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Award Addresses

Biosynthesis of Starch : ADPGlucose Pyrophosphorylase, the Regulatory Enzyme of Starch Synthesis : Structure-Function Relationships

Jack PREISS

*Department of Biochemistry, Michigan State University
(East Lansing, Michigan 48824-1319, U. S. A.)*

The synthesis of starch is believed to involve 3 enzymes, ADPGlucose pyrophosphorylase (ADPGlc PPase, [EC 2.7.7.27] ; reaction 1), starch synthase, [EC 2.4.1.21] ; reaction 2) and branching enzyme ([EC 2.4.1.18] ; reaction 3).

There are many genetic studies to indicate that all three reactions contribute to the synthesis of both starch components, amylose and amylopectin. Maize amylose extender (*ae*) mutants, containing more amylose and less amylopectin than normal maize have been shown to be defective in one of the branching enzyme isoforms (*Plant Physiol.*, **67**, 1141-1145 (1981)). Maize waxy (*wx*) mutants which contain no amylose are deficient in the starch granule bound starch synthase (*Plant Physiol.*, **62**, 383-386 (1978)) indicating that the granule bound starch synthase is involved in amylose synthesis. Starch deficient mutants of pea (*Plant Physiol.*, **89**, 1279-1284 (1989)), maize (*Science*, **151**, 343-348 (1966)) and *Arabidopsis* (*Plant Physiol.*, **86**, 1131-1135 (1988)) are deficient in ADPGlc PPase activity. Thus, genetic and biochemical evidence in plants strongly indicate that ADPGlucose (ADPGlc) is synthesized predominantly, if not solely, via ADPGlc PPase catalysis.

The cyanobacterial, algal and higher plant enzymes are activated by 3-P-glycerate (3PGA) and inhibited by orthophosphate. An algal mutant in which the regulatory sites of the ADPGlc PPase have been affected (*Planta*, **185**, 17-26 (1991)) and control analysis (Kacsar-Burns) of the plant ADPGlc PPase, indicate that the allosteric regulation observed of the enzyme in plants reflects the *in vivo* regulation of plant starch synthesis. Thus, the above studies emphasize the importance of the ADPGlc PPase in starch synthesis and the need for understanding the structure-function relationships of the regulatory and catalytic domains of the ADPGlc PPase. A number of chemical modification and site-directed mutageneses studies have identified the active and substrate binding sites both in the spinach leaf and bacterial enzymes. There is considerable conservation between the bacterial and higher plant enzymes particularly at the substrate binding sites. High conservation is also observed for the 3PGA activator sites of the cyanobacterial and higher plant ADPGlc PPases. These results suggest that the two subunits of the higher plant ADPGlc PPase evolved from the bacterial enzyme subunit. Transformation of the plant systems with the bacterial enzyme gene results in an appreciable increase in starch synthesis ranging from 1.25- to 1.6-fold in potato tubers to 10-fold in tobacco calli. These results confirm that the ADPGlc PPase activity in plants is rate-limiting for starch synthesis and that the allosteric regulation is functional in the plant.

It is a great pleasure for me to be asked to give this lecture in view of the many former eminent scientists previously asked to address this honored society. Many of those awardees

which include, Drs. D. J. Manners, S. Hizukuri, Y. Takeda, H. Fuwa, D. French, W. J. Whelan, Z. Nikuni, K. Kainuma and R. L. Whistler, were involved in the study of starch, either its

chemical properties, its structure or the enzymes involved in its degradation to which they have made outstanding contributions. It is also a great privilege for me to be honored in the same way as they have. Since my research efforts have been with the processes involved in the synthesis of starch, my address will be concerned with a discussion of the various enzymes involved, the genetic evidence that supports their function in starch synthesis, the regulation of starch synthesis, via allosteric effects on ADPglucose pyrophosphorylase and the structure-function relationships of the regulatory sites. Finally, I will discuss how transformation of plants, with an ADPglucose pyrophosphorylase gene, will increase their starch levels.

THE BIOSYNTHETIC REACTIONS OF STARCH SYNTHESIS

The sugar nucleotide donor of starch synthesis is adenosine diphosphate glucose (ADPGlc). The pathway leading to synthesis of starch consists of three enzymatic reactions, ADPGlc pyrophosphorylase ([EC 2.7.7.27]; reaction 1), starch synthase ([EC 2.4.1.21]; reaction 2) and branching enzyme ([EC 2.4.1.18]; reaction 3). Biochemical, genetic and molecular biology data indicate that synthesis of ADPGlc via ADPGlc pyrophosphorylase (ADPGlc PPase) is the predominant if not the sole route towards starch synthesis in leaf as well as in nonphotosynthetic tissues such as endosperm or tubers.

The data supporting this view are from studies of isolated mutants deficient in starch levels from maize endosperm,^{1,2)} developing pea embryos³⁾ and from *Arabidopsis thaliana*^{4,5)} that also have deficient levels of ADPGlc PPase. One *Arabidopsis* mutant, containing less than 2% of the leaf starch content seen in the normal strain has less than 2% of the normal leaf ADPGlc PPase activity. Other evidence is from application of anti-sense RNA to the

gene of the small subunit of the potato tubers ADPGlc PPase to potato tubers. This resulted in the production of tubers having only 3% of the starch observed in the normal or untreated tuber.⁶⁾ Thus, results from four diverse species of plants strongly indicate that the ADPGlc PPase pathway must play a major role in starch synthesis. Other genetic and molecular biology data support *in vivo* functions for the activity of the above starch biosynthetic enzymes, starch synthase and branching enzyme, in various plants and have been previously reviewed.⁷⁻¹⁰⁾ The maize waxy (*wx*) mutation¹¹⁾ is defective in activity of one of the starch synthase isoforms, the starch granule-bound starch synthase, which results in a starch granule devoid of amylose. This strongly suggests that the granule bound starch synthase participates in the synthesis of amylose. Another maize endosperm mutant, is the amylose extender (*ae*), mutant and it has been shown to be defective in branching isoenzyme II activity.¹²⁾ The mutation results in a starch granule having a greater percentage of amylose and in addition to the normal amylopectin fraction another amylopectin fraction with a lesser number of branch points and longer exterior chain lengths.¹³⁾ Thus, genetic evidence also supports the role for the branching enzyme activity in the *in vivo* synthesis of the α -1,6 branch linkages of amylopectin. It is anticipated that in the future, upon isolation of the genes of the branching enzyme and starch synthase isoforms, that anti-sense RNA-type experiments will be performed to determine the direct effects of loss of isoenzyme activity on starch structure and in possible practical applications these experiments would be used to form starch structures more useful for industrial application.

REGULATION OF THE ADPGlc PPASE PATHWAY

The *in vitro* evidence suggests that ADPGlc synthesis is regulated by activation of the plant ADPGlc PPase by 3-phosphoglycerate (3PGA) and inhibition by inorganic phosphate (P_i). This is consistent with the *in vivo* or *in situ* evidence showing a correlation between

the concentrations 3PGA and starch and inverse correlations between P_i and starch levels (reviewed in 7 and 8).

Lately, much *in vivo* data pertaining to regulation of starch synthesis and the involvement of ADPGlc PPase has been subject to control analysis as developed by Kacser and Burns.^{14,15} Pettersson and Pettersson¹⁶ have developed a kinetic model to determine the extent that stromal metabolites, known to affect *in vitro* leaf ADPGlc PPase activity and the rate of photosynthetic starch production under conditions of light and CO_2 saturation. The model essentially agrees with reported experimental data with respect to metabolite concentrations and to CO_2 fixation and starch synthetic rates. It showed that ATP and glucose-1-P, substrates of the ADPGlc PPase, 3PGA, fructose-6-P, and P_i provided significant contributions to starch synthetic rate changes induced by increasing P_i concentrations external to the chloroplast. At low P_i concentrations, the most significant contribution to increased starch synthetic rate was ATP and to a smaller extent 3PGA. At P_i concentrations higher than 0.12 mM, 3PGA becomes the predominant regulator of starch synthesis with glucose-1-P and fructose-6-P contributing to regulation to a smaller extent. They reached the conclusion that 3PGA and P_i play an important role in regulating starch synthesis with contributions made by ATP, glucose-1-P and fructose-6-P. Since these metabolites are either substrates or effectors of the ADPGlc PPase, the analysis is consistent with the view that the 3PGA/ P_i ratio regulates starch synthesis by regulation of ADPGlc PPase.

The availability of a low activity (7% of wild type) ADPGlc PPase mutant of *Arabidopsis thaliana*,^{5,17} has allowed the analysis of the extent of control that this enzyme exerts on chloroplast starch synthesis. In low light, a 50% and 93% decrease of ADPGlc PPase activity resulted in a 23% and 74% decrease in flux of starch synthesis with a concomitant increase of 17% and 42% increase in sucrose synthetic rate. Thus diminution of ADPGlc synthesizing activity not only significantly affected starch synthesis but also affected the partitioning of photosynthetic carbon causing more to be directed to-

wards sucrose biosynthesis. In high light, reduction of the ADPGlc synthesizing activity to 50% and 93% resulted in a 39% and 90% decrease in starch synthesis flux. The flux control coefficient¹⁷ for the ADPGlc PPase for starch synthesis was calculated to determine its magnitude of control and compared with previous results obtained with the *Clarkia xantiana* phosphoglucoisomerase and *Arabidopsis thaliana* P-glucomutase. Of the enzymes under study, the flux to starch synthesis is only regulated by ADPGlc PPase in low light and in high light and CO_2 , ADPGlc PPase activity exerts major control. Other enzymes such as plastid phosphoglucomutase and phosphoglucoisomerase exert a lesser control.¹⁷

Of interest are the control analyses using the cytosolic mutants of phosphoglucoseisomerase of *Clarkia xantiana* P-glucoseisomerase.¹⁸ Lowering the cytosolic enzyme activity had little effect on either starch or sucrose synthesis in saturating light but did increase starch synthetic rate in low light and decreased sucrose synthetic rate. Partitioning of carbon between sucrose and starch was thus affected by variation of the cytosolic phosphoglucoisomerase levels. Metabolite levels were also affected in these mutants. In the mutant containing only 18% of the wildtype cytosolic phosphoglucoisomerase activity both fructose-2,6-bis-phosphate and 3PGA levels increase about 100%. Neuhaus *et al.*¹⁸ indicated that reduced sucrose synthesis rate was due to the increased Fru-2-6-P₂ level which caused increased inhibition of cytosolic fructose-1,6-bisphosphatase which is on the pathway towards sucrose synthesis. The data also strongly support the view that increased starch synthesis in the mutants with reduced levels of phosphoglucoisomerase is due to activation of the ADPGlc PPase by the increased 3PGA concentration and 3PGA/ P_i ratio.

Of most important significance are the findings of Ball *et al.*¹⁹ showing that a starch deficient *Chlamydomonas reinhardtii* contained an ADPGlc PPase that could not be activated by 3PGA. The mutant was starch deficient whether it was grown in the light, autotrophically, or in the dark with acetate as the sole carbon source.¹⁹

In summary, various analyses of the starch

biosynthetic system of a number of plants utilizing *in vivo* data obtained from different plants and applying the Kacser and Burns control analysis method^{14,15)} as well as mutant data,¹⁹⁾ point out that the major site of regulation of starch synthesis is at ADPGlc PPase and that 3PGA and P_i are important regulatory metabolites of that enzyme.

STRUCTURAL AND REGULATORY PROPERTIES OF ADPGlc PPase

The properties of the plant and bacterial ADPGlc PPases have been extensively reviewed and for detailed information on the plant systems the reader is referred to references.^{7-9,20)} For the bacterial systems the reader is referred to references.^{21,22)} Almost every plant ADPGlc PPase studied has as its major activator, 3PGA and inhibitor, P_i , whether the leaf source is from a plant utilizing C_3 or C_4 pathways or Crassulacean metabolism. In non-photosynthetic systems studied (*e.g.*, maize endosperm, potato tuber) the ADPGlc PPase activity is highly dependent on the presence of 3PGA and is inhibited by P_i . Activation of ADPGlc PPase of carrot roots, avocado mesocarp,²³⁾ of cassava tubers²⁴⁾ and of lilly pollen²⁵⁾ by 3PGA have been reported.

A number of ADPGlc PPases have been studied with respect to their structural properties. The spinach leaf enzyme,²⁶⁻²⁹⁾ has a molecular mass of 206,000 and is composed of two different subunits, molecular masses of 51 and 54 kD. These subunits are distinguished not only by differences in their molecular mass but also by different amino acid compositions, aminoterminal sequences, peptide patterns on HPLC of tryptic digests and antigenic properties. Thus, the two subunits are distinct and probably are the products of two different genes. In contrast, the bacterial ADPGlc PPase is composed of only one subunit, 50 to 55 kD in mass, depending upon the source and the native enzymes are homotetrameric.²¹⁾

Other plant enzymes have been shown to be composed of two dissimilar subunits. The maize endosperm ADPGlc PPase, which has a molecular mass of 230,000, is composed of 2 subunits, 55 and 60 kD.³⁰⁾ Analyses of the maize

endosperm mutants, *shrunken 2* and *brittle 2* via Western blotting experiments with native and subunit antibodies of the spinach leaf enzyme, show that the mutant *bt 2* endosperm lacks the 55 kD subunit and the mutant *sh 2* endosperm lacks the 60 kD subunit.³⁰⁾ These results strongly suggest that the maize endosperm ADPGlc PPase is composed of two immunologically distinctive subunits and that the *sh 2* and *bt 2* mutations cause reduction in ADPGlc PPase activity through the lack of one of the subunits. Consistent with this is the isolation of an ADPGlc PPase cDNA clone from a maize endosperm library^{30,31)} and showing that it hybridized with the small subunit cDNA clone from rice.³¹⁾ This maize ADPGlc PPase cDNA clone is found to hybridize to a transcript present in maize endosperm but absent in *bt 2* endosperm. Thus, the *bt 2* mutant appears to be the structural gene of the 55 kD subunit of the maize ADPGlc PPase. Most important is that the above data certainly point out that the non-photosynthetic tissue ADPGlc PPase is also composed of two subunits and on the basis of immunoreactivity there is corresponding homology between the subunits in the leaf enzyme with the subunits of a reserve tissue enzyme.

The potato tuber ADPGlc PPase has been highly purified and by 2-dimensional polyacrylamide gel electrophoresis, two different peptides could be distinguished by their slight differences in molecular mass, 50 and 51 kD and in net charge.^{32,33)} Thus, as found with the spinach leaf and maize endosperm enzyme, the potato tuber enzyme is composed of two distinct subunits and not one as previously thought.^{32,33)} It appears that other plant ADPGlc PPases may also be composed of 2 subunits on the basis of Western blotting experiments. Using anti-sera towards native spinach leaf ADPGlc PPase the presence of two immunoreactive bands in gel electrophoresis of extracts of wheat, rice and maize leaves was observed.³⁴⁾ Recently, characterization of the purified barley endosperm ADPGlc PPase showed it also was composed of two distinct subunits of 51 and 60 kD.³⁵⁾ Similar data were obtained with *Arabidopsis thaliana* ADPGlc PPase.^{4,36)} It is composed of two subunits, molecular masses of 48 and 54

kD. One of the *Arabidopsis thaliana* ADPGlc PPase mutants, TL46, lacks the larger, 54 kD subunit³⁶⁾ and has been partially purified. Its kinetic properties have been studied and was shown to be activated by 3PGA and inhibited by P_i as the normal enzyme. However, there was about a 29-fold lower affinity for 3PGA and a 4 to 5-fold higher affinity for P_i for the larger subunit deficient mutant enzyme. Moreover, in the presence of saturating concentrations of 3PGA, the mutant enzyme had K_m values for ATP, glucose-1-P and Mg^{2+} , 6-, 5-, and 2-fold higher, respectively, than those of the normal enzyme. Thus, changes in the kinetics of the mutant enzyme may be due to the absence of the large subunit. This mutation provides further evidence that the larger subunit is a necessary component of the native ADPGlc PPase for optimal activity as the mutant enzyme has only 7% of the wild-type activity. The mutant synthesizes starch at 9% the rate seen for the normal strain in high light and only at 26% of the rate at low light.¹⁷⁾

It is of interest to know why two subunits are required for optimal catalytic activity of the higher plant ADPGlc PPase. The bacterial ADPGlc PPase from at least 7 organisms contains only one subunit and the native structure is homotetrameric.^{20, 21)}

THE ALLOSTERIC 3PGA ACTIVATOR BINDING SITE OF ADPGlc PPASE : DETERMINATION OF THE AMINO ACID SEQUENCES AT THE 3PGA ALLOSTERIC BINDING SITE

Studies were designed to determine the nature and location of the 3PGA binding site. These studies were facilitated by the finding that pyridoxal-5-phosphate was an activator of spinach leaf ADPGlc PPase.^{26-28, 37)} Pyridoxal-P is not as effective as 3PGA with regard to activation of velocity; only a 6-fold stimulation is observed compared to a 25-fold stimulation caused by 3PGA. Three other ADPGlc PPases, (maize endosperm and the cyanobacteria, *Synechocystis* 6803 and *Anabaena* PCC 7120) are also stimulated by pyridoxal-5-P.^{38, 39)} The apparent affinity, or $A_{0.5}$ value (concentration of

activator giving 50% of maximal stimulation), for the spinach leaf enzyme for pyridoxal-5-P is about 15 μM and is somewhat lower than the $A_{0.5}$ of 45 μM for 3PGA. Thus, there is a higher apparent affinity for pyridoxal-5-P even though there is a lesser stimulation of maximal velocity. Pyridoxal-5-P can be covalently bound to the spinach leaf ADPGlc PPase by reduction with $NaBH_4$ ^{26-28, 37)} producing a modified enzyme that is highly active in the absence of activator. The activator, 3PGA, and the inhibitor, P_i , prevents the reductive covalent binding of the pyridoxal-5-P and reduces the increase in unactivated activity caused by the reductive phosphopyridoxylation.^{26-28, 37)} The modified enzyme is also quite insensitive to P_i inhibition.^{27, 27, 37)} All the above results are consistent with the view that pyridoxal-5-P binds to the activator site and that the covalent modification with pyridoxal-5-P places the enzyme in a conformational state having higher catalytic activity and where it is resistant to P_i inhibition.

Incorporation of [3H]-pyridoxal-5-P into the spinach leaf enzyme ADPGlc PPase has been observed and there is a relationship between the amount of pyridoxal-5-P incorporated and the increase in activity caused by the modification.^{26, 37)} Many metabolites and substrates were tested and only P_i and 3PGA inhibited incorporation of the labeled pyridoxal-5-P into the enzyme. Labeled pyridoxal-5-P is incorporated about equally into both the 54 and 51 kD subunits^{37, 40, 41)} and this is shown in Table 1. The activator, 3PGA, prevented incorporation of the pyridoxal-5-P to the same extent into both subunits,³⁷⁾ (Table 1). Both subunits were separated as described³⁷⁾ and subjected to trypsin digestion. The labeled peptides isolated using reverse-phase HPLC and sequenced.^{27, 37)} Only one sequence was obtained from the 51 kD subunit and is SGIVTVIKDAL,³⁷⁾ (Table 1) with Lys covalently linked to the pyridoxal-P. The amino acid sequence found is different to that corresponding to the fructose 1,6-bis P activator site in the *E. coli* ADPGlc PPase.⁴²⁾ There are many more basic amino acid residues in the *E. coli* sequence than in the spinach leaf 51 kD activator sequence.⁴²⁾ Study of the amino acid sequence of the spinach leaf 54 kD subunit

Table 1. Protection by 3-P-glycerate and by P_i of reductive phosphopyridoxylation of spinach leaf ADPGlc PPase large and small subunits

A. Large Subunit			
Condition	Peptide 2	Peptide 1	Peptide 3
	K*WFQG	IKDAII	SGITVIFK*
	TADAVR	DK*NAR	NATIKDGVV
	nmol of [3H]-P-pyridoxyl-peptide/nmol of 54 kD peptide		
No Effector	0.33	0.25	0.45
+3-P-glycerate	0.07	0.045	0.095
+ P_i	0.28	0.006	0.41
B. Small Subunit			
	DWFQG	IKRAIID	SGIVTVIK*D
	TADAVR	KNAR	ALIPSGTVI
	nmol of [3H]-P-pyridoxyl-peptide/nmol of 51 kD peptide		
No Effector	0.00	0.00	1.3
+3-P-glycerate	—	—	0.2
+ P_i	—	—	0.25

The reductive phosphopyridoxylation was done as described.³⁷⁾ The concentrations of P_i and 3-P-glycerate used for protection of the ADPGlc PPase from reductive phosphopyridoxylation were 1 mM. The peptides containing the labeled pyridoxal-P were obtained by tryptic digestion and then via separation by HPLC (K. Ball and J. Preiss, unpublished results). Sequence analysis was done by automated Edman degradation with an Applied Biosystems 477A Protein sequencer.

activator sites shows labeling of 3 radioactive peptides from tryptic digests (Table 1). The amino acid sequences surrounding these labeled pyridoxylated Lys residues of isolated tryptic peptides are seen in Table 1. The 54 kD tryptic peptide 3 has a high similarity to the 51 kD activator site I and thus because of sequence similarity must be at the C-terminus. Comparisons of sequences suggest peptide 1 is situated near peptide 3 about 20 to 30 amino acids farther away from the C-terminal. Peptide 2 aligns with a 51 kD amino acid sequence that contains a Trp residue which is about 115 amino acids away from the N-terminal. Thus, the third Lys binding residue is closer to the N-terminal. All four lysine residues are protected about 85% from reductive pyridoxylation by the activator, 3PGA and thus, they must interact with the activator at the allosteric binding domain. In contrast, only site I of the 51 kD peptide and site II (peptide 1) of the 54 kD peptide are effectively protected by P_i (81-to 98%) from the reductive phosphopyridoxylation. It is quite possible that the inhibitor binding site overlaps with the

activator sites of Site I and II. The overall protection of the small subunit by P_i is 81% while that of the three sites of the large subunit is only 32%.

The chemical modification studies show that both subunits participate in the binding of the activator, Pyridoxal-P and that 4 lysine residues, three in the large subunit and one in the small subunit are either part of or close to the binding domain. Since 3-P-glycerate protects all 4 lysine residues from the chemical modification, these lysine residues are also involved in the binding of the physiological activator, 3-P-glycerate. Only two of the lysine residues are protected from the modification by the inhibitor, P_i . These residues may participate in the binding of the inhibitor or these lysine residues may overlap with the inhibitor site.

There is great identity in the spinach leaf ADPGlc PPase 51 kD activator site sequence with that observed for the cyanobacterium, *Anabaena* PCC 7120 and *Synechocystis* PCC 6803 ADPGlc PPase sequences at the C-terminal.^{43,44)} Since the *Anabaena* enzyme has been cloned and expressed in *E. coli*, the

Table 2. Plant ADPGlucose pyrophosphorylase activator binding sites

Site I	
	439
Spinach 51 kD	SGIVTVIKDALIPSGTVI
Potato 50 kD	SGIVTVIKDALIPSGIII
Maize 54 kD	GGIVTVIKDALLPSGTVI
<i>Anabaena</i>	SGIVVVLKNAVITDGTII
<i>Synechocystis</i>	NGIVVVIKNVTIADGTVI
Spinach 54 kD	SGITVIFKNATIKDGVV
Potato 51 kD	SGIIHLEKATIRDGTVI
Maize 60 kD	SGIVVILKNATINECLVI
Site II (P _i Binding site?)	
Spinach leaf 51 kD/Potato-50 kD	IKRAIIDKNAR
Maize 54 kD	IRRAIIDKNAR
<i>Anabaena</i>	QRRRAIIDKNAR
<i>Synechocystis</i>	IRRAIIDKNAR
Spinach 54 kD	IKDAIIDKNAR
Potato 51 kD	IRKCIIDKNAK
Maize 60 kD	IRNCIIDMNAR
Site III	
Spinach leaf 51 kD/Potato-50 kD	DWFQGTADAVRQ
Maize 54 kD	NWFQGTADAVRQ
<i>Anabaena</i>	NWFQGTADAVRQ
<i>Synechocystis</i>	DWFQGTADAVRQ
Spinach leaf 54 kD	KWFQGTADAVRQ
Potato 51 kD	KWFQGTADAVRK
Maize 60 kD	GWFOGTODSIRK

The sequences are listed in one letter code and are obtained from Ref. 45. The Lys residues that are in outline indicate that they are covalently modified pyridoxal-P and the chemical modification of the Lys residue is protected by 3-phosphoglycerate. The chemical modification studies are described in.^{37,41)} The number 439 corresponds to the Lys residue in the spinach leaf ADPGlc PPase.

analogous Lys residue (residue 419) in the *Anabaena* enzyme has been subjected to site-directed mutagenesis and a glutamate residue has been substituted. Preliminary results show that the K419E mutant has a greatly reduced affinity (ca 40- to 80-fold) for the activator, 3PGA (Y. Y. Charng and J. Preiss, unpublished results). This provides more evidence for the Lys residue near the C-terminal participating in the binding of the activator. This sequence around Lys 439 of the spinach leaf 51 kD subunit is highly conserved in many of the plant ADPGlc PPase small subunits.⁴⁵⁾ Likewise, as seen in Table 2, the

sequence around the Lys residue of Site I in the large subunit is highly conserved.⁴⁵⁾ In contrast, Table 2 shows that the pyridoxylated Lys of Sites II and III of the spinach leaf ADPGlc PPase large subunit are not as conserved and so far Site III Lys is seen only in the potato tuber ADPGlc PPase sequences.⁴⁵⁾

Further studies including site-directed mutageneses are therefore needed to characterize these various sites that appear to be involved in binding the activator, 3PGA as well as the inhibitor, P_i (Site I of small subunit and Site II of the large subunit).

It would be of interest to know if the

Table 3. Comparison of the ATP and α -glucose-1-phosphate binding sites in *E. coli* with sequences in the cyanobacterial and higher plant ADPGlc pyrophosphorylases.

	112 (ATP site)	189 (Glc-1-P site)
<i>E. coli</i>	NWYRGTADAVTQN	IIEFVEKP
<i>Anabaena</i> (96)	FQ R Y (172)	V D S
<i>Synechocystis</i> (95)	D FQ R Y (172)	T D S
Spinach-51 kD (120)	D FQ R Y (187)	A
Spinach-54 kD	S FQ R F	V L S S
Potato tuber 50 kD	D FQ R Y	A
Potato tuber 51 kD	K FQ R K F	V V Q A
Maize endosperm 55 kD	FQ R Y	A
Maize endosperm 60 kD	G FQ Q S I R K F	V L Q F
Rice endosperm (ss)	FQ R Y	V A
Wheat (WE3, (ls))	G F R W R K I	V V Q S

The amino acid sequences are designated in one letter codes. The numbers in parentheses indicate the amino acid residue in the sequence. (ss) and (ls) signify small and large subunit, respectively. Y and K in outline form indicate the modified residues in the *E. coli* ADPGlc PPase. The amino acid sequences were obtained from Ref. 43-45.

subunit activator sites are independent in causing the enzyme to go into an active conformation upon binding of 3PGA or if both peptides interact to provide the active conformation upon 3PGA binding.

STUDIES ON THE CATALYTIC SITE OF THE SPINACH LEAF ADPGlc PPASE USING THE PHOTOAFFINITY SUBSTRATE ANALOG, 8-AZIDO-ATP

8-azido-ATP (8-N₃-ATP) has been shown to be a substrate for the spinach leaf ADPGlc PPase with a K_m larger than that of the natural substrate, ATP.⁴⁶⁾ The K_m for 8-N₃-ATP is 0.81 μ M and is 8-times higher than the K_m for ATP, 0.1 μ M. The V_{max} with 8-N₃-ATP is only 1% of that observed with ATP. The product of the reaction, 8-N₃-ADPGlc, has a K_m for the spinach leaf ADPGlc PPase of 80 μ M, lower than the K_m for ADPGlc, 225 μ M. The maximal velocity rate for 8-N₃-ADPGlc is only 0.3% of that observed for ADPGlc. When UV light (257 nm) irradiates 8-azido compounds, a nitrene radical is formed which can react with electron-rich residues. Preliminary experiments⁴⁶⁾ have shown that labeled 8-N₃-ADPGlc can covalently

link to the spinach leaf ADPGlc PPase and inactivate it upon UV irradiation. The substrate, ADPGlc, is very effective in inhibiting the chemical modification by the azido compound while UDPglucose, a non-substrate, did not prevent the inactivation. The labeled 8-azido-ADPGlc is incorporated mainly if not solely in the 54 kD subunit.⁴⁶⁾ ADPGlc inhibits the incorporation by at least 67%. It thus appears that the substrate binding site is on the 54 kD subunit. It is probable, however, that the substrate/catalytic site is shared between the two subunits and that the nitrene radical reacts with a strong nucleophilic residue on the larger subunit causing the incorporation to be mainly on the larger subunit.

Table 3 shows the amino acid sequences of the *E. coli* ADPGlc PPase surrounding tyrosine residue 114 modified by 8-N₃-ADPGlc⁴⁷⁾ and lysine residue 195 modified by pyridoxal-P and protected by ADPGlc plus Mg.⁴⁸⁾ In site-directed mutageneses experiments by Hill *et al.*,⁴⁹⁾ showed that substitution of the Lys residue by other amino acids (Arg, His, Gln, Ile, Glu) would specifically change the K_m of the substrate, glucose-1-P from 100- to 10,000-fold. The K_m or K_a values of the substrate, ATP, or Mg²⁺ or the activator, fructose-1,6-bis-P

were not affected. Thus, it is postulated that Lys 195 is specifically binding to the phosphate residue of glucose-1-P or the β -phosphate of ADPGlc.⁴⁹⁾

Of interest is that there is great conservation of those sites in the ADPGlc PPases of the cyanobacteria and of higher plants (Table 3). In the Glc-1-P binding site the reactive Lys residue is conserved in the large and small subunits seen in the higher plants as well as in the cyanobacterial ADPGlc PPase subunits. It would be of interest to initiate site-directed mutagenesis experiments either with the cyanobacterial or the higher plant ADPGlc PPase subunits at the Lys residue equivalent to residue Lys 195 of the *E. coli* ADPGlc PPase. This would provide information whether the higher plant "equivalent Lys residue", is involved in glucose-1-P binding. Recently the cDNAs representing the large and small subunits of the potato tuber ADPGlc PPase have been expressed in *E. coli* and highly purified.⁵⁰⁾ Thus the ability to do site-directed mutagenesis and chemical modification experiments with the higher plant ADPGlc PPase subunits from non-photosynthetic plant tissue are now feasible. In addition, the putative ATP binding site can now be studied by chemical modification and site-directed mutagenesis and could prove definitively, that binding of the substrates and the catalytic site(s) are shared by the large and small subunits. The notion that the catalytic sites are shared by the subunits is due to the finding of small (5 to 10% of the normal activity) but significant activity in the *sh 2*, *bt 2* mutants of maize,^{1,2,30)} a *rb* locus mutant of pea embryo³⁾ and an *adg 2* mutant of *Arabidopsis thaliana*.^{5,36)} These are starch deficient mutants where one of the subunits, large or small, of the ADPGlc PPase is missing. Thus, the finding of a small amount of activity suggests that the remaining subunit, either large or small, must retain the catalytic sites to give some activity.

The *Arabidopsis thaliana adg 2* ADPGlc PPase mutant lacks the large subunit but still has catalytic activity³⁶⁾ and as discussed above in the section on the structural and regulatory properties of ADPGlc PPase, it appears that both subunits are necessary to have optimal

affinity constants for the substrates as well as the activator and optimal catalytic activity. Therefore the subunits must interact with each other and this interaction may entail the sharing of the substrate and ligand binding sites.

ISOLATION OF THE ADPGLUCOSE PPASE GENE. CLONING OF ADPGLC PPASE GENES AND THEIR SEQUENCE COMPARISONS

cDNA or genomic clones for the small subunit ADPGlc PPase gene of rice endosperm,^{26,34,51,52)} spinach leaf,⁴⁶⁾ of *Arabidopsis thaliana* (B. J. Smith-White, private communication), for the large and small subunits of maize endosperm,^{31,53,54)} and potato tuber^{33,55,56)} have been isolated. cDNA clones of ADPGlc PPase have been isolated from wheat leaf and wheat endosperm.⁵⁷⁾ The deduced amino acid sequence derived from the wheat endosperm cDNA is more similar to the deduced amino acid sequence of the maize endosperm cDNA of the large (*Sh 2*) subunit than the rice seed cDNA of the ADPGlc PPase small subunit gene suggesting that they are representative of the large subunit peptide.

At the DNA level the isolated genes are quite dissimilar. For wheat leaf and wheat endosperm there is only 59% identity^{45,57)} and on the basis of Southern hybridization analyses and restriction enzyme mapping, it is concluded that there are at least two distinct gene families in wheat. For spinach leaf and rice endosperm cDNAs there is only about a 77% identity.⁴⁵⁾ The deduced amino acid sequence of the cDNAs from rice and wheat endosperm and spinach, wheat and *Arabidopsis thaliana* leaf and from potato tuber have been compared. Two wheat endosperm cDNA clones were isolated⁵⁷⁾ and they represented two closely related gene subfamilies in wheat endosperm. One, AGA. 3, had a DNA insert of 1272 bp in length with an open reading frame of 888 bp encoding 296 amino acids. The other, AGA. 7, is 1798 bp in length comprising of an open reading frame of 1500 bp encoding 500 amino acids. The clones are considered incomplete but it was thought that the AGA. 7 insert was lacking only

some of the transit peptide portion. These wheat endosperm clones are 96% identical in shared regions of their open reading frames and differ in only 10 of the 296 amino acid residues. In contrast, there is only a 54% identity between the wheat leaf ADPGlc PPase cDNA clone, AGA.1 DNA insert deduced amino acid sequence and AGA.7. AGA.1 is 947 bp in size and encodes for 301 amino acids. Comparisons of the various clones were made with the deduced amino acid sequence derived from the rice endosperm cDNA clone which is a full length clone of 1647 bp and coded for 483 amino acids⁵¹⁾ AGA.1, AGA.3 and AGA.7 show only about 51, 44, and 43% identity with respect to amino acids, respectively, with the rice seed amino acid sequence.⁴⁵⁾ If functionally similar amino acids are considered (Lys $\approx$ Arg $\approx$ His $\approx$ Asn and Gln; Asp $\approx$ Glu; Ser $\approx$ Thr; Leu $\approx$ Val $\approx$ Ile $\approx$ Phe $\approx$ Tyr and Trp), the similarity between the sequences to the rice sequence is about 72, 64, and 65%, respectively. Greater identity is seen with the potato, spinach and *Arabidopsis* leaf small subunit clones cDNA clones with respect to their amino acid sequences. The cDNA clone of potato tuber is almost a full length clone which codes for 444 amino acids^{55,56)} while the spinach leaf clones are incomplete with two of them encoding for a total of 450 amino acids which appears to be the mature subunit without the transit peptide.^{45,46)} The *Arabidopsis* genomic clone is incomplete and its sequence encodes 240 amino acids (A. Lonneborg, private communication). For the potato tuber 50 kD, spinach leaf 51 kD and *Arabidopsis* leaf small subunit and the maize *bt 2* deduced amino acid sequences, there are about 90, 86, 88 and 85% identity, respectively, with the rice seed amino acid sequence. If similar amino acids are considered, the similarity is then, respectively, 94, 93, 93, and 89%. All of these five clones have been shown to be representative genes of the smaller subunit of the ADPGlc PPase. In contrast, less identity is seen with the derived amino acid sequences of the cDNA clones of the larger subunits of maize, *Shrunken-2* (60 kD), and potato tuber (51 kD) ADPGlc PPases with the rice seed and spinach leaf cDNA clones derived amino acid sequences.

The *Shrunken-2* sequence⁵³⁾ codes for 542 amino acids. Included in the comparison are the sequences of the 54 kD, high molecular mass subunit of the spinach leaf enzyme obtained by automated Edmann degradation procedures and then aligning the peptide sequences to the derived sequences of the rice seed and spinach leaf lower molecular mass subunit.⁴⁵⁾ There is only a 44, 46, 49, and 45% identity seen in the sequences of *Shrunken-2* with those of rice seed, spinach leaf, potato tuber small subunit and maize *brittle-2* respectively. If similar amino acids are considered, then the similarity is 67, 68, 72, and 67%, respectively. Similarly, about 59 and 60% identity is observed for the spinach leaf peptides (total of 303 amino acids) of the 54 kD subunit compared to the amino acid sequences of rice seed and spinach leaf small subunits. If similar amino acids are considered then similarity is about 76 to 77%. Comparison of the *Sh-2* sequence with the spinach leaf 54 kD subunit shows about the same identity. About 288 amino acids can be compared and 56% identity is seen. When similar amino acids are considered, 75% similarity is found. Also, the comparison of the amino acid sequences between the wheat endosperm cDNA clone, AGA.7 and the *Shrunken-2* cDNA clone shows a 66% identity in sequence and a similarity of 78% when similar amino acids are considered.

Thus, there is greater conservation between the lower kD subunits of the ADPGlc PPase from different plants than what is observed between the larger kD subunits. This is expected as the spinach leaf lower molecular weight subunit antibody reacts very well with the equivalent subunits of maize endosperm,³⁰⁾ rice seed,^{34,51)} *Arabidopsis*^{4,5,36)} and potato tuber³²⁾ enzymes. The lower molecular weight subunit antibody does not react as well with the higher molecular mass subunit of the ADPGlc PPase of these various plants. Therefore it is not expected to see as much similarity between the lower and higher molecular weight subunits. However, there appears to be low, but extensive identity between the 54 and 51 kD subunits of the spinach leaf ADPGlc PPase. Because of the significant similarity between the large and small subunits

of the ADPGlc PPase it can be speculated that they may have arisen originally from the same gene. The bacterial ADPGlc PPase has been purified to homogeneity from 9 different and diverse species and in each case has been shown to be homotetramers composed of only one subunit.^{21, 22, 38, 43, 44)} The cyanobacterial ADPGlc PPase, which has 3PGA as an allosteric activator and P_i as an inhibitor, similar to the higher plant enzyme,^{38, 58)} is homotetrameric in structure unlike the higher plant enzymes. It is quite possible therefore, that during evolution, there was gene duplication in the higher plant photosynthetic systems of the PPase gene and then divergence of the genes to produce two different peptides for the native ADPGlc PPase. Both subunits are required for optimal activity of the native plant enzyme. The reason for this gene duplication is presently unknown and the reason why association of two peptides are required for optimal activity at present is not known. It is possible that one of the peptides, the large subunit peptide may be more tissue specific because of its greater diversity in amino acid sequence while the small subunit gene, being more highly conserved in plants, is present in all the plant tissues.⁴⁵⁾

The above comparisons of the primary structures of thirteen ADPGlc PPases thus indicate that many domains of the protein are conserved from both the enteric- and cyanobacteria and angiosperm plants because of catalytic activity as well as the mode of allosteric regulation. As mentioned earlier, previous structure-function studies with the *E. coli* enzyme have identified primary structure regions associated with the substrate binding site (Table 3), the allosteric activator binding site and the inhibitor binding site.^{9, 26, 28, 40, 41, 45, 51, 55, 59, 60)} There is a conservation of the primary structure of the protein for the substrate and allosteric activator binding sites. Moreover, the proteins from angiosperm plants may be classified into two main groups on the basis of sequence identities and one of the main groups can be further divided into two subgroups. The two main groups of the plant proteins are based upon the two subunits, large and small, of the plant enzyme while the subgroups of the

large subunit are based on the tissue in which the particular gene is expressed. Additionally, The small subunit group shows a slight, but distinct division into a classification based upon whether the protein is from a monocot or dicot.

In addition, the nucleotide sequences of the genes of thirteen of these proteins are compared for relationships between them and the analysis suggests that the protein for the small subunit has been subjected to greater selective pressure to retain a particular primary structure.

INCREASING THE LEVELS OF STARCH BY EXPRESSION IN PLANT TISSUES OF A BACTERIAL ADPGlc PPASE GENE

Starch after cellulose is the most abundant polysaccharide in nature and is the major reserve polysaccharide in green plants. About 2/3 of the total starch production is used in the food and beverage industries. It is directly applied to sauces, custards and desserts as a thickener and after enzymatic hydrolysis it is used as a sweetener in drinks and confectionary. One third of the starch production however, is used for sizing of paper and board and as an adhesive in the paper, packaging and textile industries. About 10% of the production is used as a raw material in the chemical industry for production polyols, amino acids, acids, cyclodextrins, fructose, antibiotics and related compounds through various fermentation processes. Within the last 10 years, the demand for starch has dramatically increased for both industrial and specialized food uses.⁶¹⁾ Further application of starch in the chemical industry may also result from effective competition with cellulose and crude oil, two other raw materials applied in the production of a number of the aforementioned compounds. Indeed, the use of starch relative to cellulose and crude oil may increase due to ; 1) the crude oil sources are dwindling as well as its price is increasing subject to political situations and accessibility ; 2) the price of cellulose fibres is more than double the price of starch and its supply is decreasing as long as pollution reduces the growth rate of the forests all over the world and 3) there is great potential in future

basic and application research in that the starch granule properties can be changed or modified via molecular engineering techniques producing a starch more suitable for chemical and/or biotechnological processes. In order to improve starch for these means more investigation is needed to determine the factors affecting the properties of starch; for *e.g.*, the amount of protein, or lipid or covalent phosphate associated with the granule, the ratio of amylose to amylopectin, the two polysaccharide entities of the granule, as well as the structural features of these components in the granule.

Because of the increased demand for starch it was of interest to see if starch levels in a plant could be increased by expression of activity of one of the enzymes that could be involved in starch biosynthesis. As indicated above there is a preponderance of evidence to strongly suggest that the rate-limiting and regulatory enzyme of starch or bacterial glycogen synthesis was ADPGlc PPase.^{7-9, 17, 19-22)} However, in view of the difficulties involved in the expression of 2 distinct genes to reconstitute the Plant ADPGlc PPase activity, an *E. coli* ADPGlc PPase gene, *glgC16*, of an allosteric mutant,⁶²⁾ that was not dependent on the presence of activator for activity, was used by the Monsanto Company group to transfect some plant systems to see if the starch content of the plant could be increased.⁶³⁾ Since starch synthesis occurs in the plastid a nucleotide sequence encoding transit peptide of the *Arabidopsis* ribulose 1,5-bisphosphate carboxylase chloroplast transit peptide was fused to the translation initiation site of the *glgC19* gene (Fig. 1). The chimeric gene was then cloned behind the cauliflower mosaic virus (CaMV) enhanced 35S promoter or a tuber-specific patatin promoter and a polyadenylation signal from the nopaline synthase gene (Nos) fused on at the 3' end of the chimeric gene⁶³⁾ (Fig. 1). The CaMV-chimeric gene was placed in a 'cloning vector, pMON20104 having a 35S-neomycin phosphotransferase gene as a selectable marker,⁶³⁾ mated into *Agrobacterium* strain ASE and used for transformation of tobacco plants.⁶³⁾ Calli and shoots, resulting from transformation and also kanamycin resistant, were analyzed for expression of the *glgC* gene and starch

Fig. 1. Schematic representation of the promoter chloroplast transit peptide *glgC16* gene.

A modified *Arabidopsis thaliana* chloroplast transit peptide which contained a duplicated cleavage site to also eliminate the 23 amino acids of the mature N-terminal small subunit⁶³⁾ so it would not interfere with the catalytic or regulatory activity of the expressed *glgC16* gene product. The Nos terminator is the nopaline synthase 3' poly A signal.

content. The *glgC16* gene product was detected in the transformed plant calli via immuno-blotting.⁶³⁾ Where the *glgC* gene product was detected the starch levels were increased over the controls lacking the *glgC* gene product by about 1.7- to 8.7-fold in tobacco calli.⁶³⁾ When the CaMV-chimeric gene and polyadenylation signal was subjected to electroporation into tobacco TXD protoplasts ADPGlc synthesis was observed in which was resistant to P_i inhibition and activated by FBP (Table 4). ADPGlc synthesis in the control protoplast extract was totally inhibited by P_i as expected since the tobacco and most plant ADPGlc PPases are quite sensitive to P_i .

Examination and comparison via the light microscope of transgenic tobacco with control calli, show a tremendous increase of starch granules.⁶³⁾ Similarly, I_2 staining of transgenic

Table 4. ADPGlc PPase in electroporated tobacco protoplasts. *

Conditions	ADPGlc formed (nmol)
Control, 10 mM P_i	0.0
Transformed, 10 mM P_i	6.4
Transformed, 2.5 mM Fru-1, 6-bis-P + 10 mM P_i	18.0
Transformed, 20 mM 3-PGA	18.4
Transformed, 2.5 mM Fru-1, 6-bis-P	20.2

* Data obtained from.⁶³⁾

Table 5. Starch content in tubers expressing the chloroplast transit peptide (CTP) *glgC16* gene.

Tuber type	Plant lines (#)	Average starch content (% wet weight)
CTP- <i>glgC16</i>	11	16.0 ± 2.00%
Control	11	12.3 ± 1.15%

tomato shoots containing the transit peptide-*glgC16* gene caused them to stain black while the controls were essentially negative.⁶³⁾

These results certainly indicate that the bacterial enzyme can be expressed in plant tissues and that it stimulates greater production of starch further suggesting that it is a rate limiting enzyme for starch synthesis.

Similar results have been obtained for potato tubers where the chimeric gene with transit peptide, the polyadenylation signal, under control of a tuber-specific patatin promoter, increases starch in the tuber, 25- to 60% over controls not containing the bacterial enzyme (Table 5).⁶³⁾ If the bacterial ADPGlc PPase *glgC16* gene was expressed in the tuber lacking the transit peptide gene portion, no increase in starch content was noted⁶³⁾ and this most probably is due to the expressed ADPGlc PPase activity not being present in the amyloplast where starch is synthesized via starch synthase and branching enzyme catalysis.

This indicates that transfection of a plant with a bacterial ADPGlucose PPase will increase the level of starch in important crop product. Thus, via biotechnological methods the increase of starch content in plants can be accomplished. It is conceivable therefore, that similar methods can be used to change in addition to quantity, starch quality via expression/transformation of the isoforms of starch synthase and branching enzymes in plants. Various isoforms of the starch synthase and branching enzyme have been isolated from various plants^{7-9, 64-67)} and recently, with respect to the maize branching enzyme isoforms, they have been shown to have different catalytic properties with respect to size of oligosaccharide chains transferred.⁶⁸⁾ It is quite possible that the starch synthase isoforms also have different properties with respect to elongation of various branch chains

in amylopectin and in amylose synthesis.⁹⁾ Thus introduction of these various isoenzymes separately, may result in obtaining starches with modified structures in both amylopectin and amylose. These "new starches" may have greater utilization in food and industrial processes. The production of modified starches via molecular biology techniques are thus promising, may be more beneficial and even more economical than producing modified starch via chemical methods. The future of basic starch science remains bright.

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