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Deciphering the kinetic mechanisms controlling selected plant ADP-glucose pyrophosphorylases

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ABSTRACT

ADP-Glc pyrophosphorylase (AGPase), a rate-limiting enzyme in starch biosynthesis, is controlled by thermostability and allosteric regulation. Previous studies suggested that redox affects turnover number and heat stability of AGPases. Here, we investigated how allostery and redox state affect kinetic mechanisms of the reduced, heat labile and the oxidized, heat stable potato tuber enzymes; the heat labile maize endosperm enzyme and a chimeric maize/potato heat stable enzyme that lacks the cysteine responsible for redox changes. With 3-PGA, all AGPases followed a Theorell-Chance Bi Bi mechanism with ATP binding first and ADP-Glc releasing last. 3-PGA increases the binding affinity for both substrates with little effect on velocity for the maize and MP isoforms. By contrast, 3-PGA increases the velocity and the affinity for G-1-P for the potato enzymes. Redox state does not affect k_{cat} of the two potato isoforms. Without 3-PGA the oxidized potato enzyme exhibits a rapid equilibrium random Bi Bi mechanism with a dead end ternary complex. This fundamental change from rapid, ordered binding with little buildup of intermediates to a mechanism featuring relatively slow, random binding is unique to the oxidized potato tuber enzyme. Finally, ADP-Glc the physiologically relevant product of this enzyme has complex, isoform-specific effects on catalysis.

Introduction

ADP-Glc pyrophosphorylase (AGPase) catalyzes the formation of ADP-glucose (ADP-Glu) and pyrophosphate (PPi) from Glc-1-phosphate (G-1-P) and ATP. ADP-Glu is then used for the synthesis of starch in plants and glycogen in bacteria (Review, [37], [2,3]. Plant AGPases are heterotetramers composed of two identical small and two identical large subunits; whereas, most bacterial enzymes are homotetramers. Virtually all AGPases are allosterically regulated by metabolic intermediates derived from the prevailing carbon metabolism pathway. Orthophosphate (P_i) and 3-phosphoglycerate (3-PGA) are effectors that have received the most attention in plant AGPase investigations. Crystal structures of inactive forms of a non-native potato tuber small subunit homotetramer [28] and a bacterial AGPase [12] have been published.

The importance of this enzyme and, in particular, its allosteric properties has been documented by variants of this enzyme that overproduce glycogen and starch. This is especially relevant since the majority of the dry weight of the agriculturally important potato tuber and cereal seed is starch. Transgenic plant studies with allosterically altered or over-expressed AGPases have led to a 35% increase in potato yield [43], a 23% [42] and an 11% [40] increase in rice yield, a 38% increase in wheat yield [41], and an 11–18% [19], a 24% [45] and up to a 68% yield increase in maize [23]. In addition, elevated *Arabidopsis* leaf transitory starch turnover and improved growth characteristics [35,18] as well as enhanced fresh weight of aerial parts of lettuce plants [31] resulted from expression of allosterically altered AGPase.

Heat lability of AGPase is another important control mechanism. Cereal endosperm AGPases are particularly heat labile and variants with enhanced heat stability increase cereal yield [23,41,42]. In addition, formation of a disulfide bridge involving an *N*-terminal cysteine at position 81 in the potato tuber small subunit enhances heat stability [15,44,33].

Reversible formation of a disulfide bridge involving two Cys81 small subunit residues may also be associated with controlling rates of starch synthesis. Tiessen et al. [44] showed that tuber starch synthesis was inhibited by detachment from the mother plant. However, this inhibition did not occur in potatoes in which the tuber AGPase was replaced with a bacterial AGPase gene. Furthermore, as judged by non-reducing SDS gels, tuber detachment from the mother plant was associated with oxidation of the tuber AGPase via formation of a disulfide bridge between the *N*-terminal cysteines of two AGPase small subunits. Enzyme preparations from

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attached potatoes, which are composed of both oxidized and reduced small subunits, contained more AGPase activity than did preparations from detached tubers. Attached-potato AGPase was more sensitive to the activator 3-PGA and the substrate, ATP. However, the difference in AGPase activities from the attached and detached potatoes was quite transient and disappeared rapidly upon incubation in the absence of substrates.

Leaf AGPase in potato, pea and Arabidopsis exists in both reduced and oxidized forms during the day but only as the oxidized form at night, as judged by mobility on non-reducing SDS gels [25]. Sugar addition to leaves exposed to the dark as well as illumination of isolated chloroplasts lead to the detection of disulfide-reduced AGPase. Assays of non-dialyzed, crude preparations showed that AGPase activity is increased and the activity is more sensitive to 3-PGA and ATP. Interestingly, while removal of Cys81 from the Arabidopsis leaf small subunit blocks the oxidation of AGPase, levels of ADP-Glc are still reduced at night [22]. Leaf starch levels are wild-type or higher depending on growth conditions, implying that redox control of AGPase is not required for starch synthesis although its perturbation does alter diurnal regulation of starch content. Contrasting results for starch synthesis in Arabidopsis leaves, however, were recently published by Li et al. [32]. This group concluded that rates of starch synthesis and degradation through a 24 h day/night cycle were not significantly altered by replacement of the Arabidopsis enzyme by a redox insensitive *Escherichia coli* enzyme, by replacement with the Arabidopsis lacking the *N*-terminal cysteine involved in redox regulation or by mutationally removing enzymes required for reduction of the AGPase heterotetramer.

Despite the importance of AGPase, and particularly its allosteric, heat stability and possibly redox properties, relatively little is known about its kinetic mechanism. Complete descriptions have been reported for only three isoforms: the bacterial *Rhodospirillum rubrum* enzyme [36], the barley leaf enzyme [29] and the maize endosperm enzyme [4]. While all three AGPases follow an ordered sequential mechanism in the presence of 3-PGA, the barley leaf scheme contained an additional isomerization step following ADP-Glc release, a step that was not observed in the *R. rubrum* or maize mechanisms. The latter followed a Theorell Chance mechanism, a specialized ordered bi bi mechanism where the central complex concentrations are essentially zero.

Here, we investigated how allostery and redox state affect the kinetic mechanism for three important AGPase isoforms: the reduced, heat labile potato tuber enzyme; the oxidized, heat stable potato tuber enzyme; and a chimeric maize/potato heat stable enzyme (MP). The last is a hybrid AGPase composed of portions from the maize endosperm and the potato tuber AGPases in the small subunit paired with a wild-type maize large subunit. In previous studies [11,6,7,8], this small subunit chimera in which the *N*-terminal amino acids (1-198) were derived from the small subunit of the maize endosperm enzyme, while the carboxyl terminal 277 amino acids came from the potato tuber AGPase small subunit, exhibited properties not apparent in either parental enzyme. In addition, the MP enzyme lacks the *N*-terminal cysteine involved in disulfide formation in the potato enzyme. The MP construct also enhanced seed yield of transgenic maize (Hannah, unpublished). Previously, we showed that the k_{cat} value of MP, in the absence of the activator 3-PGA, is some 10 times greater than that of the maize endosperm (hereafter termed simply maize) enzyme. Thus, in the absence of activator, MP AGPase, behaves like the maize AGPase that is partially activated by 3-PGA. The presence of 3-PGA minimizes or negates this difference. Additionally, MP AGPase is more heat stable than the maize enzyme in the absence of the activator [8]. Understanding the detailed kinetic mechanism in the presence and absence of the physiological effectors should provide further insights into this agriculturally important chimera. Additionally, since it has been proposed [15,44,25,33,22] that the activity of the heterotetrameric potato tuber AGPase is greatly influenced by its oxidation state, a thorough examination of the kinetic mechanism of the oxidized and reduced potato tuber AGPases was carried out. Each mechanism was elucidated in both the presence and absence of 3-PGA, and in the presence of P_i.

All AGPases exhibited a Theorell-Chance Bi Bi mechanism in the presence of 3-PGA with ATP binding first and ADP-Glc releasing last. 3-PGA enhances AGPase activity in two fundamentally different ways depending on the AGPase isoform. For both the maize and MP enzymes, 3-PGA increases the substrate binding affinities but has little effect on V_{max}. By contrast, this allosteric activator increases the reaction velocity, as well as the affinity for G-1-P for the two potato enzymes. Importantly, the disulfide redox state does not affect the V_{max} value of the potato enzyme. Unexpectedly the oxidized potato enzyme exhibits a rapid equilibrium random Bi Bi mechanism with a dead end ternary complex in the absence of 3-PGA. We also discovered that ADP-Glc, the reaction product, serves as an allosteric activator of its own synthesis for both the maize and MP enzymes when 3-PGA is absent.

Results

Activation kinetics

Before elucidating the kinetic mechanisms in the presence of the activator 3-PGA, it was essential to determine its binding affinities (K_a values) for each enzyme. Concentrations of ATP and G-1-P were held constant at 4 mM while 3-PGA was varied, yielding the K_a values shown in Table 1. In agreement with our previous work [8], MP has substantial activity in the absence of 3-PGA. Potato AGPase activity was not detectable in the absence of 3-PGA at the protein quantity employed (60 ng). The 3-PGA K_a values for the two potato enzyme forms (oxidized, 0.52 mM and reduced, 0.20 mM) were significantly higher than those of the maize (0.06 mM) and MP (0.03 mM) enzymes. Despite the lack of detectable activity in the absence of 3-PGA, the 3-PGA activated form of both potato enzymes had greater total activity than the maize or MP counterparts. Similar to our findings, Ballicora et al. [1] also described a small increase (~2-fold) in the $A_{0.5}$ for the reduced form of the potato enzyme.

F-6-P is an effective activator of maize AGPase, with an activation constant similar to its physiological concentration [5,7,34]. Hence, it was of interest to see how MP and the two forms of the potato enzyme would respond to this activator (Table 1). The extent of F-6-P activation was comparable to 3-PGA activation for the maize enzyme and slightly less for the MP enzyme. Even though the F-6-P K_a values were higher than those of 3-PGA, they were still similar to the calculated physiological concentration for this metabolite in the maize endosperm (0.5 mM; [34]. In contrast,

Table 1
Activation constants, K_a, for 3-PGA and F-6-P.[a]

	Maize	MP	Pot − DTT	Pot + DTT
K_a 3-PGA	0.06 ± 0.007	0.03 ± 0.007	0.52 ± 0.12	0.20 ± 0.017
V_{min}	1.1 ± 0.2	11.3 ± 0.5	0	0
V_{max}	9.1 ± 0.3	7.7 ± 0.6	36.4 ± 2.1	34.0 ± 0.7
V_{total}	10.2	19.0	36.4	34.0
K_a F-6-P	0.53 ± 0.08	0.15 ± 0.07	9.8 ± 5.2	20.0 ± 3.9
V_{min}	0.8 ± 0.2	5.0 ± 0.3	0	0
V_{max}	12.8 ± 0.5	4.20 ± 0.4	4.5 ± 1.2	10.5 ± 1.1
V_{total}	13.6	9.2	4.5	10.5

[a] V_{min} represents the velocity in the absence of effector, while V_{max} is the difference between the total activity and V_{min}. V_{total} is equal to $V_{max} + V_{min}$. The ATP and G-1-P were held constant at 4 mM when varying 3-PGA and at 2 mM when varying F-6-P. Velocity is presented in μmol/min/mg and K_a is in mM.