Regeneration of Peroxiredoxins during Recovery after Oxidative Stress

ONLY SOME OVEROXIDIZED PEROXIREDOXINS CAN BE REDUCED DURING RECOVERY AFTER OXIDATIVE STRESS

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Peroxiredoxins (prx) are redox enzymes using an activated cysteine as their active site. This activated cysteine can be easily overoxidized to cysteine sulfenic acid or cysteine sulfonic acid, especially under oxidative stress conditions. The regeneration of peroxiredoxins after a short, intense oxidative stress was studied, using a proteomics approach. Important differences in regeneration speed were found, prx2 being the fastest regenerated protein, followed by prx1, whereas prx3 and prx6 were regenerated very slowly. Further study of the mechanism of this regeneration by pulse-chase experiments using stable isotope labeling and cycloheximide demonstrated that the fast-regenerating peroxiredoxins are regenerated at least in part by a retroreduction mechanism. This demonstrates that the overoxidation can be reversible under certain conditions. The pathway of this retroreduction and the reasons explaining the various regeneration speeds of the peroxiredoxins remain to be elucidated.

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Among the enzymes detoxifying reactive oxygen species, peroxiredoxins (prx)1 appear as a special class both for their catalytic mechanism and for their ubiquitous localization in cells. Furthermore, although their catalytic activity appears to be inferior to the more classical peroxidases (1), they seem to play a key role in the detoxification of reactive oxygen species (2). In addition to their catalytic role, they also bind to various cellular proteins such as proto-oncogenes (3, 4) and modulate their activities.

One of the peculiarities of peroxiredoxins is their catalytic mechanism. They represent a special type of peroxidases, as the protein is the reducing substrate itself. In the general case (e.g. glutathione peroxidase) the enzyme reduces the peroxide while oxidizing a co-substrate (e.g. glutathione). In the case of peroxiredoxins, the enzyme contains an activated cysteine at the active site (5) and can contain another conserved cysteine (6, 7). In the normal catalytic cycle, the activated cysteine is oxidized to cysteine sulfenic acid (5) or to a disulfide (8) when a peroxide is reduced. To complete the enzymatic catalytic cycle, the peroxiredoxins are then reduced back to their active thiol form, for example by the thioredoxin-thioredoxin reduction system for 2-Cys peroxiredoxins (9), (8). However, peroxiredoxins are also prone to overoxidation phenomena, in which the active site cysteine is oxidized to a sulfonic acid (10–12) or even to a sulfonic acid (12, 13). This of course leads to the inactivation of the enzyme, even in the presence of thioredoxin-based regeneration system (11, 14).

Such overoxidation phenomena are encountered in various situations in vivo, but are very prominent under oxidative stress conditions, where more than 90% of all peroxiredoxins can be converted to the overoxidized forms (12, 13). This poses in turn the problem of cell survival after a strong oxidative stress, once all the peroxiredoxins have been overoxidized. If the active peroxiredoxins are indeed essential to aerobic life, as suggested by some experiments (2), the cell needs to regenerate active peroxiredoxins after oxidative stress. It has been recently suggested that overoxidized peroxiredoxins can be reduced back to the active form during recovery after oxidative stress, prx1 being taken as an example (15). We thus decided to investigate the regeneration of peroxiredoxins after oxidative stress in more detail using the proteomics toolbox, which is able to deconvolute the overoxidized forms from those involved in the normal catalytic cycle (11–13, 15).

MATERIALS AND METHODS

Cell Culture and Oxidative Stress—HeLa S3 cells were cultured in suspension in Dulbecco’s modified Eagle’s medium containing 1 mm pyruvate, 10 mm Hepes-NaOH, pH 7.5, and 10% fetal calf serum. Oxidative stress was performed by adding to the culture medium 75 μM tert-butyl hydroperoxide for 2 h.

In the first series of experiments using labeling with stable isotopes, the cells were grown for 2 weeks in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 1 mm pyruvate, 10 mm Hepes-NaOH, pH 7.5, and 1 mm L-lysine 3,3,4,4,5,5,5,6,6-d8 (Isotec). The cells were stressed with butyl hydroperoxide as described above then recovered by centrifugation, rinsed twice with phosphate-buffered saline, and left to recover in the same medium but without deuterated lysine for various periods of time prior to final harvesting.

In the second series of experiments using labeling with stable isotopes, the cells were grown in normal culture medium, stressed with butyl hydroperoxide, and left to recover in a Dulbecco’s modified Eagle’s medium supplemented with 2-depleted medium (Sigma) reconstituted with all missing amino acids in normal form except lysine, which was used as its 4,4,5,5-tetradeuterated form (Isotec). The medium was also supplemented with...
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1 mM pyruvate, 10 mM Hepes-NaOH, pH 7.5, and 10% dialyzed fetal calf serum. The recovery phase took place in this medium for 1–3 h in the absence or presence of 1 mM cycloheximide.

Cells were then harvested by centrifugation, rinsed in phosphate-buffered saline and resuspended in homogenization buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA). A buffer volume approximately equal to the packed cell volume was used. The suspension was transferred to a polyallomer ultracentrifuge tube, and the cells were lysed by the addition of 4 volumes (relative to the suspension volume) of 8.75 M urea, 2.5 M thiourea, 25 mM spermine base, and 50 mM dithiothreitol. After 1 h at room temperature, the extracts were ultracentrifuged (30 min at 200,000 × g). The supernatant was collected and the protein was determined by a Bradford assay using bovine serum albumin as a standard. Carrier ampholytes (0.4% final concentration) were added, and the protein extracts were stored at −20 °C.

Two-dimensional Electrophoresis—Two-dimensional electrophoresis was performed with immobilized pH gradients for isoelectric focusing. Non linear 4–10 pH gradients were used (16). Homogeneous pH gradient plates were cast and cut into 4-mm-wide strips (17). The samples were applied onto the strips by in-gel rehydration overnight (17) using a thiourea-urea mixture as a denaturing agent (18). The gels were then migrated in a Multiphor apparatus (with a voltage limited to 3500 V) up to 60,000 Vh. The immobilized pH gradient strips are then equilibrated in SDS buffer and placed on top of a 10% SDS-acrylamide gel. After migration, the gels were stained either with silver (19) or with colloidal Coomassie Blue (20).

In Gel Digestion—Excised gel slice rinsing and reduction/alkylation steps were performed by the Massprep (Micromass, Manchester, UK) as described previously (12). Gel pieces were completely dried with a Speed Vac before digestion. The dried gel volume was evaluated and three volumes of enzyme, either trypsin (Promega, Madison, WI) or AspN (Roche Diagnostics GmbH, Mannheim, Germany), 12.5 ng/μl freshly diluted in 25 mM NH₄HCO₃, were added. The digestion was performed at 35 °C overnight. Then, the gel pieces were centrifuged for 5 min in a Speed Vac and 5 μl of 35% H2O/60% acetonitrile/5% HCOOH were added. The digestion was then performed according to a previously described procedure was repeated once.

MALDI-TOF-MS Analysis—Mass measurements were carried out on an Ultraflex™ MALDI-TOF/TOF mass spectrometer (Bruker-Daltonik GmbH, Bremen, Germany). This instrument was used at a maximum accelerating potential of 20 kV and was operated either in reflector-positive or reflector-negative mode. Sample preparation was performed in positive or reflector-negative mode. Sample preparation was performed on the molecular weight of this peptide, the isotopic contribution was estimated at 10%. For MS experiments with the d4 lysine labeling, the ratio d4/d0 calculated for the control before oxidative stress was used as a reference to estimate the experimental isotopic contribution at the mass of the peptide of interest plus 4 Da. Depending on the molecular weight of this peptide, the isotopic contribution was

FIG. 1. Variation of peroxiredoxins during recovery after oxidative stress. Only the zone of the two-dimensional gels containing the peroxiredoxin is shown. A, prx1 has a much more basic pI, the prx1 zone is shown as an inset for space reasons. Whole cell extracts prepared from HeLa cells cultured under control conditions (A) or treated for 2 h with 75 μM butylperoxide (B) were analyzed by two-dimensional electrophoresis. The extracts obtained from cells treated as in B but left to recover are shown in the other panels. C, 1 h recovery; D, 3 h recovery; E, 6 h recovery; F, 12 h recovery; G, 24 h recovery. H, 48 h recovery.

FIG. 2. Bar chart representation of peroxiredoxin regeneration after oxidative stress. The white bar represents the abundance (in ppm of the total spots integrated) of the basic form of each peroxiredoxin. The hatched bar represents the abundance of the acidic form, the total height of each bar representing the sum of the two forms. For simplicity reasons, the standard deviations are not represented. They are always inferior to 5% of the mean value. C, control; S, immediately after stress (t-butylhydroperoxide, 2 h); I, 1 h of recovery in peroxide-free medium after oxidative stress; 3, 3 h of recovery; 6, 6 h of recovery; 12, 12 h of recovery; 24, 24 h of recovery.
taken into account or ignored. When necessary, the corresponding "natural" \( d_4/d_0 \) ratio was subtracted from the \( d_4/d_0 \) ratios calculated at different times of the chase.

**RESULTS**

The starting point of this study lies in the fact that peroxiredoxins appear as doublet spots, which are oxidative stress-sensitive. The basic spot of the doublet appears to correspond to the forms involved in the normal catalytic cycle (12), as the thiol and disulfide forms are indistinguishable in our separation system. The acidic spot of the doublet corresponds to the overoxidized form of the peroxiredoxin, where the active site cysteine is converted to a sulfinic or sulfonic acid (11–13). We can thus use this resolving power to determine the quantitative evolution of the normal and oxidized forms of peroxiredoxins during recovery after oxidative stress. Typical results are shown in Fig. 1 and summarized in Fig. 2. Although it appears that prx4 is not very strongly oxidized in our stress conditions, prx1, 2, 3, and 6 are overoxidized at more than 90% (prx5 was not detected in these experiments). However, the evolution of the relative amounts of the oxidized versus normal forms showed important differences between peroxiredoxins, as shown in Fig. 2. Although prx2 was quickly regenerated, with more than 50% of the original amount of normal prx2 recovered in 1 h and complete regeneration in 3 h, the regeneration of prx3 and prx6 appeared much slower, with 50% regeneration in only 12 h for prx6 and complete regeneration in 24 h or more. Regeneration of prx1 was intermediate between these two extremes, with 50% regeneration in 6 h. Moreover, the quantitative results obtained from two-dimensional gels also showed major differences among the different peroxiredoxins. Although the total prx1 amount stayed more or less constant throughout the complete regeneration process, the amount of prx3 and prx6 showed a transient decrease. This decrease is driven by a gradual disappearance of the oxidized form, which is larger and earlier than the reappearance of the normal form. Conversely, prx2 showed a transient increase in its total amount during recovery after oxidative stress. This phenomenon is driven by a fast increase in the normal form, which is larger than the decrease of the oxidized form.

To investigate the regeneration process in more detail, we used a pulse-chase-like setup, using deuterated lysine as the label. In the first set of experiments, the cells were grown in a medium enriched in deuterated lysine up to steady state labeling. The cells were then submitted to an oxidative stress, rinsed, and left to recover in a medium containing only normal lysine. The quantitative ratio between the labeled and unlabelled peptides arising from the trypsin digestion of the peroxiredoxins spots can be determined easily with a mass spectrometer. Typical results are shown in Fig. 3 and summarized in Fig. 4. Here again, the order prx2-prx1-prx6 is found. For the latter protein, the very low \( d_8/d_0 \) ratio obtained after a 24 h regeneration period is clearly compatible with a regeneration of the active form by simple de novo synthesis, and slow degra-

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**FIG. 3.** Mass spectra of tryptic peptides after a labeling by deuterated lysine (\( d_8 \)) up to the steady state, an oxidative stress, and a chase in a medium containing only unlabelled lysine for various times. For prx2, the peptide shown in the figure is the 92–109 peptide, sequence KEGGLPLNLILLADVTR and \( m/z \) (monoisotopic, unlabelled: 1863.1). For prx6, the peptide shown in the figure is the 162–173 peptide, sequence VVISLQLTAEKR and \( m/z \) (monoisotopic, unlabelled: 1356.8). A, prx2, oxidized spot, starting of the chase. B, prx2, normal spot, starting of the chase. C, prx6, oxidized spot, starting of the chase. D, prx6, normal spot, starting of the chase. E, prx2, oxidized spot, 3 h chase. F, prx2, normal spot, 3 h chase. G, prx6, oxidized spot, 3 h chase. H, prx6, normal spot, 3 h chase. I, prx2, oxidized spot, 24 h chase. J, prx2, normal spot, 24 h chase. K, prx6, oxidized spot, 24 h chase. L, prx6, normal spot, 24 h chase.
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Between the two hypotheses, we performed a proteomics experiment to discriminate between very fast destruction of the oxidized prx2, or the retroreduction of the oxidized peptide with an uncleaved AspN site at the oxidized cysteine and, for the normal, sulfinic and sulfonic forms could be identified, except for prx3 where only the sulfonic acid form was identified. Moreover, the ratio of the sulfinic to sulfonic forms did not change during the early stages of recovery for all peroxiredoxins, suggesting either that the sulfinic form is produced from the sulfonic form during the separation process, as suggested elsewhere (11, 15) or that the cell is able to destroy both forms at the same speed.

However, it must be noted that the spots co-migrating with the oxidized spots but present before oxidative stress did not show the oxidized peptide but the peptide containing a normal cysteine instead. This demonstrates that these spots do not correspond to the oxidized forms of peroxiredoxins, but to another post-translational modification such as phosphorylation (22). The two phenomena can even superimpose, as shown by the example of the acidic prx2 and prx1 spots after 3 h of recovery.

The most surprising results, however, came from the analysis of the spots migrating to the position of the normal forms. Although the expected peptide containing a cysteine is found on the unstressed forms of all peroxiredoxins that can be analyzed by this method (i.e. excluding prx6), the same spot obtained during the recovery process can either show only the cysteine in its thiol form (prx2 and 3) or show mainly the cysteine in its oxidized form (prx1). In the latter case, the spot migrating to the position of the normal one is indeed not a bona fide reduced peroxiredoxin, but a modified oxidized form co-migrating with the normal one. In addition to that, additional peptide masses are frequently encountered close to the mass predicted for the normal peptide with its alkylated cysteine, with monoisotopic masses at −25, −9, and −7 Da compared with the normal, alkylated peptides. These masses correspond exactly to the masses of the non-alkylated peptide +32, 48, and 64 Da, i.e. +2, +3, and +4 oxygen atoms. They can be interpreted as an oxidized peptide with an uncleaved AspN site at the oxidized cysteine and, for the +64 Da form, with an extra hydroxylolation on the peptide. Alternatively, the +64 Da form can be a sodium adduct of an acetylated but unalkylated peptide. Interestingly enough, these peptides are encountered in the oxidized spots and in the basic prx1 spot during the early recovery phase, further evidencing the presence of oxidized cysteine in this spot. They are also encountered, at a much weaker extent, in the basic prx1 spot in the control conditions. This demonstrates that the situation is not very clear for prx1, with some oxidized protein co-migrating with the normal one for a still unknown reason. Unfortunately, with the protein amounts available.

![Bar chart representation of the evolution of the d8/d0 ratio during a pulse-chase experiment. C, control (no oxidative stress, no chase); S, stress (2 h oxidative stress in d8-containing medium, no chase); Rn, recovery in d0-only medium for n hours after steady-state labeling and oxidative stress in d8-containing medium. Numatrin is used as a control protein. A lower d8/d0 ratio than numatrin means a higher de novo synthesis, whereas a higher d8/d0 ratio means a lower de novo synthesis.](Image)

To get further details of this retroreduction, we carried out a detailed characterization of the two peroxiredoxins forms, using the negative MALDI-MS approach described previously (12). The results are shown in Figs. 6 and 7 and summarized in Table II. They point to several important phenomena. On the spots corresponding to the oxidized forms, both the sulfinic and sulfonic forms could be identified, except for prx3 where only the sulfonic acid form was identified. Moreover, the ratio of the sulfinic to sulfonic forms did not change during the early stages of recovery for all peroxiredoxins, suggesting either that the sulfinic form is produced from the sulfonic form during the separation process, as suggested elsewhere (11, 15) or that the cell is able to destroy both forms at the same speed.

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from two-dimensional gels, these peptides could only be observed in negative MALDI mode and neither in positive MALDI nor in electrospray ionization. This prevented us from gaining additional knowledge in the detail of the modifications by peptide fragmentation.

**DISCUSSION**

Peroxiredoxins are a growing family of proteins involved in the redox regulation of cells, together with peroxidases, catalase, and superoxide dismutases. As a matter of fact, many of these redox control proteins are themselves very sensitive to the redox status of the cell. This is not surprising, as these proteins have active centers that are very reducing and therefore very sensitive to overoxidation, leading to the inactivation of these proteins (11, 13, 14). If the cells are submitted to a strong oxidative stress for a long time, they die massively. However, if the oxidative stress is short enough, the cells can recover and resume growth. This recovery phase was studied previously (13, 15). These two reports demonstrate that two different phenomena account to explain the regeneration of peroxiredoxin 1 and 2, namely *de novo* synthesis (13) and, more surprisingly, retroreduction from the overoxidized form (15). However, both studies rely only on spot position on two-dimensional gels to evidence the mechanisms of peroxiredoxin regeneration. To get more details on the phenomena taking place for peroxiredoxin regeneration during recovery after oxidative stress, we used HeLa cells, which display a wider panel of peroxiredoxins (12), and a combination of protein labeling *in vivo* with stable isotopes, mass spectrometry, and protein synthesis blocking with cycloheximide.

**TABLE I**

*Variation in the d4/d0 ratio in mass spectrometry signal during a chase experiment in the presence of d4 lysine after oxidative stress.*

| Condition | Protein | prx1 | prx2 | prx2 |
|-----------|---------|------|------|------|
| T0        | 0.00    | 0.00 | 0.00 | 0.00 |
| T3        | 0.127   | 0.00 | 0.116| 0.108|
| T3 + CHX  | 0.00    | 0.00 | 0.00 | 0.00 |
| S3        | 0.099   | 0.132| 0.048| N.D. |
| S3 + CHX  | 0.01    | 0.00 | 0.00 | N.D. |

CHX, cycloheximide; T0, without stress, no chase; T3, without stress, 3 hours chase; S3, with stress, 3 hours chase.

Our results demonstrate a wide variety of regeneration speeds for the various peroxiredoxins. At one end stands prx2, which regenerates very fast by a mixed mechanism coupling *de novo* synthesis (as shown by the increase in amount of total prx2) and retroreduction. At the other end stand prx6 and prx3, for which the regeneration speed, the transient decrease in the total amount of the protein, and the labeling data are not...
consistent with a retroreduction mechanism, but rather with a regeneration mechanism using only de novo synthesis. The situation is more complex for prx1, which shows an intermediate speed. As mentioned in previous studies (15) protein synthesis blocking with cycloheximide suggested that prx1 was retroreduced, but any parasite, reversible post-translational modification could also induce shuttling of the protein spots between the ones corresponding to the normal and oxidized form positions. As a matter of fact, any modification decreasing the number of negative charges or unmasking a positive charge could give the same migration pattern as cysteine sulfinic or sulfonic acid retroreduction, this being true for prx1 and prx2.

To exclude this phenomenon and to ascertain for retroreduction, we tried to characterize in detail the peptide bearing the active site cysteine in all peroxiredoxin spots arising either before oxidative stress, immediately after oxidative stress, or during the recovery phase. These experiments clearly showed that only prx2 met the requirements for a true retroreduction, both as the regeneration speed and as the disappearance of the oxidized cysteine and reappearance of the normal cysteine in the normal form. The fact that oxidized cysteine could be found in the normal prx1 spot clearly shows that simple co-migration as cysteine sulfenic acid or sulfonic acid retroreduction, this being true for prx1 and prx2.

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This evidence for retroreduction clearly breaks the dogma of the irreversibility of the oxidation (11, 13, 23) and leads to important questions. The first one is of course the nature of the retroreducer in vivo. It is clear that neither glutathione (15) nor classical thioredoxins are able to perform this task, as shown by in vitro experiments (11, 14). However, the fact that peroxiredoxins can be linked to different thioredoxin subtypes (24) clearly leaves the field open, as suggested by recent results obtained on plants (25).

The second important point to understand is why the retroreducibility of peroxiredoxins varies so widely. For prx3, which is located in the mitochondria, it can be argued that the retroreducing system is absent or very poorly efficient in mitochondria. However, the difference between the three cytosolic proteins (prx1, 2, and 6) is more puzzling. The first hypothesis is that prx6 is not retroreduced because it is a peroxiredoxin that cannot form a disulfide bridge in its catalytic cycle, oppositely to prx1 and prx2. This does not explain, however, why prx2 is more easily retroreduced than prx1. An explanation is that prx2 has a high affinity for the retroreducer, whichever it is, whereas this affinity would decrease for prx1. It is quite clear that the various peroxiredoxins are able to bind different proteins (22), which can support this hypothesis. When combined with our labeling data on the oxidized form, prx2 appears as the most dynamic peroxiredoxin, going very easily back and forth on the complete oxidation-reduction cycle from the thiol to the sulfinic acid states. Oppositely, prx3 and prx6 appear much more static and classical in their redox behavior. The status of prx1 is still less clear but probably intermediate between the two extremes represented by prx2 and prx6.

Whatever the reasons for the easy retroreducibility of prx2 are, this characteristic differentiate prx2 as the very last cellular defense line within peroxiredoxins. Although prx2 is clearly inactivated under oxidative stress, as the other peroxiredoxins are, the quick reversibility of this inactivation makes it the first
peroxiredoxin to be active again to protect the cell from oxidative damage after the oxidative stress episode is over. The variability in retroreducibility, coupled with the presence of peroxiredoxins in almost all cell compartments, to bind a variety of other cellular partners (22), to perform other functions than simple destruction of peroxides (26, 27) may explain the variety of peroxiredoxins observed in upper eukaryots.

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