Functional contribution of a conserved, mobile loop histidine of phosphoribulokinase

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Abstract

In the *Rhodobacter sphaeroides* phosphoribulokinase (PRK) structure, there are several disordered regions, including a loop containing invariant residues Y98 and H100. The functional importance of these residues has been unclear. PRK is inactivated by diethyl pyrocarbonate (DEPC) and protected by the substrates ATP and Ru5P, as well as by the competitive inhibitor, 6-phosphogluconate, suggesting active site histidine residue(s). PRK contains only three invariant histidines: H45, H100, and H134. Previous mutagenesis studies discount significant function for H134, but implicate H45 in Ru5P binding. PRK mutant H45N is inactivated by DEPC, implicating a second active site histidine. To evaluate the function of H100, as well as another invariant loop residue Y98, PRK mutants Y98L, H100A, H100N, and H100Q were characterized. Mutant PRK binding stoichiometries for the fluorescent alternative substrate, trinitrophenyl-ATP, as well as the allosteric activator, NADH, are comparable to wild-type PRK values, suggesting intact effector and substrate binding sites. The K_{mRu5P} for the H100 mutants shows modest eight- to 14-fold inflation effects, whereas Y98L exhibits a 40-fold inflation for K_{mRu5P} . However, Y98L's K_i for the competitive inhibitor 6-phosphogluconate is close to that of wild-type PRK. These observations suggest that Y98 and H100 are not essential Ru5P binding determinants. The V_m of Y98L is diminished 27-fold compared with wild-type PRK. In contrast, H100A, H100N, and H100Q exhibit significant decreases in V_m of 2600-, 2300-, and 735-fold, respectively. Results suggest that the mobile region containing Y98 and H100 must contribute to PRK's active site. Moreover, H100's imidazole significantly influences catalytic efficiency.

Keywords: phosphoribulokinase; phosphotransferases; Calvin cycle; site-directed mutagenesis; histidine

Phosphoribulokinase (PRK; EC 2.7.1.19) catalyzes the formation of ribulose 1,5-bisphosphate (Hurwitz et al. 1956) by the in-line attack (Miziorko and Eckstein

1984) of ribulose 5-phosphate (Ru5P) on ATP. This reaction is crucial to Calvin's reductive pentose pathway for CO₂ assimilation, and, thus, PRK is found in a variety of prokaryotic and eukaryotic organisms. There are significant differences between plant/algal and bacterial PRK proteins (Tabita 1988; Miziorko 2000). For example, the plant enzymes are regulated by a thio-redoxin-mediated thiol/disulfide exchange (Buchanan 1980; Porter et al. 1986). In contrast, many bacterial PRK enzymes are allosterically regulated. Plant and algal PRKs exhibit a subunit mass of ≈ 45 kDa, while bacterial PRK subunits are considerably smaller (≈ 33 kDa). Additionally, amino acid sequences are very different. Only $\approx 13\%$ of total PRK residues are invariant.

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Abbreviations: DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; IPTG, isopropyl β -D-1-thiogalactopyranoside; PRK, phosphoribulokinase; Ru5P, ribulose 5-phosphate; RuBP, ribulose 1,5-bisphosphate; 6-PG, 6-phosphogluconate; TNP-ATP, 2'(3')-O-(2,4,6-trinitrophenol) adenosine 5'-triphosphate; NADH, β -nicotinamide adenine dinucleotide reduced form.

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These differences underscore the potential importance of those residues conserved between plants, algal, and bacterial PRK proteins.

A high-resolution structure of the protein is available only for prokaryotic (*Rhodobacter sphaeroides*) PRK A. Structures of unliganded PRK (Harrison et al. 1998) and PRK with a nucleotide bound in the regulatory site (Kung et al. 1999) have added to our insight into some aspects of catalytic function. Since PRK's protein fold indicates it to be a member of the nucleoside monophosphate kinase family of enzymes, even the structure of the open, unliganded form of PRK allows components of the ATP binding site to be assigned. Recognition of Walker A and Walker B motifs in the PRK structure confirms the ATP-Mg²⁺ binding site assignment and has led to the identification of functionally important residues (S19 and E131) in each of these ATP binding motifs (Charlier et al. 1994; Runquist et al. 2001).

Mutagenesis experiments identified conserved residues H45 and R49 as important Ru5P binding determinants (Sandbaken et al. 1992). D42, situated at the edge of the β sheet core domain of this enzyme, has been assigned as the general base catalyst that deprotonates the C1 alcohol of Ru5P prior to attack on ATP (Charlier et al. 1994). Located in the "lid" domain, which is not closely juxtaposed to the β sheet core in the unliganded PRK structure (Harrison et al. 1998), are conserved residues D169 and K165. Mutagenesis of these residues to eliminate side chain charge decreases catalytic rates by 10⁴- and 10³-fold, respectively (Charlier et al. 1994; Runquist et al. 1999). These residues are expected to become more closely juxtaposed upon PRK binding of substrates, since movement of "lid" domains of nucleoside monophosphate kinases toward the β sheet core is well preceded (Schlauderer et al. 1996).

Several dynamic regions are not well defined in the published PRK structure. Thus, limited functional information can be deduced without pursuit of more direct investigation of the protein. Included among these dynamic regions of PRK is the loop containing the conserved residues Y98 and H100. Protein modification data, included in this report, suggest that investigation of the function of invariant loop residues H100 and Y98 would be worthwhile. PRK has been mutated at these positions, and the mutant proteins have been isolated and characterized, allowing evaluation of the contribution of this loop as part of the PRK active site.

Results

Diethylpyrocarbonate inactivation of phosphoribulokinase

The group selective modification reagent diethylpyrocarbonate modifies histidine residues (Miles 1977) and

Figure 1. DEPC inactivation of wild-type phosphoribulokinase. Reaction mixtures (100 μ L) were prepared containing 50 mM HEPES buffer (pH 7.5), 10 mM MgCl₂, 1.3 μ M PRK, and 0 DEPC (■), 40 μ M DEPC (●), 40 μ M DEPC + 1 mM ATP (▲), 40 μ M DEPC + 1 mM Ru5P (▼), or 40 μ M DEPC + 1 mM ATP + 1 mM phosphogluconate (□). Incubations were initiated by DEPC addition. Aliquots were withdrawn at 10-min intervals, and enzyme activity was determined by a spectrophotometric assay.

inactivates PRK in a time-dependent fashion (Fig. 1). Protection against inactivation is afforded by either of the substrates ATP or ribulose 5-phosphate, or by the competitive inhibitor 6-phosphogluconate (Gardemann et al. 1983; Runquist et al. 1999). These observations are compatible with the contribution of a histidine residue to PRK function. Only three invariant histidines exist in PRK proteins; in the *R. sphaeroides* enzyme these correspond to H45, H100, and H134. Previous mutagenesis work (Sandbaken et al. 1992) has demonstrated that H45 influences ribulose 5-phosphate binding, but H134 has little effect on PRK function. When the H45N mutant was subjected to diethylpyrocarbonate treatment, the enzyme retained sensitivity to the reagent, but, as observed with wild-type enzyme, inactivation was retarded by the presence of substrates (ATP or ribulose 5-phosphate) or the competitive inhibitor, phosphogluconate (Table 1). The results suggested that invariant H100 (Fig. 2) might

Table 1. DEPC inactivation of wild-type PRK and the histidine mutant H45N

Incubation mix components	Residual activity (wild-type PRK)	Residual activity (H45N)
Control (minus DEPC)	100%	100%
DEPC	27%	33%
DEPC + 1 mM Ru5P	63%	50%
DEPC + 1 mM ATP	66%	61%
DEPC + 1 mM ATP + 1 mM phosphogluconate	78%	71%

Reaction mixtures (100 μ L) were prepared containing 50 mM HEPES buffer (pH 7.5) and 10 mM MgCl₂. The enzyme concentrations were 1.3 μ M for PRK and 3.5 μ M for H45N. Incubations were initiated by addition of DEPC (40 μ M for wild-type PRK; 80 μ M for H45N). The residual enzyme activity reflects the measurements after 30-min incubations.

	98	100
rsprka	QGRTRT. YVH	DDAEAARTGV
aepark	TGMHRH. YLH	SPEEAAPFGQ
rcapsprk	KGRTRR. YVH	DANESAKYGV
nitroprk	TGNTRY. YVH	DDAESALHGV
xflavusprk	SGRFRH. YVH	DAGEAKLYNT
sinomelilotiprk	VGRTRH. YVH	DDAEAVKFGS
mescrprk	KAVEKPI YNH	VTGL
peaprak	KSVQKPI YNH	VTGL
bvulgarisprk	KPVDKPI YNH	VSGL
spprk	KAVDKPI YNH	VSGL
whtrprk	KAIEKPI YNH	VTGL
arabprk	IAVEKPI YNH	VTGL
cprk	KSVDKPI YNH	VSGL
syprk	QSIMKPI YNH	ETGL
vaucheriaprak	ESVMKPI YNH	VNGT
odontellaprak	ESIEKPI YNH	VNGT

Figure 2. Alignment of prokaryotic and eukaryotic PRK deduced amino acid sequences showing the conservation of residues Y98 and H100. The invariant Y98 and H100 are shown in boldface font. Sixteen representative prokaryotic or eukaryotic sequences are shown, corresponding to PRKs from *Rhodospseudomonas sphaeroides*, *Alcaligenes eutrophus*, *R. capsulata*, *Nitrobacter vulgaris*, *Xanthobacter flavus*, *Sinorhizobium meliloti*, ice plant, pea, *Beta vulgaris*, spinach, wheat, *Arabidopsis*, *Chlamydomonas*, *Synechocystis*, *Vaucheria litoris*, and *Odontella sinensis*. Residue numbering appearing above the sequence alignments corresponds to the *R. sphaeroides* enzyme.

be sensitive to the reagent and prompted a mutagenesis approach to evaluate the function of H100 and another invariant residue (Y98) that is situated near H100 in a highly mobile PRK loop.

Structural characterization of PRK mutants Y98L, H100A, H100N, and H100Q

PRK mutants Y98L, H100A, H100N, and H100Q were expressed and purified at levels and homogeneity comparable to wild-type enzyme (Fig. 3). To ensure that the mutant PRK proteins retain substantial structural integrity (and therefore are useful for detailed character-

Figure 3. SDS-PAGE of wild-type and mutant *R. sphaeroides* PRKs. Lanes 1–5 contain H100A, H100N, H100Q, Y98L, and wild-type PRKs, respectively. Lane 6 contains molecular mass markers phosphorylase, 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.

Figure 4. Fluorescence titration of mutant PRK H100A with TNP-ATP. Sequential additions of TNP-ATP were made to a fluorescence cuvette containing 4.0 μ M H100A in 25 mM Tris (pH 8.0). The relative fluorescence enhancement is plotted versus the ratio of TNP-ATP added to the PRK mutant present in the cuvette. Binding stoichiometry is estimated from the intersection point of lines fit to the low and high occupancy regions of the titration by linear regression analysis.

ization), the binding of a fluorescent alternative substrate, TNP-ATP (Runquist et al. 1996), and the allosteric activator, NADH, were evaluated. *R. sphaeroides* PRK forms stable binary complexes with ATP and ATP analogs that dissociate only after prolonged dialysis (Runquist et al. 1996). Figure 4 shows the TNP-ATP titration of the PRK mutant, H100A. Tight, stoichiometric nonequilibrium binding is observed (1.18 per PRK subunit), an observation that is comparable to results for wild-type enzyme (1.05 per subunit). Table 2 documents that each of the Y98 and H100 mutants retains a full complement of functional substrate sites (1.05–1.18 per subunit), as well as allosteric activator (NADH) sites (0.92–1.25 per subunit). The results suggest that mutant enzyme tertiary structure is not substantially impaired. Based on these results, the data from kinetic characterization of these PRK mutants can be interpreted without concerns that protein structure has been seriously compromised.

Table 2. Substrate and effector binding by PRK mutants

Enzyme	$n_{\text{TNP-ATP}}^a$	n_{NADH}^a
Wild-type	1.05 ± 0.05	1.04 ± 0.04
Y98L	1.07 ± 0.08	0.93 ± 0.08
H100A	1.18 ± 0.10	1.25 ± 0.20
H100N	1.05 ± 0.05	0.92 ± 0.08
H100Q	1.05 ± 0.05	1.05 ± 0.05

^a n denotes the calculated number of binding sites per enzyme subunit. Stoichiometries for TNP-ATP and NADH binding were determined from the intersection point of lines fit by linear regression to the low-occupancy and plateau regions of the titration data (Runquist et al. 1996) for the various PRK proteins with fluorescent TNP-ATP substrate analog or allosteric activator NADH, as described in Materials and Methods.

Table 3. Steady-state kinetic parameters of wild-type and mutant PRK proteins

Enzyme	K_{mRu5P} (mM) ^a	$S_{1/2ATP}$ (mM) ^b	η_H ^c	V_{max} (units/mg)
Wild-type	0.10 ± 0.01	0.55 ± 0.16	1.98 ± 0.15	338 ± 18
Y98L	4.00 ± 1.40	0.38 ± 0.01	1.50 ± 0.18	12.5 ± 2.1
H100A	0.78 ± 0.31	0.43 ± 0.03	1.42 ± 0.41	0.13 ± 0.02
H100N	1.37 ± 0.26	0.40 ± 0.01	1.88 ± 0.23	0.15 ± 0.01
H100Q	1.35 ± 0.24	0.55 ± 0.05	1.39 ± 0.32	0.46 ± 0.03

^a K_{mRu5P} is the ribulose 5-phosphate concentration at half-maximal velocity, as calculated from a fit of the Ru5P saturation data to the Michaelis-Menten equation.

^b $S_{1/2ATP}$ is the ATP concentration at half-maximal velocity, as calculated from a fit of the ATP saturation data to the Hill equation.

^c η_H is the Hill constant.

Kinetic characterization of Y98 and H100 mutants

A radioisotopic assay (Paulsen and Lane 1966) that involves trapping the PRK reaction product RuBP via the RuBP carboxylase-dependent incorporation of $^{14}CO_2$ to form acid-stable [^{14}C]-3-phosphoglycerate was utilized for kinetic characterization of the PRK mutants; the results are summarized in Table 3. The mutants exhibit $S_{1/2ATP}$ values varying between 0.40 and 0.55 mM; the Hill coefficients for ATP binding (1.39–1.98) indicate that the mutants retain cooperativity in ATP binding, as observed for wild-type PRK. These results suggest that the ATP binding sites are structurally intact and that the H100 and Y98 mutations do not compromise the substrate nucleotide binding site. The K_{mRu5P} values for the H100 mutants show only modest eight- to 14-fold inflation effects, whereas Y98L exhibits a 40-fold inflation for K_{mRu5P} (Table 3). Yet, the Y98L K_i ($0.54 \pm 0.024 \mu M$) for 6-phosphogluconate, a competitive inhibitor with respect to Ru5P, is close to that of the wild-type PRK K_{iPG} ($0.22 \pm 0.02 \mu M$) (Runquist et al. 1999). Since K_i for a competitive inhibitor correlates well with an equilibrium binding constant, the results suggest that metabolite binding to Y98L's phosphoryl acceptor site is not substantially impaired. In comparison with Y98L, PRK mutants H45N and R49Q exhibit inflation in both K_{iPG} and K_{mRu5P} (Sandbaken et al. 1992; Runquist et al. 1999). The steady-state kinetic observations that are now reported suggest that Y98 and H100 are not essential for Ru5P binding, although they do affect its K_m values.

Mutant Y98L exhibits a catalytic rate reduction of 27-fold, whereas mutants H100A, H100N, and H100Q are characterized by larger rate reductions of 2600-, 2300-, and 735-fold, respectively. Corresponding V/K_{Ru5P} effects are 2.1×10^4 , 3.1×10^4 , and 9.9×10^3 . The significant reduction in V_m or V/K for each of the three H100 mutants implies that, during catalysis, the mobile region containing Y98 and H100 must be positioned as part of

the PRK active site. Moreover, the diminished catalytic rates observed in mutants containing alanine, asparagine, or glutamine substitutions suggest that the ionizable imidazole side chain of residue H100 makes a specific contribution to catalytic efficiency.

Discussion

The availability of an X-ray structure for *R. sphaeroides* phosphoribulokinase and the sequence differences between prokaryotic and eukaryotic PRK proteins provide some insight into those regions of the protein that are important for function. For example, predicted ATP binding motifs are closely juxtaposed in the three-dimensional protein structure (Harrison et al. 1998), and the functional importance of specific residues in these regions has been demonstrated (Charlier et al. 1994; Runquist et al. 2001). The absence of active-site ligands in the protein structure somewhat limits precise functional predictions, but precedent for other nucleoside monophosphate kinase family proteins (Schlauderer et al. 1996; Leipe et al. 2003) has supported development of several hypotheses concerning phosphoryl donor and acceptor binding regions (Runquist et al. 1999, 2001) in PRK.

Dynamic properties of the unliganded enzyme, even in crystalline form, account for gaps in the structural model (Fig. 5). Thus, the structure provides less insight into possible functional importance of these segments of the protein. These gaps include residues in the N-terminal region that corresponds to the "Walker A" or "P-loop" motif associated with the phosphoryl groups of

Figure 5. Structure of the *R. sphaeroides* phosphoribulokinase monomer. Labeling of secondary structural elements includes α helices A–H, and β strands 1–7 as well as 1' and 2'. There are breaks in the peptide backbone in the regions labeled "P-loop" and "H-100 loop." The two invariant residues Y98 and H100 are harbored within a disordered region (including residues 96–117) that connects β strands 1' and 2'.

substrate ATP. Another gap in the structure is located within a loop between β strands 1' and 2' and situated near H45- and R49-containing helix B, which has been implicated as part of the Ru5P binding region (Sandbaken et al. 1992). H100 is part of that section of the mobile loop (residues 100–105) that could not be modeled into the structure. Residues H45 and R49 are reasonably close (≈ 6 Å) to Y98, the last loop residue modeled before the gap in the structure. This observation supports the possibility that this mobile loop contributes to the active site. The significant ($>10^3$ -fold) effect on V_m ($>10^4$ -fold for V/K) that is observed upon mutation to eliminate the imidazole side chain of H100 represents an important indication of the functional contribution of this loop region.

The program DALI (Holm and Sander 1993) detects several proteins that exhibit a fold similar to that of PRK. The most similar fold corresponds to *Escherichia coli* panthothenate kinase (Yun et al. 2000). Interestingly, this comparison indicates that PRK Y98 and H100 correspond to pantothenate kinase residues Y175 and H177. A liganded structure of pantothenate kinase (Ivey et al. 2004) implicates these residues as part of the phosphoryl acceptor (i.e., pantothenate) site. However, it is unclear that functional tests have been conducted to more quantitatively evaluate the influence of these amino acids on catalysis by pantothenate kinase. In view of the results for PRK's Y98 and H100, comparable data for pantothenate kinase's Y175 and H177 would be interesting.

Mutation of H100 results in effects on catalytic rate that are much more notable than effects on K_m values for either PRK substrate. The PRK reaction is known to proceed by an "in-line" attack of Ru5P's C1 hydroxyl oxygen on ATP (Miziorko and Eckstein 1984); this requires formation of a ternary complex of PRK with both ATP and Ru5P substrates aligned appropriately for phosphoryl transfer. Interaction (either direct or via an indirect network of hydrogen bond interactions) of the imidazole side chain of H100 with substrate could contribute to optimization of substrate orientation for such an "in-line" attack and would represent a plausible explanation for the contribution of this active-site residue to the rate of catalysis.

Materials and methods

Materials

Deoxyoligonucleotides used for site-directed mutagenesis studies and sequencing primers were purchased from Operon. For site-directed mutagenesis, Stratagene's QuikChange Mutagenesis Kit was utilized. Isolation of plasmid DNA, grown in competent *E. coli* JM109 cells (Promega), was accomplished using Sigma's

Plasmid Pure Miniprep Kit, and Qiagen's Plasmid Mini and Midi Kits. Restriction enzymes were purchased from New England Biolabs. For recombinant protein expression, ampicillin was purchased from Fisher Scientific, isopropyl β -D-thiogalactoside (IPTG) from Research Products International Corp., and competent *E. coli* BL21(DE3) cells from Novagen. Adenosine 5'-triphosphate, β -nicotinamide adenine dinucleotide (reduced form, NADH), phospho(enol)pyruvate (PEP), pyruvate kinase, lactic dehydrogenase, D-ribulose 5-phosphate, 6-phosphogluconate, and HEPES and Tris buffers were obtained from Sigma Chemical Co. Sodium [14 C] bicarbonate, 56 mCi/mmol, was a product of American Radiolabeled Chemicals, Inc. Chromatography media used for PRK isolation included reactive green-19 agarose from Sigma Chemical Co. and Q Sepharose Fast Flow from Pharmacia Biotech. For fluorescence experiments, TNP-ATP [2'(3')-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate] from Molecular Probes was utilized. Inactivation experiments required diethyl pyrocarbonate provided by Alfa Aesar. The RuBP carboxylase required for the CO_2 fixation assay of PRK activity was isolated after expression in *E. coli* JM103 using a plasmid generously provided by C.R. Somerville (Stanford University, CA).

Construction of *prkA* mutant enzymes

Plasmid pETbprkw (Charlier et al. 1994) was used for mutagenesis of conserved residues Y98 and H100. Stratagene's QuikChange Mutagenesis Kit was utilized for the site-directed mutagenesis protocol. In the case of the Y98L mutation, TAC was changed to TTA. In the case of H100, three mutations were undertaken: H100A, H100N, and H100Q. For H100A, CAT was changed to GCT; for H100N, CAT was changed to AAT; and for H100Q, CAT was changed to CAA. DNA sequencing in both directions confirmed that the selected expression plasmids encoded the anticipated Y98L, H100A, H100N, and H100Q substitutions and verified that no additional mutations had been introduced during PCR amplification.

Expression and purification of PRK

E. coli BL21(DE3) cultures containing the *prkA* mutant plasmids were grown at 25°C in 2 L of ampicillin-containing LB media to an OD_{600} of ~ 0.5 . Expression of PRK was induced by addition of IPTG to a final concentration of 1 mM IPTG followed by 3–4 h of additional growth. The cells were collected by low-speed centrifugation; cell pellets were suspended and disrupted using a French pressure cell. A 100,000g supernatant was prepared and subjected to Q Sepharose anion exchange chromatography followed by affinity chromatography on reactive green-19 agarose. Homogeneous PRK was eluted from reactive green-19 agarose affinity resin with a buffer containing 25 mM Tris-HCl (pH 8.2), 10 mM mercaptoethanol, 1 mM EDTA, and 10 mM ATP as described previously (Charlier et al. 1994). ATP removal from ATP-eluted enzyme was accomplished by prolonged dialysis. For quantitative protein concentration estimates, an extinction coefficient of $50,303 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm was used (Runquist et al. 1996).

TNP-ATP and NADH binding stoichiometry measurements

TNP-ATP binding to PRK was evaluated by fluorescence measurements using a spectrofluorometer (Photon Technology

International). Relative fluorescence was measured at the fluorescence emission peak of 545 nm for PRK-bound TNP-ATP as described previously (Runquist et al. 1996). NADH binding to PRK was also measured using fluorescence methods. In this case, fluorescence was measured at the emission peak of 440 nm for PRK-bound NADH. The binding stoichiometry of mono- and dinucleotides to PRK was determined from the intersection point of lines fit by linear regression to the low-occupancy and plateau regions of the titration data (Runquist et al. 1996). Calculated stoichiometries reflect binding sites per 32-kDa PRK subunit.

Kinetic characterization of PRK

A radioisotopic assay that involves trapping the PRK reaction product RuBP via the RuBP carboxylase-dependent incorporation of ^{14}C to form acid-stable [^{14}C]-3-phosphoglycerate (Paulsen and Lane 1966) was utilized. In standard assays, the final concentrations of reaction mixture components were 100 mM HEPES (pH 7.5), 1 mM DTT, 20 mM MgCl_2 , 20 mM $\text{KH}^{14}\text{CO}_3$ (~1000 dpm/nmol), 5 mM ATP, 1 mM Ru5P, 1 mM NADH, and 100 units of recombinant RuBP carboxylase. Enzyme assays were conducted at 30°C. For kinetic characterization of the mutant proteins, ATP concentration ranges varied from 0.05–15 mM; Ru5P concentration ranges varied from 0.05–8 mM. Kinetic data were fit by non-linear regression analysis using the Grafit program (Erithacus software). Inhibition studies used 6-phosphogluconate in a range from 0.2–4 mM for K_i determinations (Runquist et al. 1999). For the DEPC inactivation experiments, a spectrophotometric assay for residual PRK activity was employed. This assay couples Ru5P-dependent ADP formation to NADH oxidation with pyruvate kinase and lactate dehydrogenase (Kreiger and Mizioroko 1986). Assay components included 100 mM Tris-HCl (pH 8.2), 10 mM MgCl_2 , 0.5 mM DTT, 5 mM ATP, 0.2 mM PEP, 0.2 mM NADH, 2 units of pyruvate kinase, 2 units of lactic dehydrogenase, and 1 mM Ru5P.

DEPC inactivation

Diethyl pyrocarbonate solutions were freshly prepared by dilution of the reagent into acetonitrile. Protein histidine modification due to formation of N-carbethoxyimidazole was measured by changes in $A_{240\text{ nm}}$ and employed an extinction coefficient of $3200\text{ M}^{-1}\text{ cm}^{-1}$ (Miles 1977). Prior to use in DEPC inactivation experiments, wild-type and H45N PRKs were dialyzed to remove residual ATP from affinity resin-purified enzymes. For a typical (30°C) DEPC inactivation experiment, 1–4 μM PRK was added to 50 mM HEPES (pH 7.5) and 10 mM MgCl_2 in a final volume of 0.1 mL, and enzyme activity was established. Subsequently, DEPC was added to the inactivation sample for a final concentration of 40–80 μM ; the added volume of inactivator was not more than 3% of the sample's total volume. Enzyme activity was measured at appropriate intervals by the spectral assay that follows NADH oxidation at 340 nm. Control experiments established that in the absence of DEPC there was no loss of enzyme activity within the time period of the experiment and that the small amount of acetonitrile added with the DEPC reagent also does not affect enzyme activity. Protection against DEPC inactivation by enzyme substrates was tested in experiments that utilized 1 mM ATP or 1 mM Ru5P. Protection by 1 mM phosphogluconate, a competitive inhibitor with respect to Ru5P (Gardemann et al. 1983; Runquist et al. 1999), was also assessed. The enzyme inactivation data were fit to a first-order exponential decay using the Grafit program.

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