria were essentially chlorophyll free, intact, and coupled. RC varied between 2 and 3 depending on the preparation and the substrate added. To minimize spontaneous activation of the PDC complex, care was taken that all purification steps were conducted on ice. The mitochondria were either used immediately after preparation or freeze-thawed (−20 °C then transferred to 4°C) one cycle before use. The freeze-thawed mitochondria exhibited the same parameters of respiration, PDC activity and activation state as freshly isolated mitochondria (not shown).

2.2.2. Barley chloroplasts

Barley chloroplasts were prepared by differential centrifugation of disrupted protoplasts. These were isolated enzymatically from primary leaves as described earlier (Palmqvist et al., 1994). The intact protoplasts were ruptured by passage twice over a 15 μM nylon net. The broken cells were centrifuged 4 min at 400 g. The pellet, enriched in intact chloroplasts (for results of marker enzyme measurements see Lernmark and Gardeström, 1994) was resuspended once and recentrifuged to further decrease mitochondrial contamination.

2.3. Assays

2.3.1. Mitochondrial PDC activity

Mitochondrial PDC was assayed as NADH production at 340 nm in 80 mM TES-KOH (pH 7.6 unless indicated otherwise), 1 mM $MgCl_2$, 2 mM NAD^+, 0.2 mM TPP, 0.12 mM CoA (dissolved in the presence of cysteine) and 0.1% Triton X-100 using a Biochrom 4060 open-lid spectrophotometer (Pharmacia LKB Biochrom Ltd, Cambridge, UK). PDC activity, determined from the initial slope of the reaction after addition of 1 mM pyruvate, was in all cases totally dependent on pyruvate, NAD^+ and CoA.

Mitochondrial protein for the mtPDC assay was sampled in three different ways: 1) From intact mitochondria incubated in respiration medium; 2) Directly from freshly isolated or freeze-thawed mitochondria to measure partially activated mtPDC; 3) From mitochondria incubated at 25 °C for 10 min in 20 mM TES-KOH, pH 7.5, 0.01% Triton X-100 and 20 mM $MgCl_2$ to activate mtPDC. In 1), the PDC reaction was initiated with mitochondria, in 2) and 3), pyruvate was used.

The time needed to record a stable mtPDC activity in mitochondria respiring malate varied due to background NADH oxidation, which was transient and only occurred for a maximum of 1 min. As the PDC substrates (NAD^+, pyruvate and CoA) were added in excess amounts, the overall mtPDC activity was not affected by an increased assay time. Thus, mtPDC activity was stable for at least an additional 3 min. mtPDC was not spontaneously activated during the spectrophotometric assay, since addition of 10 mM NaF inhibiting P-PDH-phosphatase (Miernyk and Randall, 1987b) had no effect.