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THE THIOREDOXIN BINDING SITE OF PHOSPHORIBULOKINASE OVERLAPS THE CATALYTIC SITE

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DISCLAIMER

THE THIOREDOXIN BINDING SITE OF PHOSPHORIBULOKINASE OVERLAPS THE CATALYTIC SITE

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1. INTRODUCTION

Phosphoribulokinase (PRK[1], E.C. 2.7.1.19) is one of a class of enzymes whose activity is linked to the light/dark cycle by thioredoxin, ferredoxin-thioredoxin reductase, and ferredoxin (1). Although enzyme activation is known to require disulfide reduction, the location of the disulfide at the primary structural level has not been established for PRK or for any other plant enzyme that is regulated in this fashion. Previous studies with BrAcNHEtOP, a potential active-site probe, identified an essential cysteinyl residue within the ATP-binding domain of PRK. Two observations suggested a connection between the active-site cysteine and a thioredoxin-sensitive cysteine. Oxidized PRK is not alkylated by BrAcNHEtOP (2) and ATP retards the oxidative deactivation of PRK (3,4). More recent data, summarized here, confirm that the active-site sulfhydryl also participates in the regulation of kinase activity.

2. PROCEDURES

2.1. Materials: PRK was purified from spinach leaves using successive gel filtration, ion exchange chromatography, and affinity chromatography on reactive red agarose (4). BrAcNHEtOP was synthesized as described earlier (5). [^{14}C]IAA from ICN was diluted with unlabeled IAA (Aldrich) as needed.

2.2. Methods: PRK activity was determined by a coupled spectrophotometric assay (4). PRK was alkylated at 4°C in 45 mM bicine-KOH, 4.5 mM $MgCl_2$, 0.9 mM EDTA, 10% v/v glycerol with the adjustments described. Oxidations were conducted in the same basic buffer but at room temperature. Prior to oxidation experiments, the kinase was desalted by gel filtration in the desired buffer to remove the exogenous DTT present in the storage buffer. Tryptic digestions, HPLC chromatography, amino acid compositions and automated sequence analyses were accomplished by standard methods (4). The carboxymethylation procedure (6) was altered to use IAA in only a 10 mM excess over exogenous thiol, and guanidine was not included in the reaction mixture when carboxymethylation of the native enzyme was desired.

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[1]Abbreviations: PRK, phosphoribulokinase; Ru5P, ribulose 5-phosphate; IAA, iodoacetic acid; DTT, dithiothreitol; BrAcNHEtOP, bromoacetyl-ethanolamine phosphate; DHA, dehydroascorbate.

3. RESULTS AND DISCUSSION

BrAcNHEtOP completely inactivates PRK concomitant with the incorporation of only one molar equivalent of reagent, corresponding to the alkylation of a cysteinyl residue. This implied high degree of specificity is confirmed by HPLC of a tryptic digest and by gel electrophoresis under non-denaturing conditions of the partially modified dimeric protein. Because ATP provides protection against alkylation and because the concentration dependency of the protection indicates a K_D (29 μM) similar to its K_m (50 μM), the reactive cysteine appears to be located within the ATP-binding domain of the active site. The tryptic peptide containing the reactive cysteine has been purified by ion exchange chromatography followed by reverse phase HPLC. Edman degradation on a gas phase sequencer (Applied Biosystems) establishes the following primary structure (the asterisk denotes the modified residue),

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                    5                   10                  15
Ser-Gln-Gln-Gln-Thr-Ile-Val-Ile-Gly-Leu-Ala-Ala-Asp-Ser-Gly-

Cys*-Gly-Lys
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which corresponds exactly to the amino terminal region of the protein (4), in which cysteine was positively identified at position 16. Prior oxidation of the kinase prevents incorporation of BrAcNHEtOP, suggesting that the reactive cysteine is a possible thioredoxin-regulatory target residue. This supposition is supported by the observation that ATP retards the oxidation of the kinase by oxidized glutathione, molecular oxygen (3,4), and oxidized thioredoxin. We investigated this effect in detail by determining the oxidation rate of the kinase by DHA in the presence of various concentrations of ATP. The calculated K_D for ATP is 23 μM, a value similar to that revealed by the kinetics of ATP protection against the alkylation of PRK. A single ATP binding site is thus invoked to account for the protective effects observed in both processes. Because both alkylation and oxidation of sulfhydryls require the ionized form, these reactions will show pH dependencies which reflect the pK_a of the target sulfhydryl. Both alkylation and oxidation rates of PRK were determined from pH 6.8–8.3 at fixed ionic strength. Both processes reveal an inflection centered at about pH 7.8, reinforcing the above postulate that the same sulfhydryl group is the target of both oxidation and alkylation. The parallels between the alkylation and oxidation of PRK are summarized in Table 1.

TABLE 1. Parallels between alkylation and oxidation

Measurement	Reaction examined	
	Alkylation	Oxidation
K_D (ATP) of protection	29 ± 4 μM	23 ± 5 μM
Apparent pK_a of reactive group	7.9 ± 0.2	7.7 ± 0.15
ADP protection	complete	complete
Ru5P protection	partial	partial

The proposal that the alkylating reagent and oxidant are reacting at a single sulfhydryl in PRK was tested directly by observing the carboxymethylation pattern after various treatments. Carboxymethylation of the enzyme under denaturing conditions with [^{14}C]IAA reveals four peaks of radioactivity upon HPLC of a tryptic digest (Fig. 1B). Prior alkylation with BrAcNHEtOP prevents the appearance of peak II (Fig. 1F), establishing that this peak represents the active-site cysteinyl residue. As expected from the protection pattern, inclusion of ATP during carboxymethylation prevents labeling of the cysteinyl residue represented by peak II (Fig. 1D). Prior oxidation of the kinase by DHA also diminishes the level of peak II (Fig. 1C,E), confirming a role of one sulfhydryl group in both catalytic and regulatory functions. The level of peak III in tryptic digests is also less in the DHA-oxidized kinase (Fig. 1E), thereby identifying the second cysteinyl residue involved in disulfide bond formation. This finding excludes the possibility that the disulfide bond is comprised of Cys-16 from two different subunits. A final interesting observation is that one of the regulatory sulfhydryls (Cys-16, represented by peak II) is exposed to solvent (i.e. accessible to IAA, Fig. 1A) while the other (represented by peak III) is buried (i.e. not accessible to IAA, Fig. 1A).

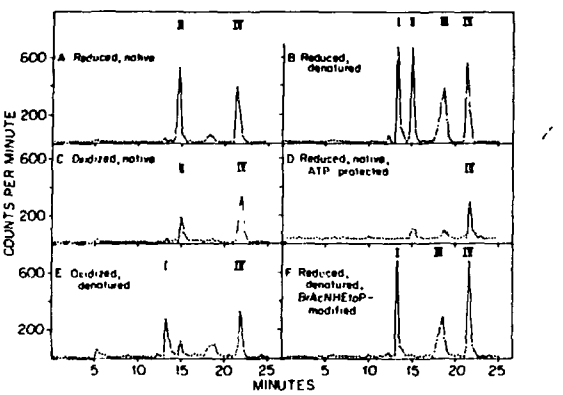

FIGURE 1. Radioactivity profiles of [^{14}C]carboxymethyl-PRK after tryptic digestion. PRK was alkylated with [^{14}C]IAA, digested with trypsin, and subjected to reverse phase HPLC (4,6). Treatments were: A, no guanidine, 10 mM DTT; B, guanidine, 10 mM DTT; C, no guanidine, 10 mM DTT (carboxymethylated in the presence of ATP); E, guanidine, prior oxidation; F, guanidine, 10 mM DTT, (prior alkylation with BrAcNHEtOP).

The finding that two cysteines per subunit undergo oxidation during deactivation of PRK (Fig. 1E) is consistent with earlier evidence (4) that the disulfide bond formed is intrasubunit, rather than intersubunit or intermolecular. Further support excluding intermolecular oxidation is found in the failure of gel filtration in the presence of 6 M urea to resolve oxidized and reduced kinase.

4. CONCLUSIONS

4.1. BrAcNHEtOP alkylates a single essential sulfhydryl (Cys-16) in PRK.

4.2. The reactive sulfhydryl is within the ATP-binding domain of the enzyme and is exposed to solvent.

4.3. The reactive sulfhydryl is involved in redox regulation of kinase activity.

4.4. The catalytic/regulatory site is comprised, in part, of the amino-terminal region of the protein.

4.5. The regulatory disulfide is intrasubunit.

5. REFERENCES

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6. ACKNOWLEDGMENT

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