Temperature Effects on Phosphoenolpyruvate Carboxylase from a CAM and a C₄ Plant

A COMPARATIVE STUDY

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ABSTRACT

The effect of temperature in the range from 10 to 35°C on various characteristics of phosphoenolpyruvate carboxylase from the leaves of a CAM plant, Crassula argentea and a C₄ plant Zea mays shows a number of different effects related to the environment in which these distinct types of metabolic specialization normally operate. The Arrhenius plot of Vₘₐₓ for the two enzyme forms shows that the CAM enzyme has a linear increase with temperature while the C₄ enzyme has an inflection at 27°C implying a conformational or aggregational change in the enzyme or a shift in reaction mechanism to one requiring a lower activation energy. The Arrhenius plot of Kₘₐₓ for the two enzymes reveals the startling fact that at temperatures above 20°C an increasing temperature causes an increase in Kₘₐₓ for the CAM enzyme while the C₄ enzyme displays a decreased Kₘₐₓ as the temperature increases. The inhibitory effect of 5 millimolar malate also shows opposite trends for the two enzymes. For the CAM enzyme the percent inhibition by malate increases from essentially none at 15°C to 70% at 35°C. For the C₄ enzyme the percent inhibition drops from about 60% at 20°C to 2% at 30°C. Similar opposite behavior of the two enzymes is found with the Kₘₐₓ for malate. Pretreatment at high temperatures for periods up to 2 hours was found to result in differences similar to those described above if the treated enzyme were subsequently assayed at 25°C.

PEPC¹ (EC 4.1.1.31) occurs in all plants (29). In CAM and C₄ plants, it plays an important role in catalyzing the fixation of CO₂ for the systems which are characteristic of these two metabolic specializations.

It has been shown that PEPC from CAM plants exists in two interconvertible forms. A night form with a higher affinity for the substrate PEP is also resistant to malate inhibition. A day form with a lower affinity for substrate is very sensitive to inhibition by malate (35, 36). In C₄ plants, recent studies have indicated that PEPC is also under a day/night system of regulation and exists in two enzyme forms with different sensitivities to inhibition by malate. In comparison with the CAM enzyme, the diurnal functioning of the C₄ enzyme operates in a reverse fashion. At night the enzyme is reported to be more sensitive to malate inhibition than the day form (9). PEPC extracted from preilluminated corn leaf shows some increase in total enzyme activity and is significantly altered in sensitivity to effectors. Malate inhibition was reduced and G6P activation was increased.

It is believed that many factors such as light, temperature, pH, and the level of metabolites could be involved in the diurnal change in enzyme kinetic properties in both CAM and C₄ plants. Light modulation of PEPC in CAM and C₄ plants has received more attention recently (9–12, 14, 15) but the mechanism of PEPC regulation in vivo is still unclear. Some CAM plants continue to display an endogenous circadian rhythm of CO₂ output and changes in the sensitivity of PEPC to malate inhibition even in continuous darkness (30, 35).

One environmental factor, diurnal temperature fluctuation, should not be ignored. Wilkins (31) suggested that the rhythm of PEPC activity in CAM plants is controlled by exposure of leaves to the combined signals of light and high temperature (35°C). Recently, the work of Sellinoti et al. (24) and Buchan-Bollig et al. (3) has indicated that the effect of temperature is cooperative with light on both C₄ and CAM PEPC.

We present here data comparing the temperature effects on PEPCs purified from a CAM plant (Crassula argentea) and a C₄ plant (Zea mays). The results indicate that the changes of PEPC kinetic and regulatory properties are consistently correlated with optimum diurnal adjustments to the temperature changes encountered by C₄ and CAM plants.

MATERIALS AND METHODS

Crassula Phosphoenolpyruvate Carboxylase. Plants (Crassula argentea Thunb.) were grown in a growth chamber as described previously (36, 37) except that the temperature was controlled at 35°C during the lighted period and 15°C in darkness. The enzyme was purified from Crassula leaf after 90 s homogenization in a Polytron with an extraction buffer containing 0.2 M Tris (pH 8.4), 2 mM DTT, 1 mM EDTA, and 2% PEG 6000. Precipitation with ammonium sulfate (35–55% saturation) was followed by chromatography on a 12 × 280 mm TSK Fractogel DEA column. The active fractions were combined and loaded on a 12 × 150 mm hydroxypatite column (BioRad) and eluted by a gradient of 150 to 500 mM potassium phosphate buffer containing 1 mM DTT and 1 mM EDTA at pH 7.2. The enzyme preparation purified by this procedure showed a major band with PEPC activity and two other faint bands on PAGE gel and one major protein band with mol wt about 110,000 on SDS-PAGE gel. The specific activity of this enzyme preparation was 15 IU/mg. The protein profile on a TSK-4000 HPLC column eluted with Tris-Mes 50 mM pH 6.8, showed two peaks. The activity profile indicated that the protein peak with PEPC activity is a tetramer, while the other protein peak, which appeared at the void volume, probably is a more highly aggregated form of

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3 Abbreviations: PEPC, phosphoenolpyruvate carboxylase; Mops, 3[N-morpholinol]propanesulfonic acid; PEP, phosphoenolpyruvate.
the enzyme.

Maize Leaf PEPC. Maize leaf PEPC was obtained from Calbiochem. HPLC showed that the major peak was about M₉ = 440,000. The specific activity was 4 IU/mg. For some studies small quantities were chromatographed by HPLC and the tetramer peak collected for use in further chromatography.

HPLC Size Exclusion Chromatography. A Spherogel-TSK 4000sw column 7.5 x 300 mm provided by Beckman was used with a Beckman HPLC system as previously described (37).

PEPC Assay. The activity of PEPC was assayed in a Beckman DU-50 spectrophotometer by monitoring the change in absorbance at 340 nm in an assay system coupled with malate dehydrogenase (34). The temperature was controlled with a circulating water bath. For temperature studies, a set of cuvettes, usually six, filled with assay mixture were preincubated at a selected temperature for about 20 min and monitored by an electrical thermometer to ensure that the cuvettes were equilibrated to the designated temperature. Assay mixtures contained 50 mM Mops (pH 7.5), 5 mM MgSO₄, 10 mM NaHCO₃, 0.15 mM NADH, and 2 IU of porcine malate dehydrogenase. For routine assay 1 mM PEP was used. Assays were based on constant levels of free anions and Mg²⁺ (4). The reaction was initiated by the addition of PEPC. Kinetic parameters were determined by fitting the rates found at varying concentrations of PEP at each temperature to the Michaelis-Menten equation modified to provide estimates of the Hill number as well as Vₘₐₓ and Kₘ (34). The term Kₘ is used here to designate both Kₘ and S₀.₅. The energy of activation (Eₐ), ΔH, and Q₁₀ were calculated from the Arrhenius plots (5, 23).

The cubic spline fits used a BASIC program adapted from Pennington (21).

RESULTS AND DISCUSSION

Effects of Temperature on Vₘₐₓ. The effects of temperature on Crassula PEPC and corn leaf PEPC maximal activity is shown in Figure 1. The Vₘₐₓ of the Crassula enzyme increased with increasing temperature from 10 to 35°C with no inflection being apparent over this range. The enzyme activation energy calculated from the plot is 14 kcal/mol.

With the corn enzyme, as is also shown in Figure 1, a discontinuity in the slope of the Arrhenius plot is found at 25°C. The activation energy (2.6 kcal/mol) at temperatures above the inflection is 5-fold lower than the Ea obtained at temperatures below the inflection (Ea = 17 kcal/mol). The lower activation energy for the formation of the reaction intermediate would increase the catalytic efficiency of the enzyme at the higher leaf temperatures commonly encountered by C₄ plants during the period of the day when PEPC should be most active (1, 13). Various discontinuities in the slope of Arrhenius plots for maize PEPC have been reported. These include 25°C (17), 11°C (28), and 17 to 20°C (22). These results suggest that the corn enzyme undergoes a change in mechanism in a midtemperature range presumably as a result of some conformational or perhaps aggregational change occurring with an increased temperature.

Temperature Effects on Kₘ. The effects of temperature on the Kₘ of the CAM and C₄ PEPC are shown in Figure 2. In this case both the CAM and C₄ enzyme display inflections, but their basic slopes are in opposite directions. The maximal apparent affinity for substrate (PEP) occurs within the temperature range which closely coincides with the temperatures at which enzymes each are likely to be functional in vivo. With the Crassula enzyme the maximal affinity for PEP occurs at 17°C. This is a temperature which is frequently encountered at night by CAM plants and should be optimal for nocturnal CO₂ fixation. The C₄ enzyme shows the lowest Kₘ at the highest temperature used (35°C) which is again consistent with the need for maximal activity of PEPC in C₄ metabolism during hot days.

Bramdon (2) reported that the diurnal fluctuation of organic acids and PEPC activity in CAM plants is associated with variations in temperature. Many workers have pointed out that night carbon dioxide fixation by CAM plants was inhibited by high night temperatures (16, 18, 19). It was noted (3) that PEPC from Kalanchoe daigremontiana has a lower Kₘ value at 15°C than at higher temperatures. Our data extend and clarify these observations. The Arrhenius plot of Kₘ (Fig. 2) shows inflections for both the CAM and C₄ enzymes. The breaking point for the CAM enzyme is at 17°C, while for the C₄ enzyme the break is found at 25°C as in the plot of log Vₘₐₓ versus 1/T (Fig. 1). It should be noted that the Kₘ for the C₄ enzyme decreased with increasing temperature and that the highest observed affinity for PEP was at the highest temperature used.

The value of enthalpy change (ΔH) was calculated from Figure 2. For the C₄ enzyme ΔH values are -7.6 kcal/mol in the low temperature range (10-20°C) and -19.4 kcal/mol at temperatures above 25°C. The value for the CAM enzyme was also negative at the low temperature range (-6.8 kcal/mol at 9-15°C) but with a positive ΔH value (19.2 kcal/mol) between 25 and 35°C. These results clearly showed that for the CAM enzyme ΔH values increased at temperatures above the inflection point (25°C). For the C₄ enzyme the increased ΔH was found at the low temperature range (10-20°C). These changes of ΔH probably are due to a temperature-dependent shift of enzyme forms with different catalytic efficiencies.

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** Arrhenius plot of log Vₘₐₓ of CAM and C₄ PEPC carboxylase. Vₘₐₓ, from lines fitted PEP concentration series run at the indicated temperatures (see "Materials and Methods" for details). *Crassula* leaves (+); *Zea mays* leaves (x).

![Figure 2](https://example.com/figure2.png)

**Fig. 2.** Arrhenius plot of log Kₘ of CAM and C₄ PEPC carboxylase. Kₘ, from lines fitted PEP concentration series run at the indicated temperatures (see "Materials and Methods" for details). *Crassula* leaves (+); *Zea mays* leaves (x).
Temperature Effects on Cooperativity. The plots of Hill Number versus temperature for the two enzymes in Figure 3 reveal interesting differences. At temperatures below 25°C the CAM enzyme shows positive cooperativity which declines with increased temperature, becoming negative at 25 to 30°C and again becomes positive at higher temperatures. The C₄ enzyme is negatively cooperative at low temperatures, but above 25°C it shows marked positive cooperativity. These changes may have significance at two levels. Positive cooperativity is likely to accompany a more efficient enzyme operation, at least at high levels of substrate. At the other level, positive cooperativity may mean that more enzyme sites are interacting, perhaps because of aggregation of the enzyme. Negative cooperativity, on the other hand, may indicate higher efficiency at low substrate concentrations, and may or may not be related to aggregational status, but probably is associated with conformational changes which have occurred in the transition from one state of cooperativity to the other.

Temperature Effects on Sensitivity to Malate Inhibition. Recent studies on the regulation of PEPC in CAM and C₄ plants indicated that the enzymes from both plants exist in two forms (day form and night form) with differing sensitivities to malate inhibition (9, 10, 15, 35). The two enzyme forms from Crassula leaves can be interconverted by some treatments (33, 34, 37). In the present studies, we noted that assay temperature had a striking influence on enzyme sensitivity to malate inhibition as shown in Figure 4. At low temperatures the CAM PEPC was rather insensitive to malate, at temperatures below 20°C its activity was inhibited less than 20% by 5 mM malate. The sensitivity of this enzyme to malate inhibition increased with increasing temperature, reaching a 70% inhibition level at 35°C. The C₄ enzyme, on the other hand, showed strong inhibition (about 55%) at low temperatures, but at temperatures above 20°C it is progressively less inhibited, reaching 0% at 30°C. A further increase in temperature to 35°C results in a 20% activation of the enzyme by 5 mM malate. This bifunctional effect of malate on PEPC has occasionally been reported (6, 32).

Similar results have been obtained for the effect of temperature on $K_i$ for malate (Fig. 5). The $K_i$ for malate of the CAM enzyme decreased from 11 to 1 mM as the temperature was increased. Again, the C₄ enzyme shows the opposite relationship. Here the $K_i$ increased from 0.3 to 5.5 mM as the temperature was increased between 15 and 27°C. Figures 4 and 5 show a clear distinction between the CAM and C₄ enzymes with respect to the temperatures at which malate is an effective inhibitor. It appears that both enzymes shift to forms which are sensitive to malate inhibition under the influence of temperatures which are favorable for operation of each type of metabolism with PEPC inhibited by malate, that is, the CAM enzyme is turned off by malate during the hot days and the C₄ enzyme is inhibited most during the cool nights, since the CAM enzyme needs to be active at night, and the C₄ enzyme is required to be most productive during the day. The affinity of the enzymes for both their substrate PEP and for the inhibitor malate responds to changes in temperature in the way which will permit the activity of PEPC to be most productive during the period when its contribution to photosynthetic metabolism is most needed by the two types of plants.

These differences in the response of regulatory characteristics to changing temperature between the CAM and C₄ PEPC carboxylases seem likely to have arisen by virtue of the differing functions of the same enzyme in the two specialized metabolic systems.

We have noted that under local conditions leaf temperatures of Crassula at midday run several degrees above ambient air temperatures, and at night they may be significantly lower than air temperatures if leaves are exposed to the clear sky. The overall range of temperatures affecting the action of PEPC is thus even greater than the diurnal air temperature range.

Hysteretic Response to High Temperature. It was of interest to determine whether or not the responses to temperature seen when assays were carried out at various temperatures could also be produced by preexposure to, e.g., a high temperature, followed by assay at the normal 25°C. The results of such an experiment
are shown in Figure 6, where the CAM PEPC has been preincubated at 42°C for varying periods of time, followed by assay at 25°C. The upper line, a control assay, shows a slight (about 10%) loss of activity which is not increased by more prolonged exposure to the higher temperature. However, when 2 mm malate is included in the assay there is a progressive increase in the inhibition produced by this low level of malate, from 10% in the nonpretreated enzyme to >50% in the enzyme pretreated for 2 h. It seems clear that the change(s) in the enzyme at high temperatures which increase the sensitivity to malate are ones which persist at least for several minutes after removal from the high temperature. On the basis of our earlier experience (37, 38), a major change likely to be induced by high temperatures is the dissociation of the resistant tetramer of the CAM enzyme to the malate sensitive dimer.

It has recently (20) been reported that PEPC from day leaves of corn was less inhibited (10–20%) by malate than the enzyme prepared from night leaves. The difference was attributed to differing levels of phosphorylation of the two forms of the enzyme resulting from exposure to light. However, since the night leaves were held 10 h in darkness at 15°C and the day leaves were exposed 1 h to light at 27°C, it seems that a differential effect of temperature may be as reasonable an explanation for the differing sensitivity to malate as the postulated effect of light on phosphorylation of the enzyme.

Comparison of Qu Values. The interaction of substrate concentration and temperature effects is shown in Table 1 where Qu values have been computed for both enzymes at five different concentrations of the substrate PEP. At the lower temperature (15–25°C) the C4 enzyme showed a larger increase in Qu with an increase in PEP concentration than the CAM enzyme. At the higher temperature (25–35°C) the CAM enzyme shows a much greater increase in Qu with increasing PEP concentration, while the C4 enzyme is essentially unresponsive to PEP concentration. These results imply that an inefficient form of the CAM enzyme at high temperatures may be converted to an efficient form by the presence of PEP and are consonant with our earlier observation that PEP induces the aggregation of the CAM PEPC dimer to the tetramer (38) and our more recent finding that the corn leaf enzyme is not susceptible to such changes after preincubation with PEP (34).

Hochachka and Somero (8, 26) suggested that temperature effects on the Km of enzymes may be more important to the organism than are temperature effects on enzyme molecular activity because evolutionary changes appear to produce a changed substrate or effector affinity more readily than an altered intrinsic activity. Recently Storey (27) reported that during mammalian hibernation the Vmax of PFK is not changed, but the enzyme was shifted to a form more sensitive to inhibition by ATP and citrate. The I50 values were 4-fold lower than controls.

In the comparisons presented here, a major point is that low temperatures favor efficient activity (low Km) of the CAM enzyme, while high temperatures have that effect on the C4 enzyme. These apparent anomalies probably are best explained by the assumption that evolutionary development of CAM and C4 metabolism has included the conformational adjustments necessary to provide this efficient arrangement. While a similar nonexplanatory explanation can presumably be invoked for the other major anomaly, the reverse response to malate as a function of temperature in the two forms of PEPC, more mundane explanations, if possible, are more attractive. How can we account for this? Possibilities are: (a) the PEPCs from both plants are under a dissociation-association regulation like that we have seen with the CAM enzyme (37, 38), and the dimer of both forms is sensitive to malate inhibition, but high temperatures make the CAM enzyme dissociate, while they make the C4 enzyme aggregate. (b) High temperatures make both forms dissociate, but the CAM dimer is inhibited by malate, while the C4 dimer is resistant. (c) Temperature overrides the effect of aggregation/disaggregation and causes some other structural change which leads to inhibition in one case and the opposite in the other. (d) The sensitivity to malate is somehow related to the different effects of temperature on Km, since malate inhibition is under some circumstances competitive with PEP (33, 36) the effect of temperature on Km PEP and Km malate may reflect the same conformational change.

The C4 enzyme has been reported to be cold susceptible (7, 25) and we have observed that it loses its activity gradually at 4°C. We found that freshly dissolved corn leaf enzyme consisted

Table 1. Qu Values for PEPC from Crassula and Z. mays Leaves at Low and High Temperature Ranges and Interaction with PEP Concentration

<table>
<thead>
<tr>
<th>PEP Concentration</th>
<th>15–25°C (A)</th>
<th>25–35°C (B)</th>
<th>A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm</td>
<td>CAM</td>
<td>C4</td>
<td>CAM</td>
</tr>
<tr>
<td>0.25</td>
<td>2.566</td>
<td>2.656</td>
<td>0.508</td>
</tr>
<tr>
<td>0.5</td>
<td>2.401</td>
<td>2.972</td>
<td>0.731</td>
</tr>
<tr>
<td>1.0</td>
<td>2.595</td>
<td>3.226</td>
<td>1.541</td>
</tr>
<tr>
<td>2.0</td>
<td>2.756</td>
<td>3.359</td>
<td>1.843</td>
</tr>
<tr>
<td>5.0</td>
<td>2.890</td>
<td>3.328</td>
<td>2.018</td>
</tr>
</tbody>
</table>

Fig. 6. Effect of preincubation at 42°C for different periods of time on the activity and sensitivity to malate of CAM PEP carboxylase. Enzyme from Crassula leaves was preincubated at 40°C for the times indicated, followed by assay at 25°C. Lines fitted by cubic spline. Percent of original activity remaining (C); percent inhibition by 5 mm malate (A).

Fig. 7. Effect of temperature on percentage of corn leaf PEPC isolated as tetramer by HPLC. Separations as described in text. Line fitted by cubic spline.
of 79% tetramer when chromatographed at 25°C. When a pure tetramer from an HPLC separation carried out at 25°C was incubated at 4°C for 16 h and chromatographed at 25°C, the tetramer dropped to 53% with the altered protein appearing as 19% dimer and 28% as an inactive monomer. After 24 h treatment at 4°C, the tetramer had decreased to 32% with 17% monomer and 51% of the total appearing as an aggregate larger than tetramer which was without activity, reflecting denaturation by conversion of the tetramer to a larger inactive aggregate, perhaps via the monomer.

It is difficult to reproduce assay conditions in HPLC separations of enzymes of different sizes, both enzyme concentration and time variables must be different because of inherent differences in the procedures. We have, however, attempted to produce a profile of temperature effects on the fraction of the total enzyme present as tetramer over the range from 5 to 35°C. This is summarized in Figure 7 in which the pure core leaf PEPC tetramer isolated at 25°C (which contained 79% tetramer) was preincubated for 5 min at the temperatures indicated and chromatographed (about 10 min) with buffer and column held at the same temperature as in preincubation. The enzyme concentration was 10-fold greater than that used for assays.

As is shown in Figure 7, the percentage of tetramer at the varying temperatures increases up to 30°C, followed by a precipitous decline to 35°C. The other forms of enzyme present consisted of dimer, monomer, and a large aggregate as mentioned above. These forms seemed to have no clear association with temperature.

The trend shown in Figure 7 is in agreement with the sensitivity to malate inhibition shown in Figure 4, e.g. increasing temperatures produce an increasing percentage of tetramer and a decreasing percentage inhibition by malate, except that at 35°C the percentage of tetramer decreases while malate produces activation rather than inhibition. This may indicate that the activation response is distinct from the inhibition response which seems to be associated with the concentration of tetramer present.

These data serve to reduce the probability of some of the possible explanations listed above, but at this point selection among them is not possible.

LITERATURE CITED