Is Overoxidation of Peroxiredoxin Physiologically Significant?

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Abstract

Eukaryotic peroxiredoxins are highly susceptible to sulfinic acid formation. This overoxidation, which is thought to convert peroxiredoxins into chaperones, can be reversed by sulfiredoxins. Several organisms, including *Caenorhabditis elegans*, lack sulfiredoxins but encode sestrins, proteins proposed to be functionally equivalent. We induced peroxiredoxin overoxidation in *C. elegans* with a short peroxide pulse. We found that reduction of overoxidized peroxiredoxin 2 (PRDX-2) was extremely slow and sestrin-independent, strongly implying that worms lack an efficient repair system. Analysis of PRDX-2's overoxidation status during *C. elegans* lifespan revealed no accumulation of overoxidized PRDX-2 at any point, questioning whether PRDX-2 overoxidation in worms is physiologically relevant. *Antioxid. Redox Signal.* 14, 725–730.

Introduction

REACTIVE OXYGEN SPECIES, such as hydrogen peroxide (H_2O_2), superoxide anions ($O_2^{\bullet-}$), and hydroxyl radicals ($OH^{\bullet}$) are constantly produced by NADPH oxidases and during oxidative phosphorylation (12). At low concentrations, they play important roles as signaling molecules, regulating a multitude of different processes that range from gene regulation and metabolism to protein translation and degradation (10). At high concentrations, however, reactive oxygen species can cause damage to virtually every cellular macromolecule and, if not detoxified, will lead to apoptosis and cell death (6, 9, 11). Not surprisingly, cells harbor a machinery of antioxidant proteins to combat oxidative stress conditions, and redox-balancing systems to sustain the reducing environment of the cell (19). One large family of antioxidant proteins is peroxiredoxins, which use an active site cysteine to attack the O–O bond of ROOH substrates, thus converting harmful peroxides to harmless water (7). Some members of the peroxiredoxin family have been shown to play important physiological roles. This is illustrated for instance in *Caenorhabditis elegans*, where lack of the cytosolic homolog peroxiredoxin 2 (PRDX-2) causes progeric phenotypes and a shortened lifespan (16, 23), or in mice, where absence of Prdx1 leads to hemolytic anemia, increased tumor frequency, and shortened lifespan as well (22).

One of the unique features of eukaryotic typical 2-Cys peroxiredoxins is their high susceptibility to cysteine overoxidation (28). In the presence of high concentrations of per-oxide, the active site cysteine of peroxiredoxins, which forms a sulfenic acid ($-SOH$) intermediate during the regular detoxification cycle, is further oxidized to sulfinic acid ($-SO_2H$), which leads to the inactivation of the peroxidase function (26). This finding led to the floodgate model, which proposes that peroxide-mediated inactivation of peroxiredoxin allows localized peroxide signaling events to occur, potentially necessary to combat oxidative stress (27). This model was supported by the discovery of sulfiredoxins, enzymes that appear to specifically reduce overoxidized 2-Cys peroxiredoxins and regenerate peroxidase activity (1). *In vitro* studies conducted with yeast and human peroxiredoxins suggested furthermore that overoxidation might cause a functional switch, turning the peroxidase into chaperone-active oligomers (14, 20).

These results suggested that eukaryotic peroxiredoxins are posttranslationally regulated by the oxidation status of their active site cysteine. It came thus as a surprise when genomic studies revealed that certain organisms, including *C. elegans*, lack sulfiredoxin homologs (18). One possible explanation was that these organisms might use sestrins, a family of antioxidant proteins recently shown to also reduce overoxidized peroxiredoxins (3, 8), to regenerate the peroxidase-active state. This alternative scenario was, however, recently challenged by a study demonstrating that sestrins are unable to reduce overoxidized peroxiredoxins *in vitro* or *in vivo* (25). Our study now sheds light into the question how organisms such as *C. elegans* deal with peroxiredoxin overoxidation. Our results strongly suggest that *C. elegans* lacks an enzymatic

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system that clears overoxidized PRDX-2 and raise the general question whether overoxidation of PRDX-2 is a physiological event in worms.

Exogenous Peroxide Treatment Causes Overoxidation and Inactivation of *C. elegans* PRDX-2

Previous analysis of the physiological consequences of an acute 30 min peroxide stress treatment in *C. elegans* revealed a multitude of defects, including a decrease in motility, brood size, and pharyngeal pumping, which were highly reminiscent of alterations observed in aging animals (16). In contrast to aging organisms, however, which eventually succumb and die, we found that young wild-type *C. elegans* recover from these transient oxidative stress conditions within 24–48 h. We identified the protein that appeared to play an essential role in this recovery process to be the cytosolic peroxiredoxin homolog PRDX-2 (16). In the absence of PRDX-2, animals were unable to fully recover from the short exposure to peroxide stress, and showed persistent peroxide-mediated defects in mobility and egg laying (16). Analysis of the *in vivo* oxidation status of PRDX-2 in wild-type worms using two-dimensional (2D) gels revealed that over 50% of the total *C. elegans* PRDX-2 protein was shifted to an acidic pI within 30 min of H_2O_2 exposure, suggestive of PRDX-2 overoxidation (Fig. 1A, upper panel). Western blot analysis using antibodies against a conserved sulfinic acid peptide derived from human PRDX-2 reacted with the shifted PRDX-2 species from *C. elegans*, confirming that this shift is indeed due to sulfinic acid formation (Fig. 1A, lower panel). These results indicated that PRDX-2 is involved in the initial detoxification of exogenous peroxide, and, during this process, undergoes peroxide-mediated overoxidation and potentially inactivation. That overoxidation of PRDX-2's active site cysteine abolishes its protective peroxidase function was furthermore suggested by our finding that the steady-state concentration of one of the best known substrate proteins of peroxiredoxins, glutamine synthetase (GLN-3) (21), decreased dramatically upon peroxide treatment (Fig. 1B). The peroxidase function of peroxiredoxins has previously been shown to protect GLN-3 from H_2O_2-mediated inactivation and oxidative degradation in cell lysates, and was in fact the activity that led to the original purification of peroxiredoxins (21). Our observation that GLN-3 is rapidly oxidatively degraded agreed with our overoxidation results, suggesting that a large proportion of PRDX-2 becomes inactivated upon exogenous peroxide stress.

To test whether *C. elegans* PRDX-2, like the typical 2-Cys peroxiredoxins from yeast and humans (14, 20), functions as a peroxidase under low H_2O_2 conditions and as a chaperone under high H_2O_2 stress, we overexpressed and purified the His-tagged version of *C. elegans* PRDX-2 from *Escherichia coli* cells. We generated overoxidized PRDX-2 using cycles of thioredoxin (Trx)-mediated reduction and H_2O_2-mediated oxidation and confirmed that overoxidation leads to a loss in peroxidase activity (data not shown) (28). Next we tested the influence of reduced and overoxidized PRDX-2 on the *in vitro* aggregation of thermally denatured citrate synthase (CS) by analyzing the amount of soluble CS after 20 min of thermal inactivation in the absence or presence of PRDX-2 (2). As shown in Figure 1C, we found that PRDX-2's chaperone function was increased upon overoxidation of PRDX-2. Presence of a 20:1 ratio of overoxidized PRDX-2 monomers to CS monomers almost completely prevented the thermal aggre-
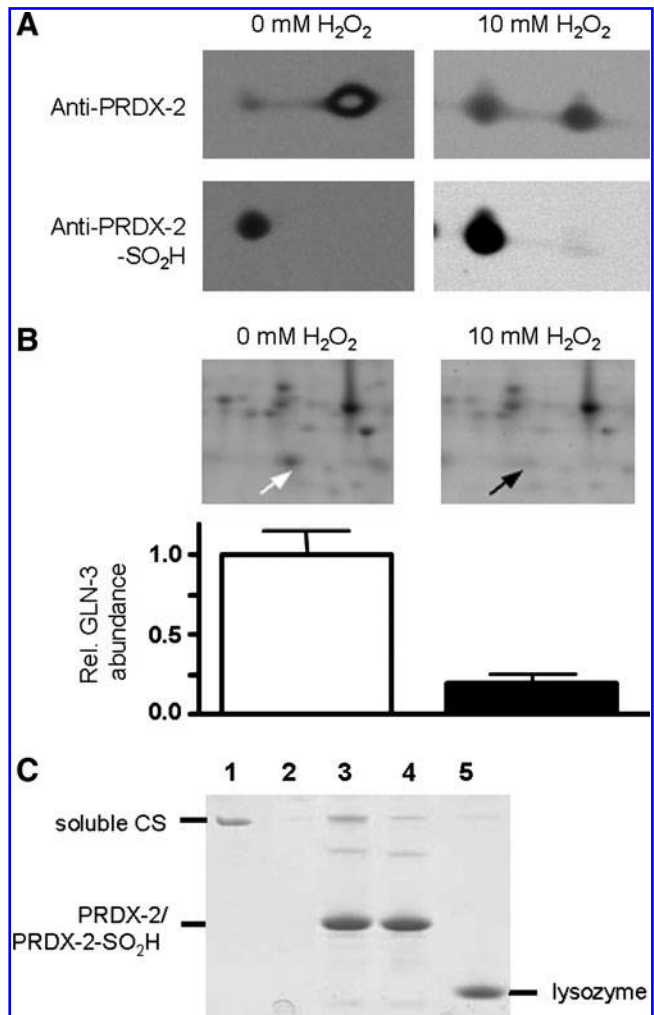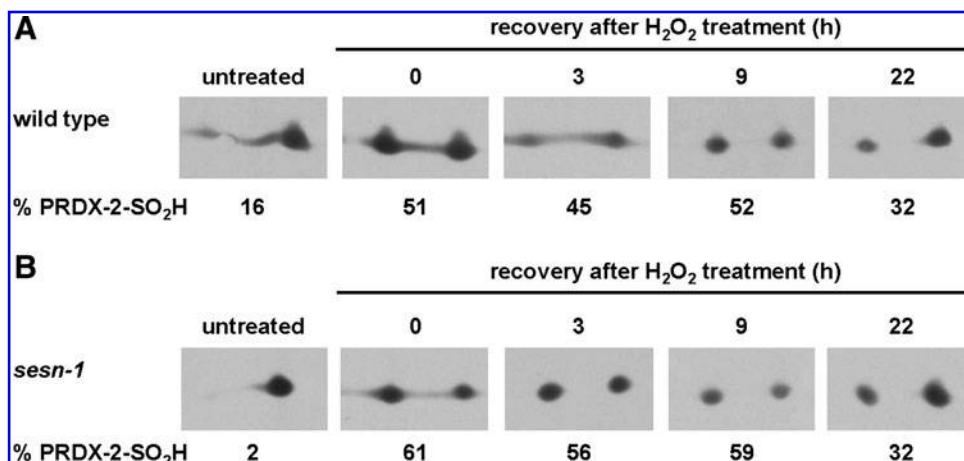

FIG. 1. Overoxidation of peroxiredoxin 2 (PRDX-2) upon exogenous oxidative stress. (A) Analysis of the overoxidation status of PRDX-2 upon exogenous oxidative stress *in vivo*. Synchronized wild-type L4 larvae were treated with 10 mM H_2O_2 for 30 min. Worms were lysed before or after the treatment, and *Caenorhabditis elegans* proteins were separated on 2D gels. Western blot analysis using antibodies against *C. elegans* PRDX-2 (upper panel) or against overoxidized PRDX-2-SO_2H (lower panel) was performed. **(B)** Glutamine synthetase (GLN-3) is rapidly degraded upon peroxide stress treatment of *C. elegans*. Synchronized wild-type L4 larvae were treated with 10 mM H_2O_2 for 30 min. Worms were lysed before or after the treatment, and *C. elegans* proteins were separated on 2D gels. Details of the Coomassie-stained 2D gels are shown. The arrows indicate the position of GLN-3, which was identified by mass spectrometry. Decodon was used to quantify changes in the steady-state levels of GLN-3. **(C)** Chaperone activity of reduced and overoxidized *C. elegans* PRDX-2 was analyzed by testing its influence on the thermal aggregation pattern of citrate synthase (CS) at 43°C. About 0.5 μM CS was incubated in the absence (*lane 2*) of chaperones or in the presence of overoxidized PRDX-2-SO_2H (*lane 3*), 10 μM PRDX-2$_{red}$ (*lane 4*), or 10 μM lysozyme (*lane 5*) in 40 mM Hepes, pH 7.5 for 20 min at 43°C. Then, samples were centrifuged and the soluble supernatant was loaded onto a 14% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The amount of soluble CS in the native protein sample is shown as control in *lane 1*.

FIG. 2. PRDX-2 recovery after exogenous oxidative stress treatment of *C. elegans*. A synchronized L4 population of wild-type **(A)** or *sesn-1* deletion worms **(B)** was treated with 10 mM H_2O_2 for 30 min, washed, and seeded on fresh plates. Aliquots of the worms were taken before and at defined time points after the stress treatment and lysed. *C. elegans* proteins were separated on 2D gels and subsequent western blot analysis with antibodies against PRDX-2 was performed. Three independent experiments were performed and a representative result is shown. ImageJ software was used to quantify the % overoxidized PRDX-2 in the worms.

gation of CS and maintained CS in a soluble form, whereas the same concentration of reduced PRDX-2 monomers had only a minor influence on the aggregation behavior of CS. These results confirmed studies with yeast and human peroxiredoxins (14, 20) and showed that overoxidation of *C. elegans* PRDX-2 causes the inactivation of its peroxidase activity and an increase in chaperone activity. Noteworthy, however, in contrast to yeast and human peroxiredoxins, significantly higher concentrations of *C. elegans* PRDX-2 were required to prevent protein aggregation, suggesting that PRDX-2 might be a less efficient chaperone.

Overoxidation of *C. elegans* PRDX-2 Appears to be an Irreversible Process *In Vivo*

To investigate the fate of overoxidized PRDX-2 in nematodes, we decided to elucidate whether this process is reversible *in vivo*. We exposed synchronized wild-type *C. elegans* to our 30 min short-term peroxide stress treatment, washed the worms, and analyzed the time course over which overoxidized PRDX-2 disappeared from the worms. As before, we lysed ~100,000 synchronized worms before and after oxidative stress treatment, separated the proteins by 2D gels, and performed western blot analysis to quantify the amount of shifted (i.e., overoxidized) PRDX-2 *in vivo*. We found that it required more than 22 h to clear the worms from overoxidized PRDX-2. This time frame was similar to the time it took peroxide-treated animals to recover from the stress treatment (24–48 h) (16), suggesting that reactivation of PRDX-2's peroxidase function might contribute to the recovery process of the animals. Our observation that clearance of overoxidized PRDX-2 is a very slow process strongly argued against an efficient endogenous repair system that reduces sulfinic acid and restores the peroxidase activity of PRDX-2. Instead, it suggested that *C. elegans* depends on the degradation of overoxidized PRDX-2 and on the new synthesis of unaltered PRDX-2 to restore the pool of peroxidase-active PRDX-2.

Consistent with the very long lifetime of overoxidized *C. elegans* PRDX-2 was the finding that *C. elegans*, like many other organisms such as *Xenopus laevis*, *Gallus gallus*, and *Neurospora*

crassa, do not encode any sulfiredoxin homologs, enzymes previously shown to reduce overoxidized peroxiredoxins (18). Further analysis of the *C. elegans* genome revealed, however, that *C. elegans* encodes the sestrin homolog Y74C9A.5. Sestrins have also been implied in the reduction of overoxidized peroxiredoxins (3, 8), although recent reports challenged these results by showing that overexpression of Sesn 2 in HeLa or A549 cells did not reduce overoxidized peroxiredoxins, and that deletion of the *sesn2* gene in mouse embryonic fibroblasts had no effect on the recovery of overoxidized peroxiredoxin (25). We decided that our ability to monitor PRDX-2's overoxidation *in vivo*, and the fact that *C. elegans* encodes only one member of the sestrin family that is nonessential for growth (data not shown), put us into an excellent position to evaluate the role of sestrins in the regeneration of overoxidized PRDX-2 in organisms that lack sulfiredoxin.

We obtained and back-crossed the Y74C9A.5 knockout strain, and, based on the homology of Y74C9A.5 to human sestrin, decided to rename the *C. elegans* gene *sesn-1* and the protein SESN-1. We exposed *sesn-1* mutant worms to our previously established peroxide stress regiment and compared the overoxidation status of PRDX-2 between wild-type and *sesn-1* mutant strains before and at distinct time points after the 30 min peroxide stress treatment. As shown in Figure 2, we did not observe any significant difference in the steady-state levels of overoxidized PRDX-2 in wild-type and mutant strains, either before or at any point after the oxidative stress treatment. As before, we found that the overoxidized form of PRDX-2 disappeared only very slowly and that the time frame in which the pool of peroxidase-active PRDX-2 was restored was similar to the time it took the *sesn-1* mutant animals to recover from peroxide stress (data not shown). These results strongly implied that SESN-1 is not involved in the regeneration of PRDX-2.

Overoxidation of PRDX-2 Appears Insignificant During the Lifespan of *C. elegans*

The slow regeneration of PRDX-2 under conditions where exogenous peroxide has long been removed suggested that worms lack an enzymatic system that reduces overoxidized
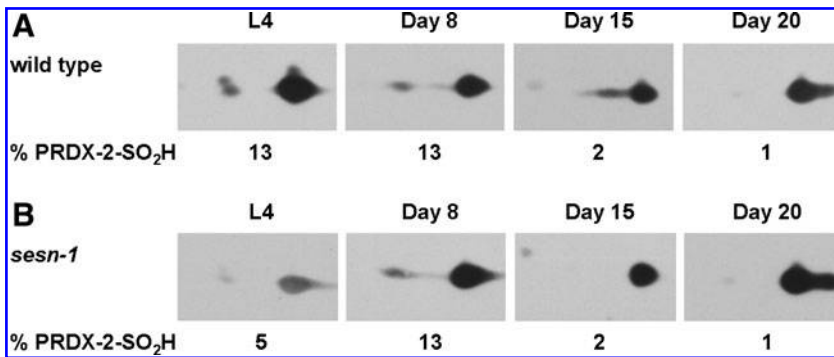
FIG. 3. Analysis of PRDX-2 over-oxidation during the lifespan of *C. elegans*. Synchronized populations of wild-type N2 **(A)** and *sesn-1* deletion worms **(B)** were cultivated at 15°C. Aliquots of worms were taken at L4, day 8, 15, and 20 of adulthood and lysed. *C. elegans* proteins were separated on 2D gels and western blot analysis was performed using antibodies against PRDX-2. A representative result is shown. ImageJ software was used to quantify the % overoxidized PRDX-2 in the worms.

PRDX-2 and rely on the slow and selective degradation of overoxidized PRDX-2 and the new synthesis of unmodified PRDX-2 to maintain their level of peroxidase-active PRDX-2. These results left us wonder whether organisms that lack sulfiredoxins are less likely to encounter peroxide stress conditions that are sufficiently high to cause overoxidation of peroxiredoxins in the first place. Alternatively, overoxidation of peroxiredoxins might provide a strategy for these organisms to generate a pool of chaperones, necessary to combat protein-unfolding conditions at distinct stages in their life. To test to what extent PRDX-2 overoxidation actually takes place during the life span of *C. elegans*, we synchronized worms, cultivated them at 15°C, and monitored the overoxidation status of PRDX-2 between the larval stage L4 and day 20 of adulthood. At day 20 under these cultivation conditions, most worms show motility defects and other age-related changes (5) (data not shown). As before, we lysed aliquots of the synchronized worms, separated the proteins on 2D gels, and performed western blot analysis with antibodies against PRDX-2. As shown in Figure 3A, we were unable to see any increase in the overoxidation status of PRDX-2 even in aged worms. Given the slow kinetic with which overoxidized PRDX-2 is cleared from the cells, these results suggest that *C. elegans* PRDX-2, at least under our growth conditions, has a very low tendency for overoxidation *in vivo*. We obtained very similar results when we analyzed the overoxidation status of PRDX-2 in *sesn-1* deletion worms (Fig. 3B), which serves as further indication that sestrin does not affect the overoxidation status of PRDX-2 in *C. elegans*.

Concluding Remarks

Eukaryotic type-2 peroxiredoxins are characterized by their high susceptibility to overoxidation. This initial finding, combined with the discovery of sulfiredoxins, enzymes specialized to reduce sulfinic acid and regenerate peroxiredoxins, led to an attractive model, in which the rapid inactivation of peroxiredoxin would permit transient, potentially compartmentalized peroxide-mediated signaling events, critical to combat oxidative stress (15). This model left unexplained, however, the fact that many organisms lack sulfiredoxin, and raised the question how these organisms deal with overoxidation and inactivation of peroxiredoxins (18). One possible explanation was that sestrins, highly conserved Nrf2-dependent antioxidant proteins, take over the function of sulfiredoxins in those organisms. We tested this hypothesis in *C. elegans*, which lacks sulfiredoxin, encodes only one member of the sestrin family, and accumulates overoxidized PRDX-2 when treated exogenously with

peroxide. Our studies showed that *C. elegans* clears overoxidized PRDX-2 only very slowly in a process that seemed unaffected by the lack of sestrin. These results are in excellent agreement with very recent studies, which demonstrated that sestrins do not play a major role in the regeneration of overoxidized human PRDX-2 (25). One possible explanation for the finding that *C. elegans* and potentially other organisms lack the need for sulfiredoxin might be that they do not encounter endogenous peroxide concentrations during their normal lifespan that lead to significant accumulation of overoxidized PRDX-2, which would require enzymatic removal. We analyzed the overoxidation status of worms until day 20 of their lifespan, where worms have been shown to suffer from oxidative stress conditions and new protein synthesis is thought to decrease (24). However, we were unable to detect any significant amount of overoxidized PRDX-2 even at this advanced stage of their life. Although we cannot exclude that increased proteolysis of overoxidized PRDX-2 factors into these low steady-state levels, we found no significant change in the overall levels of PRDX-2. Thus, very rapid proteolysis of overoxidized PRDX-2 combined with rapid new PRDX-2 synthesis would be required to maintain these high levels of unmodified PRDX-2, an unlikely event at this age. Our results suggest, however, that during the lifespan of *C. elegans*, peroxide conditions do not reach levels that are high enough to cause the overoxidation of PRDX-2, and thus to switch PRDX-2 into a molecular chaperone. While we cannot exclude the possibility that *C. elegans* will encounter stress conditions that might cause PRDX-2 overoxidation, we propose that overoxidation and its concurrent switch to molecular chaperone function does not play a significant physiological role during the normal lifespan of *C. elegans*, and potentially other organisms that lack the sulfiredoxin system.

Notes

Strains and culture conditions

The Bristol strain N2 (wild type) was provided by the *Caenorhabditis* Genetics Center and the *sesn-1* knockout strain by the Mitani Laboratory at the Tokyo Women's Medical University School of Medicine. The *sesn-1* knockout strain was back-crossed three times to the wild-type strain N2. Worms were cultured using standard protocols.

Oxidative stress treatment

Oxidative stress treatment was performed as previously described (16). In short, a synchronized L4 population

of *C. elegans* N2 or *sesn-1* was treated with 10 mM H_2O_2 for 30 min while shaking continuously. Worms were washed twice with M9 and either plated on NGM plates seeded with OP50 for recovery or shock frozen in liquid nitrogen and stored at $-80°C$. Worms were lysed in 10% trichloroacetic acid using a Fisher Scientific Power Gen 125 homogenizer.

2D gel electrophoresis, western blot analysis, and protein quantification

Samples for 2D gel electrophoresis were prepared as described (17) with the exception that protein thiols were reduced with dithiothreitol and subsequently labeled with iodoacetamide; 2D gel electrophoresis was performed using the Ettan IPGphor II and DALT II system by Amersham Biosciences as described previously (17). Western Blot analysis was performed using standard protocols using polyclonal antibodies raised against *C. elegans* PRDX-2 (Alpha Diagnostics International), or antibodies against a peptide derived from overoxidized human peroxiredoxin-SO_2H (Abcam Inc.). Protein quantification was performed using ImageJ or Delta 2D (Decodon) software.

PRDX-2 purification, reduction, and overoxidation

The plasmid pJC45 containing the His-tagged variant of *C. elegans* PRDX-2 was kindly provided by Dr. Bruchhaus (13) and transformed into *E. coli* BL21 (DE3)(pAPlacIQ). After induction with isopropyl-beta-D-thiogalactopyranoside and overexpression of PRDX-2, the protein was purified with an Ni-NTA column using standard procedures. *In vitro* reduction of purified PRDX-2 was achieved by incubating PRDX-2 with 5 mM DTT for 15 min at room temperature. To overoxidize PRDX-2, 100 μM purified PRDX-2 was incubated with 2.5 μM *E. coli* Trx, 0.08 μM *E. coli* Trx reductase, and 10 mM H_2O_2 for 1 h at room temperature (4).

Chaperone assay

Influence of PRDX-2 on the thermal aggregation of bovine CS (Sigma) was analyzed at 43°C. About 100 μM PRDX$_{red}$, PRDX-SO_2H, or lysozyme was diluted 1:10 into 40 mM Hepes, pH 7.5 at 43°C. Then, CS (final concentration 0.5 μM) was added and the samples were incubated for 20 min at 43°C. After the incubation, the samples were centrifuged (5,000 rpm, for 30 min at 4°C) and the soluble supernatant was loaded onto a 14% sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Acknowledgments

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Abbreviations Used
2D = two-dimensional
CS = citrate synthase
GLN-3 = glutamine synthetase
H_2O_2 = hydrogen peroxide
PRDX-2 = peroxiredoxin 2
sesn = sestrin

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