

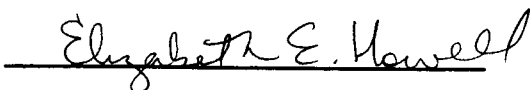
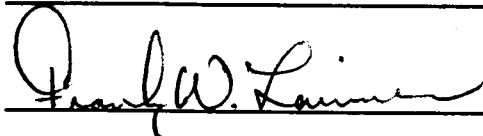
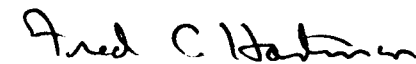
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I am submitting herewith a dissertation written by Sylvia Milanez entitled "Spinach Phosphoribulokinase: Cloning, Sequencing, and Site-Directed Mutagenesis." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.



Dr. Richard J. Mural, Major Professor

We have read this dissertation
and recommend its acceptance:



Accepted for the Council:



Associate Vice Chancellor
and Dean of The Graduate School

**SPINACH PHOSPHORIBULOKINASE: CLONING, SEQUENCING, AND
SITE-DIRECTED MUTAGENESIS**

A Dissertation

Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Sylvia Milanez

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DEDICATION

**This dissertation is dedicated to my parents, brother and sister, and to my husband
John and daughter Kathryn, who encouraged and inspired me.**

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ABSTRACT

Phosphoribulokinase (PRK) is a key light-regulated Calvin cycle enzyme which is deactivated by the formation of a disulfide bond between sulfhydryl groups. To be able to examine the enzyme's catalytic and regulatory mechanisms with the technique of site-directed mutagenesis, an expression/mutagenesis system was developed. A λ gt11 library was constructed from spinach leaf poly A⁺ RNA. Clones containing the entire mature-length enzyme were identified using rabbit polyclonal antibody raised against spinach PRK. The DNA sequence encoding mature-length PRK was determined: it encodes 351 amino acids with a calculated mass of 39,232 daltons and has a pI of 5.92. A PRK clone was modified so the mature-length protein would be expressed from the *prk* gene when inserted into an expression/mutagenesis vector. The resulting phagemid, pSM100, was introduced into *Escherichia coli* MV1190 and PRK indistinguishable in activity and mobility on SDS-polyacrylamide gels from the native enzyme was detected in crude extracts of these transformed cells.

The initial issue addressed using site-directed mutagenesis was whether any of the four PRK cysteinyl residues, particularly those involved in light-regulation, have a role in catalysis. Thus, all four cysteinyl residues were replaced (individually) by either serine or alanine. The activities of the eight mutant kinases were determined in crude cell-free extracts. Extracts of position 16 and 55 mutants were purified partially and the K_m values for the PRK substrates ATP and ribulose-5-phosphate (Ru5P) were measured. Results using both crude and partially purified extracts indicate that the sulfhydryl groups of Cys16, Cys244, and Cys250 are non-essential for structure or catalysis. PRK substituted at position 55 with either serine or alanine, however, had ten-fold lower k_{cat} and 4 to 8-fold higher K_m for Ru5P than wild-type enzyme. This

indicates Cys55 has a facilitative role in catalysis and may be part of the sugar substrate binding domain of PRK.

The Ser16 and Ser55 mutant proteins were also examined for their reactivity with the sulfhydryl reagent *N*-ethylmaleimide. The refractiveness of the Cys16 mutant protein and sensitivity of the wild-type and Cys55 mutant proteins to inactivation by NEM indicates Cys16 was the modified residue. The hyper-reactivity of Cys16 to NEM and a number of other reagents suggests that it is also the initial target for attack by the *in vivo* oxidant (oxidized thioredoxin) which causes oxidative deactivation of the enzyme.

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ABBREVIATIONS

Amino acid(s) =

Ala or A, alanine	Leu or L, leucine
Arg or R, arginine	Lys or K, lysine
Asn or N, asparagine	Met or M, methionine
Asp or D, aspartic acid	Phe or F, phenylalanine
Cys or C, cysteine	Pro or P, proline
Gln or Q, glutamine	Ser or S, serine
Glu or E, glutamic acid	Thr or T, threonine
Gly or G, glycine	Trp or W, tryptophan
His or H, histidine	Tyr or Y, tyrosine
Ile or I, isoleucine	Val or V, valine

ATP = adenosine 5'-triphosphate

Ap = ampicillin

Bluo-gal = halogenated indolyl- β -galactoside

Bicine = *N,N'*-bis(2-hydroxyethyl)glycine

bp = base pair(s)

cDNA = DNA complementary to RNA

Cm = chloramphenicol

cpm = counts per minute

DCC = dicyclohexylcarbodiimide

DNA = deoxyribonucleic acid

dNTPs = deoxynucleotide triphosphates

DTT = dithiothreitol

E. coli = *Escherichia coli*

EDTA = ethylenediaminetetraacetic acid

IPTG = isopropyl- β -D-thiogalactopyranoside

kb = kilobase(s), or 1000 bp

kDa = kilodaltons, or 1000 daltons

K_m = Michaelis-Menten constant

LB = Luria-Bertani medium

M_r = relative molecular mass

mRNA = messenger RNA

NaOAc = sodium acetate

NEM = *N*-ethylmaleimide

ORF = open reading frame

PAGE = polyacrylamide-gel electrophoresis

pfu = plaque forming unit(s)

PolIk = Klenow (large) fragment of *E. coli* DNA polymerase I

PRK = phosphoribulokinase

R. rubrum = *Rhodospirillum rubrum*

ribulose- P_2 carboxylase = ribulose biphosphate carboxylase/oxygenase

rbc gene = gene coding for ribulose- P_2 carboxylase

rbs = ribosome binding site

RF = replicative form

Ru5P = D-ribulose-5-phosphate

Rubisco = ribulose biphosphate carboxylase/oxygenase

SDS = sodium dodecyl sulfate

ss = single-stranded

tac = *trp-lac* hybrid promoter

Tris = Tris(hydroxymethyl)aminomethane

V_{max} = maximum velocity

[] = designates plasmid-carrier state

CHAPTER I

INTRODUCTION

(a) Thesis Objectives

Phosphoribulokinase (EC 2.7.1.19) (PRK) is one of two enzymes unique to the reductive pentose phosphate (Calvin) cycle, which is the primary pathway for CO₂ assimilation. PRK catalyzes the synthesis of the substrate for CO₂ fixation, ribulose-1,5-bisphosphate from ribulose-5-phosphate and ATP, requiring Mg⁺⁺ as a cofactor. The means by which PRK activity is regulated in autotrophs has been explored at length (sections (b)-1,4 of this chapter). Higher plant PRKs are light-regulated by the reversible oxidation/reduction of sulfhydryls/disulfides (12-15,22,132). In the case of spinach, the two regulatory sulfhydryls (98) are located in the ATP-binding domain of the active site (61,97,98,100,101). The means by which disulfide formation causes plant PRK to be inactivated is not known.

To complement and extend information obtained by protein chemistry techniques about PRK structure and function, a system for utilizing the technique of site-directed mutagenesis was developed. Thus the roles of the two regulatory spinach PRK cysteines, as well as that of any other desired residues (e.g. Cys244 and Cys250) could be probed by substitution at the desired positions. In order to utilize site-directed mutagenesis it was necessary to:

1. Create a spinach cDNA library in the bacteriophage λ gt11 using polyA⁺ RNA isolated from spinach leaves;
2. Isolate a cDNA clone containing the entire coding sequence for mature-length PRK from the λ gt11 library, using polyclonal rabbit anti-PRK antibody (prepared using purified spinach PRK);
3. Determine the DNA sequence of the PRK cDNA by the dideoxy chain-termination method;
4. Move the DNA fragment encoding PRK into the expression/mutagenesis vector (pFL220, (68)), correctly positioned for the production of mature-length PRK using the vector's promoter; and
5. Express the recombinant PRK in *Escherichia coli* and ascertain (biochemically) that wild-type PRK was being produced.

At the commencement of this thesis project (1985), there were no published reports of a complete PRK sequence, or of the expression of a *prk* gene in *E. coli*. The cysteinyl residues which form the regulatory disulfide in spinach PRK had not been identified, and their roles in catalysis had not been assessed. The two regulatory cysteinyl residues have recently been identified by comparing the *S*-carboxymethylation patterns of reduced *vs* oxidized PRK (98). Derivatization (and enzyme inactivation) of each of these two cysteines by several reagents was retarded by ATP, suggesting both are located at the active site of spinach PRK (62,97-101). A lysyl residue has also been located in the active site by protein chemistry techniques (84). The first reported primary structure of PRK was that for spinach (Chapter II, 109). Since then other PRK sequences have been reported, including that of wheat (105), *Chlamydomonas reinhardtii*

(110), and several prokaryotic sources (38,60,78). Some of the prokaryotic PRK genes have also been expressed in *E. coli* (59,42). A detailed account of the cloning, sequencing, expression in *E. coli*, and site-directed mutagenesis of spinach PRK is presented in Chapters II-IV. The remainder of this chapter is devoted to an overview of the properties of prokaryotic and eukaryotic PRK, with emphasis on the latter.

(b) Properties of Prokaryotic and Eukaryotic Phosphoribulokinase

1. Bacterial phosphoribulokinase

Many different types of bacteria are able to exist autotrophically by using CO₂ as their sole source of carbon. The majority of these assimilate CO₂ via the Calvin reductive pentose phosphate pathway. Bacteria not utilizing this pathway include the acetogens, the methanogens, and green photosynthetic bacteria (126), and will not be considered further. PRK, located in the cytoplasm in these organisms (77), has been characterized from a number of bacterial strains; a partial listing of these is presented in Table I¹. Comparison of the PRK primary structures from *A. eutrophus*, *R. sphaeroides*, and *X. flavus* shows a high degree of homology among these bacterial proteins (>60%) (38,60,78) (see Fig. I-1). The bacterial PRKs are either hexamers or octamers of subunits of 32,000 - 40,000 daltons, having specific activities ranging from about 30 to 140 units/mg PRK (1 unit of PRK converts one μ mol Ru5P into RuBP/minute). The exception is PRK from *Thiobacillus neapolitanus*, which has a reported specific activity of 450 units/mg PRK (73). For the PRKs examined

¹All tables are in Appendix A; all figures are located in Appendix B.

(*Alcaligenes eutrophus*, *Chromatium* sp. strain D, *R. acidophila*), the Ru5P and ATP saturation curves were sigmoidal, showing weak positive cooperativity for substrate binding (2,45,107,117). Many bacterial PRKs show varying degrees of allosteric activation by NADH (126) and are inhibited by AMP and phosphoenol pyruvate (PEP) (see Figure I-1). CO₂ fixation is thus regulated by the availability of reducing power and energy charge in anoxygenic bacteria. PRK from the oxygen-evolving cyanobacteria is not subject to activation by NADH, and is instead regulated by light, as are the PRKs of green algae and higher plants (76,21) (see section (b)-4 of this chapter).

Depending on the nutrients available in their environment, bacteria can adopt a variety of distinct modes of growth. The levels and activities of Calvin cycle enzymes are accordingly modulated, being highest during autotrophic growth (7,42,126). The synthesis of PRK and of ribulose biphosphate carboxylase/oxygenase (Rubisco), the two enzymes unique to this pathway, are often coordinately regulated (see ref. 126 for review). Recent studies with the chemolithotrophic bacterium *A. eutrophus* (50) and the phototrophic bacterium *Rhodobacter sphaeroides* (43, 36-38) have shown that genes for PRK and Rubisco are linked. Both strains of bacteria have two copies of the PRK gene and the DNA sequences of these genes have been determined (60,91). *A. eutrophus* has one copy of PRK on an endogenous megaplasmid (pHG1) and a second, nearly identical copy (96% amino acid homology) of the gene, in its chromosomal DNA (50,58-60). In both locations, the gene for PRK is about 3.8 kb downstream from the gene encoding Rubisco (50). The two PRK genes in *R. sphaeroides* are slightly less homologous (89% amino acid homology), and are located in separate, distinctly arranged chromosomal clusters, A and B (37,38,43), with the following structures:

A cluster: ---fbpA---prkA---cfxA---rbcL---rbcS---

B cluster: ---fbpB---prkB-----tklB-----gapB--cfxB---rbpL--

(*fbp*, fructose biphosphatase; *cfx*, unknown function; *rbcL*, *S* and *rbpL*, two types of Rubisco; *tkl*, transketolase; *gap*, glyceraldehyde-3-phosphate dehydrogenase).

It is not known why two PRKs exist in these species, though it may be related to their great nutritional versatility (126). It is unclear whether *prk* gene duplication is a common feature of prokaryotes.

PRK may also be coordinately regulated and expressed with the Calvin cycle enzyme fructose-1,6-bisphosphatase (FBP). In *R. sphaeroides*, *fbp* and *prk* from both cluster A and cluster B are co-transcribed as part of an operon with other Calvin cycle genes (37,38). A complex of PRK and FBP has been isolated from *Rhodospirillum rubrum* (53), and it has been suggested that these two enzymes form a macromolecular complex after protein synthesis in autotrophic bacteria (38). The genes for FBP and PRK are also linked in the prokaryote *Xanthobacter flavus* H4-14, as shown by sequence analysis of a genomic DNA fragment encoding both enzymes (78).

2. Physical properties and genetic organization of algal and higher plant phosphoribulokinase

In higher plants and algae, the active form of PRK is a dimer of subunits of about 40 kDa, in contrast to the prokaryotic kinases, which are hexamers or octamers of somewhat smaller subunits (see Table I-1). Higher M_r aggregates containing PRK have been isolated from darkened chloroplasts (section (b)-4 of this chapter). PRK is

maximally active at pH 7.9 (3,124,49), which is the pH of illuminated chloroplasts. The temperature optimum for catalysis has not been rigorously established, though the temperature-dependent fluorescence emission profile of reduced PRK shows a sharp transition above 30°C (Dr. F.C. Hartman, personal comm.), indicating PRK is unfolding. The enzyme is located in the chloroplast stroma, though some of it may be loosely associated with chloroplast membranes (12,30).

PRK is encoded by a nuclear gene and is synthesized on cytoplasmic ribosomes (5,27,29,54,114) with an amino-terminal transit peptide which is cleaved during or after its entry into the chloroplast (105,109,110,Chapter II). Analysis of Southern blots of wheat (19) and *C. reinhardtii* (110) DNA indicates that the *prk* gene is present in a single copy (per haploid genome). The *prk* gene does not seem to be linked to the genes of other Calvin cycle enzymes, based on evidence that in wheat, Calvin cycle genes are scattered over a number of chromosomes (19), with *prk* located on the long arms of the group 6 chromosomes.

PRK has been isolated and kinetically characterized from the green algae *Chlamydomonas reinhardtii* (110) and *Scenedesmus obliquus* (71,89), and from a number of plants including maize (6), tobacco (54), wheat (124), pea (3), and spinach (49,61,70,96). These PRKs have similar specific activities and affinities (K_m values) for their substrates, ATP and Ru5P, and the substrate saturation curves follow Michaelis-Menten kinetics, unlike those of prokaryotic PRKs (see section (b)-1 of this chapter). The K_m (ATP) is in the range of 63 - 100 μ M (34,49,67,73,99,110,124,Chapter IV) (though values from 30 to 710 μ M have been reported for spinach PRK (67,73)). Published values for the K_m (Ru5P) range from 25 - 250 μ M (33,34,49,53,99,110,124,Chapter IV). The specific activities of most of these kinases are

about 400 units/mg, somewhat higher than that of prokaryotic PRKs (see Table I-1).

3. The primary structure of eukaryotic PRKs

The primary structure of spinach (Chapter II,109), wheat (105) and *C. reinhardtii* (110) PRK has been determined by sequencing their cDNAs. The mature subunits of spinach and of wheat PRK subunits are very closely related: they both contain 351 amino acids (39.2 kDa), and have identical residues in 86% of the positions (105). Their transit peptides are also of similar size: 51 amino acids in spinach PRK, and 53 amino acids long in wheat PRK, though they have only 46% identity (105). PRK from the green alga *C. reinhardtii* is also similar in size (344 amino acids, 38.5 kDa) and structure (75% amino acid identity) with a calculated pI of 5.75, compared to that of spinach (pI=5.92) (110). Its transit peptide, however, is 20 amino acids shorter (31 amino acids) and shows little homology to that of spinach or wheat PRK.

Despite the difference in their primary structures, the transit peptides from wheat, *C. reinhardtii*, and spinach, they share some common amino acid motifs. These motifs are part of a consensus derived from an extensive analysis of transit peptide sequences (57). Thus, all three transit peptide sequences are rich in the hydroxylated residues (serine, threonine) and in the small hydrophobic residues (valine, alanine), while they have few or no acidic residues.

A comparison of available primary structures of prokaryotic and eukaryotic mature-length PRKs (Figure I-1) shows that there is very little homology (about 15%) between the two groups, unlike the high conservation seen among the members within each group (> 60% for prokaryotes, >70% for eukaryotes). This diversity is instructive, however, as conserved regions may represent functionally important parts of

the molecule. There is a conserved region at the *N*-terminal of all PRKs (residues 14-20; numbering based on aligned PRK sequences) which is homologous to a consensus ATP-binding sequence (G-X-X-G-X-GSK)(46), as noted by Porter (97) in the native spinach kinase (see Figure I-2). The cysteinyl residue within this region was demonstrated to be in the ATP-binding domain of the (spinach PRK) active site by chemical modification studies (see sections I(b)-4,5). Another, somewhat less conserved segment encompasses amino acids 54-70. Two residues in this region (Cys55 and Lys68) have also been assigned to the ATP-binding domain of the active site (84,100,101). Other regions suggested as conserved span amino acids 146-149, 178-193, and 199-213 (60).

4. Regulation of PRK activity in oxygenic autotrophs

PRK is a key control point for the flow of carbon in the Calvin cycle: it catalyzes an essentially irreversible reaction and its activity in chloroplasts is comparable to the rate of photosynthesis (31,34,67). Due to its pivotal role, the activity of PRK is highly regulated. Studies with reconstituted systems attempting to mimic *in vivo* chloroplast conditions indicate that *in vivo*, PRK is active in the light and inactive in the dark (30,31,34,67,69,130).

Light activates PRK by reducing a disulfide group (98) via the ferredoxin/thioredoxin system (12-15,22,132); DTT or reduced thioredoxin mimic the effect of light *in vitro* (30,69,132). Studies with PRK isolated from spinach identified the two intrasubunit cysteines comprising the regulatory disulfide as Cys16 and Cys55 (98); the two cysteines have been located in the nucleotide-binding domain of the PRK active site (61,97,98,100,101). The *in vivo* oxidant has not been conclusively identified, though

oxidized thioredoxin seems a logical choice (98,101,Chapter IV). The means by which oxidation (disulfide bond formation) causes PRK inactivation is not presently understood. Oxidation is not necessarily accompanied by gross conformational changes, since (a) the regulatory cysteines are both located in the ATP-binding domain of the active site and are presumably spatially proximal to each other (100), (b) the oxidized enzyme can still bind its substrates (6,99) (K_D (ATP) is about 20-fold higher for oxidized than for reduced PRK; K_D (Ru5P) for oxidized PRK has not been determined), and (c) significant differences between the oxidized and reduced enzyme have not been detected by fluorescence studies (35). Chemical modification studies (100,101) and site-directed mutagenesis (Chapter IV) indicate that a combination of steric effects, minor conformational changes, and removal of a catalytically facilitative cysteine (by its participation in a disulfide bond) account for PRK deactivation (Chapter IV).

A number of stromal metabolites and ions also regulate PRK activity. Some of the effectors are competitive inhibitors for the substrate ribulose-5-phosphate, including 6-phosphogluconate, ribulose-1,5-bisphosphate, 3-phosphoglycerate, fructose-1,6-bisphosphate, and P_i (33,34). PRK activity is also regulated by the relative concentrations of ATP and ADP (67,70), ADP being a strong competitive inhibitor of ATP (34). The pH and concentration of Mg^{++} also have an effect on PRK activity (13,48,67). Much of the pH effect is due to its influence in determining the ionic forms of the metabolites which modulate PRK activity (except for ribulose bisphosphate and ADP) (33,34). Thus, the ionic forms which predominate at pH 7 in darkened chloroplasts (3-phosphoglycerate²⁻, P_i^{1-} , fructose-1,6- P_2^{2-} , and 6-phosphogluconate²⁻) are the potent inhibitors of activity. Since the pH of illuminated chloroplasts is about 8, this provides another means to indirectly link PRK activity to environmental (light/dark)

conditions.

The level of ions and metabolites in the chloroplast stroma is also involved in promoting the dark-induced inactivation of the kinase: CO₂ fixation was observed to cease much more quickly (15 s) than PRK oxidation (100 s) in intact chloroplasts when shifted to the dark (66). Though the relative contribution of PRK oxidation/reduction vs metabolite regulation has not been defined, the importance of light-dependent oxidation/reduction in the regulation of PRK activity has been underscored by a study in which 40-fold more PRK activity was extracted from illuminated than from dark-harvested leaves (130). The combination of PRK oxidation and changes in stromal metabolites and ions have been proposed to account for the complete loss of PRK activity in the dark (31,34,130).

Several investigators have suggested that light also activates PRK *in vivo* by causing the dissociation of a high M_r form of PRK (which predominates in the dark) to an active low M_r (dimeric) form of the enzyme, found in the light (8,129,132). The ability of PRK to aggregate solely with itself has not been conclusively shown (102,132). PRK has been isolated as a non-covalent aggregate with other Calvin cycle enzymes including one or more of the following: ribulose biphosphate carboxylase/oxygenase (Rubisco), ribose-phosphate isomerase, phosphoglycerate kinase, and glyceraldehyde phosphate dehydrogenase (4,40,53,88,89,102,112,113). The proposed role in regulation has not been proven, due in part by difficulties in reproducing *in vivo* conditions experimentally. (8,71,89,129,132). Other possible functions for aggregate formation include channeling of reaction intermediates (*in vitro* studies with multi-enzyme complexes shows a higher-than-expected steady-state velocity of product appearance) (4,40), and a function in protecting against damaging forces such as heat and proteolysis

(102) have been proposed.

Light has been implicated in the regulation of a number of Calvin cycle enzymes besides PRK, including Rubisco, NADP malate dehydrogenase, fructose-1,6-bisphosphatase, seduheptulose-1,7-bisphosphatase, and NADP-glyceraldehyde-3-phosphate dehydrogenase (13,22,130). With the exception of Rubisco, all are regulated via the ferredoxin/thioredoxin system (13,22). The synthesis of these light-sensitive Calvin cycle enzymes is thought to be synchronized at the level of gene expression. These genes have similar (*cis*-acting) controlling elements which interact with common (trans-acting) protein transcription factors (63,105). The genetic controlling elements have not been identified for PRK, though they are likely similar to those described for the Rubisco small subunit gene, which is also light-regulated. The *rbcS* regulatory region contains positive and negative regulatory elements (causing down-regulation in the dark), regions required for light-responsiveness, and those needed for developmental regulation (see reference 86 for a review).

5. The PRK active site

Early studies recognized the sensitivity of PRK to the sulfhydryl-specific reagent *N*-ethylmaleimide (NEM) (103) and subsequent reports have confirmed this finding (6,93, Chapter IV). It has also been shown that the enzyme is sensitive to *N*-bromoacetyethanolamine phosphate (BrAcNHEtOP) (93,97), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (98), the ATP analog 5'-*p*-fluorosulfonylbenzoyladenosine (FSBA) (61,62), and iodoacetate (IAA) (62,63,97). DTNB stoichiometrically oxidizes (and inactivates) PRK by causing disulfide formation between Cys16 and Cys55 (98), while BrAcNHEtOP, NEM, and FSBA derivatize Cys16 in spinach PRK (62,97, Chapter

IV). ATP provides protection against inactivation by all of these reagents, indicating Cys16 is located at/near the active site (61,93,97,Chapter IV). The other cysteinyl residue comprising the regulatory disulfide (Cys55) is also located at/near the active site, based on ATP protection against modification of this residue with BrAcNHEtOP (100) and 2-nitro-5-thiocyanobenzoate (NTCB) (101) (PRK was methylated at the much more reactive Cys16 prior to modification of Cys55).

Despite the active-site location of Cys16, it has been shown to be catalytically non-essential. When Cys16 is quantitatively methylated (with methyl-4-nitrobenzenesulfonate), PRK retains 50% of its activity (99); amino acid replacement of Cys16 with a seryl residue has virtually no effect on catalysis (Chapter IV). Presumably NEM, BrAcNHEtOP, and FSBA inactivate PRK due to the bulk of the adduct introduced into the active site.

The hyper-reactivity of Cys16 suggests that this residue may have a critical role in PRK regulation *in vivo*, as the initial target for oxidative inactivation by oxidized thioredoxin (98,101,Chapter IV). Cys55, though not absolutely required, facilitates catalysis and may have a role in the binding of the sugar substrate since there is about a ten-fold decrease in k_{cat} and a 4 to 8-fold increase in the K_m for Ru5P when this residue is substituted with a seryl or alanyl residue (Chapter IV).

The location of the regulatory sulfhydryls in the active site might be expected to lead to the direct involvement of the individual residues in catalysis. The fact that the data do not support this conclusion, however, is not totally unexpected. The regulatory site has been shown to be separate from the active site in other light-regulated Calvin cycle enzymes (22,24,75,104). Catalysis and light-activation have been shown to be mechanistically distinct processes by analysis of a mutant PRK from *C. reinhardtii*, which

has a 20-fold reduction in the kinase activity, yet is still activated by light (114). The ability of oxidized PRK to bind ATP (6,99) (and possibly RuMP (6)), albeit less efficiently, is also consistent with the regulatory site only partially overlapping the catalytic site. Simultaneously replacing both cysteinyl residues might be thought to be more similar to oxidation *in vivo* and thus have a much greater inhibitory effect on catalysis. Preliminary data (R.J. Mural laboratory) do not support this assertion since PRK substituted at both Cys16 and Cys55 with serine still retains about 3% wild-type activity, which is not significantly higher than the activity of the C55S mutant PRK ($6 \pm 2\%$; see Table IV-2).

A third residue, Lys68, has been located at the active site by derivatization of spinach PRK with the ATP analog adenosine triphosphopyridoxal and is proposed to have a role in interacting with the phosphoryls of ATP (84). This lysine is conserved among most PRKs (see Figure I-1).

The roles of the other two cysteinyl residues in spinach PRK, Cys244 and Cys250, have been examined by site-directed mutagenesis: they are non-essential for structure or function (Chapter IV).

Though little is known about the tertiary structure of the active site, a structural motif has been identified at the *N*-terminus of PRK which is a consensus nucleotide-binding region (Figure I-2) (47). The consensus ATP-binding region has also been identified in adenylate kinase as part of an *N*-terminal region (aa 15-27) which forms a flexible loop (32). This loop is positioned in the middle of a cleft containing bound Mg-ATP and is proposed to have a function in controlling access to the ATP-binding site by altering its conformation. With the exception of Lys21, this loop is not directly involved in binding Mg-ATP (32). Though it is not known how similar the PRK active

site is to that of adenylate kinase, the lack of a role for Cys16 in ATP binding and catalysis, and the inactivation seen upon derivatization of Cys16 with large side-chains at this position (possibly analogous to the binding of Cys55 and its neighbors to Cys16 upon oxidation) is consistent with this *N*-terminal region having a similar role in PRK.

Determination of the structure of the PRK active site is still in its infancy, and efforts are underway to identify other residues located at the active site. Techniques such as site-directed mutagenesis, use of bifunctional chemical crosslinking reagents, and X-ray crystallography will undoubtedly elucidate the mechanism of PRK catalysis in the future.

CHAPTER II

CLONING AND SEQUENCING OF cDNA ENCODING THE MATURE FORM OF PHOSPHORIBULOKINASE FROM SPINACH

(a) Summary

Phosphoribulokinase (PRK) is a key enzyme in the Calvin cycle of autotrophic organisms. A spinach leaf cDNA library has been constructed in the phage expression vector λ gt11, and rabbit polyclonal antibody raised against spinach PRK was used to identify PRK clones. Analyses of the nucleotide sequences of two antibody-positive clones, 1.47 and 1.35 kb in length, showed that they encode a protein which contains the *N*-terminal amino acid sequence (95) of mature spinach phosphoribulokinase. The codon for the *N*-terminal serine of the mature protein occurs 170 bp from the 5' end of the open reading frame (ORF), suggesting that PRK is synthesized with a rather long transit peptide which is removed from the mature enzyme. The ORF, ending with an amber (TAG) codon at position 1054, predicts a mature enzyme of 351 amino acids with a calculated M_r of 39232 daltons.

(b) Introduction

An essential light-activated enzyme in all aerobic autotrophs, PRK (EC 2.7.1.19) catalyzes the ATP-dependent phosphorylation of D-ribulose-5-phosphate to form D-ribulose-1,5-bisphosphate, the primary CO₂ acceptor for photosynthetic carbon fixation. Although PRK is found in the chloroplast stroma of photosynthetic eukaryotes, several lines of evidence show that it is encoded in the nucleus (5,27,29,54,114).

Light activation of PRK in higher plants is achieved through the thioredoxin-mediated reduction of disulfide groups (132). The two half-cystines of this intrasubunit disulfide have been identified as residues number 16 and 55 of the mature protein (98). An active-site location for the cysteine at position 16 has been suggested by studies correlating enzyme inactivation with derivatization of this residue with *N*-(bromoacetyl)ethanolamine phosphate (97) and the ATP-analog 5'-*p*-fluorosulfonyl-benzoyl-adenosine (62). It has also been observed (98) that Cys-16 is in a sequence similar to part of the ATP-binding site found in a number of diverse ATP-binding proteins (47).

The *N*-terminal region and several internal peptides of spinach PRK were previously isolated as tryptic fragments and their amino acid sequences determined (62,96,97). To complete the amino acid sequence of PRK, and study the regulation of the *prk* gene, two cDNA copies of the spinach PRK mRNA were cloned and their nucleotide sequences were determined. Each of these clones contains a long ORF including 1053 nucleotides which encode the mature form of spinach PRK. The amino acid sequence of spinach PRK, as deduced from the PRK cDNA sequence, is presented.

(c) Materials and Methods

1. Chemicals, enzymes and isotopes

Restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase I Klenow fragment, oligo (dT) cellulose, Bluo-gal, isopropyl- β -D-thiogalactoside (IPTG), goat anti-rabbit antibody conjugated with horseradish peroxidase, λ gt11 dephosphorylated "arms", λ packaging extracts, and the M13 universal sequencing primer were obtained from Bethesda Research Laboratories (BRL) and were used in accordance with the manufacturer's recommendations. [^{35}S]-methionine (400-500 Ci/mmol), [α - ^{35}S]dATP (1000 Ci/mmol), and [α - ^{32}P]dCTP (3000 Ci/mmol) were purchased from Amersham. [α - ^{32}P]dTTP (3000 Ci/mmol) was obtained from New England Nuclear.

The rabbit reticulocyte lysate cell-free translation system and *EcoRI* methylase were purchased from Promega Biotec; nitrocellulose filters were from Schleicher and Schuell; and Sephacryl 200 and protein A Sepharose were from Pharmacia. *EcoRI* linkers (pGGAATTCC) were from New England Biolabs, and 3,3'-diaminobenzidine was from Sigma Chemical Co.

Reagents for oligonucleotide synthesis were obtained from American Bionetics.

2. Bacterial and phage strains

λ gt11 (*lac5* Δ (*shindIII* λ 2-3) *srl* λ 3⁰ *cI*857 *srl* λ 4⁰ *nin5* *srl* λ 5⁰ *S* am100) was used as the cloning vector (137). Host strains for the lambda library were *E. coli* Y1088 (Δ *lacU169 supE supF hsdR⁻ hsdM⁺ metB trpR tonA21 proC::Tn5* [pMC9]), pMC9 = pBR322-*lacI^q*, and *E. coli* Y1090 (Δ *lacU169 proA⁺ Δ lon araD139 strA supF trpC22::Tn10* [pMC9]) (138). The host for M13 vectors was *E. coli* JM107 (Δ *lac-pro*

endA1 gyrA96 thi-1 hsdR17 supE44 relA1[F' *traD36 proAB*⁺, *lacI*^qZΔM15]) (133).

3. RNA Preparation and characterization

Total RNA was extracted from 5 week old spinach (*Spinacea oleracea* L., cv. Melody hybrid) essentially by the method of Dean et al. (23). Polyadenylated RNA was selected by passage of the total RNA over an oligo(dT) cellulose column.

Before the poly(A)⁺ RNA pool was used for cDNA synthesis, it was tested for the presence of the phosphoribulokinase mRNA by cell-free translation and subsequent immunoprecipitation (see Fig. II-1).

4. Construction of the cDNA expression library

cDNA was synthesized from the poly(A)⁺ RNA pool using a cDNA synthesis kit from Amersham, which is based on the method of Gubler and Hoffman (41). Prior to its insertion into the unique *EcoRI* site of λgt11 DNA, the cDNA was methylated with *EcoRI* methylase and 5'phosphorylated *EcoRI* linkers were added by blunt-end ligation. Following its digestion with *EcoRI* endonuclease, this material was passed over two successive Sephacryl-200 spun columns (74) to remove excess linkers and very small cDNAs (i.e. <300 bp). This cDNA was then ligated to λgt11 dephosphorylated "arms" (λgt11 ligated at the *cos* sites and cut with *EcoRI*) in a molar ratio of arms:inserts of approximately 1:3. The ligated DNA was packaged into phage heads and plated on *E. coli* Y1088, in the presence of IPTG (0.5 mM) and Blueo-gal (40 μg/ml). Since the plaques of recombinant phage are white, the quality of the library was judged by the ratio of blue to white plaques.

5. Immunoscreening and analysis of cDNA clones

The cDNA library was screened for clones which express PRK-related antigens using the Clontech λ gt11 Immunoscreening Kit, following the provided protocol. Approximately 1/50th (10^3 pfu) of the cDNA library was plated per 82-mm plate on *E. coli* Y1090 at 37°C. Phage plaques were adsorbed onto IPTG-saturated nitrocellulose filters and incubated an additional 3 hours before being lifted. After washing and blocking, the filters were exposed to rabbit anti-PRK serum. In order to decrease its background reactivity with bacterial and phage components, this serum had been incubated with a nitrocellulose filter covered with *E. coli* Y1088 infected with λ gt11. Goat anti-rabbit antibody conjugated with horseradish peroxidase was then bound as secondary antibody, and positive clones were visualized by detecting the horseradish peroxidase with H_2O_2 and 3,3'-diaminobenzidine. Positive clones produced colored spots on the filters.

Antigen-positive λ gt11 plaques were purified, and their DNA was prepared using a plate lysate method (74). *Eco*RI and *Cla*I digestions of the DNA followed by gel electrophoresis were used to confirm the presence of inserts into the lambda phage DNA.

The *Eco*RI inserts were subcloned into the *Eco*RI site of the coliphages M13mp18 or M13mp19 and single-stranded DNA was used as a template for sequencing by the dideoxy chain-termination method (115) with [α - ^{32} P] dTTP. Some of the sequence was determined using [α - ^{35} S]dATP and the "Sequenase" Kit (U.S. Biochemical Corp.). Both ends of the antibody-positive clones were sequenced, using the M13 universal primer (BRL). Clones derived from PRK cDNA were identified as those whose nucleotide sequence encoded any of the PRK peptides whose amino acid

sequences have been published (62,96).

6. Sequence analysis by computer

Computer assisted analysis of sequence data utilized a set of programs developed by Stephens (1985), the IBI-Pustell Sequence Analysis Programs (International Biotechnologies, Inc.), and the National Biomedical Research Foundation (NBRF) Protein Identification Resource Data Base software.

(d) Results and Discussion

1. Construction and characterization of the cDNA expression library

Translation of spinach poly(A)⁺ RNA with a rabbit reticulocyte lysate system resulted in the synthesis of proteins with a wide range of sizes (from about 10 to 100 kDa), including several in the size range of PRK, about 45 kDa (61,70,96) (results not shown).

Immunoprecipitation of the products of *in vitro* translation with rabbit anti-PRK yielded two polypeptides with slightly slower mobility on SDS-PAGE than mature PRK (Fig. II-1). The ability to detect polypeptides which cross-react with antibody against PRK led us to conclude that the message for PRK was present in the mRNA pool. The slightly larger size of these polypeptides suggests that they are unprocessed precursors of mature spinach PRK.

The cDNA made from this mRNA pool ranged in size from about 0.1 to greater than 4 kbp, the majority being 0.5 to 2 kbp long. Following the addition of linkers this

cDNA was ligated into the *EcoRI* site of λ gt11, creating an expression library. A portion of the library was plated with IPTG and Blueo-gal on *E.coli* Y1088. The library contained about 6×10^4 plaque forming units, 98% of which contained inserts (i.e. 98% white plaques).

2. Identification of PRK clones

Approximately 10,000 plaques were screened with rabbit anti-PRK (see section (c)-5 of this chapter), and six antibody-positive plaques were identified. The sizes of the cDNA inserts ranged from 0.5 to 1.5 kb.

By comparing the amino acid sequence, deduced from the DNA sequence of the ends of the antibody positive clones, with the published *N*-terminal portion of mature PRK (97), two of the clones were established to contain at least a part of the PRK coding sequence. The size of the cDNA in one of the clones was estimated by agarose gels to be about 1.47 kb (clone 1) and in the other about 1.35 kb (clone 3). Since each of these clones encoded the *N*-terminal protein sequence near its 5' end, both were potentially long enough to specify a protein at least the size of that estimated for mature spinach PRK (about 45,000 kDa) (61,70,96). The identity of the other four antibody-positive clones was not established, and their analysis has not been pursued.

3. Sequence analysis of PRK DNA

The strategy for sequencing the two PRK clones is shown in Figure II-2A. Both strands of clone 3 were independently sequenced, while the sequence of clone 1 was assembled from overlapping sequences of both strands. The two clones are identical except for their termini, where the larger one (clone 1) is about 100 bp longer on the

5' end and 10 bp shorter on the 3' end. Their common region contains the entire coding sequence of mature PRK. Fig. II-3 shows a composite of the DNA sequences of the two clones which is numbered from the first base of the codon for the amino-terminal serine of the mature protein.

The sequence includes 1411 nucleotides of spinach cDNA with an ORF of 1223 nucleotides followed by 188 nucleotides of 3' untranslated sequence. This ORF encodes a polypeptide whose predicted sequence matches the first 64 amino acids from the *N*-terminus of mature spinach PRK (98). Preceding the protein's *N*-terminal serine codon is a region of DNA potentially encoding 56 amino acids, which is presumably part of a leader peptide which is removed during the maturation of PRK. The length of the spinach PRK leader peptide has been recently determined to be 51 amino acids (R.J. Mural, personal communication; 109). 45 of the 51 amino acids are encoded by Clone 1, the DNA encoding the 11 non-PRK amino acids at the very 5' end of this clone was presumably randomly incorporated during cDNA synthesis. Following the termination codon for the mature protein at nucleotide 1054, all of the reading frames are blocked by stop codons from 2 to 6 times (Fig. II-2B) in the remaining 188 base pairs of the 3' untranslated region of the cDNA.

In addition, the sequence of two internal peptides (aa residues 226-256 and 298-308) are encoded in this reading frame. The only discrepancy in these 106 assignments was at amino acid position 301 of the mature polypeptide where the sequence published for the tryptic peptide indicates alanine (62) and the DNA sequence of both clones encodes glutamic acid. Though we currently have no explanation for this difference, it should be noted that the reported sequence (62) was of an impure peptide. It is also possible that a different strain of spinach was used to isolate the PRK used in

this study so that the difference represents a natural polymorphism at residue 301.

From the analysis of two independently isolated cDNA clones it can be concluded that mature spinach PRK contains 351 amino acids, has a calculated pI of 5.92, and an M_r of about 39,232 daltons. This represents about a 10% difference from the published apparent molecular weight of about 45,000 (61,70,96), as estimated by SDS-PAGE. PRK is perhaps post-translationally modified and/or it behaves anomalously on SDS-PAGE (91). The amino acid composition, as deduced from the DNA sequence, reflects the previously reported amino acid composition for this protein (97). The presence of only two tryptophans and four cysteines is confirmed. In addition to the two regulatory cysteines near the *N*-terminal, there are two other cysteines at amino acid positions 244 and 250.

4. The PRK precursor

The *prk* reading frame extends 170 bp 5' of the codon for the *N*-terminal serine of the mature enzyme. Several nuclear-encoded chloroplast proteins of higher plants are synthesized as larger precursors with an additional 37-66 amino acids at their *N*-terminus. These amino acids constitute a transit sequence which is cleaved after the entry of the protein into the chloroplast (for example see 56,83,121). There are several lines of evidence supporting the conclusion that PRK is a nuclear-encoded protein. PRK continues to be synthesized in plant cells in which chloroplast protein synthesis has been inhibited by either heat (29) or antibiotics (5,27). There is direct genetic evidence (114) from a PRK mutant of the green alga *Chlamydomonas reinhardtii* for a nuclear location for the gene for PRK. Thus, one might expect that the 56 amino acids coded by the DNA 5' to the coding sequence of the mature protein represents part of the PRK

transit peptide. The length of the putative transit sequence cannot be determined from the two sequenced PRK clones since neither contains the initiation AUG. A cDNA clone containing the entire spinach PRK transit peptide has been recently isolated (R.J. Mural, personal communication); its sequence corresponds to that published by another group (109). Since chloroplast transit peptides are not conserved, a comparison to other transit peptides of nuclear-encoded chloroplast proteins reveals few identities.

5. Structural motifs of PRK and other ATP-binding proteins

A computer search for similarities between the deduced amino acid sequence of PRK and the protein sequences contained in the NBRF Protein Identification Resource Data Base reveals regions of PRK which resemble sequences found in a group of diverse ATP-binding proteins. PRK has a region which is similar to the "type A" ATP-binding segment found in a large number of ATP-binding proteins (26,47) (see Figure I-2, Chapter I (b)-5), as noted previously (42,98). The data base search, in addition to reiterating this finding, identified a second region of PRK which is similar to a domain characteristic of the β -subunit of F_1 -ATPases (Fig. I-3) (116,134). The conserved glutamic acid in this region has been determined to be catalytically essential in *E. coli* F_1 -ATPase: replacement of this residue (Glu181) with glutamine greatly impaired catalytic activity (2). ATPase inactivation is also caused by derivatization of the homologous glutamic acid of the thermophilic bacterium PS3 with dicyclohexylcarbodiimide (DCC) (134). DCC targets a different glutamic acid (11 residues towards the C-terminus) in the F_1 -ATPase β subunit from bovine mitochondria (28) and *E. coli* (135) (corresponding to Thr45 in spinach PRK), also inactivating the enzyme. The reason for the different reactivities of the two glutamic acid residues with

DCC is not known, though they are speculated to be involved in the binding of Mg^{2+} of F_1 -ATPase (136). PRK also requires Mg^{2+} as a cofactor (48), though the lack of conservation with other PRKs at Glu34 (see Figure I-1) does not support an analogous function (in Mg^{+2} binding) in PRK. The function of Glu34 in PRK, and the reason for the homology of this region with the ATPases, remains to be determined.

6. Conclusions

There have been two recent reports (36,42) of the cloning of the genes for two different PRKs from the prokaryote *Rhodobacter sphaeroides*, and the DNA sequence for one of these genes has been completed (P. L. Hallenbeck, personal communication). Comparing the deduced amino acid sequence of PRK from *R. sphaeroides* with that determined for spinach reveals a striking lack of similarity. With the exception of some conservation previously reported around the ATP-binding site (42,98), there are very few identities throughout the remainder of the two sequences. Though this disparity may reflect the known differences between the two enzymes in both their regulatory and kinetic parameters (36,42,96,107), it precludes identifying conserved regions that might indicate functional domains of the protein. As PRK sequences from other organisms become available, a pattern of conserved primary structure will likely emerge, identifying functionally critical residues of the enzyme.

The availability of the DNA and corresponding amino acid sequence of the entire PRK protein will facilitate further experiments to explore the properties of this key photosynthetic enzyme. The PRK clones are currently being modified so that they can be expressed in *E. coli* and used for studies involving site-directed mutagenesis. In this way the precise role of various amino acids implicated as essential for the regulation or

catalytic function of PRK can be studied. In addition these clones will be used to study the localization of this enzyme to the chloroplast, and to isolate the gene for phosphoribulokinase and study its regulation.

CHAPTER III

EXPRESSION OF SPINACH PHOSPHORIBULOKINASE IN *Escherichia coli*

(a) Summary

In order to study the function and regulation of the Calvin Cycle enzyme phosphoribulokinase (PRK) by site-directed mutagenesis, the gene for the spinach enzyme has been expressed in *E. coli*. A λ gt11 cDNA clone encoding mature spinach PRK (79) was modified so that a transcriptional fusion could be made with the *tac* promoter and ribosome binding site of the high-expression vector pFL220 (68). When the resulting plasmid, pSM100, was introduced into *E. coli* MV1190 and induced with IPTG, PRK activity was detected in crude cell extracts. The extracts contained a protein which comigrated with authentic spinach PRK on SDS-polyacrylamide gels and cross-reacted with polyclonal anti-PRK IgG on Western blots. PRK accounted for <0.1% of the total soluble cellular protein in these extracts, and Western blot analysis revealed substantial degradation of PRK was occurring. An expression regimen was developed which minimized degradation and increased the yield of PRK to 0.7% of the total soluble protein. High levels of active PRK have been found to be deleterious to *E. coli*, which may explain some of the difficulties encountered in attempts to express high levels of PRK.

(b) Introduction

An enzyme unique to the reductive pentose phosphate cycle, phosphoribulokinase (PRK, EC 2.7.1.19) catalyzes the ATP-dependent phosphorylation of D-ribulose-5-phosphate to form D-ribulose-1,5-bisphosphate, the primary CO₂ acceptor in photosynthesis. Affinity labeling studies have identified several amino acids located in the active site of spinach PRK, including Cys16 (62,97), Cys 55 (100) and Lys-68 (84). PRK is activated indirectly by light in all aerobic autotrophs via the ferredoxin/thioredoxin-mediated reduction of a disulfide bond (13,22,98). The PRK of non-aerobic autotrophs is not regulated in this manner, and activity in some cases is dependent on NADH (see 126 for a review). A comparison of the primary structures of PRK determined from a number of both prokaryotic and eukaryotic sources shows that there is a high degree of sequence conservation among the members within each group ($\geq 65\%$), though the percent identity between the two groups is low ($< 15\%$) (38,60,79,105,109,110). This perhaps reflects the differences of the corresponding kinases in their quaternary structure, regulation, and kinetic parameters (126).

A technique widely used to address questions of enzyme structure/function is site-directed mutagenesis. This method enables the systematic examination of the roles of selected amino acids, such as those in evolutionarily conserved regions of the molecule and those implicated as essential by chemical methods, in enzyme regulation and catalysis. As a prerequisite to mutagenesis, the *prk* gene has been subcloned into the high-expression phagemid vector pFL220 (68). This vector has the advantage of directly producing single-stranded template for primer-extension mutagenesis and DNA sequencing. A description of the PRK phagemid, a characterization of the cloned

protein, and the effect of PRK production on *E. coli* growth are presented.

(c) Materials and Methods

1. Chemicals, enzymes and isotopes

Restriction endonucleases, *Poll*k, T4 DNA ligase, IPTG, Blue-gal, prestained molecular weight standards, goat anti-rabbit antibody conjugated with horseradish peroxidase, M13 "universal" primer, and low-melting point (LMP)-agarose were obtained from Bethesda Research Laboratories, and were used in accordance with the manufacturer's recommendations. 5' phosphorylated *Bam*HI linkers were from New England Biolabs. Reagents for the synthesis of 5'-phosphorylated oligodeoxynucleotides were obtained from American Bionetics, Glen Chemicals, and Sigma Chemical Co. L(+)-arabinose, Ap, Cm, leupeptin, radiometric and spectrophotometric assay reagents, Reactive Red 120 agarose-CL, and 3,3'-diaminobenzidine were from Sigma Chemical Co. DTT and Bicine were from Research Organics Inc.. Other commonly used reagents were obtained at the highest level of purity readily available. Isotopes were purchased from Amersham Corp. : 5'-[α -³⁵S]dATP (1000 Ci/mmol) and NaH¹⁴CO₃ (60 Ci/mmol).

Phosphoribulokinase (E.C. 2.7.1.19) was purified from spinach leaves as previously described (96). Spinach ribulose-P₂ carboxylase (E.C. 4.1.1.39) purified to apparent homogeneity and rabbit polyclonal anti-PRK IgG (102) were the generous gifts of Dr. Fred C. Hartman.

2. Plasmids, phage, and bacterial strains

The host for M13mp18 containing PRK was *E. coli* JM107 $\Delta(lac-pro)$ *endA1 gyrA96 thi-1 hsdR17 supE44 relA1* [*F'**traD36 proAB*⁺ *lacI*^f Δ M15] (133). *E. coli* strain MV1190 $\Delta(lac-proAB)$ *thi supE* $\Delta(srl-recA)$ 306::*Tn10*(*tet*^r) [*F'**traD36 proAB*⁺, *lacI*^f Δ M15] (Bio-Rad) was used as the host for the *prk* gene cloned into pBR325 and the PRK expression phagmid (pSM100, see Fig. III-1). *E. coli* strain CJ236 *dut ung thi relA* [pCJ105 *Cm*^r] was used to make dU-substituted template for site-directed mutagenesis (65). Phagmid PFL220 is an Ap-resistant ColE1-based plasmid containing the *tac* promoter and the phage f1 intergenic (IG) region; pFL200 is PFL220 containing the *R. rubrum rbc* gene in the *Bam*HI restriction site (68).

3. Bacterial transformation and media

E. coli JM107, MV1190, and CJ236 were made competent for transformation or transfection by the method of Hanahan (44). JM107 cells (transfected with M13) were plated on B agar (10 g tryptone, 8 g NaCl, and 15 g agar/liter) with Bluo-gal (40 μ g/ml) and IPTG (0.5 Mm), and RF DNA made from colorless plaques was screened for the PRK *Eco*RI fragment. MV1190 (transformed with PBR325 or PSM100) was plated on LB (74) containing 50 μ g/ml Ap. CJ236 transfected with M13-PRK was plated on LB-Cm; when transformed with PSM100 was plated on LB-Ap,Cm. Liquid cultures of the same cells were grown in the same medium (lacking agar), except JM107 was grown in LB without antibiotics. 1% glycerol was added for growth of large-scale cultures ($\geq$ 500 ml). Other additions to the media were made while optimizing PRK yields (see Results).

4. DNA preparation

Double-stranded DNA was isolated from cells by the alkaline lysis procedure (74). pBR325-PRK plasmid DNA was additionally purified by CsCl-ethidium bromide equilibrium density gradient centrifugation (74). DNA from diagnostic restriction digests was separated electrophoretically in (0.7 - 1.2%) agarose gels in 90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8.3 buffer. DNA was visualized after staining with ethidium bromide (0.5 μ g/ml in H₂O). Preparative gels were made from (1%) LMP-agarose in the same buffer; DNA was extracted from gel slices by agarase (Calbiochem) digestion (16).

5. DNA sequencing and mutagenesis

Single-stranded pSM100 was prepared utilizing the helper phage M13KO7 (128). DNA was sequenced across regions of interest by the dideoxy chain-termination method using 5'-[α -³⁵S]dATP, with the aid of the Sequenase DNA sequencing kit (United States Biochemical Corp). The M13 "universal" primer (GAATTGTTATTC) was used to sequence the M13 PRK template, while single-stranded pSM100 was sequenced (across the *tac*-PRK junction) using an oligodeoxynucleotide primer (CAAGCTTACTCCCCAT), which was synthesized by phosphoramidite chemistry (119) with a Microsyn model 1450 DNA synthesizer (Systec). The presence of the ATG and the *Nde*I site in the resulting phagmid, pSM100, was confirmed by *Nde*I digestion and DNA sequencing.

Mutagenesis was performed by the method of Kunkel (65) using dU-substituted (PRK) template and a "Muta-Gene" kit (Bio-Rad). Primers made for mutagenesis were phosphorylated on the 5'-hydroxyl while still attached to the synthesizer (127).

6. Preparation and characterization of PRK extracts

Cells were harvested by centrifugation, washed (50 mM Bicine, 0.1 Mm EDTA, 5 mM DTT, pH 8.0), frozen in liquid nitrogen, and stored at -70°C until use. To prepare cell extract, the pellets were resuspended in 2 volumes of buffer (50 mM Bicine, 1 mM EDTA, 10 μ M leupeptin, pH 8.0) and disrupted by three passages through a French pressure cell (Aminco) at 4°C. Centrifugation at 100,000 x g for 45 minutes removed particulates; the supernatant (cell-free extract) was brought to 20% glycerol and stored at -70°C.

PRK activity in crude extracts was measured by a modified (80) ribulose-P₂ carboxylase-coupled ¹⁴CO₂-fixation filter disc assay (90,102). Total protein concentrations of the samples were determined using the Coomassie Blue dye-binding method (9), with a reagent from Pierce.

(d) Results and Discussion

1. Construction of the *prk* expression phagmid (pSM100)

An *Eco*RI fragment encoding mature spinach PRK, a portion of the PRK transit (leader) peptide and a 3' untranslated region (79) was used as the source of the PRK gene for expression phagmid construction. After a number of manipulations (Fig. III-1) a *Bam*HI fragment containing the coding sequence for mature PRK and an initiator ATG was ligated into the *Bam*HI cloning site of the expression vector pFL220 (68), making a transcriptional fusion with its *tac* promoter and ribosome binding site. DNA sequencing across the *tac*-PRK junction confirmed the intended sequence was made.

2. Optimization of *prk* expression in *E. coli*

Initially, the *prk* gene was expressed in *E. coli* MV1190 (pSM100) under conditions used for the expression of the *Rhodospirillum rubrum rbc* gene from the same vector (85,90). Briefly, a fresh overnight culture of phagmid-bearing cells (grown at 37°C in LB-Ap and 1% glycerol) was diluted 1:100 into the same media, and grown to an $A_{600} = 0.2$. PRK synthesis was then induced by addition of 0.1 mM IPTG, and the cells were incubated an additional 2.5 hours before harvesting. In extracts prepared using this protocol, PRK represented < 0.1% of the total soluble cell protein (based on activity measurements of extracts and assuming that pure PRK has a specific activity of 400 units/mg PRK). In contrast, the *R. rubrum* Rubisco accounted for 5.4% of the total protein when expressed under similar conditions (68). Additionally, Western blots of extracts of cells expressing pSM100 (Fig. III-2A) revealed substantial levels of smaller immunoreactive (PRK degradation) products. To increase PRK yields (and to minimize its proteolysis), the growth conditions of cells bearing pSM100 were varied. Among the parameters varied were the length of the pre- and post-induction periods, the growth temperature (20°C, 30°C, 37°C), pH (7.0, 7.5, 8.0), NaCl concentration (82 mM, 200 mM), and supplemental carbon source (glucose, glycerol, arabinose).

Two factors proved critical to improving the yield of PRK : temperature and supplemental carbon source. Lowering the growth temperature from 37°C to 30°C increased the yield $\geq$ 5-fold. This may be explained by evidence suggesting that at 37°C spinach PRK is mostly unfolded *in vitro*, while at 30°C it is in its native conformation (F.C. Hartman, personal comm.). Unfolded proteins have been shown to be more susceptible to intracellular proteolysis (94). Additionally, accumulation of denatured proteins can induce the heat shock response in *E. coli* (82,87), which has been shown

to cause low levels of (cloned) protein yield (39). Perhaps certain cellular proteases are less active at 30°C than at 37°C. The heat-shock-inducible protease La (20) may be included in this group, though lowering its activity does not likely play a significant role, since moving pSM100 into the *lon*⁻ strain Y1090 (138) did not improve PRK yields at 37°C (not shown).

The addition of arabinose eliminated most of the smaller (PRK degradation) products still seen on immunoblots of extracts prepared from cells grown at 30°C (Fig. III-2A) and nearly doubled the yield of active PRK. The basis for this phenomenon is unclear, though we speculate that arabinose could exert its effect after being converted by the cells into ribulose-5-phosphate (Ru5P), as *E. coli* are able to import arabinose and contain enzymes to convert it into Ru5P. Enzymes are often protected by their substrates from proteolysis (82), and binding RuMP may similarly protect PRK. The imported arabinose could also be serving to supplement the *E. coli* pentose phosphate pool, which PRK might be depleting (see section (d)-3 of this chapter).

The optimized protocol for the production of PRK from pSM100 is as follows: a fresh overnight culture (grown at 30°C in LB-Ap) is diluted 1:1000 into LB-Ap supplemented with 1% glycerol and 0.2% arabinose. After 12 hours of growth at 30°C (at which point the cells are in late- logarithmic growth), PRK production is induced by addition of IPTG to 0.1 mM, and growth is continued for an additional hour (at 30°C) before harvesting. Under these growth conditions PRK represents about 0.7% of the total soluble protein and the majority of the degradation products seen on Western blots are eliminated (Fig. III-2A). Uninduced cells grown under the same conditions produce 20 - 40% as much PRK, which is comparable to uninduced levels of Rubisco from the same vector harboring the *rbc* gene (pFL200) (F.W. Larimer, personal communication).

This may be due to titration of the *lac* repressor with this high-copy phagmid or to incomplete repression of the *tac* promoter due to its operator structure (92).

The maximal PRK yield achieved is still 8 to 10-fold lower than the yield of *R. rubrum* carboxylase (5.4%) expressed from same vector (pFL200; (68)). The difference in protein yields obtained from pSM100 and pFL200 seems to be specific to the cloned proteins and their interaction with the cells. Others have observed similar disparities in the levels of expression of different cloned proteins which are under control of identical transcription and translation signals (18).

3. Cytotoxic effect of PRK

The relatively low yields of PRK in *E. coli* prompted the examination of the effect of high levels of pSM100 expression on cell growth. First, the effect of growing *E. coli* MV1190 [pSM100] in the presence or absence of inducer (IPTG) on LB-Ap agar plates was assessed. In a second experiment, a comparison was made of the efficiencies of pSM100 vs control DNA (pFL220 containing PRK in the wrong orientation) in transforming two strains of *E. coli*, CJ236 and MV1190. Among other traits, these strains differ in their content of Lac repressor : CJ236 contains the wild-type repressor gene, *lacI*, while MV1190 has the gene which overexpresses Lac repressor, *lacI^q*. One can speculate that CJ236 will therefore contain a lower amount of Lac repressor and higher uninduced PRK levels than MV1190, and if PRK is toxic, fewer transformants should be found (relative to using control DNA) in CJ236.

Results of the $\pm$ IPTG plating experiment (Table III-1) show that $\geq 10^2$ -fold fewer colonies grew on plates containing IPTG than when the same number of colonies was plated without IPTG. Plasmid DNA was prepared from a number of the + IPTG

survivors, and restriction analysis showed that $\geq 80\%$ of these colonies had rearranged DNA. Extracts prepared from cells with altered pSM100 had no detectable PRK activity, and Western blots indicated no PRK was being made (Fig. III-2B). Clones which appeared normal by restriction analysis produced ≥ 10 -fold less PRK, as determined by activity measurements and Western blots of cell-free extracts (Fig. III-2A). When the *Bam*HI PRK fragment isolated from low-activity clones was subcloned into (wild-type) pFL220, the resulting constructs were fully active. This indicates that the vector, and not PRK, was being altered. DNA sequencing across the *tac* promoter region of the low-activity clones showed that in some instances the -35 region of the promoter was mutated (not shown).

Results linking high levels of PRK expression with lowered cell viability cell survival were also obtained from the transformation experiment (Table III-1). For CJ236, ≥ 30 -fold fewer transformants resulted when using pSM100 vs control DNA, while MV1190 survival was the same using either DNA. pSM100 DNA from the rare CJ236 transformants was usually rearranged, as determined by restriction analysis. Taken together, these results suggest that high levels of PRK are toxic to *E. coli*, while low levels seem to be tolerated (e.g. as in uninduced MV1190).

When the transformation and $\pm$ IPTG plating experiments were performed using pSM100 in which PRK had been altered by site-directed mutagenesis (see Table III-1), pSM100 containing the low-activity (position 55) mutant PRKs had no effect on *E. coli* CJ236 or MV1190 survival (and their DNA was not altered), while pSM100 with the more active mutants had essentially the same effect as wild-type pSM100. This further indicates that synthesis of PRK *per se* is not lethal, rather, high levels of PRK activity are detrimental to cell growth. The nature of the injury by PRK is presently unknown,

though the requirement for active protein indicates unfavorable codon usage (10,108), induction of the heat shock response (39), and toxicity of the gene product (itself) (11,25) are not totally responsible for the observed differential in cell survival. We cannot rule out, however, that these factors have some effect on the overall PRK yield.

One possible explanation for the difference in survival of *E. coli* in which *prk* is fully induced vs *E. coli* in which *prk* expression is repressed is based on the putative interaction of (active) PRK and the endogenous pool of Ru5P. Active PRK may be converting the Ru5P into ribulose-1,5-bisphosphate, using cellular ATP. Depleting the Ru5P could in turn deplete xylulose-5-phosphate, which is central to the metabolism of pentoses and other sugars. Lowering the level of ribose-5-phosphate (normally derived from Ru5P by phosphoriboisomerase), in particular, may be detrimental to cell survival: *E. coli* mutants lacking phosphoriboisomerase are ribose auxotrophs (119). Interfering with the *E. coli* pentose metabolism or depleting the intracellular ATP pool could account for the observed inhibition in growth associated with *prk* expression.

4. Characterization of PRK expressed from pSM100

Cloned PRK appears to be indistinguishable from the native spinach enzyme. The size of cloned and native PRK is the same on SDS-polyacrylamide gels (see Fig. III-2). The PRK content of samples on Western immunoblots, as estimated using purified spinach PRK standards and densitometry (not shown), correlates quite well with estimates of PRK concentration from activity measurements. This indicates that the specific activity of the cloned protein is the same as that of native PRK. The kinetic characterization of PRK from enriched (10-fold) extracts has been recently reported (81); the values obtained for the $V_{\max}$ and K_m for both PRK substrates were nearly

identical to that determined for the native enzyme (96,99).

5. Conclusions

The availability of the expression phagmid pSM100 fulfills the prerequisite for study of PRK functionality with the powerful technique of site-directed mutagenesis. pSM100 expresses mature-length enzyme indistinguishable from authentic spinach PRK in sufficiently high yields for biochemical characterization.

This is the first report of the development of an expression system for a higher plant PRK; thus far, only PRKs from the prokaryotes *R. sphaeroides* (36,37,42) and *A. eutrophus* (50,59) have been expressed in *E. coli*. Although the yields of these prokaryotic PRKs were (2 to 16-fold) greater than those of spinach PRK, their specific activities are lower than that of spinach PRK, so that the total amount of PRK activity (units) in the cells was in all cases less than that obtained by expressing pSM100. The toxicity to *E. coli* by the prokaryotic PRKs was not examined. Interestingly, when the *A. eutrophus prk* gene was expressed (PRK represented 11.4% of the total soluble protein), the specific activity of the cloned PRK was reduced 10-fold from that of the native enzyme (which has a specific activity of about 40 units/mg PRK) for unknown reasons (50). This suggests *E. coli* cannot tolerate high levels of PRK activity, irrespective of the source of PRK.

pSM100 has proven useful for efficient site-directed mutagenesis--eight PRK mutants have been made with this host-vector system and characterized (81). Using this technique makes possible an independent assessment of the structure-function relationships of residues previously targeted by affinity labeling, in some cases making more subtle changes and modifying sites inaccessible to chemical reagents. A

comparison of PRK primary sequences which have recently become available for both prokaryotic and eukaryotic kinases has located several conserved regions, providing additional guidance for selection of potentially critical residues to be targeted for amino acid replacement.

CHAPTER IV

ROLES OF CYSTEINYL RESIDUES OF PHOSPHORIBULOKINASE AS EXAMINED BY SITE-DIRECTED MUTAGENESIS

(a) Summary

The Calvin Cycle enzyme phosphoribulokinase is activated in higher plants by the reversible reduction of a disulfide bond, which is located at the active site. To determine the possible contribution of the two regulatory residues (Cys16 and Cys55) to catalysis, site-directed mutagenesis has been used to replace each of them in the spinach enzyme with serine or alanine. The only other cysteinyl residues of the kinase, Cys244 and Cys250, were also replaced individually by serine or alanine. A comparison of specific activities of native and mutant enzymes reveals that substitutions at positions 244 or 250 are inconsequential. The position 16 mutants retain 45-90% of the wild-type activity and display normal K_m values for both ATP and ribulose-5-phosphate. In contrast, substitution at position 55 results in 85-95% loss of wild-type activity, with less than a 2-fold increase in the K_m for ATP and a 4- to 8-fold increase in the K_m for ribulose-5-phosphate. These results are consistent with moderate facilitation of catalysis by Cys55 and demonstrate that the other three cysteinyl residues do not contribute significantly either to structure or catalysis. The enhanced stability, relative to wild-type enzyme, of the Ser16 mutant protein to a sulfhydryl reagent supports earlier suggestions

that Cys16 is the initial target of the oxidative deactivation process.

(b) Introduction

Phosphoribulokinase (EC 2.7.1.19), an enzyme unique to the reductive pentose phosphate cycle, catalyzes the ATP-dependent phosphorylation of D-ribulose-5-phosphate to form D-ribulose-1,5-bisphosphate, the requisite CO₂ acceptor in photosynthetic organisms. The light-coupled regulation of PRK activity in higher plants via the thioredoxin-mediated reversible oxidation/reduction of sulfhydryls/disulfides is well established (13,22). Among several chloroplast enzymes which respond to the thioredoxin system, PRK is unusual in that the site for modulation of activity constitutes a part of the catalytic site (97). Chemical modification studies of the homodimeric enzyme from spinach have shown that both participants of the regulatory intrasubunit disulfide, Cys16 and Cys55, are located at or near the nucleotide-binding domain of the active site (62,63,97,99,100,101).

The loss of kinase activity concomitant with disulfide bond formation could be due to oxidation of a sulfhydryl group that facilitates catalysis, to steric blockage of the active site, or to conformational changes that accompany the oxidation. Major conformational changes would appear unlikely given the proximity of Cys16 and Cys55 as predicted from their common domain location. Furthermore, the demonstrated binding of ATP by the oxidized form of PRK suggests that steric effects are not the sole basis of catalytic incompetence (6,99). In this study, attention is focused on the possible contributions, direct or indirect, of Cys16 and Cys55 to catalysis.

The cysteinyl-16 sulfhydryl group of PRK appears catalytically passive, as its selective methylation reduces k_{cat} only 2-fold (99). The hyperreactivity of Cys16 complicates the evaluation of the role of Cys55 through chemical modification. Preferential cyanylation of Cys55, after methylation of Cys16, has been achieved resulting in >95% loss of kinase activity inherent to the methylated enzyme (101). Whether PRK derivatized solely at Cys55 would exhibit one-half of wild-type activity or would be inactive is not clear. To address this issue directly, we have applied site-directed mutagenesis to a cDNA clone (79) encoding spinach PRK. The study is extended to include single amino acid substitutions for each of the four cysteinyl residues of PRK. Mutant proteins are analyzed with respect to kinetic parameters and, in some cases, sensitivity to NEM.

(c) Experimental Procedures

1. Chemicals and biologicals

Commonly used reagents were purchased at the highest level of purity readily available. Additional supplies and vendors were as follows: reagents for the synthesis of 5'-phosphorylated oligodeoxynucleotides, American Bionetics, Glen Chemicals, and Sigma Chemical Co.; prestained molecular weight standards, IPTG, and restriction endonucleases, Bethesda Research Laboratories; ribose 5-phosphate, ribose 5-phosphate isomerase, reagents for the spectrophotometric assay for PRK, ampicillin, leupeptin, Reactive Red 120 agarose-CL, and 3,3'-diaminobenzidine, Sigma Chemical Co.; DTT and Bicine, Research Organics Inc.; NEM, Aldrich Chemicals; 5'-[α - ^{35}S]dATP (1000

Ci/mmol) and $\text{NaH}^{14}\text{CO}_3$ (60 Ci/mmol), Amersham Corp.

Ribulose-bisphosphate carboxylase/oxygenase and PRK were purified from spinach and stored at -80°C according to published procedures (96,131). One unit of PRK activity represents phosphorylation of 1 μmol of Ru5P; the purified spinach PRK, used as a standard, had a spec. act. of 400 units/mg in both the spectrophotometric assay and the ^{14}C -fixation assay.

2. Strains, plasmids, and phage

Escherichia coli strain MV1190 $\Delta(\text{lac-proAB})$ *thi* *supE* $\Delta(\text{srl-recA})$ 306::Tn10(*ter*') [*F*':*traD36 proAB*⁺*lacI*^qZ Δ M15] (Bio-Rad) was used as the host for expression of wild-type and mutant *prk* genes. The wild-type vector, pSM100, was derived from the high-expression phagemid pFL220 (68) by insertion of the *prk* gene into the *Bam*HI restriction site (79,80). Single-stranded sequencing template was prepared from pSM100 by use of M13K07 helper phage (128). *E. coli* strain CJ236 *dut ung thi relA* (pCJ105 *Cm*^r) was used to make dU-substituted template for oligonucleotide-directed mutagenesis (65).

3. Synthesis of oligonucleotides

Oligodeoxynucleotide primers were synthesized by phosphoramidite chemistry (119) on a Microsyn Model 1450 DNA synthesizer from Systec. The polymers were phosphorylated on the 5'-hydroxyl while attached to the matrix (127).

4. Mutagenesis of the *prk* gene

All mutations were introduced into dU-substituted template as described by Kunkel (65) with the aid of a "Muta-Gene" kit obtained from Bio-Rad. The primers (see Table IV-1) were designed to change the targeted cysteine codon (TGT) to codons for either serine (TCT) or alanine (GCT). Following mutagenesis, phagmids were used to transform *E. coli* MV1190 (44). Cells from ampicillin resistant colonies were infected with M13KO7 helper phage, and single-stranded templates of the candidate mutants were isolated and sequenced across the region of interest by the dideoxy chain-terminator method (115). Two different primers for sequencing were used: CAAGCTTACTCCCCAT for the region encompassing the codons for Cys16 and Cys55 and GAATCCAGAAAGCC for the region encompassing the codons for Cys244 and Cys250. Typically, 25-100% of the transformants contained the desired mutation.

5. Expression of phosphoribulokinase in *E. coli*

All transformed *E. coli* cultures were maintained at 30°C, as this temperature was optimal for both PRK production and stability (80). To prepare large-scale cultures, 500 ml of medium (L-broth supplemented with 50 µg/ml ampicillin, 1% glycerol, and 0.2% arabinose) was inoculated with 0.5 ml of an overnight culture (grown in the same medium but lacking arabinose and glycerol) of *E. coli* MV1190 transformed with the phagmid harboring the appropriate *prk* gene. After a growth period of 12 h, PRK biosynthesis was induced by the addition of IPTG to 0.1 mM. After an additional hour, cells were harvested by centrifugation, washed (50 mM Bicine-KOH, 0.1 mM EDTA, 5 mM DTT, pH 8.0), frozen in liquid nitrogen, and stored at -80°C.

For small-scale cultures (3-ml), the protocol was modified as follows: the same

medium that was used for large-scale cultures was inoculated with cells from a freshly plated colony. After 16 h of growth, cells were induced with 0.5 mM IPTG; one hour later, extract was prepared from the collected cell pellet. Such extracts were used for electrophoretic analyses and for specific activity determinations of wild-type and mutant PRKs.

6. Preparation of extracts of *E. coli*

Pelleted cells from large-scale cultures were resuspended in 2 volumes of pH-8.0 buffer (50 mM Bicine-KOH, 1 mM EDTA, 10 mM DTT, 10 μ M leupeptin) and lysed by three passages through a French pressure cell (Aminco) at 4°C. Particulate matter was removed by centrifugation at 100,000 x g for 45 minutes. The supernatant (crude extract) was brought to 20% glycerol and stored at -80°C.

Cells from small-scale cultures were lysed by treatment with lysozyme. Freshly-harvested, pelleted cells were frozen at -80°C for 10 minutes and thawed by resuspension in 50 μ l of pH-8.0 buffer consisting of lysozyme (0.6 mg/ml), 10 mM EDTA, 25 mM Tris-HCl, 50 mM glucose, and 20 μ M leupeptin. After 15 min at room temperature, 50 μ l of a pH-8.0 buffer (100 mM Bicine-KOH, 30 mM MgCl₂) that contained DNAase I (25 μ g/ml) was added. Within 5-10 min, the apparent viscosity was greatly reduced, and the lysate was centrifuged for 6 min in a microfuge. The collected supernatant was assayed immediately for PRK activity. Based on protein concentration and PRK activity of wild-type samples, this procedure was as efficient as the French pressure cell in achieving lysis.

7. Partial purification of PRK

Reactive Red agarose (a hydrophobic affinity matrix) was used to purify partially PRK from *E. coli* extracts; the protocol was adapted from one applied to the purification of the native spinach enzyme (96). Crude extract (from a 500- or 1000-ml culture) was prepared as described above, except the lysis buffer was adjusted with KOH to pH 6.4. After centrifugation, the extract (6-12 ml) was diluted 2-fold with equilibration buffer (10 mM Bicine-KOH, 10 mM DTT, pH 6.5) and applied immediately to the agarose column (1×15 cm). After extensive washing of the column with equilibration buffer followed by ~250 ml of 10 mM potassium phosphate (pH 6.8) containing 10 mM DTT, PRK was eluted with a pH-7.2 buffer containing 1 mM ATP, 10 mM K-phosphate, and 10 mM DTT. The peak fractions were pooled (~30 ml) and first dialyzed against a buffer of 50 mM Bicine-KOH (pH 8.0), 10 mM K₂HPO₄, 1 mM EDTA, and 10 mM DTT to remove ATP; dialysis was then continued against the same buffer saturated with (NH₄)₂SO₄ to precipitate protein. The precipitated protein was pelleted, resuspended in a minimal volume (<1 ml) of 40 mM Bicine-KOH (pH 8.0), 0.8 mM EDTA, 10 mM DTT, and 20% glycerol, and dialyzed against the same buffer prior to storage at -80°C.

8. Protein and enzyme assays

Protein concentrations were determined by the Coomassie Blue dye-binding method (9); the necessary reagent was obtained from Pierce. The concentration of PRK in crude extracts or partially purified preparations was estimated from Western immunoblots (see below).

Crude extracts and processed samples were assayed for kinase activity as described by Porter (102), in which the phosphorylation of Ru5P is coupled to the

$^{14}\text{CO}_2$ -fixation filter disc assay for ribulose-bisphosphate carboxylase/oxygenase (90). Reactions were initiated by the simultaneous addition of ATP plus $\text{NaH}^{14}\text{CO}_3$ or Ru5P plus $\text{NaH}^{14}\text{CO}_3$. The carboxylase from spinach (300 $\mu\text{g}/\text{assay}$) was substituted for the *Rhodospirillum rubrum* enzyme. In some cases, the activity of partially purified PRK preparations was measured using a modification of (96) the enzyme-coupled spectrophotometric assay of Racker (103). Although the two assays gave comparable results with partially purified samples, the spectrophotometric assay could not be used with crude extracts because of unacceptable background levels.

The concentration of the Ru5P, formed by the action of ribose 5-phosphate isomerase on its aldehyde substrate, was calculated from the reported equilibrium constant (95).

9. Electrophoresis and immunoblotting

Crude extracts and partially purified samples of PRK were examined by vertical SDS-PAGE (15% polyacrylamide, 0.5% bisacrylamide) on minigels (100 mm wide x 90 mm high x 0.5 mm thick) using the buffer system of Laemmli (66) and an apparatus from Idea Scientific, Corvallis, OR. To visualize protein, gels were stained with Coomassie Brilliant Blue (0.1%) in methanol:acetic acid:water (5:1:5). PRK was detected by a Western immunoblotting procedure (51,52), as detailed previously (102). Proteins from the minigels were transferred (17) to nitrocellulose sheets (Schleicher and Schuell) using the GENIE electroblotter (Idea Scientific, Corvallis, OR). The nitrocellulose membrane was impregnated with rabbit polyclonal anti-PRK IgG (101) and an *E. coli* extract lacking PRK (to minimize cross-reactivity to bacterial proteins). Following exposure of the membrane to secondary antibody (goat anti-rabbit IgG conjugated with horseradish

peroxidase, Bio-Rad) and thorough washing, the immunoreactive proteins were detected by the color produced upon reaction of the horseradish peroxidase with H_2O_2 and 3,3'-diaminobenzidine in 50 mM Tris-HCl (pH 7.2).

As quantified by scans of photographic negatives of the nitrocellulose membranes with an LKB model 2202 laser densitometer, the band density was proportional to the amount of purified spinach PRK applied to the acrylamide gel over the range of 5-60 ng. In all cases, the concentration of PRK in *E. coli*-derived samples was based on comparison with the signal given by a known quantity of spinach PRK applied to the same gel. Coomassie-stained gels of partially purified preparations of PRK, in which the kinase bands were clearly resolved from contaminants, could be scanned directly with the laser densitometer. The two methods gave comparable results.

10. NEM treatment of PRK

Samples of PRK (both wild-type and mutant proteins) were freed of DTT (which otherwise would quench NEM) by passage over 1-ml columns (tuberculin syringes) of Sephadex G-50 equilibrated with a pH-8.0 buffer consisting of 50 mM Bicine-KOH, 5 mM $MgCl_2$, 0.5 mM EDTA, and 10% glycerol. The proteins were then treated with NEM at 4°C; aliquots of the reaction mixtures were assayed periodically for kinase activity. Stock solutions of NEM were prepared in acetonitrile and diluted at least 1000-fold with reaction buffer prior to combining with the protein solutions. Final reactant concentrations are provided in the pertinent figure legend.

(d) Results

1. Concentration and activity of PRK in crude extracts

Wild-type PRK represented about 0.5% of the total protein in crude mini-extracts based on measured kinase activity and a specific activity of 400 units/mg for pure spinach PRK. Because of this relatively low level of expression and the presence of *E. coli* proteins similar in size to the PRK subunit, the latter was difficult to detect on Coomassie-stained SDS-PAGE minigels (Fig. IV-1A). However, Western blots of gels developed with rabbit polyclonal antibody raised against spinach PRK showed that all eight mutant proteins and the cloned wild-type enzyme comigrated with pure PRK isolated from spinach (Fig. IV-1B). Generally, the C16A and C55A proteins² were present in the respective extracts at levels 2- to 4-fold lower than the other kinases examined. These lower levels of full-length translation products were often accompanied by immunodetectable, smaller polypeptides, perhaps reflective of enhanced susceptibility to proteolysis.

The eight mutant proteins retained varying levels of kinase activity relative to wild-type enzyme (Table IV-2). Replacement of Cys244 or Cys250 with either serine or alanine had negligible effects on activity, and hence no further analyses were performed on these proteins. Substitution of Cys16 had only modest effects, with C16S retaining 90% and C16A 45% of wild-type activity. Cys55 mutant proteins were more impaired, retaining only 5-15% of wild-type activity.

²The single-letter code for amino acids is used to describe mutant proteins. The first letter denotes the amino acid present in the wild-type enzyme at the numbered position. The final letter denotes the amino acid present at the corresponding position in the mutant protein.

The lowest level of kinase activity which could be measured in any crude extract with the radiometric assay was 0.5% of that observed in extracts containing the wild-type enzyme. The sensitivity of this assay was partially limited by the lack of direct proportionality between kinase activity and amount of extract added if the total protein exceeded about 30 μ g (10 μ l of extract).

2. Partial purification and kinetic parameters of mutant proteins

Chromatography on Reactive Red agarose as the initial step in partial purification of PRK from crude extracts of *E. coli* was similar to its effectiveness as the last step in obtaining pure PRK from spinach (96); in both cases, recoveries approached 50%. Affinities of the four mutant proteins (C16S, C16A, C55S, and C55A) for the affinity matrix appeared comparable to that of wild-type PRK expressed in *E. coli* from the cloned gene based on similar recoveries and similar degrees of enrichment (~10-fold).

The preparations, obtained by elution of the affinity column with ATP (see section (c)-7 of this chapter), were sufficiently enriched with respect to PRK that it could be discerned on SDS-polyacrylamide gels with Coomassie blue (Fig. IV-1A). Densitometry of Western immunoblots (Fig. IV-2) provided an estimation of purity (2-5%), which in conjunction with kinase assays, enabled calculations for specific activity of pure proteins.

Kinetic parameters of the partially purified kinases, as derived from Lineweaver-Burk and Eadie-Hofstee analyses, are presented in Table IV-3. The cloned, impure wild-type PRK and the purified spinach PRK were indistinguishable; the correspondence of their $V_{\max}$ values validates densitometry of immunoblots as a measure of purity.

Furthermore, the relative $V_{\max}$ values of the four mutant proteins were in close agreement with the activity measurements carried out with crude extracts (Table IV-2). Among the mutant and wild-type proteins, the K_m values for ATP differed by only 2-fold. The position 55 mutant proteins did exhibit significantly elevated K_m values for Ru5P, some 4-fold higher than wild-type in the case of the seryl substitution and 8-fold higher with the alanyl substitution.

3. Sensitivity of wild-type and mutant PRKs to NEM

Wild-type cloned kinase was extremely susceptible to inactivation by NEM as reported previously for PRK from spinach (93). This sensitivity extended to the C55S protein, which like wild-type, was almost completely inactivated by incubation with 10 μ M NEM for only 2.5 min (Fig. IV-3). ATP afforded substantial protection to both proteins. In contrast, the C16S protein was quite resistant to NEM-induced inactivation, losing only one-third of its activity during a 20-min treatment with 100 μ M reagent.

(e) Discussion

Oxidative deactivation of PRK entails intrasubunit disulfide bridging of Cys55 with Cys16 (98). The major purpose of this study was to evaluate the contribution of Cys55 of PRK to catalysis, as this regulatory residue is located at the active site (101) and specific targeting of Cys55 in the fully-reduced, active kinase by chemical modification has not been possible due to the hyperreactivity of Cys16. However, the recent cloning and sequencing of the spinach *prk* gene (79) provided the opportunity to

probe Cys55 directly by site-directed mutagenesis. Serine was chosen as one replacement residue, because it is the closest analogue, both sterically and chemically, of cysteine among amino acids that occur in proteins. If the nucleophilicity or hydrogen-bonding potential of Cys55 were important, the seryl side chain might prove to be a functional substitution. In the case of alanine, the net effect is replacement of a cysteinyl sulfhydryl with a hydrogen atom, thereby generating a chemically inert side chain.

Replacement of Cys55 by either serine or alanine results in an approximate 10-fold reduction in the k_{cat} of the kinase. A substantial effect by the most conservative substitution possible illustrates the stringency for a cysteinyl residue at this position for optimal activity. Equivalence of a seryl or an alanyl residue at position 55 in supporting kinase activity indicates that the seryl side chain does not effectively fulfill the role of Cys55; thus, the maximal contribution of Cys55 to the overall rate enhancement of the kinase is a factor of ten. The K_m for ATP is insensitive to structural alteration at position 55, but the 8-fold increase in the K_m for Ru5P that occurs upon loss of H-bonding potential (cysteinyl $\rightarrow$ alanyl) at this position could reflect a role of the sulfhydryl group in the binding of the sugar substrate.

As predicted by an earlier chemical modification study in which selective methylation of Cys16 by methyl nitrobenzenesulfonate was accompanied by only partial inactivation (99), the kinase activity is not particularly sensitive to amino acid substitution at position 16. The k_{cat} for the C16S protein is within 10% that of the wild-type kinase, and the k_{cat} for the C16A protein is only 2-fold less than the wild-type value. Interestingly, the catalytic consequences of methylation of Cys16 and its replacement by an alanyl residue are virtually identical. This may reflect the hydrophobic nature of the

change in each case, because Cys16 is located within a hydrophilic environment and is freely accessible to solvent as judged by reactivity toward several charged reagents (63,97,98). Substitution of Cys16 with serine or alanine results in little change in the K_m for either ATP or Ru5P, while its methylation results in a 5-fold increase in the K_m of ATP (99). This difference may be explained by the increased bulk of the methylated cysteine.

Based on lack of perturbation of k_{cat} by amino acid substitutions, the only other cysteinyl residues of PRK (Cys244 and Cys250) are not involved in catalysis nor are they required for conformational integrity. This finding is consistent with reports of chemical modification of Cys244 without catalytically detrimental effects (62,63,101); modifications of Cys250 have not been described previously.

The *in vitro* oxidation of PRK by stoichiometric amounts of 5,5'-dithiobis-(2-nitrobenzoic acid) completely inactivates the enzyme by virtue of intrasubunit disulfide bond formation between Cys16 and Cys55; the fully reversible chemistry is exceptionally selective, as no other modifications can be detected (97). Inasmuch as the *in vitro* oxidation is a faithful mimic of the *in vivo* process, regulatory oxidation within the chloroplast gives rise to a catalytically incompetent kinase. The 90%-diminution of wild-type activity by the conservative replacement of Cys55 by serine indicates that oxidative deactivation, to a large extent, is a direct consequence of the loss of an active-site sulfhydryl group. Steric effects of disulfide bond formation in conjunction with induced conformational changes or slight reorientation of catalytic groups could explain the absence of activity associated with the oxidized protein in contrast to the 10% residual activity displayed by the C55S kinase.

The extreme sensitivity of reduced, fully-active PRK to sulfhydryl reagents

(6,63,93,98) is retained by the C55S protein, whereas the C16S protein exhibits only sluggish reactivity toward NEM. These observations are instructive in several ways: the sulfhydryl group of Cys16 is verified as the prime target for modification by exogenous reagents; the possibility that the proximal sulfhydryl at position 55 in the wild-type enzyme significantly influences the chemical reactivity of the sulfhydryl at position 16 is discounted; similar reactivity of Cys16 in both the wild-type and C55S proteins argues against substantial conformational disruptions being caused by the replacement of Cys55 with serine. Inactivation by derivatization of the Cys16 sulfhydryl with bulky reagents (62,97) appears to be due to direct steric effects or to induced conformational changes, as this sulfhydryl can be removed by mutagenesis or methylated without substantial loss of kinase activity. Given the enhanced reactivity of Cys16 relative to Cys55 as confirmed with pertinent mutant proteins, Cys16 would appear the likely target of the *in vivo* sulfhydryl-disulfide exchange between the reduced kinase and the oxidized form of thioredoxin (13,22,98).

In summary, none of the four sulfhydryl groups of PRK are required for catalysis. Cys244 and Cys250 are clearly without effect on structure or function, as their replacements do not alter catalytic parameters. Likewise, as the Cys16 mutant retains essentially wild-type activity level, Cys16 contributes only marginally, if at all, to catalysis. However, the accessibility and reactivity of this residue are obligatory to regulation of PRK activity *in vivo*; Cys16 presumably provides the "handle" for engagement of PRK by oxidized thioredoxin, thereby forming a mixed disulfide. Intramolecular attack by Cys55 on the mixed disulfide would then yield the inactive, oxidized form of PRK. To a considerable extent, inactivation reflects the loss of an active-site sulfhydryl (Cys55) which facilitates catalysis, as demonstrated by the detrimental effects of its substitution

by mutagenesis. The concomitant loss of the active site sulfhydryl at position 16 via oxidation probably does not contribute directly to the inactivation process, but thioredoxin could not serve as a physiological modulator of PRK were it not for this sulfhydryl providing the avenue for oxidation. This assertion is reinforced by the observed resistance of position 16 mutants to sulfhydryl reagents. Specific chemical modification of Cys16 by bulky reagents does result in virtually complete loss of kinase activity (97,62), but in conjunction with the present mutagenesis studies, this inactivation can only be explained by invoking steric and/or conformational effects. Inasmuch as pairing of Cys16 with Cys55 to form a disulfide mimics modification with bulky reagents, indirect effects would also appear to contribute to oxidative inactivation of PRK.

CHAPTER V

CONCLUSIONS

The prerequisite steps to attain the goal of developing an expression/mutagenesis system for spinach PRK have been fulfilled:

- A spinach cDNA λ gt11 library was created, from which a PRK-containing clone was isolated,
- The primary sequence of PRK was determined,
- An expression/mutagenesis phagmid, pSM100, was constructed and PRK indistinguishable from the native enzyme was produced from it in *E. coli*.

The roles of the four PRK cysteine residues have been examined using a facile and efficient PRK expression/mutagenesis vector system. The effect of substituting Cys16 and Cys55 (with Ser or Ala) on PRK function is consistent with and extends previous data from chemical modification studies. The role of Cys55 was previously difficult to assess due to the presence of its very reactive neighbor, Cys16. By preparing and characterizing the appropriate PRK mutant proteins (C55S and C55A), Cys55 was determined to have a role in facilitating catalysis and may represent the first residue to be located at the PRK sugar substrate-binding domain.

The potent combination of protein chemistry approaches and site-directed mutagenesis will undoubtedly locate other residues comprising the PRK active site and determine their roles. When the 3-D structure of PRK is determined (using X-ray crystallography) site-directed mutagenesis and protein chemistry techniques can be used

to help establish the function of selected residues.

Knowledge of the mechanism of oxidative deactivation of PRK is presently incomplete. Studies presented here, as well as early chemical modification studies, suggest that a combination of conformational changes and removal of a catalytically facilitative residue are involved in this regulation. Crystal structures of oxidized and reduced PRK, as well as the mixed disulfide intermediate (between PRK and thioredoxin) will be particularly instructive in elucidating the mechanism of oxidative deactivation *in vivo*. PRK might thus provide a model for studying the regulation of other enzymes which are regulated via the ferredoxin/thioredoxin system.

LIST OF REFERENCES

1. Abdelal, A.T.H. and Schlegel, H.G.: Separation of phosphoribulokinase from enzymes of the Calvin cycle in *Hydrogenomonas eutropha* H 16. Arch. Microbiol. 95 (1974) 139-143.
2. Al-Shawi, M.K., Parsonage, D. and Senior, A.E.: Thermodynamic analyses of the catalytic pathway of F₁-ATPase from *Escherichia coli*. J. Biol. Chem. 265 (1990) 4402-4410.
3. Anderson, L.E.: Regulation of pea leaf ribulose-5-phosphate kinase activity. Biochim. Biophys. Acta. 321 (1973) 484-488.
4. Anderson, L.E.: Ribose-5-phosphate isomerase and ribulose-5-phosphate kinase show apparent specificity for a specific ribulose-5-phosphate species. Eur. J. Biochem. 212 (1987) 45-48.
5. Armstrong, J. J., Surzycki, S. J., Moll, B. and Levine, R. P.: Genetic transcription and translation specifying chloroplast components in *Chlamydomonas reinhardtii*. Biochemistry 10 (1971) 692-701.
6. Ashton, A.R.: Ribulose-5-phosphate kinase from *Zea mays*: regulation of enzyme activity by oxidation and reduction. (1983) In Thioredoxins, Structure and Function, proceedings of the conference held June 21-24, 1981, Berkeley, CA, pp.245-250, Centre National de la Recherche Scientifique, Paris.
7. Bowien, B., Friedrich, B. and Friedrich, C.G.: Involvement of megaplasms in heterotrophic derepression of the carbon-dioxide assimilating enzyme system in *Alcaligenes* spp. Arch. Microbiol. 139 (1984) 305-310.
8. Bradbeer, J.W., Ruffer-Turner, M.E., de Ferrer, E.J., Wara-Aswapati, O. and Kemble, R.J.: The regulation of phosphoribulokinase and glyceraldehyde-phosphate dehydrogenase (NADP⁺) in C3 and C4 plants. In Akeyunoglou, G. (Ed.), Photosynthesis IV: Regulation of Carbon Metabolism. Balaban International Science Services, Philadelphia, Penn., 1981, pp. 389-394.
9. Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1976) 248-254.
10. Brinkmann, U., Mattes, R.E. and Buckel, P.: High-level expression of recombinant genes in *Escherichia coli* is dependent on the availability of the *dnaY* gene product. Gene 85 (1989) 109-114.
11. Brosius, J.: Toxicity of an overproduced foreign gene product in *Escherichia coli* and its use in plasmid vectors for the selection of transcription terminators. Gene 27 (1984) 161-172.

12. Buchanan, B.B., Wolosiuk, R.A. and Schurmann, P.: Thioredoxin and enzyme regulation. *Trends in Biochem. Sci.* 4 (1979) 93-96.
13. Buchanan, B.B.: Role of light in the regulation of chloroplast enzymes. *Ann. Rev. Plant Physiol.* 31 (1980) 341-374.
14. Buchanan, B.B.: The ferredoxin/thioredoxin system: a key element in the regulatory function of light in photosynthesis. *BioScience* 34 (1984) 378-383.
15. Buchanan, B.B.: Regulation of CO₂ assimilation in oxygenic photosynthesis: the ferredoxin/thioredoxin system. *Arc. Biochem. Biophys.* (1991), *in press*.
16. Burmeister, M. and Lehrach, H.: Isolation of large DNA fragments from agarose gels using agarase. *Trends in Genet.* 5 (1989) 41.
17. Burnette, W.N.: "Western blotting" : electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112 (1981) 195-203.
18. Butt, T.R., Jonnalagadda, S., Monia, B.P., Sternberg, E.J., Marsh, J.A., Stadel, J.M., Ecker, D.J. and Crooke, S.T.: Ubiquitin fusion augments the yield of cloned gene products in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 86 (1989) 2540-2544.
19. Chao, S., Raines, C.A., Longstaff, M., Sharp, P.J., Gale, M.D. and Dyer, T.A.: Chromosomal location and copy number in wheat and some of its close relatives of genes for enzymes involved in photosynthesis. *Mol. Gen. Genet.* 218 (1989) 423-430.
20. Chung, C.H. and Goldberg, A.L.: The product of the *lon* (*capR*) gene in *Escherichia coli* is the ATP-dependent protease, protease La. *Proc. Natl. Acad. Sci. U.S.A.* 78 (1981) 4931-4935.
21. Crawford, N.A., Sutton, C.W., Yee, B.C., Johnson, T.C., Carlson, D.C. and Buchanan, B.B.: Contrasting modes of photosynthetic enzyme regulation in oxygenic and anoxygenic prokaryotes. *Arch. Microbiol.* 139 (1984) 124-129.
22. Crawford, N. A., Droux, M., Kosower, N. S., and Buchanan, B. B.: Evidence for function of the ferredoxin/thioredoxin system in the reductive activation of target enzymes of isolated intact chloroplasts. *Arch. Biochem. Biophys.* 271 (1989) 223-239.
23. Dean, C., Van den Elzen, P., Tamaki, S., Dunsmuir, P. and Bedbrook J.: Differential expression of the eight genes of the petunia ribulose biphosphate carboxylase small subunit multi-gene family. *EMBO J.* 4 (1985) 3055-3061.

24. Decottignies, P., Schmitter, J.-M. Miginiac-Maslow, M., Le Marechal, P., Jacquot, J.-P. and Gadal, P.: Primary structure of the light-dependent regulatory site of corn NADP-malate dehydrogenase. *J. Biol. Chem.* 263 (1988) 11780-11785.
25. Deng, T., Noel, J. and Tsai, M.-D.: A novel expression vector for high-level synthesis and secretion of foreign proteins in *Escherichia coli*: overproduction of bovine pancreatic phospholipase A₂. *Gene* 93 (1990) 229-234.
26. Doolittle, R. F., Johnson, M. S., Husain, I., Van Houten, B., Thomas, D. C. and Sancar, A.: Domainal evolution of a prokaryotic DNA repair protein and its relationship to active-transport proteins. *Nature* 323 (1986) 451-453.
27. Ellis, R. J. and Hartley, M. R.: Sites of synthesis of chloroplast proteins. *Nature* 233 (1971) 193-196.
28. Esch, F. S., Bohlen, P., Otsuka, A. S., Yoshida, M. and Allison, W. S.: Inactivation of the bovine mitochondrial F₁-ATPase with dicyclohexyl[¹⁴C]carbodiimide leads to the modification of a specific glutamic acid residue in the β subunit. *J. Biol. Chem.* 256 (1981) 9084-9089.
29. Feierabend, J. and Schrader-Reichhardt, U.: Biochemical differentiation of plastids and other organelles in rye leaves with a high-temperature-induced deficiency of plastid ribosomes. *Planta* 129 (1976) 133-145.
30. Fischer, K.H. and Latzko, E.: Chloroplast ribulose-5-phosphate kinase: light-mediated activation, and detection of both soluble and membrane-associated activity. *Biochem. Biophys. Res. Comm.* 89 (1979) 300-306.
31. Flugge, U.I., Stitt, M., Freisl, M. and Heldt, H.W.: The participation of phosphoribulokinase in the light regulation of CO₂ fixation. *Plant Physiol.* 69 (1982) 263-267.
32. Fry, D.C., Kuby, S.A. and Mildvan, A.S.: ATP-binding site of adenylate kinase: mechanistic implications of its homology with *ras*-encoded p21, F₁-ATPase, and other nucleotide-binding proteins. *Proc. Natl. Acad. Sci. U.S.A.* 83 (1986) 907-911.
33. Gardemann, A., Stitt, M. and Heldt, H.W.: Regulation of spinach ribulose 5-phosphate kinase by 3-phosphoglycerate. *FEBS Lett.* 137 (1982) 213-216.
34. Gardemann, A., Stitt, M. and Heldt, H.W.: Regulation of spinach ribulose-5-phosphate kinase by stromal metabolite levels. *Biochim. Biophys. Acta.* 722 (1983) 51-60.
35. Ghiron, C.A., Eftink, M.R., Porter, M.A. and Hartman, F.C.: Fluorescence studies of phosphoribulokinase, a light-regulated, chloroplastic enzyme. *Arch. Biochem. Biophys.* 260 (1988) 267-272.

36. Gibson, J.L. and Tabita, F.R.: Organization of phosphoribulokinase and ribulose biphosphate carboxylase/oxygenase genes in *Rhodopseudomonas (Rhodobacter) sphaeroides*. J. Bact. 169 (1987) 3685-3690.
37. Gibson, J.L. and Tabita, F.R.: Localization and mapping of CO₂ fixation genes within two gene clusters in *Rhodobacter sphaeroides*. J. Bacteriol. 170 (1988) 2153-2158.
38. Gibson, J.L., Chen, J., Tower, P.A. and Tabita, F.R.: The form II fructose 1,6-bisphosphatase and phosphoribulokinase genes form part of a large operon in *Rhodobacter sphaeroides*: primary structure and insertional mutagenesis analysis. Biochemistry 29 (1990) 8085-8093.
39. Goff, S.A. and Goldberg, A.L.: Production of abnormal proteins in *E. coli* stimulates transcription of *lon* and other heat shock genes. Cell 41 (1985) 587-595.
40. Gontero, B., Cardenas, M.L. and Ricard, J.: A functional five-enzyme complex of chloroplasts involved in the Calvin cycle. Eur. J. Biochem. 173 (1988) 437-443.
41. Gubler, U. and Hoffman B. J.: A simple and very efficient method for generating cDNA libraries. Gene 25 (1983) 263-269.
42. Hallenbeck, P.L. and Kaplan, S.: Cloning of the gene for phosphoribulokinase activity from *Rhodobacter sphaeroides* and its expression in *Escherichia coli*. J. Bacteriol. 169 (1987) 3669-3678.
43. Hallenbeck, P.L. and Kaplan, S.: Structural gene regions of *Rhodobacter sphaeroides* involved in CO₂ fixation. Photosyn. Res. 19 (1988) 63-71.
44. Hanahan, D.: Techniques for transformation of *E. coli*. In Glover, D. (Ed.), IRL Press, Oxford. DNA cloning. Vol 1 (1985), pp. 109-135.
45. Hart, B.A. and Gibson, J.: Ribulose-5-phosphate kinase from *Chromatium* sp. strain D. Arch. Biochem. Biophys. 144 (1971) 308-321.
46. Hartman, F. C., Soper, T. S., Niyogi, S. K., Mural, R. J., Foote, R. S., Mitra, S., Lee, E. H., Machanoff, R. and Larimer, F. W.: Function of Lys-166 of *Rhodospirillum rubrum* ribulosebisphosphate carboxylase/oxygenase as examined by site-directed mutagenesis. J. Biol. Chem. 262 (1987) 3496-3501.
47. Higgins, C.F., Hiles, I.D., Salmond, G.P.C., Gill, D.R., Downie, J.A., Evans, I.J., Holland, I.B., Gray, L., Buckel, S.D., Bell, A.W. and Hermodson, M.A.: A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. Nature 323 (1986) 448-450.
48. Huber, S.C.: Inhibition of O₂ evolution by inorganic phosphate and magnesium. Biochim. Biophys. Acta. 545 (1979) 131-140.

49. Hurwitz, J., Weissbach, A., Horecker, B.L. and Smyrniotis, P.Z.: Spinach phosphoribulokinase. *J. Biol. Chem.* 218 (1956) 769-783.
50. Husemann, M., Klintworth, R., Buttcher, V., Salnikow, J., Weissborn, C. and Bowien, B.: Chromosomally and plasmid-encoded gene clusters for CO₂ fixation (*cfx* genes) in *Alcaligenes eutrophus*. *Mol. Gen. Genet.* 214 (1988) 112-120.
51. Jahn, R., Schiebler, W. and Greengard, P.: A quantitative dot-immunobinding assay for proteins using nitrocellulose membrane filters. *Proc. Natl. Acad. Sci. U.S.A.* 81 (1984) 1684-1687.
52. Johnson, D.A., Gautsch, J.W., Sportsman, J.R. and Elder, J.H.: Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal. Techn.* 1 (1984) 3-8.
53. Joint, I.R., Morris, I and Fuller, R.C.: Purification of a complex of alkaline fructose 1,6-bisphosphatase and phosphoribulokinase from *Rhodospirillum rubrum*. *J. Biol. Chem.* 247 (1972) 4833-4838.
54. Kagawa, T.: Isolation and purification of ribulose-5-phosphate kinase from *Nicotiana glutinosa*. In Edelman, E., Hallick, R.B. and Chua, (Eds.), *Methods in Chloroplast Molecular Biology*. Elsevier, New York, 1982, pp. 695-705.
55. Karin, N. J. and Cook, J. S.: Turnover of the catalytic subunit of Na,K-ATPase in HTC cells. *J. Biol. Chem.* 261 (1986) 10422-10428.
56. Karlin-Neumann, G.A. and Towbin, E.M.: Transit peptides of nuclear-encoded chloroplast proteins share a common amino acid framework. *EMBO J.* 5 (1986) 9-13.
57. Keegstra, K. and Olsen, L.J.: Chloroplastic precursors and their transport across the envelope membranes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40 (1989) 471-501.
58. Klintworth, R., Husemann, M., Salnikow, J. and Bowien, B.: Chromosomal and plasmid locations for phosphoribulokinase genes in *Alcaligenes eutrophus*. *J. Bact.* 164 (1985) 954-956.
59. Klintworth, R., Husemann, M., Weissborn, C. and Bowien, B.: Expression of the plasmid-encoded phosphoribulokinase gene from *Alcaligenes eutrophus*. *FEMS Microbiol. Lett.* 49 (1988) 1-6.
60. Kossmann, J., Klintworth, R. and Bowien, B.: Sequence analysis of the chromosomal and plasmid genes encoding phosphoribulokinase from *Alcaligenes eutrophus*. *Gene* 85 (1989) 247-252.
61. Krieger, T.J. and Miziorko, H.M.: Affinity labeling and purification of spinach leaf ribulose-5-phosphate kinase. *Biochemistry* 25 (1986) 3496-3501.

62. Krieger, T.J., Mende-Mueller, L. and Miziorko, H.M.: Phosphoribulokinase: isolation and sequence determination of the cysteine-containing active-site peptide modified by 5'-*p*-fluorosulfonylbenzoyladenosine. *Biochim. Biophys. Acta* 915 (1987) 112-119.
63. Krieger, T.J. and Miziorko, H.M.: Spinach leaf ribulose-5-phosphate kinase: examination of sulfhydryls by chemical modification and spin-labeling. *Arch. Biochem. Biophys.* 256 (1987) 362-371.
64. Kuhlemeier, C., Cuozzo, M., Green, P.J., Goyvaerts, E., Ward, K. and Chua, N.-H.: Localization and conditional redundancy of regulatory elements in *rbcS-3A*, a pea gene encoding the small subunit of ribulose-bisphosphate carboxylase. *Proc. Natl. Acad. Sci. U.S.A.* (1988) 4662-4666.
65. Kunkel, T.A., Roberts, J.D. and Zakour, R.A.: Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154 (1987) 367-382.
66. Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227 (1970) 680-685.
67. Laing, W.A., Stitt, M. and Heldt, H.W.: Changes in the activity of ribulosebisphosphate kinase and fructose- and seduheptulose-bisphosphatase in chloroplasts. *Biochim. Biophys. Acta.* 637 (1981) 348-359.
68. Larimer, F.W., Mural, R.J. and Soper, T.S.: Versatile protein engineering vectors for mutagenesis, expression and hybrid enzyme formation. *Protein Eng.* 3 (1990) 227-231.
69. Latzko, E., Garnier, R. and Gibbs, M.: Effect of photosynthesis, photosynthetic inhibitors and oxygen on the activity of ribulose 5-phosphate kinase. *Biochem. Biophys. Res. Comm.* 39 (1970) 1140-1144.
70. Lavergne, D. and Bismuth, E.: Simultaneous purification of two kinases from spinach leaves: Ribulose-5-phosphate kinase and phosphoglycerate kinase. *Plant Sci. Lett.* 1 (1973) 229-236.
71. Lazaro, J.J., Sutton, C.W., Nicholson, S. and Pows, R.: Characterisation of two forms of phosphoribulokinase isolated from the green alga, *Scenedesmus obliquus*. *Eur. J. Biochem.* 156 (1986) 423-429.
72. MacElroy, R.D., Johnson, E.J. and Johnson, M.K.: Control of ATP-dependent CO₂ fixation in extracts of *Hydrogenomonas facilis*: NADH regulation of phosphoribulokinase. *Arch. Biochem. Biophys.* 131 (1969) 272-275.
73. MacElroy, R.D., Mack, H.M. and Johnson, E.J.: Properties of phosphoribulokinase from *Thiobacillus neapolitanus*. *J. Bact.* (1972) 532-538.

74. Maniatis, T., Fritsch, E.F. and Sambrook, J.: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
75. Marcus, F., Moberly, L. and Latshaw, S.P.: Comparative amino acid sequence of fructose-1,6-bisphosphatases: identification of a region unique to the light-regulated chloroplast enzyme. *Proc. Natl. Acad. Sci. U.S.A.* 85 (1988) 5379-5383.
76. Marsden, W.J.N. and Codd, G.A.: Purification and molecular and catalytic properties of phosphoribulokinase from the cyanobacterium *Chlorogleopsis früschi*. *J. Gen. Microbiol.* 130 (1984) 999-1006.
77. Marsden, W.J.N., Lanaras, T. and Codd, G.A.: Subcellular segregation of phosphoribulokinase and ribulose-1,5-bisphosphate carboxylase/oxygenase in the cyanobacterium *Chlorogleopsis früschi*. *J. Gen. Microbiol.* 130 (1984) 2089-2093.
78. Meijer, W.G., Enequist, H.G., Terpstra, P. and Dijkhuizen, L.: Nucleotide sequence of the genes encoding fructosebisphosphatase and phosphoribulokinase from *Xanthobacter flavus* H4-14. *J. Gen. Microbiol.* 136 (1990) 2225-2230.
79. Milanez, S. and Mural, R.J.: Cloning and sequencing of cDNA encoding the mature form of phosphoribulokinase from spinach. *Gene* 66 (1988) 55-63.
80. Milanez, S., and Mural, R. J.: Expression of spinach phosphoribulokinase in *Escherichia coli*. *FASEB J.* 4 (1990) A1761.
81. Milanez, S., Mural, R.J. and Hartman, F.C.: Roles of cysteinyl residues of phosphoribulokinase as examined by site-directed mutagenesis. *J. Biol. Chem.* (1991) *in press*.
82. Miller, C.G.: Protein degradation and proteolytic modification in *Escherichia coli* and *Salmonella typhimurium*. In Neidhardt, F.C., (Ed.), Cellular and Molecular Biology. American Society for Microbiology, Washington, D.C., 1987, pp. 680-691.
83. Mishkind, M. L., Wessler, S. R. and Schmidt, G. W.: Functional determinants in transit sequences: import and partial maturation by vascular plant chloroplasts of the ribulose-1,5 bisphosphate carboxylase small subunit of *Chlamydomonas*. *J. Cell Biol.* 100 (1985) 226-234.
84. Miziorko, H.M., Brodt, C.A. and Krieger, T.J.: Affinity labeling of spinach leaf phosphoribulokinase by ATP analogs. *J. Biol. Chem.* 265 (1990) 3642-3647.
85. Mural, R.J., Soper, T.S., Larimer, F.W. and Hartman, F.C.: Examination of the intersubunit interaction between glutamate-48 and lysine-168 of ribulose-bisphosphate carboxylase/oxygenase by site-directed mutagenesis. *J. Biol. Chem.* 265 (1990) 6501-6505.

86. Mural, R.J.: Fundamentals of light-regulated gene expression in plants. In Harris, J.R. and Biswas, B.B., (Eds.), Subcellular Biochemistry. Plenum Publishing Co., 1991, *in press*.
87. Neidhardt, F.C. and VanBogelen, R.A.: Heat shock response in *Escherichia coli* and *Salmonella typhimurium*. In Neidhardt, F.C., (Ed.), Cellular and Molecular Biology. American Society for Microbiology, Washington, D.C., 1987 pp. 1334-1345.
88. Nicholson, S., Easterby, J.S. and Powls, R.: A single chloroplast protein with latent activities of both NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase. Eur. J. Biochem. 202 (1986) 19-22.
89. Nicholson, S., Easterby, J.S. and Powls, R.: Properties of a multimeric protein complex from chloroplasts possessing potential activities of NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase. Eur. J. Biochem. 162 (1987) 423-431.
90. Niyogi, S.K., Foote, R.S., Mural, R.J., Larimer, F.W., Mitra, S., Soper, T.S., Machanoff, R. and Hartman, F.C.: Nonessentiality of histidine 291 of *Rhodospirillum rubrum* ribulose-bisphosphate carboxylase/oxygenase as determined by site-directed mutagenesis. J. Biol. Chem. 261 (1986) 10087-10092.
91. Noel, D., Nikaido, K. and Ferro-Luzzi Ames, G.: A single amino acid substitution in a histidine-transport protein drastically alters its mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Biochemistry 18 (1979) 4159-4165.
92. Oehler, S., Eismann, E.R., Kramer, H. and Muller-Hill, B. The three operators of the *lac* operon cooperate in repression. EMBO J. 9 (1990) 973-979.
93. Omnaas, J., Porter, M.A. and Hartman, F.C.: Evidence for a reactive cysteine at the nucleotide binding site of spinach ribulose-5-phosphate kinase. Arch. Biochem. Biophys. 236 (1985) 646-653.
94. Parsell, D.A. and Sauer, R.T.: The structural stability of a protein is an important determinant of its proteolytic susceptibility in *Escherichia coli*. J. Biol. Chem. 264 (1989) 7590-7595.
95. Pontremoli, S., and Mangiarotti, G.: A simple method for the preparation of D-ribulose 5-phosphate. J. Biol. Chem. 237 (1962) 643-645.
96. Porter, M.A., Milanez, S., Stringer, C.D. and Hartman, F.C.: Purification and characterization of ribulose-5-phosphate kinase from spinach. Arch. Biochem. Biophys. 245 (1986) 14-23.

97. Porter, M.A. and Hartman, F.C.: Commonality of catalytic and regulatory sites of spinach phosphoribulokinase: characterization of a tryptic peptide that contains an essential cysteinyl residue. *Biochemistry* 25 (1986) 7314-7318.
98. Porter, M.A., Stringer, C.D. and Hartman, F.C.: Characterization of the regulatory thioredoxin site of phosphoribulokinase. *J. Biol. Chem.* 263 (1988) 123-129.
99. Porter, M.A. and Hartman, F.C.: Catalytic nonessentiality of an active-site cysteinyl residue of phosphoribulokinase. *J. Biol. Chem.* 263 (1988) 14846-14849.
100. Porter, M.A., Potter, M.D. and Hartman, F.C.: Affinity labeling of spinach phosphoribulokinase subsequent to *S*-methylation at Cys16. *J. Protein Chem.* 9 (1990) 445-451.
101. Porter, M.A. and Hartman, F.C.: Exploration of the function of a regulatory sulfhydryl of phosphoribulokinase from spinach. *Arch. Biochem. Biophys.* 281 (1990) 330-334.
102. Porter, M.A.: The aggregation states of spinach phosphoribulokinase. *Planta* 181 (1990) 349-357.
103. Racker, E.: The reductive pentose phosphate cycle. I. Phosphoribulokinase and ribulose diphosphate carboxylase. *Arch. Biochem. Biophys.* 69 (1957) 300-310.
104. Raines, C.A., Lloyd, J.C., Longstaff, M., Bradley, D., and Dyer T.: Chloroplast fructose-1,6-bisphosphatase: the product of a mosaic gene. *Nucl. Acids Res.* 16 (1988) 7931-7942.
105. Raines, C.A., Longstaff, M., Lloyd, J.C. and Dyer, T.A.: Complete coding sequence of wheat phosphoribulokinase: developmental and light-dependent expression of the mRNA. *Mol. Gen. Genet.* 220 (1989) 43-48.
106. Rindt, K.-P. and Ohmann, E.: NADH and AMP as allosteric effectors of ribulose-5-phosphate kinase in *Rhodospseudomonas spheroides*. *Biochem. Biophys. Res. Comm.* 36 (1969) 357-364.
107. Rippel, S. and Bowien, B.: Phosphoribulokinase from *Rhodospseudomonas acidophila*. *Arch. Microbiol.* 139 (1984) 207-212.
108. Robinson, M., Lilley, R., Little, S., Emtage, J.S., Yarranton, G., Stephens, P., Millican, A., Eaton, M. and Humphreys, G.: Codon usage can affect efficiency of translation of genes in *Escherichia coli*. *Nucl. Acids Res.* 12 (1984) 6663-6671.
109. Roesler, K.R. and Ogren, W.L.: Nucleotide sequence of spinach cDNA encoding phosphoribulokinase. *Nucl. Acids Res.* 16 (1988) 7192.

110. Roesler, K.R. and Ogren, W.L.: *Chlamydomonas reinhardtii* phosphoribulokinase. *Plant Physiol.* 93 (1990) 188-193.
111. Sadler, J. R., Waterman, M. S. and Smith, T. F.: Regulatory pattern identification in nucleic acid sequences. *Nucleic Acids Res.* 11 (1983) 2221-2231.
112. Sainis, J.K. and Harris, G.C.: The association of ribulose-1,5-bisphosphate carboxylase with phosphoisomerase and phosphoribulokinase. *Biochem. Biophys. Res. Comm.* 139 (1986) 947-954.
113. Sainis, J.K., Merriam, K. and Harris, G.C.: The association of D-ribulose-1,5-bisphosphate carboxylase/oxygenase with phosphoribulokinase. *Plant Physiol.* 89 (1989) 368-374.
114. Salvucci, M.E. and Ogren, W.L.: A *Chlamydomonas reinhardtii* mutant with catalytically and structurally altered ribulose-5-phosphate kinase. *Planta* 165 (1985) 340-347.
115. Sanger, F., Nicklen, S. and Coulson, A. R.: DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74 (1977) 5463-5467.
116. Shinozaki, K., Deno, H., Kato, A. and Sugiura, M.: Overlap and cotranscription of the genes for the beta and epsilon subunits of tobacco chloroplast ATPase. *Gene* 24 (1983) 147-155.
117. Siebert, K., Schobert, p. and Bowien, B.: Purification, some catalytic and molecular properties of phosphoribulokinase from *Alcaligenes eutrophus*. *Biochim. Biophys. Acta* 658 (1981) 35-44.
118. Siebert, K. and Bowien, B.: Evidence for an octameric structure of phosphoribulokinase from *Alcaligenes eutrophus*. *Biochim. Biophys. Acta* 787 (1984) 208-214.
119. Sinha, N.D., Biernat, J., McManus, J. and Koster, H.: Polymer support oligonucleotide synthesis XVIII^(1,2): use of β -cyanoethyl-*N,N'*-dialkylamino-*N*-morpholino phosphoramidite of deoxynucleosides for the synthesis of DNA fragments simplifying deprotection and isolation of the final product. *Nucl. Acids Res.* 12 (1984) 4539-4557.
120. Skinner, A.J. and Cooper, R.A.: The regulation of ribose-5-phosphate isomerisation in *Escherichia coli* K12. *FEBS Lett.* 12 (1971) 293-296.
121. Smeekens, S., de Groot, M., van Binsbergen, J. and Weisbeek, P.: Sequence of the precursor of the chloroplast thylakoid lumen protein plastocyanin. *Nature* 317 (1985) 456-458.

122. Smith, T. F., Waterman, M. S. and Sadler, J. R.: Statistical characterization of nucleic acid sequence functional domains. *Nucleic Acids Res.* 11 (1983) 2205-2220.
123. Stephens, R. M.: A sequencers' sequence analysis package for the IBM PC. *Gene Anal. Techn.* 2 (1985) 67-75.
124. Surek, B., Heilbronn, A., Austen, A. and Latzko, E.: Purification and characterization of phosphoribulokinase from wheat leaves. *Planta* 165 (1985) 507-512.
125. Tabita, F.R.: Pyridine nucleotide control and subunit structure of phosphoribulokinase from photosynthetic bacteria. *J. Bact.* 143 (1980) 1275-1280.
126. Tabita, F.R.: Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. *Microbiol. Rev.* 52 (1988) 155-189.
127. Uhlmann, E. and Engels, J.: Chemical 5'-phosphorylation of oligonucleotides valuable in automated DNA synthesis. *Tetrahedron Lett.* 27 (1986) 1023-1026.
128. Vieira, J. and Messing, J.: Production of single-stranded plasmid DNA. *Methods Enzymol.* 153 (1987) 3-11.
129. Wara-Aspawati, O., Kemble, R.J. and Bradbeer, J.W.: Activation of glyceraldehyde-phosphate dehydrogenase (NADP) and phosphoribulokinase in *Phaseolus vulgaris* leaf extracts involves the dissociation of oligomers. *Plant Physiol.* 66 (1980) 34-39.
130. Wirtz, W., Stitt, M. and Heldt, H.W.: Light activation of Calvin Cycle enzymes as measured in pea leaves. *FEBS Lett.* 142 (1982) 223-226.
131. Wishnick, M., and Lane, M. D.: Ribulose bisphosphate carboxylase from spinach leaves. *Methods Enzymol.* 23 (1971) 570-577.
132. Wolosiuk, R.A. and Buchanan, B.B.: Regulation of chloroplast phosphoribulokinase by the ferredoxin/thioredoxin system. *Arch. Biochem. Biophys.* 189 (1978) 97-101.
133. Yanisch-Perron, C., Vieira, J. and Messing, J.: Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33 (1985) 103-119.
134. Yoshida, M., Poser, J. W., Allison, W. S. and Esch, F. S.: Identification of an essential glutamic acid residue in the β subunit of the adenosine triphosphatase from the thermophilic bacterium PS3. *J. Biol. Chem.* 256 (1981) 148-153.

135. Yoshida, M., Allison, W. S., Esch, F. S. and Futai, M.: The specificity of carboxyl group modification during the inactivation of the *Escherichia coli* F₁-ATPase with dicyclohexyl[¹⁴C]carbodiimide. *J. Biol. Chem.* 257 (1982) 10033-10035.
136. Yoshida, M. and Allison, W. S.: Modulation by ADP and Mg²⁺ of the inactivation of the F₁-ATPase from the thermophilic bacterium, PS3, with dicyclohexylcarbodiimide. *J. Biol. Chem.* 258 (1983) 14407-14412.
137. Young, R. A. and Davis, R. W.: Efficient isolation of genes by using antibody probes. *Proc. Natl. Acad. Sci. USA* 80 (1983a) 1194-1198.
138. Young, R. A. and Davis, R. W.: Yeast RNA polymerase II genes: Isolation with antibody probes. *Science* 222 (1983b) 778-782.

APPENDIXES

APPENDIX A.
TABLES

Table I-1 Properties of PRK from prokaryotes and eukaryotes							
Bacteria:	Native	Subunit	Suggested Multimer	Sp. Act. ¹ (units/mg)	Positive Effector	Negative Effectors	Source(s):
<i>Rhodospseudomonas acidophila</i>	248,000	32,000	octamer	111	NADH	PEP	1
<i>Rhodobacter capsulatus</i>	220,000	36,000	hexamer	49	NADH	ND ²	2
<i>Rhodobacter sphaeroides</i> (form I) (form II)	ND	32,661 33,142	octamer ND	50 36	NADH NADH (slight)	AMP, PEP AMP, PEP	3
<i>Rhodospirillum rubrum</i>	ND	ND	ND	49	NADH	AMP	4
<i>Rhodomicrobium vannielii</i>	ND	ND	ND	ND	(not NADH)	ND	5
<i>Alcaligenes eutrophus</i> (plasmid pHG1) (chromosomal)	256,000	33,100 33,300	octamer octamer	7.8; 29; 41 36	NADH NADH	AMP, PEP AMP, PEP	6
<i>Xanthobacter flavus</i> H4-14	ND	33,409	ND	ND	ND	ND	7
<i>Chromatium</i> sp. Strain D	240,000	ND	ND	138	(not NADH)	AMP, ADP	8
<i>Chloroagloopsis fritschii</i> (cyanobacterium)	230,000	40,000	hexamer	14	light (DTT) ³	AMP, PEP, aspartate	9
<i>Thiobacillus neapolitanus</i>	ND	ND	ND	450	(not NADH)	AMP, PEP	10
Green algae and plants:							
<i>Scenedesmus obliquus</i> (Active) ⁴	83,000	42,000	dimer	204	light (DTT)	ND	11
<i>Chlamydomonas reinhardtii</i>	ND	38,500	dimer	410	light (DTT)	ND	12
<i>Nicotiana glauca</i> (tobacco leaf)	90,000	45,000	dimer	360	light (DTT)	ND	13
<i>Triticum</i> sp. (wheat leaf)	83,000	39,200	dimer	893	light (DTT)	3-P- glycerate	14
<i>Spinacea oleracea</i> (spinach leaf)	90,000	39,200	dimer	360; 410	light (DTT)	(see text) ⁵	15

Table I-1 (Continued)

¹Sp. Act. = Specific activity (units/ mg PRK)

²ND = not determined

³*Scenedesmus obliquus* PRK has also been isolated as part of a complex with glyceraldehyde phosphate dehydrogenase, of which the specific activity is 148 units/mg PRK.

⁴Light activates PRK via the ferredoxin/thioredoxin system (11-14,21). DTT (dithiothreitol) mimics the effect of light *in vitro* (29,68,130).

⁵Chapter 1, section (b)-4 lists negative metabolic effectors for spinach PRK

Sources:

- (1) Rippel, S. and Bowien, B.: Phosphoribulokinase from *Rhodospseudomonas acidophila*. Arch. Microbiol. 139 (1984) 207-212.
- (2) Tabita, F.R.: Pyridine nucleotide control and subunit structure of phosphoribulokinase from photosynthetic bacteria. J. Bact. 143 (1980) 1275-1280.
- (3) Gibson, J.L. and Tabita, F.R.: Organization of phosphoribulokinase and ribulose biphosphate carboxylase/oxygenase genes in *Rhodospseudomonas (Rhodobacter) sphaeroides*. J. Bact. 169 (1987) 3685-3690.
- Gibson, J.L., Chen, J., Tower, P.A. and Tabita, F.R.: The form II fructose 1,6-bisphosphatase and phosphoribulokinase genes form part of a large operon in *Rhodobacter sphaeroides*: primary structure and insertional mutagenesis analysis. Biochemistry 29 (1990) 8085-8093.
- Hallenbeck, P.L. and Kaplan, S.: Cloning of the gene for phosphoribulokinase activity from *Rhodobacter sphaeroides* and its expression in *Escherichia coli*. J. Bacteriol. 169 (1987) 3669-3678.
- Rindt, K.-P. and Ohmann, E.: NADH and AMP as allosteric effectors of ribulose-5-phosphate kinase in *Rhodospseudomonas sphaeroides*. Biochem. Biophys. Res. Comm. 36 (1969) 357-364.
- Tabita, F.R.: Pyridine nucleotide control and subunit structure of phosphoribulokinase from photosynthetic bacteria. J. Bact. 143 (1980) 1275-1280.
- (4) Joint, I.R., Morris, I and Fuller, R.C.: Purification of a complex of alkaline fructose 1,6-bisphosphatase and phosphoribulokinase from *Rhodospirillum rubrum*. J. Biol. Chem. 247 (1972) 4833-4838.
- Tabita, F.R.: Pyridine nucleotide control and subunit structure of phosphoribulokinase from photosynthetic bacteria. J. Bact. 143 (1980) 1275-1280.
- (5) Tabita, F.R.: Pyridine nucleotide control and subunit structure of phosphoribulokinase from photosynthetic bacteria. J. Bact. 143 (1980) 1275-1280.
- (6) Husemann, M., Klintworth, R., Butcher, V., Sahnikow, J., Weissborn, C. and Bowien, B.: Chromosomally and plasmid-encoded gene clusters for CO₂ fixation (*cfx* genes) in *Alcaligenes eutrophus*. Mol. Gen. Genet. 214 (1988) 112-120.
- Klintworth, R., Husemann, M., Weissborn, C. and Bowien, B.: Expression of the plasmid-encoded phosphoribulokinase gene from *Alcaligenes eutrophus*. FEMS Microbiol. Lett. 49 (1988) 1-6.
- Kossmann, J., Klintworth, R. and Bowien, B.: Sequence analysis of the chromosomal and plasmid genes encoding phosphoribulokinase from *Alcaligenes eutrophus*. Gene 85 (1989) 247-252.
- MacElroy, R.D., Johnson, E.J. and Johnson, M.K.: Control of ATP-dependent CO₂ fixation in extracts of *Hydrogenomonas facilis*: NADH regulation of phosphoribulokinase. Arch. Biochem. Biophys. 131 (1969) 272-275.
- Siebert, K., Schobert, p. and Bowien, B.: Purification, some catalytic and molecular properties of phosphoribulokinase from *Alcaligenes eutrophus*. Biochim. Biophys. Acta 658 (1981) 35-44.
- Siebert, K. and Bowien, B.: Evidence for an octameric structure of phosphoribulokinase from *Alcaligenes eutrophus*. Biochim. Biophys. Acta 787 (1984) 208-214.

Table I-1 (Continued)

- (7) Meijer, W.G., Enequist, H.G., Terpstra, P. and Dijkhuizen, L.: Nucleotide sequence of the genes encoding fructosebiphosphatase and phosphoribulokinase from *Xanthobacter flavus* H4-14. J. Gen. Microbiol. 136 (1990) 2225-2230.
- (8) Hart, B.A. and Gibson, J.: Ribulose-5-phosphate kinase from *Chromatium* sp. strain D. Arch. Biochem. Biophys. 144 (1971) 308-321.
- Tabita, F.R.: Pyridine nucleotide control and subunit structure of phosphoribulokinase from photosynthetic bacteria. J. Bact. 143 (1980) 1275-1280.
- (9) Marsden, W.J.N. and Codd, G.A.: Purification and molecular and catalytic properties of phosphoribulokinase from the cyanobacterium *Chlorogloeopsis fritschii*. J. Gen. Microbiol. 130 (1984) 999-1006.
- (10) MacElroy, R.D., Mack, H.M. and Johnson, E.J.: Properties of phosphoribulokinase from *Thiobacillus neapolitanus*. J. Bact. (1972) 532-538.
- (11) Nicholson, S., Easterby, J.S. and Pows, R.: Properties of a multimeric protein complex from chloroplasts possessing potential activities of NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase. Eur. J. Biochem. 162 (1987) 423-431.
- (12) Roesler, K.R. and Ogren, W.L.: *Chlamydomonas reinhardtii* phosphoribulokinase. Plant Physiol. 93 (1990) 188-193.
- (13) Kagawa, T.: Isolation and purification of ribulose-5-phosphate kinase from *Nicotiana glutinosa*. In Edelman, E., Hallick, R.B. and Chua, (Eds.), Methods in Chloroplast Molecular Biology. Elsevier, New York, 1982, pp. 695-705.
- (14) Raines, C.A., Longstaff, M., Lloyd, J.C. and Dyer, T.A.: Complete coding sequence of wheat phosphoribulokinase: developmental and light-dependent expression of the mRNA. Mol. Gen. Genet. 220 (1989) 43-48.
- Surek, B., Heilbronn, A., Austen, A. and Latzko, E.: Purification and characterization of phosphoribulokinase from wheat leaves. Planta 165 (1985) 507-512.
- (15) Gardemann, A., Stitt, M. and Heldt, H.W.: Regulation of spinach ribulose 5-phosphate kinase by 3-phosphoglycerate. FEBS Lett. 137 (1982) 213-21216.
- Gardemann, A., Stitt, M. and Heldt, H.W.: Regulation of spinach ribulose-5-phosphate kinase by stromal metabolite levels. Biochim. Biophys. Acta. 722 (1983) 51-60.
- Krieger, T.J. and Mizioro, H.M.: Affinity labeling and purification of spinach leaf ribulose-5-phosphate kinase. Biochemistry 25 (1986) 3496-3501.
- Laing, W.A., Stitt, M. and Heldt, H.W.: Changes in the activity of ribulosebiphosphate kinase and fructose- and seduheptulose-bisphosphatase in chloroplasts. Biochim. Biophys. Acta. 637 (1981) 348-359.
- Lavergne, D. and Bismuth, E.: Simultaneous purification of two kinases from spinach leaves: Ribulose-5-phosphate kinase and phosphoglycerate kinase. Plant Sci. Lett. 1 (1973) 229-236.
- Porter, M.A., Milanez, S., Stringer, C.D. and Hartman, F.C.: Purification and characterization of ribulose-5-phosphate kinase from spinach. Arch. Biochem. Biophys. 245 (1986) 14-23.

Table III-1

Correlation between intracellular PRK activity levels and cell survival

Cloned <i>prk</i> gene	Relative ^a activity	MV1190 [pSM100] PLATED COLONIES ^b		TRANSFORMANTS ^c	
		-IPTG	+IPTG	CJ236	MV1190
wild-type	100%	1800	< 10	< 5	12,000
Wrong <i>prk</i> orientation	(0)	2600	3100	200	7,000
(none)	-	3400	3100	--	--
Cys 16 mutants (Ser,Ala)	45-90%	3300	≤ 10	≤ 5	7,000
Cys 55 mutants (Ser,Ala)	5-15%	2600	1800	150	6,000
Cys 244, Cys 250 mutants (Ser,Ala)	80-100%	2700	≤ 10	≤ 5	10,000

^aSingle-site mutations were introduced into PRK by site-directed mutagenesis of pSM100. Previously determined specific activities of the mutant proteins relative to wild-type enzyme are shown (81).

^b10⁻⁶ ml of an overnight culture of MV1190 harboring either mutant or wild-type pSM100 were spread on LB-Ap agar plates in the absence or presence of 0.1 mM IPTG. Control samples were similarly plated (MV1190 containing pFL220 with PRK in the wrong orientation, and MV1190 lacking plasmid DNA). The number of colonies was determined after 16 hours of growth at 30°C.

^cAbout 30 ng pSM100 mutant, wild-type, or control (pFL220 with PRK in the wrong orientation) DNA was used to transform *E. coli* CJ236 (*lacI* gene) and MV1190 (*lacI*^q gene). The transformants were grown 18-24 hours at 30°C with selection on LB-Ap (MV1190) or LB-Ap,Cm (CJ236) agar plates.

Table IV-1

Primers used for mutagenesis of the *prk* gene^a

Desired Replacement	Primer Sequence
C16S	pGATTCAGGTT <u>CT</u> GGTAAG
C16A	pGATTCAGGTG <u>CT</u> GGTAAGAG
C55S	pCTGTTATCT <u>CT</u> TTGGATG
C55A	pACTGTTATCG <u>CT</u> TTGGATG
C244S	pGGATTCCAT <u>CT</u> GGTAGAA
C244A	pTGGATTCCAG <u>CT</u> GGTAGAAA
C250S	pAATTAACAT <u>CT</u> TCTTACC
C250A	pAAATTAACAG <u>CT</u> TCTTACCC

^aThe underlined bases have been introduced by site-directed mutagenesis to replace the cysteine codons (TGT) with codons for either serine or alanine (bold).

Table IV-2

Kinase activities of crude extracts of transformed *E. coli*

Protein	Specific Activity ^a	
	(units/mg total protein)	(% wild-type)
wild-type	2.1 ± 0.4	100 ± 18
C16S	1.9 ± 0.4	90 ± 21
C16A	1.0 ± 0.2	47 ± 11
C55S	0.13 ± 0.05	6 ± 2
C55A	0.19 ± 0.03	9 ± 2
C244S	2.1 ± 0.4	100 ± 18
C244A	1.7 ± 0.5	81 ± 22
C250S	1.9 ± 0.7	90 ± 34
C250A	1.9 ± 0.4	90 ± 20

^aEach value is the average (± standard deviation) of four determinations. Western blots were used to normalize concentrations of PRK to that of wild-type enzyme.

Table IV-3

Catalytic parameters of partially purified PRKs

Protein	$K_m(\text{ATP})^a$ (μM)	$K_m(\text{Ru5P})^b$ (μM)	V_{max} (units/mg PRK)	(% w-t)
Pure enzyme (from spinach)	ND ^c	ND ^c	400	100
Wild-type	63 ± 17	95 ± 7	$426^d \pm 50$	100
C16S	94 ± 14	165 ± 5	$371^d \pm 38$	87
C16A	70 ± 19	96 ± 3	$173^d \pm 19$	41
C55S	42 ± 5	381 ± 31	$45^d \pm 12$	11
C55A	90 ± 5	754 ± 78	$67^d \pm 10$	16

^aThe concentration of ATP was varied from 20 to 500 μM at a fixed Ru5P concentration of 1.2 mM or 4.0 mM with the position 55 mutant proteins.

^bThe concentration of Ru5P was varied from 30 to 4000 μM at a fixed ATP concentration of 2.0 mM in the spectrophotometric assay and 6.0 mM in the radiometric assay.

^cThe K_m (ATP) and K_m (Ru5P) were not determined for PRK purified from spinach. The K_m (ATP) for native spinach PRK has been reported as 59 ± 12 μM and the K_m (Ru5P) as 150 μM (99). The specific activity of native spinach PRK has been reported to be 410 (96).

^dThe concentration of PRK in the impure sample was estimated from densitometer scans of Western immunoblots so that the stated V_{max} represents that of pure enzyme.

APPENDIX B.
FIGURES AND FIGURE LEGENDS

Figure I-1. Sequence alignment of phosphoribulokinases from prokaryotic and eukaryotic sources. *Source:* Richard J. Mural, *personal communication* (1991). Asterisks have been placed above residues which are identical in at least 7 of the 8 organisms. Regions showing homology among all species are underlined.

Eukaryotic PRKs:

Psm100 = spinach PRK

Whprk = wheat PRK

Crprk = *Chlamydomonas reinhardtii*

Prokaryotic PRKs:

Synprk = *Synechocystis* 6803 (cyanobacterium)

Prka = *Rhodobacter sphaeroides* form I (or A)

Prkb = *Rhodobacter sphaeroides* form II (or B)

Aeprkc = *Alcaligenes eutrophus* chromosomal

Aeprkp = *Alcaligenes eutrophus* plasmid

Xfprk = *Xanthobacter flavus*

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1                                     50
Psm100 SQQQTIVIGL AADSGCGKST FMRRLTSVFG GAAEPPKGGN PDSNTLISDT TTVICLDDPH SLDRNGRKVE
Whprk AVEQPIVIGL AADSGCGKST FMRRLTSVFG GAAEPPKGGN PDSNTLISDT TTVICLDDPH SLDRNGRKVE
Crprk DKDKTVVIGL AADSGCGKST FMRRLTSVFG GVPKPPAGGN PDSNTLISDM TTVICLDDPH SLDRNGRKVE
Synprk QLDRLVVIGL AGDSGCGKST FLRLTDLFG EEF..... MTVICLDDPH SLDRNGRKVE
Prka MSKKHPIISV TGSSGAGTST VKHTFDQIFR REGV..... K AVSIEGDAFH RPNRADRKAE
Prkb VSKKYPISV VGSSGAGTST VKHTFEQIFR REGV..... K SVSIEGDAFH RPNRADRKAE
Aeprkc MSERYPIIAI TGSSGAGTST VTRTFENIFR REGV..... K SVVIEGDSRH RYDRAEMKVK
Aeprkp MSERYPIIAI TGSSGAGTST VTRTFENIFC REGV..... K SVVIEGDSFH RYDRAEMKVK
Xfprk MSIKHPIIVV TGSSGAGTST VKRTFEQIFY REKV..... K AAPVEGDSFH RYDREKREL

71                                     120
Psm100 KVTALDPKAM DFDLMYEQVK ALKEGKAVDK PIYNHVSG.. .....L LDPPELIQPP
Whprk GVTALDPKAM DFDLMYEQVK AIKEGKAIK PIYNHVSG.. .....L LDPPELIQPP
Crprk GVTALAPEAQ NFDLMYHVK ALKEGKSVOK PIYNHVSG.. .....L IDAPELIESP
Synprk GVTALDPKAM NFDLMYEQVK TLKSGQSIMK PIYNHVSG.. .....L LDPPEKVEPN
Prka LDRRYAAGDA TFSHFSYEAN ELKELERVFR EYGETGQGR RTTVHDDAEA ARTGVAPGNF TQWAPFEDNS
Prkb LERYAAGDA TFSHFSYEAN ELKELERVFR EYGETGQGR RTTVHDDAEA ARTGVAPGNF TQWAPFEDNS
Aeprkc MAEAERTGNN NFSHFGEENN LFGDLESLFR SYAETGTGMR RHYLHSPTEA APFGQEPGTF TANEPLPADT
Aeprkp MAEAERTGNN NFSHFGEENN LFGDLESLFR SYAETGTGMR RHYLHSPTEA APFGQEPGTF TANEPLPADT
Xfprk MAEAAGKGNK NFSHFSPETN RLDDLAQLFK DYGTGSGRPF RHYVHDAGEA KLYNTEPGRF TDWEDLEQGT

141                                     190
Psm100 KILVIEGLHP MYDARVRELL DFSIYLOISM EVKFAWKIQ.. ..RDMKERGH SLESIKASIE SRKPDQDAVI
Whprk KIFVIEGLHP MYDERVRELL DFSIYLOISM EVKFAWKIQ.. ..RDMKERGH SLESIKASIE SRKPDQDAVI
Crprk PILVIEGLHP FYDKRVAELL DFKIYLOISD OIKFAWKIQ.. ..RDMKERGH SLESIKASIE SRKPDQDAVI
Synprk KVVVIEGLHP LYDERVRELV DPGVYLOISE EVKINWKIQ.. ..RDMKERGH TYEDILASIN ARKPDFTAYI
Prka HLLFYEGHLG AVVNSEVNIA GLADLKIGVV FVINLEQIQK IHRDRATRGY TTEAVTDVIL RRMHAYVHCI
Prkb DLLFYEGHLG CVNDEVNVL RHADLKIGVA FVINLEWIOK IHRDRATRGY TTEAVTDVIL RRMHAYVHCI
Aeprkc DLLFYEGHLG GVVTSVWVA QYPHLLIGVV FVINLEWIOK LMRDKKQNGY STEAVTDVIL RRMPDYVNYI
Aeprkp DLLFYEGHLG GVVTSVWVA QYPHLLIGVV FVINLEWIOK LMRDKKQNGY STEAVTDVIL RRMPDYVNYI
Xfprk DILFYEGHLG AVVTDKLNLA QHADLKIGVV FVINLEWIOK IHRDKATRGY TTDVTDVIL RRMPDYVNYI

211                                     260
Psm100 DPQKQADVV IEVLPTLIP DDOEGKVLAV RMIQKGVKF FMPVYLFDEG ..STISWIPC GRKLTCSYPG
Whprk DPQKQYADAV IEVLPTQLIP DDOEGKVLAV KLINKEGKIF FMPVYLFDEG ..STINWIPC GRKLTCSYPG
Crprk DPQKQADAMI IQVLPTQLIP DOK.GQYLRV RLIMKEGSKM FDPVYLFDEG ..STISWIPC GRKLTCSYPG
Synprk EPQKQYADVV IQVLPTKLI. EDKESKLLAV RLKQKESKIF FEPAYLFDEG ..STIDWIPC GRKLTCTYPG
Prka VQFSTQDIN FQRVPVVDTS NPFIARWIPT PDESIVVIRF KNPRGIDFPY LTMINGSWNN SRANSIVVPG
Prkb VQFSTQDIN FQRVPVVDTS NPFIARWIPT PDESIVVIRF KNPRGIDFPY LTMINGSWNN SRANSIVVPG
Aeprkc CPQFSRTHVN FQRVPCVDTN NPFISREIPA PDESMVVIRF ANPKGIDFPY LLSMINDSPN SRANTIVVPG
Aeprkp CPQFSRTHVN FQRVPCVDTN NPFISREIPA PDESMVVIRF ANPKGIDFPY LLSMINDSPN SRANTIVVPG
Xfprk CPQFTETDIN FQRVPTVDTN NPFVARWIPT PDESMVVIRF RDPHGIDFPY LLSMINDSPN SRANSIVVPG

281                                     330
Psm100 IKFSYGPDTF YGNEVTVVEM DGMFDRLEL IYVESHLNLT STKPYGEVTO QMLKHONPPG SNNGTGFPQT
Whprk IKFSYGPDTF YGNEVTVVEM DGMFDRLEL IYVESHLNLT STKPYGEVTO QMLKHONPPG SNNGTGFPQT
Crprk IKMFGYGPDTF YGQEVSVLEM DGMFDRLEL IYVESHLNLT SAKPYGEVTO QMLKHONPPG SNNGTGFPQT
Synprk IKMFGYGPDTF YGQEVSVLEM DGMFDRLEL IYVESHLNLT SAKPYGEVTO QMLKHONPPG SNNGTGFPQT
Prka NK..... QMLKHONPPG SNNGTGFPQT
Prkb NK..... QMLKHONPPG SNNGTGFPQT
Aeprkc GK..... QMLKHONPPG SNNGTGFPQT
Aeprkp GK..... QMLKHONPPG SNNGTGFPQT
Xfprk NK..... QMLKHONPPG SNNGTGFPQT

351                                     377
Psm100 IIGLKIRDLF EQLVASRSTA TATAAKA
Whprk IIGLKIRDLF EQIIAERAGV PAZAAKV
Crprk IIGLKIRDLF EQLVAKV PV.....
Synprk IIGLKIRDLF EQLVAKV PV.....
Prka PLIDRVVRES KVA.....
Prkb PLIDRVVRES R.....
Aeprkc PFVLRHMERK KRAAQ.....
Aeprkp PFVLRHMERK KRAAL.....
Xfprk PLIMKLMDRK RRAAG.....

```

Figure I-1.

Figure I-2. Sequence similarities of phosphoribulokinase and ATP-binding proteins of diverse origins. Homologous sequences are boxed in. The abbreviations used are: PRK, phosphoribulokinase; Mal k, maltose transport protein; Pst B, phosphate transport protein; Opp D, oligopeptide transport protein; His P, histidine transport protein. *Source:* Porter, M.A., Stringer, C.D. and Hartman, F.C.: Characterization of the regulatory thioredoxin site of phosphoribulokinase. J. Biol. Chem. 263 (1988) 123-129.

PROTEIN	SOURCE	SEQUENCE	POSITION
PRK	SPINACH	A A D S G C G K S T F M R	11-23
PRK	<u>R. SPHEROIDES</u>	T G S S G A G T S T V K H	11-23
MAL K	<u>E. COLI</u>	V G P S G C G K S T L L R	15-27
PST B	<u>E. COLI</u>	I G P S G C G K S T L L R	41-53
OPP D	<u>E. COLI</u>	V G E S G S G K S Q S L R	53-65
HIS P	<u>S. TYPHIMURIUM</u>	I G S S G S G K S T L L R	38-50
ADENYLATE KINASE	RABBIT	V G G P G S G K G T Q C E	14-26
MYOSIN	RABBIT	G E S G A G K T V N T K	179-190

Figure I-2.

Figure I-3. Similarities between PRK and F₁-ATPase subunits. The amino acid sequences used in this figure are from the following *Sources*: PRK, this study;

^A sequence of the β subunit of the chloroplast ATPase from tobacco, spinach and maize is from Shinozaki, K., Deno, H., Kato, A. and Sugiura, M.: Overlap and cotranscription of the genes for the beta and epsilon subunits of tobacco chloroplast ATPase. *Gene* 24 (1983) 147-155;

^B Shinozaki, K., Deno, H., Kato, A. and Sugiura, M.: Overlap and cotranscription of the genes for the beta and epsilon subunits of tobacco chloroplast ATPase. *Gene* 24 (1983) 147-155;

^C Yoshida, M., Poser, J. W., Allison, W. S. and Esch, F. S.: Identification of an essential glutamic acid residue in the β subunit of the adenosine triphosphatase from the thermophilic bacterium PS3. *J. Biol. Chem.* 256 (1981) 148-153.

^D Corresponds to an essential glutamic acid residue in the β subunit of the ATPase from the thermophilic bacterium PS3 as identified by its derivatization with DCC (134).

<u>PROTEIN</u>	<u>SOURCE</u>	<u>SEQUENCE</u>	<u>POSITION</u>
PRK	SPINACH	T S V F G G A A E P P K G G N P	26-41
F ₁ -ATPASE β SUBUNIT	CHLOROPLAST ^A	V S V F G G V G E R T R E G N D	196-211
	E. COLI ^B	Y S V F A G V G E R T R E G N D	174-189
	THERMOPHILIC BACTERIUM PS3 ^C	A G V G E P R	

Figure I-3.

Figure II-1. Western immunoblot of immunoprecipitates from cell-free translation products of poly(A)⁺ RNA from spinach leaves. Cell-free translation was carried out using the rabbit reticulocyte lysate system (Promega Biotec) with [³⁵S]-methionine added to monitor protein synthesis. Rabbit polyclonal anti-PRK IgG (kindly provided by Dr. F.C. Hartman) and protein A Sepharose beads were used to immunoprecipitate PRK from the translation products (55). The translation products were analyzed on SDS-PAGE (15% polyacrylamide) minigels using the buffer system of Laemmli (66) and PRK-related products were detected by Western immunoblots (17). Lane A contains the pre-precipitate (using pre-immune rabbit serum) of the translation products, lane B shows the products (indicated by the two arrows on the left) precipitated by rabbit anti-PRK, which have apparent M_r s of 45 and 48 kDa. Lane C contains purified mature spinach PRK (arrow on the right) with an apparent M_r of 44 kDa.

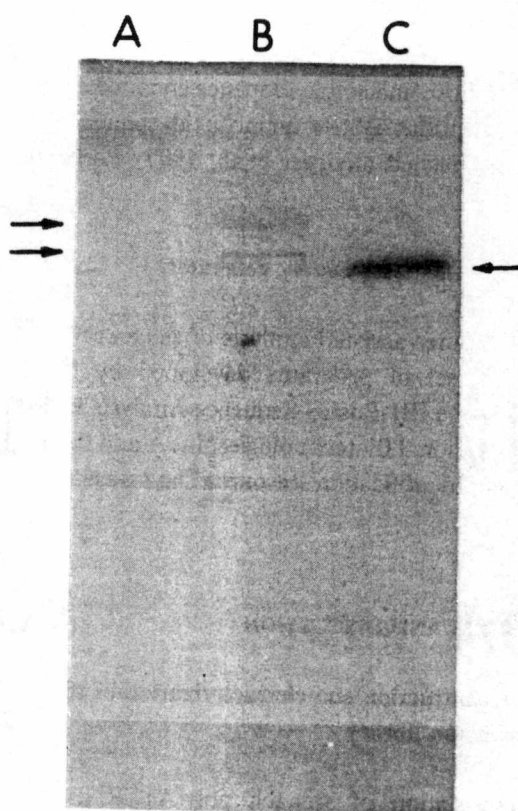


Figure II-1.

Figure II-2. Physical maps of PRK cDNA. (A) Strategy for sequencing the cDNA for spinach PRK. Clones derived from PRK cDNA were identified as those whose nucleotide sequence encoded any of the published partial amino acid sequences of PRK. The sequencing of both strands of the clones, identified as coding for PRK, was accomplished using a strategy in which oligodeoxynucleotide primers were synthesized based on newly acquired DNA sequence. The primers, 15 to 17-mers, were prepared by using phosphoramidite chemistry (46,119) on a Systec Model 1450 DNA synthesizer. Using these primers we were able to obtain most of the sequence of both strands. A deletion was made from the *Sph*I site in the PRK cDNA to the *Sph*I site in the poly-linker of M13mp18 and single stranded DNA from this construction was used as a template to complete the sequence. The top line represents the total amount of cDNA which was sequenced. The position of the coding sequence for mature PRK protein is within the boxed-in region. Several unique restriction enzyme cleavage sites are included as reference points. Cross-hatching shows regions which code for parts of the mature protein whose amino acid sequences have been previously determined (62,96,98). These include nucleotides 1 to 192 which encode the N-terminus of the mature protein, nucleotides 676 to 768 which encode the amino acid sequence of two tryptic fragments of PRK (62,98), and a peptide of 11 amino acids (62) encoded by nucleotides 892 to 924. Below this composite are the relative positions of the two clones used to construct it and the strategy used to sequence them. The arrows indicate the regions of each clone sequenced with a particular primer. Arrows beginning with a circle represent areas which were sequenced using the M13 universal primer (GAAATTGTTATCC; BRL). Regions sequenced using newly synthesized primers are represented by arrows beginning with boxes. The primers were synthesized to prime at the following sequences: 1, AACAAAGTGTTTTTCGG; 2, GAATCCAGAAAGCCA; 3, AGCTCGTTGCTAGCA; 4, TGAACAAGTTAAGGC; 5, GTTCTTACCCTGGTA; 6, GGATGATTTTCATTCCC; 7, AGCAACTGCAACAGC; 8, TCCTAAGATCTTGGT; 9, GTAGAGATGGATGGA; 10, ACAAGACCATCCTATG. (B) Position of termination codons in each of the three possible reading frames of the PRK cDNA sequence. Frame 1 can be seen as the only ORF in the region of interest. It extends from the 5' end of the composite sequence at position -170 to the TAG terminator at position 1054.

Figure II-3. Composite nucleotide sequence of the cDNA for spinach PRK with a translation of the coding region for mature spinach PRK. Several unique restriction enzyme cleavage sites are indicated in italics. The nucleotides are numbered above the DNA sequence, with an asterisk marking every tenth base, from the first base of the codon for the *N*-terminal serine of mature PRK. Corresponding amino acid residues are indicated beneath the DNA and are numbered on the right margin for each line of amino acids. The coding region of the mature protein ends 1053 nucleotides after the beginning of the *N*-terminal serine codon with a terminator, TAG (—), starting at nucleotide position 1054. This 1053 bp region encodes a protein of 351 amino acids with a calculated molecular mass of 39,232 daltons and a predicted amino acid composition closely matching that published for spinach PRK (96). The *Eco*RI linkers ligated on the ends of the cDNA clones are in lower-case letters.

-120

ScoRI *
gaattcc GTAAATAGC AATTTTATTA CCAAAAGATA GTACGTACAC AATCCAACA ACAACCAATT T

-60

AGGATCATC ATTCAACCAA AACAACAAC ANGTTTTTTT CAACTACAAA AGATCATCAT CATCAACAAA CACTTTATTT ACAACAAGAC CATCTATGT AATAACATGT
1

60

TCA CAA CAA CAA ACA ATT GTG ATT GGT TTA OCA GCA GAT TCA GGT TGT GGT AAG AGT ACA TTC ATG AGG AGG TTA ACA AGT GTT TTC GGT
S Q Q Q Q T I V I G L A A D S G C G K S T F M R R L T S V F G 30

120

XbaI *
GGC GGC GGC GAG CCA CCA AAG GGT GGT AAC CCA GAT TCA AAC ACA TTG ATT AGT GAC ACT ACT ACT GTT ATC TGT TTG GAT GAT TTT CAT
G A A E P P K G G N P D S N T L I S D T T T V I C L D D F H 60

240

TCC CTT GAT AGA AAT GGC AGG AAA GTG GAA AAA GTT ACT GCT TTA GAC CCA AAA GCT AAT GAT TTT GAT CTT ATG TAT GAA CAA GTT AAG
S L D R N G R K V E K V T A L D P K A N D F D L M Y E Q V K 90

300

GCT TTG AAA GAA GGT AAA GCT GTT GAT AAA CCT ATT TAT AAT CAT GTT TCT GGT TTG TTG GAC CCT CCT GAG CTT ATT CAA CCT CCT AAG
A L K E G K A V D K P I Y N H V S G L L D P P E L I Q P P K 120

420

BglII *
ATC TTG GTC ATT GAA GGC TTA CAC CCC ATG TAT GAC GCA CGT GTG AGG GAA TTG CTA GAC TTC AGC ATC TAC TTG GAC ATT AGC AAT GAA
I L V I E G L H P M Y D A R V R E L L D F S I Y L D I S N E 150

480

GTT AAA TTT GGC TGG AAA ATT CAG AGA GAC ATG AAA GAA AGA GGA CAC AGT CTT GAA AGC ATC AAA GGC AGT ATT GAA TCC AGA AAG CCA
V K F A W K I Q R D M K E R G H S L E S I K A S I E S R K P 180

600

GAT TTT GAT GCT TAC ATT GAC CCA CAA AAG CAG CAT GCT GAT GTA GTG ATT GAA GTA TTG CCA ACT GAA CTC ATT CCT GAT GAT GAT GAA
D F D A Y I D P Q K Q H A D V V I E V L P T E L I P D D D E 210

660

GCC AAA GTG TTG AGA GTA AGG ATG ATT CAG AAA GAA GGA GTC AAG TTT TTC AAC CCA GTT TAC TTG TTT GAT GAA GGA TCT ACC ATT TCA
G K V L R V R M I Q K E G V K F F N P V Y L F D E G S T I S 240

780

TGG ATT CCA TGT GGT AGA AAA TTA ACA TGT TCT TAC CCT GGT ATC AAA TTT TCC TAT GGC CCA GAC ACC TTC TAT GGC AAC GAG GTG ACA
W I P C G R K L T C S Y P G I K F S Y G P D T F Y G N E V T 270

840

GTA GTA GAG ATG GAT GGA ATG TTT GAC AGA TTA GAC GAA CTA ATC TAC GTC GAA AGC CAT TTG AGC AAT CTA TCA ACC AAG TTT TAT GGT
V V E M D G M F D R L D E L I Y V E S H L S N L S T K F Y G 300

960

GAA GTC ACT CAA CAA ATG TTG AAG CAC CAA AAT TTC CCA GGA AGC AAC AAT GGA ACT GGT TTC TTC CAA ACC ATA ATT GGA TTG AAG ATC
E V T Q Q M L K H Q N F P G S N N G T G F F Q T I I G L K I 330

1020

AGA GAC TTG TTC GAG CAG CTC GTT GCT AGC AGG TCT ACA GCA ACT GCA ACA GCT GCT AAA GGC TAG AAA AAGCTCGAAT TTACACAT ATGAT
R D L F E Q L V A S R S T A T A T A A K A —

1140

AAGAT ATGGAATATG TCAATCTAAA TTAATCTAC TTCATTGTGA ATTCCACTA OCTATATTAT TATCTTCTT GTATAAGTTT GTTTTGTAG GGATACTCTG ATA
1200

1180

TTTCTG CTGTAAATAG ACATGCAAGA GATGGAAGAT GCAATTCAAA AG gaattcc

1200

ScoRI
gaattcc

Figure II-3.

Figure III-1. Construction of the PRK expression phagmid, pSM100. (A) The original sequence in the *EcoRI* fragment around the codon for the *N*-terminal amino acid (serine, underlined) of mature-length PRK in M13mp18 (79). Following oligonucleotide-directed mutagenesis with the primer pGTAATAACATATGTCACAACAACA, the DNA (B) contained an ATG start codon (bold) at the 5' end of the coding sequence for mature-length PRK and a second *NdeI* restriction site (there is a naturally occurring site just distal to the 3' end of the PRK translated sequence) nested inside the *EcoRI* fragment. Because digestion of RF M13-PRK DNA with *NdeI* yielded another fragment the same size as expected for PRK, the PRK *EcoRI* fragment was gel-isolated and subcloned into the *EcoRI* site of pBR325 (interrupting the chloramphenicol acyltransferase gene). PRK-containing pBR325 DNA was digested with *NdeI*, the 5' overhanging ends were filled (using PolIk and all four dNTPs), and phosphorylated *Bam*HI linkers were ligated to the blunt-ended fragments. The 1.1 kbp PRK *Bam*HI fragment (C) was ligated into the *Bam*HI site of the phagmid expression vector, pFL220 (68) (D), creating the PRK expression phagmid pSM100 (E), which was used to transform *E. coli* MV1190.

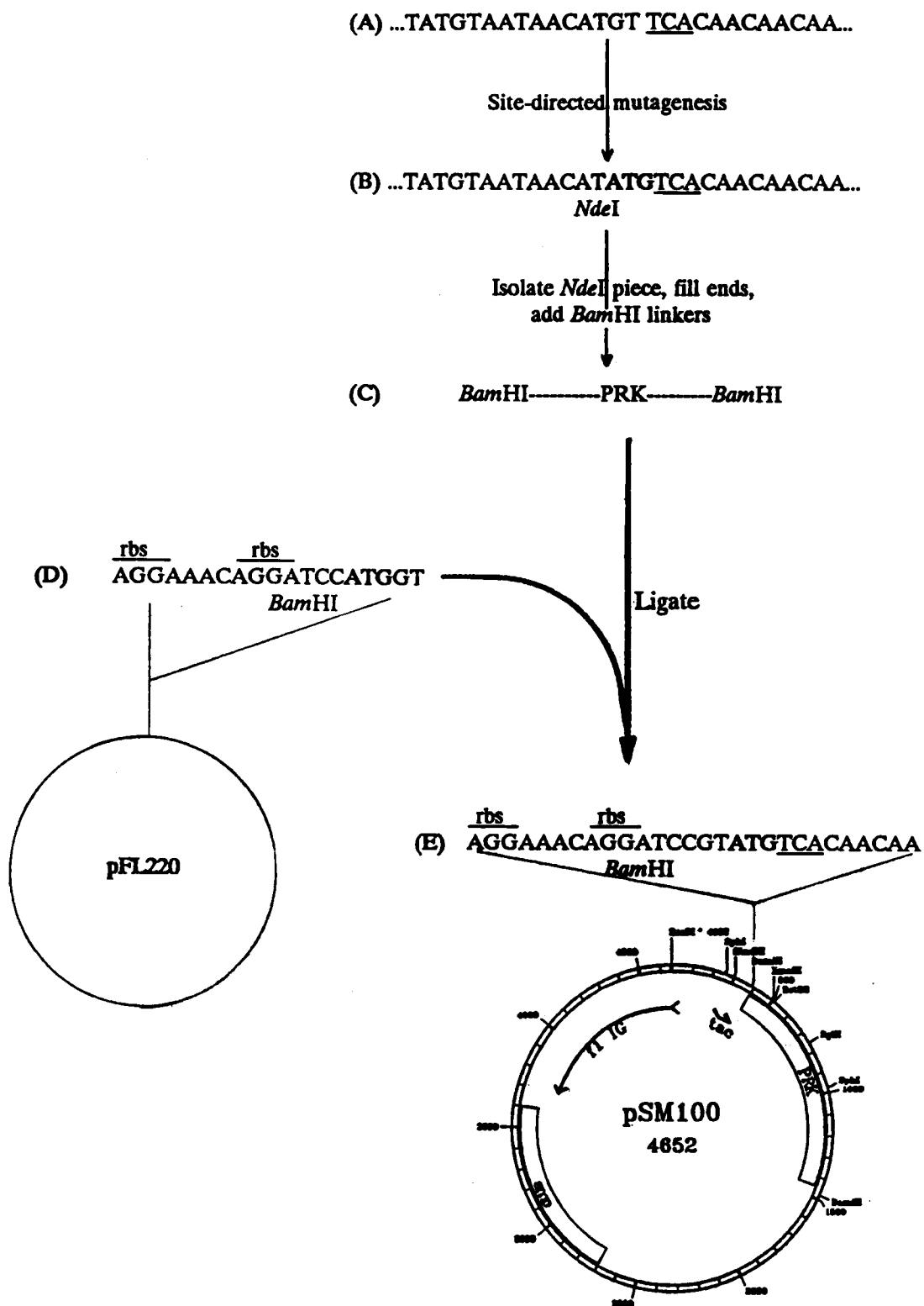


Figure III-1.

Figure III-2. Western immunoblots of crude cell-free extracts containing PRK. 10 μ g total protein from crude (*E. coli* MV1190) extracts were electrophoretically separated by vertical SDS-PAGE (15% acrylamide, 0.5% bisacrylamide) gels (100 mm wide, 90 mm high, 0.5 mm thick) using the buffer system of Laemmli (66). Proteins were transferred to nitrocellulose (17) and PRK was detected by immunoblotting as previously detailed (51,52,81) using rabbit polyclonal anti-PRK IgG (102) and goat anti-rabbit IgG conjugated with horseradish peroxidase, and the colorimetric reagent 3,3' diaminobenzidine. (A) Lane 1: extract from cells grown at 37°C without arabinose; Lane 2: extract from cells grown under optimized conditions (at 30°C with arabinose); Lane 3: extract prepared from MV1190[pSM100] colonies grown on LB-Ap agar plates in the presence of IPTG, and which had normal pSM100 DNA (as determined by restriction analysis). (B) Lanes 1-5: cell-free extracts from *E. coli* MV1190 colonies grown in the presence of inducer (0.1 mM IPTG) and had rearranged pSM100 DNA; Lane 6: 5 ng purified spinach leaf PRK; Lane 7: prestained molecular weight standards having apparent molecular weights as provided by the manufacturer are as follows: bovine serum albumin (69.3 kDa), ovalbumin (45.8 kDa), carbonic anhydrase (28.7 kDa), β -lactoglobulin (18.4 kDa) and lysozyme (15.4 kDa).

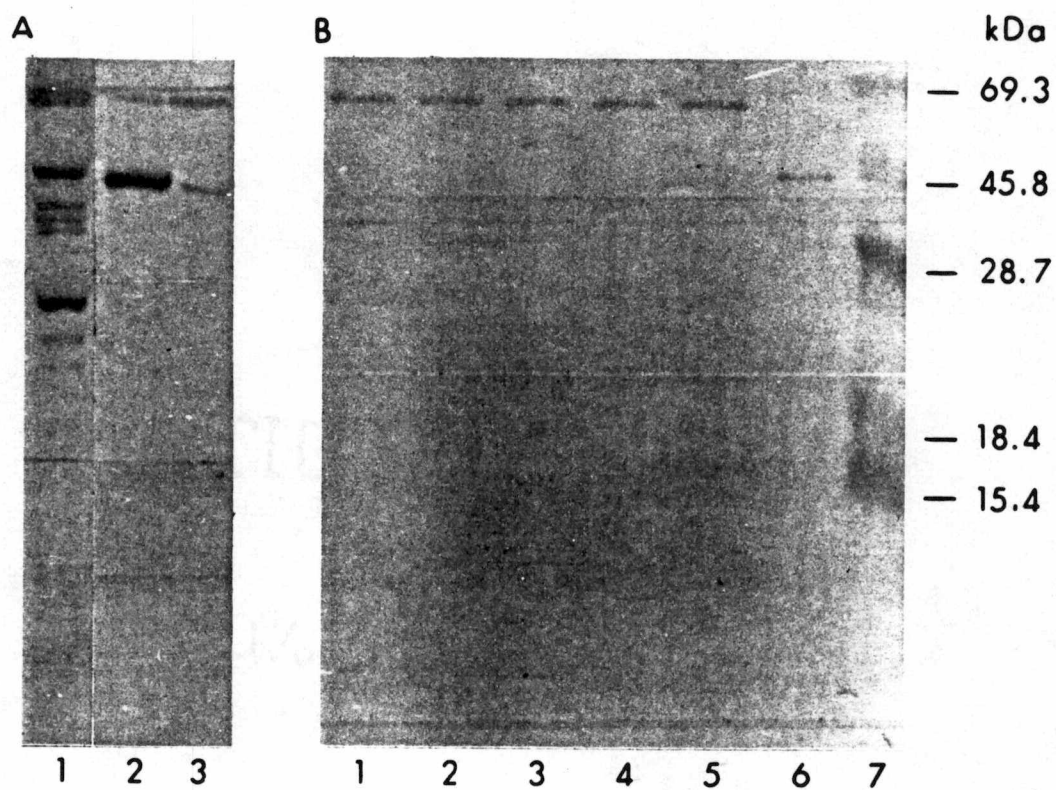


Figure III-2.

Figure IV-1. Electrophoretic analysis of crude extracts containing PRK. (A) SDS-PAGE of crude extracts. Lane 1: mixture of molecular weight markers that contains phosphorylase β (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa); Lanes 2 and 6: 100 and 200 ng, respectively, of pure PRK from spinach leaves; Lane 3: crude extract containing C16S PRK (4 μ g total protein); Lane 4: C16S PRK following its partial purification (4 μ g total protein); Lane 5: *E. coli* extract lacking PRK (4 μ g total protein). (B) Western immunoblot of an SDS-polyacrylamide gel of crude extracts. With the exception of pure spinach leaf PRK (20 ng) (Lane 1), all lanes contain 10 μ g of total protein. Lane 2: extract lacking PRK; Lane 3: C250A PRK; Lane 4: C250S PRK; Lane 5: C244A PRK; Lane 6: C244S PRK; Lane 7: C55A PRK; Lane 8: C55S PRK; Lane 9: C16A PRK; Lane 10: C16S PRK; Lane 11: wild-type PRK.

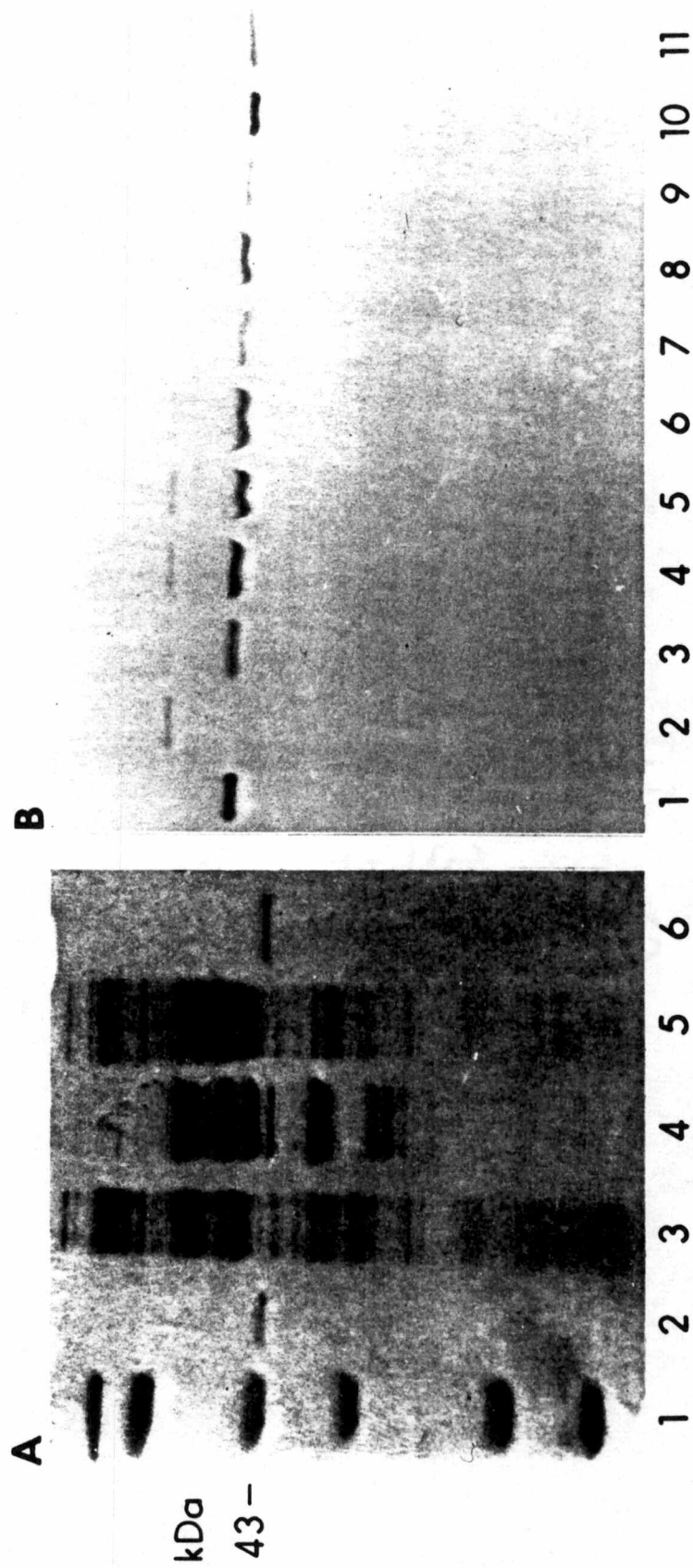


Figure IV-1.

Figure IV-2. Western immunoblot of an SDS-polyacrylamide gel of extracts enriched for PRK by chromatography on the Reactive Red affinity matrix. Lane 1: Wild-type PRK; Lane 2: C55A PRK; Lane 3: C55S PRK; Lane 4: C16A PRK; Lane 5: C16S PRK; Lane 6: purified spinach leaf PRK; Lane 7: extract lacking PRK.

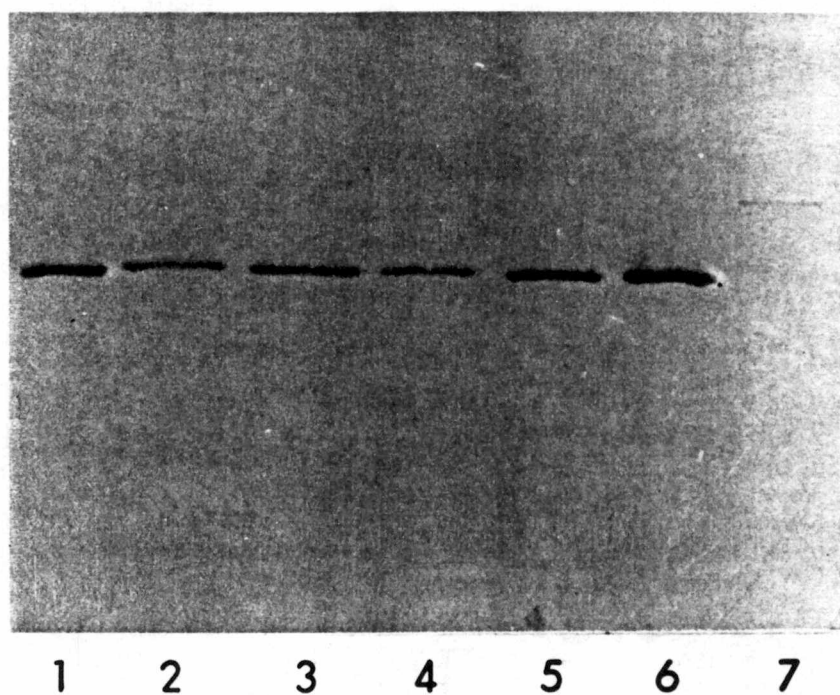


Figure IV-2.

Figure IV-3. Inactivation of wild-type and mutant phosphoribulokinase by NEM. PRK (0.02 - 0.08 units) in 50 mM Bicine, 5 mM MgCl₂, 0.5 mM EDTA and 10% glycerol was treated at 4°C with either 10 μ M (C55S, $\square$; wild-type, $\bigcirc$) or 100 μ M (C16S, Δ) NEM. Separate reaction mixtures of wild-type, C16S, and C55S PRK included 2 mM ATP ($\bullet$). Controls of C16S ($\diamond$), C55S ($\diamond$), and wild-type PRK ($\blacklozenge$) lacked NEM. Reactions were initiated by mixing 20 μ l each of pre-chilled PRK ($\pm$ 4 mM ATP) and NEM; aliquots (5 - 30 μ l) were periodically assayed for kinase activity.

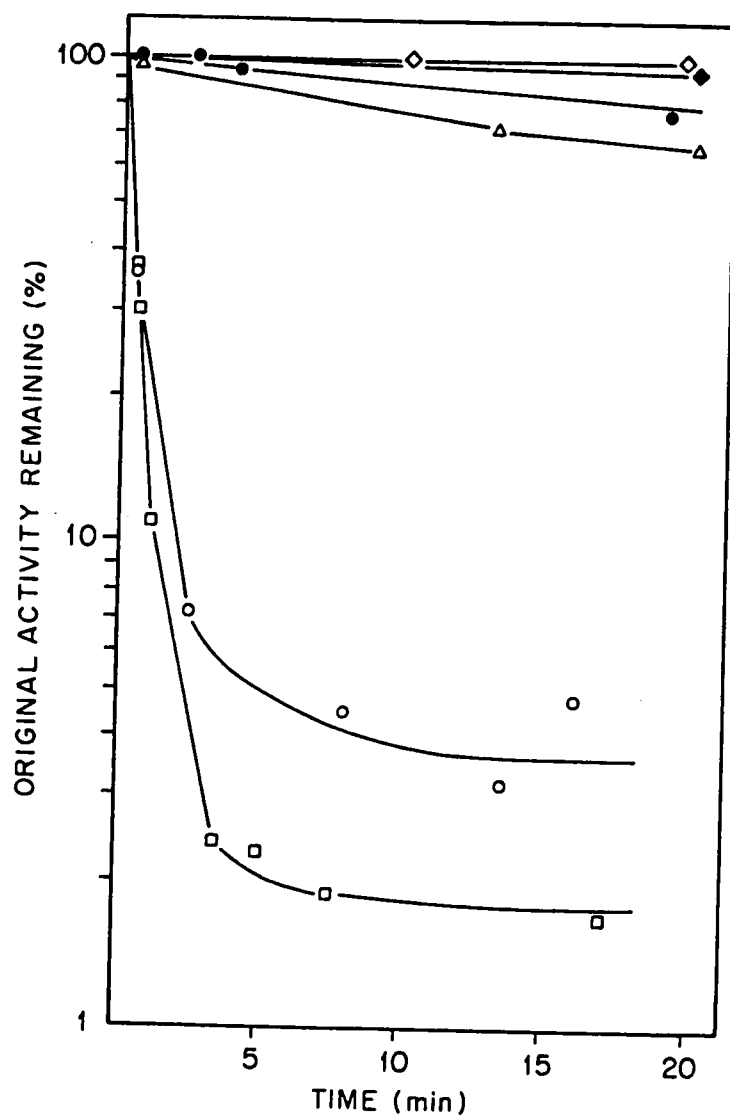


Figure IV-3.

VITA

Sylvia Milanez was born in Zagreb, Yugoslavia on December 7, 1959. She attended elementary school and high school in the Chicago area. Ms. Milanez entered Loyola University of Chicago in 1976 and in May 1980 graduated with a Bachelor of Science degree in Biology. Following a year as a Research Assistant at Rush Presbyterian St. Luke's Medical Center in Chicago, Ms. Milanez entered the University of Illinois at Urbana/Champaign, receiving a Master of Science degree in Biochemistry in 1985. From July, 1984 to September, 1985, she was a Research Associate with Dr. Fred Hartman of the Biology Division of the Oak Ridge National Laboratory. In September, 1985, Ms. Milanez was accepted into the University of Tennessee doctoral program in the Biomedical Sciences, receiving the Doctor of Philosophy degree with an emphasis in Biochemistry in August 1991 under the direction of Dr. Richard J. Mural.

Ms. Milanez is married to John D. Galambos, Ph.D., and has one daughter, Kathryn, age three and a half.