Conformational Flexibility of the Regulatory Site of Phosphoribulokinase As Demonstrated with Bifunctional Reagents†

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ABSTRACT: Phosphoribulokinase (PRK) is one of several chloroplastic enzymes whose activity is regulated by thiol–disulfide exchange via thioredoxin. Activation entails reduction of an active-site disulfide bond between Cys16 and Cys55. Bifunctional cross-linking reagents have been used to approximate the interresidue distance between Cys16 and Cys55, an issue which impinges on the relative conformational states of the activated and deactivated forms of the enzyme. Spinach PRK is rapidly inactivated by stoichiometric levels of 4,4′-difluoro-3,3′-dinitrodiphenyl sulfone (FNPS) or 1,5-difluoro-2,4-dinitrobenzene (DFNB), which span 9 and 3.5 Å, respectively. ATP, but not ribulose 5-phosphate, retards the rate of inactivation, suggesting that modification has occurred at the nucleotide binding domain of the active site. Sulfhydryl modification is indicated by partial reversibility of inactivation as effected by exogenous thiols. Tryptic mapping by reverse-phase chromatography of [^{14}C]carboxymethylated enzyme, subsequent to its reaction with either FNPS or DFNB, demonstrates modification of Cys16 and Cys55 by both reagents, and formation of only one major chromophoric peptide in each case. On the basis of the sequence analysis of the purified chromophoric peptides, Cys16 and Cys55 are cross-linked by both FNPS and DFNB. Thus, the intrasubunit distance between the β-sulfhydryls of Cys16 and Cys55 is dynamic rather than static. Diminished conformational flexibility upon oxidation of the regulatory sulfhydryls to a disulfide may be partially responsible for the concomitant loss of enzymatic activity.

The Calvin cycle enzyme phosphoribulokinase (EC 2.1.7.19) catalyzes the ATP-dependent phosphorylation of D-ribulose 5-phosphate to form D-ribulose 1,5-diphosphate, the requisite CO_2 acceptor in autotrophic organisms. As with several enzymes localized within the stroma of chloroplasts, the activity of PRK[1] is modulated during the light/dark cycle by reversible reduction/oxidation (Buchanan, 1991; Wolosiuk & Buchanan, 1978). In the light, electrons flow from ferredoxin to thioredoxin *f* (as mediated by ferredoxin–thioredoxin reductase), which reduces an intrasubunit disulfide of PRK resulting in its activation. The intrasubunit, regulatory disulfide of the homodimeric PRK from spinach is constituted from Cys16 and Cys55 (Porter et al., 1988), which are located at the active site as deduced by chemical modification (Krieger & Miziorko, 1986, 1987; Porter & Hartman, 1986, 1990; Porter et al., 1990). Thus, either direct consequences of removal of a catalytic group or indirect consequences of conformational changes could account for the complete loss of PRK activity that accompanies oxidation. Site-directed mutagenesis has been used to explore the former possibility. Replacement of Cys16 by serine is without significant effect on catalytic activity, while identical substitution for Cys55 decreases k_{cat} ~10-fold (Hudson et al., 1992; Milanez et al., 1991). Since neither sulfhydryl is required for catalysis, the lack of a free sulfhydryl at the active site can only partially explain the catalytic incompetence of the oxidized enzyme. Even though intrinsic fluorescence of both oxidized and reduced PRK is very similar (Ghiron et al., 1988), the microenvironment of the only two tryptophanyl residues (Trp155 and Trp241) would not necessarily be sensitive to localized conformational changes restricted to the regulatory site. As an alternate approach to detecting conformational differences between the two activation states of PRK, we have examined the interresidue distance between Cys16 and Cys55 in the reduced enzyme with chemical cross-linking reagents.

EXPERIMENTAL PROCEDURES

Materials. Commercial materials and vendors were as follows: IAA, Aldrich; [^{14}C]-labeled IAA, ICN Radiochemicals; FNPS and DFNB, Pierce Chemical Co.; ammonium bicarbonate, Research Organics Inc.; solvents for HPLC, Burdick and Jackson; Spectra/Gel AcA202, Spectrum; L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, Worthington. Ru5P was generated in situ from ribose 5-phosphate and phosphoriboisomerase (Pontremoli & Mangiarotti, 1962).

Spinach PRK was purified to electrophoretic homogeneity as previously described (Porter et al., 1986, 1988). The purified PRK was stored at −80 °C in a pH 8.0 buffer of 50 mM Bicine–1 mM EDTA–20% (v/v) glycerol–10 mM DTT at a protein concentration of ≥5 mg/mL. The kinase, qantified on the basis of the extinction coefficient (1%, 1-cm cell) at 280 nm of 7.2 (Porter et al., 1986), displayed a specific activity of at least 400 units/mg in a spectrophotometric assay in which generation of ADP is coupled to NADH oxidation via phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase (Porter et al., 1986; Racker, 1957). The Cys16 → Ser site-directed mutant of PRK was prepared and partially purified as previously described (Milanez et al., 1991).

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[1] Abbreviations: IAA, iodoacetic acid; PRK, phosphoribulokinase; Ru5P, ribulose 5-phosphate; FNPS, 4,4′-difluoro-3,3′-dinitrodiphenyl sulfone; DFNB, 1,5-difluoro-2,4-dinitrobenzene; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol.

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Treatment of PRK with Cross-Linking Reagents. Stock solutions of PRK were freed of exogenous thiols by gel filtration on a 1-mL (Bio-Gel P10) or a 5-mL (AcA202) column equilibrated at pH 8.0 with 50 mM Bicine–1 mM EDTA–10% (v/v) glycerol. $MgCl_2$ (5 mM) was also included in the buffer when the protective effects of substrates were to be examined. Derivatization of PRK by the cross-linking reagent was carried out in the same buffer as used for gel filtration. In the cases of kinetic experiments, the concentrations of reactants and additives (if any) are specified in the figure legends. In larger-scale experiments designed to identify the sites of modification, the PRK concentration was 2 mg/mL (50 μM in active sites); the reagent was present at a <10% molar excess. All reaction mixtures contained 1% (v/v) acetonitrile, the solvent used to prepare stock solutions of reagents. Periodically, aliquots of reaction mixtures were assayed for PRK activity.

Gel Electrophoresis. PRK that had been inactivated with the cross-linking reagent was examined by electrophoresis on a denaturing (12.5% acrylamide) gel with a PhastGel apparatus from Pharmacia. Sample and electrophoretic buffers were devoid of thiols to avoid reversibility of derivatization. Protein bands were visualized with Coomassie blue.

Carboxymethylation of PRK. Subsequent to inactivation of PRK by the cross-linking reagent, the remaining sulfhydryl groups were carboxymethylated as follows. The reaction mixture was cooled to 4 °C and, in rapid succession, adjusted to 1 mM 2-ME, 15 mM IAA (from a pH 8.0 stock of 0.5 M [^{14}C]IAA, 0.9 Ci/mol), and 4 M guanidine (from a pH 8.0 stock of 8 M guanidine hydrochloride–3 mM EDTA–100 mM K_2HPO_4). After 15–30 min on ice in the dark, the solution was dialyzed overnight against 50 mM NH_4HCO_3–1 mM 2-ME (pH 8.0). Thereafter, extensive dialysis was continued against 50 mM NH_4HCO_3 lacking added thiol; the sample was subsequently lyophilized and then digested with trypsin (3% the weight of PRK) in 25 mM NH_4HCO_3 (pH 8.0) for 24 h at 37 °C. The digest was stored at –80 °C.

Peptide Analysis and Purification. HPLC was accomplished with a unit from Laboratory Data Control. To gauge the selectivity of the cross-linking reagents and to ascertain which cysteinyl residues were targeted, small aliquots of tryptic digests were examined by reverse-phase chromatography on a Lichrosorb RP8 (5μ, C_8) column (4.6 × 250 mm, Alltech Associates Inc.). The column effluent was monitored for radioactivity to identify peptides that contained carboxymethylcysteinyl residues and monitored at 315 or 328 nm to identify peptides that were labeled with FNPS or DFNB, respectively.

To obtain sufficient quantities of chromophoric peptides for sequence analysis, a two-column purification regimen was devised. A 400–600-μg aliquot of the tryptic digest was applied to a TSK-DEAE 5PW column (7.5 × 75 mm, Supelco, Inc.), equilibrated with 20 mM NH_4HCO_3 (pH 8.0)–20% (v/v) CH_3CN, and eluted with a gradient of NH_4HCO_3 from 20 to 500 mM. The chromophoric peptide was collected and concentrated to ~200 μL under a stream of N_2 (at 40 °C). The residual solution was diluted with H_2O to ~2 mL and then lyophilized. The material was redissolved in ~400 μL of 50 mM NH_4HCO_3–4 M urea (pH 8.0) and chromatographed on a Lichrosorb RP8 column (4.6 × 250 mm) (see legend to Figure 5). The chromophoric fraction was concentrated to ~200 μL under a stream of N_2 (at 40 °C) just prior to sequence analysis. Edman degradation was performed with an automated Applied Biosystems 470A gas-phase sequencer equipped with a 120A analyzer/900A control module.

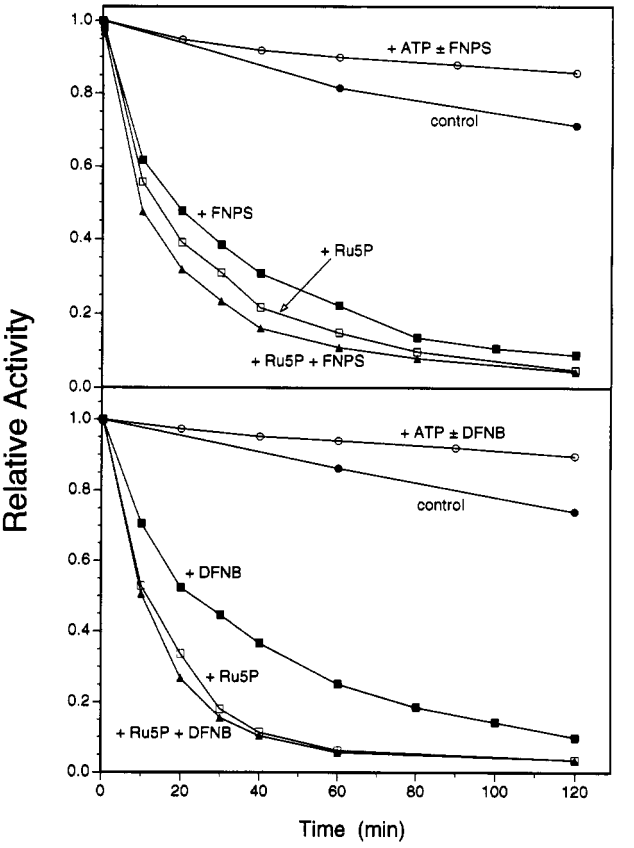


FIGURE 1: Inactivation of PRK (8 μg/mL; 0.2 μM active site) at 4 °C by 0.22 μM FNPS (top panel) and 0.22 μM DFNB (bottom panel). Final concentrations of MgATP and Ru5P are 1.2 and 3 mM, respectively.

RESULTS

Characteristics of the Reactions of PRK with Cross-Linking Reagents. Incubation of PRK with stoichiometric levels of FNPS or DFNB at room temperature results in essentially complete inactivation within 4 min (data not shown). To follow the time courses of inactivation more reliably, reaction mixtures have been examined at ice-bath temperatures (Figure 1). ATP affords substantial protection against inactivation by either reagent and also minimizes the spontaneous loss of kinase activity that occurs in the absence of exogenous thiols, due to oxygen sensitivity. By contrast, Ru5P appears to accelerate the rate of inactivation of PRK by the cross-linking reagents; however, these effects are due primarily to the enhanced sensitivity of PRK to oxygen in the presence of Ru5P (Figures 1 and 2). Since the Ru5P-induced inactivation is substantially reversed by DTT (Figure 2), it probably reflects normal oxidative deactivation entailing disulfide bond formation between Cys16 and Cys55.

Thiolysis of S-arylated cysteine is known to occur under mild conditions with respect to pH and temperature (Shaltiel, 1967). Thus, to gauge in preliminary fashion whether sulfhydryl groups of PRK are targeted by FNPS or DFNB, samples of the derivatized enzyme have been exposed to high concentrations of thiols. In the presence of 10 mM DTT at pH 8.0 and 4 °C during a 68-h incubation period, about 30% and 90% of the original PRK activity is regained by the FNPS- and DFNB-inactivated samples, respectively (data not shown).

Given the goal of deducing the intrasubunit distance between the sulfhydryl groups of Cys16 and Cys55, any cross-links

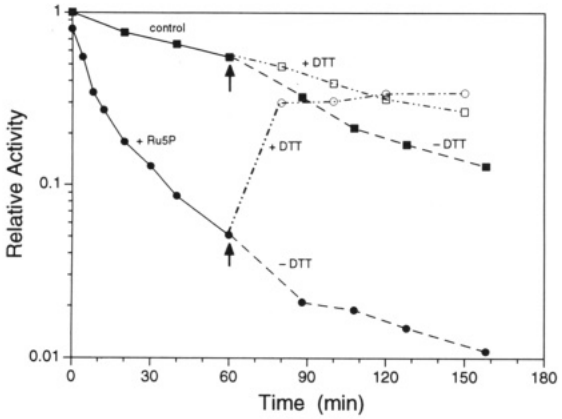


FIGURE 2: Ru5P (3.0 mM) enhanced oxidation of PRK (2 μM) at 25 °C. Arrows denote addition of DTT (10 mM final) or buffer (-DTT).

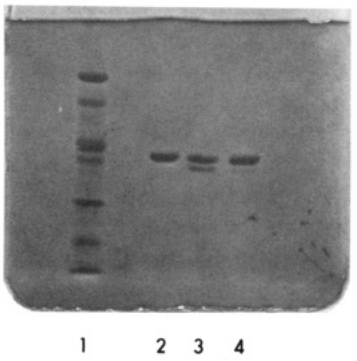


FIGURE 3: SDS–PAGE of PRK samples (0.1 μg/lane). Lane 1, molecular markers; lane 2, control; lane 3, FNPS-inactivated; lane 4, DFNB-inactivated. Molecular markers (Bio-Rad) are phosphorylase *b*, 92.5 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa.

formed between subunits or between molecules would be irrelevant or misleading. Hence, prior to characterization of inactivated PRK, samples were inspected by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. To avoid the possibility of partial reversal of the chemical derivatizations and thereby obscuring cross-linking that might have occurred, thiols were excluded from all solutions used in conjunction with sample preparation and electrophoresis. As seen in Figure 3, samples of PRK inactivated by either reagent lack species larger than the monomeric subunit (40 000 Da), which would have reflected intersubunit or intermolecular cross-linking.

Site of Derivatization. The previously established unusually high nucleophilicity of Cys16 forecasts this residue as one of the likely targets for the cross-linking reagents (Omnaas et al., 1985; Porter & Hartman, 1988; Porter et al., 1988). Consistent with the realization of this expectation, the Cys → Ser site-directed mutant of PRK is resistant to both reagents, even at 5-fold molar excesses (data not shown). To extend this indirect and partial indication of reagent selectivity, the entire array of sulfhydryl groups remaining after derivatization of PRK by the cross-linking reagents has been examined. The inactivated PRK is carboxymethylated with [^{14}C]IAA to label sulfhydryl groups; subsequently, a tryptic digest is resolved by reverse-phase chromatography, and the profile is compared to that from a control in which all four sulfhydryls are carboxymethylated. The upper panel in Figure 4 illustrates the complete separation of the four carboxymethylcysteinyl-containing peptides derived from the control; the denoted residue identification is based on previous characterization of

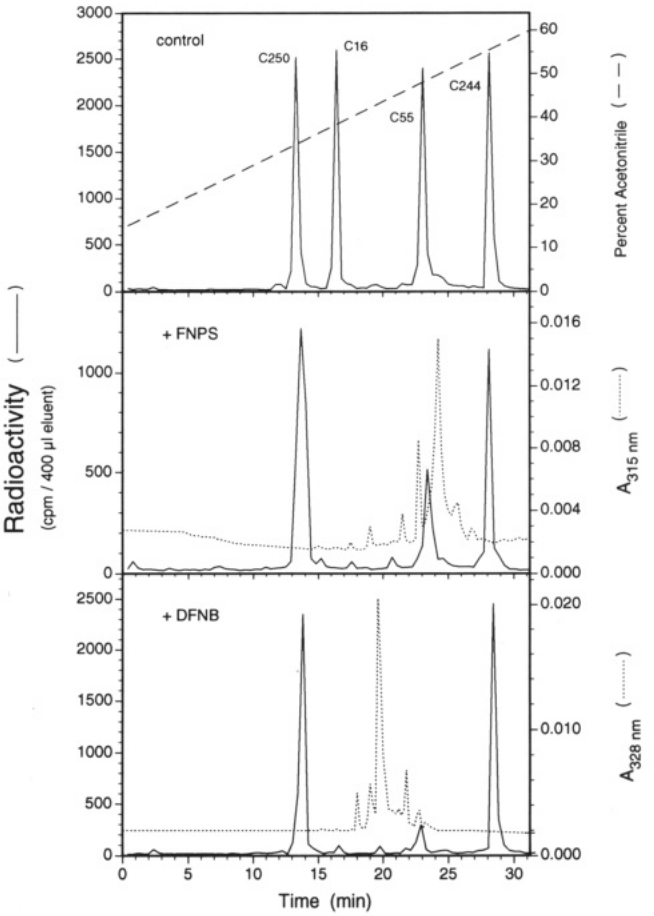


FIGURE 4: [^{14}C]Carboxymethylcysteinyl tryptic maps of PRK (~50 μg) (top panel), FNPS-labeled PRK (~30 μg) (middle panel), and DFNB-labeled PRK (~50 μg) (bottom panel). The peak labels in the top panel denote the position of the cysteinyl residue in the primary structure, contained within the radiolabeled peptide. Each sample was applied to the C_8 column equilibrated with 15% (v/v) CH_3-CN–0.1% (v/v) TFA and eluted with a linear gradient (displayed only in the top panel) up to 60% (v/v) CH_3CN–0.1% (v/v) TFA at a flow rate of 1 mL/min. The selectivity of derivatization by each reagent is indicated by the absorbancy profile at the specified wavelength (center and bottom panels).

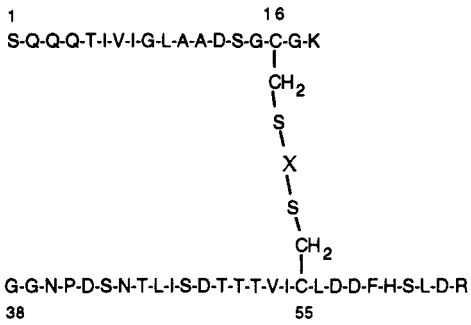
each labeled peptide (Porter et al., 1988). Digests of both FNPS- and DFNB-inactivated PRK are completely lacking in the peptide that represents carboxymethylated Cys16, confirming the sulfhydryl of Cys16 as one site of modification. Relative to control sample, the quantity of the peptide that represents carboxymethylated Cys55 is also greatly diminished in the digests, 3-fold in the case of the FNPS-treated PRK and 5-fold with the other reagent. These profiles are compatible with the quantitative modification of Cys16 and efficient, but incomplete, cross-linking to Cys55 by each of the two reagents.

A high degree of selectivity in modification of PRK by both reagents, as judged by monitoring for chromophoric peptides (Figure 4), is not surprising in view of the stoichiometric levels of reagent that were used. The minor chromophoric peaks observed could reflect low-yield cross-links of Cys16 to residues other than Cys55 and/or partial monofunctional modification of Cys16 as a consequence of displacement of one fluorine atom of the reagent by water.

Preparative isolation of chromophoric peptides for sequence analysis was achieved by consecutive anion-exchange and reverse-phase chromatography. An anion-exchange chromatogram of the total tryptic digest of the DFNB-labeled sample is illustrated in Figure 5 (top panel). The corresponding

chromatogram for the digest of FNPS-treated PRK appears virtually identical and is thus not shown. Reverse-phase profiles of the major chromophoric peptide, obtained from the anion-exchange column, are displayed in the center and bottom panels of Figure 5. Recoveries of chromophoric peptides were ~50% from each column.

Automated Edman degradation of the FNPS- and DFNB-labeled peptides (Tables I and II), in conjunction with the known sequence of PRK (Milanez & Mural, 1988; Roesler & Ogren, 1988), are consistent with the following structure in which X denotes the dinitrophenyl or the dinitrodiphenyl sulfone moiety:



The sequence data indicate one segment to be derived from residues 1–18 (position 18 is occupied by a lysyl residue and thus provides a site for tryptic cleavage) and the other segment to represent residues 38–64 (consistent with tryptic cleavages at Lys37 and Arg64). Similar amounts of two different PTH-amino acids are observed at each cycle through cycle 17; absence of a second identifiable PTH-amino acid of cycles 16 and 18 presumably reflects the presence of the modified cysteinyl residue.

DISCUSSION

Among chloroplastic enzymes that are regulated by thioredoxin, PRK appears unusual in that both sulfhydryls are located in the active-site region but are rather far removed (by 39 residues) in the primary sequence. By contrast, the regulatory cysteinyl residues of fructose-1,6-bisphosphatase (Marcus et al., 1988), NADP-dependent malate dehydrogenase (Decottignies et al., 1988; Scheibe et al., 1991), and the γ-subunit of chloroplastic coupling factor (Miki et al., 1988) are distinct from the active site, yet proximal in sequence. In the case of the well-characterized fructose-1,6-bisphosphatase, the pertinent sulfhydryls are only five residues apart (RCVVNVCG), a situation similar to the redox site of thioredoxin (WCGPCK). Given the remoteness of the regulatory and catalytic site of the bisphosphatase, the modulation of enzymatic activity via oxidation–reduction is most readily explained by the intersite transmission of conformational changes. Indeed, significant conformational alterations of the bisphosphatase, as monitored by intrinsic fluorescence, are known to accompany regulatory thiol–disulfide exchange (Pradel et al., 1981; Stein et al., 1989).

The present study represents a continuation of our efforts to define the relative contributions of direct and indirect effects to the oxidation-dependent activation state of PRK. Site-directed mutagenesis has shown that Cys55 modestly facilitates catalysis (Hudson et al., 1992; Milanez et al., 1991), thereby providing a partial explanation for the catalytic deficiencies of the disulfide form of PRK. By deduction, oxidation-induced conformational changes may be invoked to account for the remaining portion of the deactivation. Heretofore, however, observations that would be indicative of conformational differences between the oxidized and reduced enzyme have not been reported.

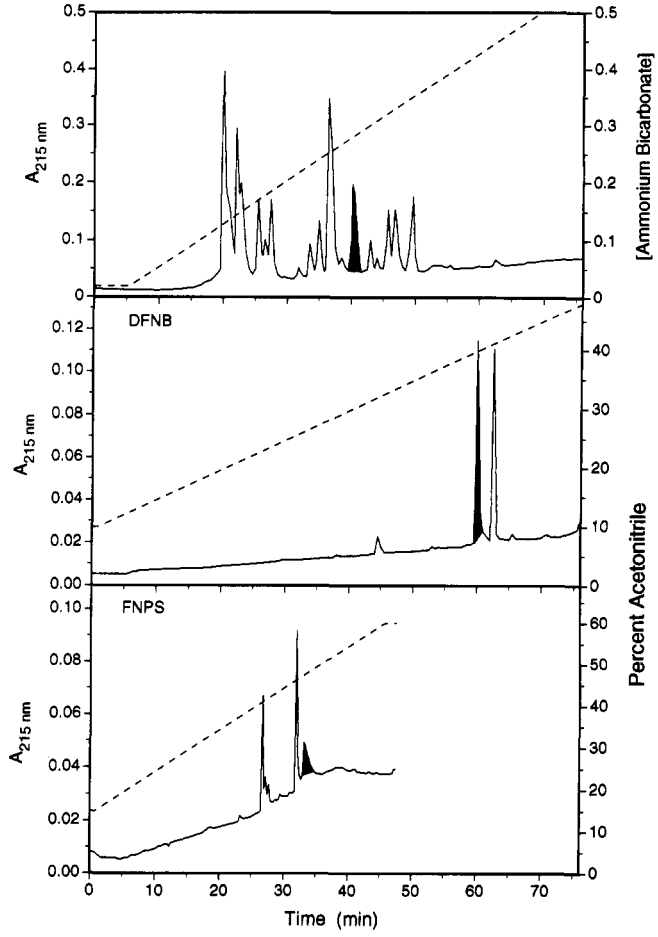


FIGURE 5: Purification of chromophoric peptides for sequence analysis. Anion-exchange chromatography of DFNB-labeled PRK digest (~500 μg) is shown in the top panel. The major chromophoric peak obtained from the DEAE column was subsequently chromatographed on a C_8 column (see Experimental Procedures) equilibrated with 10% CH_3CN(v/v)–0.1% (v/v) TFA and eluted with a linear gradient developed at 0.5%/min with a flow rate of 1 mL/min (center panel). The major chromophoric peak obtained from chromatography of the FNPS-labeled digest on the DEAE column (profile not shown) was also subsequently processed on the C_8 column (bottom panel). In this case, the reverse-phase column was equilibrated with 15% CH_3CN (v/v)–0.1% (v/v) TFA and eluted with a linear gradient developed at 1%/min at a flow rate of 1 mL/min. In each panel, the chromophoric peptide is indicated by a solid peak.

On the basis of a variety of chemical modification data, Cys16 is more nucleophilic and more solvent-accessible than is Cys55 (Porter & Hartman, 1990; Porter et al., 1988, 1990). Thus, the initial step in the oxidation process would seem to be "activation" of the β-sulfhydryl at position 16. Oxidation by molecular oxygen is envisioned to proceed via a sulfenic acid intermediate. Oxidation by a disulfide, thioredoxin in vivo (Buchanan, 1984, 1991; Crawford et al., 1989) or 5′,5-dithiobis(2-nitrobenzoate) in vitro (Porter et al., 1988), entails formation of a mixed disulfide intermediate. Irrespective of oxidant, completion of the disulfide in PRK dictates that the participant sulfur atoms in the activated intermediate must be positioned within bonding distance (<3.5 Å). The question then becomes whether activation of Cys16 induces a conformational change, which lessens the interresidue distance between Cys16 and Cys55, or whether the two sulfhydryls in the reduced enzyme are already within van der Waals radii.

As judged by highly selective and extremely rapid chemical modification of Cys16, general refractiveness of Cys55, and

Table I: Sequence Analysis of the Major FNPS-Labeled Peptide[a]

cycle	PTH-amino acid	pmol	cycle	PTH-amino acid	pmol	cycle	PTH-amino acid	pmol
1	Ser	550[b]	10	Leu	878	19	Leu	176
	Gly	1279		Ile	583			
2	Gln	1034	11	Ala	747	20	Asp	138
	Gly	1155		Ser	147[b]			
3	Gln	1062	12	Ala	876	21	Asp	190
	Asn	1067		Asp	261			
4	Gln	944	13	Asp	456	22	Phe	110
	Pro	872		Thr	212			
5	Thr	580	14	Ser	143[b]	23	His	29
	Asp	630		Thr	285			
6	Ile	783	15	Gly	285	24	Ser	24[b]
	Ser	289[b]		Thr	278			
7	Val	779	16	–	–	25	Leu	79
	Asn	544		Val	203			
8	Ile	785	17	Gly	273	26	Asp	77
	Thr	396		Ile	194			
9	Gly	599	18	Lys	132	27	Arg	12
	Leu	622		–	–			

[a] An estimated 2 nmol of peptide was loaded into the sequencer reaction flask. [b] Due to its partial decomposition, the yield of PTH-Ser is diminished.

Table II: Sequence Analysis of the Major DFNB-Labeled Peptide[a]

cycle	PTH-amino acid	pmol	cycle	PTH-amino acid	pmol	cycle	PTH-amino acid	pmol
1	Ser	156[b]	10	Leu	508	19	Leu	78
	Gly	319		Ile	234			
2	Gln	344	11	Ala	213	20	Asp	53
	Gly	336		Ser	62[b]			
3	Gln	352	12	Ala	287	21	Asp	72
	Asn	333		Asp	132			
4	Gln	308	13	Asp	184	22	Phe	32
	Pro	485		Thr	116			
5	Thr	196	14	Ser	45[b]	23	His	12
	Asp	235		Thr	115			
6	Ile	237	15	Gly	75	24	Ser	9[b]
	Ser	92[b]		Thr	115			
7	Val	256	16	–	–	25	Leu	43
	Asn	239		Val	71			
8	Ile	248	17	Gly	77	26	Asp	34
	Thr	174		Ile	61			
9	Gly	175	18	Lys	27	27	–	nd[c]
	Leu	404		–	–			

[a] An estimated 0.7 nmol of peptide was loaded into the sequencer reaction flask. [b] Due to its partial decomposition, the yield of PTH-Ser is diminished. [c] Not detected.

resistance of the Cys16 → Ser mutant to inactivation by sulfhydryl reagents, we conclude that Cys16 is the initial target of both bifunctional reagents. Completion of the Cys16–Cys55 cross-link by DFNB (with a 3.5-Å span) may be viewed as a predictable consequence of the fact that, in the reduced enzyme and/or in the intermediate (i.e., mixed disulfide) leading to the disulfide form of the enzyme, the sulfhydryls from Cys16 and Cys55 must approach bonding distance. Significantly, Cys16 and Cys55 are also efficiently cross-linked by FNPS (despite its considerably greater span of 9 Å), thereby providing direct demonstration of a previously unrecognized conformational flexibility of reduced (activated) PRK. Even if the initial targeting of Cys16 by FNPS induces a conformational change necessary for cross-linking to a group 9 Å removed, it is reasonable to envision similar conformational change resulting from mixed disulfide formation between Cys16 and thioredoxin as occurs in vivo. Futhermore, any conformational change induced by arylation of Cys16 must be reversible based on the observed restoration of kinase activity during incubation of the cross-linked, inactivated enzyme with DTT. Thus, irrespective of distinct conformers of fully reduced PRK versus induced conformational changes concomitant with modification of Cys16, the variability of inter-residue distances as now shown with crosslinkers must reflect conformational flexibility.

The demonstrated conformational flexibility in the polypeptide segment that encompasses both Cys16 and Cys55 may be crucial to both regulation and catalytic turnover. In vivo modulation of the kinase activity requires interaction and subsequent mixed disulfide formation between PRK and thioredoxin *f*. Flexibility at the regulatory site may be a prerequisite to accommodate productive binding of thioredoxin. Efficient cross-linking of Cys16 and Cys55 by reagents of greatly different lengths, reflective of a mixed population of conformers, also provides additional insight into the underlying basis for the absence of kinase activity associated with the oxidized form of the enzyme. The conformer in which the regulatory sulfhydryls are within bonding distance may be inherently inactive; completion of the disulfide would then "lock" the protein into the catalytically incompetent conformation.

On the basis of protection by ATP against derivatization and against oxidation, Cys16 and Cys55 of PRK have both been assigned to the nucleotide binding domain of the active site (Krieger et al., 1987; Krieger & Miziorko, 1986; Porter & Hartman, 1986, 1990). As expected, then, ATP retards the rate of inactivation of PRK by the bifunctional reagents. However, Ru5P (which binds at the sugar phosphate domain of the active site) accelerates spontaneous oxidtion of PRK. The latter observation is also consistent with conformational

flexibility of the regulatory site, whereby binding of Ru5P stabilizes the conformation in which the reactive sulfhydryls are proximal.

Since the regulatory sulfhydryls of PRK are located in the active-site region, conformational flexibility could play a role in catalytic turnover. The importance of mobile loops in controlling ligand access to the active site and in dictating the proper reaction pathway has been recognized with a number of enzymes (Clarke et al., 1986; Gerstein & Chothia, 1991; Hedstrom et al., 1992; Joseph et al., 1990; Knight et al., 1990; Pompliano et al., 1990; Tanaka et al., 1992). Cys16 of PRK is located within a consensus sequence defining the P-loop, GXXXXGK(S/T), common to many ATP-binding proteins (Saraste et al., 1990). Two crystal structures of adenylate kinase, which correspond to free and substrate-bound forms of the enzyme, show that the P-loop can adopt different conformations (Dreusicke & Schulz, 1988). In addition, algorithms of secondary structure prediction, executed by DNASTAR software, according to the rules of Chou–Fasman (Chou & Fasman, 1974), Garnier–Robson (Garnier et al., 1978), and Karplus–Schuylz (Karplus & Schulz, 1985), all idicate mobilty or a turn in the region of position 34–40 in the primary sequence of PRK. If viewed as a hinge, this region could account for conformational flexibility that allows for cross-linking of the Cys16 and Cys55 sulfhydryls by reagents of substantially differing dimensions. Collectively, our new results, in conjunction with secondary structure speculations, argue strongly for the concept of conformational flexibility within the regulatory/catalytic domain of PRK.

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