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Activation of Higher Plant Phospho*enol*pyruvate Carboxylases by Glucose-6-Phosphate[1]

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ABSTRACT

Studies of the response of phospho*enol*pyruvate carboxylase from C_3 (wheat [*Triticum aestivum* L.]), C_4 (maize [*Zea mays* L.]), and Crassulacean acid metabolism (CAM) (*Crassula*) leaves to the activator glucose-6-phosphate as a function of pH showed that the binding of the activator and the response path to activation were essentially identical for all three enzymes. The level of affinity for the activator differed, with the CAM enzyme having the highest affinity and the maize enzyme the lowest. The observed pK values suggest that histidine and cysteine groups may be involved in activation by glucose-6-phosphate. The presence of glucose-6-phosphate protected the enzyme against inactivation of the activation response by *p*-chloromercuribenzoate. The maximal activation response to glucose-6-phosphate showed differences among the three enzymes including different pH optima and different pH profiles. Here the maize leaf enzyme showed a potential response about twice as great as that of the C_3 and CAM enzymes.

Regulation of PEP[2] carboxylase in CAM and C_4 plants is often attributed to an interaction of two effectors—activation by Glc-6-P and inhibition by malate (1, 8, 12, 14–16, 24, 27, 28). Although other factors such as enzyme phosphorylation (3, 7, 11), aggregation/disaggregation of the enzyme (29, 30, 32), temperature (2, 21, 28, 31), and other allosteric effectors (8, 9, 14–17, 19, 22, 28) seem to be involved in at least some cases, it is clear that these two effectors have powerful influences on the post-translational activity of PEPC regardless of whether other factors may also be involved. The need for regulation is readily apparent in the case of plants with CAM metabolism, where turning off PEPC during the day is required to avoid a futile CAM cycle. In the case of C_4 plants, beneficial conservation of some high-energy ligands can be assumed if the enzyme is most active during the brightest part of the day when CO_2 concentration may be limiting the normal photosynthetic apparatus and is reduced in activity at night. Andreo *et al.* (1) have postulated that Glc-6-P acting on PEPC may be important in regulation of C_4 photosynthesis by producing, in collaboration with other regulatory factors, a controlled flux of metabolites across the chloroplast envelope. The PEPCs of C_3 plants are mostly responsible to these same effectors, and Latzko and Kelly (10) have listed 11 possible functions of PEPC in C_3 plants, although these seem less likely to need tight regulation.

Many studies (8, 9, 14–16, 22, 28) have reported the activation of a variety of PEPCs by Glc-6-P, and a number of possibilities have been postulated for the way in which the activity of the enzyme is stimulated by this sugar phosphate. It seemed potentially beneficial to understanding the mechanism of this activation to study the effect of pH on the activation of PEPC by Glc-6-P as a means of providing clues to the groups involved in binding of Glc-6-P and in the expression of its activating effect. The study was extended to include examples of three different types of plants, C_3, C_4, and CAM, both because a direct comparison of Glc-6-P activation of the PEPC of these various types of plants has not been available, and because differences in the activating mechanism might be revealing of the underlying characteristics of the process which results in activation and of the metabolic patterns of the plants themselves.

MATERIALS AND METHODS

Enzymes

Three different forms of PEPC (EC 4.1.1.31) were compared in these studies. The first was the enzyme prepared in our laboratory from field-grown *Crassula argentea* by the procedure previously described (19). The specific activity of this preparation was 19 μmol/min/mg, and it appeared homogeneous on the basis of SDS gel electrophoresis and protein-stained native gels.

The commercial maize (*Zea mays* L.) leaf enzyme from Calbiochem-Behring was used as supplied, with small quantities of the freeze-dried preparation being dissolved just before use. The specific activity of this preparation was about 5 IU/mg, and on the basis of the relative amount of protein corresponding to the 100 kD MR monomer of PEPC (30) it was estimated as between 80 and 90% pure.

The wheat leaf (*Triticum aestivum* L.) leaf PEPC was supplied by Boehringer as an ammonium sulfate suspension. It was used after dialyzing small quantities overnight at 4°C. in 50 mM Aces buffer (pH 7.2) plus 1 mM DTT. The specific activity of this preparation was about 4 IU/mg and on the basis of SDS gels it appeared to be about 60% pure.

Although both the commercial PEPC preparations were relatively impure, we found that it was not readily possible to improve their specific activities, and since the preparations did not appear to contain significant competing or interfering

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[2] Abbreviations: PEP, phospho*enol*pyruvate; PEPC, phospho*enol*pyruvate carboxylase; Aces, *N*(2-acetamido)-2-aminoethanesulfonic acid; Ches, 2(*N*-cyclohexyl-amino)ethane sulfonic acid; PCMB, *p*-chloromercuribenzoate; Glc-6-P, glucose-6-phosphate.

activities, including phosphatase, the interspecies comparisons should still be valid. More importantly, the way in which activity is expressed in these studies (as a ratio of activated and unactivated activities) minimizes the probability that differing specific activities as such could result in differing interpretations of pH effects on the processes studied.

Buffers

The buffers used for varying assay pH over the range from pH 6.0 to pH 9.0 was a mixture of 50 mM Mes, 50 mM Aces, and 50 mM Ches. The buffers were dissolved in 200 mM each and aliquots adjusted to the desired pH and diluted to make each buffer component 100 mM. This stock buffer mixture was used to prepare the assays at the desired pH by dilution with other assay components to give a total of 150 mM of the three buffers (50 mM each).

Assays

Enzyme activity was measured in 1 mL cells held at 25°C in the sample-changing cell compartment of a Cary model 219 spectrophotometer. The assays followed the disappearance of the 340 nm absorbance of NADH using malate dehydrogenase and lactate dehydrogenase as coupling enzymes (13). Each assay contained 150 mM mixed buffer as indicated above, 0.2 mM NADH, 0.1 mM MgPEP (which corresponds to a total of 0.3 mM PEP) or, for the high level PEP assays, 8.5 mM MgPEP (20 mM total PEP), 5 mM HCO_3, 5 mM Mg^{2+}, and 2 IU each of malate dehydrogenase and lactate dehydrogenase. At each pH, concentrations of Glc-6-P of 0, 0.22, 0.65, 1.74, and 4.33 mM MgGlc-6-P were used. The assays were assembled from protocols written by a BASIC program which permits construction of assays with specified concentrations of free or complexed anions and Mg^{2+}. The concentrations of PEP and Glc-6-P complexed with Mg^{2+} were used because of earlier indications that these are the forms bound by PEPC (25, 26). Assays of five different Glc-6-P concentrations at a single pH were measured at the same time. The assays were removed for pH determination 3 min after the reaction was initiated by adding PEPC and in the few cases where the final pH differed by more than 0.05 pH unit from the initial value, the final pH was used. At some pH values the observed rate changed during the 3 min period of the assay, and in these cases only the initial rate was used.

Data Analysis

The experimental value used in analysis of activation in these studies is the fractional activation defined as:

$$(v_a - v_o)/v_o = \mathrm{ACT}_{max} \times A/(K_a + A) \quad (1)$$

where v_a = the observed rate in the presence of Glc-6-P, v_o = the observed rate in the absence of activator, ACT_{max} = maximal activation at infinite Glc-6-P concentration, K_a = activation constant, and A = concentration of Glc-6-P.

The K_a and ACT_{max} values, together with the v_o rates found at two levels of PEP at each pH, provided data for a secondary analysis of the influence of pH on the enzyme and its activation. For the activation constant a model which assumes that only a singly protonated enzyme species is capable of binding the activator and carrying out the activation process (20) is used. This model is:

$$K_{a(app)} = K_a(1 + H/K_{Ei} + K_{Ea}/H)/ \cdot(1 + H/K_{EAi} + K_{EAa}/H) \quad (2)$$

where K_a is the true value of the activation constant; K_{Ea} and K_{Ei} are the dissociation constants for protonation of free enzyme; and K_{EAa} and K_{EAi} are dissociation constants for protonation of the enzyme-activator complex on the activating and inhibiting sides of the pH profile, respectively; H = the concentration of protons.

For analysis of the ACT_{max} (apparent maximal activation) data a model patterned after that of Dixon (4, 20) was used. The equation is:

$$\mathrm{ACT}_{max} = ACT_{true}/([1 + K_a^{n_a}/H^{n_a}] + [H^{n_i}/K_i^{n_i}]) \quad (3)$$

here ACT_{max} = apparent maximal activity at a given pH; ACT_{true} = true maximal activation; K_a and K_i are the dissociation constants for activation and inhibition by protonation; and n_a and n_i are the slopes of the bell-shaped curve on the activating (right) and inhibiting (left) sides, respectively. These values provide an estimate of the net number of protons involved in activation or inhibition of the Glc-6-P activation.

RESULTS

pH Profiles of Glc-6-P Activation Constants

Evidence from direct measurement of Glc-6-P binding to PEPC (25) is that the binding and expression of activation is probably overall a steady state phenomenon, or a complex process involving several reactions. The K_a therefore cannot be treated as a simple dissociation constant of an enzyme-Glc-6-P complex. Indeed, there is some evidence (19) that PEP itself also can bind at the Glc-6-P site and induce activation. The K_a values obtained here thus must be considered complex constants, and in some respects it is surprising to find that the response of K_a to pH is quite similar for the three forms of PEPC from diverse plants. This is illustrated in Figure 1 and Table I where it may be seen that there is a significant difference in the values of the true K_as. The CAM form shows the highest affinity for Glc-6-P and the maize form the lowest, with wheat intermediate between the two. On the other hand, the pK values for both the free enzyme and the EA complex do not differ significantly among the three forms with the optimum pH about pH 7.5.

Within the experimental error, which is rather large in some cases, these data appear to suggest that the characteristics of the Glc-6-P binding site are the same for the three forms of PEPC indicating conservation of the activating site and the processes associated with activation. The presence of the activator in that site also has the same influence on the characteristics of the enzyme in all three forms. The binding of Glc-6-P to the enzyme gives an additional spread between pK_{Ei} and pK_{Ea} which are about pH 7.3 to 8.0 for the free enzyme but increase to pH 6 to 9.6 when the activator is bound. This expansion of the region between the pKs suggests a substantial conformational change on activator binding, revealing groups which are otherwise not accessible, and, as

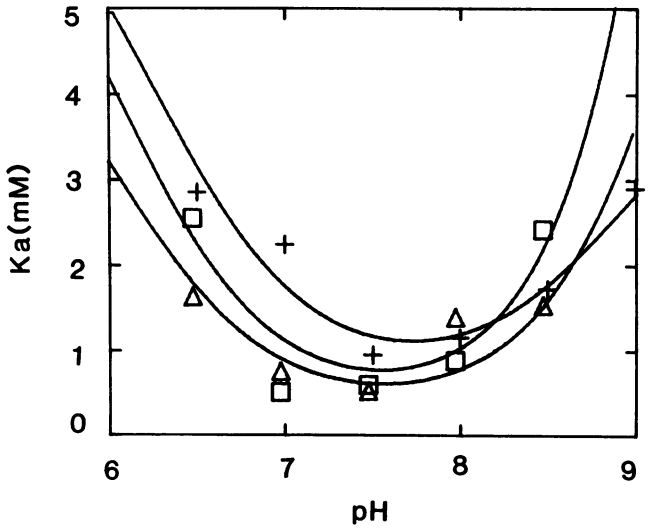

Figure 1. pH effects on the activation constants of three different PEPC enzymes for Glc-6-P. K_a values determined from Equation 1 as described in text, and pH profiles fitted to Equation 2. (+), Maize leaf PEPC; (□), wheat leaf PEPC; (△), *Crassula* leaf PEPC.

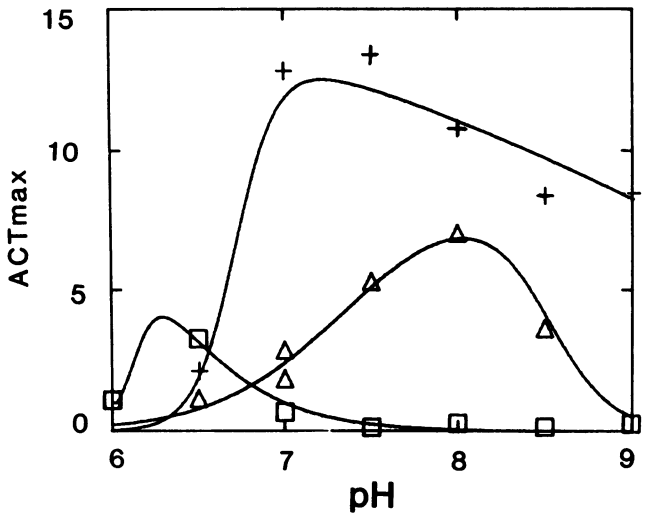

Figure 2. pH effects on the maximal activation induced by Glc-6-P in three different PEPC enzymes. ACT_{max} values determined from Equation 1 as described in text, and pH profiles fitted to Equation 3. (+), Maize leaf PEPC; (□), wheat leaf PEPC; (△), *Crassula* leaf PEPC.

Table I. *Kinetic Parameters Obtained from the Fit of K_a for Glc-6-P with Phosphoenolpyruvate Carboxylase from the Leaves of Maize, Wheat, and Crassula*

K_a values from fit of the activated fraction, $(V_a - V_o)/V_o$, to varying concentrations of Glc-6-P at different pH values to Eq. (2).

Kinetic Parameter	Maize Leaf PEPC	Wheat Leaf PEPC	*Crassula* Leaf PEPC
K_a (mM)	0.68 ± 0.12	0.33 ± 0.09	0.28 ± 0.10
pK_{EAa}	8.90 ± 0.37	14.37 ± 3.77	9.62 ± 2.79
pK_{EAi}	6.23 ± 0.58	6.02 ± 0.55	6.03 ± 0.65
pK_{Ea}	8.05 ± 0.22	7.72 ± 0.15	7.77 ± 0.19
pK_{Ei}	7.27 ± 0.22	7.39 ± 0.15	7.35 ± 0.18

Table II. *Kinetic Parameters Obtained from the Fit of Maximal Activation (ACT_{max}) by Glc-6-P of Phosphoenolpyruvate Carboxylases from the Leaves of Maize, Wheat, and Crassula*

ACT_{max} values obtained from fit of activated fraction, $(V_a - V_o)/V_o$, at varing concentrations of Glc-6-P and different pH values to Equation 1. Parameters below are from fit to Equation 3.

Kinetic Parameters	Maize Leaf PEPC	Wheat Leaf PEPC	*Crassula* Leaf PEPC
ACT_{true}	15.69 ± 0.08	9.01 ± 0.12	8.50 ± 0.05
n_a	0.33 ± 0.39	1.39 ± 0.18	2.33 ± 0.27
pK_a	9.10 ± 0.88	6.31 ± 0.14	8.46 ± 0.10
n_i	3.48 ± 0.52	5.69 ± 0.24	1.15 ± 0.16
pK_i	6.71 ± 0.27	6.14 ± 0.08	7.21 ± 0.15
Optimum pH	6.91 ± 0.49	6.17 ± 0.16	8.06 ± 0.12

mentioned below, may result from a change in the aggregation state of the enzyme.

pH Profile of Maximal Glc-6-P Activation

The relatively low level of PEP used in these studies (0.1 mM MgPEP) should provide an excellent opportunity for the expression of activation by Glc-6-P, which is under most circumstances competitive with PEP (16, 28, 29). This strong activation permits comparison of the relative effectiveness of Glc-6-P in activating the three enzymes. As Figure 2 and Table II show, the three different forms of PEPC are distinguishable from one another in several different ways when the maximal activation by Glc-6-P is examined. The maize leaf enzyme, although it has the lowest affinity for Glc-6-P, is capable of about twice as large an activation response at saturating levels of activator as the C_3 and CAM enzymes. The maize enzyme has a rather flat plateau between pH 7 and pH 9, but descends precipitously below pH 7. At pH 6 the activation is not merely zero, as shown by the fitted line in Figure 2, but Glc-6-P actually inhibits the rather low level of the reaction at that pH. The very high value of n_i, representing the steep slope at low pHs with the corn enzyme, is exceeded by the n_i value for the wheat enzyme, but this enzyme shows a sharp peak in the maximal activation response rather than the broad plateau of the corn enzyme.

The CAM enzyme shows a sharp decline between pH 8 and 9 and a normal slope on the inhibiting side which also leads to a very low level of activation at pH 6. The optimal pH, which is calculated taking into account the spread between pK_a and pK_i (20), probably best expresses the influence of pH on the response of these three forms of PEPC to Glc-6-P activation. The wheat leaf enzyme is most responsive at pH 6.17 and the corn enzyme at pH 6.91, although the breadth of the plateau for this enzyme makes this a less critical parameter. The CAM enzyme is clearly different, with a more or less normal bell shaped curve and a pH optimum of about 8.

One of the kinetic parameters which can be useful in pH studies is ACT_{max}/K_a which estimates the on rate for substrate binding to the enzyme (4). The analogous parameter in this study, ACT_{max}/K_a, was calculated and fitted to Equation 3 for the three different forms of PEPC.

Not surprisingly, all three enzymes had similarly shaped

profiles of ACT_{max}/K_a to pH (not shown), with optima around pH 7.5, tending to confirm the conclusion regarding conservation of the activation site suggested by the K_a data. More interesting are the maximal values for the three forms which by analogy with the usual pH profile of enzyme reaction with substrate should estimate k_{on} for Glc-6-P. For the three forms these values are: maize, 15.25 ± 0.13; wheat, 2.30 + 0.06; and *Crassula,* 12.46 + 1.08. These data indicate a much slower rate of binding for the wheat enzyme.

Response of Control Velocity of CAM Enzyme to pH

The control, unactivated, rate of the *Crassula* enzyme displayed an unexpected response to pH in the range studied (from pH 6–pH 9). The optimum pH was out of this range, <6.0, the pK_a was 6.94 + 0.19 and n_a was 1.07 ± 0.21. This response is thus that of activation by a single proton over the entire range of pH, and the pK_a suggests that the group responsible may be a cysteine. The possible significance of this is discussed below.

DISCUSSION

Perhaps the most surprising aspect of this study of pH effects on the activation of PEPC by Glc-6-P is the degree to which the binding site for the activator has been conserved in the three rather disparate plants studied. The PEPC of each of these plants has different major functions for which an activating response to Glc-6-P would be helpful but the data obtained (Fig. 1; Table I) indicate that the characteristics of both the binding site and of the groups which may be involved in expression of the activation response are similar, if not identical, in all three forms. This conclusion is further supported by examination of the ratio of ACT_{max}/K_a, which should give an estimate of the on rate for Glc-6-P and which also shows similar responses to pH for all three enzymes.

In spite of these similarities, the enzymes show distinct differences with respect to the true K_a or the intrinsic affinity for the activating ligand. These may arise from minor conformational differences between the enzymes and probably indicate adaptations to differing levels of endogenous Glc-6-P in the different plants.

The pK_{Ea} and pK_{Ei} values for all three enzymes fall in the range of pH 7 to 8 (Table I). At this point little is known of the groups involved in binding of Glc-6-P, but these values suggest participation of cysteine and histidine. The involvement of an arginyl residue, which often has a role as an anion binding group, has been ruled out in the case of the PEPC activation (Glc-6-P) site by earlier studies (19). The role of a thiol group in the binding of PEP by PEPC has been demonstrated (5, 6, 23) and we have found that Glc-6-P protects PEPC against inactivation by thiol reagents as well as protecting against inactivation of the response to Glc-6-P. Specifically, when *Crassula* PEPC is assayed after being incubated 20 min at pH 7.0 with 50 μM PCMB, the enzyme is activated 9.6% by 4.33 mM MgGlc-6-P. If 4.33 mM MgGlc-6-P is included in the incubation mixture with PCMB, then 4.33 mM MgGlc-6-P in the assay activates the reaction 47.1%. Magnesium must be present for this effect, suggesting that the —SH group is involved in binding of the MgGlc-6-P ligand, which appears to bind as the complex (25). The magnesium might also be required for some reaction necessary for expression of the activation, such as, *e.g.* aggregation.

The maximal response to a competitive activator like Glc-6-P is not a completely independent parameter. It involves, among other things, the K_m of the enzyme for the substrate responding to the activator, the concentration of the substrate present, and the concentration of the activator relative to its own affinity for the enzyme (K_a). While recognizing that these and other factors may be operating in this study, it seems likely that the twofold difference in maximal activation of the maize leaf enzyme compared with the wheat leaf and CAM enzymes (Table II) represents a real difference in the way the enzymes respond to Glc-6-P. The maize leaf enzyme, although its affinity for the activator is low, is capable of a substantial response given adequate amounts of Glc-6-P.

The C_3 and C_4 enzymes show a lower pH optimum and a sharp cutoff on the low pH side which suggests the possibility that cellular pH may play more of a role in controlling the response to Glc-6-P than would be the case in the CAM leaf enzyme, which has a higher pH optimum and slope of 1 with increasing protonation. Thus, even though considerable diurnal changes in cellular pH may occur in the CAM plant, the response to G-6-P may be isolated from any likely regulation by pH by its relatively high pH optimum.

The extended increase in velocity of the CAM PEPC at low PEP concentration from pH 9 to pH 6 seems to indicate that at least at low PEP concentrations, the CAM enzyme may be more sensitive to changes in pH than to activation by Glc-6-P. Although it is well known that large diurnal changes in cellular pH occur in CAM plants, it is not at all certain whether these changes, occurring mostly in the vacuole (16), may be accompanied by changes in cytosolic pH sufficient to produce the potential changes in PEPC activity indicated, but see below.

The high level of CAM enzyme activity at low pH is correlated with and doubtless associated with the lower level of response to Glc-6-P in the same pH range. It may be that in the CAM enzyme the two factors are associated through their effect on regulation by aggregation/disaggregation of PEPC perhaps by way of a pH effect on the indicated cysteine group. We have observed (32) that the CAM PEPC is primarily in the tetrameric form at pH 6.2, while preincubation for 60 min at pH 8.2 and separation by HPLC size exclusion chromatography at pH 7.5 alters this to predominantly the dimer. It has also been noted (29, 30, 32) that the tetramer is both more active and more resistant to inhibition by malate, while the dimer is less active—some data suggest that it may not be active at all and that observed rates are due to reconversion to the tetramer in the presence of PEP (32)—and more sensitive to malate inhibition. We have also found that a 20-fold dilution of PEPC prior to assay resulted in the loss of about half of the original activity. While the undiluted enzyme gave a fractional activation (see Eq. 1) in response to 4.33 mM MgGlc-6-P of 1.25, the diluted enzyme responded to the same activator treatment with a value of 2.94. This suggests that Glc-6-P is capable of inducing a change in the aggregational equilibrium toward the tetramer which has been found to be more active (32). The CAM enzyme, with a pH

optimum less than 6, is in contrast to the maize and wheat enzymes, which have broad plateaus between 7 and 9. The degree to which the low pH optimum of the CAM enzyme reflects the differences in the pH of its environmental mileu arising from the large diurnal flux of malate is a matter of speculation, but it does suggest the possibility of greater pH changes in *Crassula* cells or organelles than is usually thought to occur. However, as Roberts (18) has pointed out, it is exactly in situations of a qualitative change in metabolism such as the diurnal shifts in CAM metabolism that one would expect to find larger than usual pH changes.

The fact that responses to pH which can with some confidence be attributed to changes in aggregation of the enzyme seem to be limited to the CAM enzyme may be due to the fact that more information on such changes exists for that enzyme than for the maize or wheat enzymes. However, this is suggestive that the other PEPC forms are not necessarily subject to activity regulation by shifting aggregation state, and that question should be investigated.

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