Plant Physiol. (1975) 56, 370-372

The Influence of Temperature on Malic Acid Metabolism in Grape Berries

I. ENZYME RESPONSES[1]

Received for publication February 5, 1975 and in revised form May 15, 1975

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ABSTRACT

Phosphoenolpyruvate (PEP) carboxylase activity in immature 'Carignane' grape berries (*Vitis vinifera* L.) had a temperature optimum of about 38 C, whereas malic enzyme activity rose with increasing temperature between 10 and 46 C. *In vitro* temperature inactivation rates for the PEP carboxylase were markedly greater than for the malic enzyme activity. From the simultaneous action of malic acid-producing enzymes (PEP carboxylase and malic dehydrogenase) and malic acid-degradating enzyme (malic enzyme) systems at different temperatures, the greatest tendency for malic acid accumulation in immature grape berries was at 20 to 25 C. Time-course measurements of enzymic activity from heated, intact berries revealed greater *in vivo* temperature stability for the malic enzyme activity than for the PEP carboxylase activity.

Tartaric and malic acids generally account for 69 to 92% of all organic acids in grape berries and leaves (5). Malic acid levels vary greatly as the berries develop and mature, whereas tartaric acid levels vary considerably less (4). Kliewer (6-8), using temperature-controlled growth rooms, confirmed the field observation that cool regions typically produce grapes with higher concentrations of organic acids and, conversely, that warm regions produce grapes with lower acidity. The negative temperature correlation has been demonstrated for malic acid, and its optimum temperature for accumulation was estimated to be about 20 C (6-8).

The enzymic pathways of malic acid synthesis and degradation in immature grape berries have not been determined. Most of the work on malic acid synthesis indicates that β-carboxylation of pyruvate or of phosphoenolpyruvate is the most important pathway (3, 12-14). Meynhardt (13) and Hawker (3) found significant levels of PEP[3] carboxylase and malic dehydrogenase activities in grape berries, and the rates of dark CO_2 fixation by immature grape berries were found by Kriedemann (10) to be highest during the period of rapid malic acid accumulation.

The degradation of malic acid in grape berries is not as well understood as is its synthesis. An NADP-dependent malic enzyme, considered to be an important malic acid degradative enzyme in other plants (15), has been reported in grapes (3, 13). Other pathways, such as diffusion of malic acid into the mitochondria and its subsequent degradation through the action of malic dehydrogenase or PEP carboxykinase enzymes, may also be important.

The purpose of the present investigation was to examine how temperature may affect the balance between malic acid synthesis and degradation through the relative activities of PEP carboxylase and malic enzyme in immature grape berries.

MATERIALS AND METHODS

Plant Material. The grape berries (*Vitis vinifera* L. cv. 'Carignane') used for enzyme extraction were excised from greenhouse-grown vines. Vines were pruned to two or three shoots, depending on vigor, and one cluster per shoot. No differences in enzymic properties were found for berries harvested from 2 to 6 weeks after anthesis; therefore, 2- to 3-week-old berries were chosen for extraction because of the higher levels of enzymes present at the earlier ages (3).

Enzyme Extractions. The extraction procedure was modified from that of Meynhardt (13). Berries were cut from the pedicels, weighed, frozen in dry ice, and ground to a frozen powder in a mortar with 100 mg of polyethylene glycol 4000/g fresh weight of tissue. The frozen powder was transferred to an ice-chilled mortar and homogenized with a pestle in 10 ml of cold extraction buffer/g fresh weight. The buffer consisted of 0.5 M tris-Cl, pH 8.4; 0.2 M KCl; 20 mM cysteine-HCl; and 3 mM EDTA. The homogenate was centrifuged at 4 C and 18,500g for 20 min. It was then filtered through four layers of cheesecloth, and 3.9 g of polyethylene glycol 4000 were added per 10 ml of extract. The mixture was stirred for 0.5 hr in an ice bath, then centrifuged at 4 C and 18,500g for 20 min. The supernatant fraction was discarded, and the pellet was resuspended in 1 ml of 0.1 M tris-Cl, pH 7.8/g fresh tissue weight, using a glass homogenizer chilled in ice. After 0.5 hr, the suspension was centrifuged at 4 C and 18,500g for 20 min. The resultant supernatant liquid, hereafter referred to as the enzyme extract, was decanted and used in all enzyme assays.

Enzyme Assays. The activity of the PEP carboxylase enzyme (EC 4.1.1.31) was determined by coupling the PEP carboxylase reaction to the malic dehydrogenase (EC 1.1.1.37) reaction and measuring the oxidation of NADH at 334 nm with a Zeiss PMQ II spectrophotometer. No exogenous malic dehydrogenase was added to the reaction. The coupled activity is presumed to be

[1] This work was supported in part by a National Science Foundation Traineeship to A.N.L. This report is from a dissertation submitted by A.N.L. in partial fulfillment of the requirements for the Ph.D. degree at the University of California, Davis.

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[3] Abbreviation: PEP: 2-phosphoenolpyruvate.

an important mechanism for malic acid synthesis in grape berries. Malic dehydrogenase activity alone was determined by the procedure of Wolfe and Neilands (17), and was always found to be at least 10 times that of the PEP carboxylase activity. The coupled PEP carboxylase-malic dehydrogenase activity of the enzyme extracts was primarily attributable to the PEP carboxylase activity present. The coupled activity will be referred to as PEP carboxylase activity. The reaction medium consisted of 100 μmoles of tris buffer at pH 8, 50 μmoles of $NaHCO_3$, 4 μmoles of $MgSO_4$, 5 μmoles of PEP, 5 μmoles of GSH, 0.5 μmole of NADH, and enzyme extract representing 0.2 g fresh weight of tissue. The final volume was 3 ml. The reaction medium without PEP served as a blank, and the reaction was started by the addition of the PEP.

The activity of malic enzyme (EC 1.1.1.40) in the extracts was determined by measuring the reduction of NADP at 334 nm with a Zeiss PMQ II spectrophotometer, as described by Meynhardt (13), except the reaction was started by the addition of malate. The reaction mixture was composed of 100 μmoles of tris-Cl, at pH 7.4, 3 μmoles of $MnCl_2$, 0.4 μmole of NADP, 12 μmoles of L-malate, at pH 7.4, and enzyme extract representing 0.2 g fresh weight of tissue. The final volume was 3 ml. Before starting the reaction, blank readings were made to determine endogenous NADP reduction. NADPH oxidase activity in the enzyme extract was determined by omitting the malate and NADP and adding 0.4 μmole of NADPH.

The protein contents of the enzyme extracts were determined by the biuret method of Gornall *et al.* (2), with BSA (Nutritional Biochemicals Corp.) as the standard.

The enzymic activities are expressed as μmoles of product/mg of protein·hr.

Temperature-Response Curves. Enzymic activities were determined at 10, 15, 20, 30, 35, 40, and 45 C in a Zeiss PMQ II spectrophotometer with a temperature-controlled cell. The pH values of the reaction buffers were standardized at the temperatures used for the assays.

***In Vitro* Temperature Inactivation.** PEP-carboxylase and malic enzyme activities were determined immediately after the extraction of the enzymes. Aliquots of the extract were then placed in water baths at 25, 35, and 45 C. Samples were analyzed for enzymic activities 0.5, 1, 2, and 4 hr after the beginning of the temperature treatment. The assay temperature was 25 C for activities of both enzymes.

***In Vivo* Temperature Inactivation.** Ten vines, each with two clusters, were placed in a controlled temperature and humidity growth chamber (Sherer Gillett Model Cel 512-37) and equilibrated for 1 hr at 25 C. Then the temperature was raised in 2 C increments over the next 4 hr, at which time cluster temperature was 40 C (air temperature 41 to 42 C). At that time, three representative clusters, taken as a sample representing time 0, were immediately cooled and the enzymes were extracted as described above. Three-cluster samples were also taken 0.5, 1, 2, and 4 hr after the cluster temperature reached 40 C. Within 0.5 hr after completion of the extraction, the PEP carboxylase and malic enzyme activities were determined. The light intensity was approximately 3500 ft-c at the canopy level.

***In Vitro* Temperature Inactivation.** The procedure described for the *in vivo* temperature treatment of clusters was repeated. However, samples were taken only at 0 and 4 hr. After enzyme extraction, the *in vitro* temperature inactivation rates of malic enzyme and PEP carboxylase were determined at 45 C by the procedure previously described.

RESULTS AND DISCUSSION

The *in vitro* temperature responses of PEP carboxylase and malic enzyme activities are shown in Figure 1. Below 30 C, Q_{10} values for the malic enzyme and PEP carboxylase activities were 2.8 and 3, respectively. The activity of malic enzyme continued to rise with increasing temperature between 10 and 46 C. Brandon (1) similarly reported that malic enzyme activity from *Bryophyllum tubiflorum*, a crassulacean acid metabolism type plant, increased with increasing temperature between 15 and 53 C, indicating high thermal stability. In contrast, the temperature-response curve for PEP carboxylase activity had a temperature optimum at about 38 C (Fig. 1). Above 38 C, there was a sharp decrease in activity, indicating rapid heat inactivation of PEP carboxylase above that temperature. Similarly, a temperature optimum of about 35 C was reported by Brandon (1) for the PEP carboxylase-malic dehydrogenase activity from *B. tubi-*

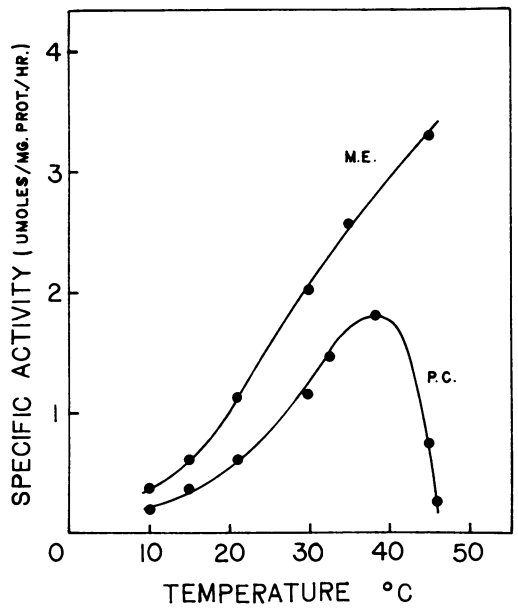

FIG. 1. *In vitro* temperature responses of PEP carboxylase (P.C.) and malic enzyme (M.E.) activities extracted from immature grape berries.

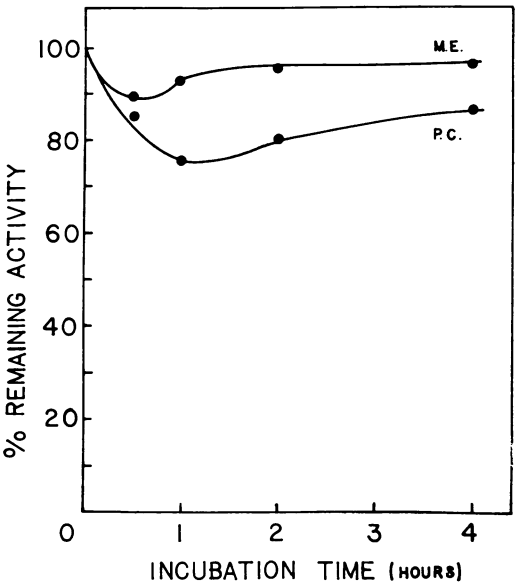

FIG. 2. Effects of a 40 C heat treatment of intact berries on the PEP carboxylase (P.C.) and malic enzyme (M.E.) specific activities after various times.

florum. The values for enzymic activities of both PEP carboxylase and malic enzyme reported by Brandon were only slightly higher than those we found in immature grape extracts.

To help understand the possible significance of the different enzyme temperature response curves, the malic enzyme response curve (representing malic acid degradation) was subtracted from the PEP carboxylase response curve (representing malic acid synthesis). The resulting curve indicates that the optimal tendency for malic acid accumulation (synthesis greater than degradation) is between 20 and 25 C, and that above 38 C there is a sharp tendency for loss of malate with increasing temperature. The relationship is similar to that reported by Brandon for *Bryophyllum* (1).

Although the relationship is between two extracted enzymic activities, and thus must be properly qualified, the findings correlate with the known effects of temperature on malic acid levels in grapes (6–8, 16). This suggests that the temperature responses of PEP carboxylase and of malic enzyme are involved in regulating malic acid levels in grape berries.

Differences between the activities of PEP carboxylase and malic enzyme at high temperatures (Fig. 1) indicate widely different rates of temperature inactivation of the activities of the two enzymes. The *in vitro* temperature inactivation rates found for the enzymic activities over a 4-hr period at 25, 35, and 45 C were, respectively, 0, 4, and 34%/hr loss for malic enzyme and 11, 39, and 96%/hr loss for PEP carboxylase. As expected from the temperature-response curves, the malic enzyme activity had greater *in vitro* stability at all temperatures than did the PEP carboxylase activity, especially at 45 C.

In vitro inactivation may or may not reflect the actual *in vivo* inactivation, because thermal protection mechanisms, such as binding to membranes or heat-protective compounds, may be operative *in vivo* (11) as well as resynthesis or reactivation of enzyme activity.

When intact berries attached to the vines were kept at 40 C for 4 hr, the responses of the two enzymes were considerably different from those *in vitro*. Not only were the rates of losses similar for the two enzymic activities during the first 30 min of heat treatment, but in both cases much of the original activity lost was recovered by 4 hr (Fig. 2). There were two important differences in the response of the enzymes to temperature. The malic enzyme activity was recovered sooner, and to a greater extent, than was the PEP carboxylase activity.

The greater loss and lesser recovery of PEP carboxylase (representing malate synthesis activity) over the 4-hr period at 40 C compared to the loss and recovery of malic enzyme (representing malate degradation activity), may explain, at least in part, the lower levels of malic acid observed in grapes at high temperatures (6–8). Sun-exposed grape berries have been found to reach 46 C in the field (9).

The possibility that the recovery of activity noted in Figure 2 was attributable to synthesis of new heat-stable isozymes was tested by examining the *in vitro* heat stabilities of the two enzyme activities before and after the *in vivo* heat treatment. The differences between the temperature inactivation of enzymes extracted before and after heat treatment were very small. These results indicate that immature grape berries do not synthesize new heat-stable isozymes of PEP carboxylase and malic enzyme in response to high temperature. The mechanisms by which the activities are recovered in grape berries are still unknown.

Temperature effects on malic acid accumulation or loss may be attributed to several factors. Langridge and McWilliam (11) pointed out that low temperatures increase CO_2 solubilities, which would tend to suppress decarboxylations and favor CO_2 fixation. Also, the production of malic acid is a very exothermic reaction, and thus would be more favored at lower temperatures in comparison to the degradation of malic acid. Substrate availability is also dependent on other related metabolic pathways and their own regulation. Probably these factors, as well as inherent temperature stability differences, affect the balance between synthesis and degradation.

Acknowledgments—The authors gratefully acknowledge the advice and assistance of R. C. Huffaker, T. Kosuge, L. W. Peterson, and R. Morenzoni.

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