Communication

Oligomerization and Regulation of Higher Plant Phosphoenolpyruvate Carboxylase

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ABSTRACT

The specific activity of phosphoenolpyruvate (PEP) measured at a saturating level of substrate diminishes as the enzyme is diluted at about the same rate that specific light scattering by the diluted enzyme decreases. The presence of PEP in the assay causes an increase in activity with increasing dilution. This is accompanied by an increase in light scattering of the diluted enzyme. The reverse situation obtains with the addition of malate to assays: the activity decreases with increasing dilution but light scattering is not substantially changed, indicating that the enzyme is already brought to a smaller aggregate by the dilution itself. In this case, the inhibition by malate in the assay probably is the noncompetitive type not involved in regulatory control by malate. Glucose-6-phosphate in the range from 1 to 6 millimolar causes an increase in activity of the enzyme run at a substrate level less than $K_m$, and an associated increase in light scattering is found, indicating an increase in the mean size of the enzyme. When PEP is added to a 1/80 diluted enzyme, light scattering increases and is associated with a more rapid activity of the enzyme. When malate is added to the same cuvette, the activity decreases and the light scattering diminishes, thus showing that the ligand response is immediately reversible. When malate is added first, followed by PEP, the reverse sequence of activity and light scattering change is observed.

PEPC (EC 4.1.1.31) is universally found in plants and bacteria, where it apparently plays an anaplerotic role in most species (1, 4). In the case of plants that use special metabolic systems such as CAM or C4 metabolism, the enzyme is of crucial significance. Because of the nature of these metabolic schemes, the regulation of the enzyme, particularly in CAM plants with their ability to stop its activity completely, is uniquely important.

In spite of rather extensive studies (1, 4, 12–15, 18, 19), no completely satisfactory explanation of the means by which PEPC is regulated is yet available. Malate as an allosteric inhibitor and Glc-6-P as an allosteric activator of PEPC do have pronounced effects on the activity of the enzyme and may play roles in its regulation. Uncovering the fact that small oligomers of PEPC, e.g. monomer and dimer, have little or no activity, whereas tetramer and larger molecules possess considerable activity (7, 9, 15–19), has suggested oligomerization as a means of controlling the activity of the enzyme. Recently, many cases have been reported that show that maize PEPC can be phosphorylated by a kinase found in leaves with PEPC. This has been interpreted as revealing the mechanism by which PEPC is regulated (2, 3).

All of these postulated systems are flawed in various ways with respect to their ability to explain the regulation of PEPC observed in nature. Glc-6-P is capable of inducing activity when PEPC is running at low PEPC levels. This is potentially useful in vivo but does not provide for shutting down PEPC during the period when decarboxylation is the primary function of a CAM system, for example. Malate inhibition is so pervasive (1, 4, 12, 14) that it must have some role in turning off PEPC. Under some circumstances, the inhibition can be large (12, 14), but using $^{13}$C as a tracer to follow the in vivo activity of PEPC (8) has shown that the CAM enzyme is completely shut down in daylight. No such complete inhibition of isolated PEPC by malate has been reported.

Regulation by oligomerization has been the subject of a variety of criticisms. Some workers do not find changes in the size of the molecule under conditions that are thought to result in different activities. We and others using HPLC and light scattering have found that PEPC has a diurnal change in size (1, 17, 18) and aggregates or disaggregates under the influence of ligands and other treatments that result in differing activities (10, 12–14).

The fact that in vitro studies use enzyme concentrations lower than those probably found in the cell has been advanced as a reason for assuming that the observations of aggregation are without value for the in vivo operation of PEPC (3). This criticism may have some validity when applied to the dissociation of large forms likely to occur on dilution, but it is not appropriate to the association of small forms that have also been shown to occur in diluted enzymes under the influence of PEP and Glc-6-P. The size change has been accompanied by an increased activity of the enzyme (15, 16). It has been suggested that producing a highly purified PEPC requires such a long preparation time that it may result in the loss by action of proteinases of the N-terminal segment that contains the seryl group that is phosphorylated (3), thus eliminating the possibility of control by phosphorylation. An opposing sug-

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2 Abbreviations: PEPC, phosphoenolpyruvate carboxylase; C4, four carbon photosynthetic mode; Glc-6-P, glucose-6-phosphate; PEP, phosphoenolpyruvate.
gestion is that incubation of the CAM PEPC with trypsin causes a decrease in activity (13), but the residual activity is still inhibited by malate and activated by Glc-6-P, making it unlikely that some crucial regulatory site has been removed.

The evidence for an exclusive or "cardinal" role of phosphorylation in regulation of PEPC is somewhat variable and appears to be limited to changes of rather small increments of PEPC activity as a result of phosphorylation (2, 3). In some cases, minor changes in the sensitivity of the enzyme to inhibition by malate, such as changes in the $K_{i,inact}$ determined only at low substrate concentrations, have been reported (3).

For C. PEPC, a strong case has not been made for the need to turn off PEPC at night, and the small effects reported for phosphorylation may be sufficient for the diurnal changes required. For the CAM enzyme, the need is for complete control, and the absence of this has led some workers to conclude that the "physiological role of phosphorylation is uncertain" (5). These workers also state that currently there is no evidence "... that phosphorylation of PEPC by the Sorghum kinase alters its kinetic properties." It may be that the role of phosphorylation in the regulation of PEPC is headed toward the situation described by Zhao et al. (20) for phosphofructokinase, where "after more than 12 years of investigation ... the physiological significance of the phosphorylation of phosphofructokinase remains unknown."

This paper reports studies intended to show that the effects of ligands known to affect PEPC do rapidly cause aggregational changes in the direction expected on the basis of a role for aggregation in control of PEPC, and these changes are accompanied by the expected changes in activity. Most importantly, data presented here also show that the linked changes in activity and size are reversible without any changes other than addition of a ligand expected to produce the contrary reaction.

MATERIALS AND METHODS

Chemicals

The trisodium salt of PEP was obtained from Sigma, as was the disodium salt of Glc-6-P. Malate was from Aldrich. NADH was supplied by Boehringer-Mannheim. All chemicals were of the highest quality commercially available.

Enzymes

Lactic dehydrogenase (from hog muscle) and malate dehydrogenase (from pig heart) were purchased from Boehringer-Mannheim.

Enzyme Purification

The PEPC was purified from leaves of maize (Zea mays L.) grown in the greenhouse as described earlier (15) using ammonium sulfate fractionation, DEAE chromatography, and hydroxyapatite. The specific activity, originally 20 units/ mg, had been reduced to about half that level after 12 months storage at −70°C. Assays were carried out using the method previously described (15) with 50 mM Aces, levels of MgPEP as indicated in the text, 5 mM HCO$_3^-$, 0.2 mM NADH, 1 unit each of malate dehydrogenase and lactic dehydrogenase in 1 mL volume, pH 7.0, and run at 25°C.

Light Scattering

Light scattering measurements were performed at 530 nm using a SPEX fluorimeter. The measuring chamber was thermostated with a water bath and measurements were made at 25°C.

Sample Preparation

All samples and buffers were filtered through a 0.22-μm membrane (Millipore Millex-GV4) directly into the 1-cm light path cuvette and sealed with Parafilm to prevent the introduction of dust. The sample was allowed to equilibrate for 10 min at the chamber temperature before beginning measurements.

RESULTS AND DISCUSSION

Dilution Effects on Activity and Size

The close association of the activity of PEPC with the enzyme size as indicated by light scattering at 530 nm is illustrated in Figure 1. Here, maize PEPC has been diluted over the range from 1/40 to 1/240. Light scattering and activity were determined immediately after dilution. The expected loss of specific activity due to this dilution is shown, and this is closely accompanied by a decrease in light scattering, indicating a decrease in the mean size of the enzyme molecule. The slopes of the two changes are comparable. Although it is not calibrated, the direct light scattering method was used to permit instantaneous readings. The undiluted PEPC was also run on a Malvern light scattering instrument that estimates particle size (15) and was found to be mostly in the tetrameric form.

The relations shown in Figure 1 indicate the ability of the method used in this study to detect an instantaneous change

![Figure 1. Effects of dilution on PEPC activity and size. Light scattering determined for 5 min starting 10 min after dilution. Sample for activity taken at beginning of light scattering measurement and measured immediately. Lines fitted by linear regression; +, activity, $r = 0.985; \Delta$, light scattering, $r = 0.957$.](http://www.plantphysiol.org)
dilution alone has reduced the size of the enzyme to the level that could be produced by malate. This suggests that the decreased activity is due to noncompetitive inhibition by malate not associated with regulation (11).

Another allosteric ligand thought to have some role in regulating PEPC is Glc-6-P. To test whether Glc-6-P has an effect on both velocity and aggregation, PEPC diluted 1/80 was used at 1 mM MgPEP (approximately = $K_m/3$) at three levels of Glc-6-P. The results are shown in Figure 3, where it is seen that on dilution the velocity relative to the rate without Glc-6-P is progressively increased by the activator. The second line shows that the total amount of light scattering increases similarly. Because these data are not confounded with dilution, the association of activity and size is more clearly delineated.

Reversibility of Ligand Effects

The data presented here and in earlier studies (7, 15–17) have shown a close association of changes in PEPC activity with shifts in its oligomeric equilibrium. In the past, because of the slow methods used for measuring molecular size and because velocity and size are not tightly coupled, it has been difficult to be certain of the causality involved. Using the direct light scattering measurements in conjunction with rapid activity assays has made it possible to show that the response to allosteric ligands is instantaneous and is in the direction required for practical regulation of PEPC activity. This is shown in Table I, where the velocity and size of PEPC, as influenced by the sequence of administration of PEP and malate, is summarized. The upper portion of the table shows the responses to PEP and malate added in that order. Activity is increased about 10% by 10 mM PEP and this is accompanied by a 22% increase in light scattering. When 10 mM malate is added to the same cuvette, the effect of PEP is reversed, with the activity reduced 50% and the light scattering 37%.

This clearly shows that the effect of PEP is reversible by addition of malate even in the presence of the original PEP

Figure 2. Effects of PEP and malate on PEPC activity and size over a range of dilution of the enzyme. A. Effect of 10 mM PEP at each level of dilution on activity (+) and light scattering (Δ). B. Effect of 10 mM malate at each level of dilution on activity (+) and light scattering (Δ). Lines fitted with cubic spline program.

in activity associated with a decrease in the mean size of PEPC molecules.

Ligand Effects on Diluted Enzyme

Malate and Glc-6-P have been shown to have influences on both oligomeric size and activity of PEPC (9, 15, 16), but the quantitative effect of dilution of the enzyme on the responses to these ligands has not been carefully studied. The interaction of PEP with PEPC is summarized in Figure 2A, where it can be seen that the presence of 5 mM PEP causes an increase in velocity relative to the enzyme without PEP of more than 50%, which increases even more at a dilution of 240-fold. The other line shows a similar response in light scattering. The greater response at high dilutions may represent the formation of aggregates from the monomeric enzyme that we have previously seen (18, 19) to be without activity.

Figure 2B shows that the presence of malate causes a decrease in the velocity of the reaction that reaches a minimal level at maximal dilution. The upper line, which represents light scattering under the influence of malate, shows little change over the dilution range used, likely indicating that
addition, and the lower part of the table shows a similar reversibility when malate is added first, followed by PEP. Here, 10 mM malate reduces the activity by 25% and the light scattering by 4%, whereas subsequent addition of PEP causes a 65% increase in activity and a 30% increase in size. The small effect of malate on light scattering when added first shows that the 1/80-diluted PEPC is near the dimeric equilibrium. The somewhat larger effect on activity is due to the noncompetitive inhibition of malate (6, 12, 14) and is not related to a change in aggregation state.

It is of interest that the addition of either PEP or malate directly to the 1/80-diluted enzyme causes a smaller change than the subsequent addition of the other ligand. We interpret this as an indication that the first addition has shifted the aggregational equilibrium in the direction associated with a particular ligand, so a larger change is possible when the second ligand is added.

**LITERATURE CITED**