Hysteresis and Reversible Cold-Inactivation of ADP-Glucose Pyrophosphorylase From Barley Seeds

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ADP-Glucose Pyrophosphorylase, Cold Lability, Hysteresis, Starch

ADP-glucose pyrophosphorylase (AGP) from barley (*Hordeum vulgare* L.) seed endosperm showed a lag in activity when assayed after storage at −20 °C. The cold-stored enzyme could regain most, or all, of its activity during 40–60 min following exposure to ambient temperatures. The lags were not observed when 2 mM $MgCl_2$ was added to the storage buffer before freezing. Storage at −20 °C, in the absence of $MgCl_2$, led to the appearance of a low activity AGP form which was activated up to 3-fold by 3-phosphoglycerate (PGA) and had high K_m values with ATP of 0.3 and 1.2 mM (with and without PGA, respectively). In contrast, storage at −20 °C in the presence of $MgCl_2$ or incubation at +20 °C resulted in an active enzyme which was only weakly activated by PGA (up to 30%) and had the respective K_m values with ATP of 0.1 and 0.3 mM. It is suggested that low temperature may induce a change in the conformation and/or oligomerization state of the AGP protein, resulting in a low activity enzyme form which has distinct regulatory and kinetic properties.

Introduction

ADP-glucose pyrophosphorylase (AGP) (EC 2.7.7.27) catalyzes the first enzymatic step of starch biosynthesis in all plants (reviewed in [1]). The plant enzyme is a heterotetramer composed of two different subunit types [1, 2]. With the exception of the recently described AGP from barley seed endosperm [3], the plant enzyme is tightly regulated by PGA (activator) and inorganic phosphate (inhibitor) [1, 4–6], with the ratio of the two effectors playing a major role in modulating AGP activity. The enzyme from barley seed endosperm is relatively insensitive to these effectors, having high activity in the absence of PGA [3].

Oligomeric enzymes have frequently been shown to undergo reversible conformational and/or aggregational changes induced by shifts in temperature [7, 8]. For some of these proteins, incubation at low temperatures leads to a loss of activity and/or to a change in regulatory and kinetic properties [7–10]. Evaluation of mechanisms underlying such cold-induced changes (reviewed in [8]) may help in better understanding of factors involved in conformational and protein-protein interactions during catalysis and regulation.

Abbreviations: AGP, ADP-glucose pyrophosphorylase; PGA, 3-phosphoglycerate.

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In the present study, AGP from barley seed endosperm was shown to undergo a reversible inactivation upon storage at low temperatures. Both catalysis and regulatory properties of the enzyme were affected. Changes in kinetic parameters of barley seed AGP upon cold-storage are discussed.

Materials and Methods

Reagents

AGP from barley seed endosperm was purified as described in [3]. Purification procedure included ammonium sulfate fractionation, heat treatment, as well as hydrophobic (aminopropyl agarose) and ion exchange (DEAE-cellulose) chromatography. Specific activity of the final preparation (using assay B) was *ca.* 30 μmol of glucose-1-phosphate produced per min per mg protein. Phosphoglucomutase (rabbit muscle) and glucose-6-phosphate dehydrogenase *(Leuconostoc mesenteroides)* were from Sigma Chemical Co.

Storage conditions

AGP was stored in a buffer containing 20 mM Mops (pH 7.4), 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 200 mM NaCl, and 40% glycerol. Where indicated, 2 mM $MgCl_2$ was included for storage.

Assays of AGP

In the direction of ADP-glucose synthesis (assay A), assays contained, in 0.2 ml, 200 mM Tes

(pH 8.0), 7 mM $MgCl_2$ and, unless otherwise indicated, 0.3 mM [U-^{14}C]glucose-1-P and 1 mM ATP. Where indicated, PGA was included to assays at 2.5 mM. After 10–15 min at 37 °C, the reactions were stopped by boiling for 30 to 40 s. The amount of ADP-glucose, product of the reaction, was determined as previously described [3, 4].

In the direction of pyrophosphorolysis (assay B), AGP was assayed spectrophotometrically by monitoring the formation of NADH at 340 nm and 25 °C. A standard assay mixture (1 ml) contained 100 mM Mops (pH 7.4), 1 mM inorganic pyrophosphate, 0.5 mM ADP-glucose, 5 mM $MgCl_2$, 0.6 mM NAD, and 2 units each of phosphoglucomutase and glucose-6-phosphate dehydrogenase.

Kinetic studies

Kinetics of AGP were studied (assay A) by double reciprocal plots, having either ATP or glucose-1-phosphate as a varied substrate and keeping the other substrate at a fixed concentration. When ATP was varied (0.08–2.5 mM), glucose-1-phosphate was kept at 0.4 mM. For determination of kinetic constants with glucose-1-phosphate (varied at 0.05–1.0 mM), ATP was fixed at 2.5 mM. Where indicated, PGA was included to assays at 2.5 mM. K_m and V_{max} values were determined directly from the double reciprocal plots.

Results

Barley seed AGP was potently inactivated by storage (−$MgCl_2$) at −20 °C. Following thawing and an exposure to +20 °C, the enzyme regained most, or all, of its activity, showing a time-dependent lag in activation (Fig. 1). This hysteretic effect was biphasic, following a sigmoidal activation curve. At the beginning of the lag period, the enzyme was up to 3-fold activated by PGA. After 40–60 min at +20 °C, AGP was relatively insensitive to PGA (only about 20% activation) and its activity increased by 4 and 10-fold for assays with and without PGA, respectively (Fig. 1). The lags were found both for the forward (synthesis) and reverse (pyrophosphorolysis) reactions of AGP (data not shown).

Incubation at +20 °C or storage at −20 °C in the presence of magnesium did not cause any appreciable changes in AGP activity (Table I). The

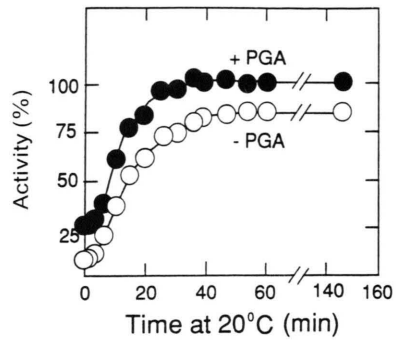

Fig. 1. Time dependence of recovery of AGP activity after storage at −20 °C. AGP was frozen overnight at −20 °C. Storage medium for AGP contained no magnesium. Assays at time zero were carried out immediately after thawing and placing the enzyme at +20 °C. Assay A was used for measurements of AGP activity. The value of 100% corresponded to *ca.* 0.8 nmol of ADP-glucose formed per min.

frozen enzyme was highly active immediately after thawing. Also, the enzymatic activity was relatively insensitive to PGA (about 30% activation). Freezing the enzyme without magnesium resulted in a low activity, which was sensitive to PGA regulation (Fig. 1, activities at time zero; Table I).

Storage without magnesium at −20 °C considerably increased K_m values of AGP with ATP (Table II). Kinetics of the cold-stored (−$MgCl_2$) enzyme with ATP followed hyperbolic response, regardless of the presence of PGA. Storage at −20 °C with $MgCl_2$ included [3], or incubation at +20 °C (Table II), resulted in an enzyme which

Table I. Effects of storage conditions (magnesium and temperature) on activity of AGP from barley seed endosperm. The enzyme (+/−$MgCl_2$) was frozen overnight at −20 °C and then assayed immediately after thawing of after 5 h at +20 °C. Assay A was used for measurements of AGP activity. The value of 100% corresponded to *ca.* 0.9 nmol ADP-glucose produced per min.

Assay conditions	Storage conditions			
	−$MgCl_2$		+$MgCl_2$	
	−20 °C	+20 °C	−20 °C	+20 °C
	Activity (%)			
−PGA	10[a]	96	100[a]	107
+PGA	32[a]	120	131[a]	133

[a] Assays were carried out immediately after thawing of the enzyme.

Table II. Kinetic constants for AGP from barley seed endosperm determined just after thawing of the enzyme stored at −20 °C or following incubation at +20 °C for 60 min. Storage medium for AGP contained no magnesium. Assay A was used for measurements of AGP activity.

Kinetic constants	Storage	
	−20 °C	+20 °C
K_m ATP [mM]		
−PGA	1.2[a]	0.3[b]
+PGA	0.3[a]	0.1
K_m glucose-1-P [mM]		
−PGA	0.1[a]	0.1
+PGA	0.1[a]	0.1

[a] Assays were carried out immediately after thawing of the enzyme.
[b] Sigmoidal kinetics.

had low K_m values with ATP and showed sigmoidal kinetics with ATP in the absence of PGA. The K_m values of AGP with glucose-1-phosphate were not significantly affected by different storage conditions nor by presence of PGA in the assays (Table II) (see also [3]).

Freezing at −20 °C for 3 h, without $MgCl_2$ in the storage medium, was sufficient for up to 90% inactivation of AGP. Storage of the AGP enzyme at 0−4 °C did not procduce as dramatic inactivation as freezing; however, about 50% of activity was lost during an overnight storage (−$MgCl_2$) at 0−4 °C. Following rewarming at +20 °C, most of the activity was recovered (data not shown).

Discussion

AGP from barley seed endosperm showed reversible changes in activity and regulatory properties that were induced upon storage at −20 °C (Fig. 1, Table I). The lags in recovery of activity observed upon exposure of the cold-stored enzyme to +20 °C (Fig. 1) are characteristic of a hysteretic enzyme [11], which undergoes slow changes between different activation states. Similar lags were observed for the cold-stored (−$MgCl_2$) AGP from barley leaves (data not shown), suggesting that cold-inactivation and hysteresis may represent common characteristics of a plant AGP enzyme.

The data in Fig. 1 and Table II suggest that the lags reflect, in part, a time-dependent change in kinetic properties of AGP, from the high K_m to low K_m forms of the enzyme. Judging by the K_m values in Table II, assays containing 1 mM ATP in the absence of PGA will detect less than 50% of V_{max} of the high K_m form, and about 80% of that of the low K_m form. In the presence of PGA, the high and low K_m forms will be assayed at about 80 and 95% of their V_{max} values, respectively. These changes in kinetic parameters can not, however, fully explain the increases of activity of about 4 and 10-fold, depending on PGA presence, observed during the lag period (Fig. 1, Table I). Thus, most of this effect has to be due to a change from an inactive to active enzyme form.

It should be emphasized that the strong activation by PGA seen for the cold-stored AGP (Fig. 1, activities at time zero; Table I), represents the property of the low activity (high K_m) form of the enzyme, stored in cold without magnesium. With magnesium included for storage at −20 °C [3], the enzyme had the same low K_m values with ATP as those determined for AGP incubated at +20 °C (Table II) and was similarly insensitive to PGA regulation (Fig. 1, Table I). The same properties were found for the crude barley endosperm AGP, when assayed immediately after isolation: it had high activity without PGA and was only slightly activated by this effector [3]. These results were obtained regardless of whether the extraction was carried out at 0−4 °C or +20 °C, and regardless of presence of magnesium (data not shown). Thus, the insensitivity to PGA and high activity without this effector represent physiological properties of AGP from barley endosperm, while properties of the cold-stored enzyme (−$MgCl_2$) appear to reflect a nonphysiological phenomenon. The high activity and insensitivity to PGA was also observed for AGP from crude wheat seed endosperm extracts (C. M. Duffus, personal communication).

An increase in K_m values upon cold-storage was previously reported for phosphoenolpyruvate carboxylase from maize leaves [10]. This change in kinetic properties has been ascribed to a reversible conformational change and dissociation of subunits comprising the native enzyme [10, 12]. Analogous processes are also likely to occur upon transition from ambient to cold temperatures of storage for barley seed AGP. The reversible changes in kinetic and regulatory properties of AGP, depending on storage conditions (Tables I and II), cer-

tainly suggest that the enzyme may exist in different stable conformational and/or aggregational states. Isomerization of stable free enzyme has previously been proposed for barley leaf AGP, based on studies on kinetic mechanism of AGP reaction [6]. Possible presence of different conformational forms, depending on *assay* temperatures, has also been discussed for AGP from *Rhodospirillum rubrum* [13]. The requirement for magnesium to prevent the formation of the low activity (high K_m) form of AGP (Table I) is consistent with the well-known role of this divalent cation to stabilize aggregational states of oligomeric enzymes [7, 9]. Magnesium may also stabilize conformational forms of proteins [14].

AGP joins a growing number of enzymes which undergo inactivation at low temperatures [7–10, 12]. The inactivation is usually caused by reversible dissociation and/or conformational changes induced by cold-treatment [7, 8]. It is believed that low temperature decreases the stability of hydrophobic bonds involved in subunit-subunit interactions, promoting dissociation of an oligomeric protein [8]. The inactivation may also reflect a temperature-dependent shift in the pK of a ionizable group which controls the active-inactive form conversion [7]. These factors need to be carefully addressed in further studies on mechanism(s) involved in cold-inactivation of AGP.

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