Evidence Supporting Catalytic Roles for Aspartate Residues in Phosphoribulokinase†

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ABSTRACT: The DNA encoding *Rhodobacter sphaeroides* phosphoribulokinase (PRK) has been modified to allow ligation into pET-3d. Using the resulting expression plasmid, PRK was overexpressed in *Escherichia coli* and isolated in milligram quantities. Homogeneous preparations of the enzyme exhibit properties comparable to those of PRK expressed using a previously described pUC19-derived construct [Sandbaken et al., *Biochemistry 31*, 3715–3719]. Mutagenesis experiments have been designed to produce conservative substitutions that eliminate the carboxyl groups of each of four conserved acidic residues (D42, E131, D169, and E178). Using the newly developed expression system, the resulting PRK variants have been expressed, isolated, and characterized. Expression levels and recoveries upon affinity chromatography purification are similar to the results obtained with wild-type PRK. Apparent substrate affinities of these mutant proteins do not differ greatly from values observed for wild-type PRK. In contrast, these PRK variants display a wide range of V_{max} values, ranging from wild-type activity (~200 units/mg; E178A) to levels that are diminished by 4 (D169A) to 5 (D42A, D42N) orders of magnitude. That the large diminutions in catalytic activity are significant and do not merely reflect gross perturbations in protein structure is suggested not only by the modest effects on substrate affinity but also by the allosteric properties of D169A, D42A, and D42N. The activities of these proteins, like that of wild-type PRK, are markedly stimulated by the positive effector NADH. The magnitude of the V_{max} perturbations suggests that D42 and D169 are candidates for the role of active site base or activator cation ligand. In contrast to the marked diminution of V_{max} observed upon mutation of D42 or D169, only a 2 order of magnitude decrease is observed with E131A; much of this effect may be attributed to the fact that this variant no longer is sensitive to allosteric stimulation by NADH.

Phosphoribulokinase (PRK;[1] EC 2.7.1.19) catalyzes the in-line transfer (Miziorko & Eckstein, 1984) of ATP's γ-phosphoryl to the C1 hydroxyl of ribulose 5-phosphate (Ru5P) to form ribulose 1,5-bisphosphate (Hurwitz et al., 1956), the CO_2 acceptor in Calvin's reductive pentose phosphate cycle. Since this reaction is critical to CO_2 assimilation, the enzyme is operative in a wide variety of autotrophic organisms. A consideration of structural and regulatory properties underscores the contrast between prokaryotic and eukaryotic PRK proteins. Most bacterial PRKs exist as octamers of 32-kDa subunits (Siebert & Bowien, 1984; Tabita, 1988) and are subject to allosteric regulation (Rindt & Ohmann, 1969; Abdelal & Schlegel, 1974; Siebert et al., 1981). Plant and algal PRKs are dimers of ~40-kDa subunits (Kagawa, 1982; Porter et al., 1986; Krieger & Miziorko, 1986) and are interconverted between inactive and active species by reversible oxidation/reduction (Buchanan, 1980) of cysteinyl sulfhydryls (Porter et al., 1988; Porter & Hartman, 1990).

While PRKs differ in structural and regulatory properties, the basic elements of the catalytic apparatus are expected to be similar; the available data confirm this expectation. For example, the N-terminal portion of the plant PRK has been affinity labeled by a reactive ATP analog (Krieger & Miziorko, 1986; Krieger et al., 1987); this region is recognized as a consensus ATP binding domain in *both* prokaryotic and eukaryotic PRKs. Similarly, basic amino acids that are conserved in *both* classes of PRKs have been implicated by mutagenesis studies (Sandbaken et al., 1992; Roesler et al., 1992) as elements of the Ru5P binding site. While some success in mapping those regions of PRK that support substrate binding is evident, little progress has been made in identifying amino acid functionalities that are crucial to reaction chemistry. The sulfhydryls that are involved in the regulation of eukaryotic PRK (i.e., Cys-16, Cys-55) have been investigated by mutagenesis approaches (Milanez et al., 1991; Hudson et al., 1992); neither is required for catalysis, although Cys-55 may have a facilitative role. Similarly, Su and Bogorad (1991) identified Ser-222 of *Synechocystis* PRK as the site of a mutation that correlates with a 10-fold diminution in catalytic activity; an effect of such a magnitude does *not* suggest the direct participation of this residue in the chemistry of phosphoryl transfer.

With the objective of facilitating the identification of catalytic residues in PRK, we have developed a plasmid that permits convenient overexpression of PRK. In this report, we describe the utility of a T7 expression system in the production of both wild-type and mutant PRKs that have been engineered to eliminate conserved carboxyl groups. The isolated enzymes have been the focus of studies that implicate two of the conserved acidic amino acids in the chemistry of RuBP formation. A preliminary account of this work has appeared in print (Runquist et al., 1994).

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[1] Abbreviations: PRK, phosphoribulokinase; Ru5P, ribulose 5-phosphate; RuBP, ribulose 1,5-bisphosphate; IPTG, isopropyl thiogalactoside; MCS, multiple cloning site.

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EXPERIMENTAL PROCEDURES

Materials

Deoxyoligonucleotides were synthesized by the Protein/Nucleic Acid Shared Facility at the Medical College of Wisconsin and were purified using C_{18} Sep-Pak cartridges from Waters (a Division of Millipore Corporation). Vent (exo$^-$) DNA polymerase and restriction endonucleases were provided by New England Biolabs and T4 DNA ligase by Promega. Deoxynucleotides were purchased from Pharmacia (Molecular Biology Division). DNA markers were obtained from International Biotechnologies, Inc., a subsidiary of Eastman Kodak Co. For the isolation of DNA fragments, Geneclean and Mermaid kits from Bio101 Inc. were used. All plasmid DNA was isolated using Qiagen's plasmid mini and midi kits. The T7 transcription/expression plasmid, pET-3d, and *Escherichia coli* strain BL21 (DE3) were purchased from Novagen. The Sequenase version 2.0 kit from United States Biochemical Corp. was used for DNA sequence analysis. New England Nuclear/Dupont provided the [α-^{35}S]dATP (1329 Ci/mmol) used for DNA sequencing and the $KH^{14}CO_3$ (40–60 mCi/mmol) used for enzyme assays.

For protein expression, ampicillin and isopropyl β-D-thiogalactoside (IPTG) were purchased from United States Biochemical Corp. Reactive Green-19 agarose and all other biochemicals and enzymes were obtained from Sigma Chemical Co., except for recombinant *Rhodospirillum rubrum* ribulose 1,5-bisphosphate carboxylase, which was generously provided by Drs. G. Lorimer and S. Gutteridge (DuPont).

Methods

Construction of a T7 Expression System for prkA: Generation of the NcoI Restriction Site. A pUC19-derived plasmid pKP1565b, containing the wild-type phosphoribulokinase gene from *Rhodobacter sphaeroides* on a *Pst* fragment inserted at the multiple cloning site (MCS), was generously provided by S. Kaplan (Hallenbeck & Kaplan, 1987). Anticipating the need for an improved protein source for the characterization of PRKs mutated to eliminate functional groups involved in catalysis, we modified the DNA flanking *prkA*'s start codon to create an *Nco*I restriction site that would be convenient for cloning the gene into the T7 transcription/expression region of a pET-3d plasmid (Novagen). A single base change (CCATGA→CCATGG) would have sufficed. However, in making this single change, the second amino acid of PRK would have been altered from a serine to a glycine. In order to make a more conservative substitution (S→A), two bases were changed (CCATGAGC→CCATGGCC). Site-directed mutagenesis using the PCR overlap extension protocol (oePCR) of Ho et al. (1989) generated the desired *Nco*I site. A pair of internal complementary oligonucleotides encoding the desired base changes was used (Table 1). Flanking deoxyoligonucleotide primers (Table 1) complementary to DNA adjacent to the region of interest were also required. The upstream primer was an M13/pUC19 reverse sequencing primer from Operon, and the downstream primer was custom synthesized. The ~732-bp PCR-amplified mutagenic fragment was purified, digested with the restriction enzymes *Hind*III(MCS) and *Bgl*II(*prkA*), repurified, and ligated to a *Hind*III/*Bgl*II-digested pKP1565b from which the corresponding wild-type coding region had been removed (Figure 1, pKP1565). The ligation mixture was used to transform *E. coli* strain JM105, and the plasmid was propagated and purified from six selected transformants. Digestion of the plasmids with the restriction enzyme *Nco*I demonstrated that each contained the newly created site. DNA sequence analysis of the transformants established that no additional nucleotide changes had been generated in the PCR-generated 5′-terminus of the gene (84 bp from ATG to *Bgl*II).

Cloning into pET-3d. For cloning into the Novagen pET-3d T7 expression system, an *Nco*I/*Nco*I fragment of 1061 bases was generated utilizing the newly created *Nco*I site at the start codon and a second *Nco*I site found 180 bp beyond *prkA's* TGA stop codon. The purified fragment was ligated into *Nco*I-digested, purified pET-3d plasmid and the resultant plasmid, (Figure 1, pETbprkwt) was transformed into *E. coli* JM105. Twelve transformants were isolated and digested with *Nco*I. This analysis showed the presence of *prkA* in three transformants; restriction analysis with *Bgl*II confirmed the orientation of the gene relative to the transcription start site. Finally, pETbprkwt carrying *prkA* in the appropriate orientation was transformed into *E. coli* strain BL21(DE3) for expression of the enzyme.

Construction of prkA Mutant Alleles: Overlap Extension PCR Mutagenesis. The mutant alleles of *prkA* encoding the single amino acid substitutions D42A, D42N, and E131A were constructed in pKP1565b by site-directed mutagenesis, using the PCR overlap extension protocol described previously (Sandbaken et al., 1992). For each mutant, a pair of internal complementary oligonucleotides encoding the desired amino acid substitution (Table 1) was used. Sequences of flanking primers required for the overlap extension procedure are also found in Table 1. The PCR-amplified mutagenic fragment of ~0.52-kb was purified, digested with *Bgl*II/*Bst*EII, repurified, and ligated to *Bgl*II/*Bst*EII-digested pKP1565b (Figure 1) from which the corresponding wild-type coding region had been removed. The *Bgl*II–*Bst*EII region generated by oePCR was sequenced in both directions in order to identify those transformants that carried *only* the desired mutation. The efficiency of mutagenesis ranged from 40% to 100%.

Several strategies were used to transfer D42A, D42N, and E131A from pKP1565b into the pET-3d expression system. For the D42A mutation, a purified *Hind*III(MCS)/*Bgl*II-(*prkA*) fragment of about 0.6 kb from pKP1565b carrying the *Nco*I site at the 5′-terminus of *prkA* was ligated to the similarly digested, purified 3.7-kb fragment from pKP1565b-D42A (Figure 1) carrying most of the *prkA* gene. Next, the *Nco*I/*Nco*I fragment recovered after the above ligation and carrying the entire *prkA* gene was combined with *Nco*I-digested pET-3d. Clones carrying an additional 1.1-kb DNA insert relative to the parent pET-3d plasmid were identified by a Quick Screen Stratagene protocol, and efficiencies were found to be in the 1:30 to 1:50 range. Restriction analysis of isolated plasmids verified the integrity of the gene and established its orientation.

For transfer of the DNA sequence encoding D42N and E131A into the T7 expression system, a 0.43-kb *Pvu*I/*Bst*EII fragment encoding either D42N or E131A was isolated from pKP1565b. A *Pst*I(pET-3d)/*Pvu*I(*prkA*) fragment of about 3.5 kb carrying the 5′-terminus of *prkA* was isolated from pETbprkwt (Figure 1). Finally, a *Pst*I(pET-3d)/*Bst*EII-(*prkA*) fragment of about 1.8 kb encoding the 3′-terminus of *prkA* was isolated from pETbprkwt. After ligation of these three pieces and transformation into JM105, numerous transformants were generated. DNA sequence analysis and restriction enzyme digestion verified that all transformants isolated contained the desired constructs in the proper orientation.

Table 1: Mutant Oligonucleotides

Enzyme	Oligonucleotides for Overlap Extension PCR	
	1 2 3 4 5 M S K K H	
Wild Type	GA ATA TCC ATG AGC AAG AAG CAT	5′-CAGCTATGACCATGATTACG (upstream primer)
1. S2A	GA ATA TCC ATG **GC**C AAG AAG CAT	5′-GAAGTGCGAGAAGGTGG (downstream primer)
	40 41 42 43 44 E G D A F	
Wild Type	C GAG GGC GAC GCC TTC C	5′-CCTCGACGGTGAAGCAC (upstream primer)
2. D42A	C GAG GGC G**C**C GCC TTC C	5′-AGCATGGCATCCGGCGC (downstream primer)
3. D42N	C GAG GGC **A**AC GCC TTC C	
	129 130 131 132 133 134 F Y E G L H	
Wild Type	TG TTC TAC GAG GGG TTG CA	(primers the same as for 2 & 3)
4. E131A	TG TTC TAC G**C**G GGG TTG CA	

Oligonucleotides for Mutagenic Cassettes

		163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179
		I Q K I H R D R A T R G Y T T E A
5.	D169A	G ATC CAG AAG ATC CAC CGC G**C**C CGC GCG ACC CGC GGT TAC ACG ACC GAG GCG
		GTC TTC TAG GTG GCG CGG GCG CGC TGG GCG CCA ATG TGC TGG CTC CGC CAG TG
6.	E178A	G ATC CAG AAG ATC CAC CGC GAC CGC GCG ACC CGC GGT TAC ACG ACC G**C**G GCG
		GTC TTC TAG GTG GCG CTG GCG CGC TGG GCG CCA ATG TGC TGG CGC CGC CAG TG

Cassette Mutagenesis. The mutant alleles of *prkA* encoding the single amino acid substitutions D169A and E178A were constructed in pETbprkwt using cassette mutagenesis and a three-way ligation strategy. First a *Pst*I(pET-3d)/*Bam*HI-(*prkA*) fragment of about 3.8 kb, including the 5′-end of the prkA gene, was isolated from pETbrpkwt (Figure 1). Second, a *Pst*I(pET3d)/*Bst*EII(*prkA*) fragment of about 1.8 kb, including the 3′-end of the gene, was isolated from a similar plasmid. This left about 60 bp of the *prkA* gene that could be replaced by the cassettes documented in Table 1. Six transformants were isolated for each mutation. Restriction cutting of the isolated plasmids verified the overall integrity of the constructs. DNA sequence analysis confirmed that the expected sequence was found in all six transformants.

Bacterial Growth. E. coli BL21 cultures (250–500 mL) containing the appropriate pET-3d plasmids were grown in ampicillin-containing LB with shaking at 25 °C to an OD_{600} between 0.5 and 0.8. Expression of PRK was induced by the addition of IPTG to a final concentration of 1 mM, followed by 3–4 h of growth at 25 °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). For preparation of the enzymes used in kinetic characterization experiments, 250 mL of induced bacterial cultures sufficed as the starting material. Cell pellets were disrupted using a French pressure cell. A 100000*g* supernatant was prepared and dialyzed twice against 4 L of 25 mM Tris Cl, pH 8.2, containing 10 mM β-mercaptoethanol and 1 mM EDTA. The dialysate was loaded onto a 0.7 × 5.0 cm affinity column of agarose–Reactive Green 19 equilibrated with the same buffer specified for dialysis. Affinity elution was performed using buffer supplemented with 10 mM ATP. Protein was determined by the method of Bradford (1976), with bovine serum albumin used 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. For a more precise kinetic characterization of PRK, product formation was estimated by a radioisotope assay, which employs RuBP carboxylase to incorporate $^{14}CO_2$ into RuBP to form acid-stable [^{14}C]-3-phosphoglycerate (Paulsen & Lane, 1966). In standard assays, the final concentrations of components in the reaction were 100 mM Hepes, pH 8.0, 1 mM DTT, 20 mM $MgCl_2$, 20 mM $KH^{14}CO_3$ (1000 dpm/nmol), 300 munits of bacterial RuBP carboxylase, 1 mM NADH, and unless

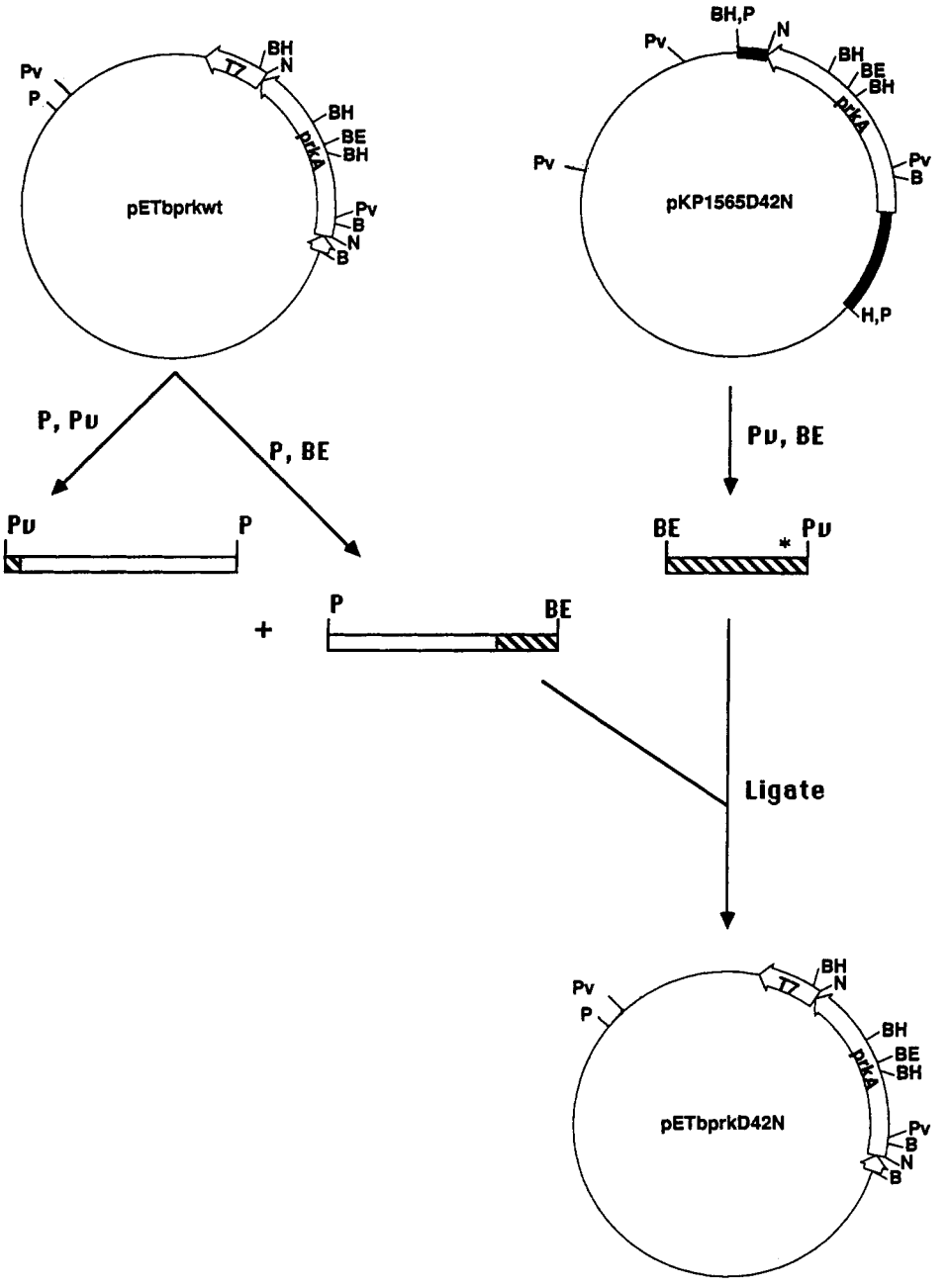

FIGURE 1: Plasmids utilized for T7-based expression of wild-type and mutant phosphoribulokinase proteins. In the pKP1565 series of plasmids (Hallenbeck & Kaplan, 1987), the black bands indicate *R. sphaeroides* genomic DNA that flanks the *prkA* gene. The crosshatching indicates the sections of *prkA* contained in each restriction fragment used in the three-way ligation: B, *Bgl*II; BE, *Bst*EII; BH, *Bam*HI; H, *Hind*III; N, *Nco*I; P, *Pst*I; Pv, *Pvu*I.

otherwise indicated, 5 mM ATP and 2.5 mM Ru5P. Estimates of the magnitude of allosteric stimulation by NADH were performed using NADH levels optimized for each PRK mutant. For the kinetic characterization of the mutant proteins, ATP concentration ranges varied from 0.05 to 6.4 mM; Ru5P concentration ranges varied from 0.012 to 1.6 mM. Kinetic data were fit by a nonlinear regression analysis algorithm (Marquardt, 1963).

RESULTS

Expression and Isolation of R. sphaeroides Phosphoribulokinase. For routine isolation of wild-type and mutant PRKs at levels required for the characterization documented in this report, it proved sufficient to start with 250 mL of an IPTG-induced culture of freshly transformed *E. coli* BL21. SDS gels of unfractionated cell extracts displayed an intense 32-kDa PRK band that dominates the array of endogenous *E. coli* proteins that individually are difficult to visualize due to their low relative abundance. Densitometry analysis suggested that PRK represents about 28% of total cellular protein and approximately 21% of the protein recovered in the 100000*g* supernatant (Figure 2, lanes 2 and 3). The specific activity of PRK in the 100000*g* supernatant is, however, only 10% of that measured with the homogeneous enzyme, raising the possibility that there is some inactive PRK protein in the high-speed supernatant. Thus, even though the expression of protein at 25 °C minimizes the production of insoluble PRK, the expressed material includes some sedimentable enzyme and possibly some inactive soluble enzyme that is subsequently resolved from active PRK upon affinity purification. The enhanced expression level supported by pETbprkwt facilitates the use of the convenient continuous

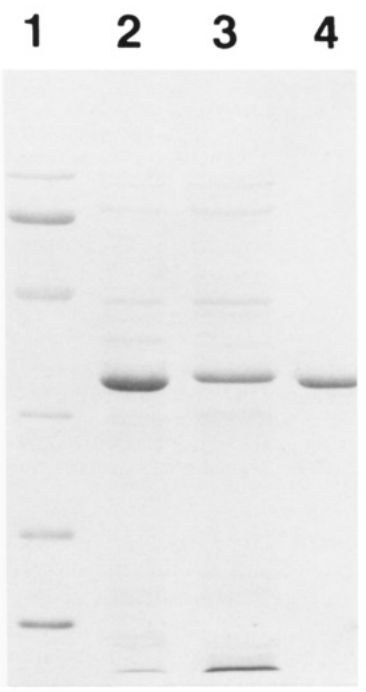

FIGURE 2: SDS–PAGE of recombinant *R. sphaeroides* phosphoribulokinase at various stages of purification. Lane 1 contains molecular weight standards, including phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Lanes 2–4 contain crude bacterial cell extract, the supernatant from centrifugation at 100000*g* for 1 h, and affinity-purified (Reactive Green-19–agarose) PRK, respectively.

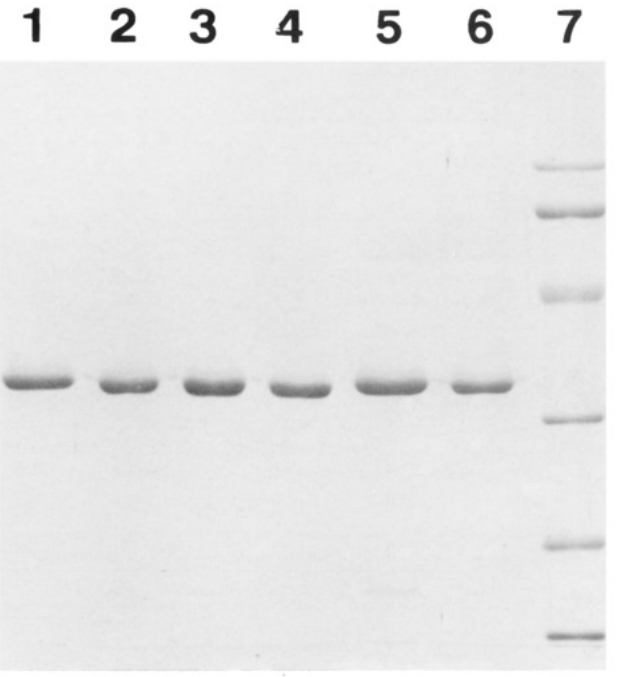

FIGURE 3: SDS–PAGE of wild-type and mutant PRKs. Lanes 1–6 contain purified wild-type PRK, PRK-D42A, PRK-D42N, PRK-D169A, PRK-E131A, and PRK-E178A, respectively. Lane 7 contains the same molecular weight standards described in the legend to Figure 2.

spectrophotometric PRK assay, despite the low, endogenous ATPase activity in the bacterial extracts. Certainly, in extracts containing wild-type PRK, as well as in mutants that possess significant activity, background corrections due to ATPase are minimal. From the supernatant recovered from 250 mL of bacterial culture, one affinity chromatography step involving ATP elution of an agarose–Reactive Green 19 resin suffices for the recovery of approximately 2 mg of homogeneous protein (Figure 2). Insertion of an additional chromatographic step facilitates scaled up preparations that allow the isolation of larger amounts of protein. The high-speed supernatant from a 1.5-L bacterial cell culture is subjected to Q-Sepharose anion exchange chromatography (1.5 × 30 cm column; 0–0.6 M KCl gradient in 25 mM Tris Cl, pH 8.0, containing 10 mM β-mercaptoethanol and 1 mM EDTA). This step affords an adequate reduction in protein contaminants, so that all of the eluted PRK can be loaded onto a 7-mL affinity column. The recovery of over 15 mg of homogeneous PRK is typical from preparations of this scale.

Similar methodology is used to isolate PRK variants (D42A, D42N, E131A, D169A, and E178A). These proteins are expressed at levels comparable to those of the wild-type enzyme and are eluted from an affinity resin in yields and purities (Figure 3) comparable to those experienced with wild-type PRK. While all of the *R. sphaeroides* PRK proteins produced using the pET construct contain alanine instead of serine at position 2 in the primary sequence, this substitution appears to be inconsequential. Such an observation is not unexpected; while serine 2 is conserved in prokaryotic PRKs, the mature forms of eukaryotic PRKs show considerable heterogeneity at this position. pET-expressed wild-type PRK containing S2A is indistinguishable from the recombinant enzyme produced using a pUC-derived plasmid (Hallenbeck & Kaplan, 1987; Sandbaken et al., 1992) in terms of V_m, K_{ATP}, K_{Ru5P}, allosteric activation, or any other parameter (*vide infra*) examined to date.

Strategy in the Design of PRK Mutants. pH–rate profiles published for both prokaryotic (Hart & Gibson, 1971) and eukaryotic (Hurwitz et al., 1956; Roesler et al., 1992) PRKs are compatible with the hypothesis that activity is dependent upon the deprotonation of an acidic amino acid. A survey of related kinases for which high-resolution structural data are available, e.g., phosphofructokinase (Hellinga & Evans, 1987), hexokinase (Anderson et al., 1978), and glycerol kinase (Hurley et al., 1993), indicates good precedent for acidic amino acids supporting the chemistry of phosphoryl transfer either by functioning as a general base or by liganding the cation–ATP substrate complex. *R. sphaeroides* PRK activity is eliminated upon incubation of the enzyme with millimolar levels of water-soluble carbodiimides (e.g., EDC, ethyl-[(dimethylamino)propyl]carbodiimide; H. Charlier, unpublished observations). However, relatively poor protection against inactivation is afforded by substrate, suggesting that this group specific reagent may modify PRK at multiple sites. In such a case, detailed structural analysis of the inactivated protein probably would not be productive. As an alternative, we utilized the wealth of primary structure information that has become available for a variety of prokaryotic and eukaryotic PRKs. Different sequence alignment analyses confirm the homology predictions of Kossmann et al. (1989) and identify four acidic amino acids that are invariably indicated as being conserved in the PRKs for which sequence data are available (Figure 4). Thus, D42, E131, D169, and E178 were targeted for conservative substitution by alanine. In the case of D42, the carboxylate was also replaced by an amide functionality.

Properties of the PRK Mutants D42A, D42N, E131A, D169A, and E178A. The levels of PRK activity in extracts of bacteria used for expression of the various PRK mutants suggested that there was some validity in the strategy outlined above. The expression levels of the mutants reflected

FIGURE 4: Locations of mutated aspartic and glutamic acids. The upper numbers designate the location in the mature spinach protein, and the lower numbers designate the location in the *R. sphaeroides* protein. The residues indicated with a star were mutated previously (Sandbaken et al., 1992). The shaded columns identify invariant residues. Deduced sequences have been reported by the following: [a]Milanez & Mural, 1988; Roesler & Ogren, 1988; [b]Horsnell & Raines, 1991; [c]Raines et al., 1989; [d]Michalowski et al., 1992; [e]Roesler & Ogren, 1990; [f]Su & Bogorad, 1991; [g]Gibson et al., 1991 (*prkA*); [h]Gibson et al., 1990 (*prkB*); [i]Kossman et al., 1989 (prk_c and prk_p sequences are listed); [j]Meijer et al., 1990.

Table 2: Kinetic Characterization of *R. sphaeroides* PRK Mutants[a]

PRK	V_{max} (units/mg)	K_{Ru5P} (μM)	K_{ATP} (mM)
WT	203 ± 11	99.5 ± 17	0.55 ± 0.16
D42A	0.0021 ± 0.00032	275 ± 47	1.93 ± 0.6
D42N	0.00140 ± 0.00005	107 ± 13	0.87 ± 0.13
D169A	0.026 ± 0.004	680 ± 135	1.3 ± 0.48
E131A	0.99 ± 0.032	570 ± 54	0.16 ± 0.043
E178A	211 ± 3	113 ± 24	0.22 ± 0.087

[a] Kinetic measurements employed the radiochemical assay, performed at 30 °C under the conditions described in Methods. Half-saturation values are reported for ATP concentration dependence, following the conventions established in previous reports on bacterial PRKs. K_m values are reported for Ru5P.

expectations based on wild-type PRK behavior; purification of the mutants (Figure 3) was accomplished without any modification of the protocol developed for wild-type enzyme. The ability to successfully use the same affinity purification protocol (ATP elution) for each of a series of PRKs that varied drastically in catalytic activity was encouraging, as it suggested that the tertiary structure of the PRKs did not differ appreciably from that of the wild-type enzyme. Detailed kinetic analysis of the PRK variants (Table 2) suggested that the conservative substitutions that were engineered resulted in proteins with, at most, modest perturbations in apparent substrate affinity. Replacement of the aspartate at positions 42 or 169 by alanine weakens the apparent ATP affinity by less than 4-fold. A more isosteric replacement of aspartate 42 by asparagine results in a protein with very little change in ATP affinity. A modest increase (<4-fold) in apparent ATP affinity is observed for E131A and E178A. In the context of Ru5P affinity, there is, again, little difference between wild-type PRK and D42N, nor does E178A show any significant perturbation. The other mutants exhibit weaker interactions with Ru5P, with the 6-fold perturbations exhibited by D169A and E131A being the most notable.

In contrast to the relatively modest changes in apparent substrate binding parameters, analysis of kinetic data, generated using recombinant ribulose bisphosphate carboxylase as a coupling enzyme in the sensitive radioactive assay, indicated that large changes in V_{max} characterize three of the five PRK mutants. Two of these proteins no longer contain a negatively charged group at residue 42. Both D42A and D42N exhibit a 10^5-fold diminution in activity (Table 2). This effect appears to be significant, especially in the case of D42N, which displays apparent substrate affinities that are virtually indistinguishable from that of the wild-type enzyme. The perturbation in the activity of D169A is also notable, representing a 10^4-fold diminution from wild-type PRK activity. The modest weakening in substrate affinity for this enzyme variant does not preclude accurate V_{max} determinations, and thus, the reduction in activity must also be regarded as significant. In contrast, the carboxyl group of E178 appears to be uninvolved in the chemistry of RuBP formation. The 2 orders of magnitude effect produced upon elimination of the carboxyl of E131 is certainly appreciable and may be rationalized in the context of the additional characterization data presented below.

The diminution in activity apparent upon characterization of D42A, D42N, and D169A does *not* appear to be attributable to gross structural perturbations. The expression levels of these mutant proteins are not appreciably different from those of wild-type PRK, and affinity chromatography involving elution with substrate ATP affords isolation with efficiencies comparable to those achieved in preparations of wild-type PRK. The prokaryotic PRKs are allosterically stimulated by NADH, and this trait offers another test of conformational integrity. As shown in Table 3, the activity of the wild-type enzyme, measured at saturating substrate concentrations, is stimulated over 30-fold in the presence of NADH. PRK mutants exhibiting the largest diminution in V_{max} retain the ability to respond to this allosteric activator. In the case of D169A, which exhibits a 10^4-fold depression in activity under optimal (NADH-supplemented) assay conditions, it is possible to accurately measure activity in the absence of NADH; almost 30-fold stimulation by the effector is observed, approaching the effect reported for wild-type enzyme. In the case of D42A and D42N, the experiment becomes technically more difficult due to the 10^5-fold drop in activity, even when NADH is present. Little activity over background levels is measurable in the absence of NADH; the approximate 15-fold stimulation of activity measured in the presence of NADH must thus be regarded as a lower limit of the intrinsic effect. On this basis, these mutant PRKs are judged to retain sensitivity to allosteric stimulation that reflects conformational integrity. These observations enhance the argument for the significance of the observations of diminished catalytic activity.

While the allosteric stimulation by NADH observed for E178A is 4-fold lower than that observed with wild-type PRK, this mutant retains good catalytic efficiency (Table 3). In comparison with the other observations of NADH stimulation, the lack of response exhibited by E131A is striking. It would appear that a significant contribution to the 10^2-fold diminution in catalytic activity can be ascribed to the protein's insensitivity to allosteric stimulation, suggesting that glutamate 131 is not crucial to catalysis. Interestingly, this glutamate is the first residue in a stretch of four conserved amino acids (Figure 4).

Table 3: Allosteric Stimulation of Phosphoribulokinases by NADH

PRK species	-fold stimulation
WT	37.5
D42A	>17.5[a]
D42N	>13.5[a]
D169A	29.0
E131A	0.0
E178A	8.6

[a] Due to the low catalytic activity of this mutant PRK under optimal conditions, activity measurements in the absence of the allosteric activator reflect background levels, and thus, the estimate of allosteric stimulation must be regarded as a lower limit of the intrinsic effect.

The H135N variant has also been expressed, isolated, and partially characterized (M. Sandbaken, unpublished); this enzyme approaches wild-type PRK in its characteristics. The structural consequences of the E131A mutation and their correlation with the lack of allosteric activation remain a subject for future study, awaiting the development of appropriate physical probes. Such investigation may benefit by the parallel study of another engineered mutant that exhibits, in the *absence* of NADH activator, catalytic activity approaching wild-type levels (J. A. Runquist, unpublished observations).

DISCUSSION

Assignments of function to enzyme residues based on the kinetic characterization of mutant proteins have been made for a variety of enzyme-catalyzed reactions. In the case of kinase reactions, there is clear precedent for assigning roles to acidic amino acids in either liganding the activator cation or deprotonating the phosphoryl acceptor. A survey of several kinases in which specific acidic amino acids are known to be involved in one or the other of these functions reveals trends in the magnitude of V_{max} effects caused by elimination of the carboxylate side chain. Consideration of these precedents provides some perspective in interpreting the large observed diminution in PRK's V_{max} upon replacement of aspartate 42 or aspartate 169 with nonacidic residues. In the case of the homologous phosphoryl transfer reaction catalyzed by phosphofructokinase, Hellinga and Evans (1987) have demonstrated a 2×10^4-fold diminution in V_{max} upon elimination of aspartate 127 in the *E. coli* enzyme. Green et al. (1993) observed a $\sim 10^5$-fold effect in a similar experiment on the analogous aspartate in the pyrophosphate-dependent *P. freundreichii* enzyme. When the acidic residues that have been implicated in cation liganding were replaced in those phosphofructokinases, the observed effects on V_{max} ranged from 10^2- to 10^3-fold (Berger & Evans, 1992; Green et al., 1993). These more modest effects on V_{max} are comparable in magnitude to the effects ($\sim 10^3$-fold) observed by Chen et al. (1993) upon elimination of cation ligands in porcine fructose bisphosphatase (Zhang et al., 1993). In all of these cases, perturbations in the K_m's were modest in comparison with the observed diminution in catalytic efficiency.

The elimination of PRK's aspartate 42 carboxyl correlates with a 10^5-fold reduction in V_{max}, while the parallel experiment on aspartate 169 produces an effect that is 1 order of magnitude smaller. The modest changes in substrate affinity of these mutant proteins, coupled with the observation that they retain sensitivity to PRK's allosteric activator, NADH, suggest that these enzymes retain substantial structural integrity and that the observed changes in catalytic efficiency are significant. The magnitude of the effect produced upon mutagenesis of aspartate 42 is compatible with its assignment as the catalytic base that deprotonates the C1 hydroxyl of Ru5P. Only 1 order of magnitude distinguishes the effects observed for aspartate 42 from those measured upon substitution of aspartate 169. Thus, the candidacy of aspartate-169 as the active site base should not be dismissed, although a role for this residue in liganding the divalent cation may represent an attractive alternative.

The selection of acidic residues that were mutated in these experiments is based on a consensus set that emerged on the basis of sequence alignment. Different sequence sets and different alignment algorithms might indicate other choices that could ultimately qualify as candidates for participation in the chemistry of Ru5P formation. Some caution is also warranted in assigning key catalytic roles to specific residues primarily on the basis of the observation that their substitution by mutagenesis correlates with dramatic rate reductions. Recent work with aconitase (Zheng et al., 1992) identified both aspartate and serine residues as sites where mutagenesis correlated with $\sim 10^5$-fold diminution in activity. Only detailed structural analysis of the protein (Lauble et al., 1992) allowed discrimination between these candidates and the ultimate assignment of serine as the catalytic base. Nonetheless, there is some self-consistency between data in this report and the results of earlier work (Sandbaken et al., 1992) that supports the candidacy of aspartate 42 as PRK's catalytic base. In reporting the importance of histidine 45 and arginine 49 in stabilizing the binding of PRK's sugar phosphate substrate, we speculated that these conserved basic residues might be aligned on adjacent turns of an amphiphilic α-helix. If this prediction of secondary structure were correct, aspartate 42 would also be positioned on the polar face of the helix, approximately aligned with, but two turns (~ 10 Å) removed from, arginine 49. This distance is compatible with the space between Ru5P's C1 hydroxyl and C5 phosphoryl. Thus, the hypothesis that aspartate 42 would be appropriately positioned to function in the abstraction of a proton from the C1 hydroxyl of an Ru5P that interacts with histidine 45 and arginine 49 through its phosphoryl group does not seem unreasonable. However, similar secondary structure analyses of the corresponding regions of eukaryotic PRKs do not argue strongly for α-helicity, so this rationale must be considered as speculative. At present, there is no reason to discount the possibility that other residues far removed in the primary sequence may also be properly juxtaposed, on the basis of the native PRK tertiary structure, to qualify for the assignment as catalytic base. There are, however, *no* data currently available that implicate a candidate residue better qualified for such a role than the acidic residues identified in this report.

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