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Non-reductive modulation of chloroplast fructose-1,6-bisphosphatase by 2-Cys peroxiredoxin

Daniel Caporaletti, Ana C. D'Alessio, Roberto J. Rodriguez-Suarez, Alejandro M. Senn, Paula D. Duek, Ricardo A. Wolosiuk *

Instituto Leloir, Patricias Argentinas 435, C1405BWE Buenos Aires, Argentina

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Abstract

2-Cys peroxiredoxin (2-Cys Prx) is a large group of proteins that participate in cell proliferation, differentiation, apoptosis, and photosynthesis. In the prevailing view, this ubiquitous peroxidase poises the concentration of H_2O_2 and, in so doing, regulates signal transduction pathways or protects macromolecules against oxidative damage. Here, we describe the first purification of 2-Cys Prx from higher plants and subsequently we show that the native and the recombinant forms of rapeseed leaves stimulate the activity of chloroplast fructose-1,6-bisphosphatase (CFBPase), a key enzyme of the photosynthetic CO_2 assimilation. The absence of reductants, the strict requirement of both fructose 1,6-bisphosphate and Ca^{2+}, and the response of single mutants C174S and C179S CFBPase bring forward clear differences with the well-known stimulation mediated by reduced thioredoxin via the regulatory 170's loop of CFBPase. Taken together, these findings provide an unprecedented insight into chloroplast enzyme regulation wherein both 2-Cys Prx and the 170's loop of CFBPase exhibit novel functions.

Keywords: 2-Cys peroxiredoxin; Fructose-1,6-bisphosphatase; Enzyme activation; Thioredoxin; Chloroplasts; Oxidative stress

2-Cys peroxiredoxins (2-Cys Prx) are ubiquitous peroxidases devoid of heme and selenium prosthetic groups that participate in numerous biological processes [1–3]. Typical proteins of this family are obligate homodimers (subunit *ca.* 23 kDa), in which the conserved cysteine of one polypeptide is linked via a disulfide bond to the complementary cysteine located at the other subunit [4]. In solution, five homodimers associate noncovalently each other yielding a doughnut-shaped decamer [5]. Although the peroxidase and the recently described chaperone activity are linked to this redox-governed process [6,7], the unsolved question is whether 2-Cys Prx regulates the function of other proteins. Therefore, the following experiments were designed to investigate more thoroughly this issue with the counterpart of higher plant chloroplasts. In this scenario, chloroplast fructose-1,6-bisphosphatase (CFBPase) is an exemplary enzyme of photosynthetic CO_2 assimilation whose catalytic capacity is markedly stimulated by light via the ferredoxin–thioredoxin system [8–11]. Site-directed mutagenesis revealed that the constituent polypeptide (*ca.* 40 kDa) of the homotetrameric rapeseed enzyme bears seven conserved cysteines of which three [Cys157, Cys174, and Cys179] participate in the reductive stimulation while the other four [Cys53, Cys96, Cys191, and Cys307] are irrelevant for catalytic activity [12,13]. Regulatory cysteines are located at an intrapeptide, named the 170's loop, that is absent in orthologs devoid of light-dependent activation [14,15]. In the present study, we conducted the first purification of a 2-Cys Prx from higher plants leaves and subsequently found that the native and the recombinant form stimulate the activity of CFBPase concertedly with fructose 1,6-bisphosphate (FBP) and Ca^{2+}. We further

Abbreviations: 2-Cys Prx, 2-Cys peroxiredoxin; CFBPase, chloroplast fructose-1,6-bisphosphatase; DTT, dithiothreitol; FBP, fructose 1,6-bisphosphate; MCO, metal catalyzed oxidation; Trx, thioredoxin.

* Corresponding author. Fax: +54 11 5238 7501.
E-mail address: rwolosiuk@leloir.org.ar (R.A. Wolosiuk).

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demonstrated that the stimulation requires strictly not only the integrity of the intrinsic disulfide bond of the 170's loop but also the third cysteine residue. Taken together these results uncover novel roles in the field of enzyme modulation not only for 2-Cys Prx but also for the 170's loop of CFBPase.

Materials and methods

Materials. Recombinant rapeseed CFBPase and thioredoxin-f (Trx-f) were prepared and purified as described previously [16,17]. All but two variants of CFBPase were prepared by site-directed mutagenesis. Exceptions, I271T, and N154Y/P159T/I271T CFBPase, originated from a CFBPase library prepared by error-prone PCR and screened for low Ca^{2+} affinity [18].

Purification of native rapeseed 2-Cys Prx. Rapeseed leaves were homogenized in 50 mM Tris–HCl buffer (pH 7.9), 1 mM EDTA, and filtered through cheesecloth. The filtrate was adjusted to pH 4.5 and centrifuged (10 min at 5000*g*). The supernatant fraction was discarded and the precipitate was resuspended in 30 mM Na-acetate (pH 5.5), 5 mM EDTA and clarified by centrifugation (15 min at 5000*g*). The addition of solid ammonium sulfate (20–90% saturation) to the supernatant fraction yielded a protein precipitate that was resuspended in 30 mM Tris–HCl buffer (pH 7.9), 30 mM NaCl, 1 mM EDTA containing protease inhibitors [1 mM benzamide, 1 mM ε-aminocaproic acid, 2 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol, and 10 μM E-64] and dialysed overnight against the same buffer. After centrifugation (30 min at 105,000*g*), the supernatant fraction was loaded on a DEAE-cellulose column equilibrated with 30 mM Na-acetate (pH 5.5), 30 mM NaCl, 0.2 mM EDTA and chromatographed with a linear gradient of NaCl [30–800 mM]. Fractions analyzed by Western blot that reacted with 2-Cys Prx polyclonal antibodies were pooled and dialysed overnight against 30 mM Na-acetate buffer (pH 5.5) and 30 mM NaCl. At this stage, the precipitate obtained by centrifugation of the cloudy dialysate (10 min at 13,000*g*) contained mainly CFBPase and 2-Cys Prx. The supernatant fraction was applied to a DEAE-Fast Flow column (Pharmacia, Uppsala) equilibrated with 30 mM Na-acetate buffer (pH 5.5), 30 mM NaCl, and eluted with a linear gradient of NaCl [30–630 mM]. Fractions immunoreactive to the 2-Cys Prx antibody but insensitive to the CFBPase antibody were pooled, dialysed overnight against 30 mM Na-acetate buffer (pH 5.5) and stored at −20 °C.

Edman microsequentiation of native rapeseed 2-Cys Prx. The protein band (*ca.* 23 kDa) isolated from rapeseed leaves (Fig. 1A, Suppl. Info.), immunoreactive to the 2-Cys Prx antibody but insensitive to the CFBPase antibody, was transferred from a SDS–PAGE to a PVDF membrane by electroblotting and subjected to two independent Edman microsequentiations at LANAIS-PRO (Univ. Buenos Aires) and Protein Structure Core Facility (Univ. Nebraska).

Modulation of CFBPase activity. CFBPase activity was analyzed at 25 °C by the two-stage assay [19]. CFBPase (1 μg) was incubated in 100 mM Tris–HCl buffer (pH 7.9); 0.75 mM FBP; 0.05 mM $CaCl_2$; and 2-Cys Prx (20–40 μg). After 30 min, an aliquot was injected into the assay solution [20 mM Tris–HCl buffer (pH 7.9); 1 mM FBP; 1 mM $MgCl_2$; and 0.02 mM EGTA] and catalysis proceeded for 1–3 min. CFBPase activity: μmoles Pi released (min mg CFBPase)$^{-1}$.

Further experimental details are given in the electronic Supporting Information.

Results and discussion

Structural and functional aspects of native rapeseed 2-Cys Prx

The screening of a cDNA library from rapeseed leaves yielded a clone (1045 bp) containing a full-length open reading frame (813 bp) that coded for a polypeptide (270 amino acids, 29,498 Da) whose C-moiety (200 amino acids, 22,316 Da) was 94 %, 93%, 56%, and 53% identical to 2-Cys Prx from spinach chloroplasts, rye chloroplasts, human mitochondria, and *Trypanosoma cruzi* mitochondria, respectively (Fig. 2, Suppl. Info.). Given that 2-Cys Prx had not been isolated from higher plants, we undertook the purification of the rapeseed counterpart to establish unequivocally the N-terminus of the chloroplast mature form and, more importantly, to assess the ability in the modulation of CFBPase activity. After removal of a persistent contamination with CFBPase, the isolated protein exhibited the structural features of counterparts from other sources; i.e. the quaternary structure of homogeneous preparations of native rapeseed 2-Cys Prx fluctuated between a homodimer linked via disulfide bonds [α_2] and a decamer associated through hydrophobic interactions [$(\alpha_2)_5$] (Fig. 1, Suppl. Info.). Moreover, the purified leaf protein protected supercoiled DNA from single strand breaks caused by the concerted action of Fe^{3+}/thiol/O_2.

To investigate the regulation of CFBPase, we used the two-stage assay that clearly separates the conversion of the inactive enzyme to an active form (modulation) from the transformation of substrates to products (catalysis) [19]. Notably, the incubation of CFBPase with native 2-Cys Prx in the presence of both 0.75 mM FBP and 0.05 mM Ca^{2+} markedly enhanced the specific activity (Table 1). The intentional absence of reductants and oxidants allowed the clear-cut assignment of the time-dependent stimulation of CFBPase to a function of 2-Cys Prx that was not directly linked to the intrinsic peroxidatic activity.

Heterologous expression of mature 2-Cys Prx and characterization of the interaction with CFBPase

On the basis of the Edman microsequentiation of native rapeseed 2-Cys Prx [A-Q-(A/T)-D-D-L-P-L-V-G-], we cloned and expressed in *Escherichia coli* cells the DNA fragment coding for the mature form of rapeseed 2-Cys Prx and additionally holding a C-terminal His-tag for purification via immobilized metal affinity chromatography. Using this preparation, the enhancement of CFBPase activity again (i) required both 1 mM FBP and 0.05 mM Ca^{2+}, (ii) showed a slow time-dependence ($t_{0.5}$: 10 min) (Fig. 1A), and (iii) was dose-dependent and saturable at

Table 1
The reductant-independent enhancement of CFBPase activity by native rapeseed 2-Cys Prx

Treatment	CFBPase activity
Complete, 30 min	32.5 ± 0.9
Minus 2-Cys Prx	11.0 ± 0.4
Minus FBP	0.6 ± 0.2
Minus Ca^{2+}	1.5 ± 0.5
Complete, no incubation	0.7 ± 0.1

CFBPase (1 μg) was first incubated with FBP, Ca^{2+} and 2-Cys Prx, as indicated, and subsequently assayed as described in Materials and methods.

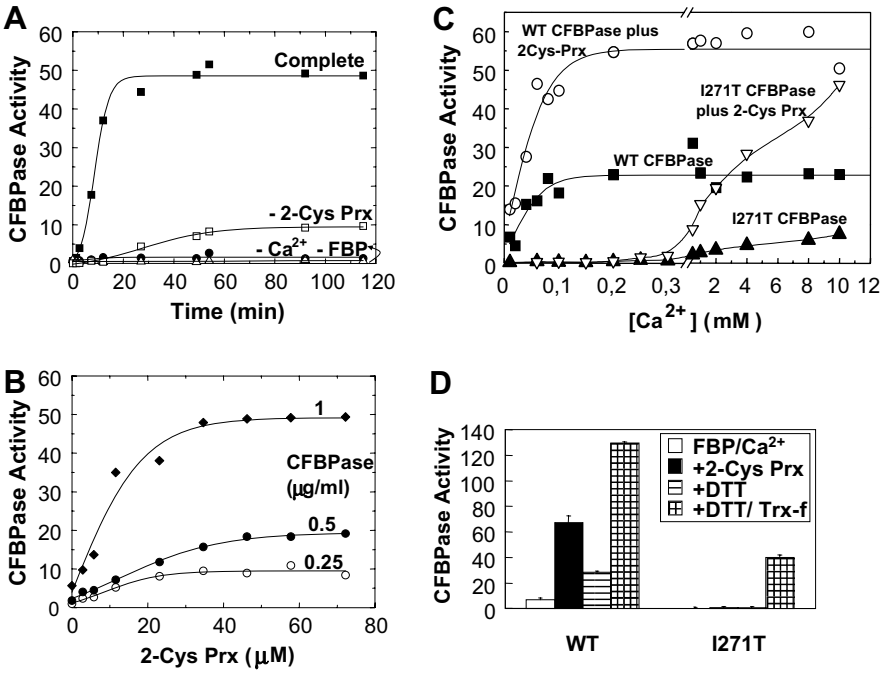

Fig. 1. Stimulation of CFBPase activity by recombinant rapeseed 2-Cys Prx. (A) Time course. CFBPase (0.64 μM) was incubated at 25 °C in 0.04 ml of 20 mM Tris–HCl buffer (pH 8) containing, as indicated, 1 mM FBP, 0.05 mM $CaCl_2$, and recombinant 2-Cys Prx (18 μM). At indicated times, an aliquot was injected into the assay solution, catalysis was halted after 2 min and released Pi was measured as described in Materials and methods. (B) Concentration dependence. The reaction conditions were essentially as in (A), except that the preincubation time was fixed at 20 min. (C) Ca^{2+} dependence. The reaction conditions were identical to (A), except that the preincubation time was fixed at 20 min and the concentration of the mutant enzyme was 3.2 μM. (D) Stimulation of I271T CFBPase. Wild type and I271T CFBPase (0.64 μM) were incubated for 20 min at 25 °C in the solution described in (A) containing 2-Cys Prx (18 μM) or DTT (0.4 mM)/Trx-f (2 μM), as indicated.

concentrations higher than 30 μM 2-Cys Prx (Fig. 1B). The Ca^{2+}-requirement for the stimulation of CFBPase suggested that cation binding to specific protein sites may play a critical role in the interaction with 2-Cys Prx. Consequently, information on this issue was sought with I271T CFBPase, a variant selected for lower Ca^{2+} affinity in studies of directed evolution [18]. Consistent with the diminished response to Ca^{2+}, 2-Cys Prx stimulated significantly the activity of I271T CFBPase in the millimolar range but it was ineffective in the micromolar range (Fig. 1C). At variance, the well-known activation of CFBPase elicited by reduced Trx was functional in the stimulation of I271T variant at micromolar concentrations of Ca^{2+} (Fig. 1D). The activation was not a consequence of global modifications of catalytic constants because 2-Cys Prx lowered the $S_{0.5}$ of Mg^{2+} while neither the $S_{0.5}$ of FBP (Table 1, Suppl. Info.) nor the pH maximum (8.8) were affected (Fig. 3, Suppl. Info.). These kinetic data contrasted with the Trx-mediated activation that enhances the affinity for FBP and Mg^{2+} in catalysis and additionally shifts the optimum pH from 8.8 to 8.0 [20].

The finding that 2-Cys Prx modulated the activity of oxidized CFBPase prompted us to investigate the action on the reduced form of the enzyme. To evaluate this issue, (i) CFBPase was activated with 10 mM DTT at 25 °C, (ii) the reductant was removed at 4 °C by ultrafiltration while maintaining both FBP and Ca^{2+}, and (iii) the enzyme was analyzed under aerobic conditions at 25 °C. We performed the step (ii) at 4 °C because it was difficult to keep the high specific activity of CFBPase at 25 °C. Fig. 2 shows that the highest activity of CFBPase persisted in the pres-

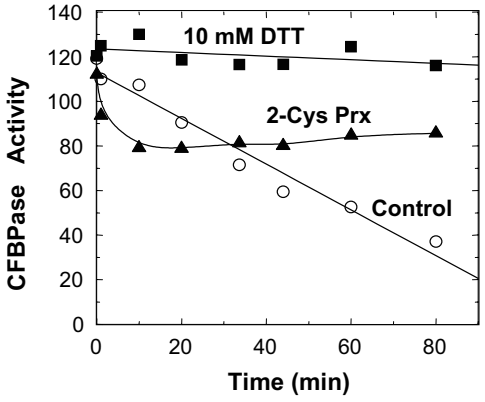

Fig. 2. Autooxidation of reduced CFBPase. CFBPase (1 μg) was incubated at 25 °C in 100 mM Tris–HCl buffer (pH 8), 1 mM FBP, 50 μM $CaCl_2$, and 10 mM DTT. After 20 min, DTT was removed at 4 °C by successive ultrafiltration (Amicon YM-10 membrane) washing with 100 mM Tris–HCl buffer (pH 8), 1 mM FBP, and 50 μM $CaCl_2$. The final preparation devoid of DTT, as assessed with 5,5′-dithiobis (2-nitrobenzoate), was stored frozen. After thawing, the protein solution was incubated at 25 °C in the absence or in the presence of either 10 mM DTT or 18 μM rapeseed 2-Cys Prx and subsequently the CFBPase activity was estimated as described in Materials and methods.

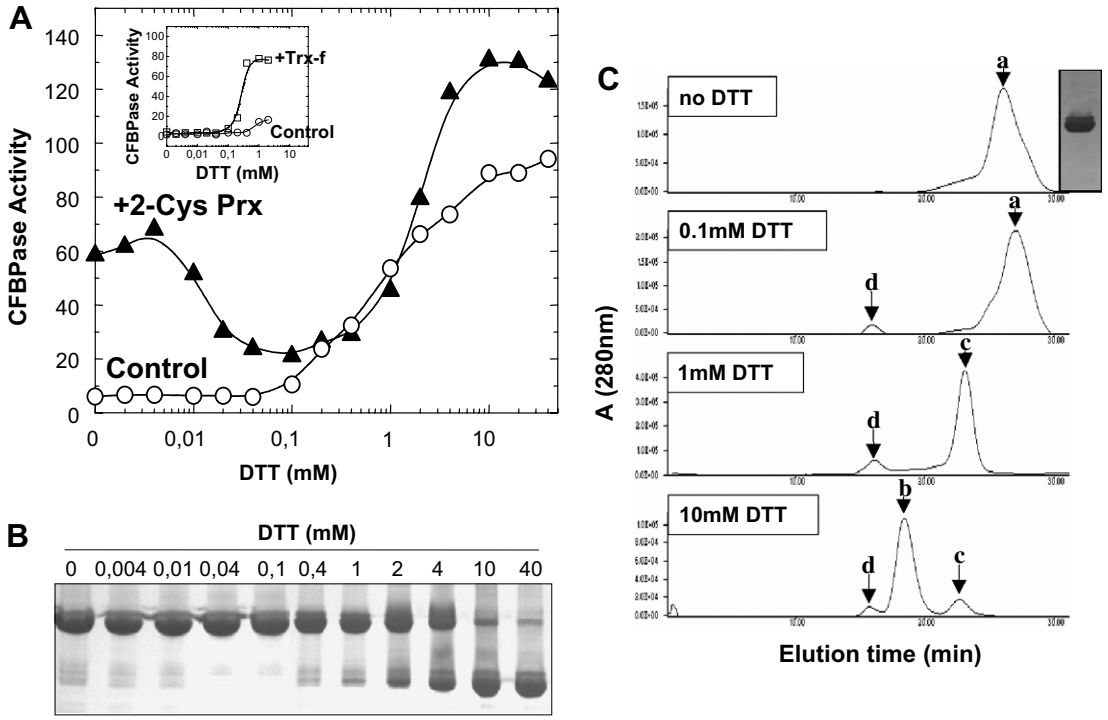

Fig. 3. Effect of DTT on rapeseed 2-Cys Prx. (A) Stimulation of CFBPase. CFBPase (1 μg) was incubated with DTT at 25 °C in 0.04 ml of 100 mM Tris–HCl buffer (pH 7.9), 1 mM FBP, 0.05 mM Ca^{2+}, and 2-Cys Prx (27 μg). After 30 min, the activity was assayed as described in Materials and methods. The reductive activation was performed under similar conditions using 2 μM Trx-f in place of 2-Cys Prx (inset). (B) Non-reductant SDS–PAGE. 2-Cys Prx was incubated with DTT as described in (A), mixed with the cracking buffer devoid of reductants and immediately loaded onto the gel (6.8 μg 2-Cys Prx/lane). (C) Redox-dependent oligomerization equilibrium of 2-Cys Prx. 2-Cys Prx (200 μg) was incubated at 25 °C for 20 min in 100 mM Tris–HCl buffer (pH 8) containing the indicated concentrations of DTT. Gel filtration was carried out in a Superdex HR-200 column equilibrated with the same buffer and connected in tandem with Light Scattering Equipment. The calculated molecular masses of the peaks were: (a) 46 kDa, (b) 232 kDa, (c) 85 kDa intermediate, and (d) undetermined aggregation (at V_0). The experiment was repeated three times. The SDS–PAGE of the loaded recombinant protein is shown in the inset.

ence of DTT but slowly decayed ($t_{0.5}$: 60 min) upon the removal of the reductant, due to cysteines oxidation as assessed by the reactivity of sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoate). Contrasting with the slow transition of the reduced state to the oxidized form, the deactivation in the presence of 2-Cys Prx proceeded through a rapid step ($t_{0.5}$ less than 1 min) followed by the stabilization of the activity at a value close to that obtained when the oxidized form of CFBPase was stimulated with 2-Cys Prx. From this kinetic analysis, we inferred that 2-Cys Prx accelerated the oxidation of reduced CFBPase and once the latter was oxidized it became activated by the former. The ability to modulate CFBPase could not be ascribed to an unspecific chaperone function because 2-Cys Prx was unable to prevent the denaturation or facilitate the refold when the enzyme lost the catalytic capacity by short incubations at high temperatures or high concentrations of NaCl (Fig. 4 and Fig. 5, Suppl. Info.) [6,7].

2-Cys Prx interacts with the 170's loop of CFBPase in the stimulation of catalytic activity

The Trx-mediated activation of CFBPase is assessed *in vitro* in the presence of DTT or any other low potential reductant (Fig. 3A, inset) because the cleavage of the disulfide bond linking Cys153 to Cys174 in the 170's loop is responsible for the enhancement of catalytic activity [12,13]. However, 0.01 to 0.1 mM DTT impaired the stimulation elicited by 2-Cys Prx (Fig. 3A). This unexpected loss of activity could not be attributed to major structural alterations of any of both proteins because concentrations that lessened the stimulation (0.01–0.02 mM) did not follow *vis-à-vis* those ($\geqslant$1 mM) that cleaved the intercatenary disulfide bond of 2-Cys Prx (Fig. 3B) and aggregated the dimers after reduction (Fig. 3C). Nevertheless, the acceleration of reduced CFBPase deactivation strongly suggested that cysteines might be critically in promoting the protein–protein interaction. 2-Cys Prx elicited in C96S CFBPase, as well as in C53S and C307S CFBPase, an activation similar to the cognate wild type enzyme (Figs. 4 and 6, Suppl. Info.) while the limited solubility of C191S CFBPase made impracticable this analysis. Thus, like the Trx-mediated activation, the response to 2-Cys Prx is not affected by substitutions of conserved cysteines located off the 170's loop. On the other hand, the insensitivity of C157S, C157S/C174S and C174S/C179S CFBPase to Trx-f confirmed the absence of the Cys157–Cys174 cystine while the reductive stimulation of C179S and C174S CFBPase was consistent with the presence of the intrinsic disulfide bond and the

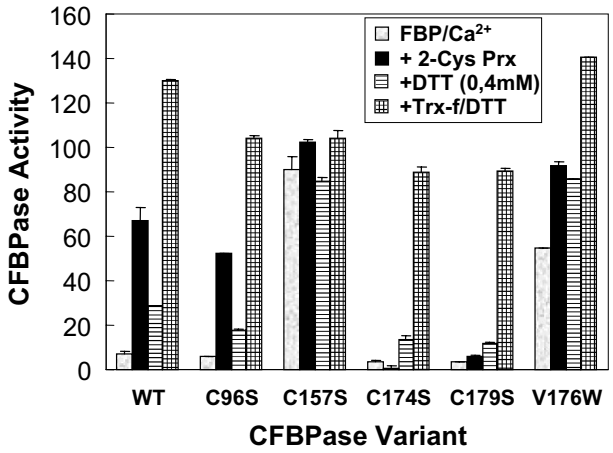

Fig. 4. Reductive- and 2-Cys Prx-mediated stimulation of CFBPase. The wild type (WT) and mutant CFBPases (1 μg) were incubated at 25 °C in 0.04 ml of 100 mM Tris–HCl buffer (pH 7.9), 0.75 mM FBP, and 0.05 mM Ca^{2+} (white bars), containing either 30 μM recombinant 2-Cys Prx (black bars), 0.4 mM DTT (hatched bars) or 0.4 mM DTT and 3 μM recombinant rapeseed Trx-f (squared bars). After 20 min, the CFBPase was assayed as described in Materials and methods.

nonphysiological (Cys157–Cys179) cystine, respectively. In sharp contrast, these single and double mutants at the 170's loop turn CFBPase insensitive to 2-Cys Prx. This finding led us to suspect that the interaction between 2-Cys Prx and CFBPase strictly requires the presence of the intrinsic disulfide bond and the third conserved cysteine in the 170's loop. To corroborate this contention, we constructed V176W CFBPase, a variant of the 170's loop that in titrations with 5,5′-dithiobis(2-nitrobenzoate) exhibited a number of sulfhydryl groups similar to the oxidized form of the wild type CFBPase. The most striking difference of this mutant with the wild type counterpart was the higher basal activity due to an enhanced affinity for Mg^{2+} which perhaps originated from an alteration in the conformation of the 170's loop. Fig. 4 shows that 2-Cys Prx and Trx enhanced the basal activity of V176W CFBPase confirming that the stimulatory action of the former requires a CFBPase whose 170's loop retains both the intrinsic disulfide bond and the third conserved cysteine. Overall, these results demonstrated that 2-Cys Prx formed with CFBPase a functional complex in which the stimulation of enzyme activity departs from the mechanism elicited by reduced Trx. Notably, the finding that 2-Cys Prx enhances the catalytic capacity via the 170's loop provides compelling evidence for a new insight into the regulation of CFBPase because this intrapeptide was hitherto considered the target for the light-actuated ferredoxin-Trx system in oxygenic photosynthetic organisms.

Conclusions

Most studies have characterized extensively the peroxidatic activity of 2-Cys Prx and recent reports extend the functional capacity of yeast and human orthologs to the suppression of protein aggregation [2,6,7,21]. The present results show that rapeseed 2-Cys Prx plays a critical role in the regulation of CFBPase activity in which the unability to prevent denaturation or allow refolding of CFBPase when the enzyme lost the catalytic activity by temperature, ionic strength, or pH perturbation suggests that the activation of CFBPase are not in line with a chaperone activity. Four aspects make the regulation of CFBPase by 2-Cys Prx unique relative to the Trx-mediated activation that dominated the scenario of chloroplast enzyme modulation for 25 years. First, redox compounds are not necessary to trigger the activation of oxidized CFBPase and low concentrations of reductants definitely prevent the enhancement of activity. Second, the stimulation of CFBPase strictly requires the concerted action of FBP and Ca^{2+} whose synergistic effect is further substantiated by higher cation requirements of I271T CFBPase. Third, the kinetic constants V_{max} and the affinity for Mg^{2+} are higher than those of the oxidized CFBPase but lower than those of the reductively activated form. Fourth, the mutation of Cys153 yields a constitutively activated CFBPase that neither 2-Cys Prx nor Trx further activate while Cys174 and Cys179, residues that are not strictly necessary for the Trx-dependent activation, are however essential to activation via 2-Cys Prx. Hence, 2-Cys Prx, like Trx, needs the integrity of the intrinsic disulfide bond of the 170's loop to stimulate CFBPase but, unlike Trx, requires strictly the three conserved cysteines. These two mechanisms for the regulation of CFBPase may reflect the modulation of a key enzyme of photosynthetic CO_2 assimilation under normal functioning and oxidative stress (Fig. 7, Suppl. Info.) [22,23]. Altogether the data convey the idea that 2-Cys Prx contributes to the specific regulation of enzyme activity and, more importantly, indicates that this unprecedented process may pervade to other areas of biology because the ubiquity of 2-Cys Prx makes plausible the regulation of functions via noncovalent interactions in nonphotosynthetic organisms [21].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.013.

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