Despite little supportive data, differential target protein susceptibility to redox regulation by thioredoxin (Trx) f and Trx m has been invoked to account for two distinct Trxs in chloroplasts. However, this postulate has not been rigorously tested with phosphoribulokinase (PRK), a fulcrum for redox regulation of the Calvin cycle. Prerequisite to Trx studies, the activation of spinach PRK by dithiothreitol, 2-mercaptoethanol, and glutathione was examined. Contrary to prior reports, each activated PRK, but only dithiothreitol supported Trx-dependent activation. Comparative kinetics of activation of PRK showed Trx m to be more efficient than Trx f because of its 40% higher $V_{\text{max}}$ but similar $S_{0.5}$. Activations were insensitive to ribulosebisphosphate carboxylase, which may complex with PRK in vivo. To probe the basis for superiority of Trx m, we characterized site-directed mutants of Trx f, in which unique residues in conserved regions were replaced with Trx m counterparts or deleted. These changes generally resulted in $V_{\text{max}}$ enhancements, the largest (6-fold) of which occurred with T105I, reflective of substitution in a hydrophobic region that opposes the active site. Inclusive of the present study, activation kinetics of several different Trx-regulated enzymes indicate redundancy in the functions of the chloroplastic Trxs.

The discovery (1, 2) that Trx f mediates the light regulation of various chloroplastic enzymes provided an explanation at the molecular level for the correlation between light intensity and the capacity for CO$_2$ assimilation by photosynthetic organisms (for reviews see Refs. 3 and 4). In the light, electron flow through ferredoxin and ferredoxin-thioredoxin reductase maintains Trx in its reduced state, in which vicinal active site sulphydryls prevail. Reduced Trx can then reduce the redox-regulated enzymes but inactivates susceptible biodegradative enzymes, thereby minimizing the simultaneous functioning of opposing metabolic pathways (5).

Shortly after the discovery of the ferredoxin-thioredoxin pathway for enzyme regulation, two distinct chloroplastic thioredoxins (denoted Trx f and Trx m) were isolated and characterized (6). The physiological need for two thioredoxins appeared to be a consequence of their preferential selectivity among the array of target enzymes. For example, under the assay conditions described, Trx f was more effective in the activation of NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, and PRK, whereas Trx m displayed a preference for NADP-dependent malate dehydrogenase and glucose-6-phosphate dehydrogenase (3, 4, 6–9). Armed with this background, we initiated an effort to identify recognition and selectivity determinants of Trx f by utilization of site-directed mutagenesis. During the course of our earlier study (10), we demonstrated that Trx f is kinetically superior to Trx m as an activator for both fructose-1,6-bisphosphatase and NADP-dependent malate dehydrogenase. Because this unexpected finding contradicted the generally accepted explanation for the presence of two chloroplastic thioredoxins, we felt it only prudent to extend kinetic analyses to another redox-sensitive enzyme and accordingly have selected PRK.

PRK catalyzes the ATP-dependent phosphorylation of d-ribulose 5-phosphate to form d-ribulose-1,5-bisphosphate, the requisite acceptor of CO$_2$ in photosynthetic carbon fixation. This chloroplast enzyme, a homodimer with a subunit molecular weight of ~40,000 (11), is reversibly inactivated by formation of an intrasubunit disulfide between Cys$^{16}$ and Cys$^{55}$ (12). Even though both of these regulatory cysteinyl residues are located in the ATP-binding domain of the active site, neither is crucial to activity. The free sulphydryl of Cys$^{55}$ is only moderately facilitative of catalysis, whereas that of Cys$^{16}$ has no effect whatsoever on activity (13). Total loss of kinase activity, which accompanies oxidation, reflects a combination of masking the sulphydryl of Cys$^{55}$ and introducing a conformational constraint as imposed by the disulfide (13, 14).

Several observations provide evidence that PRK is indeed redox-regulated in vivo by Trx. These include Trx dependence of light activation of PRK in reconstituted systems (2, 6), correlative increases and decreases in the level of the kinase activity in isolated chloroplasts during successive light-dark cycles (15–19), activation of purified preparations of PRK by Trx, and covalent complexation by highly selective disulfide bridging of Cys$^{46}$ of Trx f with Cys$^{55}$ of PRK (20). However, the relative kinetic efficiency of Trx f and Trx m in the activation of purified PRK has not been determined. Herein, we report such a comparison and also the effectiveness of several site-directed
mutants of Trx f, designed to more closely resemble Trx m, as activators of PRK. Several of the mutant thioredoxins (K58E, ΔN74, N74D, Q75D, and ΔN77) were described in our earlier report with respect to their ability to activate NADP-dependent malate dehydrogenase and fructose-1,6-bisphosphatase (10). Additionally, two newly engineered variants of Trx f are also examined: T105I and the double mutant V89I/T105I.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the sources indicated: DTNB, Fisher Scientific; DTT and buffers, Research Organics; and PRK assay reagents, bovine serum albumin, and BME, Sigma. Spinach Rubisco was purified according to a published protocol (21). Construction, expression, and purification of recombinant Trx f and many of the site-directed mutants thereof used in the present study have been described (10). Two new site-directed mutants were generated using this previously published protocol and the following primers: pGGCATCAGAATTCCTACTTCAAGATT for V89I conversion and the aforementioned primer in conjunction with pGGTGGAGAAGT-TATAGGGCGAAAATATGAT for V89IT105I. These electrophoretically pure protein preparations (>5 mg/ml) were stored at −80 °C in 50 mM Bicine-NaOH (pH 8.0), 0.1 mM EDTA, 20% (v/v) glycerol, 5 mM DTT. Purified recombinant spinach Trx m was kindly provided by Professor Peter Schürmann of the Université de Neuchâtel in Switzerland. The sample, received in lyophilized form, was resuspended in 50 mM Bicine-NaOH (pH 8.0), 0.1 mM EDTA, 20% (v/v) glycerol, 5 mM DTT to a final concentration of ~5 mg/ml, dialyzed overnight against the same buffer, and stored at −80 °C prior to use. During the course of the present study, authentic PRK isolated from spinach leaves (11, 12) and recombinant spinach PRK isolated from transformed Pichia pastoris (13, 22) were both used. In all cases, the preparations were virtually homogenous and indistinguishable as judged by denaturing polyacrylamide gel electrophoresis. However, their specific activities were somewhat variable, ranging from 260 to 358 units/mg. Prior to DTNB-oxidative inactivation of PRK to prepare the substrate for the Trx activation studies (see below), the preparations of PRK (>20 mg/ml) were stored at −80 °C in 40 mM Bicine-KOH (pH 8.0), 0.8 mM EDTA, 20% (v/v) glycerol, 10 mM DTT. Typically, the inactive DTNB-oxidized samples regained 90–95% of their original activity upon incubation with 50 mM DTT for 15 min.

Oxidation of PRK—Prior to oxidation of PRK, exogenous thiol was removed from the purified preparation by gel filtration on Sephadex G-25 equilibrated with 40 mM Bicine-KOH (pH 8.0), 0.8 mM EDTA. Oxidation was then performed by treatment of PRK (5–10 mg/ml) with a 20% molar excess (relative to subunit concentration) of DTNB at 25 °C until PRK activity could no longer be detected (~5 min). The DTNB reaction mixture was then exhaustively dialyzed against 50 mM Bicine-KOH (pH 8), 1 mM EDTA, 20% (v/v) glycerol. Stock solutions of the oxidized kinase were stored at −80 °C at a concentration of approximately 5 mg/ml. Previous studies have demonstrated that the loss of kinase activity upon incubation with DTNB is concomitant with the formation of a single disulfide bond per subunit of PRK between Cys 16 and Cys 55, with no other modifications detected (12). Thus, DTNB-oxidized PRK can be regarded as comparable with the enzyme present in the darkened chloroplast.

Activation of PRK—PRK (0.08 mg/ml, 2 μl subunit) was activated at 25 °C by the indicated concentrations of DTT, BME, GSH, or Trx (in the presence of low molecular weight thiol) in buffered solutions (25 μl) of 40 mM Bicine-KOH (pH 8.0) and 0.8 mM EDTA. Periodically, 5-μl aliquots were withdrawn for the determination of kinase activity at 25 °C. Assay solutions (1 ml) consisted of 50 mM Bicine-KOH (pH 8.0), 40 mM KCl, 10 mM MgCl2, 1 mM ATP, 3 mM phosphoenolpyruvate, 0.3 mM NADH, 5 units of pyruvate kinase, 6 units of lactate dehydrogenase, 0.8 mM ribulose 5-phosphate. The latter was prepared by incubation of 150 mM ribose-5-phosphate and approximately 2 units of the phosphoribosylamine at room temperature for 30 min in 1.3 ml of 50 mM Bicine-KOH (pH 8.0), 40 mM KCl, 10 mM MgCl2; 20 μl of this solution was used per 1-ml assay. The formation of NADH was followed spectrophotometrically at 340 nm. One unit of PRK activity corresponds to an absorbance change of 6.22/min. When activation rates are expressed as milliunits/min, the reflected amount of protein is 2.0 μg.

RESULTS

Activation of PRK by Low Molecular Weight Thiols—Several considerations prompted the inclusion of a low molecular weight thiol in reaction mixtures for the examination of the activation of PRK by Trx. Because the redox potentials of PRK, Trx f, and Trx m are virtually identical (23), complete activation of oxidized PRK will not occur upon incubation with Trx. Furthermore, even with rather stringent precautions to mitigate oxidation, aqueous solutions of reduced Trx tend to accumulate some of the oxidized form, which will further impede activation of PRK. Finally, maintaining all of the Trx in its reduced state is necessary to achieve pseudo first-order kinetics of activation of PRK and thus accurate determination of those kinetic parameters being sought.

Inclusion of an exogenous thiol in Trx-PRK reaction mixtures does, however, introduce an unavoidable complication because of the potential for PRK activation by the added thiol. PRK is known to undergo activation by DTT (2, 15, 18), but the associated kinetics have not been thoroughly examined. Thus, prerequisite to analyses of Trx-dependent activation of PRK, the rate at which DTT activates PRK was quantified. The abilities of BME and GSH to activate PRK were also determined in an effort to identify a reagent that was unable to activate PRK but would nevertheless maintain Trx in its reduced state. Three high concentrations, GSH (100 mM) and the nonphysiological reductants DTT (20 mM) and BME (50 mM) are all capable of fully activating PRK (Fig. 1). The somewhat slower rates observed with the two monothiols are consistent with their lower reduction potentials relative to that of DTT (24–26). Lower concentrations of the thiols (e.g. 2 mM), despite ~1000-fold molar excess over PRK, lead to only partial activation, presumably because of establishment of equilibrium. With DTT and BME, the initial rates of activation were directly
order kinetics); the calculated second-order rate constants ($k_{2\text{nd}}$) and presence ($f$) of GSH in the presence of DTT have been corrected for the apparent number of binding sites. The activation data points shown with GSH in the presence of DTT have been corrected for the extent of activation brought about by 4 mM DTT alone.

proportional to the concentration of reductant (i.e. pseudo first-order kinetics); the calculated second-order rate constants ($k_{2\text{nd}}$) were 5.4 m$^{-1}$ min$^{-1}$ for DTT and 0.7 m$^{-1}$ min$^{-1}$ for BME. By contrast, activation of PRK by GSH, either alone or in the presence of a fixed concentration of DTT, exhibits saturation kinetics with respect to the concentration of GSH (Fig. 2). In the absence of DTT, $k_{2\text{nd}}$ was 0.6 m$^{-1}$ min$^{-1}$ with a $V_{\text{max}}$ of 20 milliunits/min and an $S_{0.5}$ of 24 mM. In the presence of 4 mM DTT, $k_{2\text{nd}}$ was 2.9 m$^{-1}$ min$^{-1}$ with a $V_{\text{max}}$ of 12 milliunits/min and an $S_{0.5}$ of 5 mM. The plot in Fig. 2, from which these latter values are derived, is corrected for the contribution of DTT to the activation.

Because each of the thiols tested is able to activate the oxidized form of PRK, the enhancements of activation rates by Trx $f$ against a background of DTT (0.5 mM) or BME (2 mM) were compared. As shown in Fig. 3, very little differential activation by Trx occurs in the presence of BME, whereas dramatic stimulation of activation takes place in the presence of DTT. Apparently, the reduction potential of BME is insufficient to maintain all of the Trx in the reduced form. Likewise, Trx $f$ provided little enhancement of PRK activation as induced by GSH (data not shown). Thus, subsequent activation kinetics of PRK by Trx were determined in the presence of 4 mM DTT to ensure that Trx remained fully reduced.

**Comparative Kinetics of the Activation of PRK by Trx $f$ and Trx $m$—**To determine the dependence of activation rate on the concentration of Trx, molar ratios of [Trx]/[PRK] ≥ 5 were used so that [Trx]$_{\text{pmax}}$ would approximate [Trx]$_{\text{total}}$ and activation kinetics would be pseudo-first-order. Estimation of initial rates of activation was based on data collected over the range of 0–30% of the full activation level having been achieved. This range represents the linear phase of the activation curves. The Trx-dependent rate of activation (i.e. the activation rate corrected for the basal rate because of DTT alone) did not vary significantly in the presence of 1–4 mM DTT, thereby verifying that Trx remained fully reduced throughout the course of PRK activation.

Initial rates of activation, extracted from time courses and corrected for the contribution of DTT, are plotted as a function of Trx $f$ or Trx $m$ concentration in Fig. 4. The Trx concentration dependence curves exhibit sigmoidicity, as typifies interactions of Trx with target proteins (10, 27–29). The plot shown for Trx $f$ yields an $S_{0.5}$ of 17 μM and a $V_{\text{max}}$ of 7 milliunits/min. Trx $m$ is modestly more efficient than Trx $f$ as an activator of PRK because of its higher $V_{\text{max}}$ (11 milliunits/min) but virtually identical $S_{0.5}$ (15 μM). In another kinetic study with Trx $f$ in which the activation mixture contained a 10-fold lower concentration of oxidized PRK (i.e., 0.2 μM subunit), very similar $S_{0.5}$ and $V_{\text{max}}$ values were obtained (data not shown).

Because of literature reports that implicate in vivo complexation of PRK with various chloroplast proteins, including D-ribulose-1,5-bisphosphate carboxylase/oxygenase (reviewed in Ref. 30), the potential influence of this protein on the activation of PRK by Trx $f$ and Trx $m$ were determined. In a separate experiment, bovine serum albumin was used as a generic protein to substitute for the carboxylase. Neither the carboxylase (at 2-fold molar excesses relative to PRK subunits) nor bovine serum albumin (at 8-fold molar excess) significantly stimulates the activation of PRK by either thioredoxin (data not shown).

The site-directed mutants of Trx $f$, designed to be more akin to Trx $m$, were also analyzed kinetically in the activation of PRK; in all cases the Trx concentration dependence curves were sigmoidal as with the wild-type proteins and qualitatively analogous to those illustrated for T105I and V89I/T105I in Fig. 5. The derived kinetic parameters for all of the mutants are compiled in Table I. All of the mutants are somewhat impaired with respect to binding of PRK, as judged by their $S_{0.5}$ values, which range from 1.4- to 5-fold greater than that of wild-type Trx $f$. Except for K58E, the mutants display enhanced $V_{\text{max}}$ values. However, in the cases of N74D, ΔN74, ΔN77, and Q75D, the increased rates of reduction of PRK are counterbalanced by the weakened interactions with PRK, whereby the overall efficiencies of activation (i.e. $V_{\text{max}}/S_{0.5}$ values) are virtually unchanged or decreased about 2-fold. Only with T105I and V89I/T105I are the efficiencies of activation increased.

**DISCUSSION**

The overarching goal of the present study was to compare the kinetic efficiencies of Trx $f$ and Trx $m$ as activators of oxidized

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**FIG. 2.** Initial rates of PRK activation by GSH in the absence (●) and presence (○) of 4 mM DTT. Data were fit to the simplified Hill equation of $v = V_{\text{max}}[S]^{n}/[S] + [S]^{n}$, whereby $n$ is the calculated value for the apparent number of binding sites. The activation data points shown with GSH in the presence of DTT have been corrected for the extent of activation brought about by 4 mM DTT alone.

**FIG. 3.** Impact of wild-type Trx $f$ on the activation rate of PRK in the presence of low molecular weight thiols. Time courses of activation of PRK by 0.5 mM DTT (○); 0.5 mM DTT + 50 μM Trx $f$ (■); 2 mM BME (○); and 2 mM BME + 50 μM Trx $f$ (●) are shown.

**FIG. 4.** Activation of PRK by wild-type chloroplastic thioredoxins. Initial rates of PRK activation by the specified type and concentration of Trx are represented by individual data points. These initial rates are thioredoxin-dependent rates of activation, calculated by subtracting the background rate of activation by 4 mM DTT alone from the rate in the presence of both Trx and DTT. Curve fittings were performed as in Fig. 2. ○, Trx $f$; ○, Trx $m$.
PK. If initial rate determinations are to be valid and reproducible, the Trx must be maintained in its fully reduced form, which can be achieved by the addition of an exogenous low molecular weight thiol to the Trx reaction mixtures. However, the incomplete and contradictory literature concerning activation of PK by thiols compelled us to screen several to identify appropriate conditions for the Trx experiments. Of the three examined, only DTT met the criterion of clearly discernible Trx-dependent activation of PK at a rate independent of the concentration of the added thiol. Despite counter literature reports (31, 32), both BME and glutathione can also fully activate PK; they do not, however, support Trx-dependent activation, presumably because of their weaker reduction potentials, which allow build-up of oxidized Trx, thereby driving the equilibrium in favor of oxidized PK.

We were prompted to examine the activation of PK by GSH in some detail because of its high natural abundance in chloroplasts (4 mM) (33) and hence the obvious question of whether it might play some role in the regulation of PK. The rate saturation kinetics relative to the concentration of GSH were unexpected. Reduction of the PK disulfide by a monothiol entails two consecutive bimolecular reactions: formation of a protein-S-SG mixed disulfide followed by intermolecular displacement by GSH to form GSSG and the reduced protein. Irrespective of the relative rates of the two reactions, the overall rate of PK activation should be directly proportional to the concentration of GSH. Our counterintuitive finding of rate saturation argues that GSH and PK form a noncovalent complex prior to the initial sulfhydryl/disulfide exchange reaction. Although the apparent affinity of GSH for PK is rather weak (\(K_{d} = 24 \text{ mM}\)), the activation \(V_{\text{max}}\) (20 milliunits/min) is about 2-fold greater than the corresponding values for either Trx. In these experiments, the concentration of GSSG should increase stoichiometrically with PK reduction. However, in chloroplasts, the action of NADP-dependent glutathione reductase maintains GSH predominantly in its reduced form. Thus, we also examined the reduction of PK by GSH in the presence of a fixed concentration of 4 mM DTT. Under these conditions, the \(S_{0.5}\) is decreased substantially to 5 mM, closely matching the concentration of GSH in chloroplasts. This decrease likely reflects relief of inhibition of PK activation by GSSG. We attribute the apparent 2-fold drop in \(V_{\text{max}}\) to competition between DTT and GSH in their interaction with PK. This conclusion follows from the observation that the \(V_{\text{max}}\), uncorrected for the contribution of DTT alone, is about the same as the GSH-dependent \(V_{\text{max}}\) determined in the absence of DTT. The kinetic parameters as determined for the activation of PK by GSH are not incompatible with the latter influencing the PRK activity levels in chloroplasts; however, this possibility must be tempered by measurements that indicate little fluctuation in the in vivo concentration of GSH or the \([\text{GSH}]/[\text{GSSG}]\) molar ratio (33).

With respect to kinetic characterizations of the activation of PK by Trx, our data show that the two thioredoxins are quite similar in their effectiveness with modest superiority of Trx being attributable to an ~2-fold greater \(V_{\text{max}}\). In the initial study of target enzyme selectivity of chloroplast thioredoxins at the time of their discovery, Trx was judged to be more effective than Trx in the activation of PK (6). This judgement was based on a 2.5-fold greater end point activity that was attained with a single concentration of Trx in comparison to Trx. Separate experiments, again at single concentrations of thioredoxins, actually showed only slightly faster rates of activation of PK by Trx than by Trx. Thus, the perpetuation of the notion of clear-cut superiority of Trx seems to have been based on undue focus on one data set to the exclusion of the other. We would also note that the use of impure preparations of thioredoxins and an unstable form of PK complexed with NADP-dependent glyceraldehyde-3-phosphate dehydrogenase in the prior investigation could have influenced the activation kinetics.

Although our present study is the first to provide a detailed comparison of activation kinetics of PK by Trx and Trx, activation kinetics with Trx have been determined previously (34, 35). An apparent dissociation constant of 0.8 \(\mu\text{M}\) was reported for the PK-Trx complex as calculated from the Trx concentration dependence of levels of activated PK reached at equilibrium. However, the rates of PK activation increased over the range from 1 to 10 \(\mu\text{M}\) (the highest concentration examined) Trx and thus appear consistent with our \(S_{0.5}\) of 17 \(\mu\text{M}\).

In our prior study (10) of the relative effectiveness of Trx and Trx as activators of fructose-1,6-bisphosphatase and NADP-dependent malate dehydrogenase, we included a number of site-directed mutants of Trx patterned after Trx. The residues targeted for change or deletion at that time (Lys\(^{58}\), Asn\(^{74}\), Gln\(^{76}\), and Asn\(^{77}\)) appear unique to Trx; despite conservation of the corresponding residues among most thioredoxins including Trx and *Escherichia coli* Trx (36). We reasoned that such residues are good candidates for participation in target protein recognition and selectivity. Indeed, the mutants examined were generally more effective than wild-type Trx in the activation of malate dehydrogenase but less effective in the activation of fructose-1,6-bisphosphatase. Comparative structural considerations also prompted the inclusion of the T105I and V89I/T105I mutants of Trx in the present study of PK activation.

Three-dimensional structures show Lys\(^{58}\) to be near the active site and Asn\(^{74}\), Gln\(^{76}\), and Asn\(^{77}\) to be near a conserved, putative hydrophobic contact surface (37–39). Residues 89 and

| Thioredoxin | \(S_{0.5}\) | \(V_{\text{max}}\) | \(V_{\text{max}}/S_{0.5}\) |
|-------------|--------|--------|----------------|
| Wild-type Trx \(f\) | 17 | 7 | 41 |
| Wild-type Trx \(m\) | 15 | 11 | 73 |
| Trx \(f-K58E\) | 23 | 4 | 17 |
| Trx \(f-Q75D\) | 52 | 20 | 38 |
| Trx \(f-N74D\) | 32 | 10 | 32 |
| Trx \(f-Q75D\) | 92 | 15 | 6 |
| Trx \(f-A77\) | 47 | 18 | 38 |
| Trx \(T105I\) | 66 | 42 | 64 |
| Trx \(V89I/T105I\) | 47 | 37 | 79 |
and Glu63 of NF
more responsive to Trx
photosynthetic and heterotrophic growth (48, 49). We speculate
highly homologous to higher plant Trx
thioredoxins. Thus, any indispensable biological need for chlo-
Trx activate target enzymes.

Therefore, residues at positions 89 and 105 in spinach Trx
S
Trx
m
increases in
V
f
values in the activation of PRK, thus
rendering the mutants more akin to Trx
m. However, these
improvements are offset by increased S
f
values, whereby the
efficiencies differ little from that of wild-type Trx
f.

In contrast, the efficiencies displayed by Trx
f/T105I and Trx
f/V89I/T105I closely resemble that of wild-type Trx
m because of substantial
increases in V
max.

Interestingly, these two mutants also mimic
m
Trx
m with respect to activation of fructose-1,6-bisphosphatase, but the altered efficiencies primarily reflect S
f
values (46). In fact, the affinity of the double mutant for the
phosphatase is reduced nearly 50-fold relative to wild-type Trx
f.

Therefore, residues at positions 89 and 105 in spinach Trx
f clearly impact the ability of the protein to interact with and activate target enzymes.

In addition to our studies on target protein selectivity of chloroplast thioredoxins, another laboratory reported a 6-fold more rapid rate of activation of chloroplast ATP synthase by Trx
f than by Trx
m (47). Of the four target proteins whose activation kinetics have thus far been quantified, three are more responsive to Trx
f, and one is similarly responsive to both thioredoxins. Thus, any indispensable biological need for chloroplast Trx
m does not appear to reflect redox regulation of enzymes. Indeed, a thioredoxin of cyanobacteria, which is highly homologous to higher plant Trx
m, is required for both photosynthetic and heterotrophic growth (48, 49). We speculate that both major roles of Trx
m in plants remain to be elucidated.

Beyond the usual caveat of extending in vitro findings to events that occur in vivo, PRK presents a particular challenge. As emphasized, our studies were carried out with highly purified, homodimeric PRK from spinach. Although numerous independent studies suggest that in vivo chloroplast PRK is part of a multienzyme complex, an accurate description of the complex as present in chloroplasts is rendered problematic because of qualitative variation of the composition of the isolated complexes (2, 50–60). The PRK within the 5-protein complex PRK-
Rubisco-phosphoribosomase-glyceraldehyde-3-phosphate dehydrogenase-phosphoglycerate kinase has been reported to be more readily activated by Trx
f than is free PRK (34, 35). To explore the possibility that the association of PRK with other proteins might substantially alter the relative efficiencies of activation by the two thioredoxins, we tested the effect of Rubisco (the most abundant chloroplast protein) on activation of PRK by Trx
f and Trx
m. The absence of altered activation kinetics in the presence of Rubisco argues against any unique requirement for Trx
m, even if activation occurs only at the complex level in vivo. However, the presence of homodimeric PRK in fresh plant extracts (61), the inherent instability of the PRK-containing multienzyme complexes (Ref. 58; additional reports reviewed extensively in Ref. 30), the dissociation of these complexes by thiols (32, 53, 57–59) and NADPH (59, 62), and the calculated dissociation constant of the PRK-glyceraldehyde-3-phosphate dehydrogenase complex (58) indicate that at least a portion of the chloroplast PRK exists in uncomplexed form and would be available for interaction with thioredoxins. Thus, we believe that our studies on the activation of purified PRK are justified and that the derived data will provide a relevant basis of comparison in future studies with complexed PRK once the in vivo status of this enzyme is better understood.

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