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The potato tuber, maize endosperm and a chimeric maize-potato ADP-glucose pyrophosphorylase exhibit fundamental differences in Pi inhibition

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ABSTRACT

ADP-glucose pyrophosphorylase (AGPase) is highly regulated by allosteric effectors acting both positively and negatively. Enzymes from various sources differ, however, in the mechanism of allosteric regulation. Here, we determined how the effector, inorganic phosphate (Pi), functions in the presence and absence of saturating amounts of the activator, 3-phosphoglyceric acid (3-PGA). This regulation was examined in the maize endosperm enzyme, the oxidized and reduced forms of the potato tuber enzyme as well as a small subunit chimeric AGPase (MP), which contains both maize endosperm and potato tuber sequences paired with a wild-type maize large subunit. These data, combined with our previous kinetic studies of these enzymes led to a model of Pi inhibition for the various enzymes. The Pi inhibition data suggest that while the maize enzyme contains a single effector site that binds both 3-PGA and Pi, the other enzymes exhibit more complex behavior and most likely have at least two separate interacting binding sites for Pi. The possible physiological implications of the differences in Pi inhibition distinguishing the maize endosperm and potato tuber AGPases are discussed.

Introduction

ADP-glucose pyrophosphorylase ([1]AGPase EC 2.7.7.27) has been studied from a variety of sources and its major importance stems from its rate-determining role in starch biosynthesis in plants and glycogen in bacteria. AGPases exhibit a heterotetrameric $\alpha_2\beta_2$ structure in higher plants. It is also highly regulated, subject to various layers of control depending on the tissue and cellular location. The first level of control is transcriptional activation by sugars and light [34,30,26,5,11], and deactivation by phosphate and nitrogen [31,27]. Post-translational redox activation [36,21,28,14], allosteric regulation [4], thermo-lability [6] and rapid protein turnover [29] are also control mechanisms for a number of AGPases.

There is an ongoing dispute whether post-translational redox modification of AGPases is a common regulatory property in photosynthetic tissues. While some reports in *Arabidopsis* suggest there is an AGPase regulatory redox response to light and sugars [15,37], other evidence shows that the fine tuning in transitory starch accumulation in response to light by redox regulation is not a determinant [23].

The potato tuber AGPase exhibits different properties in vitro based on its oxidation state [12]. While many reports describe redox activation, very few address the actual isolated enzyme activity, but rather refer to its activation state as being a monomer or a dimer even though enzyme activity is derived from a heterotetramer. Some suggest that reductive activation results in an enzyme with an increased affinity for the activator and substrates [36], while others report that there is little or no difference in these parameters in response to redox [3,20,8]. While AGPase from cereal endosperms lack the cysteine residues necessary for redox regulation, heat lability is a common concern with the AGPases isolated from this tissue [6] and inactivation of this enzyme due to heat stress may cause major loss in agricultural yield [35,24,33,38].

Although some forms of regulation appear specific to particular tissues and species and remain controversial, allosteric regulation is accepted to be common to almost all AGPases. The higher plant enzyme is primarily activated by 3-phosphoglyceric acid (3-PGA), and inhibited by inorganic phosphate (Pi). Much confusion in the literature comes from reports that some enzymes are more highly activated by 3-PGA than are others. While this is true under limiting substrate conditions, it has recently been shown that the binding of

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[1] *Abbreviations used:* AGPase, ADP-glucose pyrophosphorylase; Pi, inorganic phosphate; PP_i, pyrophosphate.

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the substrates (ATP and G-1-P) and effectors (Pi and 3-PGA) are tightly coupled [7]. In addition, G-1-P, ADPG and Pi actually activate the maize endosperm enzyme in the absence of 3-PGA [7,8]. Thus the "activation rate" displayed in many reports cannot be compared directly, as each is a snapshot at one concentration of each substrate and one concentration of 3-PGA.

Since 3-PGA and Pi are considered the primary physiological effectors of plant AGPases, their effects have been studied intensively (for review see [16]). Our recent kinetic analysis revealed that the mechanism of action of 3-PGA is fundamentally different for the maize endosperm, MP (a maize potato small subunit mosaic paired with a wild-type maize large subunit) and potato tuber enzymes [8]. 3-PGA increases the affinity for both substrates for the maize and the MP enzymes, while it primarily increases the velocity of the potato enzymes, with some enhancement of G-1-P binding [8]. Pi inhibits the maize enzyme mainly by reversing the effect of 3-PGA; in the absence of 3-PGA, Pi has minimal effect on this enzyme [7]. In contrast, the effect of Pi on the wheat endosperm enzyme is just the opposite. Here, 3-PGA only activates the enzyme in the presence of Pi and shows no activation in the absence of Pi. The behavior of 3-PGA might best be described as an anti-inhibitor for this enzyme [13]. The potato enzyme, in contrast, is strongly inhibited by Pi, in the presence or absence of 3-PGA [2,17]. Because of this complexity and multiple phenomena associated with Pi modulation, we sought to more accurately define the Pi inhibition of the maize enzyme, the oxidized and reduced potato enzymes and the MP chimeric enzyme, and determine the coupling patterns for the effectors and the substrates.

Materials and methods

Plasmids: The plasmids, growth conditions and purification schemes for the maize, MP and potato enzymes are described in [8].

Kinetic measurements: The oxidized potato enzyme was converted to the reduced form by adding 3 mM DTT for 30 min at 4 °C and confirmed by migration on non-reducing gels SDS gels (data not shown).

Each purified enzyme preparation was desalted twice into 50 mM HEPES pH 7.4, 5 mM $MgCl_2$, 0.5 mM EDTA to remove contaminating Pi, using Zeba Micro Desalt Spin Columns (Pierce). Protein concentrations were determined using the BioRad (Hercules, CA) protein assay kit using a known concentration of AGPase. BSA (0.5 mg/ml) was added to protein stocks to increase enzyme stability.

Assay: AGPase activity was determined from the amount of pyrophosphate (PP_i) formed during the assay period, which was coupled to a decrease in NADH concentration [6]. Standard reaction mixtures contained 50 mM HEPES pH 7.4, 15 mM $MgCl_2$, in a total volume of 300 μl. When held constant in the absence of 3-PGA, the following substrate concentrations were used: ATP, 0.5 mM; G-1-P, 5 mM. When 3-PGA was added (5 mM), ATP and G-1-P concentrations were fixed at 0.4 and 0.2 mM respectively. Substrate and Pi concentration ranges when varied in the presence of 3-PGA were as follows:

Enzyme	Varied substrate	Varied substrate range, mM	Varied Pi range, mM
Maize	ATP	0.05–5	0.1–5
	G-1-P	0.025–5	0.1–10
Potato (oxidized)	ATP	0.05–1.5	0.1–2.5
	G-1-P	0.025–1.5	0.25–1.5
Potato (reduced)	ATP	0.025–1.5	0.1–1.5
	G-1-P	0.05–1	0.5–2.5
MP	ATP	0.025–2.5	1–20
	G-1-P	0.025–2.5	1–20

Substrate and Pi concentration ranges when varied in the absence of 3-PGA were as follows:

Enzyme	Varied substrate	Varied substrate range, mM	Varied Pi range, mM
Maize	ATP	0.75–15	0.5–20
	G-1-P	1.5–20	0.5–5
Potato, (oxidized)	ATP	0.05–2.5	0.025–0.25
	G-1-P	2.5–25	0.005–0.5
Potato (reduced)	ATP	0.05–2.5	0.005–0.25
	G-1-P	2.5–25	0.005–0.5
MP	ATP	0.02–5	0.025–5
	G-1-P	2.5–25	0.01–5

Reactions were initiated by adding enzyme and terminated after 10 min at 37 °C by boiling for 1.5 min. The reactions were developed by adding 200 μl of coupling reagent (25 mM imidazole pH 7.4, 4 mM $MgCl_2$, 1 mM EDTA, 0.2 mM NADH, 0.725 U aldolase, 0.4 U triose phosphate isomerase, 0.6 U glycerophosphate dehydrogenase, 1 mM fructose 6-phosphate and 0.8 μg purified PP_i-PFK per reaction) to each tube and determining the absorbance at 340 nm after 30 min. Blank samples contained complete reaction mixtures without AGPase. Reactions were linear with time and enzyme concentration. All kinetic constants were obtained using the forward reaction, unless otherwise stated.

Data analysis: Inhibition data were fitted to Eqs. 1–4, which correspond to partial mixed type inhibition (Eq. (1)), noncompetitive inhibition (Eq. (2)) cooperative noncompetitive inhibition (Eq. (3)) and cooperative mixed type inhibition (Eq. (4)) using GraphPad Prism software. v is the measured velocity, V_m is the maximum velocity, S is the substrate concentration, K_i is the inhibition constant, K_s is the dissociation constant for the ES complex, I is the inhibitor concentration, α is the factor by which K_i changes when the inhibitor is present and β is the factor by which k_p changes when the inhibitor is present.

$$v = V_m(S/K_s + (\beta SI)/(\alpha K_i K_s)/(1 + S/K_s + I/K_i + SI/\alpha K_i K_s)) \quad \text{P-MT} \quad (1)$$

$$v = V_m(S/K_s)/(1 + S/K_s + I/K_i + SI/K_i K_s)) \quad \text{NC} \quad (2)$$

$$v = V_m(S/K_s)/(1 + S/K_s + I^n/K_i^n + S^n/K_i^n K_s)) \quad \text{C-NC} \quad (3)$$

$$v = V_m(S/K_s)/(1 + S/K_s + I^n/K_i^n + SI^n/\alpha K_i^n K_s)) \quad \text{C-MT} \quad (4)$$

Results

While the maize, MP, and reduced and oxidized potato AGPases exhibit an ordered Theorell–Chance Bi Bi mechanism in the presence of 3-PGA, the effect of a single concentration of phosphate on the mechanism was difficult to interpret [8]. Here, we performed a detailed study of how this regulator affects the rate of the reaction, both in the presence and in absence of 3-PGA.

Experiments to determine the concentration range of Pi, as well as the completeness of the inhibition are shown in Fig. 1. Under the specified conditions, the maize enzyme showed only partial inhibition at Pi concentrations up to 15 mM. In contrast, the oxidized potato enzyme was very sensitive to Pi inhibition. Activity was less than 5% at 1.5 mM Pi. The oxidized enzyme was completely inhibited at 2.5 mM Pi when [ATP] was maintained at K_m levels (Fig. 1) and inhibition was still 95% when [ATP] was 10-fold greater than the K_m value (data not shown). Pi inhibited the reduced potato enzyme at a much lower Pi concentration compared to the maize enzyme but slightly greater Pi concentrations were required for the oxidized potato enzyme. At 2.5 mM Pi, over 70% of the reduced potato enzyme activity was inhibited when [ATP] was near K_m.