Calcium sensing via EF-hand 4 enables thioredoxin activity in the sensor-responder protein calredoxin in the green alga *Chlamydomonas reinhardtii*

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Calcium (Ca\(^{2+}\)) and redox signaling enable cells to quickly adapt to changing environments. The signaling protein calredoxin (CRX) from the green alga *Chlamydomonas reinhardtii* is a chloroplast-resident thioredoxin having Ca\(^{2+}\)-dependent activity and harboring a unique combination of an EF-hand domain connected to a typical thioredoxin-fold. Using small-angle X-ray scattering (SAXS), FRET, and NMR techniques, we found that Ca\(^{2+}\)-binding not only induces a conformational change in the EF-hand domain, but also in the thioredoxin domain, translating into the onset of thioredoxin redox activity. Functional analyses of CRX with genetically altered EF-hands revealed that EF-hand 4 is important for mediating the communication between the two domains. Moreover, we crystalized a variant (C174S) of the CRX target protein peroxiredoxin 1 (PRX1) at 2.4 Å resolution, modeled the interaction complex of the two proteins, and analyzed it by cross-linking over, we crystallized a variant (C174S) of the CRX target protein via a bimolecular nucleophilic substitution reaction.

Changes in the cellular Ca\(^{2+}\) concentration. Type I “sensor-responder” proteins possess both Ca\(^{2+}\)-binding and enzymatic “effector” domains. Type II components, including calmodulin (CaM), are able to bind Ca\(^{2+}\), but do not exhibit enzymatic activity and are designated as “sensor-relay” proteins. CaMs are small proteins that possess a total of four Ca\(^{2+}\)-binding EF-hand motifs organized in two lobes, each with two EF-hands (1, 2). Remarkably, this motif is highly conserved and EF-hand proteins are encoded in all eukaryotic genomes (3). On the other hand, glutaredoxins, thioredoxins (TRXs), or peroxiredoxins (PRXs) constitute part of an intricate network that impacts the redox state especially within plant cells and their compartments (4). In particular, TRXs are protein oxidoreductases that harbor a redox-active dithiol/disulfide motif in their active sites. In its reduced state, this motif allows cleavage of a disulfide bond of a target protein via a bimolecular nucleophilic substitution reaction. Oxygenic photosynthesis in plants and green algae, including *Chlamydomonas reinhardtii*, is known to be regulated both by Ca\(^{2+}\) and redox signaling (5). Calredoxin (CRX) is a novel chloroplast-localized protein from *C. reinhardtii* that is able to combine and integrate these two signaling pathways, as it comprises a Ca\(^{2+}\)-binding CaM domain with four EF-hands and a thioredoxin domain that exhibits a typical thioredoxin-fold and Ca\(^{2+}\)-dependent thioredoxin activity (6). Therefore, CRX was recognized as a novel sensor-responder protein in plants (6). Interestingly, CRX efficiently reduced *C. reinhardtii* peroxiredoxin-1 (PRX1) in *vitro* in a Ca\(^{2+}\)-dependent manner, in line with enhanced reactive oxygen species stress in a *C. reinhardtii* mutant with decreased amounts of CRX as evidenced by lipid peroxidation (6).

In this work, we addressed (i) how the Ca\(^{2+}\)-dependent activation of the TRX function in CRX is mechanistically regulated and (ii) how this relates to the Ca\(^{2+}\)-dependent reduction of PRX1 in *vitro*. Conformational changes of CRX upon Ca\(^{2+}\)-binding in

The abbreviations used are: CaM, calmodulin; TRX, thioredoxins; PTX, peroxiredoxins; CRX, Calredoxin; SAXS, small angle X-ray scattering; R\(_g\), radius of gyration; CFP, cyan fluorescent protein; HSQC, heteronuclear single quantum correlation; DTNB, 5,5′-dithiobis(nitrobenzoic acid); RMSD, root mean square deviation; B3S, bis(sulfosuccinimidyl)suberate; TrxD, thioredoxin domain.

Ca\(^{2+}\) and redox signaling play major roles in the acclimation of cells to changing environments. Ca\(^{2+}\) signaling in plants is mediated by two types of signaling components that decode changes in the cellular Ca\(^{2+}\) concentration. Type I “sensor-responder” proteins possess both Ca\(^{2+}\)-binding and enzymatic “effector” domains. Type II components, including calmodulin (CaM), are able to bind Ca\(^{2+}\), but do not exhibit enzymatic activity and are designated as “sensor-relay” proteins. CaMs are small proteins that possess a total of four Ca\(^{2+}\)-binding EF-hand motifs organized in two lobes, each with two EF-hands (1, 2). Remarkably, this motif is highly conserved and EF-hand proteins are encoded in all eukaryotic genomes (3). On the other hand, glutaredoxins, thioredoxins (TRXs), or peroxiredoxins (PRXs) constitute part of an intricate network that impacts the redox state especially within plant cells and their compartments (4). In particular, TRXs are protein oxidoreductases that harbor a redox-active dithiol/disulfide motif in their active sites. In its reduced state, this motif allows cleavage of a disulfide bond of a target protein via a bimolecular nucleophilic substitution reaction. Oxygenic photosynthesis in plants and green algae, including *Chlamydomonas reinhardtii*, is known to be regulated both by Ca\(^{2+}\) and redox signaling (5). Calredoxin (CRX) is a novel chloroplast-localized protein from *C. reinhardtii* that is able to combine and integrate these two signaling pathways, as it comprises a Ca\(^{2+}\)-binding CaM domain with four EF-hands and a thioredoxin domain that exhibits a typical thioredoxin-fold and Ca\(^{2+}\)-dependent thioredoxin activity (6). Therefore, CRX was recognized as a novel sensor-responder protein in plants (6). Interestingly, CRX efficiently reduced *C. reinhardtii* peroxiredoxin-1 (PRX1) in *vitro* in a Ca\(^{2+}\)-dependent manner, in line with enhanced reactive oxygen species stress in a *C. reinhardtii* mutant with decreased amounts of CRX as evidenced by lipid peroxidation (6).

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This article contains Figs. S1–S5, Tables S1–S3, and Data file S1. The atomic coordinates and structure factors (code 6J13) have been deposited in the Protein Data Bank (http://wwpdb.org/).

NMR data for CRX with and without Ca\(^{2+}\) can be found at Biological Magnetic Resonance Data Bank (BMRB) with accession numbers 27865 and 27860.

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solution were monitored by small angle X-ray scattering (SAXS) (7), nuclear magnetic resonance (NMR) (8), and a Förster resonance energy transfer (FRET) approach. To assay the role of individual EF-hands on Ca\(^{2+}\)-dependent activation of the TRX function, each of the four EF-hands was altered by site-directed mutagenesis. For a better understanding of the molecular mechanisms of Ca\(^{2+}\)-dependent electron transfer from CRX to PRX1, we solved the structure of PRX1 (PDB ID 6J13) and built a hypothetical complex model between CRX and PRX1 from *C. reinhardtii*. The correct prediction of this complex was further strengthened by the mass spectrometric identification of chemically cross-linked peptides between the two proteins.

**Results**

**The overall structure of calredoxin with and without Ca\(^{2+}\) in solution**

To analyze the conformational change of CRX with and without Ca\(^{2+}\), we measured SAXS (Table S1). The scattering intensity \(I(q)\) was collected from samples at a protein concentration of 10.0, 7.0, 5.0, 2.5, and 1.0 mg/ml in the presence of either Ca\(^{2+}\) or EGTA (Fig. 1, *a* and *b*). The Guinier plot (Fig. 1c) shows that the CRX protein in solution did not aggregate. The linear relationship between the radius of gyration \(R_g\) and protein concentration indicates that there are no strong interparticle effects either in the absence or presence of Ca\(^{2+}\) (Fig. S1). The \(D_{\text{max}}\) estimated from the distance distribution function \(P(r)\) of the Ca\(^{2+}\)-bound CRX was larger than that of the Ca\(^{2+}\)-free form as shown in Fig. 1d. Moreover, the Porod volume of CRX with Ca\(^{2+}\) is increased, which suggests a more elongated and flexible conformation of CRX with Ca\(^{2+}\) as compared with CRX without Ca\(^{2+}\).

The \(I(0)\) of the Ca\(^{2+}\)-bound form of CRX is higher than the Ca\(^{2+}\)-free CRX, and the molecular weight calculated from \(I(0)\) of the Ca\(^{2+}\)-free CRX is lower than expected from the CRX sequence (Table S1), opening up the possibility of protein degradation in the absence of Ca\(^{2+}\). However, analysis of protein integrity by SDS-PAGE confirmed no proteolytic cleavage of CRX after SAXS measurements (Fig. S2), so that it is not clear...
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Figure 2. FRET-based analysis of Ca\(^{2+}\)-induced conformational change of CRX. CFP and mVenus (YFP) were cloned N- and C-terminal of CRX and expressed heterologously in E. coli. (a) A conformational change upon Ca\(^{2+}\)-binding would alter FRET from CFP to YFP as shown schematically. (b) Emission of the CRX-based Ca\(^{2+}\) reporter after excitation at 435 nm. CFP and mVenus emission peaks are indicated by blue and yellow arrows, respectively. Emission spectra with (black) and without (blue) free Ca\(^{2+}\) are shown. (c) The amount of FRET is shown as the ratio of YFP to CFP emission and in dependence on the free Ca\(^{2+}\) concentration.

why the value of /(0) and the molecular weight for the Ca\(^{2+}\)-free form of CRX are lower than expected.

Dummy atom models for CRX with and without Ca\(^{2+}\) were calculated from SAXS experimental data using GASBOR (9) and DAMAVER (10) (Fig. 1e). As anticipated from the Porod volume analysis, the models show a more elongated shape of Ca\(^{2+}\)-bound CRX compared with a more globular shape of the Ca\(^{2+}\)-free form. The experimental SAXS data of CRX with Ca\(^{2+}\) did not completely fit the corresponding SAXS data computed from its crystal structure determined previously (6) (PDB ID 5E37), as shown in Fig. S3a. In the original crystal structure, the first 17 amino acids at the N terminus, 21 C-terminal amino acids, and 5 amino acids of the linker between the CaM and the TRX domain were not well-resolved due to their flexibility. Therefore, the conformations in the missing regions were modeled by MODELLER. Then, the best fitting conformation was picked and the corresponding SAXS profile was calculated using the AllosMod-FoXS (11, 12) server. Superimposing the SAXS profile calculated from this slightly modified structure of CRX, representing the full-length CRX, with the experimentally determined SAXS profile (Fig. 1e) using SUPCOMB (13) yields a good correlation of \(\chi = 1.33\) (Fig. S3b). Anticipating that CRX seems to elongate during Ca\(^{2+}\)-binding, we designed a Ca\(^{2+}\)-reporter construct similar to Yellow Cameleons based on CRX instead of calmodulin as the Ca\(^{2+}\)-sensing linker between the two fluorophores. We used an N-terminal cyan fluorescent protein (CFP) as donor fluorophore and a C-terminal mVenus as an acceptor fluorophore as shown schematically in Fig. 2a. As expected, addition of Ca\(^{2+}\) led to a decrease in mVenus fluorescence when CFP was excited; as CRX expands, the gap between the two fluorophores is enlarged and FRET, detected as the amount of mVenus fluorescence emission, is decreased compared with the Ca\(^{2+}\)-free state (Fig. 2b). A longer conformation was detected with increasing concentration from 50 to 1000 nM free Ca\(^{2+}\), beyond which the CRX conformation stayed stable (Fig. 2c). In sum, SAXS and FRET results confirm that the three-dimensional domain arrangement of CRX is Ca\(^{2+}\)-dependent and the structure without Ca\(^{2+}\) is more compact.

NMR experiments confirm the structural change upon Ca\(^{2+}\) binding

We observed severe broadening and intensity differences in the peaks of two-dimensional (2D) \(^{1}H-^{13}N\) heteronuclear single quantum correlation (HSQC) spectra, and we failed to recognize all the expected amide peaks. We, therefore, decided to explore the possibility of structural changes upon Ca\(^{2+}\) binding through chemical shift changes of the assignable side chains of the methionine (Met) residues instead of those of amide groups in the main chain. A Met has a methyl group in the terminal \(\epsilon\) position. The methyl group generally has a high NMR sensitivity because its fast rotation about the 3-fold axis (S-C bond) reduces the transverse relaxation rate and the \(^{13}C\) spin in the methyl group has no \(\text{J}_{\text{CC}}\) scalar coupling, which would otherwise split or broaden the \(^{1}H-^{13}C\) peak. Fifteen Met residues of CRX are shown in Fig. 3a. We measured the \(^{1}H-^{13}C\) HSQC spectra of \(^{13}C\)-Met-CRX with and without Ca\(^{2+}\) (Fig. 3, b and c). To identify the Met peak positions, we mutated each Met independently to leucine (Leu) and compared each mutant NMR spectrum with the WT CRX spectrum. Large chemical shift differences in the presence and absence of Ca\(^{2+}\) were observed particularly for Met residues that distribute in the CaM domain and the interface between the two domains, indicating that the calcium binding changes the conformation of these regions.
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**CRX redox activity and interaction with PRX1 depends on EF-hand 4**

The unique multiscale Ca\(^{2+}\)-dependent structural change of CRX was shown from the molecule to residue levels by SAXS, FRET, and NMR. To investigate the independent functional importance of the four Ca\(^{2+}\)-binding sites present in CRX, we constructed site-directed mutants for each of the four EF-hands by mutating the glutamate responsible for stabilizing the coordination of Ca\(^{2+}\) ions. The Ca\(^{2+}\)-dependent reduction of PRX1 via CRX in the distinct mutants was assessed and revealed the most significant increase in \(K_{Ca2}\) for the EF-hand 4 mutant when compared with WT CRX after nonlinear curve fitting by the Hill equation (\(v = \frac{v_{max} \times [S]^{nH}}{(K_{Ca2} + [S]^{nH})}\)) (Fig. 4, a and b). Parameters of the fitting analysis are summarized in Table S2. To reach half-maximal activity, 33.3 \(\mu\)M Ca\(^{2+}\) is needed compared with 120 nm for WT CRX. Whereas the EF-hand 2 mutant exhibited a minor \(K_{Ca2}\) increase to 170 nm, the mutation of EF-hands 1 or 3 had no significant effect on the \(K_{Ca2}\) value (both 120 nm). Analysis of a possible cooperative interconnection between the four EF-hands by determination of the Hill coefficient \(n_H\) indicated negative cooperativity only for the EF-hand 4 mutant, as \(n_H\) was < 1 for EF-hand 4 mutant (\(n_H = 0.4\)) and around 1 (indicating no cooperativity) for the remaining CRX variants (WT, 0.8; EF-hand 1, 1.8; EF-hand 2, 1.5; EF-hand 3, 0.9). However, for EF-hand mutants 1 and 2, the \(n_H\) value should be interpreted with caution, due to high standard deviation and subsequent incomplete refinement within the 95% confidence interval.

In addition, electron transfer between CRX and DTNB was measured (Fig. 4c). As observed for electron transfer between PRX1 and CRX, EF-hand 4 mutant required a Ca\(^{2+}\) concentration in the micromolar range (1600 nM ± 790 nM) to reach full activity, whereas the WT protein reduced DTNB already in the presence of nanomolar concentrations of Ca\(^{2+}\) (60 ± 40 nM).

**The predicted structure complex between CRX and PRX1**

Following up on our preliminary structural work done on PRX1 (14), we now have resolved the crystal structure of the PRX1(C174S) named C2S mutant at 2.4 Å resolution (PDB ID 6I13). The crystallographic data and refinement statistics are listed in Table 1. The crystal of the C2S mutant of PRX1 contains 10 molecules in the crystallographic asymmetric unit, which form two half-ring structures along the crystallographic 2-fold axis. In the crystal lattice, the two half-ring structures in the crystallographic asymmetric unit form a pentamer of dimers with crystallographic symmetry mates transformed by the crystallographic 2-fold symmetry operation, thus representing two decameric ring structures (Fig. 5a). Two independent decameric ring structures are quite similar with RMSDs between Ca atoms in each monomer of 10 molecules seen between 0.102 and 0.199 Å. The overall structure of a monomeric PRX1 shows structural similarity to those of the other typical 2-cysteine peroxiredoxins such as those of human erythrocytes (PDB ID 1QMV) (15) and plant *Pisum sativum* (PDB ID 2PWJ) (16) containing seven \(\beta\)-sheets and five \(\alpha\)-helices. Two PRX1 monomers form a head-to-tail dimer (Fig. S4) with the
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Figure 4. CRX redox activity and interaction with PRX1 depend on EF-hand 4. Recombinant WT (black squares) and single point mutated EF-hand mutants (triangles) of CRX were reduced by E. coli thioredoxin reductase (TrxR) and NADPH in defined Ca2+ concentrations. 1 µM oxidized recombinant PRX1 and 80 µM H2O2 (a and b) or 200 µM DTNB (c) were added as a substrate for reduction by CRX. NADPH oxidation (a and b) and DTNB reduction (c) were tracked at 340 and 412 nm, respectively. To calculate the redox activity, NADPH consumption (a) or 80 s of stable slope after addition of DTNB (c) were plotted against the Ca2+ concentration and fitted by the Hill equation \(v = \frac{v_{\text{max}} \times [S]^n}{(K_{\text{Ca}^2+})^n + [S]^n}\). Error bars give S.D. of three to four independent measurements. Panel b shows the same data as in panel a but in semi-log representation (NADPH oxidation versus logCa2+ of Ca2+ concentration). Assays were modified as described in Refs. 32 and 33.

dimer interface located at the antiparallel β-strand 7 (residues 137–145) and the α-helix 5 (residues 153–168) from each monomer. The active site Cys-174 at the C terminus of each monomer is located close to Cys-53 from another monomer (Fig. S4). In this case of the C2S mutant, Cys-174 was mutated to Ser to simplify crystallization and was invisible due to its high flexibility. This may have caused missing of the corresponding electron density.

The protein-protein interaction in the complex between CRX and PRX was already predicted previously based on the decameric structure of mouse PRX4 (PDB ID 3VWU) (6). In Fig. 5b, the new structure of PRX is superimposed on the previous complex model. In addition, an in vitro cross-linking approach using the chemical cross-linker BS3 (bis(sulfosuccinimidyl)suberate) was performed to evaluate the interaction sites between CRX and PRX1. We identified two different cross-links between the two proteins (Table S3). The position of the cross-linked lysines, Lys-273 in CRX and Lys-94 in PRX1 are indicated in the predicted protein-protein interaction complex (shown in Fig. 5c). The distance of 1.9 Å between the tips of their side chains fulfills the distance constraints of the BS3 spacer arm length of 11.4 Å. The cross-linked lysines highlight the corresponding area as the interaction site between CRX and PRX1, which is in close proximity to the active cysteine pair of both CRX and PRX1. Unfortunately, the second cross-link site cannot be visualized in our structure, because the C-terminal part of PRX1 including Lys-193 is not modeled due to its flexibility. However, it is of note that it is again Lys-273 of CRX, which is cross-linked, indicating that this lysine is indeed located near the CRX-PRX1 interaction site. Furthermore, negatively charged amino acid residues (aspartate and glutamate, Fig. S5) surrounding these lysines would likely promote electrostatic interactions between these two proteins.

Discussion

In this work, we provide evidence that the overall structure of CRX in solution changes in multiscale of molecule, domain, and residues upon Ca2+-binding by employing SAXS, FRET, and NMR techniques. The results from SAXS and FRET concordantly revealed that the structure of CRX is more compact and globular in the absence of Ca2+ and elongates when Ca2+ is bound (Figs. 1 and 2). It is suggested that this elongation leads to a conformational change affecting the active cysteine pair of the TRX domain, ultimately stimulating the redox activity of CRX.

| Table 1 | Data collection, phasing and refinement statistics for C2S mutant of PRX1 |
|---------|----------------------------------------------------------------------|
| Data collection | C2S mutant of PRX1 |
| Wavelength (Å) | 0.90000 |
| Space group | C2221 |
| Cell dimensions | |
| a, b, c (Å) | 134.9, 419.23, 94.53 |
| α, β, γ (°) | 90.0, 90.0, 90.0 |
| Resolution (Å) | 47.37–2.4 (2.45–2.40) |
| No. of observed reflections | 380,886 |
| No. of independent reflections | 103,254 |
| Rmerge (%) | 11.1 (97.2) |
| Completeness (%) | 98.9 (99.9) |
| Redundancy | 3.69 (3.77) |

Table 1: Data collection, phasing and refinement statistics for C2S mutant of PRX1. Values in parentheses are for highest-resolution shell.
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Figure 5. Chlamydomonas PRX1 crystal structure. (a) The decameric ring shape of C174S PRX1 (PDB ID 6J13). Monomers are labeled from A to E and A’ to E’. (b) Model of the C. reinhardtii CRX-PRX1 complex. The C-terminal peptides of the PRX-TRX complex (PDB ID 3VVU) is shown in ball-and-stick and light-green ribbon models, respectively. PRX1 is colored in red, and mouse PRX4 is colored in light blue. CRX is colored in orange, magenta, and blue. (c) Close-up view on cross-linked region identified by MS analysis after in vitro cross-linking of recombinant CRX and PRX1. The distance between the nitrogen atoms of the cross-linked lysines Lys-273 (CRX) and Lys-94 (PRX1) is 1.9 Å. Colors are as described under b.

Analysis of $^1$H-$^{13}$C Met-labeled CRX by NMR revealed structural changes within the two domains of the protein. From the observed data, the CaM domain and the interface between CaM and TRX domains are structurally changed when Ca$^{2+}$ is removed (Fig. 3), which strengthens the earlier proposed hypothesis, that a hydrogen bond network between EF-hand 4 and the active cysteine pair is responsible for the transmission of the Ca$^{2+}$-binding signal to the TRX domain (6). In one of the two proposed networks, the fourth EF-hand motif, localized in helix 7, was linked to Lys-242 located under the disulfide bridge (6). The other was through Helix 12 of the TRX domain. Importantly, the crx mutant K242L was less efficient in electron transfer to PRX1 at low Ca$^{2+}$ concentrations (6).

In the same line, the presented CRX-PRX1 interaction assay as well as the CRX activity measurements suggest that the communication between the two domains of CRX is mediated via EF-hand 4 (Fig. 4). Notably EF-hand 4 is the EF-hand located closest to the TRX domain. It is therefore reasonable to suggest that this EF-hand possesses a key regulatory function in transmitting the Ca$^{2+}$-signal from the CaM domain to the TRX domain. In the CRX-PRX1 reduction assay, changes in the Ca$^{2+}$-dependent electron transfer rate, as seen in the EF-hand 4 mutant, could be related to impact in PRX1 binding and/or electron transfer. However, the ability of the DTNB reduction via CRX should be primarily related to electron transfer efficiency, as DTNB is a small molecule interacting with the surface-exposed TRX-active Cys residues of CRX. The impact of the EF-hand 4 mutation on DTNB reduction indeed suggests that the CRX redox activity itself is regulated by the presence/absence of Ca$^{2+}$. Alternatively, the EF-hand 4 mutation could hamper reduction of CRX via NTR. How exactly the active cysteines are oriented in the presence and absence of Ca$^{2+}$ remains to be investigated in the future. Notably, EF-hand 4 is particularly important in improving Ca$^{2+}$-binding by CRX, as its deletion confers negative cooperativity among the remaining EF-hands (Fig. 4, Table S2). The high $K_{Ca^{2+}}$ value of the EF-hand 4 mutant could therefore also be explained by less efficient Ca$^{2+}$ binding not only to EF-hand 4 but also to all other EF-hands, thereby increasing the need for higher Ca$^{2+}$ amounts to reach full activity. Alternatively, Ca$^{2+}$-induced conformational changes at these other sites are decoupled from conformational changes needed to activate the CRX domain in the EF-hand 4 mutant.

The importance of EF-hand 4 in CRX is also reflected in an evolutionary survey shown in Fig. 6. Using the CRX as a query, we searched the TARA oceans data (17) and EggNOG version 4.5.1 (18) to retrieve all members of the corresponding protein family KOG0027 (signal transduction mechanisms, specifically: calcium-binding proteins). Additionally, we used tBlastX to query the nucleotide core repository and all shotgun genome-sequencing collections of NCBI for records with similarity to CRX, filtering for hits with an e-value of at least $1 \times 10^{-5}$. To analyze the evolutionary history of CRX, we considered a total of 3813 proteins from 231 species across all domains of life. Of these, we eliminated all sequences with a length of less than 153 residues, corresponding to the combined length of EF-hands 3 and 4 plus the thioredoxin domain (TrxD). As a result, CRX homologs could be recognized in members of the Chlorophyceae, as seen before (6), and surprisingly in members of Dino-phyceae, including Symbiodinium species. Importantly, EF-hands 2, 3, and 4 are more conserved across the CRX family than EF-hand 1, again underpinning the prominent role of the EF-hands located near the two-domain interface. As mentioned, the signal transmission from the Ca$^{2+}$-binding domain to the TRX domain of CRX was proposed to occur via two hydrogen bond networks stretching from EF-hand 4 to cysteine 238 of the active site (6). Comparison of the involved amino acids between the different species of the CRX family reveals that the majority of them (i.e. Asp-157, Glu-216, Thr-236, Lys-242, Lys-263, Asn-267, and Asn-269) are highly conserved.
Figure 6. Evolutionary origin of EF-hand containing thioredoxins. (A) Full phylogenetic tree of the protein family KOG0027, unrooted. Members from the plant kingdom (Viridiplantae) are marked green, whereas the magenta-colored clade highlights EF-hand containing thioredoxins. (B) Close-up of the EF-hand containing thioredoxins. Scale bars in A and B indicate substitutions per site. (C) Simplified representation of EF-hand containing thioredoxins. EF-hands 1, 2, 3, and 4, as well as the active core of the thioredoxin domain are highlighted in lighter gray. Sequences are presented in phylogenetic order, as in A and B. Numbers indicate the position of amino acids in CRX from Chlamydomonas as described in Ref. 6 (dark gray sequence). The second sequence from Chlamydomonas originates from an older version of the BLAST database. Conserved amino acids involved in the proposed H-bond network are marked with a star. Note that the data are shown 5'- and 3'-truncated, zooming in on the core domains only.
among the CRX family (Fig. 6). Considering the variability of all other amino acids (except conserved structures like the WCRPC active site), the outstanding conservation supports the idea that these amino acids are necessary in all CRX homologs to stabilize the hydrogen bond networks and therefore mediate signaling between the CaM domain (especially EF-hand 4) and the CRX active site.

The crystal structure of the PRX1 C2S mutant was solved at 2.4 Å resolution, showing a decameric ring shape (Fig. 5a). Although, the crystal structure of WT PRX1 is not available, High-Speed AFM images revealed that the WT PRX1 particles form rings with pentagonal rotational symmetry as the mutant does (14). The structure of WT PRX1 is, therefore, expected to be similar to that of the C2S mutant. The modeled complex structure between CRX and PRX1 (Fig. 5b) revealed that the elongated Ca$^{2+}$-bound form of CRX is able to form a complex with PRX1 with the active cysteines oriented toward each other, a structure that is further supported by the cross-linking data.

**Experimental procedures**

**Protein expression and purification**

The recombinant plasmid of WT CRX was constructed as previously described (6). The methionine mutants of CRX for NMR measurements were made using the In-Fusion Cloning (Clontech Laboratories, Inc.) technique. The sequence of the mutated plasmids was confirmed by DNA sequencing. Protein expression and purification were performed as previously described (6). For expression of CRX for NMR measurements, cells were grown in M9 minimal medium (1 liter) consisting of 20 mg of thymidine, adenosine, guanosine, cytidine, thiamine, and biotin, 0.01 mM FeSO$_4$, 2 mM MgSO$_4$, 0.05 mM MnCl$_2$, 1 g of NH$_4$Cl, 4 g of glucose, 0.1 mM CaCl$_2$, and 100 ml of 10× M9 medium (70 g of Na$_2$HPO$_4$, 30 g of KH$_2$PO$_4$, and 5 g of NaCl). Cells were grown in M9 medium at 37 °C until the OD$_{600}$ reached 0.3. Then 100 mg of lysine, phenylalanine, threonine, 50 mg of isoleucine, leucine, valine, and [13C]methionine were added. Cells continued culturing at 37 °C until the OD$_{600}$ reached 0.5–0.7, and protein overexpression of CRX was induced with 0.1 mM isopropyl 1-thio-$\beta$-D-galactopyranoside at 20 °C for 16–18 h. Cell pellets were collected by centrifugation at 8000 $\times$ g, 10 min, 4 °C. The obtained cell pellets were frozen and kept at −80 °C. Protein purification was performed as described (6).

**SAXS and model fitting**

SAXS measurements were done with a BioSAXS-1000 system (Rigaku, Japan) with the PILATUS 100k detector. Sample buffer for SAXS measurements was 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM DTT. 5 mM CaCl$_2$ was added for analysis of the CRX with Ca$^{2+}$, and 5 mM EGTA was added instead for analysis of the Ca$^{2+}$-free form of CRX. The exposure time was 2 h. The data were collected with 1, 2.5, 5, 7, and 10 mg/ml CRX. Ovalbumin (44.3 kDa) was used to estimate the molecular mass of CRX. All data were analyzed with the ATSAS software package (19) including PRIMUS, GNOM, GASBOR, and DAMAVER. The scattering data of the buffer were subtracted from those of the protein sample and the radius of gyration ($R_g$) and $P(r)$ distribution function were calculated with GNOM and PRIMUS. GASBOR was used for ab initio reconstruction of dummy atom models of protein structure. DAMAVER (10) was used for alignment and averaging of the 10 obtained dummy atom models to get the most probable model.

The FoXS (20, 21) and AlllosMod-FoXS (11, 12) web server was used to model the full-length structure of CRX with Ca$^{2+}$ with MODELLER (22), calculate the theoretical scattering profile from the crystal structure, and compare with the SAXS experimental profiles.

**FRET**

CFP and mVenus gene sequences were amplified by using the primers NdeI_CFP_f, 5′-GCATATTGGAAGCCACCG-CAGTTCCG-3′; CFP_NdeI_r, 5′-ATAATTCCATATGCGG-GCGGCGGTAC-3′; and BamHI_mVenus_f, 5′-ATGGATC-CAGACGGCGGTCGTGACG-3′; as well as mVenus_Sacl_r, 5′-CGGAGCTCCTCGATGTGTGGCCGATC-3′. cfp was cloned 5′ of the crx sequence in the already described pET22b (+) expression vector (6), whereas mVenus was cloned between crx and the C-terminal His tag. The fusion protein was expressed in E. coli BL21(DE3) by addition of 0.5 mM isopropyl 1-thio-$\beta$-D-galactopyranoside and purified in native form via a nickel-nitritoltriacetic acid column.

125 mM of the purified CFP-CRX-mVenus construct was used in 2 ml of 30 mM MOPS, 100 mM KCl, pH 7.2, and specified concentrations of free Ca$^{2+}$ calculated with maxchelator buffered by 5 mM EGTA. CFP was excited at 435 nm and the emission spectra were recorded in a Jasco fluorescence spectrometer from 450 to 550 nm.

**NMR spectroscopy**

The 2D $^1$H-$^1$C HSQC spectra were recorded on a Bruker Avance III spectrometer with a $^1$H resonance frequency of 950 MHz with a TCI cryoprobe operating at 25 °C. The concentration of CRX was ~0.3 mM in 20 mM HEPES buffer, pH 7.5, containing 150 mM NaCl, 0.5 mM DTT, 2.5 mM CaCl$_2$, and 10% D$_2$O. After collecting the spectra of CRX with Ca$^{2+}$, 10 mM EGTA was added into the sample to collect the spectra in the same way as the sample with Ca$^{2+}$. The data were processed and analyzed using TopSpin (Bruker).

**EF-hand mutant and activity**

Site-directed mutagenesis of CRX was performed as described before (6) using the following primers for the mutagenesis reaction of EF-hands 1–4 (Glu-50–Glu-168): E50Q_f, 5′-CACATCGATCGTGTACGACTCGTGTACGATCGATGTG-3′; E50Q_r, 5′-GCAGTTACGAGCTGCAGTCAAGCAGTTCATCGATGTG-3′; E95Q_f, 5′-CATAGATGATCTGCTTGAAC-3′; E95Q_r, 5′-CGCGGTTCATCGAAGGCGCAACCT-3′; E132Q_f, 5′-CATAGATGATCTGCTTGAAC-3′; E132Q_r, 5′-AGCTTGTCAGCTGGTGGCCGCAATG-3′; E132Q_f, 5′-CATAGATGATCTGCTTGAAC-3′; E132Q_r, 5′-AGCTTGTCAGCTGGTGGCCGCAATG-3′. Natively purified CRX versions were used in the PRX1 interaction assay as described before (6). Also the DTNB reduction assay was carried out as described in Ref. 6 but with natively purified protein as well. As after this type of...
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purification a contaminating protein also exhibited redox activity (data not shown), an additional ion exchange chromatography was carried out by using a 1-ml Mono Q anion exchange column in an ÄKTA FPLC system at pH 7.2.

Briefly, the redox activity of CRX was determined photometrically by reducing 10 (or 5) μM CRX with Escherichia coli TRXR by 10 (or 2) min incubation in 30 mM MOPS, 100 mM KCl, pH 7.2, with NADPH (200 (or 120) mM) at room temperature. The reduction rate of the target was determined at defined free Ca²⁺ concentrations for 80 (or 60) s after addition of 200 mM DTNB (or 1 mM recombinant PRX1) at 412 (or 340) nm (Ultriospec 3000, Amersham Biosciences). The activity (ν) was plotted against the free Ca²⁺ concentration ([S]) and modeled according to the Hill equation (ν = (νmax × [S]nH)/(K_H + [S]nH)) with GraphPad Prism 8.2.1 software.

X-ray structure determination of PRX1

The WT and the C2S mutant of C. reinhardtii PRX1 was over-expressed, purified, and crystallized as described (14). Only the crystal of the C2S mutant diffracted to 2.4 Å resolution suitable for atomic structure analysis. For phasing the structure factors of the C2S mutant, we carried out molecular replacement with the program Phaser MR in the CCP4 program suite (23, 24). The best homologous model for the molecular replacement calculation was thioredoxin peroxidase B from human red blood cells (PDB code 1QMV) (15) with 63% sequence identity to PRX1. Molecular replacement was successfully done and there are 10 molecules per asymmetric unit as expected from the Matthews coefficient (14). The model was built using COOT software along with the NCS averaged electron density map calculated using DM in the CCP4 package. Crystallographic refinement was performed using REFMAC5 (25). The values of Rwork and Rfree were 21.53 and 25.08%, respectively. The final structure was validated using PDB validation server.

In vitro cross-linking of CRX and PRX1

10 μM recombinant CRX were cross-linked to 10 μM recombinant PRX1 by 500 μM BS3, d5d6 (Thermo Scientific) in 30 mM MOPS, 100 mM KCl, pH 2.5, 2.5 μM free Ca²⁺ at pH 7.2 and 25 °C. To activate CRX, 1 μM thioredoxin reductase from E. coli and 120 μM NADPH as well as 40 μM H2O2 were included in the reaction mixture. After 20 min the reaction was quenched by addition of 20 μM NH4HCO3 and loaded onto an SDS-PAGE gel. Cross-linked bands appeared at a higher molecular weight than the single proteins and were cut and prepared for MS according to established protocols (26).

Peptide analysis was carried out on an LC-MS system consisting of an Ultimate 3000 nanoLC (Thermo Scientific) coupled to a Q Exactive Plus mass spectrometer (Thermo Scientific). LC setup and mobile phases were identical with the ones described previously for the identification of CRX interaction partners (6). Peptides were eluted at a flow rate of 300 nl min⁻¹ using the following gradient: 2.5–7.5% B over 4 min, 7.5–40% B over 21 min, 40% B for 3 min, 40–99% B over 3 min, 99% B for 10 min. MS data were acquired in a data-dependent mode, dynamically choosing the six most abundant precursor ions from the survey scans (scan range m/z 400–2,500, resolution 70,000, AGC target value 1e6, maximum injection time 50 ms) for fragmentation (MS/MS) by higher-energy C-trap dissociation (27% normalized collision energy, isolation window 2.0 m/z, resolution 17,500). AGC target value for MS/MS was 1e5 at 100 ms maximum injection and an intensity threshold of 4e4. Dynamic exclusion was enabled with an exclusion duration of 15 s. Singly and doubly charged ions, ions with charge state 8 and above as well as ions with unassigned charge states were excluded from fragmentation. Internal lock mass calibration on m/z 445.1203 was enabled.

For the identification of cross-linked peptides MS raw data were searched using Kojak (version 1.6.1 (27)) against a target-decoy database containing the polyepptide sequences of recombinant CRX, PRX1 as well as common contaminants (cRAP, https://www.thegpm.org/crap/index.html). Decoys were generated by sequence reversal. The following settings were applied: cross-linker masses, 138.0681 (BS3 d5) and 142.0931 (BS3 d6); mono-link masses, 156.0786 (BS3 d4) and 160.0137 (BS3 d5); fragment bin offset, 0; fragment bin size, 0.03; precursor mass tolerance, 5 ppm; maximum number of missed cleavages, 2. Carbamidomethylation of cysteine and oxidation of methionine were set as fixed and variable modifications, respectively. Spectrum-level q-values and posterior error probabilities were determined using Percolator (version 2.10, (28)).

Evolutionary origin of CRX

All TARA oceans (17) and EggNOG version 4.5.1 (18) data belonging to members of the protein family KOG0027 (signal transduction mechanisms, specifically: calcium-binding proteins) were considered for reconstructing the evolutionary history of CRX. Additionally, we used tBlastX to query the nucleotide core repository and all shotgun genome-sequencing collections of NCBI for records with similarity to CRX, thereby filtering for hits with an e-value of at least 1e-5. Forty contigs or scaffolds of the retrieved best hits, all belonging to Chlorophyta, were subjected to a functional annotation using the EggNOGmapper in HMmer mode to identify protein domains with help of Hidden Markov Models. The database search and functional transfer was limited to data of Viridiplantae. We prioritized precision over coverage by preferring one-to-one orthologs and experimental gene ontology evidence. Of the entered sequences, only one record of Chlamydomonas sp. was placed in the KOG0027 family, which was, however, not investigated further.

To analyze the evolutionary history of CRX, we eliminated all sequences of the original 3827 proteins from 234 species that were shorter than 153 residues, a length cutoff corresponding to the combined length of EF-hands 3 and 4 plus the TrxD. Records with identical sequences but of differing lengths from the same organisms and other forms of bioinformatic duplicates were removed. The resulting data set of 1,384 sequences from 167 species was aligned with mafft version 7 (29) under the FFT-NS-i method with two refinements, employing the BLOSUM62 scoring matrix, a gap penalty of 1.53, and an offset value of 0.01. The resulting alignment was subjected to phylogenetic analysis with FastTree version 2.1.5 (30). A maximum likelihood search was conducted, for which we used the model

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of Whelan and Goldman (31) with 20 site rate categories and an optimized Gamma20 likelihood. Support values were computed based on local bootstrapping and the minimum evolution criterion; pseudocounts were employed to account for the largely gapped alignment. All raw data are available as Data file S1.

To investigate the origin of the combination of EF-hands and a potentially functional TRX, we first investigated all protein sequences for the presence of sequences with a high similarity to EF-hand 2, EF-hand 3, and EF-hand 4, enforcing a conserved spacing, i.e. not allowing for any insertions or deletions within or between these. To explore the additional presence of an intact TrxD, we queried all EF-hand 2-, 3-, and 4-containing sequences for the presence of an N-terminal TRX by limiting the search string to its actual active site (twCRPCk). To relax the search, we used as queries TWCXCK, where X represents any residue, and TWCRCPC (as characterized in C. reinhardtii). These searches revealed the presence of EF-hand 2, 3, and 4 plus TRX only in species belonging to Chlorophyta or dinoflagellates. In the phylogenetic tree of the protein family KOG0027, the occurrence of such EF-hand containing thioredoxins is confined to a single clade (Fig. 6). Searches for only CXXC, CRPC, or CKPC (as found in Symbiodinium sp.) revealed isolated occurrences of this motif in eight members of the KOG0027 protein family, however, neither of which are in correct positions to EF-hands, if present, and none belonging to Viridiplantae or other plastid-bearing organisms. We extracted the chlorophyte and dinoflagellate sequences, among which the validated CRX, and re-aligned these data with mafft as above, but enabling a maximum of 1000 iterative refinements. This alignment was used to further characterize the conservation of the domain structure and similarities between the phylogenetically distinct dinoflagellates and chlorophytes by eye.

Data availability

SAXS data have been uploaded to the SASDBD data bank with the accession SASDE99 for the Ca2+-free and SASDEA9 for the Ca2+-bound form of CRX. Coordinates and structure factors have been deposited in the worldwide Protein Data Bank with the PDB ID 6j13. NMR data for CRX with and without Ca2+ can be found at the Biological Magnetic Resonance Data Bank (BMRB) with codes 27865 and 27860, respectively. Raw data for the CRX evolutionary analysis are available as Data file S1. All other data that support the findings of this study are available from the corresponding author on reasonable request.

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