

The Molecular Pathway for the Regulation of Phosphoribulokinase by Thioredoxin *f**

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Phosphoribulokinase (PRK) is one of several plant enzymes that is regulated by thiol-disulfide exchange as mediated by thioredoxin, which contains spatially vicinal, redox-active cysteinyl residues. In an earlier study (Brandes, H. K., Larimer, F. W., Geck, M. K., Stringer, C. D., Schürmann, P., and Hartman, F. C. (1993) *J. Biol. Chem.* 268, 18411–18414), our laboratory identified Cys-46 of thioredoxin *f* (Trx), as opposed to the other candidate Cys-49, as the primary nucleophile that attacks the disulfide of target proteins. The goal of the present study was to identify which of the two redox-active cysteinyl residues of PRK (Cys-16 or Cys-55) is paired with Cys-46 of Trx in the interprotein disulfide intermediate of the overall oxidation-reduction pathway. Incubation of a mixture of the C16S mutant of PRK and the C49S mutant of Trx with Cu²⁺ results in covalent complex formation as detected by SDS-polyacrylamide gel electrophoresis. Complexation is fully reversible by dithiothreitol and is retarded by ligands for PRK. Under the same conditions, Cu²⁺ induces very little complex formation between the following pairs of mutants: C16S PRK/C46S Trx, C55S PRK/C49S Trx, and C55S PRK/C46S Trx. When either 5-thio-2-nitrobenzoate-derivatized C16S or C55S PRK, as mimics of the oxidized (disulfide) form of the enzyme, is mixed with C49S Trx, stable covalent complex formation occurs only with the C16S PRK. Thus, two independent approaches identify Cys-55 of PRK in the intermolecular disulfide pairing with Trx.

Thioredoxins are small (12–14 kDa) ubiquitous proteins that control the redox state of diverse target proteins by the mediation of thiol-disulfide exchanges (1, 2). The redox-active sulfhydryls of thioredoxin are located in the highly conserved active-site sequence -Trp-Cys-Gly-Pro-Cys-. The pathway for the

reduction of a protein disulfide by thioredoxin entails nucleophilic attack by one of the active-site sulfhydryls to form a protein-protein mixed disulfide followed by intramolecular displacement of the reduced target protein with concomitant formation of oxidized thioredoxin (Fig. 1). As the mixed disulfide is disfavored thermodynamically, its direct characterization has not been possible. However, the active-site cysteinyl residue of thioredoxin that is nearest the N terminus (Cys-32 of the *Escherichia coli* protein) is well established as the primary nucleophile. Originally, this assignment was deduced from the high chemical reactivity and the low pK_a (6.7) of Cys-32 (3–6) and was subsequently supported by the three-dimensional structure of *E. coli* thioredoxin, in which Cys-32 is solvent-accessible and Cys-35 is inaccessible (7). More recently, Cys-32 of *E. coli* thioredoxin, the corresponding residue of human thioredoxin, and the corresponding residue of the closely related protein glutaredoxin were shown to engage in mixed disulfide bond formation with low molecular weight thiols or peptides (8–11). Finally, the C49S mutant of chloroplastic thioredoxin *f* (Trx)¹ retains the capacity to activate chloroplastic fructose-1,6-bisphosphatase, whereas the C46S mutant is totally inactive in this regard. Thus, Cys-46 of Trx (analogous to Cys-32 of *E. coli* thioredoxin) is verified as the primary nucleophile in the reduction of *in vivo* target proteins, thereby minimizing the possibility that protein-protein interactions might alter the relative accessibility and reactivity of the active-site sulfhydryls of thioredoxin.

In contrast to the rigorous proof that Cys-46 of Trx participates in intermolecular disulfide bond formation, the identity of the pairing residue in any target protein has not been established heretofore. We have addressed this issue with PRK by examining the potential of site-directed mutants, which lack either one of the two redox-active sulfhydryls (Cys-16 or Cys-55), to form stable mixed disulfides with the C49S mutant of Trx. This approach, which should be applicable to other target proteins, clearly identifies Cys-55 as the participant in the intermolecular mixed disulfide.

EXPERIMENTAL PROCEDURES

Materials—The following chemicals and biologicals were procured from the indicated vendors: Bicine and DTT, Research Organics, Inc.; components for kinase assays, Sigma; cupric sulfate, Fisher Scientific; DTNB, Pierce. D-Ribulose 5-phosphate was prepared from D-ribose 5-phosphate by the action of phosphoribose isomerase, and its concentration was based on the reported equilibrium constant (12). Construction and purification of the Trx and PRK mutants used in this study have been reported (13, 14).

PRK Activity Assay—PRK was assayed at 25 °C by the spectrophotometric method of Racker (15). Assay mixtures (1 ml) at pH 8.0 included 50 mM Bicine, 40 mM KCl, 1 mM ATP, 2.5 mM D-ribulose 5-phosphate, 3 mM phosphoenolpyruvate, 10 mM MgCl₂, 0.4 mM NADH, 5 units of pyruvate kinase, 6 units of lactate dehydrogenase, and <0.2 unit of PRK. One unit of PRK is defined as the formation of 1 μmol of ADP/min.

Polyacrylamide Gel Electrophoresis—Denaturing (0.5% (w/v) SDS) and nondenaturing electrophoresis was carried out at 15 °C on 8–25% polyacrylamide PhastGel Media with a PhastGel apparatus (Pharmacia Biotech Inc.). Gels were stained with silver or Coomassie Blue according to the manufacturer's recommendations.

¹ The abbreviations used are: Trx, thioredoxin *f*; Bicine, *N,N'*-bis-(2-hydroxyethyl)glycine; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid; DTT, dithiothreitol; PRK, phosphoribulokinase.

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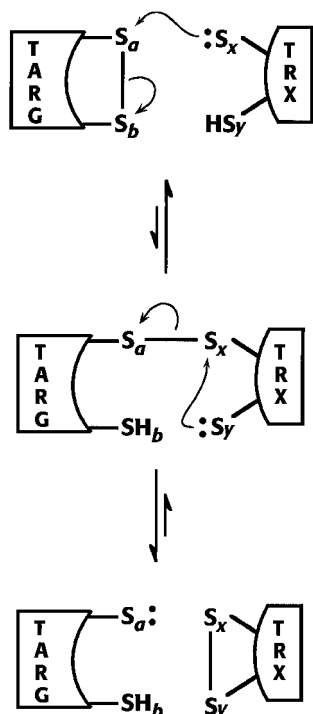


FIG. 1. Pathway for redox modulation of target proteins (TARG) by thioredoxin (TRX).

Covalent Complexation of PRK and Trx by Cupric Ion-catalyzed Oxidation—Concentrated stocks of the PRK and Trx mutants were freed of DTT by gel filtration on Bio-Gel P6 (1-ml column) equilibrated with thiol-free 50 mM Bicine (pH 8.0), 20% (v/v) glycerol. Mixtures of the pertinent mutants were prepared at a 2:1 or 5:1 molar ratio of Trx:PRK subunit and incubated at 4 °C in the presence of 2 mM CuSO_4 ; the final concentration of PRK was 0.1 mg/ml. Periodically, aliquots of the reaction mixtures were assayed for kinase activity and analyzed electrophoretically.

Covalent Complexation of PRK and Trx via a Mixed Disulfide Intermediate—Concentrated stocks of C16S PRK, C55S PRK, and C49S Trx were exhaustively dialyzed against 50 mM Bicine (pH 8.0), 1 mM EDTA, 20% (v/v) glycerol. The dialyzed samples of C16S PRK and C55S PRK at 1.5 mg/ml were inactivated by a 5- and 0.2-fold molar excess of DTNB, respectively. Loss of kinase activity and concomitant release of TNB were monitored. After the activity had decreased to <2% of its initial value, each sample was dialyzed against the Bicine buffer just described and then stored at -80 °C. A thawed aliquot of the TNB-C16S PRK or TNB-C55S PRK was titrated with a 2-fold molar excess of dialyzed C49S Trx while monitoring PRK activity and release of TNB. Aliquots preceding and subsequent to titration were analyzed electrophoretically.

RESULTS AND DISCUSSION

PRK, a homodimer with a subunit molecular weight of 39,232 (16, 17), is representative of plant enzymes that are regulated during light-dark transitions (18). Photon flux drives the reduction of thioredoxin (as mediated by ferredoxin-thioredoxin reductase), which in turn reduces the regulatory disulfide of target proteins (18–20). Having recently identified Cys-46 as the primary nucleophile of Trx (13), we wished to complete the description of the molecular pathway for activation (reduction) of PRK by ascertaining which of its two regulatory cysteinyl residues (Cys-16 or Cys-55) is involved in the protein-protein mixed disulfide intermediate. As the instability of the putative intermediate precludes its direct characterization, we reasoned that a stable complex should result by combining mutants of Trx and PRK, each retaining the cysteinyl residue necessary for intermolecular disulfide bond formation but lacking the spatially proximal cysteinyl residue responsible for intramolecular disulfide bond formation.

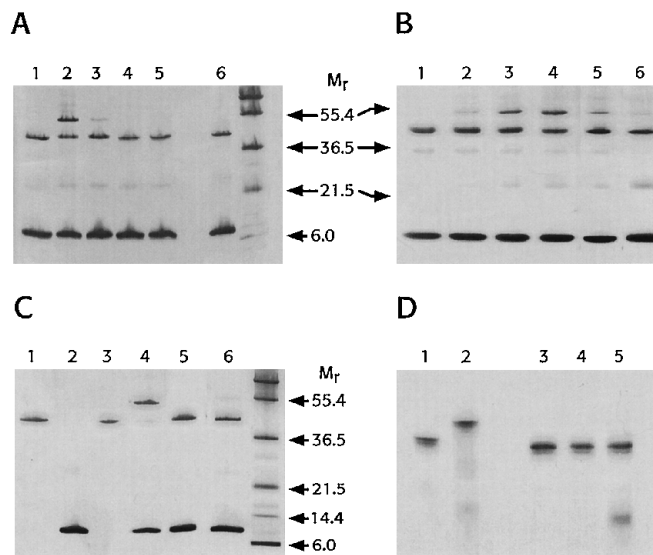


FIG. 2. Polyacrylamide gel electrophoresis of PRK/Trx reaction mixtures. Denaturing (+ SDS), silver-stained gels are shown in A, B, and C, and a nondenaturing, Coomassie Blue-stained gel is shown in D. In all cases, Trx was used at a 2-fold molar excess relative to the concentration of PRK subunits. A, incubations of mixtures of PRK mutants and Trx mutants in the presence of Cu^{2+} at time zero (lane 1) and after 24 h (lanes 2–6). Samples applied (equivalent to 50 ng of PRK) are as follows: lane 1, C16S PRK/C49S Trx (control); lane 2, C16S PRK/C49S Trx; lane 3, C16S PRK/C46S Trx; lane 4, C55S PRK/C49S Trx; lane 5, C55S PRK/C46S Trx; lane 6, C16S PRK/C49S Trx + 0.1 M DTT. B, time course for covalent complex formation between C16S PRK and C49S Trx in the presence of Cu^{2+} with or without an added ligand for PRK. Samples applied (equivalent to 40 ng of PRK) are as follows: lane 1, time zero; lane 2, 3 h; lane 3, 10 h; lane 4, 24 h; lane 5, 24 h in the presence of 1.5 mM MgATP; lane 6, 24 h in the presence of 2.6 mM D-ribose 5-phosphate (each ligand at $20 \times K_m$). C, covalent complex derived from C49S Trx plus TNB-derivatized C16S PRK. Samples applied (equivalent to 25 ng of PRK in lanes 1–6 and 25 ng of Trx in lane 2) are as follows: lane 1, C16S PRK; lane 2, C49S Trx; lane 3, TNB-derivatized C16S PRK; lane 4, C49S Trx + TNB-derivatized C16S PRK; lane 5, sample shown in lane 4 after incubation with 10 mM DTT; lane 6, C49S Trx + TNB-derivatized C55S PRK. D, covalent complex derived from C49S Trx plus TNB-derivatized C16S PRK. Samples applied (equivalent to 350 μg of PRK) are as follows: lane 1, TNB-derivatized C16S PRK; lane 2, C49S Trx + TNB-derivatized C16S PRK; lane 3, C16S PRK; lane 4, TNB-derivatized C16S PRK after treatment with 10 mM DTT; lane 5, C49S Trx-C16S PRK complex after treatment with 10 mM DTT.

Reaction mixtures were prepared with all four possible combinations of the relevant mutants of PRK and Trx: C16S PRK/C49S Trx, C16S PRK/C46S Trx, C55S PRK/C49S Trx, and C55S PRK/C46S Trx. Cupric sulfate was added to the reaction mixtures to catalyze disulfide bond formation between those pairs of sulfhydryls that become juxtaposed within the protein-protein complexes. SDS-polyacrylamide gel electrophoresis reveals a prominent 54-kDa species, consistent with covalent coupling between PRK (40-kDa subunit) and Trx (13.5-kDa), derived from the reaction mixture of C16S PRK/C49S Trx (Fig. 2A). The 54-kDa species is lacking in the reaction mixtures of C55S PRK paired with either mutant of Trx and is barely discernible in the reaction mixture of C16S PRK/C46S Trx. Thus, the observed covalent coupling involves Cys-55 of PRK and Cys-46 of Trx. When the C16S PRK/C49S Trx reaction mixture is incubated with DTT prior to electrophoresis, the complex is not present, verifying that stable complexation is via a disulfide linkage. Covalent complexation does not occur merely in the presence of oxygen (all buffers are air-saturated), so either a catalyst (e.g. Cu^{2+}) or stronger oxidant would appear to be required. Interestingly, Cu^{2+} -o-phenanthroline (generally considered a more efficient catalyst than Cu^{2+} alone for

oxidation of sulfhydryls) (21), dehydroascorbate, and oxidized glutathione are totally ineffective in promoting formation of the 54-kDa species. These negative observations provide indirect evidence that the oxidation catalyzed by Cu^{2+} occurs within the preformed noncovalent C16S PRK-C49S Trx complex in which solvent accessibility to the site of oxidation is limited.

A time course of Cu^{2+} -catalyzed oxidation of C16S PRK/C49S Trx and the inhibitory effects of ATP and ribulose-5-phosphate are shown in Fig. 2B. As both regulatory cysteinyl residues of PRK are located at the active site, the inhibition by MgATP and ribulose 5-phosphate of intermolecular disulfide bond formation between C16S PRK and C49S Trx is readily explained. The inability to drive the complexation to completion even with a 5-fold molar excess of Trx (data not shown) probably reflects undefined Cu^{2+} -catalyzed oxidations of PRK distinct from protein-protein disulfide bond formation. For example, we note inactivation of the PRK control (including Cu^{2+} but excluding Trx) at about the same rate as that of the C16S PRK/C49S Trx reaction mixture (data not shown). Furthermore, in both cases, these inactivations are fully reversible with DTT so that kinase activity cannot be used to monitor the extent of complex formation.

Even though the results of the Cu^{2+} -catalyzed oxidations clearly invoke Cys-55 of PRK as participatory in the normal mixed disulfide intermediate and confirm our earlier identification of Cys-46 of Trx as the bridging partner, a caveat arises. Is the noncovalent complex generated from mimics of the reduced forms of both Trx (C49S) and PRK (C16S) equivalent to the normal complex of the thiol-disulfide exchange pathway, in which one of the two proteins would be oxidized and the other reduced? To circumvent this concern, we prepared mixed disulfides of the C16S and C55S mutants of PRK by treatment with DTNB; the derivatized mutants can be viewed as mimics of the oxidized (disulfide) form of PRK. With each mutant, only 1 molar eq of TNB was released concomitant with complete loss of kinase activity. Original levels of activity (specific activities of C16S and C55S are 95 and 20% of the wild-type enzyme, respectively) were restored by incubating the derivatized proteins with 10 mM DTT. These data (not shown) verify the formation of the desired protein-TNB mixed disulfide. Upon combining TNB-derivatized C16S PRK with a 2-fold molar excess of C49S Trx, the former is totally consumed and replaced by a 54-kDa species, which undergoes dissociation in the presence of DTT (Fig. 2C). The absence of a band coinciding with free PRK subunit demonstrates that both binding sites for Trx of dimeric PRK are occupied in the covalent complex prior to its dissociation by SDS. In stark contrast, TNB-derivatized C55S PRK does not give rise to detectable levels of the covalent complex. Thus, an approach totally independent of Cu^{2+} -catalyzed oxidation invokes the same residue (Cys-55) of PRK in formation of the mixed disulfide with Trx.

The C16S PRK-C49S Trx complex is also well resolved from C16S PRK by polyacrylamide gel electrophoresis under nonreducing conditions (Fig. 2D). Its slower mobility, relative to

PRK, shows that dissociation of PRK subunits does not occur under the conditions used to prepare the covalent complex.

Based on analogies with thioredoxin, we had presupposed that Cys-16 of PRK, rather than Cys-55, engages in mixed disulfide bond formation. Cys-16 (like Cys-32 of *E. coli* thioredoxin) displays an abnormally low pK_a (22) and hyperreactivity toward numerous sulfhydryl reagents (23–25), whereas Cys-55 (like Cys-35 of *E. coli* thioredoxin) is far less accessible for chemical modification (26–28). Apparently, the sulfur atom of Cys-55 is either more exposed in the disulfide form of PRK than in the free sulfhydryl form or the interaction of Trx with PRK induces a conformational change that renders this sulfur susceptible to attack by Cys-46 of Trx.

In summary, this study marks the first example of a complete molecular description of the thiol-disulfide exchange pathway between any thioredoxin and a target enzyme, provides a facile avenue for large scale preparation of a PRK-Trx covalent complex for future biochemical and structural characterization, and validates an approach that could be of general utility in the characterization of other target protein-thioredoxin complexes.

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