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Enhanced heat stability and kinetic parameters of maize endosperm ADPglucose pyrophosphorylase by alteration of phylogenetically identified amino acids

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ABSTRACT

ADP-glucose pyrophosphorylase (AGPase) controls the rate-limiting step in starch biosynthesis and is regulated at various levels. Cereal endosperm enzymes, in contrast to other plant AGPases, are particularly heat labile and transgenic studies highlight the importance of temperature for cereal yield. Previously, a phylogenetic approach identified Type II and positively selected amino acid positions in the large subunit of maize endosperm AGPase. Glycogen content, kinetic parameters and heat stability were measured in AGPases having mutations in these sites and interesting differences were observed. This study expands on our earlier evolutionary work by determining how all Type II and positively selected sites affect kinetic constants, heat stability and catalytic rates at increased temperatures. Variants with enhanced properties were identified and combined into one gene, designated *Sh2*-E. Enhanced properties include: heat stability, enhanced activity at 37 °C, activity at 55 °C, reduced K_a and activity in the absence of activator. The resulting enzyme exhibited all improved properties of the various individual changes. Additionally, *Sh2*-E was expressed with a small subunit variant with enhanced enzyme properties resulting in an enzyme that has exceptional heat stability, a high catalytic rate at increased temperatures and significantly decreased K_m values for both substrates in the absence of the activator.

Introduction

ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27)[2], a critical enzyme in the starch biosynthetic pathway for plants, catalyzes the formation of PPi and ADP-glucose (ADP-Glc) from ATP and glucose-1-phosphate (G-1-P). Regulation of this rate-limiting step is controlled by a series of complex checkpoints including transcription, posttranslational modification, heat lability and allostery (Reviewed [1–5]). The relative importance of each type of control is specific to the organism and tissue of expression; however, allosteric regulation by 3-phosphoglyeric acid (3-PGA) and inorganic phosphate (Pi) is common to virtually all plant AGPases.

Most bacterial AGPases exhibit a homotetrameric structure while the plant enzymes consists of two large and two small subunits, leading to a heterotetramer arranged in an $\alpha 2\beta 2$ structure (Reviewed in [6]). The two plant subunits were derived from gene duplication [7,8]. The maize endosperm subunits share 43.2% identity and 61% similarity and loss of the large subunit *shrunken-2* (*Sh2*) or the small subunit *brittle-2* (*Bt2*) function abolishes >90% of endosperm AGPase activity [9]. Rather than interchangeable subunits, the plant AGPase subunits have undergone sequence divergence leading to two different subunits; however, both are required for allosteric and catalytic properties of the enzyme [10]. Throughout the plant kingdom, the small subunits of AGPase are strikingly conserved compared to the large subunits. Using an evolutionary approach as well as measurement of the effect of amino acid changes on the activity of maize endosperm AGPase expressed in *Escherichia coli*, the AGPase small subunit was shown to be more conserved because it was less tissue specific, less redundant, and has to form functional enzyme complexes with different large subunits expressed both in the cytosol and in the plastid [11,12].

The importance of AGPase to plant agriculture is evident from the many studies in which altered or over-expressed AGPases were placed in plants and yields were increased [13–21]. Heat stability of the endosperm enzyme is particularly important as evidenced by placement of a moderately heat stable AGPase into some cereals. This resulted in yield increases of 38% in wheat [15], 23% in rice [16] and up to a 68% in maize [21].

Previously, phylogenetic analyses have shown that duplications in the large subunit have led to 4 distinct phylogenetic groups (Supplemental Fig. 1) [12]. Group 1 consists of large subunits

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[2] *Abbreviations used:* AGPase, ADP-glucose pyrophosphorylase; PPi, pyrophosphate; 3-PGA, 3-phosphoglyeric acid; Pi, inorganic phosphate.

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expressed primarily in leaves while group 2 consists of subunits expressed in both source and sink tissues. Group 3 can be divided into two sub-groups 3a and 3b, which include large subunits expressed in the sink tissues in eudicots and monocots, respectively. Finally, group 4 includes very few members that are poorly characterized. Biochemical analyses have shown that the large subunits in different groups have functionally diverged as reflected in the kinetic and allosteric properties of the AGPase complexes in which they participate [22].

Use of branch-site models within the PAML (Phylogenetic Analysis by Maximum Likelihood) package identified several positively selected amino acid positions in the branches that followed the large subunit duplications, resulting in the groups described above [12]. These amino acid positions could lead to the functional divergence of the AGPase large subunit. Additionally, amino acid residues conserved within, but variable among AGPase large subunit groups could also lead to the functional divergence of the AGPase large subunit (type-II functional divergence; [23]). DIVERGE software [24] was used to identify such amino acid positions by pairwise comparisons of large subunit groups. Site-directed mutagenesis was used to alter several of these amino acid positions in SH2 [25]. Effects were observed in catalytic and allosteric properties of AGPase as well as heat stability.

Herein, we used the phylogenetic selection of amino acid sites to synthesize an enhanced maize endosperm AGPase large subunit. To do this, we expanded on the previous work by further site-directed mutagenesis of the type II and positively selected amino acid residues in SH2. An extensive study of kinetic parameters, heat stability and activity at 37 °C and 55 °C was completed. Selection was practiced for alterations enhancing k_{cat}, decreasing K_m and K_a values for substrates and the activator 3-PGA, 3-PGA independent activity, heat stability in absence of bound substrates and effectors and catalytic activity at increased temperatures. Variants were identified for each selected trait. Importantly, these altered phenotypes were combined successfully into a single gene (*Sh2*-E) as gleaned from studies of enzymes produced with the wildtype small subunit (BT2) and an enhanced small subunit (MP).

Experimental procedures

Amino acid selection and preparation of enzyme variants were described by Georgelis et al. [25].

Plasmid preparation

The plasmids, pMONc*Bt2* and pMONc*Sh2*, containing the small and large subunits of maize endosperm AGPase, respectively, [14] were expressed in *E. coli* AC70R1-504 cells [26] as previously described [27]. Evolutionary mutants were prepared by QuikChange site-directed mutagenesis (Agilent) using pMONc*Sh2* as template.

Sh2-E was prepared synthetically by GenScript. The insert was subcloned into the pMONc*Sh2* vector using standard cloning methods and T4 DNA Ligase (Invitrogen).

The small subunit mosaic AGPase, referred to as MP, was constructed by Cross et al. [28]. The N-terminal 199 amino acids were derived from the maize endosperm with the remaining 277 amino acids derived from the potato tuber AGPase small subunit. This chimeric enzyme was co-expressed with the maize large subunit.

Protein expression and purification

The evolutionary-based variants of the large subunit were co-expressed in *E. coli* AC70R1-504 with the wildtype maize small subunit and purified according to standard procedures [27]. SDS–PAGE was used to determine the purity of the enzymes. Since many of the enzymes did not reach homogeneity (~95%) the amount of AGPase contained in each sample was corrected by determining the percent purity of each preparation. This was accomplished by running each purified enzyme preparation on SDS–PAGE followed by analysis with ImageJ® imaging analysis software (U.S. National Institutes of Health, Bethesda, Maryland, USA; Supplemental Fig. 2). The k_{cat} values presented here are based on the corrected amount of the AGPase in each purified preparation.

Determination of kinetic constants, K_m, k_{cat} and K_a

The rate of the AGPase reaction was determined from a coupled assay in which the amount of pyrophosphate (PPi) formed during the reaction was coupled to a decrease in NADH concentration [27,29]. Each reaction was 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, 0.725U aldolase, 0.4U triose phosphate isomerase, 0.6U glycerophosphate dehydrogenase, 1 mM fructose 6-phosphate and 0.8 µg purified PPi-PFK per reaction) to each tube and determining the absorbance at 340 nm. Blank samples contained complete reaction mixtures without enzyme. The amount of pyrophosphate (PPi) produced was determined from a standard curve using PPi in complete reaction mixtures lacking AGPase. Change in absorbance between the blank and the reaction was used to calculate the amount of PPi produced. Each reaction was linear with time and enzyme concentration. Saturation plots to determine kinetic parameters were hyperbolic in all cases. Cooperative effects were not observed as a component governing substrates/activators/inhibitor kinetics. When held constant, reaction mixtures contained 50 mM HEPES pH 7.4, 15 mM $MgCl_2$, 2.0 mM ATP, and 2.0 mM G-1-P and 5 mM 3-PGA in a total volume of 300 µl. The Michaelis constants for the substrates of the various proteins were determined by incubating the purified AGPase with a varying level of substrate (ATP or G-1-P) at a constant saturating level of co-substrate. Likewise, the activation constant (K_a) was determined by varying the activator at fixed constant levels of both substrates.

Kinetic constants in the absence of 3-PGA were determined by varying one substrate at several constant concentrations of the co-substrate. The reactions were pre-warmed to 37 °C, initiated by addition of the appropriate enzyme, and terminated by boiling for 1.5 min. Reactions were then developed with coupling reagent as described above.

Catalytic activity at 37 °C and 55 °C

Activity was measured using the method for determining the kinetic constants as described above, with the following exceptions. All reactions had constant concentrations of substrates (2.0 mM) and activator (2.5 mM). Reactions mixes (1300 µl) were pre-warmed to either 37 °C or 55 °C and the assay was started with the addition of the appropriate enzyme. Assay time points (300 µl) were removed every 2.5 min and boiled, for a total of 10 min. All reactions were linear for 10 min at 37 °C. For those reactions at 55 °C that were linear with time for 10 min, a rate was calculated.

Resistance to thermal denaturation of purified maize AGPase and evolutionary mutants

Resistance to thermal denaturation in the absence of substrates or activators was determined using desalted enzymes supplemented with 0.5 mg/mL BSA in a total volume of 10 µl. Samples were incubated at 37 °C for 0–7.5 min, then immediately cooled with ice. The remaining catalytic activity of each sample was determined from the standard assay (forward direction) in the presence of 2.5 mM 3-PGA. Reactions were initiated by adding AGPase