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

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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.

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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.