Activity and quantity of ribulose bisphosphate carboxylase- and phosphoenolpyruvate carboxylase-protein in two Crassulacean acid metabolism plants in relation to leaf age, nitrogen nutrition, and point in time during a day/night cycle

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Abstract. Activity of ribulose 1,5-bisphosphate (RuBP) carboxylase in leaf extracts of the constitutive Crassulacean acid metabolism (CAM) plant Kalanchoe pinnata (Lam.) Pers. decreased with increasing leaf age, whereas the activity of phosphoenolpyruvate (PEP) carboxylase increased. Changes in enzyme activities were associated with changes in the amount of enzyme protein as determined by immunochemical analysis, sucrose density gradient centrifugation, and SDS gel electrophoresis of leaf extracts. Young developing leaves of plants which received high amounts of NO₃ during growth contained about 30% of the total soluble protein in the form of RuBP carboxylase; this value declined to about 17% in mature leaves. The level of PEP carboxylase in young leaves of plants at high NO₃ was an estimated 1% of the total soluble protein and increased to approximately 10% in mature leaves, which showed maximum capacity for dark CO₂ fixation. The growth of plants at low levels of NO₃ decreased the content of soluble protein per unit leaf area as well as the extractable activity and the percentage contribution of both RuBP carboxylase and PEP carboxylase to total soluble leaf protein. There was no definite change in the ratio of RuBP carboxylase to PEP carboxylase activity with a varying supply of NO₃ during growth. It has been suggested (e.g., Planta 144, 143-151, 1978) that a rhythmic pattern of synthesis and degradation of PEP carboxylase protein is involved in the regulation of β-carboxylation during a day/night cycle in CAM.

Key words: Crassulacean acid metabolism – Day/night cycle – Kalanchoe – Leaf age and CAM – Mesembryanthemum – Nitrogen nutrition – Phosphoenolpyruvate carboxylase – Ribulose bisphosphate carboxylase.

Introduction
Ribulose bisphosphate (RuBP) carboxylase may account for 50% and more of the total soluble leaf protein in C₃ plants (Björkman et al. 1976; Ku et al. 1979). Corresponding values are considerably lower (10 to 20%) in leaves of C₄ plants, and this difference is thought to be related to the high CO₂ partial pressures in the bundle-sheath cells of C₄ plants, where RuBP carboxylase is localized. Levels of phosphoenolpyruvate (PEP) carboxylase protein are negligible in C₃ plants and may represent 10-15% of the total soluble protein in leaves of C₄ plants (Björkman et al. 1976; Hague and Sims 1980). The activity and amount of RuBP carboxylase protein in a given C₃ species are markedly influenced by the level of irradiance during growth and by the nitrogen status of the plants (Medina 1971; Blenkinsop and Dale 1974; Wong 1979).

Thus far, there have been no reports on the amount of RuBP carboxylase- and PEP carboxylase-protein in Crassulacean acid metabolism (CAM) species. Such plants have the capacity to fix atmospheric CO₂ both in the dark via PEP carboxylase and in the light primarily via RuBP carboxylase (Osmond 1978; Osmond and Holtum 1981). The ratio of light-to-dark CO₂ fixation is dependent on a variety of
environmental factors and on leaf age. It is therefore of interest to know the extent to which the degree of dark and light CO₂ fixation is related to extractable activity and quantity of carboxylating enzymes present in tissues with CAM. There is also a vast amount of literature proposing that CO₂ fixation via PEP carboxylase is regulated by changes in the amount of enzyme protein. High levels of the enzyme at night and low levels during the day could avoid a futile refixation of CO₂ produced during decarboxylation of malate, into malate (e.g., Pierre and Queiroz 1978). These conclusions are based exclusively on determination of enzyme activities, not enzyme quantities.

In the paper presented here, we report the activity and amount of RuBP carboxylase and PEP carboxylase proteins extracted from leaves of different ages from the constitutive CAM plant Kalanchoe pinnata grown at high and low levels of NO₃ in the rooting medium. We also studied amounts of PEP carboxylase protein in leaves of Kalanchoe pinnata and in leaves of the inducible CAM plant Mesembryanthemum crystallinum during a day/night cycle. The determination of enzyme protein was done by immunochemical assay, by sucrose density gradient centrifugation, and by sodium dodecyl sulfate (SDS) gel electrophoresis.

Materials and methods

Plant material. Kalanchoe pinnata (Lam.) Pers. was propagated from leaf cuttings. Plants were grown in a non-shaded greenhouse during the summer. Irradiance was as much as 2 mmol photons m⁻² s⁻¹ at noon. Maximum air temperatures were between 30 and 38 °C and minimum temperatures between 15 and 23 °C. Plants were kept in 2-l pots (one plant per pot) and watered daily. Plants during the summer. Irradiance was as much as 2 mmol photons m⁻² s⁻¹ at noon. Maximum air temperatures were between 30 and 38 °C and minimum temperatures between 15 and 23 °C. Plants were propagated as described in the legend of Table 2. Two to 32 µl aliquots were added to 100 µl of a partially purified rabbit immunoglobulin fraction raised to 2-times recrystallized tobacco RuBP carboxylase (Ku et al. 1979). Samples were made to a constant vol. of 150 µl with extraction buffer and incubated at 30°C for 1 h and subsequently at 4°C overnight. The antibody-RuBP carboxylase-precipitate was collected by centrifugation at 10,000 g in a Beckman Microfuge B for 90 s. The supernatant was discarded and the precipitate washed twice with 25 mM Tris (hydroxymethyl) aminomethane (Tris), pH 7.5, containing 150 mM NaCl. The washed precipitate was dissolved in 10 µl 1 M NaOH and assayed for soluble protein by the method of Bradford (1976), using crystalline bovine serum albumin (BSA) as a protein standard. Purified tobacco RuBP carboxylase protein was used as an immunological standard. The concentration of tobacco RuBP carboxylase was determined by published extinction coefficients at 280 nm (Siegel and Lane 1975).

Aliquots of leaf extracts were precipitated with trichloroacetic acid (TCA) (final concentration 10%, w/v) and washed twice with 10% TCA. Precipitates were dissolved in 1 M NaOH and soluble protein was determined according to Bradford (1976) using crystalline BSA as the standard.

Sucrose density gradient centrifugation. See legend of Fig. 3. SDS gel electrophoresis. Electrophoretic analysis of the samples was conducted using the alkaline SDS-discontinuous buffer system of Laemmli (1970). Gels, polymerized between two glass plates separated by 0.75 mm thick spacers, consisted of a 1 cm, 4.5% (w/v) acrylamide stacking gel [4.62% (w/v) T, 2.6% (w/v) C], and a 9.5 cm resolving gel containing a 7.5-15% (w/v) linear acrylamide gradient [7.7-15.4% (w/v) T, 2.6% (w/v) C], stabilized by a 5-17% (w/v) linear sucrose gradient (Chua 1980). Extracts were prepared by heating them for 1 min in the sample buffer described by Laemmli (1970). Molecular weight markers, rabbit muscle phosphorylase b (92,500); bovine serum albumin (68,000); bovine liver catalase (60,000); porcine heart fumarase (49,000); rabbit muscle aldolase (40,000); porcine heart mitochondrial NAD-malate dehydrogenase (34,000); bovine erythrocyte carbonic anhydrase (29,000); soybean trypsin inhibitor (21,500); and chicken egg white lysozyme (14,000); all from Sigma, St. Louis, Mo., USA, were electrophoresed in a separate lane for molecular weight calibration. Electrophoresis was performed in the direction cathode to anode for 4 h at 25°C using a constant current of 15 mA. The gels were stained for 1 h at 40°C or overnight at room temperature in a methanol:water:acetic acid (5:1:1 by vol.) solution containing 0.1% (w/v) Coomassie brilliant blue R-250, then destained for 24 to 48 h in 10% (v/v) acetic acid. For densitometric analysis, destained gels were soaked for 30 min in destaining solution containing 2% (v/v) glycerc, dried between sheets of porous cellophane dialysis membrane, and scanned using a Joyce-Loebel double-beam recording microdensitometer with a 0.39 density wedge.

Chlorophyll. Chlorophyll was determined according to Arnon (1949).

Titrateable acidity. Leaf discs of a known area were extracted in 20% boiling ethanol for 15 min, and the extracts were titrated with 10 mM NaOH to pH 6.5.

Results

Experimental plants of Kalanchoe pinnata grown at 24 and 0.6 mM NO₃ had a total leaf area of 0.33 and 0.17 m² and an aboveground dry weight of 27 and 18 g, respectively. Plants at high NO₃ had 10 leaf pairs and plants at low NO₃ had 8 leaf pairs. The specific leaf weight (fresh weight per unit leaf area), which was similar for plants from both nitrogen-level treatments (slight trend to lower values at