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Commonality of Catalytic and Regulatory Sites of Spinach Phosphoribulokinase: Characterization of a Tryptic Peptide That Contains an Essential Cysteinyl Residue†

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ABSTRACT: Phosphoribulokinase catalyzes the ATP-dependent synthesis of ribulose bisphosphate in the illuminated, chloroplast stroma. The enzyme is regulated in vivo by thioredoxin-mediated oxidation/reduction of sulfhydryls/disulfides. One sulfhydryl group of the kinase from spinach is preferentially alkylated by (bromoacetyl)ethanolamine phosphate, leading to complete loss of enzyme activity, concomitant with the incorporation of only 1 molar equiv of reagent. The tryptic peptide containing the alkylated cysteine has been purified, sequenced, and observed to be derived from the amino terminus of the kinase. The highly reactive cysteinyl residue occupies position 16 of the primary structure. The protection against alkylation afforded by ATP corresponds to a dissociation constant of $\sim 30\ \mu M$, similar to the K_m for ATP and hence supporting an assignment of cysteine-16 to the active site. Several lines of evidence suggest that the active site sulfhydryl group is involved in the thioredoxin-mediated regulation of the kinase. The oxidatively deactivated kinase does not react with (bromoacetyl)ethanolamine phosphate. ATP, which protects against alkylation, also protects against oxidation with an apparent dissociation constant of $\sim 25\ \mu M$. The pH dependencies of both alkylation and oxidation reflect a pK_a of ~ 7.8, consistent with the involvement of the same sulfhydryl in both processes.

Phosphoribulokinase (PRK;[1] EC 2.7.1.19) catalyzes the ATP-dependent phosphorylation of ribulose 5-phosphate to yield ribulose 1,5-bisphosphate, the primary CO_2 acceptor in photosynthetic organisms. The plant enzyme is regulated by metabolites (Anderson, 1973; Gardemann et al., 1983), energy charge (Lavergne et al., 1974; Laing et al., 1981), and light (Avron & Gibbs, 1974; Fischer & Latzko, 1979; Buchanan, 1980). Regulation by light entails electron flow from ferredoxin to thioredoxin to the oxidized kinase, thereby reducing a disulfide of the enzyme and restoring catalytic activity (Wolosiuk & Buchanan, 1978). Not surprisingly, the reduced, activated kinase is quite sensitive to thiol reagents (Hurwitz et al., 1956; Omnaas et al., 1985); however, only in the case of one alkylating agent, ^{14}C-labeled (bromoacetyl)ethanolamine phosphate, was inactivation demonstrated directly to be a consequence of preferential modification of a cysteinyl residue. This highly reactive cysteinyl residue, perhaps one of those involved in the thioredoxin-mediated regulation, appears to be located at or near the catalytic site on the basis of protection against inactivation that is afforded by ATP (Omnaas et al., 1985).

Except for a single recent report (Krieger & Miziorko, 1986) in which 5′-[(fluorosulfonyl)benzoyl]adenonsine was explored as a potential site-specific reagent, chemical modification studies of the kinase have been carried out with very crude preparations. Hence, the degree of specificity of sulfhydryl group modification and the subsequent identity of the essential cysteinyl residue at the sequence level were not established. With the recent availability of homogeneous spinach PRK (Krieger & Miziorko, 1986; Porter et al., 1986), we have undertaken chemical characterization of the enzyme after its derivatization by BrAcNHEtOP. In this paper, we demonstrate a correlation between inactivation and selective alkylation of one sulfhydryl group, which is subsequently identified by isolation of the predominant labeled peptide. We also report the pH dependencies and the kinetics of ATP protection of both the alkylation reaction and the oxidative deactivation of the kinase. The results are compatible with the assignment of Cys-16 to the catalytic and regulatory sites of PRK.

Materials and Methods

Spinach PRK, a homodimer of 45 000-Da subunits, was purified as described by Porter et al. (1986). ^{14}C-Labeled BrAcNHEtOP was synthesized by the procedure of Hartman et al. (1973) and had a specific radioactivity of 1700 cpm/nmol. Commercial materials and vendors were as follows: substrates, Bicine, and coupling enzymes for assaying the kinase were from Sigma Chemical Co.; reagents for amino acid analysis were from Beckman Instruments or Pierce Chemical Co.; reagents for automated Edman degradation were from Applied Biosystems; TPCK-trypsin was from Millipore; ultrapure guanidine hydrochloride was from Schwarz/Mann; iodoacetic acid was from Aldrich; trifluoroacetic acid was from Pierce Chemical Co.; HPLC-grade water and acetonitrile were

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[1] Abbreviations: PRK, phosphoribulokinase; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; DTT, dithiothreitol; BrAcNHEtOP, (bromoacetyl)ethanolamine phosphate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; PTH, phenylthiohydantoin; Cys(Cm), *S*-(carboxymethyl)cysteine; HPLC, high-performance liquid chromatography; DEAE, diethylaminoethyl.

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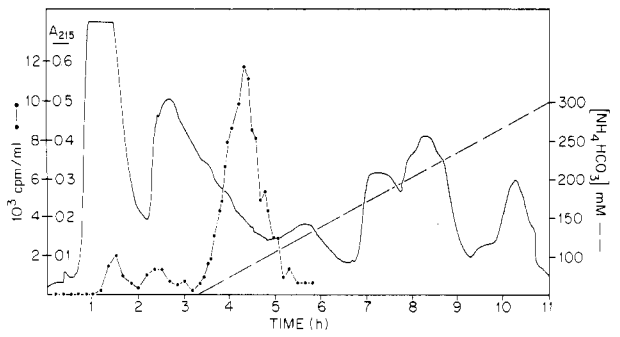

FIGURE 1: DEAE-cellulose chromatography of a tryptic digest of PRK that had been inactivated with ^{14}C-labeled BrAcNHEtOP and *S*-carboxymethylated with iodoacetic acid. Details are provided in the text.

from Burdick and Jackson; DEAE-cellulose (DE-52) was from Whatman; reagents for electrophoresis were from Bethesda Research Laboratories.

Assay of PRK. The coupled, spectrophotometric assay of Racker (1957), with slight modification (Porter et al., 1986), was used at pH 7.9 and 25 °C to monitor catalytic activity. In a standard 1-cm cuvette, one unit of enzyme activity corresponds to an absorbancy change at 340 nm of 6.22/min. The kinase used in this study had a specific activity of 450 units/mg, and its concentration was determined by the absorbance at 280 nm with $\epsilon^{1\%}$ of 7.2 (Porter et al., 1986).

Correlation of Inactivation and Incorporation of BrAcNHEtOP. Prior to alkylation, the kinase was dialyzed at 4 °C against 50 mM bicine/40 mM KCl/5 mM $MgCl_2$/0.5 mM EDTA/10 mM DTT/10% (v/v) glycerol (pH 8.0), and the protein concentration was then adjusted to 12.7 mg/mL (0.28 mM subunit). At 25-min intervals, five aliquots of 50 mM BrAcNHEtOP were added to 0.85 mL of the kinase solution at 4 °C to give a final reagent concentration of 1 mM. Periodically, appropriately diluted aliquots of the reaction mixture were assayed for kinase activity, and separate 5-μL aliquots (undiluted) were applied to 2.3-cm disks of Whatman 3MM paper (Bollum, 1968). The disks were immediately submerged in ice-cold 10% (w/v) trichloroacetic acid with stirring. Disks were successively washed twice with 300 mL each of 5% trichloroacetic acid, 1:1 (v/v) diethyl ether/ethanol, and diethyl ether. Each wash was conducted on ice with stirring for 20 min. After the final wash, the disks were air-dried, placed in scintillation vials to which 10 mL of Amersham ACS scintillation fluid was added, and counted in a Packard 3225 liquid scintillation spectrophotometer.

Inactivated PRK was treated under standard conditions with 60 mM sodium iodoacetate in the presence of 4 M guanidine to carboxymethylate protein sulfhydryl groups (Schloss et al., 1978). After exhaustive dialysis against 50 mM ammonium bicarbonate (pH 8.0), the carboxymethylated protein was digested overnight at 37 °C with 1% (w/w) trypsin; a second portion of trypsin (1% w/w) was then added, and the digestion was continued for 9 additional hours. The digest was brought to 2 mM DTT and stored at −80 °C.

Purification of Labeled Peptide. A portion (3.3 mg, 74 nmol) of the tryptic digest of the BrAcNHEtOP-inactivated kinase was applied to a 5 × 160 mm column of DE-52 equilibrated with 50 mM ammonium bicarbonate/1 mM DTT (pH 8.0). The column was then washed with 70 mL of equilibration buffer and eluted with a 100-mL linear gradient of 50–300 mM ammonium bicarbonate/1 mM DTT (pH 8.0) (Figure 1). Approximately 80% of the total radioactivity applied to the column was recovered in the major labeled peak. Fractions comprising the latter half of this peak (26 nmol) were pooled and lyophilized to dryness; the residue was dissolved in water and subjected to HPLC (Figure 2). One fraction (9 nmol) from the center of the labeled peak was used for subsequent compositional and sequence analyses.

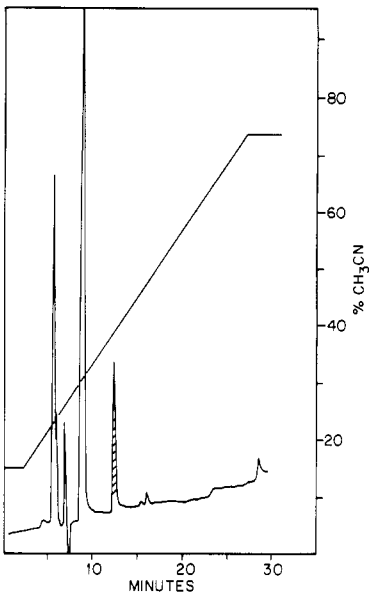

FIGURE 2: HPLC of the pooled, peak fractions obtained from the DEAE-cellulose column (see Figure 1). The labeled peptide is illustrated by cross-hatching. Absorbancy at 215 nm is shown in arbitrary units.

Amino Acid and Sequence Analyses. Total acid hydrolysis of peptides was achieved in evacuated (<50 μmHg) sealed tubes with 6 N HCl/0.01 M 2-mercaptoethanol at 110 °C for 21 h. Hydrolysates were dried on a Speed Vac concentrator (Savant Instruments Inc.) and subjected to chromatography on a Beckman 121M amino acid analyzer using Beckman's "3-hour, single-column system". The analyzer was hard wired to a PDP data acquisition system. Computer programs used in data collection and analysis were provided by S. S. Stevens and J. T. Holderman of the Oak Ridge National Laboratory.

Peptides were subjected to automated Edman degradation on a gas-phase sequencer (Applied Biosystems, Model 470A) according to the manufacturer's instructions. PTH-amino acids were identified and quantified by HPLC (Laboratory Data Control) on a C18 column (Spherisorb ODS 5 μm, 4.6 × 250 mm) run at room temperature with a flow rate of 1.5 mL/min. Two different solvent systems were used: (a) an isocratic solvent of Lottspeich (1980), comprised of 68.5% aqueous sodium acetate (0.01 M, pH 5.20), 31% acetonitrile, and 0.5% 1,2-dichloroethane; (b) a gradient of 10–50% acetonitrile in 0.1% aqueous phosphoric acid.

HPLC of Peptides. Reverse-phase chromatography of peptides was achieved with an instrument from Laboratory Data Control. Peptide mixtures (1–30 nmol) were injected onto a 0.46 × 25 cm Lichrosorb 5RP8 column (HPLC Technology) and eluted by a 15–75% (v/v) acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid (Mahoney & Hermodson, 1980). The total gradient was developed in 25 min at a flow rate of 1 mL/min. Effluents were monitored by the absorbancies at 215 nm and by determining radioactivity in collected fractions.

Protection of PRK by ATP against Alkylation and Oxidation. The rate of inactivation of PRK by BrAcNHEtOP, in the presence of different fixed levels of ATP, was examined under conditions where pseudo-first-order kinetics prevailed. Reaction mixtures (100 μL) at 4 °C contained 3.3 μM (0.15 mg/mL) kinase, 0–108 μM ATP, and 0.4 mM BrAcNHEtOP

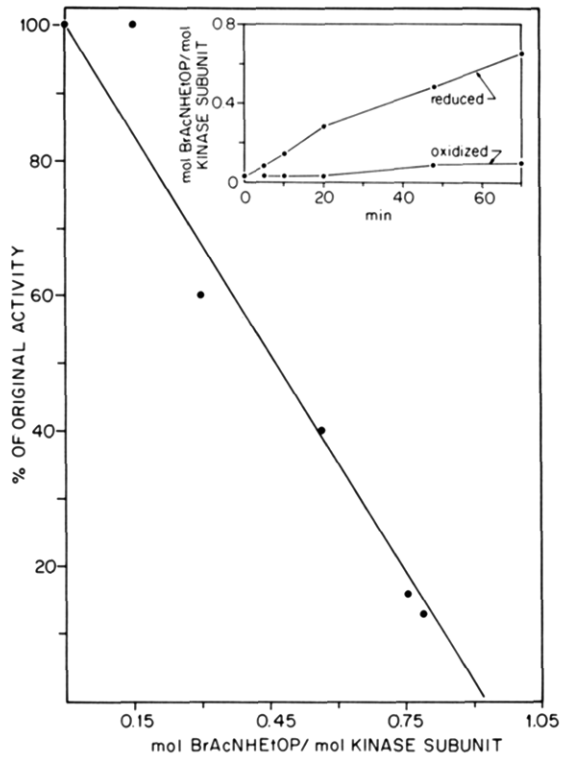

FIGURE 3: Correlation between inactivation of PRK and incorporation of ^{14}C-labeled BrAcNHEtOP. The inset shows the time courses of reagent incorporation into the fully reduced kinase (from which the correlation was determined) and into the kinase that had been deactivated by prior oxidation with dehydroascorbate. Reaction conditions are described in the text.

in 45 mM Bicine/4.5 mM $MgCl_2$/36 mM KCl/0.2 mM DTT/10% (v/v) glycerol (pH 8.0). Periodically, kinase activity was determined in appropriately diluted aliquots. The half-time of these inactivations (and those described below) was calculated by least-squares fitting of experimental data points; correlation coefficients were typically $\geq$0.95.

The protective effect of ATP against oxidation of PRK (0.5 mg/mL) by dehydroascorbate (4 mM) was examined in a very similar way, but at 25 °C due to the slow rate of oxidation as compared to alkylation. Except for the omission of DTT, the buffer was identical with that used in the alkylation experiments.

pH Dependencies of Alkylation and Oxidation Reactions. The basic buffer compositions and temperatures were the same as those described above for the ATP-protection experiments; the ionic strengths of all buffers (pH 7.2–8.3) were matched by the addition of KCl. Kinase concentrations of 0.3 and 0.8 mg/mL were used in the alkylation ([BrAcNHEtOP] = 0.4 mM) and oxidation ([dehydroascorbate] = 4 mM) reactions, respectively. Suitable aliquots of the reaction mixtures (100 μL) were periodically diluted and assayed to determine the half-times of inactivations.

RESULTS

Specificity of Modification of RPK by BrAcNHEtOP. The relationship between the loss of kinase activity and the extent of protein alkylation by BrAcNHEtOP is linear. The level of incorporation, extrapolated to total inactivation, is 0.9 molar equiv of reagent/mol of subunit (Figure 3). If the enzyme is first deactivated by oxidation with dehydroascorbate, little alkylation occurs (Figure 3, inset).

The high degree of site specificity as implied by the 1:1 stoichiometry in the reaction of ^{14}C-labeled BrAcNHEtOP with the kinase is confirmed by nondenaturing polyacrylamide gel electrophoresis of partially inactivated enzyme (Figure 4) and by peptide mapping of the inactivated enzyme (Figures 1 and 5). Since PRK is a homodimer (presumably with two active sites) and since the reagent moiety introduced contains the dianionic phosphate group, electrophoresis should resolve the fully inactivated enzyme having both subunits modified from the partially inactivated enzyme having a single subunit modified. This expectation is met upon electrophoresis, under nondenaturing conditions, of kinase samples inactivated to varying extents (Figure 4). A band of intermediate mobility between that of native and fully inactivated enzyme is readily

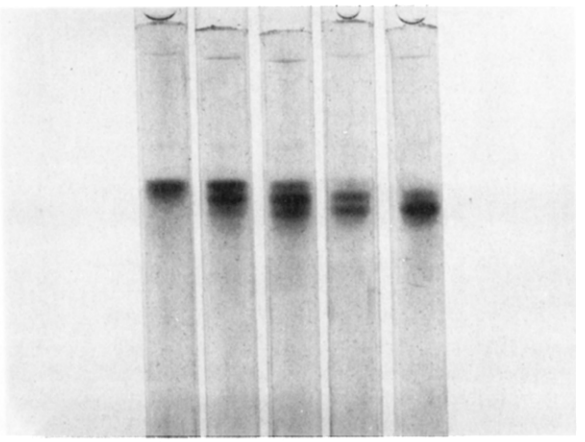

FIGURE 4: Nondenaturing polyacrylamide gel electrophoresis of PRK at various levels of inactivation by BrAcNHEtOP. Electrophoresis was conducted in 7.5% acrylamide/0.25% bis(acrylamide) gels with the buffers of Laemmli (1970) without sodium dodecyl sulfate. Samples (from the left) exhibited 100, 75, 50, 25, and 6% of original enzymic activity, respectively.

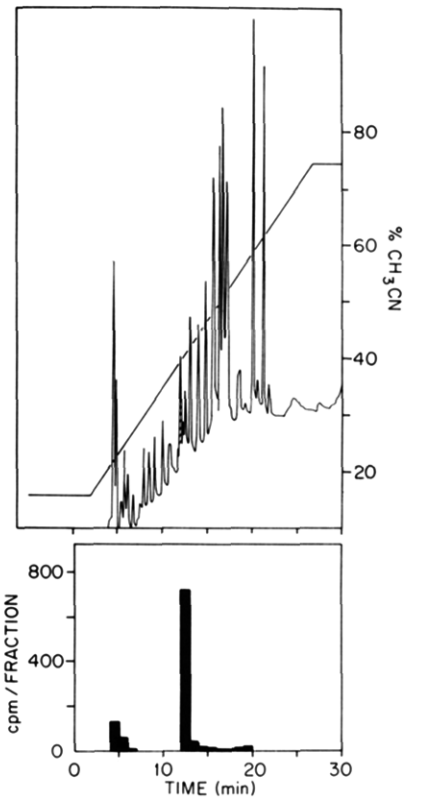

FIGURE 5: HPLC of a total tryptic digest of PRK after inactivation by ^{14}C-labeled BrAcNHEtOP followed by *S*-carboxymethylation with iodoacetate. One-minute fractions were collected directly into scintillation vials for counting. The upper pannel shows the absorbance profile at 215 nm in arbitrary units; the major labeled peptide is indicated by cross-hatching. The lower panel shows the total distribution of radioactivity in the profile.

discerned, and the relative amounts of the three bands as a function of activity remaining are clearly consistent with a dimeric protein structure. On the basis of densitometry scans of gels and subsequent determinations of radioactivity associated with each band in sliced gels, the protein species of intermediate mobility has a specific radioactivity only half as great as that of the most rapidly migrating protein species (data not shown). If the overall incorporation of 1 molar equiv of reagent/mol of subunit reflected multiple sites of derivitization in low yields, a more complex pattern of electrophoretic heterogeneity would likely prevail.

Fractionation of total tryptic digests of the labeled kinase by DEAE-cellulose chromatography (Figure 1) and by HPLC (Figure 5) reveals a single, major radioactive peptide. In the latter case, 80% of the radioactivity applied to column is recovered in the major peak, thus excluding the possibility that any significant site of modification has been overlooked.

Characterization of Labeled Peptide. The purified, ^{14}C-labeled peptide obtained by HPLC (Figure 2) has been subjected to compositional and sequence analyses. Amino acid analysis reveals the following composition: Cys-(Cm)$_1$Asx$_1$Thr$_1$Ser$_2$Glx$_3$Gly$_3$Ala$_2$Val$_1$Ile$_2$Leu$_1$Lys$_1$. By collection of fractions from the amino acid analyzer and counting them, all of the radioactivity in the hydrolysate is found in association with Cys(Cm), thus confirming the earlier conclusion that a sulfhydryl group of the kinase is the target for alkylation by BrAcNHEtOP (Omnaas et al., 1985). Since alkylation of a protein by BrAcNHEtOP introduces a substituted carboxamidomethyl moiety, acid hydrolysis liberates any alkylated residue as a carboxymethyl amino acid.

Edman degradation of the labeled peptide, which confirmed its homogeneity, establishes the sequence:

$$\overset{5}{\text{NH}_2\text{-Ser-Gln-Gln-Gln-Thr}}\text{-Ile-Val-}\overset{10}{\text{Ile-Gly-Leu}}\text{-Ala-Ala-}$$

$$\overset{15}{\text{Asp-Ser-Gly-Cys}}^{*}\text{-Gly-Lys}$$

The site of alkylation is denoted as Cys*, which is inferred by a gap in the Edman degradation due to the insolubility of PTH-Cys-AcNHEtOP in the extraction solvent. The sequence presented matches that of the NH_2-terminal region of the kinase in which Cys was positively identified at position 16 (Porter et al., 1986).

Protective Effect of ATP against Alkylation and Oxidation of PRK. Earlier work demonstrated that inactivation via alkylation or oxidation of PRK was prevented by modest concentrations (2 mM) of ATP (Omnaas et al., 1985; Porter et al., 1986). To ascertain if protection against both of these modifications reflects binding of ATP to a single site, the apparent K_D for ATP has been determined by measuring inactivation rates as a function of ATP concentration as described by Baker (1967). The apparent K_D for ATP as a protector against alkylation by BrAcNHEtOP is 29 ± 4 μM (Figure 6), and a similar value of 23 ± 5 μM is observed in the oxidation by dehydroascorbate (Figure 6). The single site inferred appears to correspond to the catalytic site, because the K_m for ATP is 50 ± 4 μM (data not shown), in agreement with a published value of 53 μM (Gardemann et al., 1983).

pH Dependency of Alkylation and Oxidation of PRK. Since both reactions involve the deprotonated form of sulfhydryls, the pH dependencies of these reaction rates can reveal the pK_a values of the participating groups. The relationship between the observed second-order rate constant (k_2) and [H^+] is provided by (Welches & Baldwin, 1981)

$$1/k_2 = [H^+]/(Kk_0) + 1/k_0$$

where k_0 is the intrinsic second-order rate constant for the deprotonated group and K is the ionization constant for that group. Plots of $1/k_2$ vs. [H^+]for both the alkylation by BrAcNHEtOP and the oxidation by dehydroascorbate of PRK kinase are linear (data not shown). From the slopes of these lines [$1/(Kk_0)$], the calculated pK_a of the reactive sulfhydryl group is 7.9 ± 0.2 in the alkylation reaction and 7.7 ± 0.15 in the oxidation reaction. Corrections for temperature differences would make these values even closer (Benesch & Benesch, 1955).

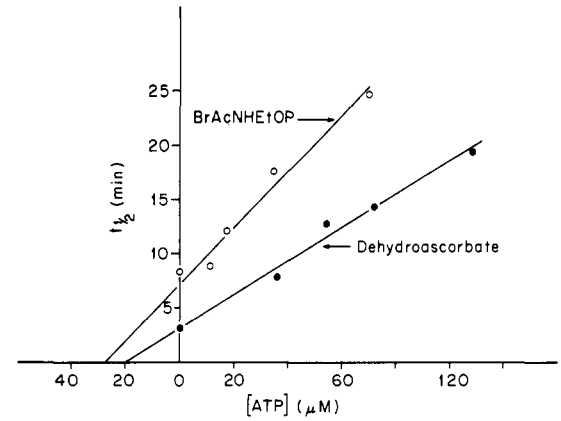

FIGURE 6: Protection of PRK by ATP against alkylation (○) or oxidation (●). Half-times of inactivation by 0.4 mM BrAcNHEtOP or 4 mM dehydroascorbate are plotted vs. the concentration of ATP present in the reaction mixtures. Experimental details are provided in the text.

Discussion

The data presented above support four major conclusions: (1) BrAcNHEtOP alkylates a single cysteinyl residue of PRK; (2) the reactive sulfhydryl is located within the ATP-binding domain of the catalytic site; (3) the active-site sulfhydryl is involved in the redox regulation of the kinase; (4) the catalytic/regulatory site is comprised, in part, of the amino-terminal region of the enzyme.

Direct correlation of inactivation of PRK with stoichiometric incorporation of reagent (Figure 3), peptide mapping of the labeled enzyme (Figure 5), and electrophoretic patterns of partially inactivated enzyme (Figure 4) are all consistent with the modification of a single residue per subunit. The anticipated identity of cysteine as the type of residue modified (Omnaas et al., 1985) is confirmed by amino acid and sequence analyses of the purified radioactive peptide. Due to identical sequences of the isolated tryptic peptide and the amino-terminal region of the kinase (Porter et al., 1986), the reactive cysteinyl residue is revealed to occupy position 16 of the intact subunit. The finding that the K_D for ATP (~30 μM) based on the protection of the kinase against alkylation is similar to the K_m for ATP (~50 μM) suggests that the reactive sulfhydryl group is located within the nucleotide binding domain of the catalytic site.

Previous work from our laboratory (Omnaas et al., 1985) had indicated the modification of two cysteinyl residues of PRK by BrAcNHEtOP. Only one of these was classified as essential (as verified in this study), because ATP prevented inactivation and reduced the level of incorporation by about half. In retrospect, it is clear that the apparent modification of a nonessential sulfhydryl was a consequence of the crude (only 15% pure) kinase preparation used and that the incorporation observed in the presence of ATP represented labeling of contaminating proteins.

Activation of PRK by reduced thioredoxin presumably entails reduction of an intrasubunit disulfide. This conclusion

follows the observations that air oxidation of the activated kinase correlates with the loss of two sulfhydryls per molecule of subunit but without change in the molecular weight that would be dictated by the formation of intermolecular disulfides (Porter et al., 1986). Several findings in this study indicate that one of the two regulatory sulfhydryls is the active site group susceptible to alkylation by BrAcNHEtOP. Oxidized, deactivated PRK is resistant to alkylation by BrAcNHEtOP under the same conditions whereby the reduced enzyme is rapidly inactivated (Figure 3, inset). Furthermore, kinetic analyses of the protective effects of ATP against both oxidation and alkylation reveal similar K_D values for ATP (Figure 6), implying that both modifications occur at the same ligand binding site. That the same sulfhydryl group within the nucleotide binding domain undergoes either alkylation or oxidation is suggested by their analogous pH dependencies, which reflect a single pK_a value of 7.7–7.9.

The picture that thus emerges for the thioredoxin-mediated activation of PRK is one in which reduction of a disulfide generates a free sulfhydryl at the active site, which is required, either directly or indirectly, for catalytic activity. Presumably, the amino-terminal region of the kinase is freely exposed to solvent and available for interaction with thioredoxin. Although the relationships between chloroplastic illumination and subsequent reductions of thioredoxin and PRK establish reduced thioredoxin as the in vivo activator (Wolosiuk & Buchanan, 1978; Buchanan, 1980), the identity of the in vivo deactivator, which could be oxidized thioredoxin, is obscure. Our decision to use dehydroascorbate as oxidant in this study was based on the suggestion that it might be an in vivo deactivator (Wolosiuk & Buchanan, 1977); furthermore, the dehydroascorbate-deactivated enzyme can be fully activated by reduced thioredoxin (data not shown). This latter observation suggests that, irrespective of the nature of the in vivo oxidant, the same sulfhydryls are involved. Therefore, we believe that our conclusions derived from oxidation experiments with dehydroascorbate are valid physiologically.

We do not know if the "essential" sulfhydryl is actually required for catalysis or whether conformational changes accompany the regulatory oxidation process and account for the loss of enzymic activity. On the basis of protection by ATP against DTT-mediated activation of PRK (Ashton, 1983), it appears that the deactivated enzyme is still functional in nucleotide binding, so perhaps the conformational differences between the two forms are not great.

This paper provides the first sequence information about the thioredoxin-binding domain of any plant enzyme that is subject to light regulation. In addition to PRK, other chloroplastic enzymes whose activities are modulated by thioredoxin (isozyme f) include fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, and NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (Buchanan, 1980). It will be of interest to determine if their common mode of regulation reflects sequence homologies adjacent to the pertinent cysteinyl residues. Among these enzymes, even partial sequences have been determined only for spinach chloroplastic fructosebisphosphatase (Harrsch et al., 1985). Although homology with the amino-terminal region of PRK is not revealed, only two of the anticipated six cysteinyl-containing peptides (Zimmermann et al., 1976) from the phosphatase were sequenced.

Registry No. PRK, 9030-60-8; ATP, 56-65-5; BrAcNHEtOP, 52011-43-5; L-Cys, 52-90-4; dehydroascorbate, 33124-69-5.

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