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Identification of the Phosphoribulokinase Sugar Phosphate Binding Domain†

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ABSTRACT: A recombinant form of *Rhodobacter sphaeroides* phosphoribulokinase (form I; NADH dependent) has been expressed in and purified to homogeneity from *Escherichia coli* that harbor the *prk*A gene in the plasmid pKP1565b. Restriction digestion of the phosphoribulokinase-encoding plasmid produces a tractable 450 bp fragment that encodes amino acid residues 28–179, which include a region (residues 42–54) highly conserved among phosphoribulokinase proteins. Using overlap extension polymerase chain reaction methodology, directed mutagenesis was performed to produce mutant proteins in which basic residues in this conserved region were replaced by neutral amino acids. Lysine-53, implicated by affinity labeling studies, has been replaced by methionine; little effect on substrate binding or catalysis is apparent. In contrast, when histidine-45 is replaced by asparagine, a 40-fold increase in the K_m for ribulose 5-phosphate results; a 200-fold increase results when arginine-49 is replaced by glutamine. Implication of this region as part of the sugar phosphate binding site is compatible with previous results that indicate targeting by an ATP analogue containing a reactive functionality esterified to the γ-phosphoryl group. The phosphoribulokinase reaction involves a single in-line phosphoryl transfer, requiring that the γ-phosphoryl of ATP be closely juxtaposed to the bound cosubstrate. It follows that any reactive group attached to the γ-phosphoryl in a nucleotide analogue that is bound to PRK in the absence of the cosubstrate will be favorably positioned to modify the sugar phosphate binding site.

Phosphoribulokinase (PRK;[1] EC 2.7.1.19) catalyzes a key step in Calvin's reductive pentose phosphate cycle, namely, the synthesis of the CO_2 acceptor ribulose 1,5-bisphosphate. The plant and algal PRKs are active as dimers and typically exhibit turnover numbers (Krieger & Miziorko, 1986; Porter et al., 1986) that are 1 order of magnitude higher than some octameric bacterial PRKs (Tabita, 1988). As might be expected for an enzyme that catalyzes a key metabolic step, PRK activity is subject to regulation. Plant and algal PRKs are interconverted between active and inactive forms by thiol/disulfide exchange (Buchanan, 1980) while bacterial PRKs are subject to allosteric regulation (Abdelal & Schlegel, 1974; Tabita, 1980; Rippel & Bowien, 1984).

The active site of spinach PRK has been investigated in some detail. The N-terminal portion of the protein has been identified as a consensus ATP binding domain and has, in fact, been affinity-labeled by reactive ATP analogues (Kreiger & Miziorko, 1986; Kreiger et al., 1987). Cysteine-16 in this domain is not catalytically essential (Porter & Hartman, 1988) and is not conserved in prokaryotic PRKs but controls activity in eukaryotic PRKs by reversibly forming a disulfide with cysteine-55 (Porter et al., 1988). Affinity labeling with lysine-directed reactive ATP analogues has recently implicated the region around lysine-68 as part of the catalytic domain (Miziorko et al., 1990). Upon mapping lysine-68 within the catalytic site, we suggested that this basic residue might function to stabilize binding of the nucleotide substrate (Miziorko et al., 1990). As initial sequence information on bacterial PRKs became available, the presence of a homologous lysine in the eukaryotic enzyme was suggested (Kossman et al., 1989) as well as discounted (Gibson et al., 1990). Thus, the significance and function of this residue remain to be established.

Systems for heterologous expression of *Rhodobacter sphaeroides* PRK have been developed by Hallenbeck and Kaplan (1987) and by Gibson and Tabita (1987). We have adopted Hallenbeck and Kaplan's expression system for the allosterically controlled PRK A (form I) in order to develop a model system useful for exploring structure/function correlations that account for PRK's regulation and catalysis. This report provides an indication of the value of this experimental

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[1] Abbreviations: PRK, phosphoribulokinase; Ru5P, ribulose 5-phosphate; RuBP, ribulose 1,5-bisphosphate; IPTG, isopropyl thiogalactoside; oePCR, overlap extension polymerase chain reaction.

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Table I: Synthetic Oligonucleotides Used for Mutagenesis of the *prk*A Gene[a]

wild-type	
	GAC GCC TTC CAC CGC TTC AAC CGC GCC GAC ATG AAG GCC GAG CTG GAC
K53M	
	C GAC ATG ATG GCC GAG C
R49Q	
	GC TTC AAC CAG GCC GAC ATG
H45N	
	GAC GCC TTC AAC CGC TTC A

[a] The indicated wild-type sequence corresponds to bases 124–171 of the coding sequence. The synthetic mutagenic oligonucleotides (coding sequence shown above with substitutions underscored), together with the appropriate complementary mutagenic oligonucleotides, were employed in the PCR overlap extension procedure of Ho et al. (1989). This allows amplification of a mutagenic duplex, which is digested with *Bgl*II/*Bst*EII prior to ligation into pKP1565b, which had been similarly digested to remove the wild-type coding region. Sequences of flanking primers used for the overlap extension procedure were 5′CCTCGACGGTGAAGCAC3′ (primer A) and 5′AGCATGGCATCCGGCGC3′ (primer B).

system and of the utility of mating it with polymerase chain reaction methodology to produce PRK mutants in a facile manner. Such mutants prove useful in addressing the function of conserved basic residues in the protein sequence corresponding to the region that flanks spinach PRK lysine-68.

A preliminary account of this work has appeared (Sandbaken et al., 1991).

Experimental Procedures

Materials

Deoxyoligonucleotides were synthesized by the Protein/Nucleic Acid Shared Facility at the Medical College of Wisconsin. Restriction enzymes and T4 DNA ligase were provided by New England Biolabs. Replinase and $KH^{14}CO_3$ (40–60 mCi/mmol) were obtained from Dupont/New England Nuclear. Geneclean was provided by Bio 101 Inc. Sequenase (United States Biochemicals) was used for DNA sequence analysis. Reagents for protein determination were obtained from Bio-Rad. Ribulose 5-phosphate (Ru5P), Reactive Green Agarose, and all other biochemicals and enzymes were obtained from Sigma Chemical Co. (St. Louis, MO).

Methods

Construction of Mutant Alleles of prkA. The plasmid encoding wild-type phosphoribulokinase from *R. sphaeroides*, pKP1565b, was provided by S. Kaplan (Hallenbeck & Kaplan, 1987). Mutant alleles of *prk*A encoding single amino acid substitutions were constructed by site-directed mutagenesis using the PCR overlap extension protocol (oePCR) of Ho et al. (1989). Flanking deoxyoligonucleotide primers (17-mers), complementary to DNA adjacent to the region of interest, were used to isolate all mutant alleles; sequences are indicated in the legend to Table I. For each mutant, a pair of internal complementary oligonucleotides encoding the desired amino acid substitution was used (Table I). The PCR-amplified mutagenic fragment was purified, digested with the restriction enzymes *Bgl*II and *Bst*EII, and ligated to *Bgl*II–*Bst*EII-digested pKP1565b from which the corresponding wild-type coding region had been removed. The ligation mixture was used to transform *Escherichia coli* strain JM105 according to Miller (1972). Mutants were identified by DNA sequence analysis of selected transformants. Template DNA was prepared according to Del Sal et al. (1989). The frequency of mutants isolated ranged from 40 to 100%. Once the desired mutant was isolated, the entire *Bgl*II–*Bst*EII fragment generated by oePCR was subjected to DNA sequence analysis to confirm that only the desired mutation was present and that no additional nucleotide changes had been generated in constructing the mutant *prk*A alleles.

Bacterial Growth. E. coli JM105 cultures (250–500 mL) containing the appropriate plasmid were grown in ampicillin-containing LB (Miller, 1972) at 37 °C overnight to stationary phase. Expression of PRK was induced in stationary-phase cultures by the addition of IPTG to a final concentration of 1 mM followed by 3–4-h growth at 37 °C. The cells were collected by low-speed centrifugation. Cell pellets were occasionally stored at −20 °C prior to cell lysis.

Preparation and Assay of Wild-Type and Mutant Phosphoribulokinases. PRK was isolated using a modification of the affinity chromatography procedure of Gibson and Tabita (1987). Protein was determined by the method of Bradford (1976) with bovine serum albumin as a standard. Enzyme activity assays were performed at 30 °C. The activity of purified PRK was routinely measured spectrophotometrically as described by Krieger and Miziorko (1986) by coupling Ru5P-dependent ADP formation to NADH oxidation with pyruvate kinase and lactate dehydrogenase. To quantitate PRK activity in high-speed supernatants of bacterial extracts (containing elevated levels of nonspecific ATPase activity), an alternate spectrophotometric assay of Andrews et al. (1973) was used in which formation of product ribulose bisphosphate (RuBP) was coupled to NADH oxidation. This coupled reaction utilized spinach ribulose-bisphosphate carboxylase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase. Exogenous RuBP was omitted from the assay. For kinetic analysis of PRK, a radioisotopic assay measuring the incorporation of $^{14}CO_2$ into acid-stable 3-phosphoglycerate was used (Paulsen & Lane, 1966). In standard assays, final concentrations of components in the reaction were 100 mM Hepes, pH 7.5, 10 mM DTT, 20 mM $MgCl_2$, 20 mM $KH^{14}CO_3$ (1000 dpm/nmol), 300 milliunits of spinach ribulose-1,5-bisphosphate carboxylase, 1 mM NADH, and, unless otherwise indicated, 10 mM ATP and 2.5 mM Ru5P. For the characterization of the mutant proteins, ATP concentration was varied from 0.1 to 6 mM; Ru5P concentration was varied from 20 to 200 μM for wild-type PRK and PRK-K53M, from 0.4 to 7 mM for PRK-H45N, and from 0.6 to 10 mM (above which level substrate inhibition becomes considerable) for PRK-R49Q.

Results and Discussion

Properties of Recombinant Bacterial Phosphoribulokinase. Upon purification of wild-type *R. sphaeroides* PRK that is expressed in *E. coli*, a homogeneous protein of 32-kDa subunit molecular mass [Figure 1, lane 1 (lanes numbered from left to right)] is recovered, as expected both on the basis of the open reading frame in the *prk*A (form I) gene and on earlier molecular mass estimates for the encoded protein (Hallenbeck & Kaplan, 1987). The specific activity (45 units/mg) measured for the purified enzyme under standard spectrophotometric assay conditions compared well with the value reported for the enzyme recovered from *R. sphaeroides* (Tabita, 1988). The recombinant PRK is stable upon storage at −80 °C, facilitating its characterization. Milligram levels of homogeneous wild-type protein (Figure 1) could be prepared from IPTG-induced bacteria recovered from 250 mL of LB nutrient broth.

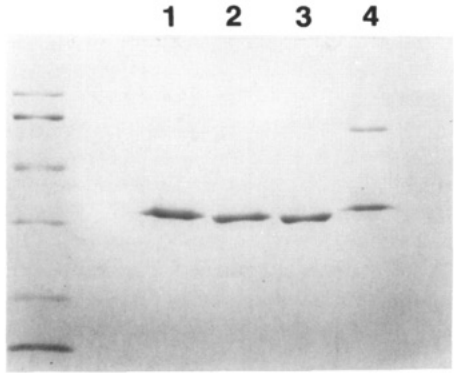

FIGURE 1: SDS gel electrophoresis of wild-type and mutant PRKs. The far left lane contains a mixture of molecular mass markers including phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). Lanes 1–4 (numbered from left to right) contain purified wild-type PRK, PRK-K53M, PRK-H45N, and PRK-R49Q, respectively.

Table II: Catalytic and Substrate Binding Parameters of Purified Recombinant PRKs[a]

protein	V_m (units/mg)	K_{ATP} (mM)	K_{Ru5P} (mM)
wild type	165	0.5	0.1
K53M	155	0.8	0.1
H45N	40	2.0	4.3
R49Q	72[b]	1.2	20

[a] Kinetic measurements employed the radioactive assay, performed at 30 °C under the conditions described under Methods. Half-saturation values are reported for ATP concentration dependence, following the convention established in previous reports on bacterial PRKs. K_m values are reported for Ru5P. [b] Corrected for a protein contaminant estimated to comprise 22% of the PRK-R49Q preparation.

These observations, coupled with availability of methodology (demonstrated below) for relatively straightforward engineering of PRK proteins, would appear to make this system an attractive alternative to recombinant systems for production of eukaryotic PRKs (Milanez et al., 1991).

While extensive kinetic data on the authentic *R. sphaeroides* enzyme are not available for comparison, substrate saturation curves measured using the recombinant enzyme indicate that the catalytic properties of this enzyme are similar to those reported for other bacterial PRKs. A sigmoidal dependency of reaction rate on ATP concentration is observed; the observed K_{ATP} = 0.5 mM (Table II) represents the concentration required for half-maximal activity and allows comparison with the ATP affinity reported for other bacterial PRKs (e.g., 1.5 mM for *Rhodobacter acidophila* PRK; Rippel & Bowien, 1984). The recombinant *R. sphaeroides* PRK exhibits a hyperbolic saturation curve with variable [Ru5P] (K_m = 0.1 mM; Table II), although at high concentrations of this metabolite, some substrate inhibition becomes apparent. The recombinant form I enzyme is markedly activated (10-fold) by NADH, as reported for the authentic protein (Rindt & Ohmann, 1969); our measured K_a = 0.11 mM agrees well with other reported values for NADH-sensitive bacterial PRKs (0.05–0.19 mM; Rippel & Bowien, 1984; Rindt & Ohmann, 1969; Siebert et al., 1981).

Rationale and Strategy in Designing PRK Mutants. As initial sequence data for plant (Milanez & Mural, 1988; Roesler & Ogren, 1988) and bacterial (Hallenbeck, 1989; Kossman et al., 1989) PRKs became available, the presence of an amino acid residue in bacterial PRKs that could be considered homologous to spinach K68 was uncertain. However, as additional plant or algal (Raines et al., 1989; Roesler et al., 1990) and bacterial (Meijer et al., 1990; Gibson et al., 1990) PRK structures were published, the suggested homologies of Kossman et al. (1989) were largely confirmed. In particular, the region (Figure 2) corresponding to bacterial PRK residues 42–54 or plant PRK residues 57–69 displays a level of identity or conservative substitution considerably higher than that observed overall. The depicted alignment suggested that basic amino acids are invariably found at residues corresponding to positions 45, 49, and 53 of the bacterial protein. Secondary structure predictions suggested a high probability of α helicity in this region. Modeling of the putative helix further suggests that it would be amphiphilic, with staggered alignment of H45, R49, and K53 on consecutive turns (Figure 3) of the polar face. While K53 had originally been proposed to stabilize binding of a phosphorylated PRK substrate, this model suggested that H45 and R49 could also contribute to such a function. Consequently, mutants PRK-K53M,[2] PRK-H45N, and PRK-R49Q, in which one basic charge has been eliminated and steric differences are minimal, were constructed, expressed, and purified.

Properties of PRK-K53M, PRK-H45N, and PRK-R49Q. Directed mutagenesis of the *Bgl*II/*Bst*EII fragment (Figure 4) which corresponds to bases 503–960 of the *Eco*RI/*Pst*I insert of pKP1565b was employed to eliminate single basic amino acid residues in the highly conserved region corresponding to residues 42–54. PRK-K53M and PRK-H45N were purified to homogeneity (Figure 1; lanes 2 and 3) by the same affinity chromatography procedure employed for the wild-type enzyme (Gibson & Tabita, 1987). Selective elution of the mutant PRKs from the green dye affinity matrix with the substrate ATP suggested that the catalytic sites of these proteins were not grossly perturbed. A similar approach was used to purify PRK-R49Q. In this case, however, a contaminant representing 20–25% of the total protein (Figure 1; lane 4) was coeluted with ATP. The unidentified contaminant did not complicate activity measurements, and calculation of maximal activity was corrected appropriately for protein levels attributable to PRK-R49Q.

Despite the nonconservative nature of the K53M mutation, the kinetic properties of this enzyme are not markedly different from those observed for wild-type protein. K and V_m values (Table II) are within 2-fold of those measured for the wild-type enzyme. In contrast, the H45N substitution results in a mutant PRK with one dramatically altered characteristic. This protein exhibits an approximately 40-fold increase in K_{Ru5P} (4 vs 0.1 mM). The corresponding binding parameter for substrate ATP is not drastically affected (Table II). Measured V_m values for PRK-H45N are reduced severalfold in comparison with values measured for wild-type PRK or PRK-K53M, but these changes are smaller than the effect on K_{Ru5P}. An even larger (200-fold) perturbation is observed in the K_{Ru5P} (20 mM) for PRK-R49Q; as in the case of PRK-H45N, other parameters are only marginally affected. Together, these mutations argue strongly for assignment of this basic region to the enzyme's sugar phosphate substrate binding site.

In eukaryotic PRKs, one of the cysteinyl sulfhydryls involved in regulation of activity by thiol/disulfide exchange is cysteine-55, which has been the focus of investigation involving mutagenesis techniques. In contrast to our observation of substrate binding perturbations with bacterial PRK-H45N and PRK-R49Q, results of mutagenesis work with spinach PRK

[2] The single-letter code for amino acids is used to designate mutants. The first letter indicates the amino acid present in the wild-type enzyme at the numbered position. The final letter indicates the amino acid present at this position in the mutant.

Source	Sequence Position		Reference
R. sphaeroides (form I)	42-54	D A F H R F N R A D M K A	Hallenbeck, 1989
R. sphaeroides (form II)	42-54	D A F H R F N R A D M K A	Gibson et al., 1990
A. eutrophus	42-54	D S F H R Y D R A E M K V	Kossman et al., 1989
X. flavus	42-54	D S F H R Y D R Y E M R E	Meijer et al., 1990
C. reinhardtii	57-69	D D Y H C L D R N G R K V	Roesler and Ogren, 1990
Wheat	57-69	D D Y H S L D R T G R K E	Raines et al, 1989
Spinach	57-69	D D F H S L D R N G R K V	Milanez & Mural, 1988; Roesler & Ogren, 1988

FIGURE 2: Partial sequence comparison of phosphoribulokinases. Shaded regions indicate conserved charged amino acids in this region of comparison. Form I PRK is sensitive to allosteric activation by NADH while form II is reportedly insensitive.

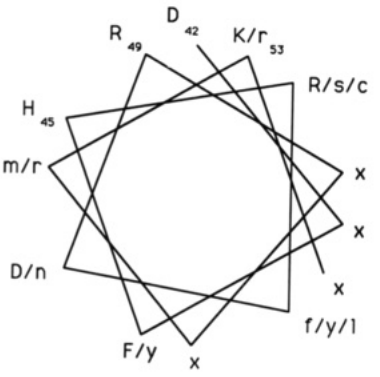

FIGURE 3: Helical wheel model depicting residues 42–54 of *R. sphaeroides* phosphoribulokinase. Positions occupied by residues D42, H45, R49, and K53 are highlighted for emphasis.

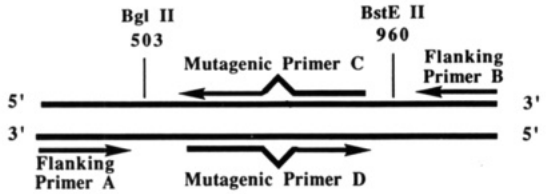

FIGURE 4: Construction of *prk*A mutants. The PCR overlap extension procedure of Ho et al. (1989) was employed to amplify mutagenic duplexes corresponding to bases 503–960 of the 1.7-kb insert of pKP1565b, which encodes *R. sphaeroides* PRK A. Primers A and B, which flank *Bgl*II and *Bst*EII sites, respectively, were used to produce all mutant alleles. Primers C and D represent complementary mutagenic oligonucleotides; appropriate pairs (cf. Table I) were synthesized for production of each mutant allele.

(Milanez et al., 1991), involving a change of the eukaryotic protein's regulatory cysteine-55 to serine or alanine, primarily indicate effects on catalytic efficiency. While regulatory cysteines are not found in the prokaryotic PRK which is the focus of this report, those observations complement our data on sugar phosphate substrate binding by PRK since the combined mutagenesis results considerably refine our understanding of the active-site domains of these heterologous regulated kinases.

The elimination of the basic charge in the sugar phosphate binding region of bacterial PRK does not drastically affect catalysis of phosphoryl transfer. In our earlier speculation concerning assignment to the active-site domain of the lysine corresponding to bacterial PRK's K53, we did not suggest a catalytic function to this residue, but suggested a possible role in substrate binding. The mutagenesis experiments presented here suggest that H45 and R49, basic residues that are likely to be in close spatial proximity to K53, do, in fact, influence substrate binding. The altered binding of the sugar phosphate substrate rather than the nucleotide substrate was unanticipated in view of our original observation that ATP afforded protection from the affinity label that targets this region of PRK (Miziorko et al., 1990). On the basis of our mutagenesis results, we conducted comparable experiments with Ru5P and observed that the sugar phosphate (5 mM) also affords protection against inactivation (4-fold increase in $t_{1/2}$). This observation is compatible with the implication of this region of PRK as part of the Ru5P binding site.

Interpretation of Mutagenesis and Affinity Labeling Results in the Context of the Stereochemical Course of the Phosphoribulokinase Reaction. In retrospect, the observations reported here are quite compatible with the composite body of enzymological and mechanistic data available for phosphoribulokinases. Previously we had shown (Miziorko & Eckstein, 1984) that spinach PRK catalyzes the transfer of the γ-phosphoryl moiety of ATP to the C1 hydroxyl of Ru5P with inversion of stereochemistry. There is no reason to expect that mechanistic features of the reaction catalyzed by the bacterial enzyme are different from those elucidated for the plant or algal enzymes. The observation of inversion of stereochemistry is typically interpreted as implying a single in-line transfer (Knowles, 1980) of the phosphoryl moiety between donor and acceptor molecules. In the case of PRK, this means that, minimally, a ternary complex forms between enzyme, Ru5P, and ATP. Furthermore, the C1 hydroxyl of the sugar phosphate substrate must be capable of juxtaposition with the γ-phosphoryl of ATP. Since the γ-phosphoryl of ATP must approach the bound sugar phosphate, it follows that any ATP analogue, which qualifies as an affinity label by virtue of incorporation of a reactive functionality tethered to the γ-phosphoryl of the nucleoside triphosphate, is capable of positioning the reactive moiety near the sugar phosphate binding site. In the specific case of adenosine triphosphopyridoxal (Miziorko et al., 1990), the targeted lysine is proposed to correspond to bacterial PRK's K53. This residue is one of three basic amino acids that may be oriented on adjacent turns of an amphiphilic helix. The charge cluster represented by such an arrangement would be attractive as a sink for the negatively charged phosphoryl group of Ru5P. In the binary complex formed by PRK and the affinity label, the reactive pyridoxal moiety must find itself juxtaposed with this positively charged region. While several residues in this region of the protein are accessible to the pyridoxal group, which may be viewed as the reactive terminus of a flexible 10-Å chain that is anchored to a bound adenosine, the chemically favored target for covalent modification via Schiff's base formation is the ϵ-amino group contributed by the residue corresponding to K53. On the basis of our mutagenesis data,

this lysine, despite its probable location within the catalytic domain, does not appear to be directly involved in binding the sugar phosphate substrate. Instead, the other basic residues in this region, H45 and R49, are clearly implicated in binding this substrate. In retrospect, the stereochemical information available for PRK has provided us with a valuable perspective as we reconcile the initial identification of this region by protein chemistry experiments involving an ATP analogue with the subsequent implication of this region in sugar phosphate binding.

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Registry No. PRK, 9030-60-8; Ru5P, 551-85-9; histidine, 71-00-1; arginine, 74-79-3.

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