Inactivation of Maize Phosphoenolpyruvate Carboxylase by Urea

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ABSTRACT
Phosphoenolpyruvate carboxylase purified from leaves of maize (Zea mays, L.) is sensitive to the presence of urea. Exposure to 2.5 mM urea for 30 min completely inactivates the enzyme, whereas for a concentration of 1.5 mM urea, about 1 h is required. Malate appears to have no effect on inactivation by urea of phosphoenolpyruvate carboxylase. However, the presence of 20 mM phosphoenolpyruvate or 20 mM glucose-6-phosphate prevents significant inactivation by 1.5 mM urea for at least 1 h. The inactivation by urea is reversible by dilution. The inhibition by urea and the protective effects of phosphoenolpyruvate and glucose-6-phosphate are associated with changes in aggregation state.

Although the effects of urea on proteins are varied and subtle (13), it is commonly considered that a major aspect of action of urea is to induce the dissociation of oligomeric proteins (3, 5, 6, 12). Although some enzymes have been found (6) to give varied responses depending on the concentration of urea applied, urea is widely used to dissociate large enzyme molecules. In some instances, urea in concentrations from 3 to 8 M has been shown to dissociate tetrameric proteins to their subunits. This general pattern is shown by fumarase (5), glutamate dehydrogenase (1), and phosphofructokinase (6), in which the active tetramer is dissociated to monomers by the presence of urea. This effect of urea, which disaggregates proteins by breaking hydrogen bonds (12), offers a different way of probing the factors involved in equilibria of enzymic oligomers. Our interest in this question arises from the aggregation and disaggregation of PEPC, which is a major factor in regulating the activity of that enzyme (15, 17).

We have studied the effect of moderate concentrations of urea on the inactivation of PEPC and have investigated the effect on the response to urea of ligands known to regulate the enzyme. We have also shown, using light scattering, that the effect of urea is to dissociate the enzyme.

MATERIALS AND METHODS

Chemicals and Enzymes
The trisodium salt of PEP and the disodium salt of glc-6-P were obtained from Sigma. Malate was from Aldrich and NADH from Boehringer-Mannheim. All chemicals were of the highest quality commercially available.

Malate dehydrogenase (from pig heart) and lactate dehydrogenase (from hog muscle), both suspended in 50% glycerol, were purchased from Boehringer-Mannheim.

Enzyme
PEPC was purified from the deribed leaves of 3- to 4-week-old seedlings of maize by the method previously described (9). At the time of harvest, the leaves had been exposed to sunlight for 4 to 6 h in a greenhouse maintained at a daytime temperature of 30°C. The specific activity at the time of purification was 22 U/mg of protein.

The activity of PEPC was determined using 1-mL assays containing 50 mM Hepes buffer, pH 7.2; 5.2 mM MgPEP; 5 mM Mg2+; 5 mM NaHCO3; 0.2 mM NADH; and 2 units each of malate dehydrogenase and lactic dehydrogenase (8).

Light-scattering measurements in 50 mM Hepes, pH 7.2, were performed at 25°C in a thermostated sample chamber at 530 nm using a SPEX spectrofluorimeter. All samples and buffers were filtered through a 0.22-μm membrane (Millipore Milllex-GV4) directly into the 1-cm light path cuvette, sealed with Parafilm to prevent introduction of dust. Samples were equilibrated 10 min before measurements.

Data Analysis
The inactivation of PEPC as a function of time was fitted to the minimal equation of Goldbeter and Koshland (2)

\[ FA = (1 - F_1)e^{-(k_1 + k_2)t} + (F_1e^{-k_2t}) \]

where \( FA \) = observed fractional activity, \( F_1 \) = fraction of activity susceptible to rapid inactivation, \( k_1 \) = rate constant for rapid inactivation, \( k_2 \) = rate for slow inactivation, and \( t \) = time in minutes. Inactivation as a function of urea concentration was fitted to either a linear regression or a second-order parabola.

RESULTS

Inactivation of PEPC as a Function of Urea Concentration
The residual activity of maize PEPC exposed to increasing concentrations of urea for 30 min is shown in Figure 1. A second-order parabola gives a reasonable fit with \( R^2 = 0.985 \). The fitted line also indicates that a 30-min exposure to 2.5 mM urea will completely inhibit the enzyme.
Within short periods, this inactivation by urea is reversible. A control PEPC preparation with a velocity of 9.5 μmol min⁻¹ mg⁻¹ is reduced to 6.3 U after a 10-min exposure to 1.5 M urea. An aliquot of this treated enzyme diluted to 0.4 M urea shows a rate of 11.6 after 3 min. The original treated enzyme is reduced to 1.5 U after 15 min, but if diluted to 0.4 M urea at that point, a rate of 10.1 U is found within 4 min, and after an additional 10 min, the rate is 10.0 U. At that point (30 min), the original urea-treated enzyme is reduced to an activity of 0.04 U.

**Time Course of PEPC Inactivation by Urea**

To further study the effects of urea, a concentration of 1.5 M was chosen as offering an intermediate level of inactivation to permit observation of the effects of treatments with ligands known to affect the oligomerization of PEPC.

The response of PEPC to 1.5 M urea for periods of up to 45 min is summarized in Figure 2. Even this low concentration of urea quickly inactivated PEPC. The control line (+), fitted to a Goldbeter and Koshland inactivation function (2) with an $R^2 = 0.972$, shows that about one-half of the activity was quickly lost ($F_i = 0.504 \pm 0.083$, with $k_i = 0.45 \pm 0.03$ min⁻¹). The second phase of inactivation was much slower with $k_2 = 0.05 \pm 0.15$ min⁻¹. When a high (20 mM) concentration of malate was present (Δ), there were no significant changes in the $F_i$ (0.46 ± 0.06) or $k_i$ (0.37 ± 0.06 min⁻¹) and $k_2$ (0.06 ± 0.13 min⁻¹). Clearly, malate had no protective effect in reducing the inactivation of PEPC by urea. When 20 mM PEP or 20 mM glc-6-P was present with 1.5 M urea, the enzyme was inactivated by only a few percent within 45 min, and the lines are best fitted to linear regressions. PEP treatment (O) has an $r = 0.768$ and a slope of −0.004 min⁻¹, and the glc-6-P line (●) has an $r = 0.643$ and a slope of −0.002 min⁻¹. Within the 45-min time frame of this experiment, neither of these slopes was significantly different from zero.

A suspension of 275 μg/mL of PEPC in buffer gave a light-scattering emission of 3.681 $\times$ 10⁷ arbitrary units when corrected for a blank consisting of 3 mL of buffer plus 563 μL of 8 M urea (final concentration 1.5 M). A second cuvette with the same concentration of PEPC showed a slow decrease in emission after 563 μL of 8 M urea were added, reaching a steady state after 20 min with a corrected emission value of 1.588 $\times$ 10⁷ arbitrary units. If the decrease in light scattering is due to that by dilution alone is caused by a decreased size of the PEPC, the mean size has been decreased 43% by exposure to 1.5 M urea. Because the light-scattering system was not calibrated for protein size, we cannot be certain what these changes signify in terms of mean aggregate dimensions, but a comparable suspension of the same initial PEPC preparation read in a Malvern 4700c was found to be predominantly in the tetrameric form (17).

Malate had little or no effect on the inactivation of PEPC by urea (Fig. 2). On the other hand, the inclusion of 20 mM PEP or 20 mM glc-6-P with the urea treatment was highly beneficial in moderating the time course of urea inactivation. Because urea does not bind to the enzyme, it is unlikely that these effects are due to displacement of urea from the enzyme. Because both PEP and glc-6-P have been shown in other studies to increase the size of the PEPC aggregate (15, 17), it is reasonable at this point to speculate that the "protective" effect of these ligands is due to their independent effect in inducing aggregation, perhaps by reforming hydrogen bonds broken by urea. Light-scattering measurements tend to confirm such an assumption, because an increase in emission was observed when either PEP or glc-6-P was added to PEPC in 1.5 M urea.

**DISCUSSION**

The use of specific ligands to influence the activity of PEPC has been informative concerning the number of sites (7, 9–11) and the character of sites (11, 13, 14) on the enzyme responsible for general activity and for activation and inhibition by glc-6-P or malate. These allosteric regulators have been shown to have an effect on the aggregation or disaggregation of the enzyme as well as on its activity. However, it...
has not been possible to distinguish between the effect of having a specific group on the enzyme rendered inactive by such an added ligand and the effect of this substitution on the ability of the enzyme to change the state of its aggregation. The slow inactivation of PEPC by urea illustrated in Figure 2 is consonant with the slow decrease in light scattering on exposure to urea. The increase in light scattering when PEP is added to an enzyme preparation containing 1.5 M urea is more rapid than the decrease on addition of urea to an enzyme preparation in the absence of PEP but is not nearly so rapid as the increase in light scattering of the enzyme solution on exposure to PEP in buffer alone (15, 17).

These observations suggest that aggregation or disaggregation in the presence of urea involves the breaking or formation of multiple bonds. Inactivation by urea is thought to involve breaking of hydrogen bonds (4, 5), a large number of which may be involved in the structure of a tetrameric enzyme. The effect of PEP or glc-6-P in reversing this change due to urea may come in response to reorientation of enzyme subunits into a conformation more favorable for reestablishment of hydrogen bonds. If this is the case, the effect of PEP or glc-6-P on aggregation may result from an initial effect of these ligands on the conformation of PEPC subunits. Malate, which is known (15, 16) to disaggregate the enzyme, is different in that its presence is without substantial effect on the inactivation of PEPC by urea.

LITERATURE CITED