Final Report for DoE Grant DoE DE-FG02-96ER20216

We focused our studies on AGPase and Pho1 not only on the structure-function relationships of these enzymes at the biochemical level but also on the physiological roles of these enzymes in influencing source-sink relationships. Five objectives were initially proposed and our progress for each objective is summarized below. Due to the reduction in funding, reduced effort was made in Objectives 2 and 3 and transgenic studies initially proposed in Objective 4 were delayed until year 3.

Objective 1. Determine the substrate and effector binding properties of AGPase homotetrameric LS and SS forms and heterotetrameric LS-SS forms.

The role of allosteric regulation of ADPglucose pyrophosphorylase (AGPase) in rice seed starch metabolism. Null and missense mutations in the cytosolic isoform of rice endosperm AGPase LS (*OsAGPL2*) were identified via analysis of a mutant population by TILLING (Targeted Induced Local Lesion in Genomes). Interestingly, rice seeds expressing a LS missense mutation had just as low seed weight and starch content as seeds null for LS but expressing a SS homotetramer. This observation suggests that the AGPase activities in the null and missense mutant seeds are similar and that the heterotetrameric enzymes containing missense mutated LSs have net catalytic activities only as high as the SS homotetramer enzyme. Analysis of the kinetic properties of purified wildtype enzyme ($L^{WT}S^{WT}$), LS missense mutant enzymes ($L^{T139I}S^{WT}$ and $L^{A171V}S^{WT}$), and small subunit homotetramer S^{WT} enzyme showed that the mutations affect both catalytic and allosteric regulatory properties. The homotetramer S^{WT} and the two LS missense mutant enzymes required 3- to 6-fold higher levels of 3-PGA for 50% activation. Although the homotetramer S^{WT} enzyme had only about 20-25% catalytic activity of the two LS missense mutant enzymes, it exhibited 9-fold more resistance to Pi inhibition, which likely compensates for its lower catalytic activity in accumulating starch levels similar to those expressing the LS missense mutants. Overall, these results indicate that the LS is essential for optimal catalysis and allosteric regulation of the heterotetrameric AGPase. These results were published [1].

Redox-regulation of the rice endosperm AGPase (). In addition to control by the allosteric effectors, 3-PGA and Pi, the leaf stem and tuber AGPases are also regulated by redox potential. Redox control is mediated via the interchain disulfide bond formed between the conserved cysteines (Cys^{12}) located at the N-terminal region of the two SSs in the heterotetrameric enzyme. The rice endosperm SS lacks this conserved cysteine but, nevertheless, the recombinant enzyme shows greater than 3-fold more affinity to the activator 3-PGA in the presence of the reducing agent DTT. In addition, the reduced enzyme possesses higher catalytic activity (~ 3-fold) at 3-PGA concentration equal to its $A_{0.5}$ value. At higher 3-PGA concentration (10 mM), however, the reduced and non-reduced enzymes display equal catalytic activities indicating that the redox regulation is only important at low activator levels, a physiologically relevant condition in storage tissues. Analysis of the LS primary sequence revealed the presence of several Cys residues located at the N-terminal region. To determine the roles of N-terminal located Cys of the large subunit in redox control, several mutants were generated and characterized. Biochemical analysis showed that truncation of the LS N-terminal by removal of 54 amino acids results in loss of the redox control and that Cys^{47} and Cys^{58} residues are essential for proper redox response of the enzyme. These results demonstrate that the rice endosperm cytosolic AGPase, the dominant AGPase activity in developing seeds, is subjected to redox control and this regulation occurs through the LS. These results were published [2].

Improving starch yield in cereals by over-expression of ADP-glucose pyrophosphorylase: Expectations and unanticipated outcomes). To compare the effects of AGPase expression in transgenic cereal plants we assembled the experimental data from the literature. Expression of up-regulated AGPase enzymes under the control of endosperm-specific promoters were expected to

increase starch production and, in turn, grain size. Current evidence indicates that AGPase transgenes, under control of putative endosperm-specific promoters, the maize *shr2* LS or rice glutelin, mediated an unexpected increase in seed number and not grain weight. The increase in seed number indicates that these endosperm promoters are also active in meristematic tissue, which gives rise to floral organs, and/or during early fertilization, which may abrogate seed abortion. To assess the spatial and temporal expression of AGPase LS sequences during rice development, the maize *shr2* and the orthologous rice promoter sequences were fused with a GUS reporter gene and transformed into rice. A detailed study will illuminate the spatial expression patterns of these promoters, which may provide new strategies in manipulating starch metabolism for increasing plant yields. A manuscript describing these results were published [3].

Objective 2. Structural studies on the potato AGPase. Despite efforts over the last nine years, the native 3-D structure of a higher plant AGPase has not been determined due to the inability to obtain fine crystals of the AGPase heterotetramer. Since addition of 2 mM ATP to purified AGPase favors a structure formation by decreasing entropy, crystallization trials were performed in different buffer condition containing ATP. In the presence of 2 mM ATP and 5 mM Mg^{2+}, fine crystals were obtained by Dr. ChulHee Kang, Director of the Biomolecular X-Ray Crystallography Center (BXRC) at WSU and a collaborator on this study. Unfortunately, the crystals had extremely low resolution diffraction to be useful.

As the attainment of AGPase 3-D structure has not been achieved despite considerable effort, other physical methods to evaluate the structure-function of this enzyme were pursued. One major question that needs to be resolved is the LS's ATP binding activity. This question was addressed by analysis of AGPase wildtype and variant forms using Isothermal Titration Calorimetry (ITC). The wildtype AGPase exhibited a biphasic response suggesting a high affinity value of 1.2 µM and 34 µM. The soluble homotetramer LS enzyme binds ATP at a K_d of 47 µM, suggesting that it is responsible for the higher K_d of the wildtype enzyme. Results from ITC analysis of AGPase was published [4].

Molecular modeling and homology approaches to define the potato AGPase large subunit active site. The potato LS and SS play unequal roles in catalysis with the SS being the dominant subunit and the LS contributing little to product formation likely due to mutations at its active site. This latter view is supported by the introduction of two amino acid replacements, $K^{41}R$ and $T^{51}K$, in the LS, which partially restores catalytic activity of AGPase ($L^{RK}S^{Si}$) containing a catalytically-silenced SS (S^{Si}) [5]. To identify other target LS residues, we used both structural and sequence homology alignment information. By modeling the LS sequence based on the ADPglc-bound crystal structure of SS homotetramer (PDB id: 1YP4), residues within 10 Å distance of the putative ADPglc binding pocket were identified. These residues were then compared in the LS and SS sequences from five different species as well as three additional LS sequences which were previously shown to have catalytic activity [6]. Using the distance from the bound ADPglc molecule in the SS structure and side-chain biochemical properties as well as conservation among different subunits, three groups of residues were identified as potential targets that would restore LS catalytic activity. 1) Within 5 Å: the SS homolog of Ser^{155} in LS is an Ala, a residue partially conserved in active LSs. The hydroxyl group of Ser^{155} of LS is modeled to be in close contact with the hydroxyl group of adenosine. Thus, this steric hindrance may adversely affect the correct orientation of substrate in the active site. 2) Within 7.5 Å: the SS homolog of Asp^{192} in LS is an Ala, which is partially conserved in active LSs. The side chain of Asp^{192} is modeled to lie close to the phosphate groups of ADPglc and might affect its binding in the active site. 3) Within 10 Å: the SS homolog of Lys^{135} of LS is a Gln, which is fully conserved among active LSs.

Addition of $S^{155}A$ mutation to L^{RK} mutant generated the triple mutant $L^{RKS135A}$ which when assembled with S^{Si} resulted in an enzyme ($L^{RKA}S^{Si}$) exhibiting twice the catalytic activity of the $L^{RK}S^{Si}$ mutant with no significant changes in 3-PGA and ATP affinities (specific activities for $L^{WT}S^{WT}$, $L^{RK}S^{Si}$ and $L^{RKA}S^{Si}$ are 49.07, 4.63 and 9.20 µmoles/min/mg respectively). The addition of a $K^{135}Q$ mutation ($L^{RKA+K135Q}S^{Si}$) further improved the activity as viewed by the enhanced accumulation of glycogen in *E. coli* cells (located below $S^{WT}L^{WT}$ in Figure 1).

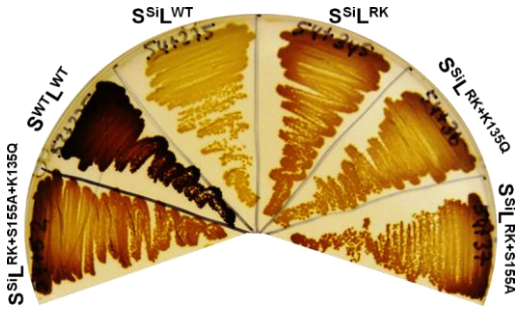

Figure 1. Iodine staining of glycogen accumulation in *E. coli* cells expressing the recombinant WT ($L^{WT}S^{WT}$) and various mutant forms. Note that cells expressing the catalytically-silenced $L^{WT}S^{Si}$ accumulates little if any glycogen, whereas those containing the $L^{RK+S144A+K135Q}S^{Si}$ accumulate significantly more levels of glycogen.

Objective 3 - Introduce Up-regulatory AGPase subunits to manipulate carbon flux in Poplar (*Populus trichocarpa* Nisqually-1)

We had earlier demonstrated a direct relationship between photosynthetic capacity, leaf starch accumulation/turnover, and plant productivity in the model plants, *Arabidopsis* and rice [7]. These successful studies were extended to the bioenergy feedstock plant poplar. DNAs encoding up-regulatory forms of the potato AGPase LS (UpReg1)[8] and SS (devo330, [9]), respectively, were transformed into poplar hybrid plant (*P. tremula* × *P. alba*) and expressed under the control of the Rubisco small subunit promoter. Qualitative measurement of starch content in transgenic poplar leaves by iodine staining, however, showed no obvious increases in starch content, suggesting a poor contribution of the transgenes to starch biosynthesis. This was verified by AGPase enzyme activity measurements where the transgenic lines showed no apparent increases in enzyme activity, indicating that the transgene was not effectively functioning in poplar leaves. Co-expression in *E. coli* of the cloned cDNAs encoding poplar's 5 LSs and one SS with the up-regulatory potato SS and LS transgenes, respectively, indicated poor expression and/or subunit compatibility as viewed by the extent of glycogen accumulation.

Objective 4 - Determine the role of the unique L80 peptide of Pho1 in starch synthesis

The higher plant Pho1 is structurally similar to the yeast and animal enzyme forms except that it contains an extra 80 residue peptide (L80), rich in acidic and basic amino acids, located near the middle of the primary sequence. The potential role of the unique L80 peptide region as an enzyme regulator was directly addressed by comparing the kinetics of the intact wildtype enzyme to the L80-deleted enzyme Pho1ΔL80. A variety of metabolic effectors such as ADPglc, ATP, AMP, etc., were tested to see if Pho1 and Pho1ΔL80 respond differently to the effectors. No significant differences were observed between Pho1 and Pho1ΔL80, suggesting that the L80 does not serve as a regulatory site by these effectors. However, Pho1ΔL80 was more susceptible to inactivation at 45°C than Pho1, indicating that the L80 region contributes to enzyme stability. Pho1 and Pho1ΔL80 showed no significant differences in substrate affinities toward Glc1-P, amylopectin, and maltohexaose. Interestingly, substrate affinities toward Glc1-P and amylopectin for both enzymes were increased by 4- to 7-fold by lowering the reaction temperature from 36ºC to 18ºC. This result indicates that the Pho1 enzyme, regardless of the presence of L80, undergoes temperature–dependent conformational changes which modify substrate accessibility at the active site.

Our previous studies of a *pho1* mutant (BMF136) indicated that Pho1 is essential for starch synthesis at low temperature in rice [10]. Purified recombinant Pho1 was used to measure enzyme

activity at varied temperatures. Interestingly, the temperature coefficient (Q10) for Pho1 was very low when measured between 10°C to 40°C (Q10 for Pho1 = 1.08; Q10 for Pho1ΔL80 = 1.10), indicating the reaction rate of rice starch phosphorylase is not significantly affected by changes in temperature. Thus, it can be postulated that the catalytic rate of Pho1 enzyme *in planta* is largely temperature independent and functions at its near maximum rate even at low temperature. The kinetic properties of Pho1 at low temperature were published [11].

To resolve the X-ray crystal structure of starch phosphorylase, proteins were purified to near homogeneity. Diverse crystallization conditions were conducted by Dr. Kang's laboratory but high-quality crystals have yet to be obtained thus far.

In order to locate an ADPglc effector binding site in rice Pho1, Ala^{314} and Arg^{315}, homologous residues located at the AMP binding site of the rabbit glycogen phosphorylase *b*, were replaced by Arg and Ala, respectively. A314R substitution had no significant effect on enzyme activity while $R^{315}A$ had only 5% of normal activity. The sensitivity of the mutant enzyme to ADPglc, however, was similar to the wildtype enzyme. These results indicate that Arg^{315} is important for catalytic activity of Pho1 but is not involved in binding of the inhibitor ADPglc.

To understand the physiological role of the L80 region found in Pho1, cDNA sequences encoding Pho1 and Pho1ΔL80 were transformed into BMF136 rice line (*pho1* null mutant). Immunoblot analysis identified 17 transgenic lines for Pho1 and 6 lines for Pho1ΔL80. The transgenic plants will be tested at low and ambient temperatures for their starch synthesis and other phenotypes, studies that we propose to carryout in the next granting period.

Objective 5: Elucidate the control of Pho1 activity by post-translational modifications and by interacting proteins.

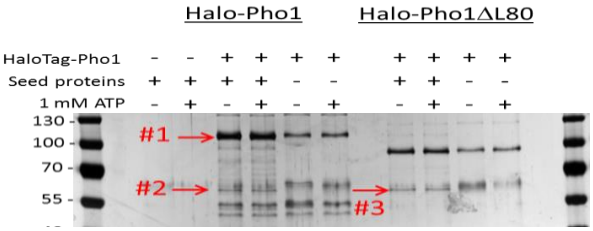

Figure 3. Pull-down experiment for identification of Pho1 interacting protein(s) in rice seeds. LC-MS/MS results showed that #1 band is Pho1 while #2 and #3 bands are α-1,4-glucantransferases (Dpe1). Presence of 1 mM ATP did not produce significant differences in elution profiles.

Genetic [10] and biochemical [12] studies indicate that Pho1 functions in association with other proteins likely in a multi-protein complex. In order to identify protein(s) that interacts with Pho1 in rice, we performed a pull-down experiment using Halo-tagged Pho1 and Halo-tagged Pho1ΔL80 and detected a 62 kD protein in developing rice seeds that interacted with either protein (Figure 3). LC-MS/MS analysis revealed that the interacting protein is α-1,4-glucantransferase, also known as disproportionating protein (D-enzyme or Dpe1). Gel permeation chromatography and native PAGE analyses showed that recombinant Dpe1 forms a homodimer and physically interacts with dimerized Pho1 at 1:1 molar ratio to form an oligomeric complex. FACE analysis showed that rice Dpe1 catalyzes cleavage and transfer of a maltose or large moiety from one oligosaccharide to another (e.g. maltotriose + maltotriose <=> maltopentaose + glucose). Size exclusion chromatography of a developing seed protein extract followed by immunoblot analysis showed that the majority of the Dpe1 and Pho1 co-eluted at about 600- to 700 kD. FACE analysis of reaction products generated with maltopentaose and Glc 1-P showed that the fractions containing Pho1:Dpe1 multi-complex preferentially synthesized longer maltodextrin chains than Pho1 alone.

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