Hysteresis and Reversible Cold Inactivation of Maize Phosphoenolpyruvate Carboxylase

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Maize (*Zea mays* L.) leaf phosphoenolpyruvate (PEP) carboxylase (PEPCase) (EC 4.1.1.31) showed a lag in activity when assayed after storage at 0–4 °C. The lag was promoted by high pH on storage (7.8–8.5) and was observed over a range of assay pH (7.1–8.5). Thermal reactivation of the cold-stored enzyme by assay temperature (18 °C) accounted for most of the hysteretic effect, but presence of PEP in the reaction mixture was required to completely eliminate the lag. Based on steady-state rates after the lag, stability of PEPCase in the cold was independent of protein concentration. It is suggested that low temperature and high pH induce a change in the oligomerization state of PEPCase, resulting in a less active but relatively stable form of the enzyme. The lag probably reflects a reversal of this process, promoted by assay temperature and presence of PEP.

Introduction

Phosphoenolpyruvate carboxylase (PEPCase) (EC 4.1.1.31) is the key enzyme of C_4 and Crassulacean acid metabolism (CAM) groups of plants, catalyzing the first reaction of carbon fixation during photosynthesis [1, 2]. The enzyme, which is absent in animal tissues, has been extensively characterized from many plants and bacteria [3–5] and found to be highly regulated, frequently exhibiting allosteric kinetics [3–7].

It is believed that kinetic and regulatory properties of PEPCase may depend on the oligomerization state of the protein. The enzyme, which is most active as a tetramer, exists also as a dimer (less active) and monomer (inactive), and the equilibrium between these states could be altered by changes in temperature, pH, salt and metabolite levels [6–10]. As a result, activity of the enzyme may depend on the conditions of extraction and subsequent storage of the isolated protein. Another problem to be considered is an apparent instability of PEPCase, especially when stored in the cold and at low protein concentration [7, 8, 11, 12]. The cold treatment causes dissociation of the active tetrameric form of PEPCase [8].

In the present study, we demonstrated that the cold-inactivated maize PEPCase could partly regain its activity during time-courses of assays, the lags probably reflecting a slow aggregation of subunits of PEPCase, and thus representing a typical hysteretic effect [13].

Materials and Methods

The enzyme was extracted from leaves of 12–14 day-old maize (*Zea mays* L., *cv.* Garland Flint) plants by homogenization (20 s) in a medium of 40 mM Hepes (pH 7.8), 2 mM $MgCl_2$, 1 mM EDTA and 5 mM DTT. The extraction as well as subsequent ammonium sulfate fractionation were carried out at 0–4 °C, using vacuum-degassed buffers. The extract was filtered through several layers of cheesecloth and centrifuged at 14,000 × *g* for 10 min. The supernatant was adjusted to 45% saturation with solid ammonium sulfate, equilibrated for 30 min and centrifuged (14,000 × *g* for 10 min). The resulting supernatant was further adjusted to 60% saturation with ammonium sulfate and then equilibrated and centrifuged as before. The pellet (45–60% ammonium sulfate fraction) was resuspended in a small volume of the homogenization medium, and constituted the source of PEPCase in the present study. The enzyme (5–9 U/mg protein) was stable for at least two months when frozen immediately after the fractionation. For all studies, aliquots of the concentrated enzyme were first incubated at 18 °C for 2–4 h, and then were diluted into the homogenizing medium (pH 7.8) or into

Abbreviations: PEP, phosphoenolpyruvate; PEPCase, phosphoenolpyruvate carboxylase; MDH, malate dehydrogenase; $t_{1/2}$, half time of activation.

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the media of either 40 mM Mes (pH 6.5), 40 mM Mops (pH 7.1) or 40 mM Tricine (pH 8.5), each supplemented with 2 mM $MgCl_2$, 1 mM EDTA and 5 mM DTT. Occasionally, the concentrated enzyme was passed through Sephadex G-25 column and then diluted into the homogenizing medium. Such preparations gave the same results as reported here for not desalted PEPCase.

PEPCase was assayed spectrophotometrically at 340 nm (18 °C), using porcine heart MDH (Sigma) and NADH to couple to oxaloacetate formation. Assays (1 ml) contained 100 mM Hepes (pH 7.8), 5 mM $MgCl_2$, 5 mM $NaHCO_3$, 1.2 mM DTT, 5 mM PEP, 0.2 mM NADH, and 5–10 U MDH. In studies intended to test the effect of assay pH on rates of PEPCase, Hepes was replaced by 100 mM Mops (pH 7.1) or Tricine (pH 8.5). Steady-state rates were routinely determined following oxidation of 0.15 mM NADH during assays. If the rates were still non-linear at that time, an additional aliquot of NADH was added to assure an accurate assay. Reactions were initiated by addition of PEPCase. A unit of PEPCase activity was expressed as one μmole NADH oxidized per min under assay conditions.

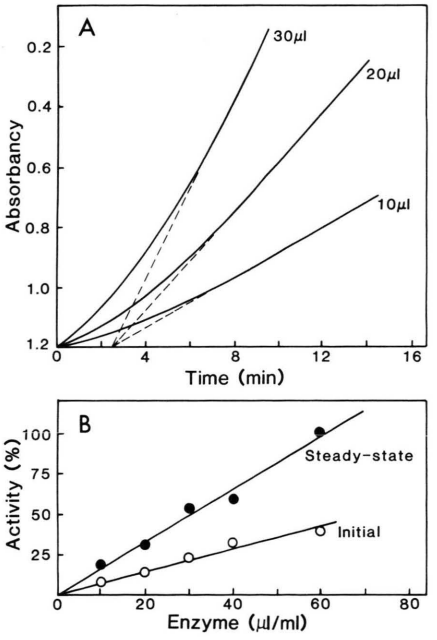

Fig. 1. Reactivation of PEPCase in the assay. The enzyme, diluted with the homogenization medium to 2.35 mg protein/ml, was stored at 0–4 °C for 130 h. (A) Time-course of PEPCase activity in the assay for different amounts of the enzyme (10, 20 and 30 μl per 1 ml of assay). Extrapolation of steady-state rates to the time axis gives $t_{1/2}$ value [13]. (B) Relationship between amount of PEPCase in assays and initial and steady-state rates.

Results

Maize leaf PEPCase showed marked lags in activity when assayed following preincubation in the cold (Fig. 1). For a given duration of the cold treatment, at a given dilution of protein on storage, the half-time of activation ($t_{1/2}$), defined as an intercept on the time axis after extrapolation of the steady-state rate [13], appeared to be independent of protein concentration in the assay (Fig. 1 A), while there was a linear relationship between protein concentration in the assay and initial and steady-state velocities (Fig. 1 B). No lags were observed for preparations stored at 18 °C. It should be stressed that the amount of MDH (5–10 U) used as a coupling enzyme in assays of PEPCase did not limit the reactions, and thus could not contribute to the hysteretic phenomenon.

The cold-induced lags in PEPCase activity were observed for preparations stored at a relatively high pH of 7.8–8.5 (Fig. 2). Storage at lower pH (6.5–7.1) promoted no lags, regardless of assay pH. Lower storage pH apparently stabilized PEPCase against cold inactivation, as judged by the fact that the enzyme kept for 12 h at pH 6.5–7.1 was two or three times more active as that stored

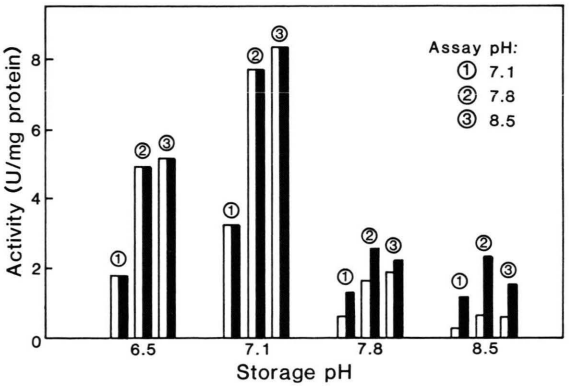

Fig. 2. Effect of storage and assay pH on activities and reactivation of maize PEPCase. The enzyme, diluted in the appropriate buffer medium to 0.76 mg protein/ml, was stored in the cold for 12 h. Open bars, initial rates; filled bars, steady-state rates.

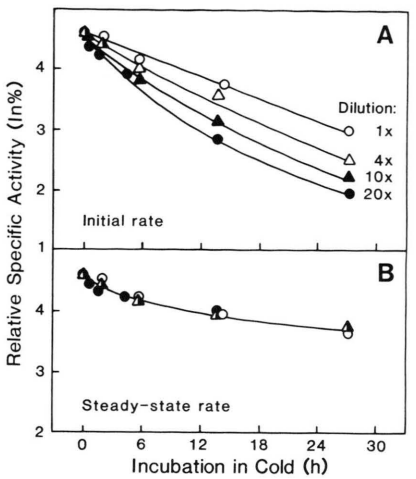

Fig. 3. Irreversible and reversible components of inactivation of PEPCase in the cold in relation to protein concentration upon storage. The enzyme (9.4 mg protein/ml) was diluted with the homogenization medium as indicated and then stored at 0–4 °C. (A) Initial rates. (B) Steady-state rates.

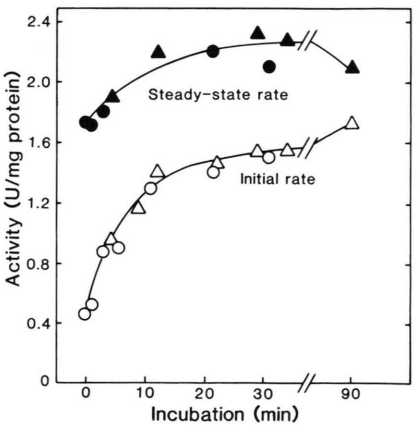

Fig. 4. Time-course of reactivation of cold-stored PEPCase during incubation at 18 °C. The enzyme (1.05 mg protein/ml) was cold-stored in the homogenization medium for 36 h, and then was diluted (100 ×) with an appropriate medium kept at 18 °C. (○–○–○) and (●–●–●), initial and steady-state rates, respectively, of PEPCase incubated at 18 °C in 130 mM Hepes (pH 7.8) alone; (△–△–△) and (▲–▲–▲), initial and steady-state rates, respectively, of PEPCase incubated at 18 °C in the medium containing all assay components but PEP.

at pH 7.8–8.5 (Fig. 2). The storage pH, however, had no effect on the general shape of the pH optimum curve of PEPCase activity (based on initial rates), with the highest rates observed at pH 7.8–8.5. Following activation of PEPCase in the assay, there was a sharper pH optimum of 7.8 (based on steady-state rates for preparations stored at pH 7.8 and 8.5) (Fig. 2).

The stability of PEPCase in the cold strongly depended on protein concentration upon storage (Fig. 3 A). The more diluted the protein, the larger the extent of the inactivation. On the other hand, when reactivation in the assay was taken into account, steady-state rates were found to be independent of protein concentration in the storage medium (Fig. 3 B). For a given duration of the cold treatment, the $t_{1/2}$ of activation of PEPCase in assays was roughly proportional to protein dilution upon storage (data not shown). Thus, in relation to data in Fig. 1, while the $t_{1/2}$ for cold-stored PEPCase appeared to be independent of protein concentration in assays, it did vary with protein level on storage.

Transfer of the cold-inactivated enzyme to 18 °C resulted in a considerable increase in activity (Fig. 4), with a time-course, based on initial rates, similar to that monitored continuously during assays of cold-stored PEPCase. Steady-state rates of PEPCase increased only slightly at 18 °C. The incubation substantially decreased the extent of lags during assays, but it did not eliminate them entirely even after 90 min treatment. Very similar time-courses of activation were observed for the enzyme incubated (18 °C) either in 130 mM Hepes (pH 7.8) alone and in the medium containing all assay components but PEP (Fig. 4), indicating a role for PEP in promoting lags in assays of the "warmed-up" enzyme. The PEP-dependent activation could sometimes be observed even 12 h after transfer of the enzyme from cold to 18 °C, but in most cases it gradually disappeared within 2–4 h of the incubation.

Discussion

Although the phenomenon of cold lability of plant PEPCase is well documented [7, 8, 11, 12], its exact mechanism is rather poorly understood. It has been demonstrated that the enzyme dissociates in the cold [7, 8], and that several factors may affect the state of oligomerization of the enzyme [6–12]. The partial reversibility of cold inactiva-

tion of plant PEPCase following an incubation at higher temperatures was also previously observed [8, 11, 12], but we are not aware of any study describing the reactivation occurring directly in assay media (Fig. 1). On the contrary, under certain storage and assay conditions, a marked loss of activity during assays was reported [9, 14] rather than activation. The lags in assays of PEPCase were previously observed for the purified enzyme from *E. coli* [15], but their possible relatedness to storage conditions apparently was not elucidated at that time.

In the present study, we have confirmed (Fig. 2 and 3) recent reports that the cold lability of PEPCase from C_4 plants is favoured by high pH [7, 12] and high protein dilution on storage [12]. Storage pH represents a major factor in controlling the cold-inactivation phenomenon for many enzymes [16]. Concerning the effect of protein dilution on cold lability of PEPCase, two components, a reversible and irreversible cold inactivation, were identified. The reversible component, based on initial rates for the cold-stored enzyme, did indeed depend on protein concentration upon storage, but the irreversible inactivation (based on steady-state rates) was independent of protein dilution (Fig. 3). In terms of lags, the more diluted the enzyme stored in the cold and high pH, the larger the extent of reactivation during assays and, as discussed in the Results section, the larger the $t_{1/2}$ value.

Of considerable importance may be the slow activating effect of PEP in assays of the cold-stored enzyme (Fig. 4). PEP has been reported to stabilize the enzyme against cold inactivation [8], presumably by preventing dissociation of the active tetrameric form [6, 7]. Our data, apart from indicating that rewarming of the cold and high pH stored enzyme may be insufficient for a complete recovery of its oligomerization state existing prior to the cold treatment, suggest also that binding of PEP (or its turnover effect) leads to an aggregation of the partly dissociated protein. It is now well established that PEP may bind to a regulatory site of PEPCase [3, 17], responsible for allosteric kinetics of the enzyme, but whether the same mechanism is involved in the slow PEP-dependent activation of the cold-inactivated enzyme (Fig. 4) is unknown at present.

Assuming that hysteresis in assays of cold-stored PEPCase reflects a reversal of the cold-induced dissociation of subunits reported for maize enzyme [8], the lags may provide a convenient means for a *continuous* monitoring of shifts in equilibrium between different oligomeric forms of the enzyme. The shifts probably represent a slow aggregation of dimers to tetramers rather than the monomer to dimer to tetramer conversion. Formation of monomers of PEPCase is apparently an irreversible process, as demonstrated for a diethylpyrocarbonate-induced dissociation of purified maize enzyme [7]. A breakdown of tetramers/dimers of PEPCase to monomers, accompanied by loss of activity, may be responsible for the irreversible component of cold inactivation observed in the present study.

It is unclear whether the cold-induced hysteresis of PEPCase, as observed in the present study, may have any significance for physiological regulation of the enzyme in leaves. However, certain cold-labile enzymes may also undergo a slow reversible dissociation at higher, more physiological, temperatures by the influence of specific ions or ligands, including coenzymes, substrates and allosteric effectors [16, 18]. Metabolites may displace the dissociation equilibria as one means of controlling catalysis [18]. Studies are in progress in search of effectors which could replace cold treatment as the means to induce lags in assays of plant PEPCase.

Acknowledgements

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