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Inactivation of Maize Leaf Phosphoenolpyruvate Carboxylase by the Binding to Chloroplast Membranes[1]

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ABSTRACT

Phosphoenolpyruvate carboxylase (PEPC) purified from maize (*Zea mays* L.) leaves associates with maize leaf chloroplast membrane in vitro. The binding of PEPC to the membrane results in enzyme inactivation. A protein isolated from a maize leaf chloroplast membrane preparation inactivated PEPC. Treatment with membrane preparation or with partially purified inactivating protein accelerates PEPC inactivation at low temperature (4°C). Interaction of PEPC with chloroplast membrane or inactivating protein may inactivate the enzyme by influencing dissociation of the enzyme active tetramer.

PEPC[3] (EC 4.1.1.31) is a key regulatory enzyme playing an important role in CO_2 fixation in C_4 plants and making possible the high photosynthetic efficiency of such plants (8, 22, 28). The enzyme is regulated by several substrates and cellular metabolites such as PEP, Glc-6-P, and malate (31–33). PEPC from maize has been shown to be phosphorylated using an endogenous kinase (14) that increases enzyme sensitivity to inhibition by malate when assayed with subsaturating MgPEP but has no effect on enzyme V_{max}.

Numerous investigators have observed the existence of oligomeric forms of this enzyme and it is considered by many that differing sizes of PEPC molecules have different levels of intrinsic activity (10, 11, 21, 33, 34, 36, 38, 39). The tetrameric form of the enzyme is reported to be the most active (22, 24, 29, 30, 35, 38). Recent studies have suggested that regulation of the catalytic activity of PEPC is accomplished by changing the aggregation states of the enzyme (24, 29, 30, 33, 35). Treatments such as dilution of the enzyme or incubation of the enzyme at low temperatures induce dissociation of PEPC to the less active or inactive dimer and monomer in vitro (3, 7, 15, 38).

Recent progress made in the study of microcompartmentation of cytosolic enzymes in mammalian tissues suggests that enzymes can be regulated via interaction with cell membranes (12, 13, 18). Previous studies from this laboratory demonstrated that PEPC is associated with the chloroplast of *Crassula* (a CAM plant) (37). Some reports also indicated that PEPC may bind with a membrane or cell particulate fraction (1, 23, 26). Some groups observed that the discontinuity of Arrhenius plots of PEPC activity in desalted maize (*Zea mays* L.) leaf extracts can be abolished by treating the enzyme extract with Triton X-100 (19, 25). This shows that PEPC in C_4 plants may be membrane bound and sensitive to low temperatures at least in some situations. The mechanism and physiological significance of binding of PEPC to the chloroplast membrane remain unclear.

PEPC is inactivated when the enzyme binds to the chloroplast membrane or when it interacts with a protein isolated from the membrane. The enzyme binding to the membrane increased the time for attainment of V_{max} during assay of the enzyme preincubated at low temperature (4°C). We propose that interaction of PEPC with a membrane or with the inactivating protein anchored in the membrane may affect PEPC conformational or aggregational changes and make the inactivation process hysteretic.

MATERIALS AND METHODS

Enzymes

PEPC purified from maize (*Zea mays* L.) leaves was prepared as described previously (33). Briefly, leaves were harvested after 6 h of daylight, deribbed, and homogenized with a Polytron; the procedure included ammonium sulfate precipitation, DEAE chromatography, and hydroxylapatite chromatography. The preparation was frozen in 50 mM phosphate buffer containing 1 mM EDTA, 1 mM DTT, and 20% (v/v) glycerol at pH 7.2. The specific activity was 22 units/mg and appeared homogeneous on the basis of SDS gels. The preparation was stored at −20°C. An AVIDCHROM desalting cartridge (BioProbe International, Inc., Tustin, CA) was used to remove salts before use.

Chloroplast Membrane Preparation

Chloroplasts were prepared from leaf extracts of maize as described earlier for *Crassula* (37), except that 20% (v/v) glycerol replaced 0.33 M sorbitol. Broken cells were removed by filtering through three layers of fine nylon cloth. Pellets were obtained following the procedure of Chen and Wildman (6) except that centrifugation was at 300*g* for 3 min. The pellet was washed three times with Hepes buffer (50 mM, pH 7.4) to elute PEPC and other soluble proteins associated with the membranes. These were then extracted with buffer (50 mM Hepes, 1 mM EDTA, 1 mM DTT, 20% [v/v] glycerol, and

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[3] Abbreviations: PEPC, phosphoenolpyruvate carboxylase; PEP, phosphoenolpyruvate; Glc-6-P, glucose-6-phosphate; Aces, *N*-(2)-acetamido)-2-aminoethanesulfonic acid.

0.5% [v/v] Triton X-100). The membrane preparation from broken chloroplasts was dialyzed overnight against the same buffer without Triton X-100. The membranes were homogenized in a Potter homogenizer for 5 min before experiments. This is designated as the chloroplast membrane preparation. Although the procedure used should enrich the preparation with membranes from intact chloroplasts, the preparation contains both external and internal chloroplast envelopes. Determination of the physiological significance of the membrane inactivation will depend on a later demonstration that the outer envelope alone can produce the inactivation of PEPC.

Partial Purification of Chloroplast Membrane Proteins

The chloroplast membrane preparation was centrifuged at 20,000*g* for 10 min. The supernatant plus 0.5% (v/v) Triton X-100 was loaded on a TSK-Gel Toyopearl DEAE column, (20 × 1.5 cm) equilibrated with 50 mM Hepes (pH 7.4). The column was eluted at 1 mL/min with a NaCl gradient from 0 to 2 M in the same buffer. The fractions with PEPC inactivating activity were pooled, concentrated, desalted, and 4.0 mL was applied to a BioGel A size exclusion column (0.5 M, 50 cm × 1.5 cm) previously equilibrated with Hepes buffer (50 mM, pH 7.4) and eluted at 1 mL/min with that buffer. The activity peaks were pooled. The column was calibrated using the following proteins as mol wt standards: yeast alcohol dehydrogenase (M_r 150,000), aldolase (M_r 158,000), catalase (M_r 240,000), and ferritin (M_r 440,000). Protein was determined by dye binding (4).

Enzyme Inactivation

Purified PEPC (about 15 μg) was diluted in Aces buffer (200 mM, pH 7.4) to a total volume of 100 μL at 4°C with or without membrane preparation or inactivating protein. Aliquots were withdrawn at intervals, and the enzyme activity was measured using a standard assay.

Addition of protease inhibitors (Aprotinin, 0.2 mg/mL; trypsin inhibitor, 0.2 mg/mL; and PMSF, 1 mM) and phosphatase inhibitor (NaF, 20 mM) were found to have no effect on the inactivation of PEPC by the membrane preparation. The membrane preparation and inactivating protein carried over to the assay mixture do not interfere with the standard PEPC activity assay system.

PEPC Activity

PEPC activity was determined spectrophotometrically as previously described (31) by monitoring the disappearance of NADH at 340 nm in a 1.0-mL assay containing 50 mM Aces (pH 7.4), 5 mM HCO_3^-, 0.2 mM NADH, 2 units of malate dehydrogenase, and 2 mM Mg-PEP. Concentrations of free and Mg-bound ligands were calculated by use of a computer program written in BASIC (33).

Measurement of PEPC Binding to Chloroplast Membranes

PEPC binding was measured by centrifugation. Enzyme was mixed with membrane preparation in Aces buffer (200 mM, pH 7.4) at 4°C for 10 min. The mixture was then centrifuged at 7000*g* for 5 min. Aliquots of the supernatant liquid and the mixture before membrane addition were assayed for PEPC activity. The difference in activity between these two samples was assessed as the fraction of the enzyme bound to the membrane preparation.

Estimation of Enzyme Size by Light Scattering

Light scattering was measured at an angle of 90° at 530 nm in a Spex spectrofluorimeter (model DM1B). Dust-free 3-mL samples of PEPC at 150 μg/mL in 200 mM Aces, pH 7.4, were followed for 6 min with and without the addition of 50 μL of buffer containing 88 μg of the PEPC inactivating protein.

Commercial Enzymes and Chemicals

Malate dehydrogenase from Boehringer was obtained from pork heart and suspended in 50% (v/v) glycerol. Malate was from Aldrich, other chemicals were from Sigma and were of the highest purity available.

RESULTS AND DISCUSSION

Inactivation of Maize Leaf PEPC in the Presence of Chloroplast Membrane Preparation

Preincubation with 200 μg of chloroplast membrane preparation for 10 min provoked complete inactivation of PEPC. The response of PEPC activity to membrane concentration is hyperbolic, as shown in Figure 1. The subsequent addition of Triton X-100 to the assay cuvette up to 1% (v/v) restored the enzyme activity to some degree. For example, nearly complete enzyme activity was recovered by the addition of Triton X-100 to a mixture that was 40% (v/v) inactivated. To determine the correlation of PEPC binding to the membrane with inactivation of the enzyme, the percentage of PEPC binding to the membrane during the preincubation treatments was measured as described in "Materials and Meth-

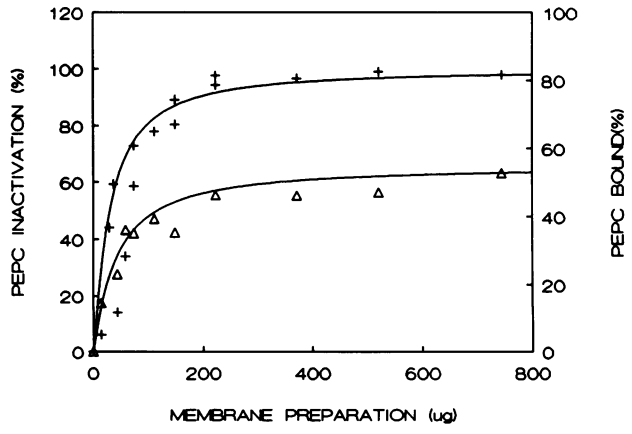

Figure 1. Effects of chloroplast membrane preparation on the inactivation and binding of PEPC from maize leaves. The inactivation and binding of PEPC were measured by standard procedures including a 10-min exposure (see "Materials and Methods"). +, Inactivation of PEPC; Δ, percentage PEPC bound.

ods." This showed that the increase of enzyme inactivation was accompanied by an increase of the enzyme binding to the membrane. Both curves give the same mean inhibition value (about 37 μg of membrane protein). It is of interest that complete inactivation of PEPC was reached with increasing concentration of membrane preparation at the point where only 50% (v/v) of the enzyme was bound to the membrane. This relationship between the inactivation of the enzyme and its binding to the chloroplast membrane is similar to the association between inactivation of phosphofructokinase and its binding to erythrocyte membranes (12). In that case, the initial rate of inactivation continued to increase with increasing membrane concentration even after the binding of phosphofructokinase had reached a plateau. These results and those shown in Figure 1 indicate that membrane binding is not the direct or sole cause of enzyme inactivation. It was suggested that for phosphofructokinase (12) the oligomerization equilibrium is being shifted by interaction of the enzyme with the membrane or a protein associated with it so that the enzyme is converted to an inactive form without being removed from solution by centrifugation. In the present case, it appears that if the bound enzyme does not contribute to activity then the activity of the PEPC not removed by centrifugation must be altered by the presence of membranes to induce loss of the activity of the PEPC that remains in solution. It is possible that conversion of the active tetramer to a less active or inactive dimer or monomer of PEPC (35) may be responsible for the results of Figure 1.

Altered Kinetic Properties of Membrane-Bound Maize Leaf PEPC

Maize leaf PEPC incubated with chloroplast membrane preparation shows significant changes in V_{max} and K_m values. The estimate of k_{on} for PEP (V_{max}/K_m) is 5-fold lower than for the control enzyme (Fig. 2).

Change in sensitivity to effectors of PEPC is one of the important mechanisms of regulation of the enzyme (9, 35). Figure 3 shows the alteration of the response of PEPC to Glc-6-P activation induced by preincubating with chloroplast membrane preparation. The K_a for Glc-6-P is higher for the enzyme in the presence of membrane (5 mM [O]) or partially purified membrane (3 mM [Δ]) compared with the control enzyme (0.8 mM [+]).

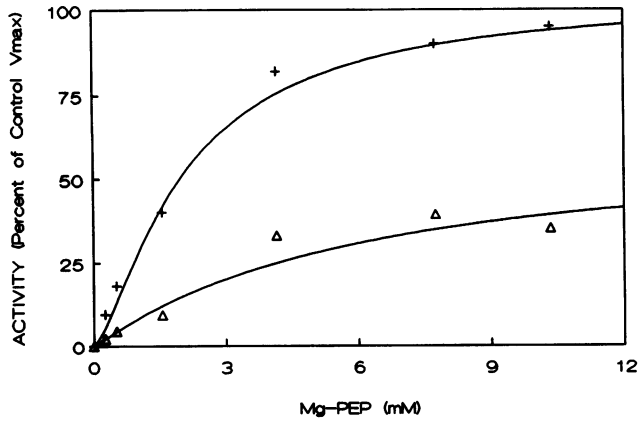

Figure 2. Effect of membrane preparation on the saturation curve of PEPC for Mg-PEP. +, Control (PEPC diluted 10-fold with 200 mM Aces buffer, pH 7.4); Δ, PEPC + membrane preparation (135 μg).

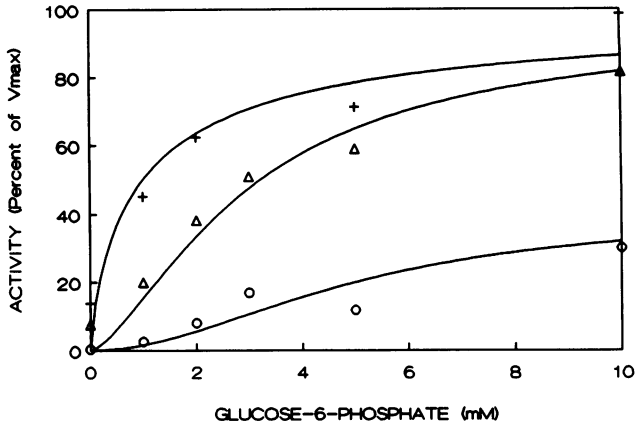

Figure 3. Effect of membrane preparation on Glc-6-P activation of PEPC. +, Control (PEPC diluted 10-fold with 200 mM Aces buffer, pH 7.4); Δ, PEPC + partially purified inactivating protein (54 μg); O, PEPC + membrane preparation (135 μg).

It also can be seen that inactivation of the enzyme decreased as Glc-6-P concentration increased in the assay cuvette (Fig. 3). Substrate PEP and activator Glc-6-P protected the enzyme from inactivation. Whether this is by binding to the enzyme or the membrane is uncertain. The presence of PEP (20 mM) or Glc-6-P (10 mM) reduced PEPC inactivation by the membrane preparation, respectively, 62 and 45% (v/v) (enzyme treated with 54 μg of membrane preparation).

Hysteretic Nature of PEPC Activity Affected by Chloroplast Membrane Preparation at Low Temperature

C_4 PEPC is known to be unstable at low temperatures (3, 7, 15, 27, 30, 39). Some of these authors have suggested that cold-induced inactivation of PEPC reflects a reversible dissociation of the enzyme. We found that incubation with chloroplast membrane preparation at low temperature (4°C) inactivated PEPC more strongly than treatment at room temperature. The percentage of PEPC inactivation by membrane preparation increased from 36 to 57% (v/v) after lowering the preincubation temperature from 25 to 4°C. Participation of the membrane preparation in accelerated cold inactivation of PEPC produced a longer lag time in assays (Fig. 4). It is seen here that although dilution alone at 20°C does not cause a significant increase in the lag, dilution at 4°C does. Dilution plus incubation with chloroplast membrane preparation causes a greater lag at both temperatures. The relationship between protein concentration of membranes and the lag time ($t_{1/2}$) in PEPC assays is shown in Figure 5. The increase of the lag time is dependent on the concentration of membrane protein, suggesting that the protein is inducing a shift in equilibrium between active and inactive forms of the enzyme, we suggest tetramer and dimer or monomer.

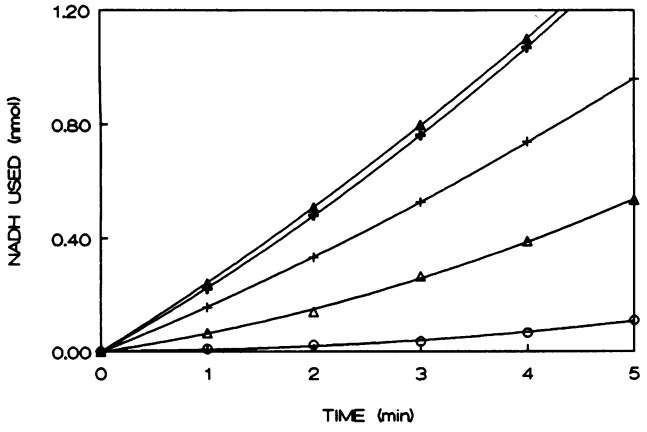

Figure 4. Hysteretic behavior of PEPC affected by cold and the addition of membrane preparation. +, PEPC preincubated at 20°C; ▲, PEPC diluted 10-fold with 200 mM Aces buffer, pH 7.4, and preincubated at 20°C; +, PEPC diluted 10-fold with 200 mM Aces buffer, pH 7.4, and preincubated at 4°C; Δ, PEPC diluted 10-fold with 200 mM Aces buffer, pH 7.4, and preincubated with 74 μg of membrane preparation at 4°C; O, PEPC diluted 10-fold with 200 mM Aces buffer, pH 7.4, and preincubated with 223 μg of membrane preparation at 4°C.

Isolation of PEPC Inactivating Protein from Maize Chloroplast Membranes

It was shown earlier that plant PEPC may bind to the outer membrane of the chloroplast (37). Now we find that the elution profile of a maize chloroplast membrane preparation from the DE-52 column yields two peaks of absorbance at 280 nm that are capable of inhibiting PEPC (Fig. 6). The main peak eluted at 0.4 M NaCl, higher than the salt concentration at which PEPC is eluted from this column (33). This peak of the inactivating protein, when applied to a gel filtration column (BioGel A 0.5 M, 50 cm × 1.5 cm), eluted at an effluent volume corresponding to a mol wt of 35,000 (Fig. 7). This partially purified inactivating protein showed a 4-fold higher specific activity than the membrane preparation

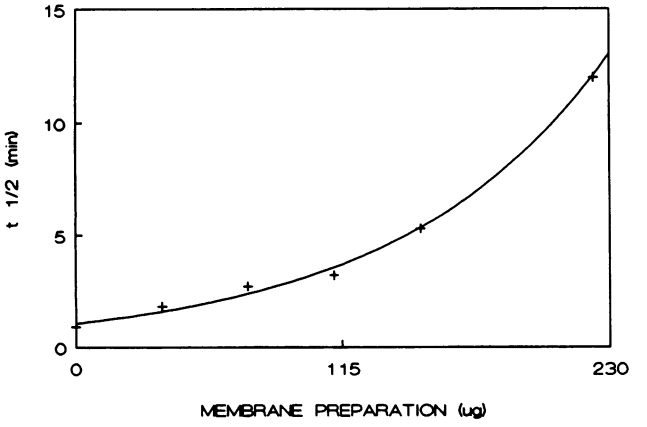

Figure 5. The effect of chloroplast membrane preparation on the lag time of maize PEPC. +, $t_{1/2}$ (min).

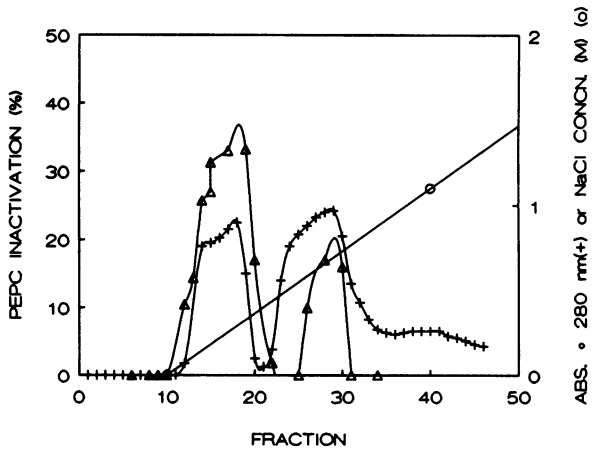

Figure 6. PEPC inactivating protein profile on DEAE column (TOYAPEARL DEAE-650 M). +, Absorbance at 280 nm; Δ, PEPC inhibition (% [v/v]); O, NaCl (M).

and has the same effect as the membrane preparation itself on hysteretic behavior of cold inactivation of PEPC. As shown in Figure 7, preincubation of this partially purified inactivating protein (58 μg) with PEPC has about 80% inhibitory effect on the enzyme activity (upper line, where inactivation percentage is calculated using the rates of control enzyme preincubated at 25°C.) The lower line uses control rates of enzyme preincubated at 4°C, thus subtracting out the effect of temperature and showing the residual effect of inactivation by the membrane preparation. The inactivation decreased gradually during the assay, indicating that the membrane inactivation at low temperature may be reversible, perhaps by change in the aggregation state of the PEPC.

The binding of some cytosolic enzymes to membranes and other particulate fractions of mammalian and plant cells has

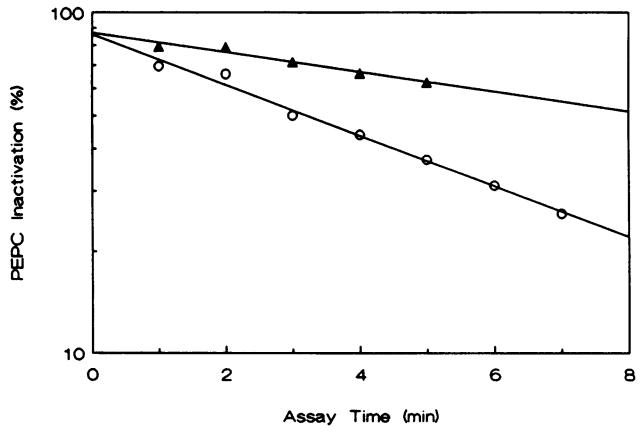

Figure 7. Hysteretic behavior of PEPC induced by PEPC inactivating protein. PEPC was preincubated with PEPC inactivating protein (58 μg) at 4°C. ▲, Total inactivation of PEPC inactivating protein (inactivation percentage calculated using control enzyme preincubated at 25°C); O, net inactivation of PEPC inactivating protein at 4°C (inactivation percentage calculated using control enzyme preincubated at 4°C).

been widely observed (12, 13, 20). Changes in the aggregation state of the enzyme have sometimes been associated with the binding of enzymes to membranes. Evidence that some similar phenomenon is involved with PEPC and the chloroplast membrane preparation is shown in Figure 8. Here, light scattering by PEPC (upper line) is dramatically decreased on the addition of 100 μg/mL of partially purified inactivating protein. The difference persists for at least several minutes even though both samples increase in intensity and thus size during the measurements.

The distribution of cytosolic enzymes between membrane-bound and free states has been associated with the metabolic and physiological states of the cell (20). Some proteins have been identified in the binding of enzymes to membranes. A transmembrane anion transporter, band 3 protein, has been identified as an inactivating and binding protein for phosphofructokinase from erythrocyte membranes (12, 13). Hexokinase and glycerol kinase have been reported to interact with porin, a pore-forming protein, on the outer surface of the mitochondrial membrane (2). Recent studies on the regulation of the enzymes indicate that some important enzymes such as phosphofructokinase interact with other proteins, causing a reversible inactivation by inducing a shift in the equilibrium from the active to an inactive form (5, 16–18, 40). We hope that further studies on PEPC inactivating protein from chloroplast membranes will show whether this protein belongs to this group.

The nature of the membrane protein that inactivates PEPC in these studies has not been determined in detail. On the basis of the strong absorbance of the partially purified protein at 280 nm, its reactivity with the Bradford reagent, and its loss of activity when heated for 3 min at 100°C, we assume that it is a protein. As described in "Materials and Methods," protease inhibitors and a phosphatase inhibitor have no effects on the enzyme inactivation by either membrane or inactivating protein. Because of the current interest in the possibility that the activity of PEPC can be altered by phosphorylation/dephosphorylation, the question of whether this protein may be a phosphatase arises. We found no effect of the presence of NaF on inactivation by chloroplast membrane preparations. We also studied the possibility that the PEPC inactivating protein preparation could be a nonspecific phosphatase, using a method coupling it with lactate dehydrogenase to determine whether the inactivating protein can hydrolyze PEP. The results showed no "PEP phosphatase" activity even at high PEP concentration (20 mM), whereas about 30% (v/v) of PEPC activity was inhibited under the same conditions by the addition of inactivating protein.

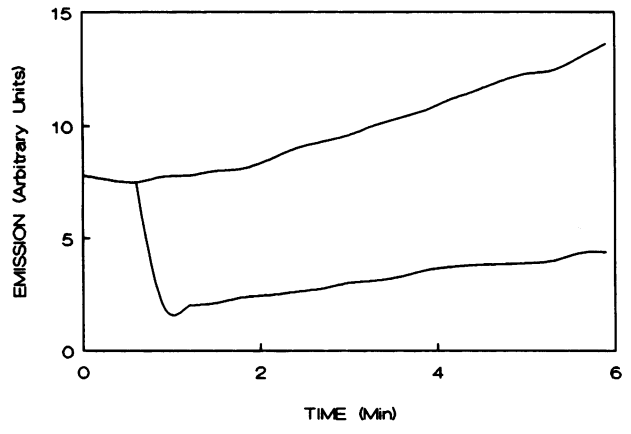

Figure 8. Light scattering of PEPC as affected by partially purified chloroplast inactivating protein. At the point where the two lines join, 300 μL of 150 μg/mL of the membrane protein was added to one 3-mL sample, producing the lower line. The line to the left of that point is the mean of two samples. Comparable dilution with buffer was without effect on the light scattering.

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