A method for detection of overoxidation of cysteines: peroxiredoxins are oxidized *in vivo* at the active-site cysteine during oxidative stress

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Peroxiredoxins are often encountered as double spots when analysed by two-dimensional electrophoresis. The quantitative balance between these two spots depends on the physiological conditions, and is altered in favour of the acidic variant by oxidative stress for all the peroxiredoxins we could analyse. Using HeLa cells as a model system, we have further analysed the two protein isoforms represented by the two spots for each peroxiredoxin. The use of selected enzyme digestion and MS

demonstrated that the acidic variant of all the peroxiredoxins analysed is irreversibly oxidized at the active-site cysteine into cysteine sulphinic or sulphonic acid. Thus, this acidic variant represents an inactivation form of the peroxiredoxins, and provides a useful marker of oxidative damage to the cells.

Key words: cysteic acid, mass spectrometry (MS), post-translational modification, proteomics.

INTRODUCTION

Organisms living under aerobic conditions need to protect themselves against the damage caused by reactive oxygen species (O_2^- , H_2O_2 , $OH^\bullet$), arising from the incomplete reduction of oxygen during cellular respiration or exposure to external agents such as light, ionizing radiations or some redox drugs [1,2]. These reactive oxygen species can damage various components of living cells such as unsaturated lipids (giving rise to deleterious organic peroxides), proteins or nucleic acids. To counter these deleterious processes, cells present various protective systems, either repairing the damage (e.g. DNA repair enzymes) or destroying the reactive oxygen species. Among the latter enzymes, peroxiredoxins 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 [3], and can contain another conserved cysteine [4,5]. The activated cysteine is oxidized to cysteine sulphenic acid [3] or to a disulphide [6] when a peroxide is reduced. In order to complete the enzymic catalytic cycle, the peroxiredoxins are then reduced back to their active thiol form, for example by the thioredoxin/thioredoxin reductase system for 2-Cys peroxiredoxins [6,7].

The peroxiredoxins are a growing and ubiquitous protein family. In addition to the classical cytosolic peroxiredoxins, named Prx1 and Prx2 [8] (but a variety of other names are also encountered), a third isoform, Prx3 (also named AOP or SP22), is present in mitochondria. Other peroxiredoxins have been described more recently, and microsomally secreted [9], peroxisomal [10] and chloroplastic [11] isoforms are now known, in addition to 1-Cys Prx [12].

Whereas the primary catalytic activity of peroxiredoxins is well characterized, they also seem to play a role in many physiological processes, including adaptation to oxygen [13], atherosclerosis [14], cancer transformation [15] and cell differentiation [16]. In this last study, Prx2 was shown to be induced

early during erythroid differentiation, and appeared as two distinct protein forms in mature red blood cells. This was correlated to the oxidative stress occurring in red blood cells, attributed itself to the large quantities of haem present in the cells. Two distinct forms of Prx3 were also detected in mitochondria [17]. Interestingly enough, the proportions of the two forms varied with the redox status of the cells, with the relative amount of the acidic form increasing with oxidative conditions. It must also be mentioned that forms of Prx2 and Prx3 oxidized on the active-site cysteine have also been described previously [18,19]. We thus suspected that the acidic form of peroxiredoxins could correspond to an oxidatively modified form, while the basic form could correspond to the normal form of peroxiredoxins. We have thus tried to demonstrate this hypothesis for several peroxiredoxins, using two-dimensional (2D) gel electrophoresis to separate the peroxiredoxin forms and MS to characterize them in detail.

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 % foetal calf serum. Oxidative stress was induced by adding to the culture medium 75–150 μ M t-butyl hydroperoxide for 1–6 h.

Cells were then harvested by centrifugation, rinsed in PBS and resuspended in homogenization buffer (0.25 M sucrose, 10 mM Tris/HCl, pH 7.5, and 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 vol. (relative to the suspension volume) of 8.75 M urea, 2.5 M thiourea, 25 mM spermine base and 50 mM dithiothreitol (DTT). After 1 h at room temperature, the extracts were ultracentrifuged (30 min at 200 000 g). The supernatant was collected and the protein was

Abbreviations used: AspN, peptidyl-Asp metalloendopeptidase; 2D, two-dimensional; DHAC, di-hydrogen ammonium citrate; DHAP, 2,6-dihydroxyacetophenone; DTT, dithiothreitol; ESI, electrospray ionization; MALDI, matrix-assisted laser-desorption ionization.

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determined by a Bradford assay, using BSA as a standard. Carrier ampholytes (0.4% final concentration) were added and the protein extracts were stored at -20°C .

2D electrophoresis

2D electrophoresis was performed with immobilized pH gradients for isoelectric focusing. Home-made linear pH 4–8 or pH 4–12 gradients were used [20], and prepared according to published procedures [21]. Immobilized pH gradient strips were cut with a paper cutter, and rehydrated in 7 M urea, 2 M thiourea, 4% CHAPS and 0.4% carrier ampholytes (pH 3–10 range), containing either 20 mM DTT (pH 4–8 gradients) or 5 mM Tris cyanoethyl phosphine (purchased from Molecular Probes; for pH 4–12 gradients) [22]. The protein sample was mixed with the rehydration solution in the case of pH 4–8 gradients, or cup-loaded at the anode for pH 4–12 gradients. Isoelectric focusing was carried out for a total of 60 000 Vh. After focusing, the strips were equilibrated for 2×10 min in 6 M urea, 2% SDS and 125 mM Tris/HCl, pH 7.5, containing either 50 mM DTT (first equilibration step) or 150 mM iodoacetamide (second equilibration step) [23]. The equilibrated strip was loaded on the top of a 10% polyacrylamide gel, and submitted to SDS/PAGE at 12 W/gel. After migration, the gels were stained with either silver [24] or colloidal Coomassie Blue [25].

MS

In-gel digestion

Spot digestion was performed essentially as described previously [26]. Stained protein spots or bands were excised and shrunk in 1 ml of 50% ethanol for 2 h. Each gel slice was cut into small pieces with a scalpel, washed with 100 μl of 25 mM NH_4HCO_3 and dehydrated with 100 μl of acetonitrile. This operation was repeated twice. Gel pieces were completely dried with a Speed Vac before enzymic digestion. The dried gel volume was evaluated and 3 vol. of 12.5 ng/ μl peptidyl-Asp metalloendopeptidase from *Pseudomonas fragi* (AspN; EC 3.4.24.33; Roche Diagnostics, Mannheim, Germany) in 25 mM NH_4HCO_3 (freshly diluted) was added. The digestion was performed at 35°C overnight. The gel pieces were centrifuged and 5 μl of 25% water/70% acetonitrile/5% formic acid was added. The mixture was sonicated for 5 min and centrifuged. The supernatant was recovered and the operation was repeated once. For matrix-assisted laser-desorption ionization (MALDI) MS analysis, the supernatant volume was reduced under nitrogen flow to 4 μl , 1 μl of water/5% formic acid was added and 0.5 μl of the mix was used for the analysis. For nano electrospray MS the sample extraction volume was adjusted to 20 μl and the supernatant was desalted using a ZipTipC18 pipette (Millipore, Bedford, MA, U.S.A.). For microbore liquid chromatography coupled online with multiple-stage tandem MS, the supernatant solvent was completely evaporated in order to remove all acetonitrile from the sample. Then, 5 μl of water/5% formic acid was added and injected in the HPLC system.

MALDI MS

For MALDI MS, mass measurements were carried out on a Bruker BIFLEX IIITM MALDI-time-of-flight mass spectrometer equipped with SCOUTTM High Resolution Optics with X-Y multisample probe and gridless reflector. This instrument was used at a maximum accelerating potential of 20 kV (in positive mode) or -20 kV (in negative mode) and was operated in reflector mode. A saturated solution of α -cyano-4-hydroxycinnamic

acid in acetone was used as a matrix. A first layer of fine matrix crystals was obtained by spreading and fast evaporation of 0.5 μl of matrix solution. On this fine layer of crystals, a droplet of 0.5 μl of aqueous formic acid (5%) solution was deposited. Afterwards, 0.5 μl of sample solution was added and a second 0.2 μl droplet of saturated matrix solution (in 50% water/50% acetonitrile) was added. The preparation was dried under vacuum. The sample was washed one to three times by applying 1 μl of aqueous formic acid (5%) solution on the target and then flushed after a few seconds. In negative mode, formic acid was removed from the aqueous solution and in order to improve the signal/noise ratio we also used another matrix containing 2,6-dihydroxyacetophenone (DHAP) together with di-hydrogen ammonium citrate (DHAC) [27]. In positive mode, internal calibration was performed with angiotensin, substance P, bombesin and corticotropin ('ACTH'), with respective monoisotopic masses of $m/z = 1046.542$, $m/z = 1347.736$, $m/z = 1620.807$ and $m/z = 2465.199$. In negative mode, the calibration was performed in external mode with the same peptides as before with monoisotopic masses of $m/z = 1044.542$, $m/z = 1345.736$, $m/z = 1618.807$ and $m/z = 2463.199$ respectively. Monoisotopic peptide masses were assigned and used for database searches.

The MS-FIT program from the ProteinProspector package (University of California at San Francisco) was used for database searching (<http://prospector.ucsf.edu>). All human proteins (taxonomy: human) present in the SwissProt database were taken into account without any pI and molecular mass restrictions. The peptide mass error was limited to 100 p.p.m., one miss cleavage might be accepted and no amino acid substitutions were allowed.

Nano electrospray ionization (ESI) tandem MS

The remaining tryptic mixture was used for nano ESI tandem MS. After MALDI MS analysis, the sample extraction volume was adjusted to 20 μl and the supernatant was desalted using a ZipTipC18 pipette. Tandem mass spectra in positive mode were acquired either on an Esquire 3000+ (Bruker-Daltonik, Bremen, Germany) or on a Q-TOF2 (Micromass, Altrincham, Cheshire, U.K.). Both instruments were equipped with a nano-spray ion source. A 5 μl sample volume was loaded in a gold/palladium-coated nanospray capillary (Protona, Odense, Denmark), previously rinsed with 5 μl of water/acetonitrile/formic acid (45:45:10, by vol.).

On the Esquire 3000+ instrument, the voltage applied to the counter electrode was set to 600 V. The voltage on the metal-glass capillary interface was optimized at 140 V and the voltage applied to skimmer 1 was set at 40 V. For tandem MS experiments, the precursor ions were fragmented by applying a resonance frequency on the end-cap electrodes (peak-to-peak amplitude 1.0 V) matching the frequency of the selected ions. Fragmentation of the precursor ions occurred in the ion trap due to collisions with helium buffer gas (pressure 5 mPa). Scanning was performed in the standard resolution mode at a scan rate of 13 000 m/z per s. A total of 50 scans were averaged to obtain a mass spectrum. Calibration of the ion trap analyser was performed by using the multiply-charged ions produced by the same peptide mixture previously indicated.

On the Q-TOF2 instrument, the tip of the nanospray capillary was set to a potential of about 1500 V and the extraction cone voltage (Vc) was optimized at 30 V. The MS data were acquired from $m/z = 50$ to $m/z = 850$. The m/z scale has been calibrated by using the synthetic PolyAlanine ions. Fragmentation of precursor was performed by collision with argon gas. The

Figure 1 2D electrophoresis of whole-cell extracts (acidic and neutral proteins)

Whole-cell extracts prepared from HeLa cells cultured under control conditions (**A**) or treated for 2 h with 75 μ M butylperoxide (**B**) were analysed by 2D electrophoresis. The pH gradient in the first dimension ranged from 4 to 8, thereby separating the acidic and neutral cellular proteins only. The proteins changing with oxidative stress were characterized by MALDI MS. The spot pairs corresponding to peroxiredoxins are shown by arrows.

collision energy was selected independently for each precursor ion by visual inspection of spectrum.

RESULTS

The starting point of this study lies in the fact that peroxiredoxins appear as doublet spots which are oxidative stress-sensitive. As can be seen in Figure 1 for the acidic peroxiredoxins and in Figure 2 for the basic Prx1, the more basic spot of the doublet is always more intense under control conditions, while the acidic spot becomes the most intense under strong oxidative stress conditions. MALDI MS confirmed that both spots of the doublet are indeed the same protein, as shown for example for Prx2 in Figure 3. This clearly showed that the difference between the two spots corresponding to the same protein is due to a post-translational process. Theoretical pI calculations and correlation with the pI values observed on the 2D gels suggested that the basic spot of the doublet corresponds to the native protein and that the acidic spot was a modified form. Furthermore, these calculations suggested that this modification corresponds to the addition of a single negative charge to the protein. Several modifications can match this charge change. However, sulphation is rather rare and phosphorylation was rather unlikely in this case. This latter hypothesis was supported by the fact that the Prx1 modification also corresponded to a single negative charge, while phosphorylation at pH 8 behaves as a double negative charge.

Furthermore, the first purification of bovine Prx3 showed that the protein, purified as a protease substrate, was indeed a modified form of the protein, with a cysteine turned into a cysteine sulphinic acid [18]. As this modification corresponded to an oxidation of the active-site cysteine, we inferred that the modification observed on the 2D gels also corresponded to an irreversible oxidation of the active-site cysteine into a cysteine sulphinic or a cysteine sulphonc acid.

In order to confirm this hypothesis, we used the fact that cysteine sulphinic and sulphonc acids, but not cysteine, are substrates for AspN from *P. fragi* [18,28]. Thus, if our hypothesis was correct, the AspN digestion profiles of the two forms should be different, as shown in Figure 4. Of special interest to us was the fact that cysteine oxidation should result after AspN digestion in the production of a conserved pentapeptide DFTFV (or DFTPV for 1-Cys Prx). Using electrospray MS and tandem MS, we looked for the presence of this peptide in the modified forms, but not in the native ones, where a much bigger peptide (over 2000 Da) was expected. We detected a m/z 628 peak in the MS spectra coming from the modified forms only, and this mass matched with that of the expected peptide. To confirm this identification, tandem MS was performed on this peptide and produced the expected DFTFV or DFTPV sequence, as shown in Figure 5. This peptide was observed for the modified forms of Prx1–Prx4 plus 1-Cys Prx, i.e. all the peroxiredoxins observed on the gel. This demonstrated that the modification was indeed the oxidation of the active-site cysteine into cysteine sulphinic or sulphonc

Figure 2 2D electrophoresis of whole-cell extracts (basic proteins)

The same analysis as in Figure 1 was performed, but a pH 4–12 gradient was used in the first dimension. This allowed analysis of basic proteins, including Prx1, which was identified by MALDI MS.

Figure 3 MALDI mass spectra of paired spots corresponding to the same peroxiredoxin

The MALDI mass spectra corresponding to the basic (A) and acidic (B) forms of Prx2 are shown. The peaks matching the predicted masses are shown with asterisks. The peptide matching the active-site-containing peptide (including cysteine modified with iodoacetamide) is indicated with an arrow.

acid. In order to be able to ascertain the modification, we tried to produce a peptide including the active-site cysteine, using either trypsin or endoproteinase Glu-C (V8 proteinase; EC 3.4.21.19) digestion. We were, however, never able to observe

the peptide encompassing the active site. This was probably due to the length and hydrophobicity of the expected peptide (≈ 3000 Da). Furthermore, the cysteine oxidation introduced a strong anionic charge to the modified peptide, thereby

decreasing dramatically its ionization efficiency in positive mode. We therefore tried to observe the complementary peptide release by the AspN digestion, i.e. the peptide beginning with the modified cysteine and ending just before the next aspartic acid in the sequence. Here again, the negative charge carried by the modified cysteine was expected to create problems in ionization and/or fragmentation, at least in the positive mode. This was indeed the case and the complementary peptides could not be observed in positive mode, neither by MALDI MS nor by ESI MS. When we switched to the negative mode in MALDI MS, however, we could observe the complementary peptides for Prx1, Prx2 and Prx3, as shown in Figure 6. This allowed us to further demonstrate the reality of the cysteine oxidation and also allowed us to determine the oxidation state of the cysteine. It could be seen that the same spot contains both the sulphinic and the sulphonic acid forms, which are electrophoretically indistinguishable. It must be mentioned here that the sulphinic form could be observed only with the dedicated matrix we have used (DHAP/DHAC) and not with more classical and acidic matrices (e.g. α -cyano-4-hydroxycinnamic acid). This was mostly due to the fact that the background was much lower with the DHAP-based matrix, enabling us to see the minor sulphinic form which is close to the detection threshold. This sulphinic form was not observed for other peroxiredoxins. This could be due to a real

In conclusion, the MS results obtained for the various peroxi-redoxins observed in this study are shown in Table 1. They allow us to conclude confidently that the basic spots correspond to the native forms or to DTT-reducible forms (i.e. thiol, disulphide or sulphenic acid), while the acidic spots correspond to DTT-resistant forms (i.e. sulphinic or sulphonic acids).

Peroxioredoxins are a growing family of proteins involved in the redox regulation of cells, together with peroxidases, catalase and superoxide dismutases. As redox regulators, they appear to play a role in key cellular processes, such as apoptosis control, redox signalling and kinase modulation. Consequently, the status of these proteins in the cell can alter significantly the cellular response to various stimuli. 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 centres which are very reducing, and therefore very

Figure 5 Tandem MS spectra of the DFTFV and DFTPV peptides

Top panel: the DFTFV peptide present in an AspN digest of modified peroxiredoxins was analysed and characterized by tandem MS, as described in the Materials and methods section. The interpretation of the spectrum is shown on the spectrum itself. This peptide was observed in modified Prx1–4. Bottom panel: the same analysis was performed for 1-Cys Prx, where a DFTPV peptide was expected.

sensitive to overoxidation, leading to the inactivation of the proteins. Interestingly, a modification in the peroxiredoxins could be detected by 2D electrophoresis in cells subjected to strong oxidative stress [17,29]. These papers have suggested that this oxidative stress-dependent modification is in fact due to the oxidation of the active, reducing site of the protein. In the case of peroxiredoxins, this active site is an activated cysteine with a low pK_a [30]. However, there has been no real dem-

onstration that the modified form observed on the 2D gels is indeed an overoxidized form. By using a combination of enzyme digestion and MS, we could demonstrate directly that the acidic variants of peroxiredoxins induced under oxidative stress are oxidatively inactivated forms of the protein. Interestingly enough, a single 2D spot usually contains both the sulphinic and sulphonic forms, which explains some discrepancies previously observed in the literature [18,19]. This implies in turn that the quantitative measurement of both the native and oxidized forms of peroxiredoxins is a measure of the oxidative status of the cell and/or the cell compartment under study. It must be mentioned, however, that measurement of the native form does not strictly reflect the real detoxification power of the cell or cellular compartment. Due to the reducing environment in which the protein extraction is made, all DTT-reducible forms will be converted into the free SH form. This means that the free SH, disulphide and cysteine sulphenic acid forms are all converted into the 'native' form during extraction. It has been mentioned that the sulphenic forms of the active cysteine are exceptionally stable for at least one peroxiredoxin [3]. However, this stability is due to special features of the native protein, including the absence of a free cysteine nearby. This is obviously not the case for other peroxiredoxins [4], in which the transiently oxidized intermediate is a disulphide bridge. As our protein extraction conditions use a high concentration of DTT in a denaturing environment, it can be safely postulated that the thiol, disulphide and sulphenic forms of the various peroxiredoxins are converted into the free thiol form. While the free SH form is the available active protein, the disulphide and sulphenic acid

Table 1 Summary of the MS results for the peroxiredoxins

Total peptide means the AspN digestion peptide encompassing the active site and calculated from the sequence. The N-terminal peptide is the short pentapeptide produced by the extra digestion at the oxidized cysteine, while the complementary peptide is the peptide beginning at the oxidized cysteine in this extra digestion (see Figure 4).

Spot	Total peptide	N-terminal peptide (628.3 Da)	Complementary peptide	Comments
Prx1 (normal)	No	No	No	
Prx1 (modified)	No	Yes	Yes	Sulphonic acid
Prx2 (normal)	Yes	No	No	
Prx2 (modified)	No	Yes	Yes	Sulphinic + sulphonic acids
Prx3 (normal)	No	No	No	
Prx3 (modified)	No	Yes	Yes	Sulphonic acid
Prx4 (normal)	No	No	No	
Prx4 (modified)	No	Yes	No	
1-Cys Prx (normal)	No	No	No	
1-Cys Prx (modified)	No	Yes (578.3 Da)	No

Figure 6 Negative MALDI spectrum of the complementary peptide containing the oxidized cysteine

The AspN digest of the native (norm.) and oxidized (ox.) forms of peroxiredoxins was analysed by MALDI MS operating in negative mode. It can be seen that the oxidized spot contains both the sulphinic acid (1480.883 m/z) and sulphonic acid (1496.839 m/z) forms. These peptides are of course absent in the native form.

forms represent transiently unavailable protein. In contrast, the DTT-resistant forms (i.e. the sulphinic and sulphonic derivatives) represent irreversibly inactivated forms. Consequently, while the measurements made by 2D electrophoresis are not sufficient to provide a precise idea of the real status of the peroxiredoxin defence line, they provide a good indication of the oxidative damage to cellular proteins and thus of oxidative stress.

Thanks to various subcellular localizations of peroxiredoxins, the parallel examination of their status via 2D electrophoresis provides a measure of the oxidative stress in the corresponding compartments. From our data with butyl peroxide, it can be seen that the secretory compartment (Prx4-containing) is less prone to oxidative stress than cytosol (Prx1-, Prx2- and 1-Cys Prx-containing) or mitochondria (Prx3-containing). While this observation is probably of moderate interest for a very broad-range agent such as butyl peroxide, it may be very interesting for more localized stresses (e.g. via drugs).

In addition, it must be mentioned that these inactivated forms are not encountered only in cells submitted to a strong experimental oxidative stress. These forms are naturally present for Prx3 in mitochondria extracted from cells or tissues [17,18,31], but also exist for other peroxiredoxins, for example in red blood cells [16]. Careful examination of the gels presented in this paper also demonstrate minor amounts of these oxidized peroxiredoxins. Thus, the balance between the native and oxidized forms of peroxiredoxins can be considered as a physiologically relevant indicator of oxidative stress. This prevalence of strongly oxidized forms, even in normal cells, can appear intriguing. However, we believe that this situation is due to the special catalytic cycle of peroxiredoxins. During this cycle, the destruction of the peroxide is concomitant with the oxidation of the peroxiredoxin in a transiently oxidized state (disulphide or sulphenic acid). This state appears to be critical, as the fate of the enzyme depends on the chemical species next entering the active site. If this species is a reducing one, e.g. reduced thioredoxin, the enzyme is reduced back to its original reduced form and is ready for destroying another peroxide molecule. However, if the species entering the transiently oxidized form is oxidizing, e.g. another peroxide molecule, then the transiently oxidized state is further oxidized to an irreversible state corresponding to a sulphinic or sulphonic acid. This stochastic mechanism explains why some oxidized peroxiredoxin can be found under normal conditions. It also explains why the oxidized form becomes dominant under oxidative stress conditions. Under such conditions, the peroxide concentration increases and the concentration of reduced thioredoxin decreases, being replaced by the disulphide form of thioredoxin. These two phenomena combine to increase the probability of overoxidation.

It must be mentioned at this stage that the stress conditions used in the present study are very intense, and lead to total cell death after 10 h of exposure to t-butyl hydroperoxide. t-Butyl hydroperoxide, being partly lipid soluble, it is able to attack polyunsaturated lipids and to give rise to a chain reaction producing both lipid peroxides and hydrogen peroxide. Thus the amount of total peroxides produced is far greater than the amount of t-butyl hydroperoxide. This explains why the peroxiredoxin defence line is overwhelmed by the peroxides generated by this treatment, although it is very likely that each peroxiredoxin molecule is able to destroy many peroxide molecules before it becomes inactivated. Good indirect evidence for this efficiency of the peroxiredoxin defence line lies in the data obtained with a less severe oxidative stressor such as glucose oxidase [19].

Last but not least, the method described here is expected to be of general use for the detection of cysteine overoxidation to

sulphinic or sulphonic acid forms. In former studies, the cysteine sulphonic form of Prx2 was detected in a peptide with several missed trypsin cleavage sites [19]. By definition, such an event cannot be expected to give reliable characterization of cysteine overoxidation. In contrast, the strategy used here has been devised for more general application. When a spot offset in terms of pI on 2D gels suggests overoxidation at a cysteine position, the AspN digestion strategy should produce indirect but strong evidence by the appearance of a cleavage peptide in the modified protein only, just before the oxidized cysteine. The data in Table 1 exemplify the specificity of this approach and confirm the fact that AspN does not produce any cleavage at unoxidized cysteines. Due to the relatively low number of cysteines normally encountered in proteins, the number of possible cleavage peptides is low, so that the results are easily interpretable. Depending on the level of confidence required, simple MS or tandem MS can characterize the cleavage peptide. It may be necessary, however, to use various MS ionization types (ESI or MALDI) to be sure of visualizing the cleavage peptide. As to the complementary peptide, which in turn allows characterization of the oxidation state, the negative charge imparted by the oxidized cysteine makes its ionization rather difficult in the classical positive mode. Its detection may therefore require more exotic MS modes, as illustrated in this paper.

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