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Prompt and Easy Activation by Specific Thioredoxins of Calvin Cycle Enzymes of *Arabidopsis thaliana* Associated in the GAPDH/CP12/PRK Supramolecular Complex

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ABSTRACT The Calvin cycle enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoribulokinase (PRK) can form under oxidizing conditions a supramolecular complex with the regulatory protein CP12. Both GAPDH and PRK activities are inhibited within the complex, but they can be fully restored by reduced thioredoxins (TRXs). We have investigated the interactions of eight different chloroplast thioredoxin isoforms (TRX f1, m1, m2, m3, m4, y1, y2, x) with GAPDH (A_4, B_4, and B_8 isoforms), PRK and CP12 (isoform 2), all from *Arabidopsis thaliana*. In the complex, both A_4-GAPDH and PRK were promptly activated by TRX f1, or more slowly by TRXs m1 and m2, but all other TRXs were ineffective. Free PRK was regulated by TRX f1, m1, or m2, while B_4- and B_8-GAPDH were absolutely specific for TRX f1. Interestingly, reductive activation of PRK caged in the complex was much faster than reductive activation of free oxidized PRK, and activation of A_4-GAPDH in the complex was much faster (and less demanding in terms of reducing potential) than activation of free oxidized B_4- or B_8-GAPDH. It is proposed that CP12-assembled supramolecular complex may represent a reservoir of inhibited enzymes ready to be released in fully active conformation following reduction and dissociation of the complex by TRXs upon the shift from dark to low light. On the contrary, autonomous redox-modulation of GAPDH (B-containing isoforms) would be more suited to conditions of very active photosynthesis.

Key words: carbon metabolism; enzymology; light regulation; metabolic regulation; photosynthesis; Arabidopsis.

INTRODUCTION

Thioredoxins (TRXs) are small ubiquitous redox proteins encoded by large gene families in oxygenic photosynthetic organisms (Lemaire et al., 2003; Buchanan and Balmer, 2005; Florencio et al., 2006; Meyer et al., 2006). *Arabidopsis thaliana* has been reported to contain at least 20 genes coding for classical TRX proteins of 10–14 kDa with a conserved WC(G/P)PC motif in the active site (Meyer et al., 2005; Lemaire et al., 2007), nine of which are targeted to plastids (Collin et al., 2003, 2004; Lemaire et al., 2007; Schürmann and Buchanan, 2008). Based on sequence similarities, plastid TRXs have been classified into four types (f, m, x, y), each including one or more isoforms. Apart from TRX y1, which is mainly expressed in non-photosynthetic organs (Collin et al., 2004), all other TRXs appear to be predominantly, but not exclusively, expressed in leaves (Schmid et al., 2005; de Dios Barajas-Lopez et al., 2007; Traverso et al., 2008).

In chloroplasts, TRXs are reduced in the light by Photosystem I via ferredoxin and ferredoxin:thioredoxin reductase (Dai et al., 2007; Schürmann and Buchanan, 2008). The active site dithiol of reduced TRXs can reduce disulfide bridges on target proteins, in most cases enzymes that are reversibly activated by reduction (Buchanan and Balmer, 2005). The multiplicity of TRX genes is paralleled by a multiplicity of TRX targets, most of which have been recently identified through redox proteomic approaches (Michelet et al., 2006; Lemaire et al., 2007). In chloroplasts, these include, for instance, all 11 enzymes of the Calvin cycle, besides Rubisco

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activase (Portis et al., 2008) and CP12 (Wedel et al., 1997), which are involved in Calvin cycle regulation. Only in few cases, however, has the specificity of TRX targets for different TRX isoforms been thoroughly investigated (Schürmann and Buchanan, 2008). In general, TRXs f and m were found to be principally involved in enzyme regulation, including light-modulation of photosynthetic metabolism, while TRXs x and y seem to serve mainly as hydrogen donors for antioxidant enzymes (e.g. peroxiredoxins: Collin et al., 2003, 2004; glutathione peroxidases: Navrot et al., 2006; methionine sulfoxides reductases: Vieira dos Santos et al., 2007).

In this work, we investigated the specificity of interactions between chloroplast TRXs and two enzymes of the Calvin cycle (glyceraldehyde-3-phosphate dehydrogenase, GAPDH, and phosphoribulokinase, PRK) which are known to form a supramolecular complex with CP12 under oxidizing conditions, those prevailing when the photosynthetic electron transport is decreased under limiting light or stress conditions or in the dark (Wedel et al., 1997; Scheibe et al., 2002; Graciet et al., 2004; Trost et al., 2006; Howard et al., 2008). All experiments were performed with recombinant proteins from *Arabidopsis thaliana*, namely eight chloroplast TRX isoforms (f1, m1, m2, m3, m4, x, y1, and y2; Collin et al., 2003, 2004) and five different targets (GAPDH-isoforms A_4, B_4, and B_8; CP12-isoform 2 and PRK; Marri et al., 2005).

PRK is a dimer with one TRX-sensitive disulfide per subunit (Porter et al., 1988) and, although disulfide formation itself causes a significant inhibition of PRK activity (Hirasawa et al., 1999; Geck and Hartman, 2000; Hutchinson et al., 2000), inclusion of oxidized PRK into the supramolecular complex with GAPDH and CP12 leads to additional inhibition of enzyme activity (Marri et al., 2005). CP12 may contain two intramolecular disulfides and, similarly to PRK, has to be fully oxidized to promote the assembly of the supramolecular complex including also GAPDH (Wedel and Soll, 1998; Graciet et al., 2003; Marri et al., 2008).

Calvin cycle's GAPDH exists in different isoforms and may either have no disulfides (isoform A_4) or contain two identical disulfides per tetramer, one in each of the C-terminal extensions specific for B-subunits (isoforms A_2B_2 or A_8B_8, Baalmann et al., 1996; Fermani et al., 2007). Recombinant GAPDH isoforms made of B-subunits only (B_4 or B_8) were never observed in vivo but found to display similar properties to native AB-GAPDH isoforms (A_2B_2 or A_8B_8), including the autonomous regulation by TRXs and metabolites such as NAD(P)(H) and 1,3-bisphosphoglycerate (Baalmann et al., 1996; Li and Anderson, 1997; Sparla et al., 2002, 2005). The A_4-GAPDH isoform is instead completely insensitive to regulation by TRXs and metabolites (Baalmann et al., 1996; Scagliarini et al., 1998; Sparla et al., 2002), although catalytic cysteines (Cys-149) of A_4-GAPDH can form mixed disulfides with glutathione under oxidative stress conditions (Zaffagnini et al., 2007). This post-translational modification leads to complete loss of enzyme activity, but can be reverted by glutaredoxins (Zaffagnini et al., 2008). On the other hand, complex formation with PRK and CP12 leads to reversible inhibition of A_4-GAPDH activity under physiological conditions (Graciet et al., 2004; Marri et al., 2005) and has been envisioned as a mechanism for light-modulation of A_4-GAPDH in land plants (Trost et al., 2006; Marri et al., 2008), similar to the regulatory system of lower photosynthetic organisms that do not contain AB-GAPDH (Wedel and Soll, 1998; Graciet et al., 2004; Tamoi et al., 2005; Oesterhelt et al., 2007). Due to the close similarity between A- and B-subunits, a similar regulation based on CP12 might also affect A_2B_2-GAPDH, which was observed to be partially complexed with CP12 and PRK in the dark or under low-light conditions in different higher plant species (Wedel and Soll, 1998; Scheibe et al., 2002; Howard et al., 2008).

Here, we show that the inhibition of the activities of enzymes complexed with CP12 (A_4-GAPDH and PRK) can be rapidly and fully reversed by specific TRX isoforms. Interestingly, this process is much faster than the reductive activation of free enzymes (B-GAPDH or PRK) and, in the case of GAPDH, is also less demanding in terms of reducing potential. Since this regulatory mechanism based on the dissociation of a supramolecular complex results in the parallel activation of two Calvin cycle enzymes, we speculate that its physiological function might be to ensure a fast and coordinated onset of the Calvin cycle upon the transition from dark to light (Howard et al., 2008).

RESULTS

A supramolecular complex comprising A_4-GAPDH, CP12 (isoform 2; Trost et al., 2006), and PRK of *Arabidopsis thaliana* was reconstituted in vitro by incubating the three purified recombinant proteins with NAD under oxidizing conditions (oxidized DTT, Marri et al., 2005). The 500-kDa complex included two GAPDH tetramers, two PRK dimers, and four CP12 monomers (Marri et al., 2008) and eluted in size-exclusion chromatography as a single symmetrical peak (Figure 1) with strongly inhibited enzyme activities. The NADPH-dependent GAPDH activity of the complex was 26 $\pm$ 6% (SD, n = 16) of the NADPH-activity of free A_4-GAPDH. PRK activity of the complex was 5 $\pm$ 2% (SD, n = 10) of the activity of the fully reduced free enzyme.

Thermodynamics of Complex Dissociation and GAPDH/PRK Reactivation

The reactivation process of GAPDH and PRK embedded in the A_4-GAPDH/CP12/PRK complex was analyzed by equilibrium redox titrations in the presence of a cocktail of different *Arabidopsis* chloroplast thioredoxins and different ratios of oxidized/reduced DTT (Figure 2). In these experiments, TRXs acted as redox mediators, favoring full equilibration of the complex with the redox potential established by DTT (Hutchinson and Ort, 1995; Hirasawa et al., 1999). The measurements were performed at pH 7.9, corresponding to the pH of the chloroplast stroma in the light.

Although A_4-GAPDH contains no redox-sensitive disulfides, the NADPH-dependent GAPDH activity of the complex