STRUCTURE-FUNCTION STUDY ON ALLOSTERIC REGULATION OF ADP-GLUCOSE PYROPHOSPHORYLASE FROM CYANOBACTERIUM *ANABAENA* PCC 7120

By

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ABSTRACT

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ADP-glucose pyrophosphorylase is a pivotal enzyme in the biosynthesis of starch in plant tissues and glycogen in bacteria. The gene of *Anabaena* ADP-glucose pyrophosphorylase was isolated from a genomic library and encodes a fully active enzyme when expressed in *E. coli* cells. Analysis of the deduced amino acid sequence indicated that the cyanobacterial enzyme is more similar to the higher-plant than to the enteric bacterial enzyme. The lysyl residue within the putative activator-binding site near the C-terminus of the higher-plant enzyme, determined by chemical modification with pyridoxal-P, is also conserved in the *Anabaena* enzyme. Site-directed mutagenesis of the corresponding lysine (Lys_{419}) of the *Anabaena* enzyme was done to determine the function of this residue. Replacing Lys_{419} with either arginine, alanine, glutamine, or glutamic acid largely reduced the apparent affinities for the activator, 3-P-glycerate, 25- to 150-fold. The mutations caused lesser or no effect on other kinetic constants or enzyme properties suggesting that Lys_{419} is primarily involved in the binding of 3-P-glycerate, probably by an ionic interaction between its positively charged ϵ-amino group and the negatively charged carboxyl or phosphate groups of the activator.

Chemical modification of the *Anabaena* enzyme with pyridoxal-P results in

enzyme more active in the absence of activator and less sensitive to the inhibition by orthophosphate. Only one lysyl residue was predominantly modified and was identified as Lys_{419}. Similar results were obtained for the K419R mutant enzyme in which Lys_{419} is substituted by arginine. In this case, an alternative lysyl residue, Lys_{382}, of the mutant enzyme was modified. The results suggest that both Lys_{382} and Lys_{419} are involved in the binding of 3-P-glycerate.

to my family

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ABBREVIATIONS

3PGA	3-P-glycerate
ADPGlc PPase	ADP-glucose pyrophosphorylase
bp	base pair(s)
BSA	bovine serum albumin
cpm	counts per minute
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
FPLC	fast performace liquid chromatography
HEPES	4-(2-hydroxethyl)-1-piperizineethane sulfonic acid
HFBA	heptafluorobutyric acid
HPLC	high performance liquid chromatography
IPTG	isopropyl-ß-D-thiolgalactopyranoside
kb	kilo-base pair(s)
kDa	kilo-dalton
LB medium	Luria-Bertani medium
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PPi	pyrophosphate
PLP	pyridoxal-P
PVDF membrane	polyvinylidene difluoride membrane
SDS	sodium dodecyl sulfate
SSC	sodium chloride + sodium citrate solution
TCA	trichloroacetic acid
TFA	trifluoroacetic acid

INTRODUCTION

Biosynthesis of starch has been one of the most interesting topics in plant physiology and biochemistry research. Not only because starch is an important agricultural product and raw material for many industries, but also is the complexity of the enzymatic functions that lead to its synthesis. Over decades of effort, nowadays, it is believed that the synthesis of starch occurs predominantly via the ADP-glucose pathway (Preiss, 1991).

In this pathway, ADP-glucose, the glucosyl donor, is synthesized from ATP and glucose-1-P by ADP-glucose pyrophosphorylase (E.C. 2.7.7.27). The glucosyl moiety of ADP-glucose is then transferred to the non-reducing end of a preexisting primer (either a component of starch amylose or amylopectin or a maltodextrin) in an α-1,4-glucosidic linkage by starch synthase (E.C. 2.4.1.21). The branch chain of starch molecule is formed by cleavage of an α-1,4-glucosyl chain and religation via an α-1,6-glucosyl linkage under the function of branching enzymes (E.C. 2.4.1.18). The bacterial version of starch, glycogen, is synthesized via the same pathway described above (Preiss and Romeo, 1989).

Among these three enzymes, ADP-glucose pyrophosphorylase is known as an allosteric enzyme regulated by effectors derived from the dominant carbon assimilation pathway in the organism. For example, the higher-plant enzymes usually are activated by the CO_2-fixation product, 3-P-glycerate (3PGA), and inhibited by orthophosphate (Pi). The enteric bacterial enzymes are activated by fructose-1,6-P_2 and inhibited by AMP. Substantial evidence from intact leaf system indicates that the [3PGA] to [Pi] ratio within the chloroplast regulates starch synthesis by affecting the activity of ADP-glucose pyrophosphorylase (Petterson and Ryde-Petterson, 1989; Neuhaus et al., 1989; Neuhaus and Stitt,

1990). Genetic studies of this enzyme also suggest that the allosteric properties of ADP-glucose pyrophosphorylase affects the synthesis of starch (Ball et al., 1991) and bacterial glycogen (Govons et al., 1973; Kumar et al., 1989; Ghosh et al., 1992; Meyer et al., 1992).

Study of the structures related to the allosteric regulation of ADP-glucose pyrophosphorylase has been a great interest and was proceeded mainly by chemical modification and site-directed mutagenesis. The primary structures of the allosteric site for the *E. coli* and spinach leaf enzymes were identified by chemical modification using a site-specific probe, pyridoxal-P (Parsons and Preiss, 1978; Morell et al., 1988; Preiss et al. 1992). The amino acid sequence and location of fructose-1,6-P_2-binding site of the *E. coli* pyrophosphorylase are very different from those of the 3PGA-binding site determined for the spinach enzyme. The activator site of the *E. coli* enzyme is close to the N-terminus of the protein. However, activator of the spinach leaf enzyme binds to a site near the C-terminus. Comparison of *E. coli* pyrophosphorylase with the higher-plant enzymes showed significant differences in the primary structures, which may reflect the distinct allosteric properties of these enzymes (Anderson et al., 1989; Smith-White and Preiss, 1992).

Cyanobacteria are considered as phylogenetic intermediates between plants and bacteria. Their photosynthetic function is similar to that of higher plant chloroplast, but they synthesize glycogen as their major carbohydrate reserve like bacteria (Shively, 1988). Interestingly, the properties of the cyanobacterial ADP-glucose pyrophosphorylases seem to be in agreement with the intermediate position occupied by these photosynthetic prokaryotes. The enzyme from cyanobacteria is activated by 3PGA and inhibited by Pi as is the higher plant enzyme (Levi and Preiss, 1976; Iglesias et al., 1991). However, unlike the heteroterameric

higher-plant enzyme, the cyanobacterial enzyme is homotetrameric in structure similar to the enteric bacterial ADP-glucose pyrophosphorylase (Iglesias et al., 1991).

Before this project was started, there was no primary structure of the cyanobacterial enzyme available to compare with that of the enteric bacterial and higher-plant enzymes. Thus, to obtain an insight into the structure-function relationships of ADP-glucose pyrophosphorylase, it was of interest to clone the cyanobacterial gene. A full length clone was isolated for ADP-glucose pyrophosphorylase from a genomic library of *Anabaena* sp. strain PCC 7120 using a probe generated by polymerase chain reaction. The deduced amino acid sequence of the *Anabaena* gene was shown to be very similar to that of the higher-plant enzymes, both large and small subunits. Expression of the *Anabaena* gene in *E. coli* cells generated an active recombinant enzyme. This work was published in the October issue of *PLANT MOLECULAR BIOLOGY* (1992) 20, 37-47. Chapter II of this dissertation is reproduced from this publication by permission of Kluwer Academic Publishers.

The second section of this project is to determine the structure-function relationships of a lysyl residue in the putative 3PGA-binding site. This residue was identified by chemical modification of the spinach leaf enzyme (Morell et al., 1989) and is highly conserved in the sequences of higher-plant and cyanobacterial ADP-glucose pyrophosphorylases. Site-directed mutagenesis experiments were performed on the corresponding lysine of the *Anabaena* enzyme (Lys_{419}) by replacing it with either arginine, alanine, glutamine, or glutamic acid. The mutant enzymes were purified and characterized by kinetic studies and were compared with the wild-type enzyme. The results suggest that the major role of Lys_{419} is involved in the binding of 3PGA. Moreover, alteration in activator specificity was

observed for the glutamine mutant enzyme. This work is included in Chapter III of this dissertation.

Previous studies have shown that pyridoxal-P can covalently modify the activator site of the cyanobacterial enzyme (Iglesias et al., 1993). However, the sequence of the modified peptide has not been identified. This structure was resolved by isolation and sequencing of the phosphopyridoxylated peptide, indicating that Lys_{419} of the *Anabaena* enzyme is the site predominantly modified by pyridoxal-P. Furthermore, an *Anabaena* mutant pyrophosphorylase (K419R) was also subject to the same chemical modification approach. It appears that pyridoxal-P specifically modify the mutant enzyme and caused an alteration in allosteric properties. In this case, Lys_{382} was found to be the amino acid residue labeled by pyridoxal-P. This work is presented as Chapter IV. Both Chapter III and Chapter IV were written in a format for publication in J. Biol. Chem.

Preceding the three chapters described above is a review of the literature on ADP-glucose pyrophosphorylase studies. It provides a general background about the physiological role and the features of this enzyme from bacterial and plant sources. A summary of the research is presented as Chapter V, as well as a discussion of possible directions for future study.

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CHAPTER I

LITERATURE REVIEW

LITERATURE REVIEW

1. The Roles of ADP-glucose pyrophosphorylase in Starch Biosynthesis

1.1. Synthesis of ADP-glucose, the precursor of starch:

Starch, a large polymer of glucose residues, is the most common form of carbon and energy reserves for the photosynthetic plants. This polysaccharide is found as water-insoluble granules confined in specialized organelles called chloroplast and amyloplast. Starch granules are composed of mainly two types of molecules, amylose and amylopectin. Amylose is basically a linear α-1,4-glucan with occasional secondary chains attached by α-1,6-glucosyl linkage, called branch points. Amylopectin has a highly branched structure which predominantly consists of α-1,4-glucosidic linkages with an average of one branch points every 20 to 26 glucosyl residues (Manners, 1985; Morrison and Karkalas, 1990).

The metabolic pathway leading to starch synthesis was solved after the discovery of nucleotide-diphosphate-sugars in the 1950s by Leloir and coworkers (Leloir, 1970). Recondo and Leloir (1961) first demonstrated that the α-1,4-glucosidic linkage of starch is produced via the transfer of the glucosyl unit from ADP-glucose to the non-reducing end of a preexisting α-1,4-glucan, catalyzed by starch synthase. The synthesis of α-1,6-linkages occur under the catalysis of branching enzyme shown by Bourne and Peat much earlier (1945). In most tissues starch synthase is specific for ADP-glucose (Preiss, 1982). The granule-bound starch synthase can use UDP-glucose as substrate, although the affinity for this compound is 15- to 30-fold lower than for ADP-glucose (Preiss, 1988). The substrate specificity of starch synthase suggests that starch is predominantly, if not solely, synthesized through the ADP-glucose pathway.

In 1962, Espada first demonstrated the enzymatic synthesis of ADP-glucose

from ATP and glucose-1-P. The reaction is catalyzed by ADP-glucose pyrophosphorylase (ATP: α-glucose-1-P adenylyl transferase) (equation 1). The enzyme is later found distributed in chloroplasts and amyloplasts in which starch synthesis occurs (Okita et al., 1979; ap Rees et al., 1984; Lin et al., 1988). Although the enzyme can catalyze in pyrophosphorylase direction, the reaction of ADP-glucose synthesis is maintained far from reversible equilibrium due to the presence of high activity of alkaline pyrophosphatase in plastids (Weiner et al., 1987)

$$\text{ATP} + \text{glucose-1-P} \quad <====> \quad \text{ADP-glucose} + \text{PPi} \qquad (1)$$

According to the data obtained from studies on various plant tissues, the activity of ADP-glucose pyrophosphorylase (ADPGlc PPase) is sufficient to account for the rates of starch synthesis (Ozbun et al., 1973; Heldt et al., 1977; Okita et al., 1979; ap Rees et al., 1984; Edwards et al., 1988). Therefore, it is believed that ADP-glucose is predominantly synthesized through the action of ADPGlc PPase *in vivo*. So far, this idea is amply supported by genetic evidences derived from various plant species.

First, mutants of maize endosperm having a reduced level of starch (25-30 % of wild-type) were shown to have ADPGlc PPase activities only 5-10 % of that in normal maize (Tsai and Nelson, 1966; Dickinson and Preiss, 1969). The *shrunken-2* mutant has normal or even higher activities of the enzymes that catalyze reactions intervening between sucrose and starch such as sucrose synthase, hexokinase, phosphoglucoisomerase, phosphoglucomutase, uridine diphosphokinase, and UDP-glucose pyrophosphorylase. Thus, the basis for the low amounts of starch in the endosperm of these mutants is due to a deficiency of

ADPGlc PPase. A similar result was reported by Smith *et al.* (1989) that the pea developing embryo with mutation at *rb* locus has altered starch content through an effect on ADPGlc PPase. The reduced ADPGlc PPase activity (3-5 % of wild-type) of the pea mutant results in reduced starch formation (38-72 % of wild-type).

Lin and his coworkers have looked for mutant lines which are completely lacking starch by performing genetic studies on *Arabidopisis thaliana* (Lin *et al.*, 1988). One starchless mutant line, generated by chemical mutagenesis, was isolated and was shown completely lacking ADPGlc PPase activity. This finding first demonstrates that starch biosynthesis in the chloroplast is entirely dependent on the pathway involving ADPGlc PPase. They also isolated a starch deficient mutant which contains only 5 % of wild-type ADPGlc PPase activity (Lin et al., 1988).

Recently, Müller-Röber *et al.* (1992) inhibited starch synthesis in transgenic potato tubers by expressing a chimeric gene encoding the antisense RNA of the small subunit of the potato tuber enzyme. The expression of ADPGlc PPase was almost completely inhibited in the starchless tubers of the transgenic plants. In contrast, the mRNA level of sucrose synthase did not change. Thus, it is likely that ADPGlc PPase also plays an exclusive role in starch synthesis in the amyloplast.

In the chloroplast, it is undisputed that the substrates of ADPGlc PPase, ATP and glucose-1-P, are generated within the plastid through the function of photosynthesis and gluconeogenesis. In the amyloplast, starch synthesis exclusively depends on the transport occurring across the amyloplast envelope. So far, it is less affirmative about how exactly the transport system works in the amyloplast. A phosphate/triose phosphate translocator similar to that present in the chloroplast has been proposed to be involved in importing carbon into the

amyloplast (Heldt et al., 1991). If this is the case, the plastid should have all the enzymes necessary for the conversion of triose phosphate to starch. The studies on amyploplasts from soybean suspension culture (Macdonald and ap Rees, 1983) and cauliflower bud plastids (Journet and Douce, 1985) are in agreement with this theory. Moreover, dihydroxyacetone phosphate is a better substrate for starch synthesis than hexose phosphates when supplied to the amyloplast preparations from developing maize endosperm (Echeverria et al., 1988). In contrast, wheat endosperm amyloplasts lack plastidic fructose-1,6-bisphosphatase activity (Entwistle and ap Rees, 1988), suggesting that triose phosphate is not the carbon source for starch synthesis in the organelle. It was shown that glucose-1-P served as an effective precursor for starch accumulation in wheat endosperm amyloplasts (Tyson and ap Rees, 1988). For pea amyloplasts, glucose-6-P, instead of glucose-1-P, is the most effective in entry into the plastids for starch synthesis (Hill and Smith, 1991). These findings are consistent with the study of Keeling et al. (1988), which showed that only partial redistribution of ^{13}C occurs between C1 and C6 atoms in starch after feeding the developing wheat endosperm with [1-^{13}C]- or [6-^{13}C]-glucose or fructose. Another unresolved question is the source of ATP for ADPGlc PPase. It is possible that ATP crosses the envelope through an adenylate translocator (Liedvogel and Kleinig, 1980; Stitt, 1990) or that it is produced within amyloplast by the glycolysis pathway.

1.2. The regulatory enzyme of starch synthesis:

In the chloroplast regulation of starch synthesis is believed to occur primarily at the ADP-glucose synthetic step (Preiss, 1984; Kruger, 1990). In the other words, the capacity of starch synthesis is determined by the activity level of ADPGlc PPase. ADPGlc PPase is the rate-limiting enzyme as 1) the enzyme

catalyzes the first committed step of starch synthesis; 2) the activity of ADPGlc PPase is allosterically regulated by metabolites.

The allosteric regulation of higher-plants ADPGlc PPase was first reported by Ghosh and Preiss (1965). They found that the spinach leaf enzyme is activated by 3-P-glycerate and inhibited by orthophosphate (Pi). Subsequently the activation and inhibition by these metabolites were also seen for ADPGlc PPases from other higher plants (Sanwal et al., 1968), green algae (Sanwal and Preiss, 1967; Ball et al., 1991), and blue-green bacteria (Levi and Preiss, 1976; Iglesias et al., 1991). In all cases, glycolytic intermediates such as fructose-1,2-P_2, fructose-6-P, glucose-6-P, and P-enolpyruvate were found to activate the enzyme to lesser extent and at much higher concentrations. For spinach leaf ADPGlc PPase, which has been studied in the most detail, 3-P-glycerate decreases the apparent *Km* values of substrates and increases the specific activity of the enzyme (Ghosh and Preiss, 1966).

It has been seen in most cases that the sensitivity of ADPGlc PPase to phosphate inhibition is modulated by 3-P-glycerate. For example, when 3-P-glycerate was present at a concentration of 1 mM , the apparent *Ki* value of Pi increases from 60 μM to 1.2 mM for the spinach leaf enzyme in ADP-glucose synthesis (Gosh and Preiss, 1966). Conversely, Pi also can exert antagonistic effect on 3-P-glycerate. Thus, it has been proposed that regulation of both ADP-glucose and starch synthesis is modulated by the ratio of [3-P-glycerate] to [Pi]. This hypothesis is in agreement with the evidence obtained from *in situ* studies performed on leaf and isolated chloroplast systems.

One of the *in situ* studies was done by Heldt *et al* (1977) in determining the role of Pi and other factors in the regulation of starch formation in leaves and isolated spinach chloroplasts. Firstly, starch synthesis in leaf discs is increased by

Pi starvation or depletion. In the isolated spinach chloroplasts, starch synthesis is almost completely inhibited in the presence of 1 mM or higher concentration of Pi in the medium. However, the inhibitory effect of Pi is overcome by 3-P-glycerate. Simultaneously measurement of metabolite concentration in the stroma and CO_2 fixation into starch indicates that the controlling factor of starch formation seems to be the ratio of [3-P-glycerate]/[Pi] rather than the concentration of hexose monophosphate. Similar results was also obtained from the studies of Gibbs and coworkers (Steup et al., 1976; Peavey et al., 1977).

Regulation of the rate of ADP-glucose synthesis in the crude extracts of spinach chloroplasts has been measured by Kaiser and Bassham (1979) under conditions simulating the metabolite levels of glucose-6-P, ATP, Pi, and 3-P-glycerate in the chloroplast during lightness and darkness. In the presence of phosphoglucomutase and ADPGlc PPase in the crude extracts, ADP-glucose synthesis was enhanced 6- to 7-fold when the 3-P-glycerate concentration was increased from 1.4 mM to 4 mM, a change occurring in the chloroplast in the dark-light transition. Essentially the same results were observed for the purified spinach leaf ADPGlc PPase under simulated conditions (Copeland and Preiss, 1981). The purified spinach leaf enzyme is more sensitive to Pi inhibition under the dark-simulated conditions in which the 3-P-glycerate concentration is lower.

Recently, the work of Ball *et al* (1991) with unicellular green alga demonstrated that 3-P-glycerate activation of ADPGlc PPase *in vivo* is necessary for starch synthesis. By X-ray mutagenesis of *Chlamydomonas reinhardtii* cells, they isolated a starch deficient mutant (less than 5 % of wild-type) in which the ADPGlc PPase is not sensitive to 3-P-glycerate and Pi. With respect to the synthesis of ADP-glucose in crude extract, the apparent *Vmax* of the mutant is about the same as that of the wild-type in the absence of 3-P-glycerate. However,

in the presence of the activator, the apparent *Vmax* of the mutant is about 5-fold lower than that of the wild-type. The other enzymes related to starch metabolism were essentially not affected.

So far, not as much is known about the regulation of starch synthesis in the amyloplasts of nonphotosynthetic reserve tissues. ADPGlc PPases purified from reserve tissues are activated by 3-P-glycerate and inhibited by Pi in the same way as the enzyme from photosynthetic tissues (Preiss, 1982). This observation implies that the regulation of starch synthesis in amyloplasts is similar to that in chloroplasts.

However, there are reports indicating that partial purified ADPGlc PPases from maize (Dickinson and Preiss, 1969), wheat (Olive et al., 1989), and barley (Kleczkowski et al., 1993) endosperms were not quite as sensitive to 3-P-glycerate and orthophosphate (Pi) as the spinach leaf enzyme. One of the reasons for these observations might be due to partial proteolysis of the enzyme under the purification condition used, which was shown to be the case for the maize endosperm enzyme (Plaxton and Preiss, 1987). When proteolysis was prevented by the addition of protease inhibitors such as phenylmethylsulfonyl fluoride and/or chymostatin, the nonproteolytic form of maize endosperm ADPGlc PPase was able to be partially purified and was shown to be sensitive to modulation by 3-P-glycerate and Pi.

A similar approach has been applied to the purification of the barley endosperm enzyme (Kleczkowski et al., 1993). The presence of protease inhibitors decreased, but did not prevent the proteolysis of the barley endosperm enzyme. In the presence of protease inhibitors, ADPGlc PPase in the crude extract of barley endosperm showed less sensitivity to 3-P-glycerate and Pi. This may be due to either the partial proteolysis of the enzyme or the interfering factors

which may exist in the crude extract. Thus, it would be necessary for the nonproteolytic enzyme to be further purified.

Overall, the idea of ADPGlc PPase as the rate limiting enzyme in starch synthesis has been examined recently by expression of an *E. coli* gene that encodes a regulatory mutant enzyme in plants (Stark et al., 1992). In both transgenic tomato and potato tissues, starch synthesis was enhanced significantly, suggesting that the ADPGlc PPase reaction is the rate limiting step. Moreover, it is the regulatory properties of the enzyme that make the reaction rate limiting.

2. Bacterial ADPGlc PPases

2.1. ADPGlc PPase and bacterial glycogen synthesis:

Bacterial glycogen is a polysaccharide containing glucosyl residues linked together by α-1,4-glucosidic linkages and branched via α-1,6-linkages similar to the glycogen of animals. In many bacterial species, the glucan accumulates when cell growth is limited and an excess of a carbon source is available (Preiss and Romeo, 1989). It is generally considered as an energy storage compound which prolong viability under certain conditions (Preiss, 1984). It has been shown that glycogen-containing bacterial cells survive longer than glycogen-less cells under starvation conditions (Strange et al., 1961; Strange 1968) It was also reported that glycogen may play a role in providing an energy source in sporulation for *Bacillus cereus* (Slock and Stahly, 1974).

The major mechanism of bacterial glycogen synthesis is similar to that which occurs in mammalian cells, in which transfer of the glucosyl unit from a nucleotide-diphosphate-glucose to an α-1,4-glucan primer is involved (Preiss and Walsh, 1981). However, glycogen synthesis in animal cells utilizes UDP-glucose as glucosyl-donor while bacterial glycogen synthesis utilizes ADP-glucose, the

same glucosyl donor for starch synthesis in plants (see section 1.1.).

All bacterial glycogen synthases that have been tested utilize ADP-glucose as a substrate much better than UDP-glucose (Greenberg and Preiss, 1964). ADP-glucose is produced from glucose-1-P and ATP by the catalysis of bacterial ADPGlc PPase (Shen and Preiss, 1965; Preiss, 1969; 1973; 1978; Preiss and Walsh, 1981). Mutants of *E. coli* and *S. typhimurium*, either glycogen-deficient or glycogen-hyperproducing, are affected in glycogen synthase and/or ADPGlc PPase (Preiss, 1984; Preiss and Romeo, 1989). Moreover, the UDP-glucose pyrophosphorylase deficient mutants of *E. coli* are still able to produce normal amount of glycogen, indicating that the glucosyl donor for bacterial glycogen synthesis is ADP-glucose, not UDP-glucose (Preiss and Romeo, 1989).

The control of glycogen formation in bacteria can be exerted in at least two ways: 1. genetic regulation of the enzymes of the glycogen biosynthetic pathway; 2. allosteric regulation of the activity of ADPGlc PPase (Preiss, 1984; Preiss and Romeo, 1989). The allosteric regulation of bacterial ADPGlc PPase has been extensively studied and will be reviewed in section 2.2., 3.1., and 3.2.. Recently, additional information also has been obtained about the genetic regulation. Considerable evidences indicate that cAMP, cAMP-receptor protein, and ppGpp induce the expression of the *glg*C (ADPGlc PPase) and *glg*A (glycogen synthase) genes. Negative control of the *glg* genes via a pleiotropic gene, *csr*A, also has been demonstrated (Romeo et al., 1993). The genetic regulation of bacterial ADPGlc PPase has been very recently reviewed by Preiss and Romeo (1994) and is beyond the scope of this literature review.

2.2. Activator and inhibitor specificities of bacterial ADPGlc PPase:

Most bacterial ADPGlc PPases, similar to the plant enzyme, are regulated

by metabolites (Preiss, 1984). For bacteria which may live in a rapidly changing milieu, this kind of regulation provides a dynamic control of energy and carbon flow. With respect to the specificity for regulators, the prokaryotic enzyme is more complex than the plant enzyme. Diverse specificities were observed for ADPGlc PPase from various bacterial species and has been classified into seven groups (Preiss, 1984). The regulators listed in Table 1 seem to correlate well with the nature of the carbon-assimilation pathway dominant in those organisms.

For the bacteria taking the Embden-Meyerhof glycolytic route, the enzymes are mainly activated by fructose-1,6-P_2 or and fructose-6-P and inhibited by AMP and ADP. The enzyme from *Serratia marcescens* and *Enterobacter hafniae* although very sensitive to AMP inhibition, are exceptions because they do not seem to be activated to any great extent by any physiological compound (Preiss, 1989). For the organisms that only employ the Entner-Doudoroff pathway, ADPGlc PPase is activated by fructose-6-P and pyruvate. The anaerobic photosynthetic bacteria such as *Rhodobacter gelatinosa*, *Rhodobacter globiformis*, and *Rhodobacter sphaeroides* are able to metabolize hexoses either via modified glycolytic or Enter-Doudoroff pathways depending on the growth condition (Preiss, 1978). Thus, it is not surprise to see that the enzymes from these organism are activated by pyruvate, fructose-6-P as well as fructose-1,6-P_2.

ADPGlc PPases from some anoxygenic photosynthetic bacteria are only activated by pyruvate. Generally these organisms such as *Rhodospirillum rubrum*, *Rhodospirillum tenue*, and *Rhodocyclus purpureus* utilize pyruvate and tricarboxylic acid cycle intermediates as carbon sources and photosynthetic donors, but do not grow on glucose or fructose. The enzymes from cyanobacteria are mainly activated by 3-P-glycerate, the first product of CO_2-fixation via the reductive pentose-phosphate pathway. These bacteria perform an oxygenic

Table 1. Regulators of ADPGlc PPase from various bacterial species

Organism	Main carbon pathway	Regulators	
		activator	inhibitor
Escherichia coli *Enterobacter aerogenes* *Salmonella typhimurium*	Embden-Meyerhof	Fructose-1,6-P_2	AMP ADP
Mycobacterium smegmatis *Micrococcus luteus* *Aeromonas formicans* *Aeromonas hydrophila*	Embden-Meyerhof	Fructose-6-P Fructose-1,6-P_2	AMP ADP
Serratia liquefaciens *Serratia marcescens*	Embden-Meyerhof	None	AMP
Rhodobacter spheroides *R. gelatinosa* *R. globiformis*	Embden-Meyerhof and Entner-Doudoroff	Fructose-6-P Fructose-1,6-P_2 Pyruvate	AMP Pi
Chromatium vinosum *Rhodopseudomonas capsulata* *Rhodomicrobium vanniellii* *Rhodopseudomonas palustris* *Chlorobium limicola* *Arthrobacter viscosus* *Agrobacterium tumefaciens*	Entner-Doudoroff	Fructose-6-P Pyruvate	AMP ADP
Rhodospirillum rubrum *R. molischianum* *R. tenue*	TCA cycle; Reductive carboxylic acids cycle	Pyruvate	None
Synechococcus 6301 *Aphanocapsa* 6308 *Synechocystis* 6803 *Anabaena* 7120	Reductive pentose-phoshate pathway	3-P-glycerate	Pi

photosynthesis similar to that of chloroplasts of plants. Thus, it is reasonable that the specificity of the regulators for the cyanobacterial enzymes are the same as that of the plant enzymes.

As shown in Table 1, the specificities of activator are overlapping in several groups. For some of the enzymes which are activated by multiple metabolites, it has been shown by kinetic studies that these metabolites probably bind to the same site. Thus, it is possible that the structure of the activator binding sites are similar or related to each other. Mutation of the part of the gene coding for the activator site has probably occurred during evolution resulting in the coordination of effective activators with the prevalent carbon-assimilatory pathway in the organisms (Preiss, 1984).

Although the carbon-assimilation pathway in each bacterial species could be different, the rate of glycogen synthesis depends on the energy charge and carbon levels in the organism nevertheless. It is reasonable that ADPGlc PPase which is sensitive to the metabolites of the major pathway can function as one of the sensors detecting the energy and carbon change. We can consider the activator of ADPGlc PPase a signal of carbon excess while the inhibitor (AMP, ADP or Pi) together with the substrate ATP as indications of the energy charge. It has been demonstrated that the sensitivity of ADPGlc PPase toward its regulator is important for regulation of the *E. coli* glycogen synthesis. In *E. coli* B mutant strains SG5 (Govons et al., 1969; 1973) and CL1136 (Preiss et al., 1976), and *E. coli* K12 mutant 618 (Creuzat-Sigal et al., 1972; Cattaneo et al., 1969), glycogen accumulates at a faster rate than the wild-type strain due to the altered regulatory properties of the ADPGlc PPase in the mutants. The mutant enzymes have a higher affinity for allosteric activators and a lower affinity for inhibitor, which make the mutant enzymes more active at physiological energy values and are

sufficient to account for the increased rate of glycogen accumulation *in vivo*.

3. Structural Studies of ADPGlc PPase

3.1. Subunit and primary structure of ADPGlc PPase:

According to all the studies to date, ADPGlc PPase from all sources is tetrameric in structure. The enzyme from enterobacteria (Haugen et al., 1976) or cyanobacteria (Iglesias et al., 1991) is a homotetramer, while the enzyme from higher-plants is more complex. The subunit composition of the enzyme from potato tuber was once reported as a homotetramer (Sowokinos and Preiss, 1982). However, the report has been shown to be incorrect (Okita et al., 1990).

Hannah and Nelson (1975, 1976) have studied mutant forms of ADPGlc PPase derived from maize *sh2* and *bt2* endosperms indicating that each enzyme has an allele-specific apparent *Km* value for glucose-1-P, and the level of enzyme activity is *Sh2* and *Bt2* dosage dependent. They suggested that the maize endosperm enzyme consists of two different polypeptides that are products of the *Sh2* and *Bt2* loci.

The heterotetrameric structure of the spinach leaf ADPGlc PPase was first described by Copeland and Preiss (1981). A more comprehensive study was later conducted by Morell et al. (1987) confirming that the spinach leaf enzyme contains two subunits which are different in mobility on SDS-PAGE, antigenicity, tryptic peptide mapping, and N-terminal sequences. The molecular masses of the subunits were determined as 51 and 54 kD proteins on SDS-PAGE. According to their apparent sizes, the 51 and 54 kD proteins sometimes are referred to as the small and large subunits, respectively.

Polyclonal antibodies raised against the spinach leaf small subunit react strongly with the spinach leaf 51 kD protein, but react weakly with the spinach

leaf 54 kD protein in Western blotting experiment, and vice versa. Thus, it was suggested that the small and large subunits of the spinach leaf enzyme are structurally different (Morell et al., 1987). These studies provide important biochemical evidences showing that the higher-plants enzyme is encoded by two different genes.

Western blotting experiments also demonstrate the presence of two subunits in the enzyme from *Arabidopsis thaliana* leaf and maize endosperm using the antibodies raised against the spinach leaf enzyme. The antibodies crossreact to two proteins of the *Arabidopsis thaliana* leaf on Western blots, which is not seen for the starchless mutant, TL25 (Lin et al., 1988a). The TL25 mutant leaves, as mentioned earlier, contain no ADPGlc PPase activity. A starch deficient mutant, TL46, which has only 5 % wild-type pyrophosphorylase activity, was found lacking the subunit cross-reactive to the antibody against the spinach leaf large subunit (Lin et al., 1988b).

The work of Preiss et al. (1990) has shown that the maize endosperm small (55 kD) and large subunit (60 kD) also crossreact to antibodies raised against the spinach leaf small and large subunits, respectively. It was shown that maize endosperms from the mutants *bt2* and *sh2* lack one of these two subunits; the *bt2* endosperm lacks the small subunit while the *sh2* endosperm lacks the large subunit by Western blot analysis.

The structural gene of ADPGlc PPase was first cloned by Okita et al. (1981) from *E. coli*, and designated as *glg*C. It is located at approximately 75 min on the *E. coli* K12 chromosome with other closely linked glycogen biosynthesis structural genes as determined by P1 transduction (Creuzet-Sigal et al., 1972; Preiss et al.,1973; Lartil-Damotte and Lares, 1977). The *glg*C gene was cloned by screening for a neighboring gene *asd* (aspartate semialdehyde dehydrogenase)

which can complement the growth of *Asd*⁻ cells in media lacking diaminopimelic acid. The deduced amino acid sequence of *E. coli* ADPGlc PPase was later determined by Baeker et al. (1983). The ADPGlc PPase gene of another enterobacterium *Salmonella typhimurium* was cloned by using a heterologous probe derived from the *E. coli glg*C gene (Leung and Preiss, 1987a and 1987b).

The structural genes of the higher-plants ADPGlc PPase have been cloned from several species and tissues using different cloning strategies. The rice seed cDNA clone was isolated from a lambda expression library using antibody against the spinach leaf enzyme as a probe (Preiss et al., 1987a; Anderson et al., 1989). From the sequence alignment (Smith-White and Preiss, 1992), it is probably the cDNA of the small subunit of rice pyrophosphorylase. The maize *Sh2* and *Bt2* gene were cloned by differential screening of a maize endosperm cDNA library with labeled cDNA probes synthesized from mutant versus wild-type endosperm RNA (Barton et al., 1986). The deduced amino acid sequences of the *Sh2* and *Bt2* was reported by Bhave et al. (1990) and Bae et al. (1990), respectively. Olive et al. (1989) isolated the cDNA clones from wheat leaf and endosperm. The primary structures of the potato tentative large and small subunits have been reported by two different groups (Müller-Röber et al., 1990; Anderson et al., 1990; Nakata et al., 1991). The amino acid sequence of the spinach leaf small subunit was deduced from a cDNA clone (Smith-White and Preiss, 1992) and N-terminal sequencing of the purified small subunit protein (Morell et al., 1987).

The exact features which indicates the correspondence between a specific subunit of the enzyme and isolated cDNA clones is uncertain except for those from maize endosperm (Preiss et al., 1990). A systematic comparison of the primary structures of ADPGlc PPase from diverse sources has been done by Smith-White and Preiss (1992). According to this study, the structures of the proteins from

higher-plants can be divided into two groups, which are based upon the two, small and large, subunits of ADPGlc PPase. The sequences of the small subunits are more homologous to each other whereas those of the large subunits are more divergent (Fig. 1). The amino acid sequences of the higher-plants enzymes are quite different to that of the enterobacterial enzymes, however, with some fairly conserved area.

So far, the results of comparison of the primary structures are consistent with the results derived from immunological studies. Thus, Western blotting seems to be a reliable and quick way for getting preliminary information about the protein structure. For example, the cyanobacterial enzyme was identified as homotetrameric protein like the enterobacterial enzyme (Iglesias et al., 1991). However, the cyanobacterial pyrophosphorylase is immunologically more related to the spinach leaf than to the *E. coli* enzyme. More precisely, the cyanobacterial enzyme is more related to the spinach leaf small subunit than to the large subunit (Iglesias et al., 1991).

Recently, the works of Iglesias et al. (1994) indicate that the algal ADPGlc PPase from *Chlamydomonas reinhardtii* is composed of two subunits with molecular mass of 50 and 53 kD, respectively, suggesting that the algal enzyme is a heterotetramer. Western blotting experiments indicate that the two subunits of the *Chlamydomonas reinhardtii* enzyme are structurally more similar to the small subunit than to the large subunit of the spinach leaf enzyme.

From the spectrum of subunit structure and immunological studies, it was hypothesizedthat during evolution there was duplication of the pyrophophorylase gene which further diverged to produce two different subunits (Iglesias and Preiss, 1992; Smith-White and Preiss, 1992). The absence of one subunit results in very low activity (Hannah and Nelson, 1976; Lin et al. 1988b; Iglesias et al., 1993) and

Fig. 1. Alignment of the primary structures of ADPGlc PPase from *E. coli*, *Salmonella typhimurium* (S.t.), maize sh-2 and bt-2 loci, wheat endosperm (we7), potato tuber large subunit (pot-l) and small subunit (pot-s), and rice seed. Gaps, indicated by dots, have been introduced to give better alignment. The number indicated is corresponding to the sequence of the *E. coli* enzyme (not including dots). The references of these sequence are described in Literature Review.

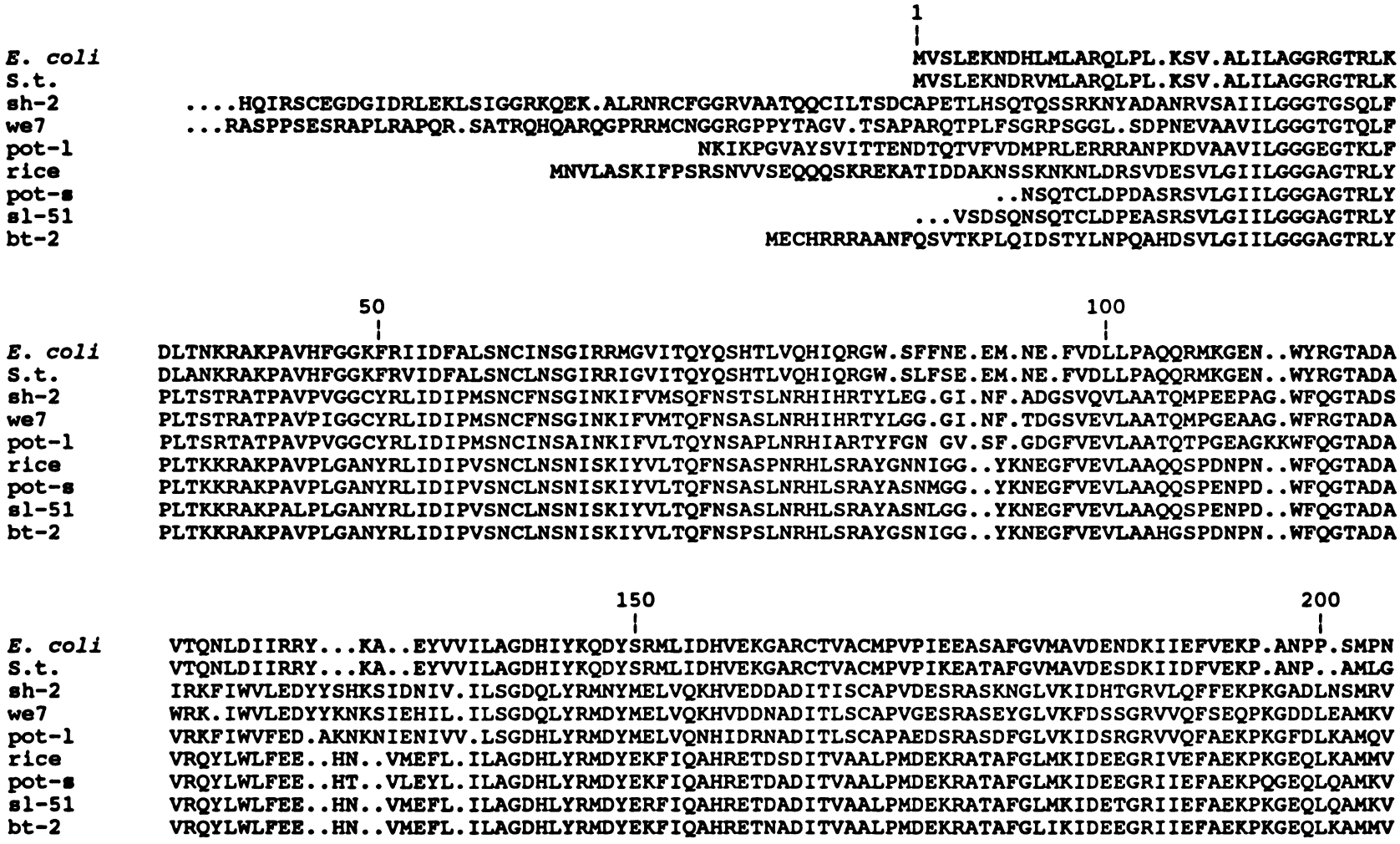
1
E. coli MVSLEKNDHLMLARQLPL.KSV.ALILAGGRGTRLK
S.t. MVSLEKNDRVMLARQLPL.KSV.ALILAGGRGTRLK
sh-2 ....HQIRSCEGDGIDRLEKLSIGGRKQEK.ALRNRCFGGRVAATQQCILTSDCAPETLHSQTQSSRKNYADANRVSAIILGGGTGSQLF
we7 ...RASPPSESRAPLRAPQR.SATRQHQARQGPRRMCNGGRGPPYTAGV.TSAPARQTPLFSGRPSGGL.SDPNEVAAVILGGGTGTQLF
pot-1 NKIKPGVAYSVITTENDTQTVFVDMPRLERRRANPKDVAAVILGGGEGTKLF
rice MNVLASKIFPSRSNVVSEQQQSKREKATIDDAKNSSKNKNLDRSVDESVLGIILGGGAGTRLY
pot-s ..NSQTCLDPDASRSVLGIILGGGAGTRLY
sl-51 ...VSDSQNSQTCLDPEASRSVLGIILGGGAGTRLY
bt-2 MECHRRRAANFQSVTKPLQIDSTYLNPQAHDSVLGIILGGGAGTRLY
50
100
E. coli DLTNKRAKPAVHFGGKFRIIDFALSNCINSGIRRMGVITQYQSHTLVQHIQRGW.SFFNE.EM.NE.FVDLLPAQQRMKGEN..WYRGTADA
S.t. DLANKRAKPAVHFGGKFRVIDFALSNCLNSGIRRIGVITQYQSHTLVQHIQRGW.SLFSE.EM.NE.FVDLLPAQQRMKGEN..WYRGTADA
sh-2 PLTSTRATPAVPVGGCYRLIDIPMSNCFNSGINKIFVMSQFNSTSLNRHIHRTYLEG.GI.NF.ADGSVQVLAATQMPEEPAG.WFQGTADS
we7 PLTSTRATPAVPIGGCYRLIDIPMSNCFNSGINKIFVMTQFNSASLNRHIHRTYLGG.GI.NF.TDGSVEVLAATQMPGEAAG.WFRGTADA
pot-1 PLTSRTATPAVPVGGCYRLIDIPMSNCINSAINKIFVLTQYNSAPLNRHIARTYFGN GV.SF.GDGFVEVLAATQTPGEAGKKWFQGTADA
rice PLTKKRAKPAVPLGANYRLIDIPVSNCLNSNISKIYVLTQFNSASPNRHLSRAYGNNIGG..YKNEGFVEVLAAQQSPDNPN..WFQGTADA
pot-s PLTKKRAKPAVPLGANYRLIDIPVSNCLNSNISKIYVLTQFNSASLNRHLSRAYASNMGG..YKNEGFVEVLAAQQSPENPD..WFQGTADA
sl-51 PLTKKRAKPALPLGANYRLIDIPVSNCLNSNISKIYVLTQFNSASLNRHLSRAYASNLGG..YKNEGFVEVLAAQQSPENPD..WFQGTADA
bt-2 PLTKKRAKPAVPLGANYRLIDIPVSNCLNSNISKIYVLTQFNSPSLNRHLSRAYGSNIGG..YKNEGFVEVLAAHGSPDNPN..WFQGTADA
150
200
E. coli VTQNLDIIRRY...KA..EYVVILAGDHIYKQDYSRMLIDHVEKGARCTVACMPVPIEEASAFGVMAVDENDKIIEFVEKP.ANPP.SMPN
S.t. VTQNLDIIRRY...KA..EYVVILAGDHIYKQDYSRMLIDHVEKGARCTVACMPVPIKEATAFGVMAVDESDKIIDFVEKP.ANP..AMLG
sh-2 IRKFIWVLEDYYSHKSIDNIV.ILSGDQLYRMNYMELVQKHVEDDADITISCAPVDESRASKNGLVKIDHTGRVLQFFEKPKGADLNSMRV
we7 WRK.IWVLEDYYKNKSIEHIL.ILSGDQLYRMDYMELVQKHVDDNADITLSCAPVGESRASEYGLVKFDSSGRVVQFSEQPKGDDLEAMKV
pot-1 VRKFIWVFED.AKNKNIENIVV.LSGDHLYRMDYMELVQNHIDRNADITLSCAPAEDSRASDFGLVKIDSRGRVVQFAEKPKGFDLKAMQV
rice VRQYLWLFEE..HN..VMEFL.ILAGDHLYRMDYEKFIQAHRETDSDITVAALPMDEKRATAFGLMKIDEEGRIVEFAEKPKGEQLKAMMV
pot-s VRQYLWLFEE..HT..VLEYL.ILAGDHLYRMDYEKFIQAHRETDADITVAALPMDEKRATAFGLMKIDEEGRIIEFAEKPQGEQLQAMKV
sl-51 VRQYLWLFEE..HN..VMEFL.ILAGDHLYRMDYERFIQAHRETDADITVAALPMDEKRATAFGLMKIDETGRIIEFAEKPKGEQLQAMKV
bt-2 VRQYLWLFEE..HN..VMEFL.ILAGDHLYRMDYEKFIQAHRETNADITVAALPMDEKRATAFGLIKIDEEGRIIEFAEKPKGEQLKAMMV

Fig. 1

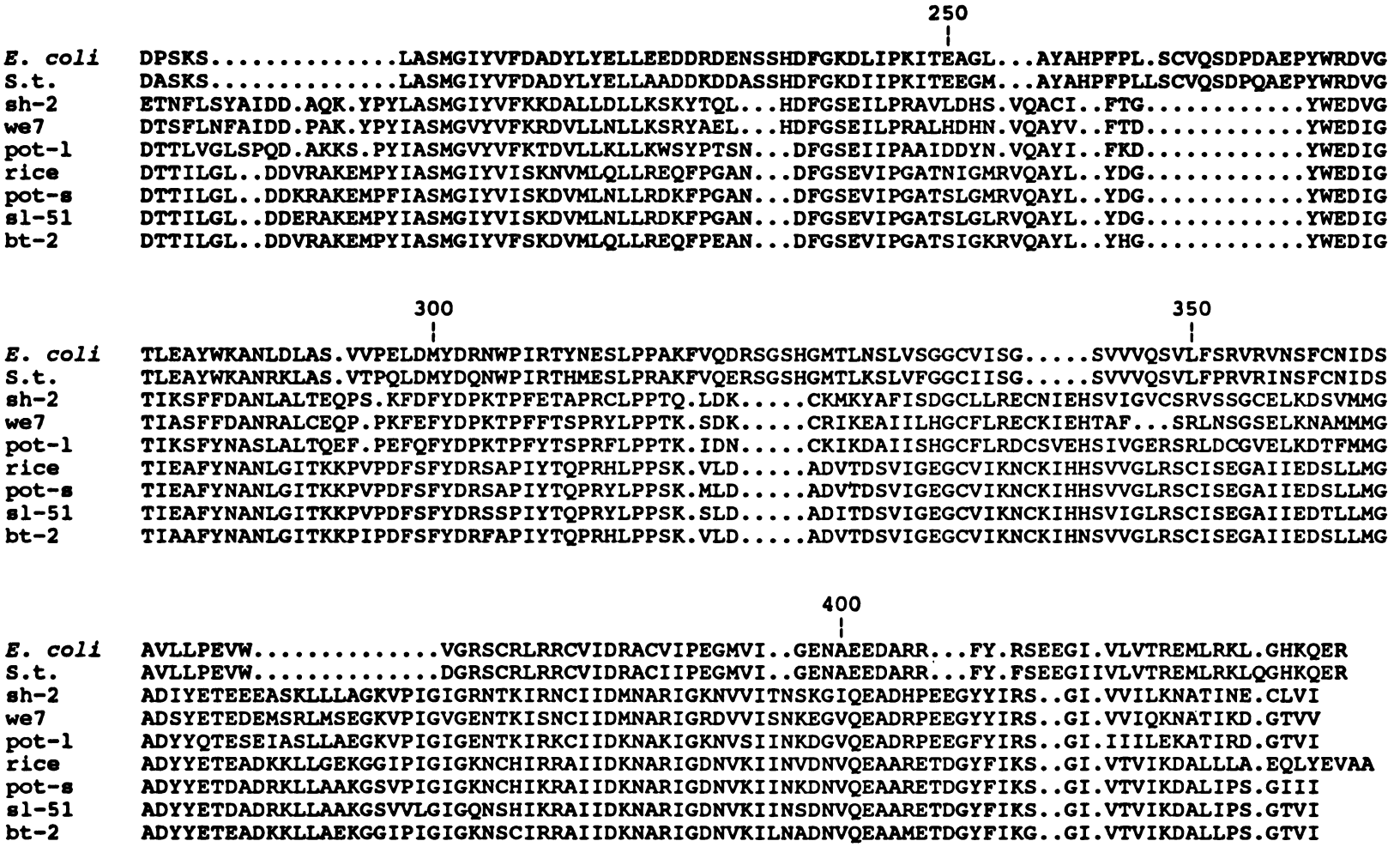
250
E. coli DPSKS.....LASMGIYVFDADYLYELLEEDDRDENSSHDFGKDLIPKITEAGL...AYAHPFPL.SCVQSDPDAEPYWRDVG
S.t. DASKS.....LASMGIYVFDADYLYELLAADDKDDASSHDFGKDIIPKITEEGM...AYAHPFPLLSCVQSDPQAEPYWRDVG
sh-2 ETNFLSYAIDD.AQK.YPYLASMGIYVFKKDALLDLLKSKYTQL...HDFGSEILPRAVLDHS.VQACI..FTG.....YWEDVG
we7 DTSFLNFAIDD.PAK.YPYIASMGVYVFKRDVLLNLLKSRYAEL...HDFGSEILPRALHDHN.VQAYV..FTD.....YWEDIG
pot-1 DTTLVGLSPQD.AKKS.PYIASMGVYVFKTDVLLKLLKWSYPTSN...DFGSEIIPAAIDDYN.VQAYI..FKD.....YWEDIG
rice DTTILGL..DDVRAKEMPYIASMGIYVISKNVMLQLLREQFPGAN...DFGSEVIPGATNIGMRVQAYL..YDG.....YWEDIG
pot-s DTTILGL..DDKRAKEMPFIASMGIYVISKDVMLNLLRDKFPGAN...DFGSEVIPGATSLGMRVQAYL..YDG.....YWEDIG
sl-51 DTTILGL..DDERAKEMPYIASMGIYVISKDVMLNLLRDKFPGAN...DFGSEVIPGATSLGLRVQAYL..YDG.....YWEDIG
bt-2 DTTILGL..DDVRAKEMPYIASMGIYVFSKDVMLQLLREQFPEAN...DFGSEVIPGATSIGKRVQAYL..YHG.....YWEDIG
300
350
E. coli TLEAYWKANLDLAS.VVPELDMYDRNWPIRTYNESLPPAKFVQDRSGSHGMTLNSLVSGGCVISG.....SVVVQSVLFSRVRVNSFCNIDS
S.t. TLEAYWKANRKLAS.VTPQLDMYDQNWPIRTHMESLPRAKFVQERSGSHGMTLKSLVFGGCIISG.....SVVVQSVLFPRVRINSFCNIDS
sh-2 TIKSFFDANLALTEQPS.KFDFYDPKTPFETAPRCLPPTQ.LDK.....CKMKYAFISDGCLLRECNIEHSVIGVCSRVSSGCELKDSVMMG
we7 TIASFFDANRALCEQP.PKFEFYDPKTPFFTSPRYLPPTK.SDK.....CRIKEAIILHGCFLRECKIEHTAF...SRLNSGSELKNAMMMG
pot-1 TIKSFYNASLALTQEF.PEFQFYDPKTPFYTSPRFLPPTK.IDN.....CKIKDAIISHGCFLRDCSVEHSIVGERSRLDCGVELKDTFMMG
rice TIEAFYNANLGITKKPVPDFSFYDRSAPIYTQPRHLPPSK.VLD.....ADVTDSVIGEGCVIKNCKIHHSVVGLRSCISEGAIIEDSLLMG
pot-s TIEAFYNANLGITKKPVPDFSFYDRSAPIYTQPRYLPPSK.MLD.....ADVTDSVIGEGCVIKNCKIHHSVVGLRSCISEGAIIEDSLLMG
sl-51 TIEAFYNANLGITKKPVPDFSFYDRSSPIYTQPRYLPPSK.SLD.....ADITDSVIGEGCVIKNCKIHHSVIGLRSCISEGAIIEDTLLMG
bt-2 TIAAFYNANLGITKKPIPDFSFYDRFAPIYTQPRHLPPSK.VLD.....ADVTDSVIGEGCVIKNCKIHNSVVGLRSCISEGAIIEDSLLMG
400
E. coli AVLLPEVW.....VGRSCRLRRCVIDRACVIPEGMVI..GENAEEDARR...FY.RSEEGI.VLVTREMLRKL.GHKQER
S.t. AVLLPEVW.....DGRSCRLRRCVIDRACIIPEGMVI..GENAEEDARR...FY.FSEEGIIVLVTREMLRKLQGHKQER
sh-2 ADIYETEEEASKLLLAGKVPIGIGRNTKIRNCIIDMNARIGKNVVITNSKGIQEADHPEEGYYIRS..GI.VVILKNATINE.CLVI
we7 ADSYETEDEMSRLMSEGKVPIGVGENTKISNCIIDMNARIGRDVVISNKEGVQEADRPEEGYYIRS..GI.VVIQKNATIKD.GTVV
pot-1 ADYYQTESEIASLLAEGKVPIGIGENTKIRKCIIDKNAKIGKNVSIINKDGVQEADRPEEGFYIRS..GI.IIILEKATIRD.GTVI
rice ADYYETEADKKLLGEKGGIPIGIGKNCHIRRAIIDKNARIGDNVKIINVDNVQEAARETDGYFIKS..GI.VTVIKDALLLA.EQLYEVAA
pot-s ADYYETDADRKLLAAKGSVPIGIGKNCHIKRAIIDKNARIGDNVKIINKDNVQEAARETDGYFIKS..GI.VTVIKDALIPS.GIII
sl-51 ADYYETDADRKLLAAKGSVVLGIGQNSHIKRAIIDKNARIGDNVKIINSDNVQEAARETDGYFIKS..GI.VTVIKDALIPS.GTVI
bt-2 ADYYETEADKKLLAEKGGIPIGIGKNSCIRRAIIDKNARIGDNVKILNADNVQEAAMETDGYFIKG..GI.VTVIKDALLPS.GTVI

Fig. 1 (Cont.)

altered kinetic constants (Li and Preiss, 1992). Recently, it was shown that the recombinant enzyme containing only the putative full-length small subunit of potato tuber enzyme has a specific activity similar to the native enzyme with two subunit (Fu, Ballicora and Preiss, unpublished data). However, this enzyme requires a much higher 3PGA concentration for optimal activity compared to the native enzyme. With only large subunit the potato enzyme seems to be relatively inactive (Iglesias et al., 1993). It is possible that the large subunit plays a regulatory role, in combination with the small subunit, restoring the sensitivity of the enzyme toward its activator.

3.2. Structures of the substrate and regulator binding sites:

3.2.1. The nature of the substrate and regulator binding:

Steady-state kinetics (Paule and Preiss, 1971; Kleczkowski et al., 1993) and equilibrium binding studies (Haugen and Preiss, 1979) have been performed to elucidate the mechanism of substrates and regulators binding to ADPGlc PPase. The substrates have been shown to bind in an ordered mechanism, *i.e.* ATP binds first, followed by the binding of glucose-1-P. Products are also released in order with pyrophosphate released first, followed by ADP-glucose. No exchange of glucose-1-P with ADP-glucose or pyrophosphate with ATP occurs in the absence of the second substrate (Gentner and Preiss, unpublished results).

Based on the results of equilibrium binding experiments on the *E. coli* enzyme (Haugen and Preiss, 1979), there appears to be four binding sites per tetramer for substrates, inhibitors, and activators. Each of the four identical subunits contains up to three potential sites, one for each type of ligands. The experiments show that chromium ATP (CrATP, an analogous inhibitor of MgATP), ATP and the activator, fructose-1,6-P_2, bind to only half of the expected

sites in the tetrameric enzyme, while ADP-glucose and the inhibitor, AMP, bind to four sites/tetrameric enzyme. Since the experimental conditions only measure high affinity sites, additional low affinity sites may exist for ATP and fructose-1,6-P_2. Nevertheless, it appears that the enzyme contains nonequivalent binding sites for certain compounds on identical subunits. In the presence of glucose-1-P, however, CrATP binds to four sites/tetramer (under the binding conditions the conversion of glucose-1-P and CrATP to ADP-glucose can be neglected). Thus, it was suggested that in the catalytically functioning enzyme there is interaction between the ATP and glucose-1-P binding sites. The enzymes first binds two ATP molecular/tetramer. This allows the binding of glucose-1-P, which do not bind to the enzyme in the absence of ATP, then permitting in turn the additional binding of two more ATP molecules.

Despite the fact that fructose-1,6-P_2 shows half of the sites binding, the other two activators, pyridoxal-P and hexanediol-1,6-P_2, display four sites binding. Incorporation of pyridoxal-P into the allosteric site of *E. coli* ADPGlc PPase by reduction with $NaBH_4$, however, show that after covalent attachment of pyridoxal-P to only two of the four sites, the enzyme is fully activated (Parsons and Preiss, 1978). It was therefore suggested that any activator probably need only be bound to two of the four sites for maximal stimulation of the enzyme (Haugen and Preiss, 1979). The higher plant enzymes, which contain two dissimilar subunits, are also likely to display this half of the sites binding property.

3.2.2. Primary structures of the substrate and regulator binding sites:

Cloning and sequencing of the various ADPGlc PPase genes have led to structure/function studies to elucidate the location of substrate and regulator binding sites. This work has mainly proceeded by chemical modification using

substrate or activator analogues, and site-directed mutagenesis of cloned ADPGlc PPase. Further insight into structure/function relationship has been aided by the cloning and sequencing of available mutant *E. coli* ADPGlc PPase with altered regulatory properties.

Pyridoxal-P has been used to modify lysyl residue of protein. A Schiff-base is formed between pyridoxal-P and the ϵ-amino group of lysine and can be converted into covalent bond by reduction with sodium borohydride. This compound has been used as a site specific probe to identify lysyl residues in enzymes that possess binding sites for sugar phosphates. These enzymes include fructose-1,6-diphosphatase (Marcus and Herbert, 1968), glyceraldehyde-3-P dehydrogenase (Ronchi et al., 1970), phosphofructokinase (Uyeda, 1969), 6-phosphogluconate dehydrogenase (Rippa et al., 1967), and phosphoglucose isomerase (Schnackerz and Noltmann, 1971).

Pyridoxal-P is an activator of many bacterial and plant ADPGlc PPases (Parsons and Preiss, 1978a; Preiss et al., 1987b; Iglesias et al., 1991). Chemical modification of *E. coli* ADPGlc PPase with [^{3}H]pyridoxal-P resulted in modification of two distinct lysyl residues, Lys_{39} and Lys_{195} (Parsons and Preiss, 1978a; Parsons and Preiss, 1978b). The presence of ADP-glucose and $MgCl_2$ prevents pyridoxylation of Lys_{195} while fructose-1,6-P_2 protects Lys_{39}. Modification of Lys_{39} results in an enzyme with high activity even in the absence of fructose-1,6-P_2. Thus, it was suggested that Lys_{39} is located in the allosteric activator site. On the other hand, modification of Lys_{195} results in a loss of catalytic activity. This lysyl residue is probably involved either in the binding of the substrates ADP-glucose, α-glucose-1-P, or PPi, or in the catalytic mechanism of the enzyme.

Fig. 2 shows the location and alignment of the amino acid sequences near

Lys_{39} and Lys_{195} with other known sequences. The putative activator binding region of the *E. coli* enzyme, protected by fructose-1,6-P_2, is highly conserved in all the ADPGlc PPase sequences known to date, although, Lys_{39} is not conserved in all the sequences. The site protected by ADP-glucose is not as conserved, but the Lys_{195} is conserved in all the sequences.

Site-directed mutagenesis experiments have been performed to further study the roles of these lysyl residues. Substitution of Lys_{39} of the *E. coli* enzyme with glutamic acid caused a decrease in apparent affinity for the allosteric activators (Gardiol and Preiss, 1990). The *Ka* values for the major activators of the K39E enzyme, 2-PGA, pyridoxal-P, and fructose-1,6-P_2 were 5-, 9-, and 23-fold higher, respectively, than those for the wild-type enzyme. The level of activation of the K39E mutant enzyme by the above activators was only approximately 2-fold compared to 15- to 28-fold respectively, for the wild-type enzyme.

Substitution of Lys_{195} of the *E. coli* enzyme with glutamate generated the largest effect on the binding of glucose-1-P (Hill et al., 1991). The Km value of the mutant enzyme for glucose-1-P is 12,000-fold greater than that of the wild-type enzyme. Although ADP-glucose protects this residue from being pyridoxylated, the apparent affinity for this substrate decreased only 6-fold, an effect much smaller than that for glucose-1-P. The kinetic constants for ATP, Mg^{2+}, and allosteric activator, fructose-1,6-P_2, are relatively unchanged. The catalytic efficiency of the mutant enzyme is similar to that measured for the wild-type enzyme. According to the kinetic studies on a series of Lys_{195} mutants, it was shown that both the size and charge of lysine are required for proper binding of glucose-1-P at the catalytic site. The binding motif, -F-X-E-K-P-, has also been found in other sugar-nucleotide synthetases, and is probably involved in the general binding of sugar-phosphate (Jiang et al., 1991; Stevenson et al., 1991;

Fig. 2. Amino acid sequences of the phosphopyridoxylated peptides of *E. coli* ADPGlc PPase and the comparison with the other known sequences. Nomenclature is the same as in Fig. 1. The lysyl residues, modified by pyridoxal-P, are marked with *. The numbers indicated correspond to the *E. coli* sequence. Gaps have been introduced to give better alignment as indicated in Fig. 1.

Fructose-1,6-P_2-protected site:

	33 | *
E. coli	LKDLTNKRAKPAV
S.t.	LKDLANKRAKPAV
sh-2	LFPLTSTRATPAV
we7	LFPLTSTRATPAV
pot-1	LFPLTSRTATPAV
rice	LYPLTKKRAKPAV
pot-s	LYPLTKKRAKPAV
sl-51	LYPLTKKRAKPAV
bt-2	LYPLTKKRAKPAV

ADP-glucose-protected site:

	182 * 200 | |
E. coli	AVDENDKIIEFVEKP.ANPP
S.t.	AVDESDKIIDFVEKP.ANPA
sh-2	KIDHTGRVLQFFEKPKGADL
we7	KFDSSGRVVQFSEQPKGDDL
pot-1	KIDSRGRVVQFAEKPKGFDL
rice	KIDEEGRIVEFAEKPKGEQL
pot-s	KIDEEGRIIEFAEKPQGEQL
sl-51	KIDETGRIIEFAEKPKGEQL
bt-2	KIDEEGRIIEFAEKPKGEQL

Fig. 2

Köplin et al., 1992; Marolda et al., 1993; May et al., 1994).

A similar approach using [^{3}H]pyridoxal-P as a modifier was also conducted for the spinach leaf ADPGlc PPase (Morell et al., 1988; Ball et al., 199?). The spinach enzyme consists of two subunits, 51 and 54 kDa. Both subunits were pyridoxylated. When the enzyme was modified with 50 μM [^{3}H]pyridoxal-P, about 62 % of the radioactivity was associated with the 54 kDa subunit and 38 % with the 51 kDa. The modified enzyme is less dependent on the presence of the activator for activity, and is more resistant to phosphate inhibition than the unmodified enzyme (Morell et al., 1988). The enzyme was protected the most by the presence of 3-P-glycerate or Pi. The substrates did not provided much protection (Morell et al., 1988). From this, the authors suggested that pyridoxal-P is covalently bound to the allosteric activator site. Four different pyridoxylated sites were identified, one in the 51 kDa (site-1) (Morell et al., 1988), and three in the 54 kDa subunit (site-2,3,4) (Preiss et al., 1993). The sequences of these sites have been determined and are indicated in Fig. 3. The amounts of [^{3}H]pyridoxal-P (PLP) incorporation are 35, 28, 16, and 21 % for PLP-site-1, site-2, site-3 and site-4, respectively. All the pyridoxal-P labeled sites are protected in the presence of 3-P-glycerate and only PLP-site-1 and site-3 are protected by Pi. The locations of PLP-site-1 and site-2 are essentially the same, which is near the C-terminus. Their sequences also are very similar.

The arginine-specific reagent phenylglyoxal has been used to covalently modify ADPGlc PPases from *E. coli* (Carlson and Preiss, 1982), spinach leaf (Ball and Preiss, 1992) and *Synechocystis* (Iglesias et al., 1992). From these studies, phenylglyoxal appears to interfere with the allosteric regulation of the enzyme. However, the arginine residue(s), modified by [^{14}C]phenylglyoxal, has not been identified.

Fig. 3. Amino acid sequences of the phosphopyridoxylated peptides of spinach leaf ADPGlc PPase and aligned with the other known sequences. Nomenclature is the same as in Fig.1. Site-1 is from the spinach small subunit. Site-2,3,4 are from the spinach large subunit. The lysyl residues, modified by pyridoxal-P, are marked with *. The numbers indicated correspond to the *E. coli* sequence. Gap have been introduced to give better alignment as indicated in Fig. 1.

PLP-Site-1

```
                    412
                     ¦      *
sl-51                SGIVTVIKDALIPSGTVI
rice                 SGIVTVIKDALLLAEQLYEVYY
pot-s                SGIVTVIKDALIPSGIII
bt-2                 SGIVTVIKDALLPSGTVI
E. coli              EGIVLVTREMLRKLGHKQER
```

PLP-Site-2

```
                    412
                     ¦      *
sl-54                SGITVIFKNATIKDGVV
pot-1                SGIIIILEKATIRDGTVI
sh-2                 SGIVVILKNATINECLVI
we7                  SGIVVIQKNATIKDGTVV
```

PLP-Site-3

```
                    379
                     ¦      *
sl-54                IKDAIIDKNAR
pot-1                IRKCIIDKNAR
sh-2                 IRNCIIDMNAR
we7                  ISNCIIDMNAR
E. coli              LRRCVIDRACV
```

PLP-Site-4

```
                              123
                     *         ¦
sl-54                KWFQGTADAVRQ
pot-1                KWFQGTADAVRK
sh-2                 GWFQGTQDSIRK
we7                  GWFRGTADAWRK
E. coli              .WYRGTADAVTQ
```

Fig. 3

The photoaffinity labeling agents 8-azido-ATP and 8-azido-ADP-glucose were verified as substrate site specific probes of the *E. coli* ADPGlc PPase (Lee *et al*, 1986). In the absence of UV light (254 nm), the substrate analogs can be utilized as substrates by ADPGlc PPase. However, the maximal activity observed with azido-ATP and azido-ADP-glucose are only 0.3 and 0.9 %, respectively, of those observed with ATP and ADP-glucose. Both compounds are the competitive inhibitors of the enzyme with respect to the natural substrates. Therefore, it was suggested that azido-ATP and azido-ADP-glucose interact specifically at the substrate site of *E. coli* ADPGlc PPase. In the presence of light, azido-ATP and azido-ADP-glucose are covalently incorporated into the enzyme, which can be prevented by the presence of ATP, ADP-glucose, and inhibitor, AMP.

The tryptic peptides that were labeled by azido-ADP-[^{14}C]glucose were subsequently purified and sequenced (Lee and Preiss, 1986). ADPGlc-Site-1 is the major binding region of azido-ADP-[^{14}C]glucose and is highly conserved in all sequences known to date (Fig. 4). ADPGlc-Site-2 accounts for 20 % of the total radioactive peptides recovered. The sequence is not as conserved as site-1. However, Lys_{195}, which is necessary for glucose-1-P binding, is conserved in the alignment (Fig. 4).

Although the importance is currently unknown, one should keep in mind that the sequences and locations of ADPGlc-site-1 and site-3 are similar to that of the PLP-site-4 and site-3 of the spinach enzyme, respectively (Fig. 3).

The inhibitor binding site of the *E. coli* enzyme was identified by using an inhibitor analogue, 8-azido-AMP (Larsen et al., 1986). The major 8-azido-AMP binding site was associated with Tyr114, which is located within the major azido-ADP-glucose-incorporated site (Fig. 4). This suggests that the binding sites for AMP, ATP and ADP-glucose might be overlapping.

Fig. 4. Amino acid sequences of azido-ADP-[^{14}C]glucose-incorporated peptides of *E. coli* ADPGlc PPase and the comparison with the other known sequences. Nomenclature is the same as in Fig.1. The numbers indicated correspond to the *E. coli* sequence. Gaps have been introduced to give better alignment as indicated in Fig. 1.

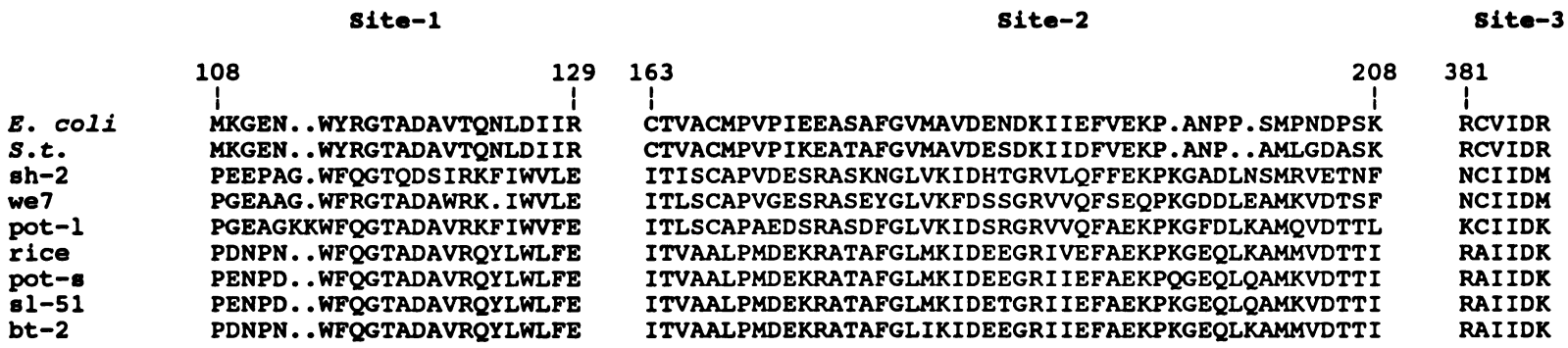
Site-1
Site-2
Site-3
108
129
163
208
381
E. coli MKGEN..WYRGTADAVTQNLDIIR CTVACMPVPIEEASAFGVMAVDENDKIIEFVEKP.ANPP.SMPNDPSK RCVIDR
S.t. MKGEN..WYRGTADAVTQNLDIIR CTVACMPVPIKEATAFGVMAVDESDKIIDFVEKP.ANP..AMLGDASK RCVIDR
sh-2 PEEPAG.WFQGTQDSIRKFIWVLE ITISCAPVDESRASKNGLVKIDHTGRVLQFFEKPKGADLNSMRVETNF NCIIDM
we7 PGEAAG.WFRGTADAWRK.IWVLE ITLSCAPVGESRASEYGLVKFDSSGRVVQFSEQPKGDDLEAMKVDTSF NCIIDM
pot-1 PGEAGKKWFQGTADAVRKFIWVFE ITLSCAPAEDSRASDFGLVKIDSRGRVVQFAEKPKGFDLKAMQVDTTL KCIIDK
rice PDNPN..WFQGTADAVRQYLWLFE ITVAALPMDEKRATAFGLMKIDEEGRIVEFAEKPKGEQLKAMMVDTTI RAIIDK
pot-s PENPD..WFQGTADAVRQYLWLFE ITVAALPMDEKRATAFGLMKIDEEGRIIEFAEKPQGEQLQAMKVDTTI RAIIDK
sl-51 PENPD..WFQGTADAVRQYLWLFE ITVAALPMDEKRATAFGLMKIDETGRIIEFAEKPKGEQLQAMKVDTTI RAIIDK
bt-2 PDNPN..WFQGTADAVRQYLWLFE ITVAALPMDEKRATAFGLIKIDEEGRIIEFAEKPKGEQLKAMMVDTTI RAIIDK

Fig. 4

Chemical mutagenesis experiments on *E. coli* have been conducted to randomly produce mutant strains with altered glycogen accumulation (Govons et al., 1969; Preiss, et al. 1976; Creuzat-Sigal et al., 1972). Characterization of the ADPGlc PPase from some of these mutants shows altered regulatory properties of the enzyme. For *E. coli* B mutant stains SG5 (Govons et al., 1969; 1973) and CL1136 (Preiss et al., 1969) and *E. coli* K12 mutant strain 618 (Cattaneo et al., 1969; Creuzat-Sigal et al., 1972), ADPGlc PPase is "superactive" having higher activity in the absence of the allosteric activator, fructose-1,6-P_2, and is less sensitive to the inhibition of AMP than the wild-type enzyme. The genes of these "superactive" mutant enzymes have been cloned from the *E. coli* mutant strains. It was shown that single amino acid substitutions occurred at position 67 of mutant CL1136, arginine to cysteine (Ghosh et al., 1992), position 295 of mutant SG5, proline to serine (Meyer et al., 1992), and position 336 of mutant 618, glycine to aspartate (Kumar et al., 1989). ADPGlc PPase from *E. coli* B mutant strain SG14, which has lower apparent affinities for substrates, activator, fructose-1,6-P_2, and the inhibitor, AMP, has a single amino acid substitution at position 44, alanine to threonine (Meyer et al., 1993). The alanine residue is close to the activator site determined by reductive phosphopyridoxylation and is highly conserved in all sequences known to date (Fig. 2).

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CHAPTER II

MOLECULAR CLONING AND EXPRESSION OF THE GENE ENCODING ADP-GLUCOSE PYROPHOSPHORYLASE FROM CYANOBACTERIUM *ANABAENA* sp. STRAIN PCC 7120

ABSTRACT

Previous studies indicated that ADP-glucose pyrophosphorylase From the cyanobacterium *Anabaena* sp. strain PCC 7120 is more similar to higher plant than to enteric bacterial enzymes in antigenicity and allosteric properties. In this paper, we report the isolation of the *Anabaena* ADP-glucose pyrophosphorylase gene and its expression in *Escherichia coli*. The gene we isolated from a genomic library utilizes GTG as the start codon and codes for a protein of 48,341 daltons which is in agreement with the molecular mass determined by SDS-PAGE for the *Anabaena* enzyme. The deduced amino acid sequence is 63, 54, and 33 % identical to the rice endosperm small subunit, maize endosperm large subunit, and the *E. coli* sequences, respectively. Southern analysis indicated that there is only one copy of this gene in the *Anabaena* genome. The cloned gene encodes an active ADP-glucose pyrophosphorylase when expressed in an *E. coli* mutant strain AC70R1-504 which lacks endogenous activity of the enzyme. The recombinant enzyme is activated and inhibited primarily by 3-phosphoglycerate and Pi, respectively, as is the native *Anabaena* ADP-glucose pyrophosphorylase. Immunological and other biochemical studies further confirmed the recombinant enzyme to be the *Anabaena* enzyme.

INTRODUCTION

ADP-glucose pyrophosphorylase (EC 2.7.7.27) catalyzes the reversible synthesis of ADP-glucose and pyrophosphate from ATP and α-glucose-1-P. It is a pivotal enzyme in the synthesis of starch in plants and glycogen in bacteria for generating the glucosyl donor, ADP-glucose (29-31, 33). Studies based on a wide range of sources have shown that ADP-glucose pyrophosphorylase is commonly modulated by allosteric effectors and is tetrameric in protein structure (29, 30). Major differences in allosteric properties and protein structure, however, exist between the higher plant and bacterial enzymes. In *E. coli*, ADP-glucose pyrophosphorylase is mainly activated by fructose-1,6-bisphosphate (FBP) and inhibited by AMP and ADP (24, 30). But for all the higher plant enzymes that have been studied, 3-phosphoglycerate (3PGA) and Pi are the most effective activator and inhibitor, respectively (29, 31, 33). The molecular mass of ADP-glucose pyrophosphorylase from different sources, either bacteria or plants, has been determined to be about 200 kDa with four subunits. In enteric bacteria, ADP-glucose pyrophosphorylase is a homotetramer encoded by a single gene locus (30), whereas the enzymes from higher plants are more complex having heterotetrameric structure with two dissimilar subunits (10, 17, 20, 23, 24, 31, 32).

Cyanobacteria are considered as phylogenetic intermediates between plants and bacteria. Their photosynthetic function is similar to that of the higher plant chloroplast, but they synthesize glycogen as their major carbohydrate reserve like bacteria (37). ADP-glucose pyrophosphorylase from the cyanobacterium *Anabaena* sp. strain PCC 7120 has been purified and characterized previously (15). As expected, the cyanobacterial enzyme has characteristics intermediate to

that of the higher plant and *E. coli* enzymes. The ADP-glucose pyrophosphorylase from *Anabaena* sp. strain PCC 7120 is similar to the plant enzymes in being allosterically activated by 3PGA and inhibited by Pi, but is homotetrameric as are the bacterial enzymes.

To obtain insight into the structure/function relationships and the evolutionary phylogeny of ADP-glucose pyrophosphorylase, we isolated a full length genomic clone from *Anabaena* sp. strain PCC 7120. The deduced amino acid sequence of the *Anabaena* gene is compared to higher plant and bacterial sequences. The *Anabaena* gene was expressed in the *E. coli* mutant strain AC70R1-504 which lacks endogenous ADP-glucose pyrophosphorylase activity. The recombinant enzyme from the transformed cells was subsequently characterized as *Anabaena* ADP-glucose pyrophosphorylase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media

E. coli strains HB101 and DH5αF' were used as hosts for the cosmid library and pUC119 constructions, respectively (5, 21). *E. coli* strain AC70R1-504, a mutant lacking endogenous ADP-glucose pyrophosphorylase activity (7), was used for the expression of *Anabaena* ADP-glucose pyrophosphorylase gene. *E. coli* strains were usually grown in LB medium supplemented with ampicillin (100 μg/ml) for selection and maintenance of plasmids (21). Cultures were grown at 37 °C overnight on a rotary shaker. For gene expression, enriched medium was used to grow the transformed AC70R1-504 cells. The enriched medium contained 1.1 % K_2HPO_4, 0.85 % KH_2PO_4, 0.6 % yeast extract, 0.2 % glucose and 100 μg ampicillin/mL (28).

DNA isolation and PCR amplification

Anabaena sp. strain PCC 7120 was grown in liquid BG-11 medium supplemented with 5 mM Tes buffer, pH 8.0 (8). *Anabaena* genomic DNA was prepared from cells at late-log phase by the method described by Porter (27). Minipreparations of plasmids were performed by the alkaline lysis method (21). DNA fragments were purified by excision from agarose gels followed by electroelution into dialysis bags, extraction with phenol/chloroform, and ethanol precipitation (21). DNA amplification with degenerate primers using Taq DNA polymerase was performed according to Compton (9). One nanogram of *Anabaena* genomic DNA was initially amplified for five cycles at an annealing temperature of 37 °C followed by 35 cycles at 55 °C. A specific PCR product of 250-bp was purified from a 1.6 % agarose gel and repaired at the 3' termini

with Klenow fragment before ligating the blunt-ended fragment into pUC119 (36).

Gene cloning and sequence determination

A probe derived from the 250-bp PCR fragment was labeled with [^{32}P]dCTP by random primed labeling (21) and used to probe total *Anabaena* sp. strain PCC 7120 DNA. The hybridization signals specifically corresponded to a 1.8 kb *Hin*dIII fragment and a 15 kb *Xba*I fragment. The probe was then used to screen a genomic library by colony hybridization (21). The genomic library was constructed in the cosmid vector pWB79 as previously described (5). Plasmid DNA from a positive clone which contained both the 1.8 kb *Hin*dIII and 15 kb *Xba*I fragments that hybridized to the PCR probe was isolated for further subcloning. Overlapping subclones were generated by using restriction sites or by the unidirectional deletion method of Dale (11). The sequence of the gene was determined on both strands by the chain termination method (35). The nucleotide sequence data were analyzed with the Genetic Computer Group's sequence analysis software of University of Wisconsin (12).

Southern analysis

Southern blotting was performed using 2 μg of total *Anabaena* sp. strain PCC 7120 DNA for each restriction digestion. The digested DNA was resolved on a 0.9 % agarose gel and transferred to a nitrocellulose filter by a capillary method (40). A 1.3 kb fragment comprising the ADP-glucose pyrophosphorylase gene was generated by PCR and used for probing the filter with 50 % formamide at 40 °C overnight (21). The filter was subsequently washed with three changes of 1 x SSC and 0.1 % SDS at 40 °C for 12 min and exposed to an X-ray film for radiography.

Construction of expression plasmids

Plasmids derived from the positive clones were digested with *Eco*RI to yield a 5.5 kb fragment (Fig. 1), which contains the entire coding region for ADP-glucose pyrophosphorylase, as well as a putative ribosome binding site and promoter sequence. The 5.5 kb fragment was introduced into pUC119 vector at the polycloning site in both orientations with respect to the *lac* promoter. The plasmids in reverse or correct orientations were designated as pAnaE3a or pAnaE3b, respectively. *E. coli* strain AC70R1-504 was used as host for the expression plasmids.

Assay of ADP-glucose pyrophosphorylase

The activity of ADP-glucose pyrophosphorylase was determined either in pyrophosphorolysis (assay A) or synthesis (assay B) directions.

Assay A. Pyrophosphorolysis of ADP-glucose was followed by the formation of [^{32}P]ATP in the presence of [^{32}P]PPi. The reaction mixture contained 20 μmol Hepes-NaOH buffer (pH 7.0), 2 μmol $MgCl_2$, 0.5 μmol ADP-glucose, 0.5 μmol [^{32}P]PPi (ca. 3000cpm/nmol), 50 μg BSA, 2.5 μmol NaF, 1 μmol 3PGA and enzyme preparation in a total volume of 0.24 mL. The reaction was carried out at 37°C for 10 min and terminated by adding 3 mL of cold 5 % TCA. The [^{32}P]ATP formed was measured as described previously (23). A unit of ADP-glucose pyrophosphorylase activity is defined as that amount of enzyme catalyzing synthesis of 1 μmol ATP/min under the reaction conditions described.

Assay B. Synthesis of ADP-glucose was measured as previously described (14). The reaction mixture contained 20 μmol Hepes-NaOH buffer (pH 8.0), 50 μg of BSA, 1.5 μmol of $MgCl_2$, 0.5 μmol of ATP, 0.1 μmol of α-[^{14}C]glucose-1-P (about 1000 cpm/nmol) and 0.15 unit of inorganic pyrophosphatase in a final

volume of 0.2 mL. Assays were initiated by addition of enzyme. The reaction mixture was incubated at 37 °C for 10 min and terminated by heating in boiling water bath for 30 sec.

Protein assay

Protein concentration was determined by using bicinchoninic acid reagent (38) with BSA as the standard.

Partial purification of the recombinant ADP-glucose pyrophosphorylase

All purification procedures, except where noted, were performed at 0-4 °C. Assay A was used to monitor enzyme activity throughout the purification. *E. coli* mutant strain AC70R1-504 cells containing pAnaE3b were grown at 37 °C overnight in 1 L enriched medium containing 0.2 % glucose and 100 μg ampicillin/mL to stationary phase and then harvested by centrifugation. Crude extract was prepared by suspension of 4.5 g cell paste in 20 mL of 20 mM potassium phosphate buffer, pH 7.5, containing 5 mM DTT and 1 mM EDTA (buffer A) followed by sonication, and centrifugation for 10 min at 12,000g. The crude extract was then brought to 60 °C within 5 min in a 125 mL flask and kept at the same temperature for an additional 4 min, then cooled on ice. The sample was centrifuged at 20,000g for 15 min. The pellet was washed once with 2 mL buffer A and centrifuged as above. The supernatants were combined and absorbed onto a DEAE-sepharose Fast-Flow column (1.5 x 13 cm, 0.18 mL bed volume/mg of protein), equilibrated with 20 mM potassium phosphate buffer, pH 7.5, containing 2 mM DTT. The enzyme was eluted with a linear gradient containing 4 bed volumes of the above buffer in mixing chamber and 4 bed volumes of 50 mM potassium phosphate, pH 6.0, containing 2 mM DTT and 0.4 M KCl in the

reservoir chamber. The fractions containing high specific activity were pooled and concentrated with PM-30 membrane in an Amicon concentrator.

Antibody neutralization of enzyme activity

Neutralization of the ADP-glucose pyrophosphorylase activity was performed basically as previously described (26). About 0.05 unit of the partially purified recombinant *Anabaena* enzyme was mixed with 3 μmol of Hepes-NaOH, pH 7.0, containing 10 μg of BSA, 1 μmol of Pi, 0.1 μmol of DTT, 5 mg of sucrose, and 45 μL of serum containing varying amounts of anti-spinach leaf or anti-*E. coli* ADP-glucose pyrophosphorylase immune serum diluted into preimmune serum in a total volume of 0.1 mL. The mixture was incubated for 30 min at 30 °C and then for 2 hr on ice prior centrifugation for 5 min in Eppendorf microcentrifuge. Enzyme activity in the supernatant was measured by using assay A.

Protein electrophoresis and immunoblotting

Disc-PAGE and SDS-PAGE were performed according to Laemmli (18). After electrophoresis, proteins on the gel were transferred onto nitrocellulose membranes according to Burnette (6). Following electroblotting nitrocellulose membranes were treated with affinity purified rabbit anti-spinach leaf ADP-glucose pyrophosphorylase IgG and the antigen-antibody complex was visualized as previously described (15).

Nucleotide sequence accession number

The DNA sequence reported here has been deposited in EMBL under accession number Z11539.

RESULTS AND DISCUSSION

Gene cloning with a PCR probe

PCR has been widely used as a tool for cloning genes related to other known sequences. We adopted the strategy described by Compton (9) to clone the ADP-glucose pyrophosphorylase gene from *Anabaena* sp. strain PCC 7120. Two degenerate primers were synthesized to amplify the *Anabaena* genomic DNA. One of the primers, 5'-GAAGCG(AGTC)GC(AGTC)AA(AG)CC(AGTC)GC (AGTC)GT-3', was derived from the conserved amino acid sequences of the FBP binding site of ADP-glucose pyrophosphorylase. The activator binding site determined from the *E. coli* enzyme is conserved in higher plant enzymes despite the fact that FBP gives only minimal activation of the higher plant ADP-glucose pyrophosphorylases (31). The second primer, 5'-ATCAGC(AGTC)GT(AGTC)CC (TC)(TC)GA(AG)(AT)CCA-3', was derived from the conserved amino acid sequences of the ADP-glucose binding site. The substrate binding site for ADP-glucose, as determined by labeling of the *E. coli* enzyme by [^{14}C]-8-N_3-ADP-glucose (19), is also highly conserved in all known sequences of ADP-glucose pyrophosphorylases (31, 33, 39).

As expected, a major PCR product of ca. 250-bp was amplified from the *Anabaena* genomic DNA (data not shown). The PCR product was cloned into pUC119 and sequenced. The deduced amino acid sequence from this fragment is highly homologous to all ADP-glucose pyrophosphorylase sequences that have been determined. To isolate a full length gene, the PCR product was used as probe to screen an *Anabaena* genomic library constructed in the cosmid vector pWB79. Positive clones were isolated, and Southern as well as restriction analysis indicated that the ADP-glucose pyrophosphorylase gene resided in a 15 kb *Xba*I

fragment. Restriction maps of this fragment and its subclone of 5.5 kb *Eco*RI fragment are given in Fig. 1. The same degenerate primers used for cloning the *Anabaena* gene also were used for cloning the ADP-glucose pyrophosphorylase gene successfully from an unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 (16). We believe that these primers are applicable to cloning ADP-glucose pyrophosphorylase genes from other sources, be they of bacterial, algal or higher plant origin.

Sequence analysis

The nucleotide and deduced amino acid sequences of the gene along with its flanking nucleotide sequence are shown in Fig. 2. The *Anabaena* gene codes for a polypeptide of 429 amino acids with a calculated molecular weight of 48,347 daltons, which is close to the size of the *Anabaena* ADP-glucose pyrophosphorylase subunit of 50 kDa observed on SDS polyacrylamide gels (15). As seen in some bacterial genes, the *Anabaena* gene utilizes GTG instead of ATG as a start codon. This GTG start codon was subsequently confirmed by sequencing the first 12 N-terminal amino acids of *Anabaena* ADP-glucose pyrophosphorylase (data unpublished). Six bases prior to the start codon, there is a putative prokaryotic ribosome binding site, GGGAGA, (Fig. 2). Sequences with homology to the -35 and -10 box sequences of *E. coli* promoters (34) were observed and probably responsible for the expression of the gene in *E. coli* for later experiments. A repeated sequence of seven bases, AGTCAAC, was observed directly downstream from the stop codon, and 240 bases before the start codon of the coding region (Fig. 2). No such repeated sequence was found within the coding region. The presence of this repeated sequence has been reported to be specifically restricted to heterocystic strains of cyanobacteria (22).

Fig. 1. Restriction map of cloned *Anabaena* PCC 7120 DNA containing the gene for ADP-glucose pyrophosphorylase. Only restriction sites for *Eco*RI (R), *Hin*dIII (H), *Cla*I (C), and *Xba*I (X) are indicated. The 5.5 kb *Eco*RI fragment from the 15 kb *Xba*I fragment is enlarged to show detail features. The coding region of the *Anabaena* ADP-glucose pyrophosphorylase is designated by an open box. The putative promoter is designated by P with arrow indicating direction of transcription.

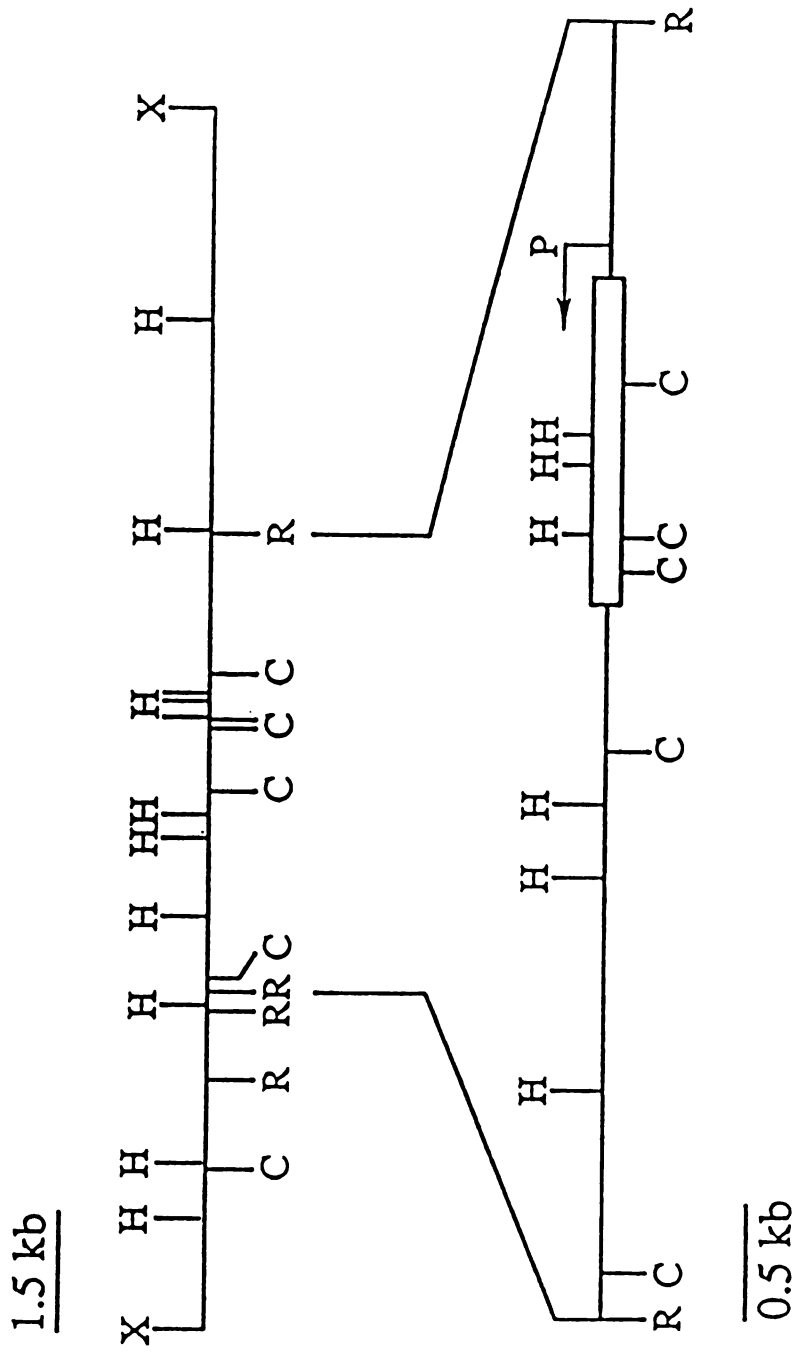

Fig.1

Fig. 2. Nucleotide and deduced amino acid sequence of the *Anabaena* ADP-glucose pyrophosphorylase gene. The putative -10 and -35 sequences are underlined. A prokaryotic ribosome binding site is shaded by ▒. The directly repeating sequences are marked by arrows.

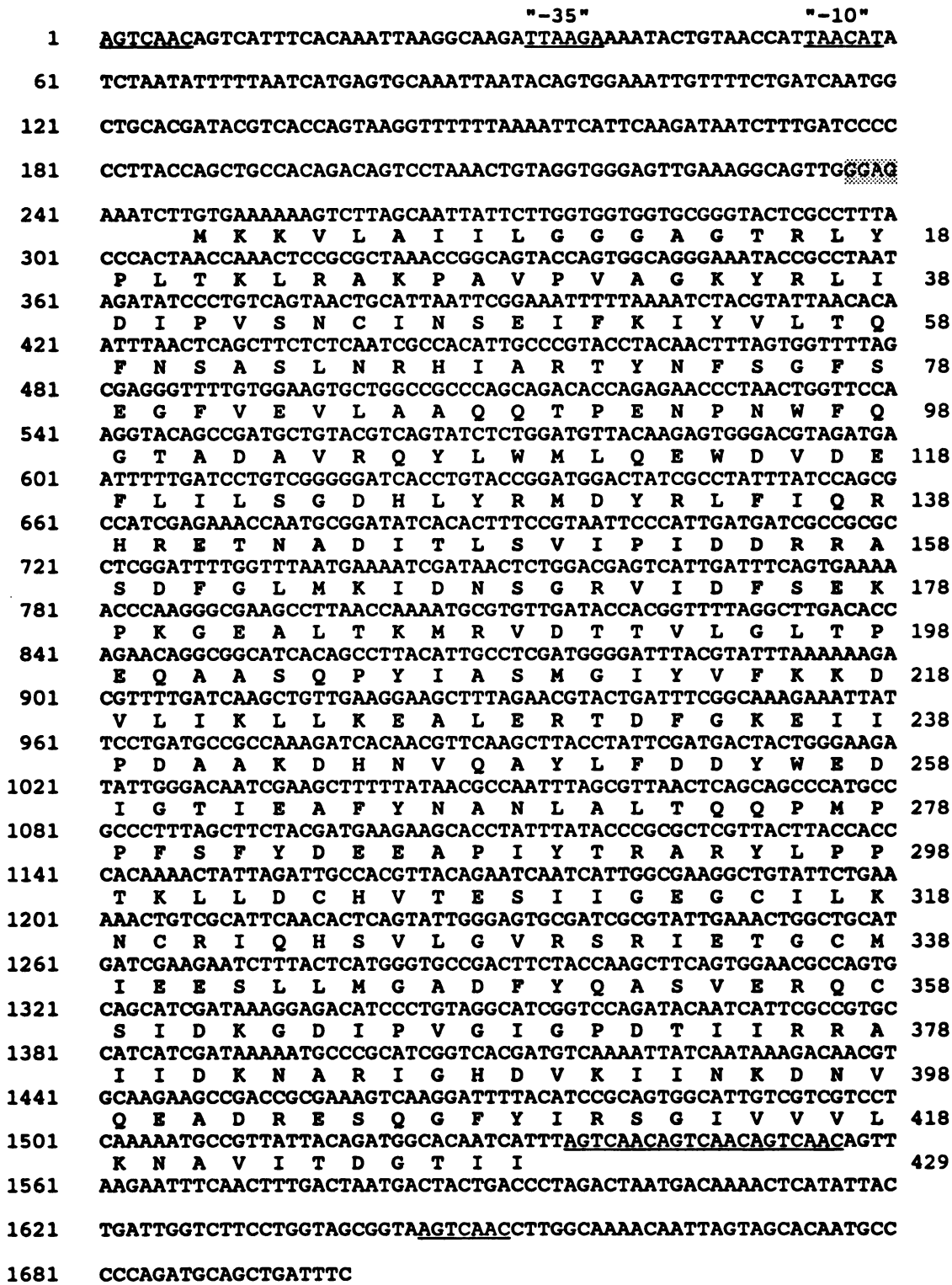
"-35"
"-10"
1 AGTCAACAGTCATTTCACAAATTAAGGCAAGATTAAGAAAATACTGTAACCATTAACATA
61 TCTAATATTTTTAATCATGAGTGCAAATTAATACAGTGGAAATTGTTTTCTGATCAATGG
121 CTGCACGATACGTCACCAGTAAGGTTTTTTAAAATTCATTCAAGATAATCTTTGATCCCC
181 CCTTACCAGCTGCCACAGACAGTCCTAAACTGTAGGTGGGAGTTGAAAGGCAGTTGGGAG
241 AAATCTTGTGAAAAAAGTCTTAGCAATTATTCTTGGTGGTGGTGCGGGTACTCGCCTTTA
M K K V L A I I L G G G A G T R L Y 18
301 CCCACTAACCAAACTCCGCGCTAAACCGGCAGTACCAGTGGCAGGGAAATACCGCCTAAT
P L T K L R A K P A V P V A G K Y R L I 38
361 AGATATCCCTGTCAGTAACTGCATTAATTCGGAAATTTTTAAAATCTACGTATTAACACA
D I P V S N C I N S E I F K I Y V L T Q 58
421 ATTTAACTCAGCTTCTCTCAATCGCCACATTGCCCGTACCTACAACTTTAGTGGTTTTAG
F N S A S L N R H I A R T Y N F S G F S 78
481 CGAGGGTTTTGTGGAAGTGCTGGCCGCCCAGCAGACACCAGAGAACCCTAACTGGTTCCA
E G F V E V L A A Q Q T P E N P N W F Q 98
541 AGGTACAGCCGATGCTGTACGTCAGTATCTCTGGATGTTACAAGAGTGGGACGTAGATGA
G T A D A V R Q Y L W M L Q E W D V D E 118
601 ATTTTTGATCCTGTCGGGGGATCACCTGTACCGGATGGACTATCGCCTATTTATCCAGCG
F L I L S G D H L Y R M D Y R L F I Q R 138
661 CCATCGAGAAACCAATGCGGATATCACACTTTCCGTAATTCCCATTGATGATCGCCGCGC
H R E T N A D I T L S V I P I D D R R A 158
721 CTCGGATTTTGGTTTAATGAAAATCGATAACTCTGGACGAGTCATTGATTTCAGTGAAAA
S D F G L M K I D N S G R V I D F S E K 178
781 ACCCAAGGGCGAAGCCTTAACCAAAATGCGTGTTGATACCACGGTTTTAGGCTTGACACC
P K G E A L T K M R V D T T V L G L T P 198
841 AGAACAGGCGGCATCACAGCCTTACATTGCCTCGATGGGGATTTACGTATTTAAAAAAGA
E Q A A S Q P Y I A S M G I Y V F K K D 218
901 CGTTTTGATCAAGCTGTTGAAGGAAGCTTTAGAACGTACTGATTTCGGCAAAGAAATTAT
V L I K L L K E A L E R T D F G K E I I 238
961 TCCTGATGCCGCCAAAGATCACAACGTTCAAGCTTACCTATTCGATGACTACTGGGAAGA
P D A A K D H N V Q A Y L F D D Y W E D 258
1021 TATTGGGACAATCGAAGCTTTTTATAACGCCAATTTAGCGTTAACTCAGCAGCCCATGCC
I G T I E A F Y N A N L A L T Q Q P M P 278
1081 GCCCTTTAGCTTCTACGATGAAGAAGCACCTATTTATACCCGCGCTCGTTACTTACCACC
P F S F Y D E E A P I Y T R A R Y L P P 298
1141 CACAAAACTATTAGATTGCCACGTTACAGAATCAATCATTGGCGAAGGCTGTATTCTGAA
T K L L D C H V T E S I I G E G C I L K 318
1201 AAACTGTCGCATTCAACACTCAGTATTGGGAGTGCGATCGCGTATTGAAACTGGCTGCAT
N C R I Q H S V L G V R S R I E T G C M 338
1261 GATCGAAGAATCTTTACTCATGGGTGCCGACTTCTACCAAGCTTCAGTGGAACGCCAGTG
I E E S L L M G A D F Y Q A S V E R Q C 358
1321 CAGCATCGATAAAGGAGACATCCCTGTAGGCATCGGTCCAGATACAATCATTCGCCGTGC
S I D K G D I P V G I G P D T I I R R A 378
1381 CATCATCGATAAAAATGCCCGCATCGGTCACGATGTCAAAATTATCAATAAAGACAACGT
I I D K N A R I G H D V K I I N K D N V 398
1441 GCAAGAAGCCGACCGCGAAAGTCAAGGATTTTACATCCGCAGTGGCATTGTCGTCGTCCT
Q E A D R E S Q G F Y I R S G I V V V L 418
1501 CAAAAATGCCGTTATTACAGATGGCACAATCATTTAGTCAACAGTCAACAGTCAACAGTT
K N A V I T D G T I I 429
1561 AAGAATTTCAACTTTGACTAATGACTACTGACCCTAGACTAATGACAAAACTCATATTAC
1621 TGATTGGTCTTCCTGGTAGCGGTAAGTCAACCTTGGCAAAACAATTAGTAGCACAATGCC
1681 CCCAGATGCAGCTGATTTC

Fig. 2

A comparison of the deduced amino acid sequence of the *Anabaena* enzyme and several other known sequences is shown in Fig. 3. The *Anabaena* sequence shared 63, 54, and 33 % identity to the sequences of the small subunit of rice seed (1), the large subunit of maize endosperm (3), and the *E. coli* enzyme (2), respectively. The *Anabaena* sequence is more similar to that of the higher plant enzymes than to the *E. coli* enzyme. Furthermore, the *Anabaena* enzyme is structurally more related to the small subunit of the higher plant ADP-glucose pyrophosphorylases. This result is in agreement with the cross-reaction of *Anabaena* ADP-glucose pyrophosphorylase with the antibodies against the spinach leaf subunits (15). Highly conserved regions were seen in the alignment. However, the 3PGA binding site at the C-terminus, determined by covalent modification (31), was only conserved in the higher plant and *Anabaena* sequences, not in the *E. coli* sequence. This is consistent with the allosteric property that the *Anabaena* enzyme was activated mainly by 3PGA (15).

Southern analysis

For Southern analysis, an 1.3-kb fragment derived from the entire coding region of the ADP-glucose pyrophosphorylase gene was generated by PCR and used as a probe. The probe hybridized to a single band in all restriction digests (Fig. 4), indicating that there is only a single copy of the ADP-glucose pyrophosphorylase gene in the genome of *Anabaena* sp. strain PCC 7120. This result as well as other biochemical evidence (15) further confirms that *Anabaena* ADP-glucose pyrophosphorylase is a homotetrameric enzyme.

Expression of *Anabaena* ADP-glucose pyrophosphorylase in *E. coli*

To examine whether the cloned gene encodes active *Anabaena*

Fig. 3. Comparison of the deduced amino acid sequence of the ADP-glucose pyrophosphorylase from *Anabaena* PCC 7120 and the sequences from other sources. AN, *Anabaena* PCC 7120; RE, the small subunit from rice endosperm (1); ME, the large subunit from maize endosperm (3); EC, *E. coli* (2). The maize sequence is truncated at the N-terminus. The residues that are identical or similar in all the sequences are designated by ♦ and ▲, respectively. Gaps have been introduced to give better alignment (indicated by dots).


1
100
AN MKKVLAIILGGGAGTRLYPLTKLRAKPAVPVA
RE MNVLASKIFPSRSNVVSEQQQSKREKATIDDAKNSSKNKNLDRSVDESVLGIILGGGAGTRLYPLTKKRAKPAVPLG
ME HQIRSCEGDGIDRLEKLSIGGRKQEK.ALRNRCFGGRVAATQQCILTSDCAPETLHSQTQSSRKNYADNARVSAIILGGGTGSQLFPLTSTRATPAVPVG
EC MVSLEKNDHLMLARQLPL.KSV.ALILAGGRGTRLKDLTNKRAKPAVHFG
101
200
AN GKYRLIDIPVSNCINSEIFKIYVLTQFNSASLNRHIARTY.N.FSG.F.SEGFVEVLAAQQTPENPN.WFQGTADAVRQYLWMLQEW...D.VDEFL.IL
RE ANYRLIDIPVSNCLNSNISKIYVLTQFNSASPNRHLSRAYGNNIGG.YKNEGFVEVLAAQQSPDNPN.WFQGTADAVRQYLWLFEE...HN.VMEFL.IL
ME GCYRLIDIPMSNCFNSGINKIFVMSQFNSTSLNRHIHRTYLEG.GINF.ADGSVQVLAATQMPEEPAGWFQGTADSIRKFIWVLEDYYSHKSIDNIV.IL
EC GKFRIIDFALSNCINSGIRRMGVITQYQSHTLVQHIQRGW.SFFNEEM.NE.FVDLLPAQQRMKGEN.WYRGTADAVTQNLDIIRRY...KA..EYVVIL
201
300
AN SGDHLYRMDYRLFIQRHRETNADITLSVIPIDDRRASDFGLMKIDNSGRVIDFSEKPKGEALTKMRVDTTVLGLTPEQ.AASQ.PYIASMGIYVFKKDVL
RE AGDHLYRMDYEKFIQAHRETDSDITVAALPMDEKRATAFGLMKIDEEGRIVEFAEKPKGEQLKAMMVDTTILGL..DDVRAKEMPYIASMGIYVISKNVM
ME SGDQLYRMNYMELVQKHVEDDADITISCAPVDESRASKNGLVKIDHTGRVLQFFEKPKGADLNSMRVETNFLSYAIDD.AQK.YPYLASMGIYVFKKDAL
EC AGDHIYKQDYSRMLIDHVEKGARCTVACMPVPIEEASAFGVMAVDENDKIIEFVEKP.ANPP.SMPNDPSKS.....LASMGIYVFDADYL
301
400
AN IKLLKEALERT....DFGKEIIPDAAKDHN.VQAYL..FDD.....YWEDIGTIEAFYNANLALTQQPMPPFSFYDEEAPIYTRARYLPPTK.L
RE LQLLREQFPGAN...DFGSEVIPGATNIGMRVQAYL..YDG.....YWEDIGTIEAFYNANLGITKKPVPDFSFYDRSAPIYTQPRHLPPSK.V
ME LDLLKSKYTQL...HDFGSEILPRAVLDHS.VQACI..FTG.....YWEDVGTIKSFFDANLALTEQPS.KFDFYDPKTPFFTAPRCLPPTQ..
EC YELLEEDDRDENSSHDFGKDLIPKITEAGL...AYAHPFPLSCVQSDPDAEPYWRDVGTLEAYWKANLDLAS.VVPELDMYDRNWPIRTYNESLPPAKFV
401
500
AN LD.....CHVTESIIGEGCILKNCRIQHSVLGVRSRIETGCMIEESLLMGADFYQASVERQCSIDKGDIPVGIGPDTIIRRAIIDKNARIGHDVKIINKD
RE LD.....ADVTDSVIGEGCVIKNCKIHHSVVGLRSCISEGAIIEDSLLMGADYYETEADKKLLGEKGGIPIGIGKNCHIRRAIIDKNARIGDNVKIINVD
ME LDK....CKMKYAFISDGCLLRECNIEHSVIGVCSRVSSGCELKDSVMMGADIYETEEEASKLLLAGKVPIGIGRNTKIRNCIIDMNARIGKNVVITNSK
EC QDRSGSHGMTLNSLVSGGCVISG.....SVVVQSVLFSRVRVNSFCNIDSAVLLPEVW.....VGRSCRLRRCVIDRACVIPEGMVI..GE
501
539
AN NVQEADRESQGFYIRS..GIVVVLKNAVITDGTII
RE NVQEAARETDGYFIKS..GIVTVIKDALLLAEQLYEVAA
ME GIQEADHPEEGYYIRS..GIVVILKNATINECLVI
EC NAEEDARR...FY.RSEEGIVLVTREMLRKLGHKQER

Fig. 3

Fig. 4. Southern blot analysis of genomic DNA from *Anabaena* sp. strain PCC 7120. Genomic DNA was digested with *Eco*RI (lane 1), *Xba*I (lane 2), *Ssp*I (lane 3), *Eco*RI + *Ssp*I (lane 4), and *Xba*I + *Ssp*I (lane 5). The digested DNA was electrophoresed on a 0.9 % agarose gel, transferred to nitrocellulose filter, and probed with a 1.3 kb PCR product comprising the entire coding region of *Anabaena* ADP-glucose pyrophosphorylase gene.

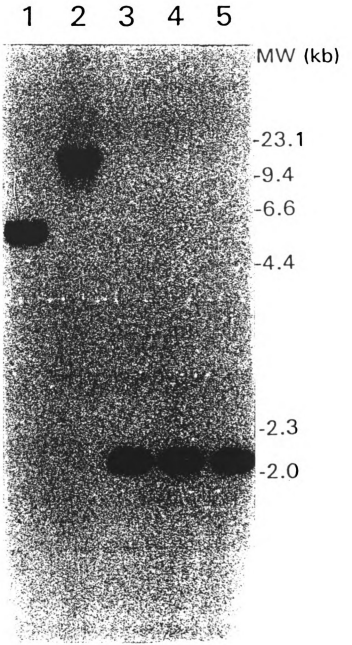

Fig. 4

ADP-glucose pyrophosphorylase, the gene was introduced into *E. coli* for expression. A 5.5 kb *Eco*RI fragment containing the entire coding region for ADP-glucose pyrophosphorylase was ligated into pUC119 in forward and reverse orientations to yield expression plasmids pAnaE3b and pAnaE3a, respectively. The 5.5 kb *Eco*RI fragment also contains about 1 kb of upstream noncoding DNA, including the putative ribosome binding site and promoter sequences. *E. coli* strain AC70R1-504, a mutant lacking ADP-glucose pyrophosphorylase activity, was used as host for the gene expression. ADP-glucose pyrophosphorylase activity was observed in the crude extract of the cells transformed with pAnaE3a or pAnaE3b, but not in the cells transformed with control plasmid pUC119 containing no insert (Table 1). The enzyme was activated by 3PGA, but not by FBP, which indicates the recombinant enzyme was not *E. coli* ADP-glucose pyrophosphorylase. The expression of the gene was independent of its orientation in pUC119, indicating that an *Anabaena* promoter preceding the coding region may be functional in *E. coli*. Promoters of *Anabaena* genes recognized by *E. coli* have been reported (4, 13). However, the promoter of *Anabaena* ADP-glucose pyrophosphorylase gene has not been identified in this study.

Partial purification of the recombinant enzyme

For further identification and characterization, the recombinant enzyme was partially purified from *E. coli* AC70R1-504 cells transformed with pAnaE3b. The transformed cells were grown in enriched medium, which gave about two fold higher expression than in LB medium (data not shown). The recombinant enzyme was partially purified to a specific activity of 2.9 units/mg (Table 2). In the crude extract, the recombinant enzyme accounted for approximately 0.2 % of the total soluble protein of the cells based on the specific activity of the pure native enzyme

Table 1. Expression of the cloned *Anabaena* ADP-glucose pyrophosphorylase gene in *E. coli* AC70R1-504*

Strains (crude extract)	Enzyme Activity**		
	None	FBP	3PGA
	glucose-1-P formed (μmol/10 min/mg protein)		
E.coli AC70R1-504/pUC119	0	0	0
E.coli AC70R1-504/pAnaE3a	0.29	0.23	1.34
E.coli AC70R1-504/pAnaE3b	0.27	0.17	1.01

* The transformed *E. coli* cells were grown in LB medium with 100 μg ampicillin/mL.

** The ADP-glucose pyrophosphorylase activity was assayed with 2 mM of indicated effectors by Assay A. The crude extract contained 6 mg protein per mL.

Table 2. Partial purification of recombinant ADP-glucose pyrophosphorylase from *E.coli* AC70R1-504/pAnaE3b

Step	Volume	Total Protein	Specific Activity	Purification	Yield
	mL	mg	units/mg		%
Crude extract	20	127	0.18	1	100
Heat treatment	21	93	0.24	1.3	100
DEAE-Sepharose/ Concentration	4.1	6.4	2.92	16.3	82

determined previously (15). During purification, the heat treatment at 60 °C for 4 min did not cause significant loss of activity, indicating that the recombinant enzyme has similar heat stability as the native ADP-glucose pyrophosphorylase (15).

Characterization of the recombinant enzyme

The specificity of the recombinant enzyme to several allosteric effectors was examined (Table 3). The recombinant enzyme was mainly activated by 3PGA and inhibited by Pi, as is the native *Anabaena* enzyme (15). Lesser but significant activation by 2-PGA was also observed. This result might be due to contamination by phosphoglyceromutase activity in the partially purified enzyme fraction. The $A_{0.5}$ and $I_{0.5}$ values for 3PGA and Pi of the recombinant enzyme were 0.15 mM and 36 μM, respectively, similar to the values of 0.12 mM and 44 μM determined previously for the *Anabaena* ADP-glucose pyrophosphorylase (15).

ADP-glucose pyrophosphorylase from *Anabaena* was shown to be antigenically related to the spinach leaf ADP-glucose pyrophosphorylase, and is inhibited by the antiserum raised against the spinach leaf enzyme (15). To determine if the recombinant enzyme retained the same antigenicity, the recombinant enzyme was incubated with antibodies specific for spinach leaf or *E. coli* ADP-glucose pyrophosphorylase. The recombinant enzyme was effectively inhibited by antibodies for the spinach enzyme, but not by those for the *E. coli* enzyme (Fig. 5). The amount of antiserum causing 50 % inhibition was about 80 μL per unit of the recombinant enzyme, similar to the value reported for the native enzyme (15).

The size of the recombinant enzyme subunit was compared to the enzyme purified from *Anabaena* sp. strain PCC 7120 on SDS-PAGE followed by Western

Table 3. Effect of different compounds on the activity of recombinant ADP-glucose pyrophosphorylase from *E. coli* AC70R1-504/pAnaE3b

Compound	ADP-glucose formed*	Relative activity
	nmol/10 min	
None	3.8	1.0
Glucose-6-P	11.3	2.9
Fructose-6-P	13.5	3.5
Fructose-1,6-P_2	7.8	2.0
P-enolpyruvate	21.9	5.7
3-P-glycerate	36.6	9.6
2-P-glycerate	28.5	7.4
ADP	2.5	0.7
AMP	6.7	1.8
Pi	0.03	0.01

* Enzyme activity was measured by Assay B with the specific effectors at a concentration of 2 mM.

Fig. 5. Neutralization of the recombinant enzyme from *E. coli* AC70R1-504/pAnaE3b by anti-spinach leaf (●) and anti-*E. coli* (○) ADP-glucose pyrophosphorylase immune serum. Assay A was used to determined the ADP-glucose pyrophosphorylase activity of the enzyme after incubation with different amounts of the corresponding antiserum.

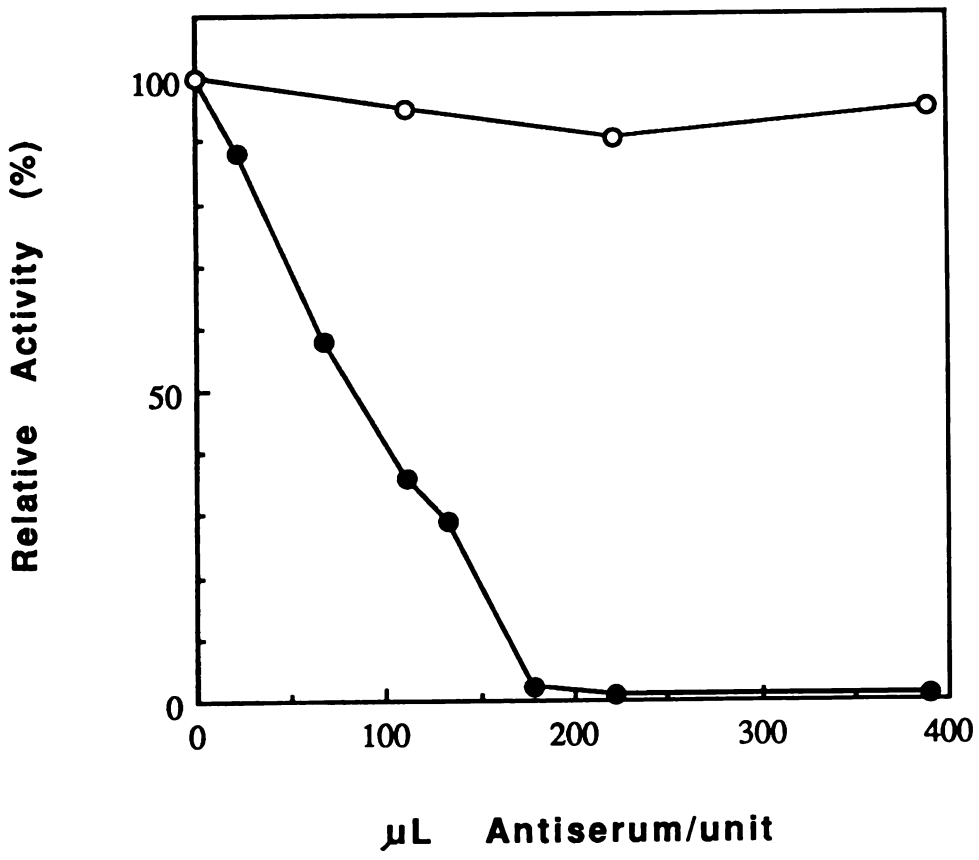
Relative Activity (%)
100
50
0
0
100
200
300
400
μL Antiserum/unit

Fig.5

blot analysis. Affinity purified antibodies raised against the spinach leaf enzyme recognized only one band in the crude extract from *E. coli* AC70R1-504/pAnaE3b, but not in control cells transformed with pUC119 (Fig. 6A, lanes 1,2). The migration of the protein band was the same as the *Anabaena* enzyme on the SDS polyacrylamide gel (Fig. 6A, lane 3) indicating that the subunit of the recombinant enzyme and of the *Anabaena* enzyme are the same size, determined previously as 50 kDa (15). A similar result was obtained by analysis on a native-PAGE followed by Western blot analysis (Fig. 6B). Again, no difference was observed between the recombinant and the *Anabaena* enzymes.

Results reported in this study indicate that a full length *Anabaena* ADP-glucose pyrophosphorylase structural gene was isolated. Only one copy of this gene exists in the *Anabaena* genome. The cyanobacterial protein is more closely related in amino acid sequence to the higher plant enzymes than to the *E. coli* enzyme. The expression of the Anabaena ADP-glucose pyrophosphorylase gene in *E. coli* yielded an active enzyme that was shown to be the same as the *Anabaena* ADP-glucose pyrophosphorylase. This result enables us to further study the structure-function relationships of the enzyme.

Fig. 6. Western blot analysis of the recombinant enzyme from *E. coli* AC70RI-504/pAnaE3b. About 35 μg of crude extract protein from AC70R1-504/pUC119 (lane 1), AC70R1-504/pAnaE3b (lane 2), and 0.2 μg of pure *Anabaena* ADP-glucose pyrophosphorylase (lane 3) were resolved on a 10 % SDS-polyacrylamide gel (A) or 7 % native-polyacrylamide gel (B) and then transferred to nitrocellulose filters. The filters were incubated with affinity purified antisera against spinach leaf ADP-glucose pyrophosphorylase prior to staining.

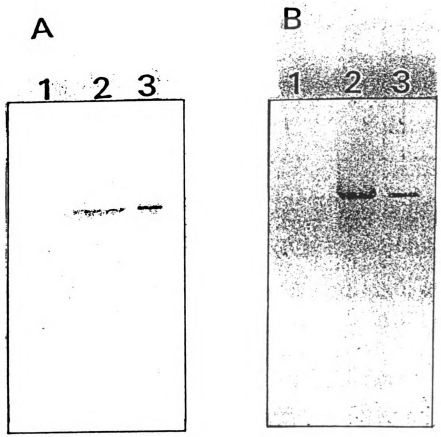

Fig.6

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CHAPTER III

MUTAGENESIS OF AN ALLOSTERIC SITE RESIDUE, LYS_{419}, OF ADP-GLUCOSE PYROPHOSPHORYLASE FROM *ANABAENA* SP. STRAIN PCC 7120

ABSTRACT

Chemical modification studies of spinach leaf ADP-glucose pyrophosphorylase have shown that a highly conserved lysyl residue near the C-terminus might be involved in the binding of 3-P-glycerate, the allosteric activator. Site-directed mutagenesis of the corresponding residue (Lys_{419}) of the *Anabaena* enzyme was done to determine the role of this conserved residue. Replacing Lys_{419} with either arginine, alanine, glutamine, or glutamic acid produced mutant enzymes with apparent affinities for 3-P-glycerate, 25- to 150-fold lower than that of the wild-type enzyme. These mutations caused lesser or no effect on the kinetic constants for the substrates and inhibitor, orthophosphate. Catalytic efficiency and thermal stability of the arginine mutant are similar to that of the wild-type enzyme. The results suggest that the major role of Lys_{419} is involved in 3-P-glycerate binding. 3-P-glycerate still is the most effective metabolite in activating all except the K419Q enzyme. For this enzyme, fructose-1,6-P_2, the physiological activator of *E. coli* ADP-glucose pyrophosphorylase, is a more effective activator than 3-P-glycerate at lower concentrations. Kinetic studies show that fructose-1,6-P_2 competitively inhibits 3-P-glycerate activation of the *Anabaena* wild-type enzyme suggesting that these two compounds bind to the same allosteric site.

INTRODUCTION

ADP-glucose pyrophosphorylase (ATP:α-glucose-1-P adenylyl transferase, EC 2.7.7.27) catalyzes the following reaction: glucose-1-P + ATP ⇔ ADP-glucose + pyrophosphate. The enzyme is responsible for the first step of the biosynthesis of starch in plants and glycogen in bacteria by providing ADP-glucose, the precursor for synthesis of the polysaccharide (1,2). ADP-glucose pyrophosphorylase from higher plants is heterotetrameric encoded by two different genes (3), while the enzyme from enterobacteria (2) or cyanobacteria (4) is homotetrameric in structure. Usually, the catalytic activity of ADP-glucose pyrophosphorylase is regulated by effectors derived from the dominant carbon assimilation pathway in the organism.

The enzyme from higher-plants (1), green algae (5,6,7) or cyanobacteria (4,8) is mainly activated by the CO_2-fixation product, 3-P-glycerate (3PGA), and inhibited by orthophosphate (Pi). The enzyme from other bacterial species, however, is inhibited by AMP and ADP and activated by other glycolytic intermediates, such as fructose-1,6-P_2, fructose-6-P or pyruvate (2). Besides increasing the catalytic efficiency of the enzyme, the activator also exerts heterotropic effects on the kinetic constants of the substrates. In the presence of the activator, ADP-glucose pyrophosphorylase has higher apparent affinities for substrates and is less sensitive to the inhibitor. Studies of *E. coli* (9-12) and *Chlamydomonas* (7) mutants have shown that ADP-glucose pyrophosphorylase with altered allosteric properties have altered levels of glycogen or starch accumulation. For example, *E. coli* mutants having ADP-glucose pyrophosphorylases, with higher apparent affinity for the activator, have higher rates of glycogen synthesis than the wild-type *E. coli* (2, 9-12). These data suggest the importance of the

allosteric properties for the regulation of the biosynthesis of starch and bacterial glycogen. It is therefore of great interest to understand the structure-function relationships of the allosteric sites.

Chemical modification of the spinach leaf ADP-glucose pyrophosphorylase has been conducted by using a site-specific probe, pyridoxal-P, to determine the location of the 3PGA-binding site(s) (13). Pyridoxal-P, an activator of the spinach leaf enzyme, was used to covalently bind to lysyl residues that might be at or near the allosteric site(s) by reducing the Schiff base with sodium borohydride. In the presence of 3PGA and Pi, the modification was effectively prevented. Once the enzyme was modified, it became highly active in the absence of activator and less sensitive to Pi inhibition.

Isolation and sequencing of the labeled tryptic peptide from the small subunit of the spinach leaf enzyme showed that the putative allosteric site is located near the C-terminus of the protein. The pyridoxal-P labeled lysyl residue and its flanking sequence are highly conserved in all the higher-plant and cyanobacteria enzymes sequenced to date (3,14). The spinach leaf large subunit was also found to be phosphopyridoxylated at the conserved C-terminus lysine in addition to two other less conserved sites (14). Thus, it is likely that this conserved C-terminus lysyl residue is important for the binding of the activator. Indeed, chemical modification of the *Anabaena* ADP-glucose pyrophosphorylase with pyridoxal-P also yield enzyme highly active in the absence of activator and less sensitive to Pi inhibitor (15). The modified lysyl residue was Lys_{419} (15).

Site-directed mutagenesis experiments were of interest to further examine the structure-function relationships of the conserved C-terminus lysyl residue modified by pyridoxal-P. Since an expression system of the *Anabaena* enzyme is available, experiments were performed on the corresponding residue, Lys_{419}, of

Anabaena ADP-glucose pyrophosphorylase. Previous studies have shown that the *Anabaena* enzyme is very similar to the higher-plant enzyme in allosteric properties and primary structure (4,16). The cyanobacterial enzyme is also encoded by only one gene and has been successfully expressed in *E. coli* (16). It can easily be used for the site-directed mutagenesis experiments on the highly conserved residues of ADP-glucose pyrophosphorylase. In this paper, we report the characterization of the Lys_{419} mutant enzymes and discuss the probable function of the lysyl residue.

MATERIALS AND METHODS

Chemicals

[α-^{35}S]dATP and the *in vitro* site-directed mutagenesis kit were purchased from Amersham Corp. [^{14}C]glucose-1-P and [^{32}P]PPi were from DuPont-New England Nuclear. Enzymes for DNA manipulation and sequencing were from New England Biolabs and United States Biochemical Corp., respectively. The coupling enzymes for the ADP-glucose pyrophosphorylase assay were purchased from Sigma Company. Oligonucleotides were synthesized by the Macromolecular Facility at Michigan State University. All other reagents were of the highest available commercial grade.

Bacterial Strains and Media

E. coli strain TG1 (K12, Δ(lac-pro), supE, thi, hsdD5/F'traD36, proA$^+$B$^+$, lacI^q, lacZΔM15) was used for site-directed mutagenesis. *E. coli* mutant strain AC70R1-504, which has no ADP-glucose pyrophosphorylase activity, was used for expression of the *Anabaena* ADP-glucose pyrophosphorylase gene (16,17). *E. coli* strain TG1 was grown in LB medium. AC70R1-504 cells were grown in enriched medium containing 1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% yeast extract and 0.2% glucose, at pH 7.0.

Site-directed Mutagenesis

For both site-directed mutagenesis and gene expression, plasmid pANAE3a was used. In pANAE3a, a 5.5 kb *EcoRI* fragment of *Anabaena* genomic DNA containing the native *Anabaena* ADP-glucose pyrophosphorylase gene and its putative promoter was ligated onto the *EcoRI* site of pUC119 plasmid (16). The

original start codon, GTG, has been changed to ATG by site-directed mutagenesis. Sequencing of the N-terminus of the recombinant enzyme has shown that the gene was translated correctly in *E. coli*. The orientation of the gene, which is opposite to the *lac* promoter of pUC119, enables TG1 cells to synthesize single-stranded DNA containing the antisense strand of the gene using helper phage M13KO7 (18).

Site-directed mutagenesis experiments were performed according to the method of Eckstein (19) using the *in vitro* site-directed mutagenesis kit from Amersham Corp. The Lys_{419} mutant enzymes with substitution of arginine, alanine, glutamine and glutamic acid are designated as K419R, K419A, K419Q and K419E, respectively. The mutant oligonucleotides used are shown in Fig. 1. The plasmids recovered in the last step of the mutagenesis were screened by dideoxy sequencing (20) in the region of the desired mutation. The entire coding region of each mutant allele was sequenced to verify the absence of unintended mutations.

Expression and Purification of Wild-type and Mutant Enzymes

Competent cells of *E. coli* mutant strain AC70R1-504 were transformed with the plasmid containing wild-type or mutant ADP-glucose pyrophosphorylase gene by the heat shock method (18). The transformed cells were grown in 5 x 1 l enriched medium at 37 °C on a rotary shaker. The cells were harvested after 24 hours of growth since the gene is expressed in late log phase (Charng and Preiss, unpublished data). The wild-type enzyme was purified by heat treatment at 60 °C, ion-exchange chromatography on DEAE-Sepharose, FPLC chromatography on Mono Q, and Phenyl-Superose columns as described previously (4,16). The mutant enzymes were purified as the wild-type enzyme except the heat treatment

Fig. 1. Nucleotide sequence and encoded peptide sequence of the ADP-glucose pyrophosphorylase gene in the region of Lys_{419} (I), and the synthetic oligonucleotide used for site-directed mutagenesis at Lys_{419} (II). The position 419 codons and anticodons are underlined, and the base substitutions are indicated with arrows.

I.

```
          V   V   L   K   N   A   V
5′ -GTC GTC CTC AAA AAT GCC GTT- 3′
3′ -CAG CAG GAG TTT TTA CGG CAA- 5′
```

II.

```
                              ↓
K419R     5′ -GTC GTC CTC AGA AAT GCC GT- 3′

                              ↓↓
K419A     5′ -GTC GTC CTC GCA AAT GCC GT- 3′

                              ↓
K419E     5′ -GTC GTC CTC GAA AAT GCC GT- 3′

                              ↓
K419Q     5′ -GTC GTC CTC CAA AAT GCC GT- 3′
```

Fig.1

step was omitted.

Assay of ADP-glucose pyrophosphorylase

Assay I. Enzyme activity was assayed in the ADP-glucose synthesis direction according to the method of Preiss *et al* (21).

(A) Activated conditions. For assay of the wild-type enzyme in the presence of activator, the reaction mixtures contained 20 μmol Hepes-NaOH buffer (pH 8.0), 0.1 μmol of [^{14}C]glucose-1-phosphate (about 1,000 cpm/nmol), 0.5 μmol of ATP, 2 μmol of $MgCl_2$, 50 μg of bovine serum albumin, 0.15 unit of inorganic pyrophosphatase, 0.5 μmol of 3PGA and enzyme in a final volume of 200 μl. The assays were initiated with the addition of enzyme and incubated at 37 °C. For assay of the mutant enzymes, the reaction mixtures were identical to wild-type, with the exception that the amounts of 3PGA and $MgCl_2$ were altered to obtain maximal activity. For K419R and K419A, 2 μmol of 3PGA was used, while 4 μmol was used for K419Q and K419E. For the K419E enzyme, 4 μmol of $MgCl_2$ was used in the presence of 3PGA.

(B) Unactivated conditions. The synthesis of ADP-glucose in the absence of activator was measured as described above except that 3PGA was omitted and the amount of ATP was increased to 1 μmol in the reaction mixtures. The enzyme is inhibited by ATP when the amount of the substrate is increased to more than 1 μmol in the reaction mixtures. The amount of [^{14}C]glucose-1-phosphate was increased to 0.25 μmol for the K419R mutant enzyme. For the K419R enzyme, 1 μmol instead of 2 μmol of $MgCl_2$ was used in the absence of 3PGA due to inhibition occurring at $MgCl_2$ concentration higher than 5 mM. The amounts of substrates or effectors mentioned above, are saturating for the enzyme indicated in the 0.2 ml reaction mixtures and were used for determination of kinetic

parameters.

Assay II. During purification, enzyme activity was assayed in the pyrophosphorolysis direction as previously described (21). The reaction mixtures contained 20 μmol of HEPES-NaOH buffer pH 7.0, 2 μmol of $MgCl_2$, 0.5 μmol of ADP-glucose, 0.5 μmol [^{32}P]PPi (about 3,000 cpm/nmol), 50 μg of bovine serum albumin, 2.5 μmol of NaF, 1 μmol of 3PGA and enzyme preparation in a total volume of 250 μl. The amount of 3PGA in the reaction mixtures was increased to 2 μmol for the K419R and K419A enzymes, and 3 μmol for the K419Q and K419E enzymes.

Kinetic Analysis

For determination of kinetic parameter, the concentration of the substrate or effector tested was systematically varied with the other substrates and effectors fixed at a saturating concentration as described in assay I. For ATP and glucose-1-P saturation curves, the substrates were varied from 0.05 to 5 mM and 0.005 to 1.5 mM, respectively. $MgCl_2$ concentration was varied from 1 to 15 mM except that for the K419E enzyme the $MgCl_2$ was varied from 1 to 30 mM in the presence of 20 mM 3PGA. For Pi inhibition, the inhibitor was varied from 0.005 to 1 mM in the absence of 3PGA and from 0.05 to 10 mM in the presence of saturating of 3PGA. For 3PGA saturation, the activator was varied from 0.01 to 5 mM for the wild-type enzyme and from 0.1 to 25 mM for the mutant enzymes. Kinetic data were plotted as initial velocity versus substrate or effector concentration and replotted as double-reciprocal plots to determine *Vmax*. Kinetic constants for hyperbolic plots were also determined by double reciprocal plots. Sigmoidal data were replotted as Hill plots to obtain kinetic constants. Interaction coefficients, n_H, were determined by Hill plots. Kinetic parameters were

expressed as $S_{0.5}$, $A_{0.5}$, and $I_{0.5}$, which correspond to the concentration of substrate, activator, or inhibitor required for 50 % of maximal velocity, activation, or inhibition, respectively. All the data were reexamined by using nonlinear iterative least-squares fitting to a modified Michaelis-Menten equation with the use of a computer program (22). The kinetic parameters obtained were in good agreement with those calculated from double reciprocal or Hill plots.

Protein Assay

Protein concentration was determined by using bicinchoninic acid reagent (23) with bovine serum albumin as the standard.

Protein Electrophoresis

SDS-PAGE were performed according to Laemmli (24). After electrophoresis, proteins on the gel were visualized by staining with Coomassie Brilliant Blue R-250.

Thermal Stability

The purified preparations of all the enzymes were diluted to give the same final protein concentration, 0.2 mg/ml. The dilution buffer was 20 mM potassium phosphate, pH 7.5, containing 1 mg/ml bovine serum albumin. The samples were heated simultaneously for 5 min in a 60 °C water bath, then immediately placed on ice. The residual activities of the heated enzymes were assayed in pyrophosphorolysis direction as described above.

RESULTS

Purification of Lys_{419} mutant enzymes

To determine whether the mutations of Lys_{419} affect the catalytic efficiency of ADP-glucose pyrophosphorylase, the mutant enzymes were purified to greater than 90 % homogeneity as estimated by SDS-PAGE (data not shown). The mutant enzymes were stable under the purification procedure. No significant difference was observed in the profiles of DEAE, Mono Q, and Phenyl-Superose chromatographies between the wild-type and the mutant enzymes (data not shown).

Kinetic characterization of Lys_{419} mutant enzymes

In the synthesis direction of assay, the apparent affinity for 3PGA decreased dramatically when Lys_{419} was replaced with either arginine, alanine, glutamine, or glutamic acid. The $A_{0.5}$ values for 3PGA of the K419R, K419A, K419Q, and K419E enzymes were about 25-, 50-, 140-, and 150-fold higher than that of wild-type enzyme, respectively (Table 1). The interaction coefficients were changed from 1.0 for the wild-type to 1.8-1.9 for the mutant enzymes. Thus, the binding of the activator for the mutant enzymes is cooperative.

Although the apparent affinity for 3PGA is largely reduced, the degree of activation increased from 17-fold for the wild-type enzyme to about 50- to 100-fold for the K419E, K419A, and K419R mutants. This is due to the lower specific activities of the mutant enzymes in the absence of 3PGA. In the presence of saturating 3PGA, the *Vmax* values of the K419R and K419A enzymes were similar to that of wild-type. The *Vmax* values however, in the absence of the activator, were 5-fold lower (Table 1). The *Vmax* of the K419Q enzyme was 2-4 % of the wild-type *Vmax* either in the presence or absence of 3PGA.

Table 1. Kinetic parameters of the *Anabaena* wild-type and mutant ADP-glucose pyrophosphorylases. Reactions were performed in the synthesis direction of assay as described in "Experimental Procedures." Data represent the mean ± standard deviation of two independent experiments.

		Wild-type	K419R	K419A	K419Q	K419E
	3PGA[a]			$S_{0.5}$, $I_{0.5}$, $A_{0.5}$ (n_H)		
ATP, mM	-	1.88 ± 0.62 (1.1)	1.44 ± 0.05 (1.0)	1.10 ± 0.21 (1.0)	1.27 ± 0.16 (1.0)	2.16 ± 0.43 (1.2)
	+	0.28 ± 0.03 (1.0)	0.27 ± 0.02 (1.6)	0.33 ± 0.04 (1.8)	0.52 ± 0.06 (1.8)	0.56 ± 0.06 (1.7)
glucose-1-P, μM	-	26 ± 4 (1.1)	131 ± 5 (1.2)	20 ± 1 (1.2)	64 ± 14 (1.1)	21 ± 1 (1.0)
	+	38 ± 6 (1.0)	44 ± 6 (1.1)	52 ± 2 (1.1)	68 ± 6 (1.0)	70 ± 3 (1.0)
Mg^{2+}, mM	-	4.47 ± 0.54 (4.2)	3.09 ± 0.22 (2.0)	4.21 ± 0.15 (5.4)	4.48 ± 0.66 (4.6)	4.68 ± 0.65 (5.0)
	+	3.36 ± 0.27 (3.8)	3.91 ± 0.26 (3.4)	3.69 ± 0.40 (3.6)	3.22 ± 0.26 (3.3)	12.6 ± 1.2 (4.4)
Pi, μM	-	51 ± 11 (1.0)	174 ± 14 (1.5)	37 ± 4 (1.1)	28 ± 5 (1.0)	45 ± 10 (1.1)
	+	1190 ± 50 (3.7)	469 ± 25 (3.2)	232 ± 8 (2.0)	127 ± 1 (1.2)	151 ± 12 (1.8)
3PGA, mM		0.04 ± 0.01 (1.0)	1.02 ± 0.08 (1.8)	2.18 ± 0.35 (1.8)	5.51 ± 1.01 (1.8)	5.97 ± 0.79 (1.9)
Vmax (unit/mg[b])	-	4.5 ± 0.6	0.8 ± 0.1	0.9 ± 0.3	0.2 ± 0.0	0.4 ± 0.1
	+	75.8 ± 5.8	103 ± 11	80.4 ± 4.4	1.5 ± 0.2	21.2 ± 2.8

a. The $S_{0.5}$, $I_{0.5}$, and *Vmax* values were determined in the absence or presence of saturating 3PGA for the wild-type and each mutant enzymes as described in "Experimental Procedures."

b. One unit of enzyme activity is expressed as the amount of enzyme required to form 1 μmol of ADP-glucose/min at 37 °C assayed in the synthesis direction as described in "Experimental Procedures."

In general, the mutations at residue 419 caused smaller or no alteration in the apparent affinities for the substrates, ATP, glucose-1-P, Mg^{2+}, and inhibitor, Pi (Table 1), indicating that the conformations of these ligand-binding sites are relatively unchanged. The only significant changes observed were the 5-fold increase in the $A_{0.5}$ for glucose-1-P, the 3-fold increase in $I_{0.5}$ for Pi of the K419R enzyme in the absence of activator, and the 4-fold increase in $S_{0.5}$ for $MgCl_2$ of the K419E enzyme in the presence of activator. For the wild-type enzyme, 2.5 mM of 3PGA desensitizes the Pi inhibition by increasing the $I_{0.5}$ value from 51 to 1190 μM. With apparent affinity relatively unchanged for Pi but largely decreased for 3PGA, the mutant enzymes are more susceptible to the inhibition by having lower $I_{0.5}$ values for Pi in the present of saturating activator. The Lys_{419} mutant enzymes seemed to maintain the heterotropic effect on the binding of ATP exerted by 3PGA (Table 1). The apparent affinities for ATP of the mutant enzymes were increased upon the binding of 3PGA.

Effect of Lys_{419} side chain on 3PGA binding

In view of the effects of size, charge, and hydrophillicity of the substitution amino acids, the apparent affinities for 3PGA was directly compared between the wild-type and the mutant enzymes. As substitutions of Lys_{419} go from basic to neutral to an acidic amino acid, the $A_{0.5}$ values for 3PGA increased correlatively except for the K419Q mutant (Table 1). The cationic property seems to be the most important factor in 3PGA binding at position 419, as K419R enzyme has the highest apparent affinity for 3PGA compared with the other mutant enzymes. However, charge alone probably is not sufficient since the arginine mutant has a significant decrease in apparent 3PGA binding. The size of the amino acid may also be important as arginine is larger than lysine and may sterically interfere with

proper binding of the activator.

The high $A_{0.5}$ value of 3PGA of the K419E mutant may be explained as a result of electrostatic repulsion between the anionic groups of glutamate and 3PGA. However, the effect of the anionic side chain of Glu_{419} probably is not completely reflected by the kinetic data, since the K419E mutant requires a much higher concentration of $MgCl_2$ for maximum activation by 3PGA (Table 1). It is possible that the magnesium ion mediates the interaction between the anionic groups of Glu_{419} and 3PGA, which could minimize the repulsion. Replacement of Lys_{419} with glutamine whose side chain is neutral in charge, hydrophilic, and similar in size to lysine resulted in lower apparent affinity for 3PGA compared to that replaced with alanine, a smaller and non-hydrophilic residue. This result seems to be contradictory with our prediction. Perhaps glutamine interacts with other amino acid(s) through hydrogen bonding and subsequently modifies the conformation of the 3PGA-binding site.

Activator specificities

Since Lys_{419} seems to be mainly involved in 3PGA binding, it was of interest to examine whether the specificity for the allosteric activator has been changed. To do so, several compounds, some of which are known as the major activators of the other bacterial enzymes (2), were used to test their effect in the enzyme assay of the mutants. For all mutant enzymes except for K419Q, 3PGA still is the major activator, while Pi still is the most effective inhibitor (Table 2).

Unexpectedly, fructose-1,6-P_2, the physiological activator for the *E. coli* ADP-glucose pyrophosphorylase, became the most effective activator for the glutamine mutant enzyme (Table 2). Further kinetic studies show that the ratio of activation by 3PGA and by fructose-1,6-P_2 is obviously changed for the K419Q

Table 2. Specificity of allosteric effectors of the wild-type and mutant enzymes. Reactions were performed in synthesis direction of assay as described in "Experiment Procedures" with the presence of effectors as indicated. Data represent the average of two identical duplications.

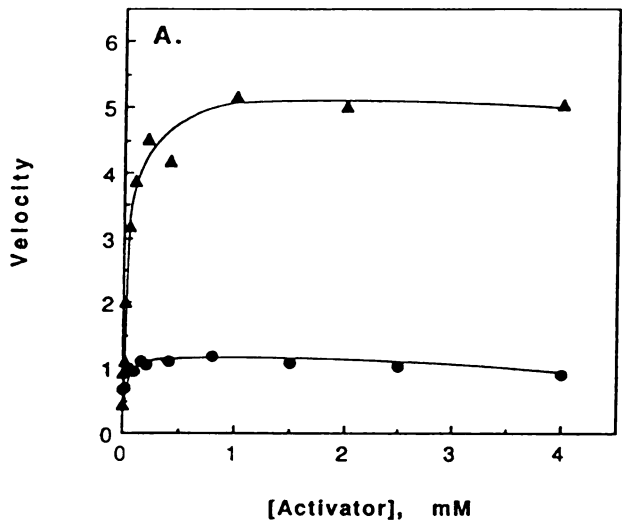
Effectors		wild-type	K419R	K419A	K419Q	K419E
		ADP-Glucose formed nmol/10 min				
None		0.71	0.28	0.34	1.04	0.67
	mM	Relative Activity				
None		1.0	1.0	1.0	1.0	1.0
fructose-6-P	2	5.3				
	5	6.1	0.6	0.8	0.8	0.3
fructose-1,6-P_2	2	1.6				
	5	1.7	10.9	5.5	6.3	0.9
glucose-6-P	2	3.6				
	5	5.2	1.4	0.9	0.9	0.5
glucose-1,6-P_2	2	0.3				
	5	0.3	1.3	0.2	0.5	0.5
P-enolpyruvate	2	6.0				
	5	6.8	34.4	4.7	3.0	0.5
pyruvate	2	1.1				
	5	1.2	0.9	1.0	1.0	0.8
2,3-P-glycerate	2	0.4				
	5	0.2	0.6	0.1	1.0	0.9
3PGA	2	9.2				
	5	10.5	63.5	35.3	3.8	9.6
NADPH	2	0.7	1.5	1.3	1.9	0.8
hexane-1,6-diol-P_2	2	0.5	1.3	4.7	2.7	0.4
ADP	2	0.9	1.6	2.2	1.9	0.8
AMP	2	1.6	9.0	10.3	2.9	1.1
Pi	2	0.1	0.2	0.1	0.1	0.1

enzyme. Usually, 3PGA is about 5-fold more effective than fructose-1,6-P_2 for the wild-type (Fig. 2A) as well as for the K419R and K419A enzymes (data not shown). However, for the K419Q enzyme, fructose-1,6-P_2 is about as effective as 3PGA (Fig. 2B). Interestingly, the mutant enzyme was activated more by fructose-1,6-P_2 than by 3PGA at lower concentrations due to a higher apparent affinity for the former. This could be due to a conformational change in the 3PGA-binding site whose specificity for activator is therefore altered. Similar to that for 3PGA, the mutant enzymes also have increased $A_{0.5}$ values for fructose-1,6-P_2. The $A_{0.5}$ values were altered from 0.06 mM for the wild-type to 0.65, 0.81 and 1.89 mM for the K419R, K419A and K419Q enzymes, respectively.

Some interesting changes were also observed for the mutant enzymes in response to the other activator analogues such as fructose-6-P and glucose-6-P. Even though not as effective as 3PGA, the compounds activated the wild-type enzyme 4- to 6-fold at 5 mM, but were not able to activate the mutant enzymes (Table 2). The wild-type enzyme has an $A_{0.5}$ value of 1.82 mM for fructose-6-P with 8-fold activation, while the K419R enzyme is not activated by up to 40 mM of the compound. The result indicates that Lys_{419} is even more important for the proper binding of the activators with only one anionic group. The mutation of Lys_{419} also affects the binding of P-enolpyruvate for which the $A_{0.5}$ value in the synthesis direction changed from 0.22 mM for the wild-type to 0.69 mM and 1.74 mM for the K419R and K419A enzymes, respectively. These data suggest that these molecules are binding to the same activator-binding site. Previous kinetic studies on spinach leaf ADP-glucose pyrophosphorylase have suggested that fructose-1,6-P_2 and P-enolpyruvate bind to the same site as 3PGA (25).

As mentioned in the introduction, the corresponding lysyl residue of Lys_{419} in the spinach enzyme was modified by pyridoxal-P, the activator analogue. It

Fig. 2. Activation of the wild-type (A) and K419Q (B) enzymes by 3PGA (▲-▲) and fructose-1,6-P_2 (●-●). Initial velocities of the enzymes (in nmol of ADP-glucose formed/10 min) were determined as described in "Experimental Procedures" with the concentrations of 3PGA and fructose-1,6-P_2 being varied. The amounts of the wild-type and K419Q enzymes used were about 0.01 and 0.45 μg, respectively.


A.
Velocity
[Activator], mM
0
1
2
3
4
5
6

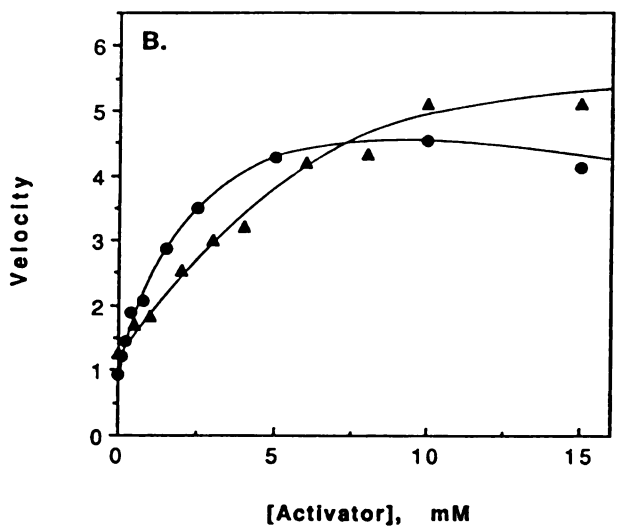

B.
Velocity
[Activator], mM
0
5
10
15

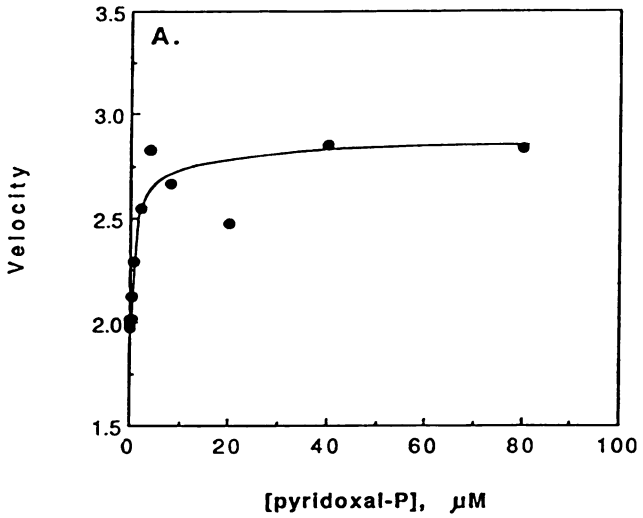
Fig.2

was of interest to see whether the mutation also affected the binding of pyridoxal-P. The *Anabaena* wild-type enzyme was activated by pyridoxal-P only about 1.5-fold but with an $A_{0.5}$ value of 1.1 μM (Fig. 3A). In contrast to the wild-type enzyme, the K419R mutant enzyme displayed a much lower apparent affinity for pyridoxal-P but with activation up to about 20-fold (Fig. 3B). The K419A mutant enzyme had a similar result but the activation level is lower, only 3-fold (data not shown). The $A_{0.5}$ values for pyridoxal-P of K419R and K419A were increased about 75 and 240-fold, 82 and 275 μM, respectively, which may be due to the inability of arginine and alanine to form a Schiff base with pyridoxal-P. However, the possibility of a Schiff base between pyridoxal-P and other lysyl residues of the mutant enzymes is certainly possible.

Competitive study of 3PGA and fructose-1,6-P_2 binding

The alteration in activator specificity of the K419Q mutant raised an immediate question of whether fructose-1,6-P_2 and 3PGA bind to the same or different sites. Fructose-1,6-P_2 activates the *Anabaena* wild-type enzyme only 2-fold with an $A_{0.5}$ value close to that of 3PGA. Therefore, if fructose-1,6-P_2 competitively binds to the 3PGA-binding site, it should decrease the 3PGA activation by increasing its $A_{0.5}$ value. Fig. 4A shows that increasing the concentration of fructose-1,6-P_2 in the assay medium elevated the 3PGA $A_{0.5}$ value in a linear relationship. When the data were replotted as double reciprocal plots (Fig. 4B), the maximal activation velocities were essentially unchanged. The concentration of fructose-1,6-P_2 that increases the 3PGA $A_{0.5}$ value 2-fold is 0.1 mM, which is close to the fructose-1,6-P_2 $A_{0.5}$ value. Moreover, fructose-1,6-P_2 also overcomes the inhibitory effect of Pi by increasing the *Ki* value as does 3PGA. The presence of 2.5 mM fructose-1,6-P_2 increased the *Ki* value of Pi from

Fig. 3. Activation of the wild-type (A) and K419R (B) enzymes by pyridoxal-P. Initial velocities of the enzymes (in nmol of ADP-glucose formed/10 min) were determined as described in "Experimental Procedures" with the concentration of pyridoxal-P being varied. The amounts of the wild-type and K419R enzymes used were about 0.07 and 0.03 μg, respectively.


A.
Velocity
3.5
3.0
2.5
2.0
1.5
0
20
40
60
80
100
[pyridoxal-P], μM

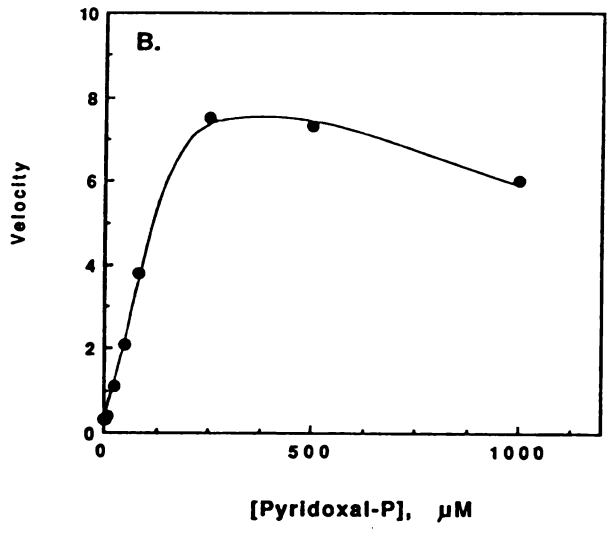

B.
Velocity
10
8
6
4
2
0
0
500
1000
[Pyridoxal-P], μM

Fig.3

Fig. 4. 3PGA saturation curves for the wild-type enzyme in the presence of fructose-1,6-P_2 (A) and the double reciprocal plot of $v-v_o$, the observed velocity at each 3PGA concentration (v) minus the velocity in the absence of 3PGA (v_o), against 3PGA concentration (B). Initial velocities (in nmol of ADP-glucose formed/10 min) were determined as described in "Experimental Procedures", except that the concentration of 3PGA was varied as indicated in the figure. Different amounts of fructose-1,6-P_2 was added to the assay to evaluate its effect on 3PGA activation. The concentrations of fructose-1,6-P_2 are 0 mM (●-●), 0.25 mM (▲-▲), 0.5 mM (○-○), 1.0 mM (Δ-Δ). The inset in panel A is the relationship between the concentrations of fructose-1,6-P_2 and the $A_{0.5}$ values of 3PGA.

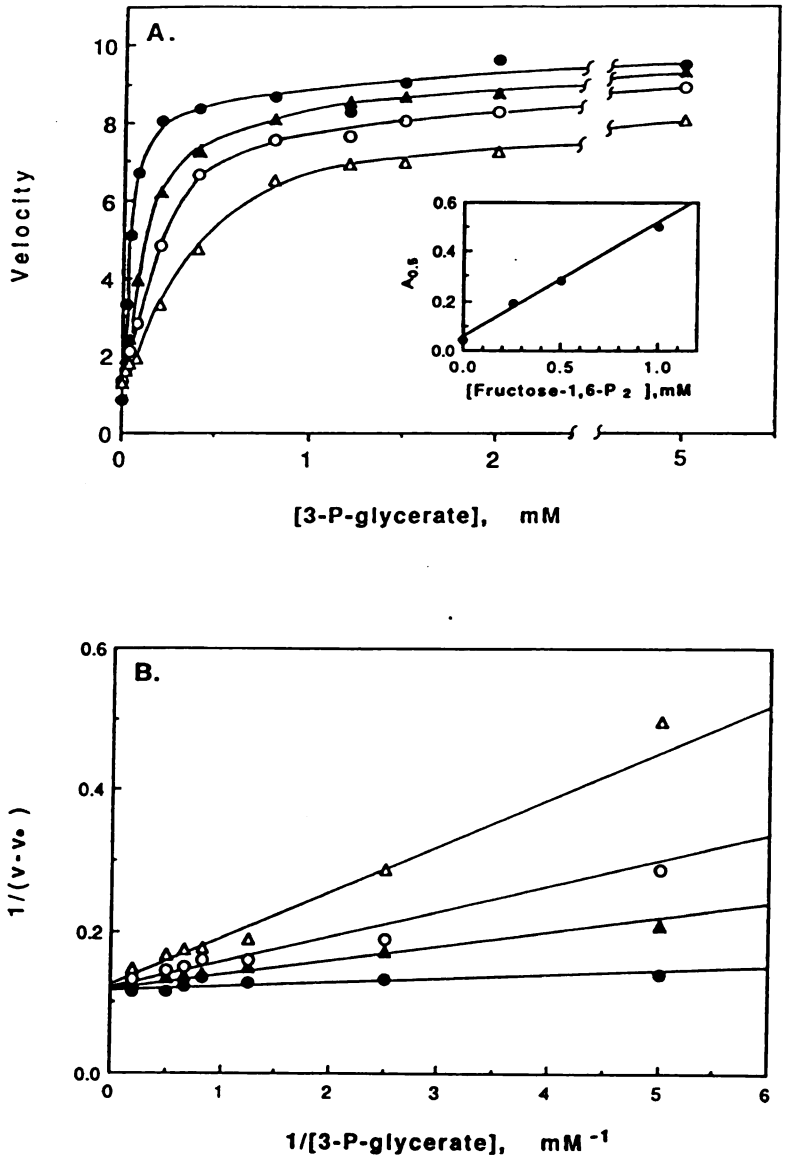

A.
Velocity
[3-P-glycerate], mM
$A_{0.5}$
[Fructose-1,6-P 2],mM
B.
1/(v-v₀)
1/[3-P-glycerate], mM -1

Fig.4

51 μM to about 470 μM. The results suggest that fructose-1,6-P_2 and 3PGA competitively bind to the same allosteric site where Lys_{419} is involved in binding of both compounds.

Thermal stability

To show whether Lys_{419} is essential for the thermal stability, the mutant enzymes was directly compared to the wild-type by heat treatment at 60 °C for 5 min. After the heat treatment, the wild-type enzyme retained 80 % of the activity, while the K419R, K419Q, K419A, and K419E enzymes retained 90, 72, 44, and 16 % activity, respectively. The result indicates that lysyl residue at position 419 is not absolutely required for the stability of the enzyme since replacement with arginine and glutamine did not significantly affect the stability. However, the tolerance for amino acid substitutions at this position is somewhat low suggesting that Lys_{419} may have a role in maintaining the optimal protein folding.

DISCUSSION

Recently, chemical modification of the *Anabaena* enzyme showed that modification of Lys_{419} with pyridoxal-P yields an enzyme no longer requiring the presence of activator for high activity (15). The allosteric activator, 3PGA, and inhibitor, Pi, were very effective in protecting the enzyme from modification (15). These data strongly suggest that Lys_{419} is involved in the binding of the activator. The corresponding lysyl residue in the spinach leaf enzyme was shown to be located at the activator-binding site, identified by chemical modification with pyridoxal-P (13).

In this paper, site-directed mutagenesis experiments have been performed to verify and probe the function of Lys_{419} of *Anabaena* ADP-glucose pyrophosphorylase. According to the results presented here, we conclude that Lys_{419} is primarily involved in 3PGA binding, probably by an ionic interaction between its positively charged ϵ-amino group and the negatively charged carboxyl or phosphate groups of the activator. The large increases of the $A_{0.5}$ values for 3PGA when Lys_{419} was replaced by other amino acids may explain the absolute conservation of lysine in all higher-plant and cyanobacterial ADP-glucose pyrophosphorylases sequenced to date. The lysyl residue probably is required for the allosteric activation of ADP-glucose pyrophosphorylase under physiological concentrations of 3PGA. For thermal stability, residue 419 might be involved, but lysine obviously is not essential. From the *Vmax* values and the kinetic constants for substrates and inhibitor, Lys_{419} is probably not involved in the rate limiting step of the catalytic mechanism or responsible for maintaining the native conformation of the enzyme.

Chemical modification studies of the small subunit of the spinach leaf

enzyme have shown that Pi, in addition to 3PGA, protects the corresponding lysyl residue of Lys_{419} from modification by pyridoxal-P (13). Similar results were obtained for the cyanobacterial enzymes showing that Pi effectively prevented the incorporation of pyridoxal-P (15,26). However, from the kinetic constants for Pi, Lys_{419} is probably not involved in the binding of the inhibitor. The interaction coefficients (n_H) of 3PGA of the mutant enzymes was increased from 1.0 to 1.8-1.9. This probably is induced by the single amino acid replacements. Indeed, it has been observed for other enzymes that single mutation which caused decreased affinity for a ligand resulted in an increase in cooperativity (27,28). A theory of preexisting cooperativity has been proposed by First and Fersht to explain this phenomenon (28).

Replacement of Lys_{419} with glutamine produces a mutant enzyme with altered activator specificity. The K419Q enzyme is activated more by fructose-1,6-P_2 than by 3PGA at lower concentrations. From the competition study, fructose-1,6-P_2 and 3PGA seemed to bind to the *Anabaena* enzyme at the same site with similar affinity. Therefore, it is possible that a slight alteration in the conformation of the activator site could have changed the specificity for activator. Similar cases have been reported before that a single amino acid mutation causes change in specificity for effectors of an enzyme (29-31). However, it is the first report for ADP-glucose pyrophosphorylase subject to such an alteration, which may provides a prospect in the structural relationships between the cyanobacterial and *E. coli* enzymes.

Since the mutant enzymes still can be activated by 3PGA, it is very likely that a second region, in addition to Lys_{419}, is required for the binding of the activator. This could be another region on the same subunit or a region on the other subunit of the homotetrameric enzyme. Chemical modification of the

Anabaena K419R enzyme has shown that another lysyl residue, Lys_{382}, was modified by pyridoxal-P (15). Consistently, the lysyl residue corresponding to Lys_{382} of the large subunit of the spinach enzyme was also modified by reductive phosphopyridoxylation (14). In both cases, the modification was prevented by 3PGA and Pi, suggesting that this lysyl residue may also play an important role in the binding of the activator.

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CHAPTER IV

CHEMICAL MODIFICATION OF THE ALLOSTERIC ACTIVATOR SITES OF *ANABAENA* ADP-GLUCOSE PYROPHOSPHORYLASE WITH PYRIDOXAL-PHOSPHATE

ABSTRACT

Chemical modification of the *E. coli* and spinach leaf ADP-glucose pyrophosphorylases with pyridoxal-P led to the identification of the activator-binding sites. The activator site of the spinach leaf enzyme is closer to the C-terminal half, while that of the *E. coli* enzyme is near the N-terminus. The enzyme from cyanobacterium *Anabaena* is activated by 3-P-glycerate as is the spinach leaf enzyme. However, its subunit structure is similar to the homotetrameric *E. coli* enzyme, and in contrast to the spinach leaf pyrophosphorylase which is heterotetrameric, with two different subunits. Pyridoxal-P, a lysine-specific reagent, has been shown to be an activator of the *Anabaena* ADP-glucose pyrophosphorylase. In the presence of $NaBH_4$ and pyridoxal-P the *Anabaena* enzyme was covalently modified. The modified enzyme was more active in the absence of 3-P-glycerate and more resistant to phosphate inhibition. Sequencing of the tryptic [^{3}H]pyridoxal-P-labeled peptide, purified by reverse phase HPLC, indicated that a C-terminus lysine (Lys_{419}) was modified. Further experiments showed that pyridoxal-P can be covalently bound to a mutant enzyme in which Lys_{419} is replaced by an arginine. The modified mutant enzyme also has greatly altered allosteric properties. The [^{3}H]pyridoxal-P-labeled tryptic peptide was purified and sequenced. In this case, an alternative lysyl residue, Lys_{382}, of the mutant enzyme was labeled. Both Lys_{419} and Lys_{382} correspond to the lysyl residues within the activator-binding site of the spinach leaf enzyme.

INTRODUCTION

ADP-glucose pyrophosphorylase (ATP:α-glucose-1-P adenylyl transferase, EC 2.2.7.37) catalyzes the reversible synthesis of ADP-glucose and pyrophosphate from ATP and α-glucose-1-P. The enzyme plays a regulatory role in the biosynthesis of glycogen in bacteria (1) and starch in plants (2).

Major differences exist between the bacterial and plant enzymes with regard to allosteric properties and subunit structure. ADP-glucose pyrophosphorylases from plant, algae and cyanobacteria are mainly activated by the CO_2-fixation product, 3-P-glycerate (3PGA), and inhibited by orthophosphate (Pi) (2-5). The *E. coli* and *Salmonella typhimurium* enzymes are activated by the glycolytic intermediate, fructose-1,6-P_2, and inhibited by AMP and ADP (1). ADP-glucose pyrophosphorylases from algae (6) and higher-plant tissues (2) are heterotetrameric composed of two subunits. In contrast, the enzymes from *E. coli*, *Salmonella typhimurium* and cyanobacteria are homotetrameric (1, 4).

Previous efforts have been made to locate the activator-binding sites of *E. coli* ADP-glucose pyrophosphorylase by chemical modification with [^{3}H]pyridoxal-P (7). Pyridoxal-P, an activator analog of the enzyme, is covalently linked to a lysyl residue presumably located at the activator-binding site by reducing with $NaBH_4$. The lysyl residue is situated near the N-terminus at position 39.

Pyridoxal-P in addition to 3PGA can activate the spinach leaf ADP-glucose pyrophosphorylase. Reductive phosphopyridoxylation has been performed on the spinach leaf enzyme, and different results were obtained (8, 9). Four lysyl residues of the enzyme are modified by [^{3}H]pyridoxal-P with asymmetrical distribution on the two subunits of the enzyme. One lysine is located on the C-terminus of the small (51 kD) subunit (8), while the other three are present on the

large (54 kD) subunit (9).

Cyanobacterial ADP-glucose pyrophosphorylase has characteristics intermediate to that of the higher-plant and *E. coli* enzyme, *i.e.*, having the same specificity for effectors as does the higher-plant enzyme and being homotetrameric similar to the *E. coli* enzyme (4). Previous studies have shown that pyridoxal-P binds to the activator site of the cyanobacterial enzyme (10). In this paper, we report the chemical modification of the cyanobacterial ADP-glucose pyrophosphorylase from *Anabaena* PCC 7120 and the sequence of the phosphopyridoxylated peptide. A lysine near the C-terminus, Lys_{419}, is specifically modified by pyridoxal-P. Chemical modification was also performed on a mutant enzyme where Lys_{419} was substituted by an arginine. This mutant enzyme, although still activated by 3PGA, required much higher concentrations of activator (11). An alternative lysyl residue, Lys_{382}, is modified by reductive chemical modification with pyridoxal-P. The data suggest that Lys_{382} in addition to Lys_{419} are involved in the binding of activator.

MATERIALS AND METHODS

Chemicals

Pyridoxal-5'-P, ATP, ADP-glucose, α-glucose-1-P, 3PGA, sequencing-grade trypsin and inorganic pyrophosphatase were purchased from Sigma Company. [^{14}C]glucose-1-P was obtained from Amersham Corp. NaB[^{3}H]$_4$ was from New England Nuclear. [4-^{3}H]Pyridoxal-P was synthesized by reduction of pyridoxal-P with NaB[^{3}H]$_4$ and reoxidized with MnO_2 by the method of Stock et al. (12). The preparation was chromatographed on Dowex 1x8-100 (12) and elution of labeled pyridoxal-P was monitored by thin layer chromatography (13). The specific activity of the stock preparation was 2.2 x 10^6 cpm/nmol and was used within a week of chromatography. All other reagents were of the highest available commercial grade.

Enzyme purification

Anabaena ADP-glucose pyrophosphorylase was purified from the *E. coli* cells containing the recombinant *Anabaena* gene (14). The K419R mutant enzyme was generated by site-directed mutagenesis as previously described (11). Both the wild-type and mutant enzymes were purified to more than 90 % homogeneity as previously described (11). Protein concentration of purified enzyme was determined by absorbance at 280 nm, using an extinction coefficient of 1.0 for 1.0 mg of protein/ml/cm or by using the bicinchoninic acid reagent (15) with BSA as the standard. Data from these two methods are in good agreement.

ADP-glucose pyrophosphorylase assay

Enzyme activity was assayed in the ADP-glucose synthesis direction

according to the method of Preiss *et al* (16).

(A) Activated conditions. For assay of the wild-type enzyme in the presence of activator, the reaction mixtures contained 20 μmol Hepes-NaOH buffer (pH 8.0), 0.1 μmol of [^{14}C]glucose-1-phosphate (about 1,000 cpm/nmol), 0.5 μmol of ATP, 2 μmol of $MgCl_2$, 50 μg of bovine serum albumin, 0.15 unit of inorganic pyrophosphatase, 0.5 μmol of 3PGA and enzyme in a final volume of 200 μl. The assays were initiated with the addition of enzyme and incubated at 37 °C. For assay of the mutant enzyme, the reaction mixtures were identical to wild-type, with the exception that 2 μmol of 3PGA was used.

(B) Unactivated conditions. The synthesis of ADP-glucose in the absence of activator was measured as described above except that 3PGA was omitted and the amount of ATP was increased to 1 μmol in the reaction mixtures. The amount of [^{14}C]glucose-1-phosphate was increased to 0.25 μmol for the K419R mutant enzyme.

Treatment of kinetic data

For determination of kinetic parameter, the concentration of the substrate or effector tested was systematically varied with the other substrates and effectors fixed at a saturating concentration as described previously (11). Kinetic data were treated as described previously (11). Kinetic parameters were expressed as $S_{0.5}$, $A_{0.5}$, and $I_{0.5}$, which correspond to the concentration of substrate, activator, or inhibitor required for 50 % of maximal velocity, activation, or inhibition, respectively.

Reductive phosphopyridoxylation

ADP-glucose pyrophosphorylase, 100 μg, in 50 mM Hepes-NaOH (pH 8.0)

was incubated with [^{3}H]pyridoxal-P of indicated concentrations (see Results) in a final volume of 1 ml, in an 1.5 ml eppendorf tube. Following incubation in the dark for 30 min at room temperature, 100 μl of $NaBH_4$ was added to a final concentration of 49 mM. The reduction was allowed to proceed for 60 min in the dark at room temperature. Aliquots of the reaction mixtures were desalted through Sephadex G-50 to remove free pyridoxal-P (17). The desalted sample was used for enzyme assay to evaluate the effect on ADP-glucose synthesis in the absence and presence of 3PGA. The specific activities of [^{3}H]pyridoxal-P was 386,000 cpm/nmol for the peptide isolation study.

Measurement of [^{3}H]pyridoxal-P incorporation

After reductive phosphopyridoxylation as described above, the incorporation of [^{3}H]pyridoxal-P into protein was measured by determining trichloroacetic acid (TCA) precipitable counts. One ml of cold 10 % (w/v) TCA was added to 200 μl of phosphopyridoxylation mixtures followed by 1 hr of incubation on ice. The precipitate was collected by centrifugation at 12,000 g for 5 min, washed three times with cold 5 % TCA, and dissolved in 400 μl of 3 % Na_2CO_3 containing 0.1 N NaOH (Sample A). The protein concentration of sample A was measured using a micro bicinchoninic acid reagent kit from Pierce. The radioactivity was determined by counting 200 μl sample A in 5 ml of Safety Solve following the addition of 15 μl of 6 N HCl. The specific activity of [^{3}H]pyridoxal-P was 25,000 cpm/nmol. The calculated molecular weight of the *Anabaena* enzyme is 48,341/subunit (48,369/subunit for the K419R enzyme).

Tryptic digestion of the modified enzyme

ADP-glucose pyrophosphorylase was first reductively phosphopyridoxylated

under the conditions mentioned above. The concentrations of [^{3}H]pyridoxal-P were 10 and 50 μM for the wild-type and K419R enzymes, respectively. The reaction mixtures were concentrated to about 0.5 mg/ml protein following dialysis against 50 mM Hepes buffer, pH 8.0. The phosphopyridoxylated protein was then digested with trypsin at a trypsin/substrate ratio (w/w) of 1:20 in 50 mM Hepes buffer, pH 8.0, at 37 °C for 20 hr. The digestion reaction was stopped by heating in a boiling water bath for 10 min. After cooling, the sample was treated with 10 μg of fresh trypsin for another 20 hr digestion at 37 °C . Completeness of tryptic digestion was monitored by running a portion of the sample on 15 % SDS-polyacrylamide gel electrophoresis (18).

HPLC fractionation of tryptic peptides

Peptides labeled with [^{3}H]pyridoxal-P were isolated by reverse phase chromatography on a Waters Nova-Pak C18 column (3.9 x 300 mm) attached to a Waters' high performance liquid chromatography (HPLC) system. Following the injection of sample, the column was washed with solvent A (0.1 % trifluoroacetic acid (TFA) in water) for 10 min and eluted with a 100 ml linear gradient of 0-80 % solvent B (0.1 % TFA in 90 % acetonitrile) with a flow rate of 1 ml/min. The elution of the peptides was monitored by absorbance at 214 nm and collected by hand. The radioactive peak was detected by counting a portion of each fraction in 5 ml of Safety Solve with liquid scintillation counter.

The [^{3}H]pyridoxal-P labeled peptides were further purified by using a second solvent system. The peaks from the first chromatograph were lyophilized and resuspended in solvent C (0.1 % heptafluorobutyric acid (HFBA)). They were rechromatographed on an Applied Biosystems C18 column (1 x 250 mm) attached to a Brownlee Labs Microgradient System (Applied Biosystems) eluted with a 3.6

ml linear gradient of 10-100 % solvent D (0.1 % HFBA in 90 % acetonitrile) with a flow rate of 40 μl/min. The radioactive fractions were absorbed to PVDF membrane and allowed to dry. The membrane was washed with water prior to N-terminal sequencing.

N-terminal sequence analysis

The radioactive peptide was sequenced by sequential automated Edman degradation using a 477A protein sequencer and the PTH derivatized amino acids were detected by a 120A analyzer from Applied Biosystems. The position of the labeled amino acid in the peptide was determined by collecting the PTH-amino acid from each cycle and measuring the radioactivity in 5 ml of Safety Solve.

RESULTS

Reductive phosphopyridoxylation of the wild-type *Anabaena* ADP-glucose pyrophosphorylase

Previous studies have shown that pyridoxal-P is an activator of *Anabaena* ADP-glucose pyrophosphorylase (10, 11). The enzyme has a higher apparent affinity for pyridoxal-P ($A_{0.5}$, 1.1 μM) than for 3PGA ($A_{0.5}$, 40 μM), the physiological activator. However, pyridoxal-P activates the enzyme only about 2-fold while 3PGA usually gives 10- to 15-fold activation. (4, 10, 11).

The *Anabaena* enzyme is covalently modified by pyridoxal-P with the reducing agent $NaBH_4$. Reductive phosphopyridoxylation yields enzyme more active in the absence of 3PGA and less active in the presence of the activator than the unmodified enzyme (Table 1). The ratio of enzyme activity in the absence and presence of 3PGA was increased upon the modification with increased concentrations of pyridoxal-P. If either pyridoxal-P or $NaBH_4$ is omitted from the modification reaction mixtures the activity ratio is essentially unchanged compared to the unmodified enzyme. Without pyridoxal-P in the mixtures, the enzyme is less stable and has decreased activity (Table 1).

In the presence of 10 and 50 μM of pyridoxal-P, 1.9 and 2.0 moles of pyridoxal-P were incorporated into one mole of tetrameric enzyme, respectively. The incorporation of pyridoxal-P was effectively inhibited by inclusion of the activator, 3PGA, or the inhibitor, Pi (Table 2). This inhibition of incorporation was concomitant with the low -3PGA/+3PGA activity ratio. Fructose-1,6-P_2, which has been shown competitively bind to the 3PGA site (11), also provided good protection. The substrates, ATP, ADP-glucose, glucose-1-P, and PPi, however, were less effective.

Table 1. Effect of reductive phosphopyridoxylation on *Anabaena* ADP-glucose pyrophosphorylase (wild-type) activity. The phosphopyridoxylation reaction was done as described under "Materials and Methods" with the pyridoxal-P concentrations indicated in the table. Controls in the absence of $NaBH_4$ were performed in parallel. Enzyme activity was assayed in the presence of 2.5 mM 3PGA (+ 3PGA) and in the absence of the activator (- 3PGA).

[Pyridoxal-P]		Enzyme Activity - 3PGA	Enzyme Activity + 3PGA	-3PGA/+3PGA Activity Ratio
		ADP-glucose formed (nmol/10 min)		
μM	$NaBH_4$			
0	-	0.88	12.3	0.08
0	+	0.82	13.7	0.06
1	-	1.64	24.3	0.07
1	+	4.30	11.7	0.37
5	-	2.14	26.1	0.08
5	+	7.78	9.38	0.83
10	-	1.97	26.9	0.07
10	+	7.34	8.77	0.84
50	-	1.83	26.0	0.07
50	+	7.22	7.95	0.90
100	-	2.15	26.5	0.08
100	+	6.71	7.10	0.94

Table 2. Effect of substrates and allosteric effectors on the reductive phosphopyridoxylation of *Anabaena* ADP-glucose pyrophosphorylase (wild-type). The enzyme was modified with 10 μM of [^{3}H]pyridoxal-P in the presence of the specified compound. Incorporation of [^{3}H]pyridoxal-P was measured by TCA precipitable count as described in "Materials and Methods" except that the protein was co-precipitated with 50 μg BSA. The incorporation in the absence of protecting compound was arbitrarily set as 100 %. Enzyme activity was assayed as was in Table 1.

Substrate or Effector	-3PGA/+3PGA Activity Ratio	Incorporation %
None, no $NaBH_4$	0.08	-
None	0.84	100
$MgCl_2$, 5 mM	0.86	97
ADPGlc, 2 mM $MgCl_2$, 5 mM	0.80	93
ATP, 2 mM $MgCl_2$, 5 mM	0.78	94
Glc-1-P, 2 mM	0.74	92
PPi, 2 mM	0.31	29
Pi, 2 mM	0.14	< 1
3PGA, 2 mM	0.12	3
Fru-1,6-P_2, mM	0.15	8

Kinetics of the phosphopyridoxylated wild-type enzyme

Reductive phosphopyridoxylation with 10 μM of pyridoxal-P resulted in an enzyme almost insensitive to activation by 3PGA (Fig. 1). The modified enzyme also exhibited less sensitivity to the inhibition by Pi compared to the unmodified enzyme (Fig. 2). The concentration of Pi required to inhibit 50 % activity of the modified wild-type enzyme is about 15-fold higher than that for the unmodified enzyme, 625 μM as compared to 40 μM.

The modification also diminished the synergistic effect of the activator on the binding of ATP. For the unmodified enzyme, the $S_{0.5}$ value for ATP was decreased from 1.88 mM to 0.28 mM in the presence of 3PGA. For the modified enzyme, the $S_{0.5}$ values of ATP are about the same, 0.72 and 0.74 mM, in the absence and presence of the activator, respectively. The modified enzyme has similar kinetic parameters for glucose-1-P and Mg^{2+} as the unmodified enzyme in the absence and presence of 3PGA (data not shown).

Isolation and sequencing of the [^{3}H]pyridoxal-P labeled peptides of the wild-type enzyme

The [^{3}H]pyridoxal-P modified enzyme was subject to tryptic digestion under non-denaturing conditions. The digestion appeared to be complete as judged from SDS-PAGE. The sample was centrifuged at 12,000 x g before loading on the C18 column. All the radioactivity was retained in the supernatant fluid. The tryptic peptides were separated by reverse phase HPLC. A single radioactive peak containing most of the eluted radioactivity was identified (Fig 3). The radioactive peptide was further purified by rechromatography in a second solvent system as described in Materials and Methods.

The amino acid sequence of the purified peptide was determined by

Fig 1. Activation of the modified (▲-▲) and unmodified (●-●) *Anabaena* ADP-glucose pyrophosphorylase (wild-type) by 3PGA. The enzyme was modified with 10 μM pyridoxal-P and assayed under activated condition as described in "Materials and Methods".

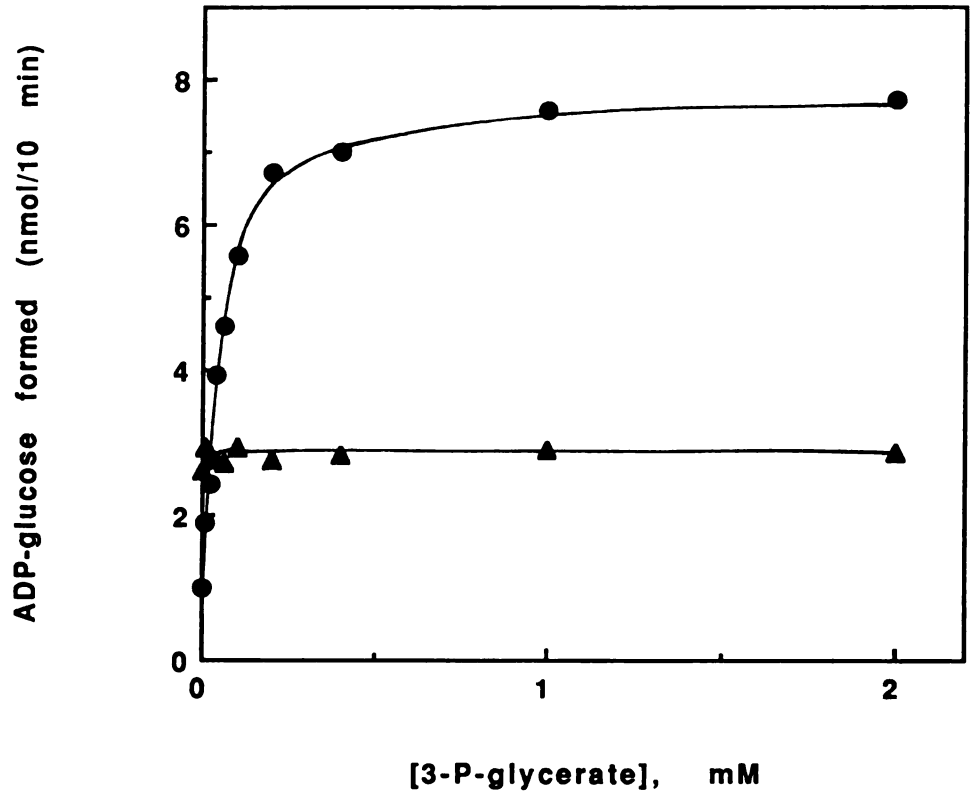

Fig.1

Fig 2. Inhibition of the modified (▲-▲) and unmodified (●-●) *Anabaena* ADP-glucose pyrophosphorylase (wild-type) by Pi. The enzyme was modified with 10 μM pyridoxal-P and assayed under unactivated condition as described in "Materials and Methods".

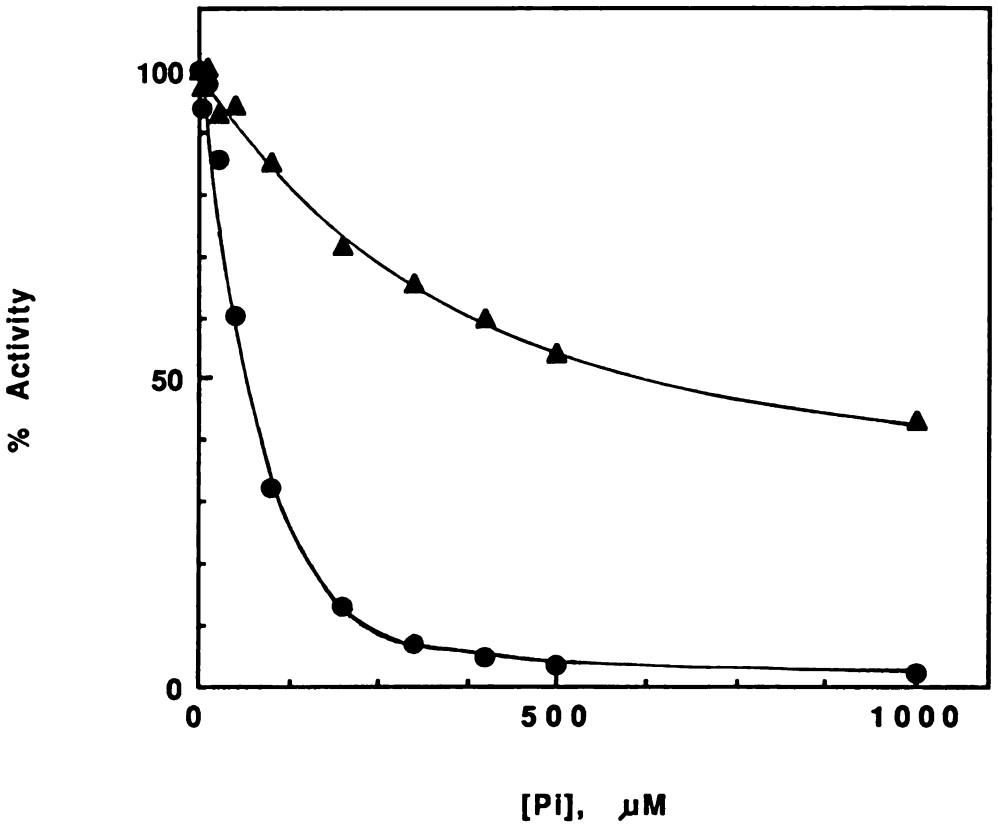

Fig.2

Fig 3. Reverse phase HPLC of the tryptic digest of the [^{3}H]pyridoxal-P labeled *Anabaena* ADP-glucose pyrophosphorylase (wild-type). The peptides (200 μg) were separated on a C18 column (3.9 x 300 mm) with 1 ml/min flow rate and a gradient of solvent A and B as described in "Materials and Methods". Fractions were collected by hand and a portion of each fraction was counted to measure radioactivity.

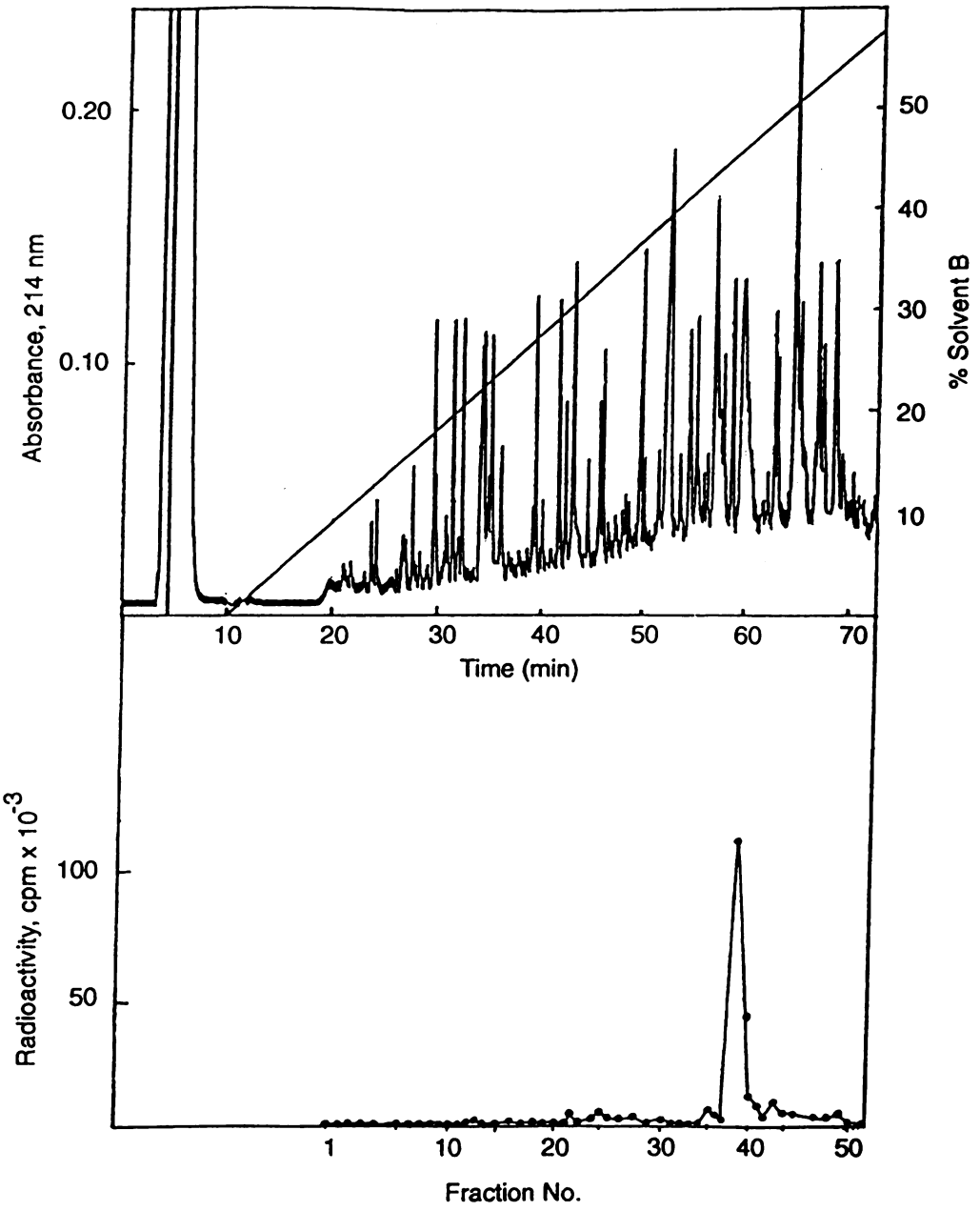
0.20
0.10
Absorbance, 214 nm
50
40
30
20
10
% Solvent B
10
20
30
40
50
60
70
Time (min)
Radioactivity, cpm x 10^-3
100
50
1
10
20
30
40
50
Fraction No.

Fig.3

automated Edman degradation and was found to be: Ser-Gly-Ile-Val-Val-Val-Leu-X-Asn-Ala-Val-Ile-Thr-Asp-Gly-. The PTH derivative obtained in cycle 8 can not be identified (indicated by X). However, it contained the radioactive residue, presumably, the phosphopyridoxylated lysine. The alignment of this sequence to the deduced amino acid sequence of *Anabaena* ADP-glucose pyrophosphorylase (14) indicated that Lys_{419} corresponds to the labeled residue in the isolated peptide.

Reductive phosphopyridoxylation of the K419R mutant enzyme

To see whether a mutation at Lys_{419} can prevent phosphopyridoxylation, we performed the same study on a mutant enzyme in which Lys_{419} was replaced by an arginine. The mutant enzyme has been characterized previously and was shown to be similar to the wild-type enzyme except that the binding of 3PGA was altered (11). The $A_{0.5}$ value of 3PGA of the K419R mutant enzyme is 25-fold higher than the wild-type enzyme. As was observed for the wild-type enzyme, pyridoxal-P also can activate the mutant enzyme but not as much as 3PGA (Fig. 4).

Incorporation studies showed that the mutant enzyme still can be covalently modified by reductive phosphopyridoxylation. As was found with the wild-type enzyme, the -3PGA/+3PGA activity ratio of the mutant enzyme was increased upon modification with increased concentrations of pyridoxal-P (Fig. 5). Unlike the modified wild-type enzyme, the modified K419R enzyme was about as active as the unmodified mutant enzyme in the presence of 3PGA (data not shown). In the presence of 50 and 100 μM of [^{3}H]pyridoxal-P, 1.4 and 1.3 moles of [^{3}H]pyridoxal-P were incorporated into one mole of tetrameric enzyme, respectively.

The modified K419R enzyme was also less sensitive to 3PGA activation

Fig 4. Activation of the K419R mutant enzyme by 3PGA and by pyridoxal-P. The enzyme was assayed under activated condition as described in "Material and Methods" with the activator of indicated concentration.

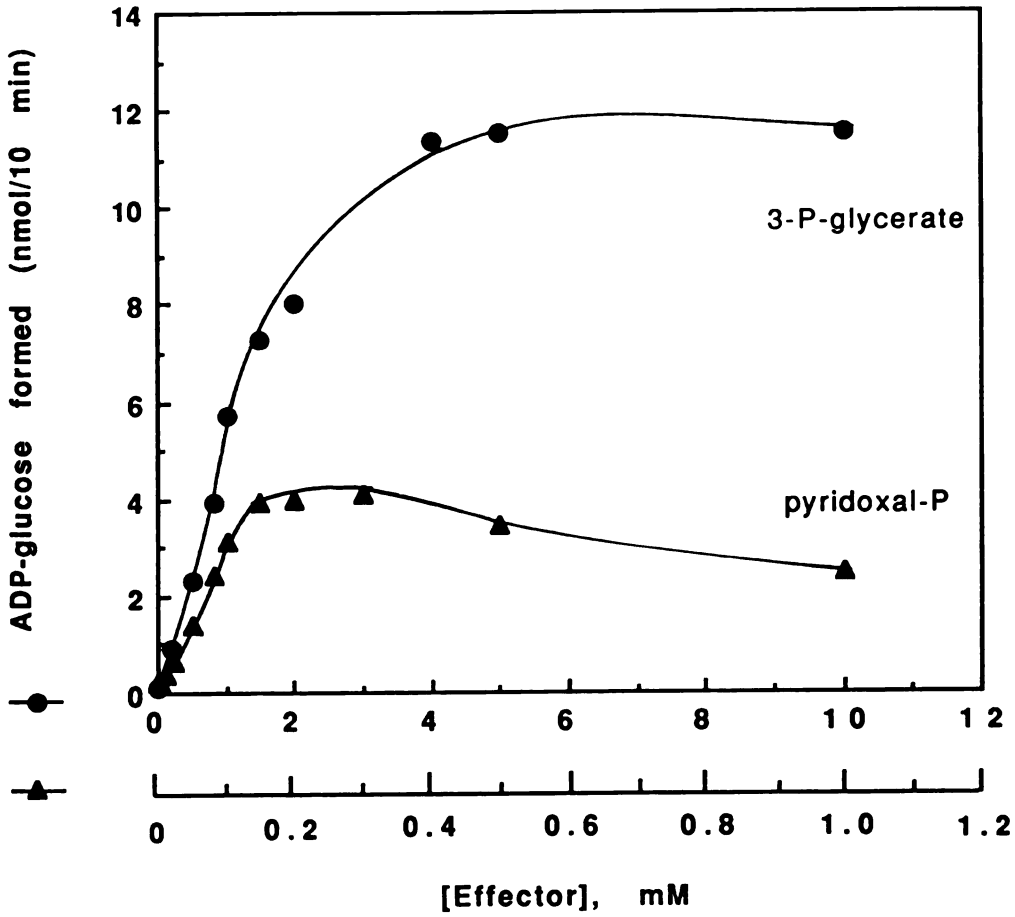
ADP-glucose formed (nmol/10 min)
14
12
10
8
6
4
2
0
3-P-glycerate
pyridoxal-P
0
2
4
6
8
10
12
0
0.2
0.4
0.6
0.8
1.0
1.2
[Effector], mM

Fig.4

Fig 5. Effect of reductive phosphopyridoxylation (▲-▲) on the -3PGA/+3PGA activity ratio of K419R mutant ADP-glucose pyrophosphorylase. The reductive phosphopyridoxylation reaction was proceeded as described under "Materials and Methods". Controls (●-●) in the absence of $NaBH_4$ were performed in parallel. Enzyme activity was assayed in the presence of 10 mM 3PGA (+3PGA) and in the absence of the activator (-3PGA).

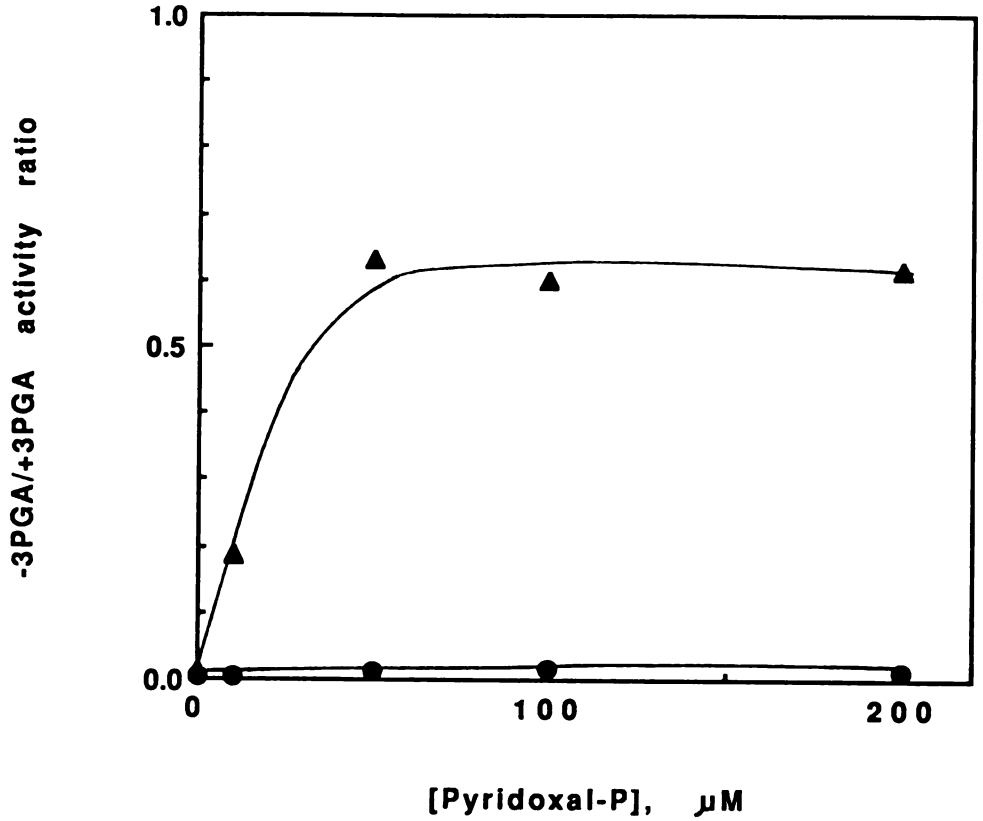

Fig.5

(Fig. 6) and almost insensitive to Pi inhibition compared to the unmodified enzyme (Fig. 7). As shown in Table 3, the incorporation of pyridoxal-P was inhibited most effectively by 3PGA. Pi and fructose-1,6-P_2 also inhibited more than 90 % of the incorporation (Table 3).

Isolation and sequencing of the [^{3}H]pyridoxal-P labeled peptides from the K419R enzyme

The tryptic [^{3}H]pyridoxal-P labeled peptide from the modified K419R enzyme was isolated and sequenced as was the peptide of the wild-type enzyme. The major radioactive peak was eluted at lower percentage of acetonitrile compared to that of the wild-type enzyme (Fig. 8). The peak was further purified in a second solvent system (as described in Materials and Methods) and subject to amino acid sequencing. The sequence was found to be: Ala-Ile-Ile-Asp-X-Asn-Ala-Arg. The PTH derivative in cycle 5 contained the labeled residue. The location of this peptide was identified by aligning it to the deduced amino acid sequence of *Anabaena* pyrophosphorylase (14). The unidentified residue in cycle 5 corresponds to Lys_{382} of the enzyme.

Fig 6. Activation of the modified (▲-▲) and unmodified (●-●) *Anabaena* ADP-glucose pyrophosphorylase (K419R mutant) by 3PGA. The enzyme was modified with 50 μM pyridoxal-P and assayed under activated condition as described in "Materials and Methods" .

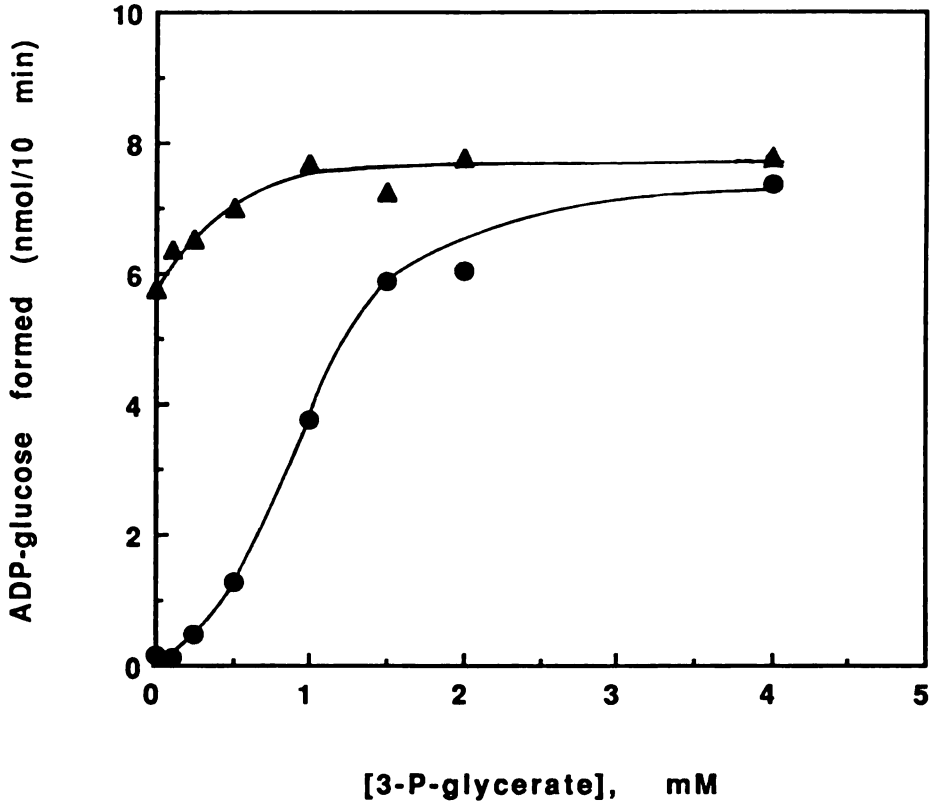
ADP-glucose formed (nmol/10 min)
10
8
6
4
2
0
0
1
2
3
4
5
[3-P-glycerate], mM

Fig.6

Fig 7. Inhibition of the modified (▲-▲) and unmodified (●-●) *Anabaena* ADP-glucose pyrophosphorylase (K419R mutant) by Pi. The enzyme was modified with 50 μM pyridoxal-P and assayed under unactivated condition as described in "Materials and Methods" .

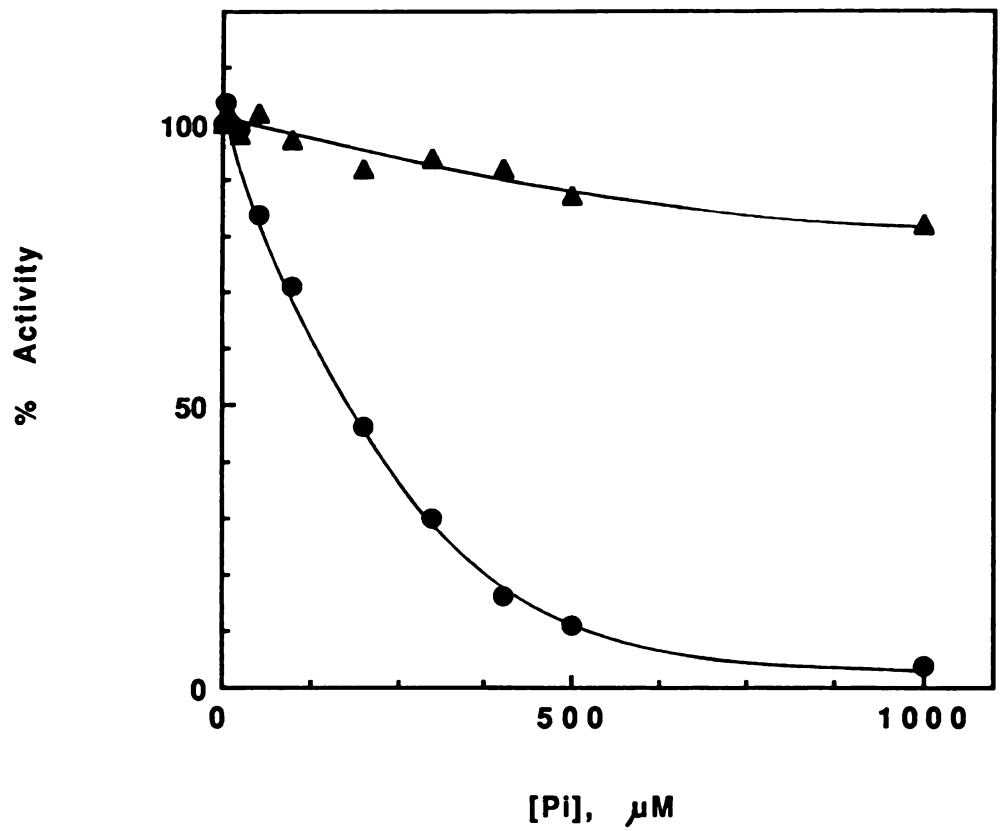

Fig.7

Table 3. Effect of substrates and allosteric effectors on the reductive phosphopyridoxylation of *Anabaena* ADP-glucose pyrophosphorylase (K419R mutant). The enzyme was modified with 50 μM of [^{3}H]pyridoxal-P in the presence of the specified compound. Incorporation of [^{3}H]pyridoxal-P was measured as described in Table II. The incorporation in the absence of protecting compound was arbitrarily set as 100 %. Enzyme activity was assayed as was in Table 1.

Substrate or Effector	-3PGA/+3PGA Activity Ratio	Incorporation %
None, no $NaBH_4$	0.02	-
None	0.63	100
$MgCl_2$, 5 mM	0.59	114
ADPGlc, 2 mM $MgCl_2$, 5 mM	0.47	99
ATP, 2 mM $MgCl_2$, 5 mM	0.20	81
Glc-1-P, 2 mM	0.25	83
PPi, 2 mM	0.12	22
Pi, 2 mM	0.04	10
3PGA, 2 mM	0.03	< 0.1
Fru-1,6-P_2, mM	0.04	< 0.1

Fig 8. Reverse phase HPLC of the tryptic digest of the [^{3}H]pyridoxal-P labeled *Anabaena* ADP-glucose pyrophosphorylase (K419R mutant). The peptides were separated under conditions the same as that for the wild-type enzyme as indicated in Fig. 3.

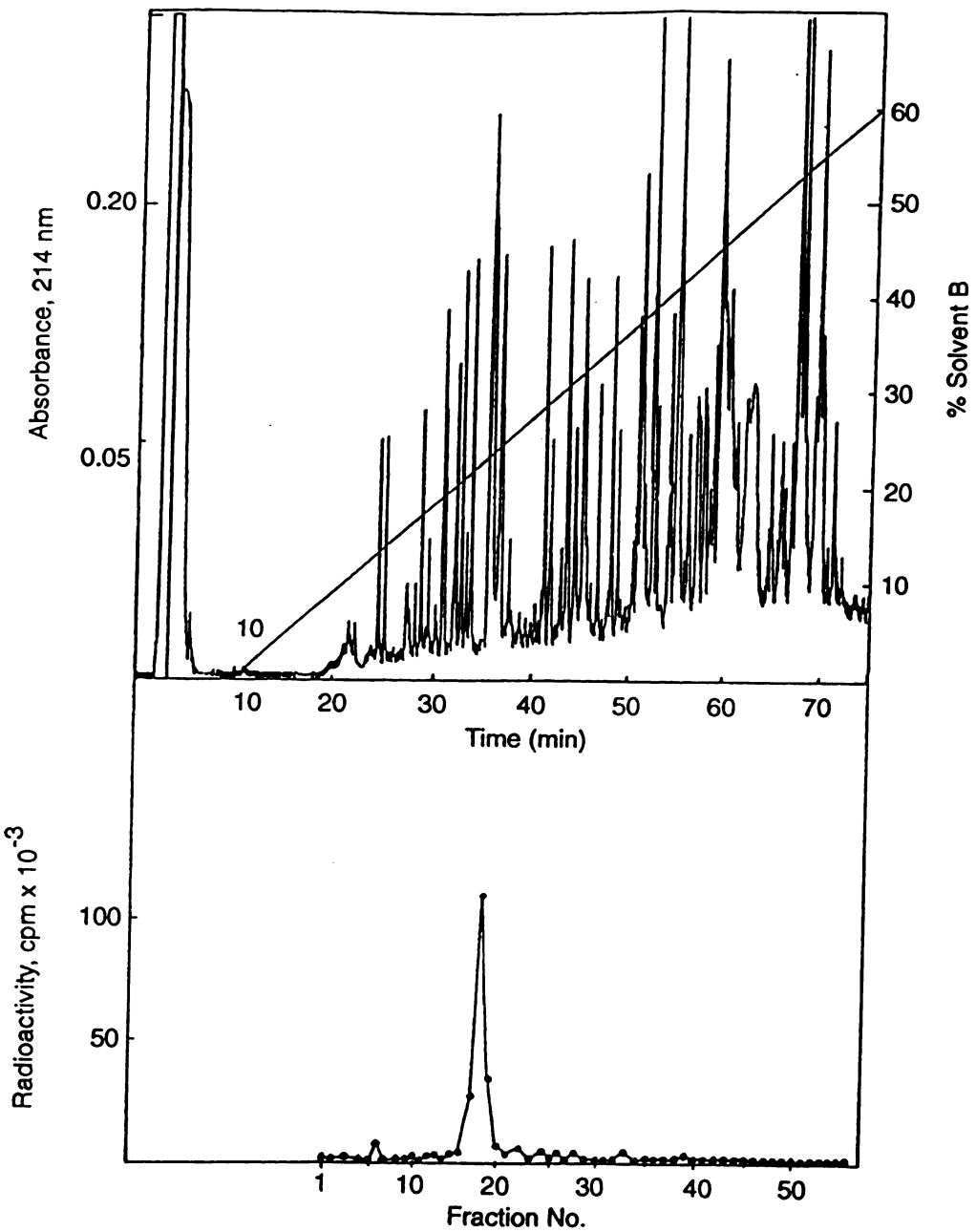
Absorbance, 214 nm
0.20
0.05
10
10
20
30
40
50
60
70
Time (min)
% Solvent B
60
50
40
30
20
10
Radioactivity, cpm x 10^-3
100
50
1
10
20
30
40
50
Fraction No.

Fig.8

DISCUSSION

Chemical modification of *Anabaena* ADP-glucose pyrophosphorylase with pyridoxal-P greatly effected the allosteric properties of the enzyme. The modified enzyme was almost insensitive to activation by 3PGA and was less sensitive to inhibition by Pi. The incorporation of radioactive labeled pyridoxal-P can be effectively prevented by the inclusion of the allosteric effectors while the substrates/products inhibited poorly except PPi which also an inhibitor of the enzyme. Thus, it can be considered an analogue of Pi that also inhibits the binding of pyridoxal-P (8). In the presence of 3PGA, the modified enzyme has lowered activity compared to the unmodified enzyme. This may be due to the inhibition of 3PGA activation by pyridoxal-P, which is a less potent activator. The increased activity in the absence of 3PGA indicates that the catalytic site was relatively intact under the conditions of reductive phosphopyridoxylation. Taken together, the results suggest that the binding of pyridoxal-P is primarily at the activator site, which is consistent with the findings for the spinach leaf (8) and *Synechocystis* enzymes (10).

Pyridoxal-P appeared to bind predominantly at a single location in the wild-type enzyme. The sequence of this peptide matches to the C-terminus of the deduced amino acid sequence of the *Anabaena* enzyme. Lys_{419} is the only lysyl residue within this peptide and appears to be trypsin-resistant due to phosphopyridoxylation. It corresponds to the C-terminus lysyl residues that were phosphopyridoxylated in the spinach leaf small (8) and large (9) subunits.

It has been shown that the lysyl residue corresponding to Lys_{419} is highly conserved in all the cyanobacterial and higher-plant pyrophosphorylases sequenced to date (9, 19). Structure-function studies of this lysine have been performed using

site-directed mutagenesis. The results indicate that Lys_{419} of the *Anabaena* enzyme is mainly involved in the binding of 3PGA (11). Replacement of Lys_{419} with Arg, Ala, Gln or Glu largely reduced the apparent affinity for 3PGA while having no or small effects on the kinetic parameters for the substrates and the inhibitor, Pi. This is consistent with our protection studies in which 3PGA is the most effective in protecting the enzyme from chemical modification. Although Pi also protected the enzyme as well as 3PGA, it is possible that the 3PGA and Pi binding sites are overlapping or Pi causes the enzyme adopt a conformation of low affinity for pyridoxal-P.

We took the advantage of having the K419R mutant enzyme at hand to see whether pyridoxal-P can covalently modify the mutant enzyme. The incorporation of [^{3}H]pyridoxal-P into the mutant enzyme was about 30 % lower than that of the wild-type enzyme. An alternative peptide of the K419R mutant enzyme was found labeled by [^{3}H]pyridoxal-P. From sequencing analysis, Lys_{382} appears to be the major target of the reductive phosphopyridoxylation of the K419R enzyme. Interestingly, the peptide corresponds to one of the modified sites of the spinach leaf large subunit (9). Alignment of all the amino acid sequences of ADP-glucose pyrophosphorylase available has shown that Lys_{382} is conserved in all the cyanobacterial, the small subunit of the higher-plant enzymes and in the large subunit of the spinach leaf, wheat leaf, and potato tuber enzymes (9, 19). These results suggests that the location and structure of the activator site of the cyanobacterial and higher-plant enzymes are quite similar. It is in good agreement with the high identity (50-60 %) of their overall primary structures and similar allosteric properties.

The kinetic and protection studies suggest that Lys_{382} is also located at the activator-binding site. This supports the suggestion made on the basis of site-

directed mutagenesis of the *Anabaena* ADP-glucose pyrophosphorylase Lys_{419} residue (11) that another basic amino acid residue is also involved in the binding of the activator. Site-directed mutagenesis experiments at Lys_{382} may further elucidate the role of this residue.

In the primary structure Lys_{382} and Lys_{419} are close to each other and near the C-terminus of the *Anabaena* enzyme. These two residues may be even closer in the three dimensional structure. Lys_{419} was preferentially labeled by pyridoxal-P, which is also the case for the corresponding lysyl residue in the spinach leaf enzyme (Ball et al., unpublished data). This may be explained either by postulating that Lys_{419} is closer to the aldehyde group of pyridoxal-P than Lys_{382} or by postulating that pyridoxal-P forms a Shiff-base with Lys_{419} which is thermodynamically more stable than with Lys_{382}. However, this may not be an indication that Lys_{419} is more important than Lys_{382} in the activator binding.

Although solving protein structure by chemical modification sometimes could be very limiting, in this paper, we demonstrated a way to widen the study. By performing chemical modification on a site-directed mutant enzyme, we were able to further identify an potentially important residue which may not have been identified in the wild-type enzyme.

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CHAPTER V

SUMMARY AND PERSPECTIVES

SUMMARY AND PERSPECTIVES

The structure gene of ADP-glucose pyrophosphorylase from cyanobacterium *Anabaena* PCC 7120 has been cloned from a genomic library. The gene codes for a protein of 429 amino acid residues. The deduced amino acid sequence is more similar to that of the higher-plant enzyme (50-60 % identical) than to the *E. coli* pyrophosphorylase (33 % identical). This result is consistent with the immuno studies showing that the cyanobacterial enzyme cross-reacts with the antibody raised against the spinach leaf enzyme, not with the antibody against the *E. coli* enzyme (Iglesias et al., 1991). Southern analysis indicated that there is only one copy of the gene in the *Anabaena* genome. The gene contains sequences with homology to the -35 and -10 box sequences of *E. coli* promoters and prokaryotic ribosome-binding site. These features are probably responsible for the expression of the *Anabaena* enzyme in *E. coli* cells. The recombinant enzyme is sensitive to 3PGA activation and Pi inhibition and is inhibited by the antiserum raised against the spinach leaf enzyme. The expression system allows structure-function study to be performed on important ligand-binding sites identified by chemical modification.

The lysyl residue corresponding to Lys_{419} of *Anabaena* pyrophosphorylase is conserved in all higher-plant and cyanobacterial ADP-glucose pyrophosphorylases sequenced to date. The lysyl residue is covalently modified by reductive phosphopyridoxylation of the spinach leaf enzyme and is believed to be involved in the binding of 3-P-glycerate, the allosteric activator (Morell et al., 1988). Replacing Lys_{419} of the *Anabaena* enzyme with either arginine, alanine, glutamine, or glutamic acid has been done by site-directed mutagenesis method. All the mutant enzymes were purified to homogeneity. The specific activity for

the arginine and alanine mutants is close to that of the wild-type enzyme and is slightly altered for the glutamate and glutamine enzymes. The apparent affinities for 3-P-glycerate are largely reduced for the mutant enzymes. The concentration of 3PGA necessary to obtain 50 % maximal activation of the K419R, K419A, K419Q, and K419E enzymes is 25-, 55-, 138- and 150-fold higher, respectively, than that for the wild-type enzyme. In general, these mutations caused no or less than 5-fold alteration on the kinetic constants for the substrates, glucose-1-phosphate, ATP, Mg^{2+} and the inhibitor, orthophosphate. Taken together, the results suggest that Lys_{419} is primarily involved in the binding of the activator. For all the mutant enzymes except K419Q, 3PGA still is the most effective activator. For the K419Q enzyme, fructose-1,6-P_2, the physiological activator of *E. coli* ADP-glucose pyrophosphorylase, is a more effective activator than 3PGA at lower concentrations. Fructose-1,6-P_2 activates the wild-type enzyme less than 2-fold but inhibits the enzyme activity in the presence of 3PGA. Kinetic studies suggest that 3-P-glycerate and fructose-1,6-P_2 competitively bind to the same activator-binding site.

A combination of techniques of site-directed mutagenesis and chemical modification was used to further elucidate the structure of the activator-binding site. Similar to the results obtained for the spinach leaf enzyme (Morell et al., 1988), pyridoxal-P can be covalently bound to the *Anabaena* enzyme in the presence of $NaBH_4$, which can be effectively inhibited by the presence of either 3PGA or Pi. The modified enzyme is more active in the absence of 3PGA and less sensitive to Pi inhibition. Sequencing of the tryptic [^{3}H]pyridoxal-P-labeled peptide, purified by reverse phase HPLC, indicated that a single lysine, Lys_{419}, was modified. This result is consistent with the site-directed mutagenesis experiment demonstrating that Lys_{419} is interacting with the activator. Chemical

modification of the K419R mutant enzyme showed an alternative lysyl residue, Lys_{382}, of the mutant enzyme was labeled. Previous studies also has shown that the corresponding lysyl residue in the spinach leaf large subunit was modified by pyridoxal-P (Preiss et al., 1992). From kinetic and protection studies, Lys_{382} is likely, joining the force of Lys_{419}, participating in the binding of 3PGA. Site-directed mutagenesis of Lys_{382} would be of interest to determine its role.

Although, the function of Lys_{419} has been identified as stabilizing the binding of 3PGA by conferring a positively charged site chain, it is still unclear about which function group of 3PGA is interacting with Lys_{419}. This might be an important question in the future when more amino acid residues are found involved in the binding of the activator and understanding of the coordination of these residues is anticipated. Hopefully, by using the techniques of NMR or Fourier transform infrared spectroscopy, the effect of amino acid side chain on the spectra of the function groups of 3PGA can be assessed.

Since the *Anabaena* enzyme is overproduced in *E. coli* cells, it is possible to obtain a satisfactory amount of protein for crystallization experiment. So far, high resolution crystallography of ADP-glucose pyrophosphorylase has not been achieved. However, progress and experience are accumulated on crystallizing the *E. coli* enzyme. Because the primary structure of *Anabaena* pyrophosphorylase is very similar to that of the higher-plant enzymes, the three-dimensional structure of the cyanobacterial enzyme will be a good model for the enzyme that is 3PGA/Pi regulated.

Further chemical modification with different agents would be of interest to probe the important residues for the regulation of ADP-glucose pyrophosphorylase. For example, the involvement of arginine residue in allosteric regulation of ADP-glucose pyrophosphorylase has been shown by using phenylglyoxal (Carlson and

Preiss, 1982; Iglesias et al., 1992; Ball and Preiss, 1992). Isolation and sequencing of the modified peptide(s) should be able to locate the labeled arginine.

Sometimes, it is very beneficial to be aware of important amino acid sequence of known function in other enzyme that also utilize similar substrate or effector as does ADP-glucose pyrophosphorylase. A triplet sequence, Asp-Phe-Gly, has been shown highly conserved in protein kinase family and is implicated in ATP binding (Hanks et al., 1988). The aspartic acid residue is a ligand to Mg^{2+} which is coordinated by ß- and τ-phosphates of ATP (Bossemeyer et al., 1993; Taylor et al., 1993). Interestingly, this sequence is absolutely conserved in all ADP-glucose pyrophosphorylase sequenced to date. Since ADP-glucose pyrophosphorylase requires the metal ion for activity, this motif probably has the same function as for protein kinase.

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