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Chloroplast FBPase and SBPase are thioredoxin-linked enzymes with similar architecture but different evolutionary histories

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The Calvin–Benson cycle of carbon dioxide fixation in chloroplasts is controlled by light-dependent redox reactions that target specific enzymes. Of the regulatory members of the cycle, our knowledge of sedoheptulose-1,7-bisphosphatase (SBPase) is particularly scanty, despite growing evidence for its importance and link to plant productivity. To help fill this gap, we have purified, crystallized, and characterized the recombinant form of the enzyme together with the better studied fructose-1,6-bisphosphatase (FBPase), in both cases from the moss *Physcomitrella patens* (Pp). Overall, the moss enzymes resembled their counterparts from seed plants, including oligomeric organization—PpSBPase is a dimer, and PpFBPase is a tetramer. The two phosphatases showed striking structural homology to each other, differing primarily in their solvent-exposed surface areas in a manner accounting for their specificity for seven-carbon (sedoheptulose) and six-carbon (fructose) sugar bisphosphate substrates. The two enzymes had a similar redox potential for their regulatory redox-active disulfides (−310 mV for PpSBPase vs. −290 mV for PpFBPase), requirement for Mg²⁺ and thioredoxin (TRX) specificity (TRX *f* > TRX *m*). Previously known to differ in the position and sequence of their regulatory cysteines, the enzymes unexpectedly showed unique evolutionary histories. The FBPase gene originated in bacteria in conjunction with the endosymbiotic event giving rise to mitochondria, whereas SBPase arose from an archaeal gene resident in the eukaryotic host. These findings raise the question of how enzymes with such different evolutionary origins achieved structural similarity and adapted to control by the same light-dependent photosynthetic mechanism—namely ferredoxin, ferredoxin-thioredoxin reductase, and thioredoxin.

Calvin–Benson cycle | sedoheptulose-1,7-bisphosphatase | fructose-1,6-bisphosphatase | redox regulation | thiol–disulfide exchange

In oxygenic photosynthesis, CO₂ fixation takes place via the Calvin–Benson cycle consisting of 13 individual reactions that can be separated into carboxylation, reduction, and regeneration phases (1). Considerable effort has focused on a description of the individual enzymes and the overall regulation of the cycle (2, 3). In chloroplasts, the activity of four enzymes of the cycle is linked to light: NADP-glyceraldehyde 3-phosphate dehydrogenase, phosphoribulokinase, fructose-1,6-bisphosphatase (FBPase), and sedoheptulose-1,7-bisphosphatase (SBPase). In some plants, Rubisco is similarly regulated indirectly by Rubisco activase. The activity of each of these enzymes is modulated by the ferredoxin/thioredoxin system—a thiol-based mechanism in which photoreduced ferredoxin provides electrons for the reduction of thioredoxin (TRX) by the enzyme ferredoxin-thioredoxin reductase (FTR) (3–5). TRX, in turn, reduces specific disulfides and thereby activates the regulatory

members by thiol–disulfide exchange. Chloroplasts contain several typical thioredoxin subtypes (*f*, *m*, *x*, *y*, and *z*) with different target preferences (6) as well as a number of proteins containing an atypical TRX active site (7). The ferredoxin/thioredoxin system was uncovered by observing the activation of FBPase by photoreduced ferredoxin (8)—a finding later extended to SBPase (9). Due to its high activity and convenient assay, FBPase was used to explore the system, eventually leading to the identification of FTR and TRX as essential components and to the finding that other photosynthetic enzymes are regulated by this mechanism (10–12). As part of this study, SBPase, which at the time was considered to be a secondary activity event of FBPases (e.g., 13, 14), was found to be a separate enzyme in chloroplasts (15). Both phosphatases function in the regeneration stage of the Calvin–Benson cycle. Their natural

Significance

We demonstrate that, although the two phosphatases of the Calvin–Benson cycle of photosynthesis [sedoheptulose-1,7-bisphosphatase (SBPase) and fructose-1,6-bisphosphatase (FBPase)] share extensive structural homology, their redox-regulatory disulfides are incorporated in strikingly different positions, in agreement with an independent evolutionary origin of each enzyme. This article compares in detail the structures of the enzymes together with their regulatory and catalytic properties as well as their phylogenies. Significantly, the substrate binding site of SBPase is larger than that of FBPase, thus allowing it to accommodate both seven- and six-carbon sugar phosphate substrates, whereas FBPase is active only with the latter. The data suggest that SBPase is of archaeal origin, whereas FBPase is descended from bacteria.

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The authors declare no conflict of interest.

Data deposition: The crystallography, atomic coordinates, and structure factors reported in this paper have been deposited in the Protein Data Bank, www.pdb.org [PDB ID codes 5IZ1 (PpFBPase) and 5IZ3 (PpSBPase)].

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monomer. The structure for oxidized *Pp*FBPase presently reported confirms the role of these two cysteines in disulfide formation. The distances between the sulfur atoms of the cysteine residues 224/246 and 241/246 in *Pp*FBPase are more than 7 Å for both pairs, so that disulfide formation would require a major conformational rearrangement, as was suggested to occur between Cys153 and Cys178 in the pea C173S mutant (16). In our X-ray structures, the redox-regulatory disulfides were shown to be surface-exposed and remote from the sugar biphosphate binding sites. Based on a crystallographic comparison with the pig kidney enzyme, it was postulated that the reduction of the disulfide of pea FBPase provoked a shift in the position of several β -strands, resulting in the reorientation of a critical glutamate side chain necessary for cofactor binding (16). At this point, the structural rearrangements leading to reductive SBPase activation are yet to be defined.

Regulation of FBPase and SBPase of *P. patens* Chloroplasts.

Assay of SBPase. The enzyme was ideally assayed by measuring P_i release from SBP. However, the lack of a reliable commercial source of SBP necessitated that we use an alternate procedure for large experiments. Therefore, in those cases, we measured activity with FBP as substrate. We found that the homogeneous enzyme could use FBP at 1/100th the rate observed with SBP. Therefore, unless stated otherwise, we monitored activity of SBPase with FBP. **Thioredoxin specificity.** For optimal catalysis, FBPase and SBPase are reduced by the light-dependent ferredoxin/thioredoxin system or its nonphysiological in vitro replacement, DTT-reduced TRX. Because chloroplasts contain multiple classical TRXs (*f*, *m*, *x*, *y*, and *z*), we tested the effect of several different TRXs on the reductive activation of the enzyme. TRX *z*, as well as the atypical chloroplast TRX-like2.2, were unable to activate either phosphatase, whereas TRXs *f* and *m* were effective in the order $f > m$ (Fig. 2A). *Pp*FBPase was activated at all levels of TRXs *f* and *m* tested, but *Pp*SBPase required relatively high levels of both redoxins and even then was only sluggishly activated by TRX *m*. Thus, *Pp*FBPase activation saturated at about 2 μ M TRX *f* and at 20 μ M TRX *m*. The results show that, under these conditions, TRX *m* activated *Pp*FBPase, in agreement with earlier reports (27, 28). We conclude that TRX *f* is more effective than TRX *m* in regulating the two phosphatases, as found originally (4), and that activation of SBPase by TRX *m* is marginal. TRXs *x* and *y* function in reactive oxygen species defense jointly with accessory enzymes and are not active with FBPase (6). Consequently, these proteins were not tested. The results demonstrate that the moss (bryophyte) phosphatase enzymes exhibit regulatory properties similar to the more advanced seed plant species. It has been proposed that redox regulation in its modern form appeared after the endosymbiotic event (29) and was later refined in land plants. In keeping with this idea, some years ago the NADP-dependent malate dehydrogenase (NADP-MDH) of *Chlamydomonas reinhardtii* was found to display regulatory properties intermediate between those of nonredox counterparts and the fully redox-controlled enzyme of land plants (30).

Redox potentials. To gain further insight into the regulation of the phosphatases, we estimated the potentials of the redox-active disulfides of both enzymes following treatment with a varying amount of oxidized and reduced DTT plus a catalytic amount of TRX *f*. The resulting band pattern indicated that *Pp*SBPase (−310 mV) has a slightly more negative reduction potential at pH 7.0 than *Pp*FBPase (−290 mV) (Fig. 2B). This difference may be a reflection of the versatility of function: FBPase functions in both the Calvin–Benson cycle and starch synthesis, whereas SBPase has a role only in the former pathway.

Redox status vs. catalytic activity. We next compared the relative reduction rates coupled with a measure of catalytic activity of the phosphatases. To this end, we reduced the proteins with a range of reductant (DTT) concentrations and stopped the reaction after different incubation times to measure the extent of reduction by gel electrophoresis and enzyme activity by biochemical assays. The

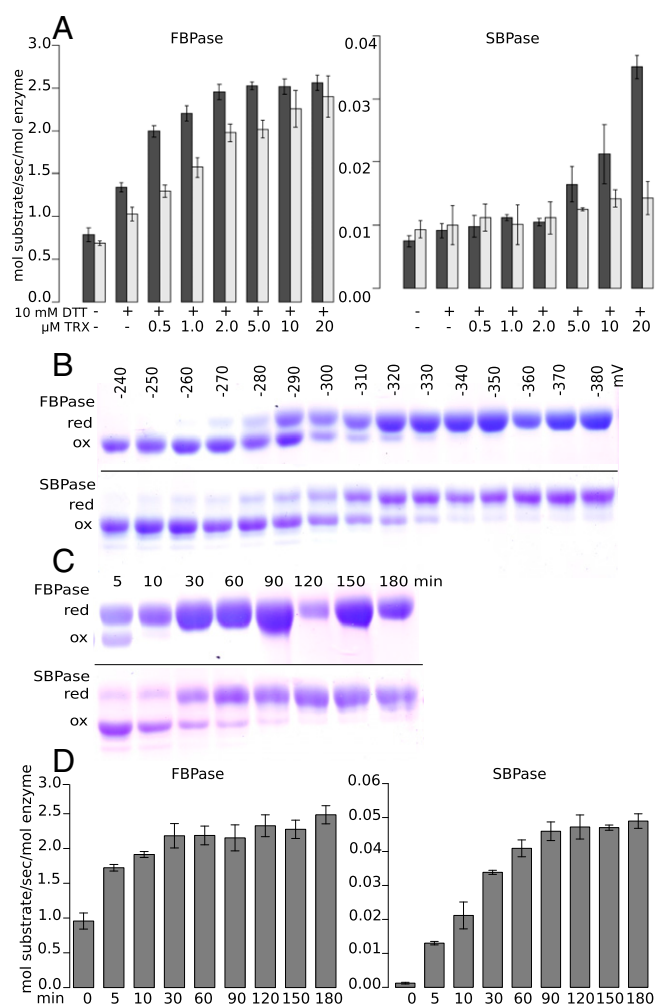


Fig. 2. Regulatory aspects of *Pp*FBPase and *Pp*SBPase. (A) Dependency of phosphatases on thioredoxin. Dark gray bars show the activity of the enzymes reduced with TRX *f*, and light gray bars show the activity with TRX *m*. Activities are depicted in mol substrate transformed per s/mol enzyme. Both FBPase and SBPase activities were evaluated using the coupled spectrophotometric assay and the “alternate” FBP substrate for SBPase. (B) Redox potential. Midpoint redox potentials estimated by SDS/PAGE following methoxy-PEG (mPEG)-maleimide labeling. Both proteins were treated with various ratios of oxidized and reduced DTT and then labeled with mPEG-maleimide. The oxidation–reduction potential was read at the point indicating that the protein was half-oxidized and half-reduced. (C) Time course of reduction. mPEG-maleimide labeling by reduction with 10 mM DTT and 3 μ M TRX *f* at pH 7.0. (D) Time course of activation. Experimental conditions were as in C. Red, reduced; ox, oxidized. Error bars in A and D represent standard deviation.

experiments were carried out at pH 7.0 to slow the reduction/activation process and the onset of activity. As seen in Fig. 2 C and D, *Pp*FBPase was almost completely reduced after 10–30 min, whereas *Pp*SBPase was only partially reduced after 90 min. The rate of reduction of both phosphatases correlated with the appearance of catalytic activity, unlike earlier observations with NADP⁺-dependent malate dehydrogenase, where reduction was substantially faster than activation (31, 32). The absence of such a hysteretic effect with the phosphatases (4) is possibly linked to a simpler mechanism of activation. Both enzymes possess only a single disulfide per subunit, compared with NADP-MDH with two regulatory disulfides that necessitate an interconversion with an additional, internal cysteine. Our experiments thus suggest that the molecular movements required to activate the phosphatases are more restricted than for NADP-MDH. Moreover, under identical experimental

opisthokonts (Fig. 3B). This conclusion is supported by the finding that the SBPase gene is present in several unicellular eukaryotes that may have acquired it by secondary endosymbiosis of phototrophic eukaryotes (40). Notably, the regulatory cysteines have been either partially or completely lost from the SBPase genes, as seen, for example, in the alveolate *Tetrahymena thermophila* (Figs. S1 and S7). Irrespective of evolutionary origin, chloroplast FBPase and SBPase subsequently independently acquired the same mechanism of redox regulation under the control of ferredoxin, FTR, and TRX, although the positions of the regulatory sites both in the amino acid sequence and in the 3D structure are radically different. It remains to be seen why evolution has chosen two distinct sites on the highly structurally homologous FBPase and SBPase to implement a very similar regulatory principle. Examining a number of chloroplast redox-regulated enzymes, we have earlier made the proposal that acquisition of redox regulation responds to structural constraints inherent to each catalyst and that there cannot be a universal regulatory module fitting all regulatory enzymes (41). There are in the literature a number of studies dealing with the evolution of structures and active sites along temperature gradients (essentially comparing psychrophilic, hyperthermophilic, and mesophilic enzymes catalyzing identical reactions). In directed evolution it has been observed that the opening of larger cavities at the active site essentially correlates with modifications in the loops bordering these positions with the possible removal of bulky amino acid chains. Interestingly, in our situation, changes of that sort occur near the active and regulatory sites. Our data suggest that the FBPase–SBPase comparison is an example of natural selection achieving results similar to those reported in directed evolution for lipase and amylase in particular (42–44).

Concluding Remarks

Two differences stand out in distinguishing chloroplast SBPase and FBPase at the protein level: (i) the solvent-accessible surface areas of their active sites, and (ii) the nature and relative positioning of their redox-active regulatory disulfides. As perhaps would be expected, the active-site solvent-accessible surface area for SBPase was found in this study to be significantly larger than for FBPase, thus allowing the accommodation of the seven-carbon sugar phosphate. This size difference is reflected in the substrate specificity of the enzymes. SBPase with the larger surface area hydrolyzes both the seven-carbon substrate SBP and the smaller six-carbon FBP, although it is much less effective with the latter. By contrast, whereas highly active with FBP, FBPase with the smaller active-site surface area is inactive with SBP. It remains to be seen whether this specificity difference has physiological consequences. Interestingly, the plant mutants with decreased SBPase activity have a much stronger phenotype than the chloroplast FBPase ones (45). We suggest that in FBPase mutants, either cytosolic FBPase with the help of either a transport system or SBPase can substitute to some extent for authentic FBPase. Obviously, our results indicate that the opposite is not true, explaining the more marked phenotype linked to the SBPase mutants. More mysterious is the basis for the difference in the regulatory sites. The two redox-active cysteines have long been known to differ not only in their adjoining amino acids but also in their placement in the proteins. Initially, we thought that knowledge of the structure of the SBPase and FBPase enzymes might help explain these differences. However, this turned out not to be the case: Despite their low amino acid sequence identity, the proteins display highly similar folds at the subunit level similar to what was observed for thioredoxin and glutaredoxin. Moreover, there were no striking differences in redox potentials or in the activity parameters altered on reduction by TRX. Our evidence suggests that FBPase was derived from bacteria in conjunction with the endosymbiotic event giving rise to mitochondria, and that SBPase was derived from an archaeal gene, putatively present in the host cell. It is remarkable that enzymes derived from genes with such different histories were adapted to embrace the same mechanism of

regulation by redox transitions—that is, catalytic activity under the control of light, ferredoxin, and a thiol/disulfide regulatory chain. It is becoming fascinating to understand the evolutionary changes in the enzymes that made this adaptation possible. Lessons learned here could apply to other enzymes of the Calvin–Benson cycle.

Materials and Methods

Preparation of Recombinant PpFBPase and PpSBPase. cDNA from PpFBPase (1sPp153_72) and PpSBPase (1sPp41_162) was amplified by PCR (primers are listed in Table S2) and cloned in pET expression vectors. The proteins were produced in *Escherichia coli* and purified by several purification steps (for details, see SI Materials and Methods).

Crystallization and Structure Determination. The crystals obtained were analyzed by X-ray diffraction, and the structure was solved by molecular replacement (see SI Materials and Methods and Table S3 for detailed information).

Enzyme Activity Assays. The TRXs used for the assays were overexpressed in *E. coli*, and the sequences were retrieved from *Pisum sativum* (TRX *f*) (46) and *C. reinhardtii* (TRX *m*) (47).

Coupled Assay for FBP Hydrolysis. The activity of the enzymes determined with FBP as substrate was measured spectrophotometrically at 340 nm in a coupled system. The reduction of NADP⁺ was followed at 340 nm and the slope values were calculated. The reaction mix (in 500 μ L) contained 0.2 mM NADP⁺, 30 mM Tris-HCl (pH 8.0), MgSO₄ (3 mM with reduced enzymes; 16 mM with oxidized enzymes), 0.6 mM FBP, 0.1 units of glucose 6-phosphate dehydrogenase, and 0.1 units of phosphoglucose isomerase. For determining K_m values, enzymes were incubated with 10 mM DTT and 3 μ M TRX *f* for 1.5 h and assayed with FBP concentrations ranging between 0 and 1.5 mM. For determining Mg²⁺ requirement, Mg²⁺ concentrations ranged between 0 and 30 mM; the Hill equation was used to calculate $S_{0.5}$. In TRX specificity assays, the phosphatase enzymes were preincubated at pH 8.0 at room temperature for 30 min with 10 mM DTT and different concentrations of the indicated TRX. For determining the time-dependent extent of reduction, assays were conducted with 10 mM DTT and 3 μ M TRX *f*. Reactions were stopped by adding 50 μ L 20% (wt/vol) TCA.

SBPase Assay. The release of P_i was measured colorimetrically. After reduction with 10 mM DTT and 3 μ M TRX *f* the activated enzyme was added to a 180- μ L reaction mix containing 5 mM Mg²⁺ in 30 mM Tris-HCl (pH 8.0). After an 8-min incubation at room temperature, 800 μ L P_i mix (2.5% sulfuric acid, 7.5 mM ammonium heptamolybdate, 100 mM FeSO₄) was added and the P_i released was measured at 660 nm. Because we could not identify a reliable commercial source of SBP, we used a 1980s Sigma product (kindly provided by Peter Schürmann, University of Neuchâtel, Neuchâtel, Switzerland) that gave reproducible results (mass spectrometric analysis confirmed that the compound was not degraded). SD of P_i release did not exceed 5%.

Midpoint Redox Potential Estimation. Midpoint redox potentials were calculated from the relative concentration of reducing agent added during titration according to the Nernst equation (for details, see SI Materials and Methods).

Time Course of Reduction of Phosphatases. Assay conditions were as described for midpoint potential measurements, except that 3 μ M TRX *f* was included to ensure complete reduction.

Phylogenetic Analysis. One portion of the sequences was selected based on the phylogenetic analysis performed by Jiang et al. (37), and the other portion was retrieved from Blast searches using the PpSBPase (Pp1s41_162) or PpFBPase (Pp1s153_72) protein sequence (48) as template with the 1KP webtool (www.onekp.com) and UniProt databank (www.uniprot.org). For alignment, Jalview (49) was used with the Muscle algorithm (default settings) and subsequently checked manually (Dataset S1). The C and N termini were trimmed manually according to the functional domains corresponding to amino acids 148–425 of PpFBPase. In total, 361 sites were used for calculation. The phylogenetic tree was built with MrBayes (version 3.1.2) software (50). The settings were adjusted to: aamodel, mixed; ngen, 1,000,000; samplefreq, 100; burn-ins, 2,500. After all generations, the SD of split frequencies was below 0.01. Numbers at branches represent posterior probabilities as inferred by MrBayes (version 3.1.2). The constructed tree was confirmed by achieving the same phylogenetic topology when using maximum-likelihood and neighbor-joining methods.

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