

Roles and Microenvironments of Tryptophanyl Residues of Spinach Phosphoribulokinase¹

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Phosphoribulokinase is one of several Calvin cycle enzymes that are light-regulated via the ferredoxin–thioredoxin system (R. A. Wolosiuk and B. B. Buchanan, 1978, *Arch. Biochem. Biophys.* 189, 97–101). Substitution of the only two Trp residues of the enzyme was prompted by the following goals: to identify each tryptophanyl residue with respect to prior classifications as exposed and buried (C. A. Ghiron *et al.*, 1988, *Arch. Biochem. Biophys.* 260, 267–272); to explore the possible active-site location and function of conserved Trp155, as suggested by sequence proximity to catalytic Asp160 (H. A. Charlier *et al.*, 1994, *Biochemistry* 33, 9343–9350); and to determine if fluorescence of a Trp residue can serve as a gauge of conformational differences between the reduced (active) and the oxidized (inactive) forms of the enzyme. Emission spectra and acrylamide quenching data demonstrate that Trp155 is solvent exposed, while Trp241 is buried. Kinetic parameters of the W241F mutant are not significantly altered relative to those of wild-type enzyme, thereby discounting any requirement for Trp at position 241. While substitution of Trp155 with Phe or Ala has little impact on $V_{\max}$, the K_m for Ru5P and ATP are increased substantially; the diminished affinity for ATP is particularly pronounced in the case of the Ala substitution. In further support of an active-site location of Trp155, its fluorescence emission is subject to quenching by nucleotides. Fluorescence quenching of reduced W241F by ATP gives a dissociation constant

(K_d) of 37 μM , virtually identical with its K_m of 46 μM , and provides for the first time a direct measurement of the interaction of the kinase with product ADP (K_d of 1.3 mM). Fluorescence quenching of oxidized W241F by ATP reveals a K_d of 28 mM; however, this weakened binding does not reflect an altered microenvironment of Trp155, as its steady-state emission and fluorescence lifetimes are unaffected by the oxidation state.

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Key Words: phosphoribulokinase; active site; tryptophan; mutagenesis; fluorescence.

Phosphoribulokinase (EC 2.7.1.19) catalyzes the final metabolic step in the regeneration of D-ribulose 1,5-bisphosphate, the primary substrate for photosynthetic carbon assimilation. Although the three-dimensional structure of PRK³ has not been reported, the active site of the enzyme has been partially mapped by chemical modification and by site-directed mutagenesis. A characteristic feature of homodimeric PRK from higher plants is the active-site location of both sulfhydryls (contributed from Cys16 and Cys55 in the spinach enzyme) (1, 2), which account for the redox regulation of the enzyme activity as mediated by thioredoxin in a light-dependent fashion (3, 4). Site-directed mutagenesis has shown that Cys55 contributes moderately to catalysis and to binding of Ru5P, whereas Cys16 is noncontributory in both parameters (5, 6). Loss of activity concomitant with regulatory oxidation of Cys16 and Cys55 to form an intrasubunit disulfide is due in part to masking the free sulfhydryl at position 55 (5, 6) and

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³ Abbreviations used: PRK, phosphoribulokinase; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); Ru5P, ribulose 5-phosphate; DTT, dithiothreitol; bicine, *N,N'*-bis(2-hydroxyethyl)glycine; tricine, *N*-tris(hydroxyethyl)methylglycine; bis-tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

	57	60		155	160
	↓	↓		↓	↓
<i>Spinacia</i>	...TVICLDDF	HSL	...VKFAW	--KIQRDM	KERGHs...
<i>Triticum</i>	...TVICLDDY	HSL	...VKFAW	--KIQRDMA	ERGHs...
<i>Arabidopsis</i>	...TVICLDDY	HSL	...VKFAW	--KIQRDMA	ERGHs...
<i>Mesembryanthemum</i>	...TVICLDDY	HSL	...VKFAW	--KIQRDMA	ERGHs...
<i>Chlamydomonas</i>	...TVICLDDY	HCL	...IKFAW	--KIQRDMA	ERGHs...
<i>Synechocystis</i>	...TVICLDDY	HSL	...VKINW	--KIQRDMA	ERGHs...
<i>Rhodobacter</i>	-----DAFH	HRF	...INLEW	IQKLH	RDRATRGYT...
<i>Alcaligenes</i>	-----DSFH	RY	...INLEW	IQKLH	RDKKQRGYS...
<i>Nitrobacter</i>	-----DAFH	RY	...INLEW	IQKLH	RDRNARGYS...
<i>Xanthobacter</i>	-----DSFH	RY	...INLEW	IQKLH	RDKATRGYT...

FIG. 1. Amino acid sequence alignments of active-site segments from eukaryotic and prokaryotic PRKs. Denoted residue numbers refer to spinach enzyme, and dashes indicate alignment gaps. Alignments, based on the entire sequences, were made with the GCG algorithm PileUp [Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI]. The sources for the amino acid sequences are as follows (database accession number): *Spinacia*, GenBank M21338; *Triticum*, GenBank X57952; *Arabidopsis*, GenBank X58149; *Mesembryanthemum*, GenBank M73707; *Chlamydomonas*, GenBank M36123; *Synechocystis*, GenBank M77134; *Rhodobacter*, GenBank M64624; *Alcaligenes*, SWISSPROT P19923; *Nitrobacter*, GenBank L22884; and *Xanthobacter*, GenBank X17252.

in part to conformational constraints imposed by the disulfide (7).

PRKs from photosynthetic bacteria (typically octamers of identical subunits) (8) are also responsive to the redox status of the cell, but regulation of activity is mediated by the allosteric activator NADH rather than by reversible oxidation of sulfhydryls (8, 9). Hence, despite their active-site locations, Cys16 and Cys55, which are conserved among eukaryotic PRKs, lack representation in the prokaryotic enzymes. However, site-directed mutagenesis of *Rhodobacter sphaeroides* PRK has identified several strictly conserved active-site residues, the counterparts of which in the spinach enzyme are adjacent to Cys55 (Fig. 1). These include His60⁴ and Arg64, which contribute substantially to the binding of Ru5P (10), and Asp57, a key catalytic residue which enhances k_{cat} by 10⁵-fold (11). Another invariant catalytic residue, Asp160, which enhances k_{cat} by 10⁴-fold, has been identified in a distal segment by site-directed mutagenesis of the *R. sphaeroides* enzyme (11).

Asp160 of PRK is located within an 11-residue segment encompassing the greatest preponderance of invariant residues that occur within any segment of similar length throughout the entire 387-residue polypeptide chain (Fig. 1) (see Refs. 12–15 for complete sequences of the spinach and *R. sphaeroides* enzymes). Only one of the two tryptophanyl residues of spinach PRK is species invariant, and it is located in this highly homologous segment. To explore the possibility of a functional role for Trp155, as implicated by its sequence proximity to Asp160, we have replaced this Trp with either Phe or Ala. We have also replaced the other

Trp (at position 241) of spinach PRK with Phe in order to classify each Trp as exposed or buried, because a prior fluorescent study (16) with wild-type PRK indicated the presence of both classes. Additionally, the availability of a mutant containing a single Trp at position 155, confirmed herein to be located at the active site, has enabled us to address the question of whether fluorescence of Trp155 might serve as a gauge of conformational differences between the reduced and the oxidized forms of PRK. In this paper, we report the characterization of the single mutants W155F, W155A, and W241F and the double mutant W155F–W241F with respect to kinetic parameters and fluorescence properties.

MATERIALS AND METHODS

Chemicals and biologicals. The following supplies were procured from the indicated vendors: dye-terminator cycle sequencing reagents from Perkin–Elmer; *Pfu* DNA polymerase from Stratagene; restriction endonucleases, alkaline phosphatase, Klenow fragment of DNA polymerase I, and T4 DNA ligase from New England Biolabs; oligonucleotides from GIBCO BRL; yeast nitrogen base without amino acids from Difco; bicine, 2-(*N*-morpholino)ethanesulfonic acid, *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid), and DTT from Research Organics, Inc.; 4-(2-aminoethyl)benzenesulfonyl fluoride from Calbiochem; other buffers, biologicals for enzyme assays, and DTNB from Sigma Chemical Co.; Toyopearl chromatographic resins from TosoHaas; ceramic hydroxyapatite type I from Bio-Rad; and acrylamide (UltraPure grade) from ICN Biomedicals.

Incubation of ribose 5-phosphate with phosphoriboisomerase gave Ru5P, and its concentration was calculated from the equilibrium constant of 0.27 (in favor of ribose 5-phosphate) (17, 18) and was verified by assay with PRK.

PRK mutants. Published procedures (6) were followed for the construction of expression cassettes encoding the mutant PRKs and for the chromosomal integration of cassettes into *Pichia pastoris*. The following oligonucleotide primers were used to introduce the desired amino acid substitution (indicated by the underlined codon) for the targeted tryptophanyl residues: ATTTGCCCTTCAAATTCAG for W155F, CCATTCATTCCATCATGT for W241F, simultaneous dual priming with both of these oligonucleotides for the double mutant W155F–W241F, and AAGTTAAATTTGCCCGCAAATTCAG-AGAG for W155A. Substitutions of the Trp codons were confirmed by dye-terminator cycle sequencing (ABI 373A DNA sequencer) of PCR-amplified *prk* template from genomic DNA of transformed *P. pastoris* (6) using the primers CTAAGATCTTGGTCATGGAAG for the W155 region and ATTCCTGATGATGATGAAGGC for the W241 region.

Purification of recombinant wild-type and mutant PRKs. Culturing of transformed *P. pastoris*, lysis of harvested cells (40–60 g from a 1-liter culture), and anion-exchange chromatography of cell extracts on SuperQ-Toyopearl 650M followed our earlier procedure (6). Typically, the kinase was recovered from this column in virtually quantitative yield at ~25% purity. Subsequent steps entailed successive hydrophobic and hydroxyapatite chromatography rather than red-agarose affinity chromatography as used earlier. As defined by the elution buffer (pH 8.0) for the SuperQ column, the solvent composition of the pool (~120 ml, ~200 mg of total protein) was 50 mM bicine, 1 mM EDTA, 10 mM DTT, 0.25 M potassium acetate, and 0.1 mM aminoethylbenzenesulfonyl fluoride. Solid (NH₄)₂SO₄ was added to a final concentration of 1.0 M to the pooled fractions; following filtration (0.8-μm Gelman Acrodisc) of the pool, it was applied to a 60-ml phenyl-Toyopearl 650S column previously equilibrated with 1.0 M (NH₄)₂SO₄ in pH 8.0 buffer (50 mM bicine, 1 mM EDTA, 10 mM

⁴ Irrespective of species, residues are numbered in accordance with the spinach enzyme.

DTT). The column was washed with equilibration buffer to remove nonbound material followed by a 720-ml inverse gradient of 1.0 to 0.0 M $(\text{NH}_4)_2\text{SO}_4$ (in the same pH 8.0 buffer). Appropriate fractions [~ 0.4 to 0.5 M $(\text{NH}_4)_2\text{SO}_4$] were pooled (~ 135 ml, ~ 75 mg of total protein), concentrated to < 50 ml (Amicon YM30 membrane), and dialyzed (Spectra/Por 2 membrane) exhaustively against a pH 7.0 buffer [50 mM tricine (titrated with solid bis-tris propane), 0.5 mM CaCl_2 , 10 mM DTT]. The sample was then filtered ($0.45\text{-}\mu\text{m}$ Gelman Acrodisc) and applied to a 25-ml ceramic hydroxyapatite type I column (bead size $20\text{ }\mu\text{m}$) previously equilibrated with a pH 7.0 buffer [20 mM tricine (titrated with solid bis-tris propane), $3\text{ }\mu\text{M}$ CaCl_2 , 10 mM DTT, 2 mM potassium phosphate]. After unbound material was removed by elution with equilibration buffer, a 450-ml gradient (2 to 100 mM potassium phosphate, $3\text{ }\mu\text{M}$ CaCl_2 , 10 mM DTT at pH 7.0) was applied to the column. Fractions containing the bulk of the kinase (40 mg, 70% overall recovery from crude extract) were pooled and concentrated to $0.5\text{--}1$ ml by ultrafiltration over a YM30 membrane. The concentrated sample was then dialyzed (Spectra/Por 2) three times against 1 liter of pH 8.0 buffer (50 mM bicine, 1 mM EDTA, 10 mM DTT, 20% glycerol). The dialyzed sample was clarified in a microcentrifuge and stored at -80°C .

The concentration of wild-type PRK was determined at 280 nm based on an extinction coefficient of 7.2 for a 1% solution (19). Concentrations of mutant PRKs were calculated from their $A_{280\text{ nm}}$ ratios under native and denaturing conditions (20, 21).

Purification of phosphoriboisomerase. The commercial enzyme used in the assay for PRK was freed of trace contamination with Ru5P epimerase by anion-exchange chromatography. One milligram of spinach phosphoriboisomerase type II (Sigma Chemical Co. Cat. No. P1780) was dissolved in 1 ml of a pH 8.0 buffer (50 mM bicine, 1 mM EDTA, 5 mM DTT) and dialyzed against the same buffer. The dialyzed sample was applied to a 4-ml TSK-DEAE 5PW column that had been equilibrated with the bicine buffer. After washing the column with 15 ml of the same buffer, an 80-ml gradient (0 to 0.5 M KCl in the bicine buffer) was applied to the column, with the isomerase eluting at ~ 0.125 M. The isomerase pool (2 ml) was dialyzed against a pH 8.0 buffer (50 mM bicine, 1 mM EDTA, 5 mM DTT, 20% glycerol) and then stored at -80°C .

Assay for PRK. Kinase activity was determined spectrophotometrically at 25°C , in which generation of ADP is coupled to NADH oxidation by use of phospho(*enol*)pyruvate, pyruvate kinase, and lactate dehydrogenase (22). Assay solutions (1 ml, pH 8.0) routinely contained 50 mM bicine, 40 mM KCl, 10 mM MgCl_2 , 0.24 mM NADH, 1 mM ATP, 2 mM Ru5P, 3 mM phospho(*enol*)pyruvate, 4 units of pyruvate kinase, 5 units of actate dehydrogenase, and < 0.2 units of PRK. One unit of activity is defined as $\Delta A_{340\text{ nm}}$ of 6.22/min, representing phosphorylation of $1\text{ }\mu\text{mol}$ of Ru5P. For determination of kinetic parameters when varying the concentration of Ru5P, the ATP concentration was held at 1 mM for wild-type and W241F, at 2 mM for W155F, at 6 mM for W155F–W241F, and at 8 mM for W155A. To determine kinetic parameters when the concentration of ATP was varied, the Ru5P concentration was held at 1 mM for wild-type and W241F and at 2 mM for W155F, W155F–W241F, and W155A. Kinetic data were analyzed by a Marquardt–Levenberg algorithm of nonlinear least-squares fitting to $v = V \cdot S/(K_m + S)$.

Oxidation of PRK. Fully active, reduced PRK was converted to the disulfide form by oxidation with DTNB (1). An aliquot of the purified stock kinase ($\sim 200\text{ }\mu\text{l}$, ~ 3 mg) was freed of DTT by rapid buffer exchange (1 ml/min) on an 8-ml Sephadex-G25 column equilibrated with 50 mM bicine, 1 mM EDTA, 10% glycerol at pH 8.0. A 20% molar excess (relative to active sites) of DTNB was added to the gel-filtered kinase (1.8 ml, 2.8 mg). After oxidation was complete ($< 0.1\%$ of initial activity remaining), the sample was dialyzed (50 mM bicine, 1 mM EDTA, 20% glycerol, at pH 8.0) exhaustively to remove free thionitrobenzoate and to prepare the sample for storage at -80°C .

Fluorescence measurements. Steady-state fluorescence measurements at 25°C were made with an SLM-AB2 fluorimeter with excita-

tion at 295 nm and both excitation and emission bandwidths of 4 nm. Protein solutions ($A_{280\text{ nm}} \leq 0.06$) contained 50 mM bicine and 20 μM EDTA at pH 8.0. Unless otherwise indicated, PRK was in the reduced form and DTT was present at 200 μM . When oxidized PRK was examined, exogenous thiol was omitted from the buffer. MgCl_2 at 10 mM was included in the buffer for titrations of reduced enzyme with ATP and at 50 mM for titrations of both oxidized enzyme with ATP and reduced enzyme with ADP. The acrylamide stock (5.0 M) used in quenching experiments was freshly prepared in 50 mM bicine and adjusted to pH 8.0 with KOH. Corrections in observed fluorescence were made for the attenuation of the incident excitation light by acrylamide or nucleotides (23).

Recorded emission spectra are corrected emission spectra, as the instrument accounts in real time for wavelength-dependent efficiencies of the light source. Controls lacking protein were always employed to subtract out background signals.

Analysis of acrylamide quenching data relied on a Marquardt–Levenberg algorithm for nonlinear least-squares fitting to either $F_0/F = 1 + K_{SV}Q$ or $F_0/F = (1 + K_{SV}Q)e^{V/Q}$, where F_0 and F are the fluorescence intensities in the absence and presence of quencher, K_{SV} is the Stern–Volmer collisional quenching constant, Q is the quencher concentration, and V is the static quenching constant (23). Analysis of ligand binding data relied on the same algorithm fitted to $F_i = F_m \cdot S/(K + S)$, where F_i is the measured fluorescence perturbation at a given concentration of ligand S , F_m is the maximal fluorescence perturbation observed under saturating concentrations of S , and K is the dissociation constant (24).

Fluorescence lifetime measurements were performed with an ISS frequency domain instrument with an argon ion laser coupled with an appropriate interference filter to give an effective excitation wavelength of 300 nm. Intensity decay data were analyzed as described elsewhere (25). Data were fit to a biexponential decay (τ_1 and τ_2), since this is considered a simplest case to explain emission from distinct tryptophan rotamers (26–28).

RESULTS

Kinetic parameters. Based on polyacrylamide gel electrophoresis under both nondenaturing and denaturing conditions, each of the PRK preparations was greater than 95% homogeneous (Fig. 2). Comigration of the mutant PRKs with the wild-type enzyme confirms full-length translation products and proper assembly of subunits into dimers. As shown in Table I, replacement of Trp241 diminishes V_{max} only slightly and does not alter the K_m for either substrate. Although replacement of Trp155 is inconsequential to V_{max} , substantial increase in the K_m of both substrates results. The detrimental impact on the binding of ATP is greater than on that of Ru5P, and this impact is far more severe with abolishment of the indole ring (as assessed with W155A) than with replacement by a phenyl ring (as assessed with W155F).

Fluorescence characteristics. The emission spectra of wild-type enzyme, W155F, W241F, and W155F–W241F are shown in Fig. 3. The blue-shifted spectrum of W155F, relative to W241F, suggests that Trp241 is located within a less polar environment than is Trp155. The spectrum of the wild-type enzyme is not simply the composite of the spectra of the two mutant enzymes. This is likely due to fluorescence resonance energy transfer from the more blue-emitting tryptophan (Trp241) to the more red-emitting tryptophan (Trp155),

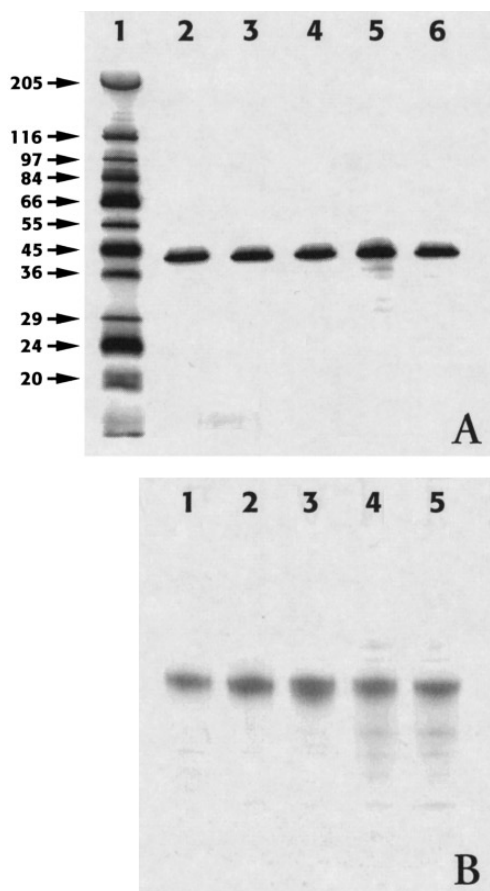


FIG. 2. (A) Silver-stained denaturing polyacrylamide gel electropherogram (8–25% PhastGel, Pharmacia) of PRKs (25 ng per lane). Lane 1, molecular-weight markers; Lane 2, wild-type enzyme; Lane 3, W155F; Lane 4, W155A; Lane 5, W241F; Lane 6, W155F-W241F. (B) Coomassie blue-stained nondenaturing polyacrylamide gel electropherogram (8–25% PhastGel, Pharmacia) of PRKs (500 ng per lane). Lane 1, wild-type enzyme; Lane 2, W155F; Lane 3, W155A; Lane 4, W241F; Lane 5, W155F-W241F.

as suggested from the phase-resolved spectra of authentic spinach PRK (16, 29). The absence of fluorescence from the double mutant confirms that the emission from the other three proteins emanates exclusively from tryptophanyl residues upon excitation at 295 nm.

TABLE I
Kinetic Parameters of PRKs

Construct	$V_{\max}$ (U/mg)	K_m MgATP (μ M)	K_m Ru5P (μ M)
Wild-type	550	46	48
W241F	480	45	42
W155F	600	290	150
W241F-W155F	450	400	230
W155A	600	3000	280

Note. Standard errors for kinetic parameters are <10%.

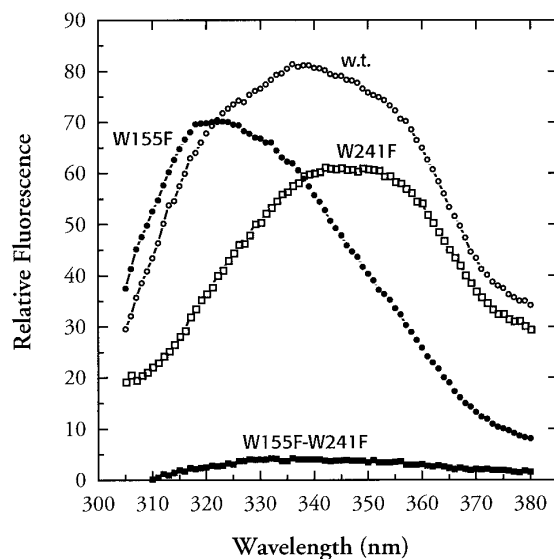


FIG. 3. Corrected emission spectra of PRKs. The final concentration of each PRK was 60 μ g/ml in pH 8.0 buffer containing 50 mM bicine, 20 μ M EDTA, and 200 μ M DTT. Spectra were scanned at 2 nm/s.

To further test the supposition that Trp241 is buried relative to Trp155, the two single mutants were subjected to acrylamide quenching and were also analyzed for fluorescence lifetimes (Fig. 4, Table II). As depicted by the curvilinear response of W241F in the Stern-Volmer plots, Trp155 exhibits both a dynamic (K_{SV} of 4.6 M^{-1}) and a static (V of 0.4 M^{-1}) component in its quenching by acrylamide. With a value of τ_1 for Trp155

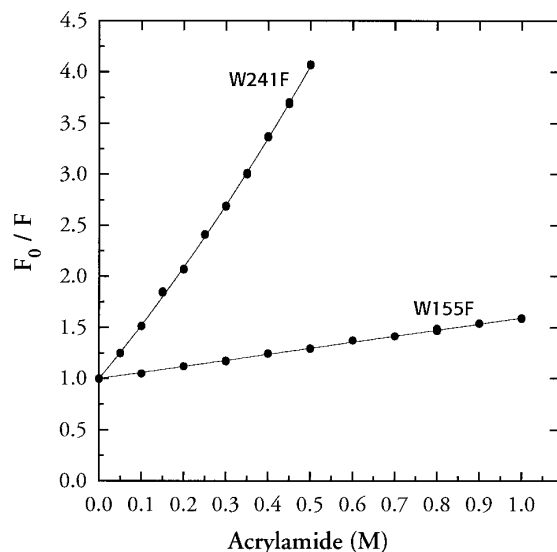


FIG. 4. Acrylamide quenching of PRK mutants. For W241F, emission was monitored at 350 nm; for W155F, emission was monitored at 325 nm. The final concentration of each PRK was 80 μ g/ml in 50 mM bicine, 20 μ M EDTA, and 200 μ M DTT at pH 8.0.

of 7.1 ns, the collisional rate constant for acrylamide (k_q), which serves as a kinetic measure of the solvent exposure of the tryptophanyl residue, is thus $0.65 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (23). For comparison, the collisional rate constant for acrylamide with a fully exposed indole side chain of a randomly coiled polypeptide is on the order of $4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, while that of a fully buried indole side chain within a protein core is $< 0.05 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (23). Additionally, the value of V also provides insight into the relative exposure of the tryptophanyl residue (23); a value of 0.4 indicates partial solvent exposure. In contrast, analysis of W155F shows that Trp241 displays a linear response to acrylamide quenching, indicative of a buried residue. Likewise, the K_{SV} value (0.6 M^{-1}) is much lower compared to Trp155; with a measured τ_1 of 3.3 ns, a collisional rate constant of $0.18 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ is calculated, also consistent with relative solvent inaccessibility of Trp241.

Steady-state emission of W241F did not appear to be dependent on oxidation state of the enzyme; this was confirmed by comparison of fluorescence lifetimes of the reduced and oxidized forms of W241F (Table II).

ATP (1 mM) or Ru5P (2 mM) did not appreciably affect the emission of W155F at 320 nm, nor did Ru5P (2 mM) appreciably affect the emission of W241F at 350 nm (data not shown). However, ATP (1 mM) resulted in a 60% reduction of W241F emission at 350 nm, as well as a blue shift in the λ_{max} from 345 to 335 nm (Fig. 5), indicative of the microenvironment of Trp155 becoming less polar upon binding of the nucleotide. Fluorescence of the wild-type PRK at 350–360 nm was quenched to a similar extent by saturating concentrations of ATP. Based on the emission spectra of the W155F and W241F (Fig. 3), the remaining fluorescence of the ATP-quenched spectrum of wild-type enzyme (Fig. 5) is derived primarily from Trp241.

The decrease in fluorescence of PRK upon binding of nucleotide was exploited to acquire a dissociation constant for MgATP (Fig. 6). The K_d for the binding of MgATP to reduced W241F was $37 \mu\text{M}$ MgATP (Fig. 6), a value virtually identical to the K_m of $46 \mu\text{M}$. The oxidized form of W241F displayed a far weaker affinity for MgATP (K_d of 28 mM) than did the reduced form. Saturating levels of MgADP perturbed fluorescence emission of W241F to the same extent as did MgATP.

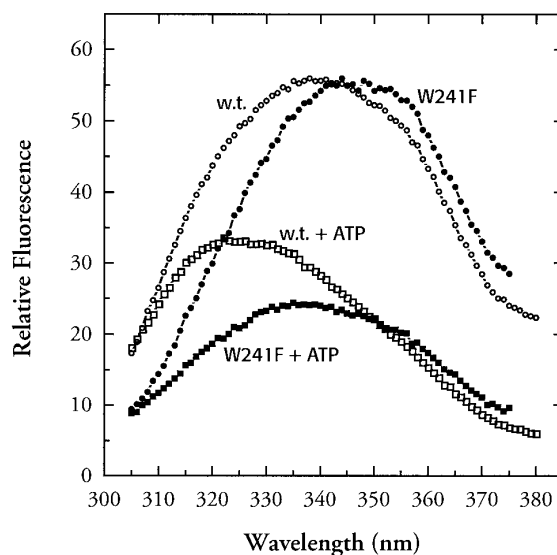


FIG. 5. Corrected emission spectra of wild-type PRK and W241F in the presence and absence of saturating levels of ATP (1 mM). Final concentrations of wild-type PRK and W241F are 50 and 80 $\mu\text{g}/\text{ml}$, respectively, in 50 mM bicine, 10 mM MgCl_2 , 20 μM EDTA, and 200 μM DTT at pH 8.0. Spectra were scanned at 2 nm/s. In each case, instrument controls are set so that emission at λ_{max} (in absence of ATP) is $\sim 60\%$ full scale.

Titration of fluorescence quenching gave a K_d for MgADP of 1.3 mM (Fig. 7).

DISCUSSION

The data presented lead to four major conclusions: (i) neither of the two tryptophanyl residues of PRK serves a catalytic role; (ii) species-invariant Trp155 is located at the active site and is required for high affinity of the enzyme for ATP; (iii) Trp155 is partially exposed to solvent, while nonconserved Trp241 is relatively buried; and (iv) despite the active-site location of Trp155, its microenvironment is independent of the oxidation state of the enzyme.

Given its active-site location, its sequence proximity to the catalytic carboxylate of Asp160, its 70-fold enhancement of the affinity of ATP, and quenching of its fluorescence by ATP and ADP, the indole ring of Trp155 may engage the adenine ring of ATP directly through

TABLE II
Fluorescence and Acrylamide Quench Parameters for W241F and W155F

Construct	$K_{sv} (\text{M}^{-1})$	$V (\text{M}^{-1})$	$\tau_1 (\text{ns})$	$\tau_2 (\text{ns})$	$k_q (\times 10^{-9} \text{ M}^{-1} \text{ s}^{-1})$	$\lambda_{\text{max}} (\text{nm})$
W241F (red)	4.6	0.4	7.1	1.5	0.65	350
W241F (ox)	—	—	6.9	1.4	—	350
W155F	0.6	0	3.3	1	0.18	320

Note. W241F (ox) was not subject to acrylamide quench studies.

hydrophobic or π - π interactions. Such an interpretation would be consistent with the lesser impact on ATP binding from replacement of the indole side chain with a phenyl group compared to a hydrogen atom. Alternatively, the contribution of Trp155 to ATP binding may be indirect, and the consequence of its replacement would then reflect modest conformational perturbations. However, the insensitivity of $V_{\max}$ to either replacement argues against the latter possibility. As Ru5P does not influence tryptophanyl fluorescence, direct interaction between this substrate and the indole ring of Trp155 is unlikely. Thus, the contribution of Trp155 to Ru5P binding is likely transmitted through other residues.

Examination of the fluorescence properties of W155F and W241F extends and clarifies earlier studies with wild-type PRK, which relied primarily on the inactive,

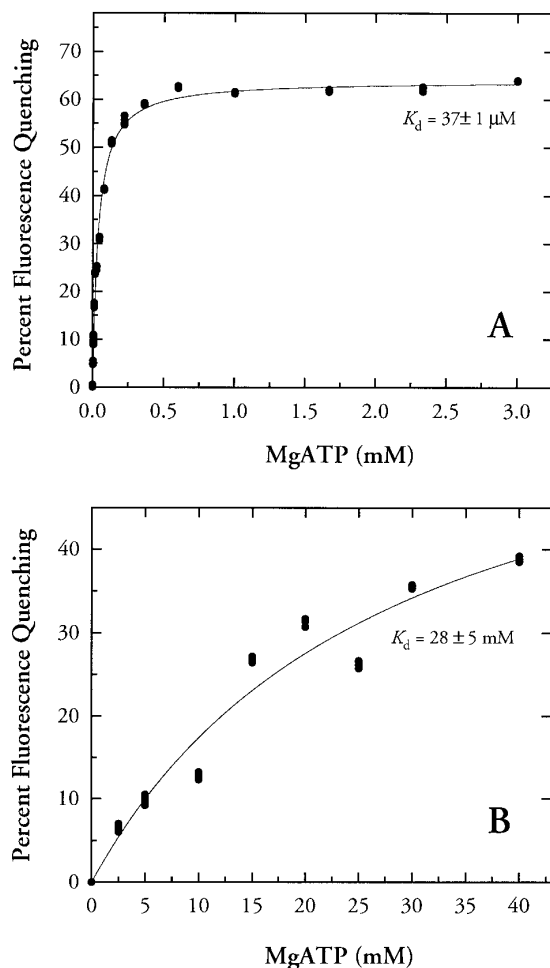


FIG. 6. MgATP titrations of reduced (A) and oxidized (B) W241F based on quenching of fluorescence emission monitored at 350 nm. The final concentration of each PRK is 80 $\mu\text{g/ml}$ in 50 mM bicine and 20 μM EDTA at pH 8.0. The sample of reduced enzyme also contained 10 mM MgCl_2 and 200 μM DTT, whereas the sample of oxidized enzyme contained 50 mM MgCl_2 .

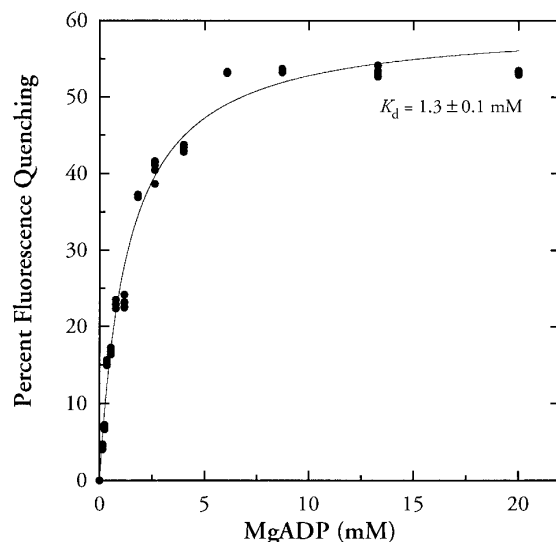


FIG. 7. MgADP titration of reduced W241F based on quenching of fluorescence emission monitored at 350 nm. The final concentration of enzyme was 80 $\mu\text{g/ml}$ in 50 mM bicine, 50 mM MgCl_2 , 20 μM EDTA, and 200 μM DTT at pH 8.0.

oxidized form of the enzyme (16). In those studies two classes of tryptophans were deduced from excitation, emission, anisotropy, and lifetime data. The more buried of the two residues fluoresced with a $\lambda_{\max}$ of 325 nm, and the more exposed fluoresced with a $\lambda_{\max}$ of 345 nm. In excellent agreement with these data, fluorescence emission spectra of W155F and W241F display $\lambda_{\max}$ values at 320 and 345 nm, respectively. The emission-based assignment of Trp155 as the more solvent accessible of the two residues is confirmed unequivocally by the acrylamide quenching experiments and the lifetime measurements.

W241F is a particularly attractive surrogate for wild-type PRK for the quantification of binding affinities of nucleotide substrates. The mutant enzyme is nearly as active catalytically as wild type, and the K_m values for both substrates are unaltered by the single amino acid substitution; furthermore, the only tryptophanyl residue of W241F is located at the active site, and fluorescence emission from the residue is quenched dramatically by ATP or ADP. Titrations of fluorescence quenching show that PRK has a 35-fold lower affinity for ADP (K_d of 1.3 mM) than for ATP (K_d of 37 μM) and that regulatory oxidation of the enzyme lowers the affinity for ATP by 750-fold (from K_d of 37 μM to K_d of 28 mM). Interaction of ATP with oxidized PRK cannot be examined on the basis of enzyme kinetics because of the total absence of activity. Hence, the present study represents the first direct demonstration that oxidized PRK retains competency in the binding of ATP, albeit with a greatly compromised affinity.

Based on fluorescence emission and lifetimes of Trp155, as assessed with the reduced and oxidized

forms of W241F, the microenvironment of this active-site tryptophanyl residue is insensitive to the oxidation state of the enzyme. This is surprising in view of a number of present and past observations. As shown in this study, Trp155 is in close proximity to bound ATP, the K_d of which is exquisitely sensitive to the oxidation state of the enzyme. Prior chemical modification studies have shown that bound ATP is also close to Cys16 and Cys55 (2, 30–34), the active-site participants in redox regulation (1, 2). Chemical crosslinking with bifunctional reagents has revealed considerable conformational flexibility in the vicinity of the regulatory sulfhydryls, which is lost upon disulfide bond formation (7). Conformational flexibility of the active site has also been implicated by characterization of the dithiol/disulfide exchange reaction between thioredoxin and PRK (35). The transient intermediate entails mixed disulfide bond formation between Cys46 of thioredoxin *f* and Cys55 of PRK (35), despite inaccessibility of the latter to traditional sulfhydryl reagents (1). Apparently, binding of thioredoxin by PRK induces conformational change to provide access to the otherwise sterically masked sulfhydryl. Despite these diverse indicators of conformational differences between reduced and oxidized PRK, the conformational constraints imposed on the latter do not restrict access of water to the active site as judged by the fluorescence properties of Trp155 but do restrict access of larger ligands as reflected by the greatly weakened affinity of oxidized PRK for ATP. Although Trp155 cannot be used as a gauge for the oxidation status of PRK, it does provide an attractive avenue for quantifying interactions of ligands with the enzyme.

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