ENERGETICS OF MALATE ACCUMULATION IN THE VACUOLES OF *KALANCHOË TUBIFLORA* CELLS

U. LÜTTGE, J. A. C. SMITH, G. MARIGO+ and C. B. OSMOND*
Institut für Botanik, Technische Hochschule Darmstadt, 6100 Darmstadt, FRG

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1. Introduction

The diurnal rhythm of crassulacean acid metabolism is characterized by dark fixation of CO_2 and accumulation of malic acid during the night. Photosynthetic fixation of CO_2 derived from decarboxylation of this malate takes place while the stomata are closed during the following light period. Photosynthetic fixation of atmospheric CO_2 occurs during the latter part of the day if the stomata open. The quantum requirements for long-term growth of CAM plants [1,2] and for photosynthesis during the light period [3] have been determined. Measurements of respiratory O_2 uptake during the dark period have been discussed in relation to a possible competition of respiration and malate synthesis by PEP-C for substrate (i.e., PEP) [4].

Until now the process of malic-acid transport into the vacuole has not been included in the assessment of the energy budget of CAM. This malic-acid transport across the tonoplast is active, probably driven by a H^+-ATPase [5,6]. Here we provide the basis for calculation of energy turnover and balance during malate accumulation. The results put some restraints on hypotheses about the transport mechanism. The data are consistent with an ATPase operating with a stoichiometry of 2 H^+/ATP hydrolyzed.

Abbreviations: ADPG, adenosinediphosphoglucose; CAM, crassulacean acid metabolism; diPGA, 1,3-diphosphoglycerate; FBP, fructose-1,6-bisphosphate; G-3-P, glyceraldehyde-3-phosphate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEP-C, phosphoenolpyruvate-carboxylase

+ Permanent Address: Centre de Physiologie Végétale, Université Paul Sabatier, 118, route de Narbonne, 31077 Toulouse, France

* Permanent Address: Research School of Biological Sciences, Australian National University, Canberra, ACT 2601, Australia

2. Materials and methods

Intact plants of *Kalanchoë tubiflora* (Harv.) Hamet and excised phyllodia were performing CAM in a growth chamber under a 12 h light:12 h dark rhythm at 25°C and 30–45 W/m² (measured with a YSI Model 65A Radiometer, Yellow Springs Instruments OH, at the level of the plants) in the light period; at 15°C in the dark period and ~60% relative humidity throughout the whole rhythm. Respiratory O_2 uptake was measured manometrically. Segments of phyllodia sampled during the dark period were placed in ~15-ml large Warburg vessels (4 segments each 20 mm long, i.e., ~1 g fresh wt/vessel) in air on 2.2 ml buffer consisting of 1.7 ml 2.5 M K_2CO_3, 0.3 ml 2.5 M $KHCO_3$, 0.2 ml carboanhydrase solution (2 mg enzyme/ml, EC 4.2.1.1), to keep CO_2 at 0.04–0.05% (v/v). Measurements extended over ~90 min, during which time linear rates of O_2 consumption were obtained. Malate was determined enzymatically as in [6].

3. Results

Respiratory O_2 uptake by phyllodia of *K. tubiflora* was constant during the whole dark period of the CAM rhythm. Similar observations were made for *K. daigremontiana* in [4,7–9]. The same rates of O_2 uptake were measured when 30 mm long phyllodium segments or twice as many 15 mm long segments

were placed in Warburg vessels. Since in the latter case there is a larger wound surface due to slicing, this makes it unlikely that our measurements comprise a substantial O_2 uptake due to wound respiration [10,11]. Whether phyllodium segments were taken from intact plants or from excised phyllodia performing CAM, also did not affect the results. At 15°C and 21% O_2, i.e., under the conditions of nocturnal malic-acid accumulation in *K. tubiflora*, the rate of O_2 uptake based on 22 measurements, was 1.06 ± 0.14 μmol $O_2 . g^{-1}$ fresh $wt^{-1} . h^{-1}$ (mean ± SD). For technical reasons CO_2 in our Warburg vessels was 0.04–0.05% (v/v), i.e., somewhat higher than atmospheric. However, above 0.03% there is little dependence of *K. daigremontiana* respiration on CO_2 concentration, although below 0.03% respiration increased with decreasing CO_2 concentration [8]. For *K. daigremontiana* at 14°C and 21% O_2 and 0.028% CO_2 an O_2 uptake of ~0.5 μmol . g fresh $wt^{-1} . h^{-1}$ has been reported [8]; i.e., a value lower than ours for *K. tubiflora*. These workers used an O_2 electrode and intact plants in a gas-exchange chamber [12]. Respiration rates for detached leaves of *K. daigremontiana* given in the literature are somewhat variable, ranging from ~0.6 μmol . g fresh $wt^{-1} . h^{-1}$ to ~3 μmol . g fresh $wt^{-1} . h^{-1}$ [4,7,9]. The latter value was measured at 20°C; respiration of *K. daigremontiana* is highly temperature-dependent between 10–30°C [4,8,13]. It is essential for the discussion that our manometric measurements are not under-estimates of O_2 uptake due to the artificial situation of phyllodium segments in the Warburg vessels. The comparison with the literature suggests that this is not the case.

Fig.1 shows the relation between malate levels obtained in *K. tubiflora* and *K. daigremontiana* at various times during the dark period and the pH of leaf tissue homogenate. *K. daigremontiana* reaches somewhat higher malate levels (≥200 μmol/g fresh wt [5,6]) than *K. tubiflora*. But lower pH values were found in *K. tubiflora*, where there is obviously less buffering.

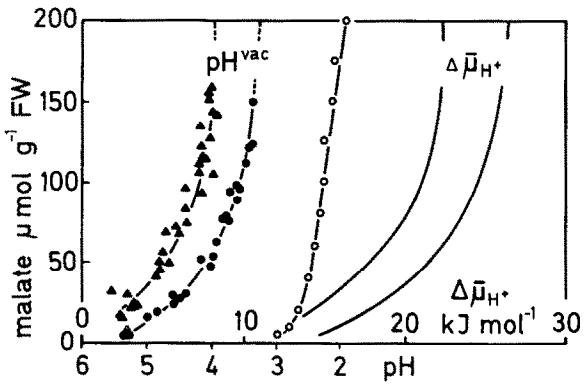

Fig.1. Relation between tissue-extract pH (taken as vacuolar pH) and malate levels in *K. daigremontiana* (▲) and *K. tubiflora* (●) and the corresponding $\Delta\bar{\mu}_{H^+}$, (left curve *K. daigremontiana*; right curve *K. tubiflora*). (○) pH of various concentrations of malic acid in distilled water.

4. Discussion

Since malate is always accumulated together with two titratable protons, it has been suggested that its uptake into the vacuole is driven by active H^+ transport across the tonoplast [5,6]. The H^+-electrochemical gradient at the tonoplast:

$$\Delta\bar{\mu}_{H^+} = RT \ln \frac{c_{H^+}^{vac}}{c_{H^+}^{cyt}} + F\Delta E = 2.303\, RT\, \Delta pH + F\Delta E$$

(where symbols have the usual thermodynamic meaning) can be calculated for all malate concentrations observed, if we assume a cytoplasmic pH of 7.5 (measured in green cells [14,15]), a vacuolar pH equivalent to that of the leaf homogenate, and a tonoplast electrical potential of +25 mV as measured for *K. daigremontiana* [16]. The result is plotted in fig.1. Taking the in vivo cytosol free energy of ATP hydrolysis as 55–60 kJ/mol [14,17] it is obvious that the $\Delta\bar{\mu}_{H^+}$ obtained at the highest malate concentrations would still allow active transport of 2 H^+ into the vacuole for 1 ATP (2 H^+-ATPase). *K. tubiflora* having more acid vacuolar sap approaches the point at which the ATPase would be close to thermodynamic equilibrium with ATP, since $\Delta\bar{\mu}_{H^+}$ is close to 1/2 ATP-free energy; i.e., 27–30 kJ/mol. This thermodynamic restriction could be why *K. tubiflora* generally does not reach levels of malate accumulation as high as *K. daigremontiana*, where >200 μmol/g fresh wt has been reported [6].

Energy turnover during malate accumulation in the dark follows from reactions 1–7, as shown in table 1. The net balance of reduction equivalents is zero, since reaction 3 is producing and reaction 6 is consuming reduction equivalents. The balance of ATP is –0.5 mol ATP/mol malate if reaction 1b operates and –1 mol ATP/mol malate if reaction 1a is effective. With a

Table 1
Energetics of nocturnal malic-acid accumulation: Reactions of the dark period involving energy turnover

1a	Hexose		+ ATP	=	Hexose-P	+ ADP	
1b	Starch	+ P_i		=	Hexose-P		
2	Hexose-P		+ ATP	=	FBP	+ ADP	
3	2 G-3-P	+ 2 P_i	+ 2 NAD^+	=	2 diPGA	+ 2 NADH	+ 2 H^+
4	2 diPGA		+ 2 ADP	=	2 PEP	+ 2 ATP	
5	2 PEP	+ 2 CO_2		=	2 OAA		+ 2 P_i
6	2 OAA	+ 2 H^+	+ 2 NADH	=	2 Malate	+ 2 NAD^+	
7	2 Malate	+ 4 H^+	accumulated in vacuole by "2 H^+-ATPase",				
			+ 2 ATP	⟶		+ 2 ADP	+ 2 P_i

Net energy balance (per mol malate accumulated):

with reaction 1a	1 ATP	= 1 ADP	+ 1 P_i
with reaction 1b	0.5 ATP	= 0.5 ADP	+ 0.5 P_i

1 H^+-ATPase at the tonoplast the requirements for ATP would increase to 1.5 or 2 mol ATP/mol malate.

Taking a P/O_2 ratio of 6, respiratory O_2 uptake produces ~6.4 μmol ATP . g fresh wt^{-1} . h^{-1}. The difference between the highest and lowest malate levels obtained in *K. tubiflora* during the 12 h dark period is 145 μmol/g fresh wt (fig.1), corresponding to an average rate of ~12 μmol . g fresh wt^{-1} . h^{-1}. In a similar way, it can be calculated for *K. daigremontiana*, using the data given in section 3, that the rate of ATP synthesis by oxidative phosphorylation is lower than the highest rates of malate accumulation. This leads to the conclusion that the energy budget of these CAM cells allows the observed accumulation of malic acid only with the following restraints on the mechanisms involved.

1. The CO_2 acceptor for dark fixation must be derived from phosphorolysis of starch and not from free hexose. This is generally accepted [18,19]. The involvement of amylase in starch hydrolysis [20] can only be of limited importance, because the energy cost would be too high.
2. Transport of 2 mol H^+ + 1 mol $malate^{2-}$ across the tonoplast cannot be driven by a 1 H^+-ATPase, but instead a 2 H^+-ATPase is probably involved. ATPases are known to exist in the tonoplast of higher-plant cells (see [21]).

The additional energy cost of malic acid transport should be considered by calculating the theoretical quantum requirement of CAM plants. However, even when this is done, and the possibility of some futile cycling through malic acid during net CO_2 fixation in the late light period is allowed, the theoretical quantum requirement remains lower than that measured for CO_2 fixation [3] and growth [1,2].

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