CHAPTER 20

REDOX PROTEOMICS – A ROUTE TO THE IDENTIFICATION OF DAMAGED PROTEINS

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Abstract: The "oxygen paradox" is that molecular oxygen is both essential for aerobic life but also can be toxic to cells largely because of the effects of oxygen-derived species collectively called "reactive oxygen species" (ROS) such as the hydroxyl radical. Cells have evolved elaborate defences against ROS but if these defences are decreased (as in ageing) or if the ROS challenge becomes too great (as in toxicity), a state of oxidative stress (OS) ensues. Proteins are the principal targets of ROS and redox proteomics uses proteomics tools to study redox-based effects on the cell's protein complement. We have long used bivalve molluscs as sentinel organisms for study of pollution effects in estuaries, in particular looking at effects on stress-response proteins such as antioxidative enzymes, detoxification enzymes and heat shock proteins. Stress-response proteins are often affected by more than one stressor so these targets are likely to be of interest in other stress contexts. We are now applying redox proteomics approaches to study stress effects in bivalves. We detect carbonylation, glutathionylation, ubiquitination, effects on disulphide bridge patterns and changes in protein expression signatures in a range of electrophoresis formats. The effects are tissue- and treatment-specific. We find that many proteins targeted by OS are associated with either actin or protein disulphide isomerase. Many of the tools we use are species-independent and are appropriate for other stress scenarios.

Keywords: bivalve; proteome; oxidative stress; redox; protein oxidation; 2D SDS–PAGE; hydroxylation; glutathione; carbonyl; racemization, ubiquitin

Introduction

The "oxygen paradox" is that molecular oxygen is both essential for life but can also cause toxicity via formation of reactive oxygen species

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(ROS; Holland, 1994; Halliwell, 1996). This may be mediated by a variety of xenobiotics (e.g. metals, polyaromatic hydrocarbons, quinones; Valavanidis et al., 2006). ROS can interact rapidly and quantitatively with lipids, DNA and proteins with rate constants approaching diffusion-controlled values (~10^8–10^{10} M^{-1} s^{-1}). Some ROS are free radicals which are produced by mitochondrial electron transport and in inflammation. Cells evolved complex defences against ROS at an early stage of aerobic evolution. These defences (Fig. 1) include antioxidant enzymes, thioredoxin-like proteins and natural antioxidants such as glutathione (GSH) and vitamin E (Masella et al., 2005). The reduced state of the cell interior is ultimately maintained by a reduced:oxidized glutathione ratio (GSH:GSSG) maintained in the range 30–100 at the expense of NADPH oxidation; a low ratio can trigger apoptosis (Mates and Sanchez-Jimenez, 2000). Much research has focused on oxidative stress, especially from the perspective of cell signalling (Bigelow and Squier, 2005), ageing (Levine and Stadtman, 2001), and the etiology of human disease (Halliwell and Gutteridge, 2007).

Proteomics studies the total complement of cell proteins or of defined protein subsets; sub-proteomes (He and Chiu, 2003). It uses high-throughput techniques to detect change in level/status of specific

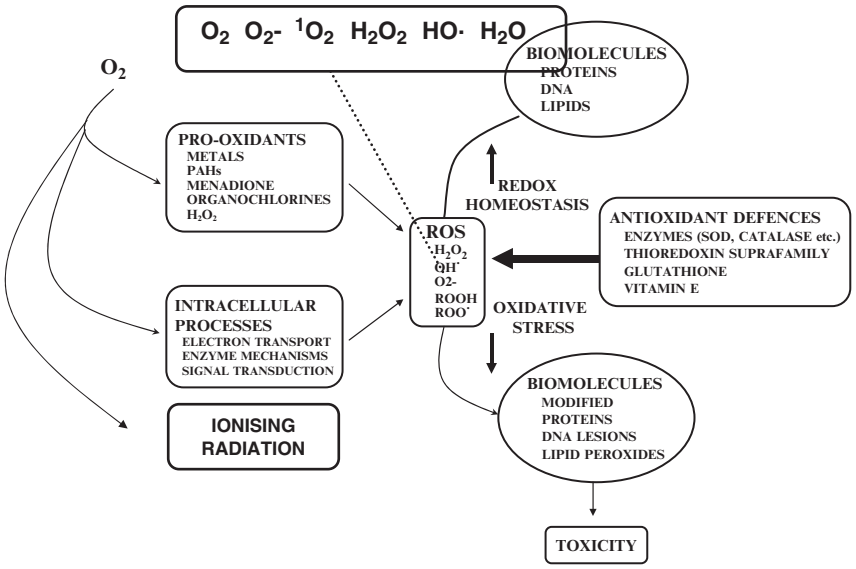


Fig. 1. Oxidative stress arises when the balance between ROS and cellular defences is upset. This can happen if the levels of ROS increase or if defences decrease as occurs in ageing

proteins. Two dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2D SDS–PAGE) is especially popular for these studies. Proteins are first separated in a pH gradient based on pI differences followed by a second dimension based on differing Mr (O'Farrell, 1975; Görg, 1991). Oxidative stress can cause change in levels of specific proteins detectable by protein staining and image analysis (Patton, 2002). Similarly, redox-based processes altering the pI or Mr of proteins (e.g. charge isomerization, protein backbone cleavage, crosslinking) are detected as altered 2D SDS–PAGE spots. In practice, a surprisingly small number of changes to the absolute amounts of individual proteins is usually observed. Notwithstanding this, redox-based modification of proteins in certain proteins is often quite extensive. This article describes how 2D SDS–PAGE separations can be probed to detect redox-based protein modification and thus to identify proteins targeted by oxidative stress.

Biological Targets of Reactive Oxygen Species

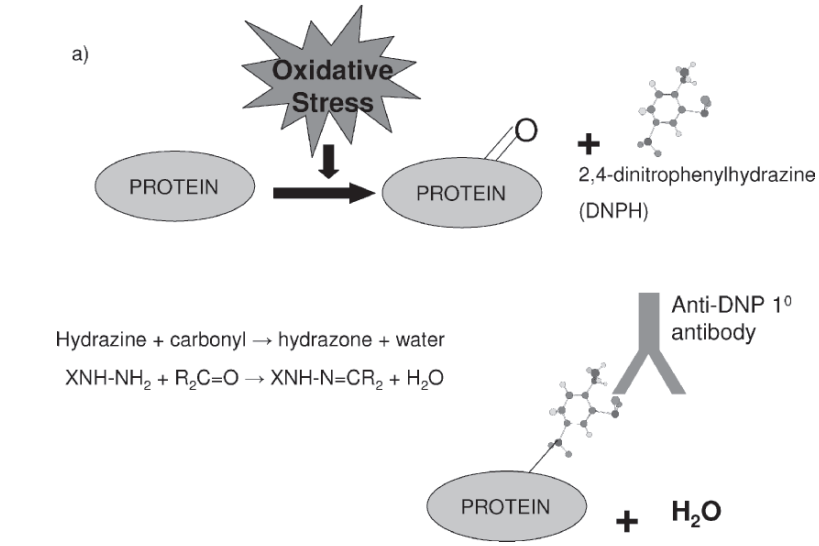
Molecular oxygen may be reduced to water by successively being converted into ROS such as H_2O_2 and the hydroxyl radical ($HO^{\cdot}$) in a strongly thermodynamically favoured process ($\Delta G \sim -110$ kCal/Mol). Individual ROS may react at differing rates with a range of biological targets (Davies, 2005). For example, the most important ROS in Biology, $HO^{\cdot}$, reacts with cysteine 1,000 times faster than with glycine. Conversely, a given biological target such as methionine can react at quite different rates with individual ROS. Fast reacting ROS are therefore thought to react with proteins rather non-specifically while slow reacting ones have the potential to react more specifically. Proteins absorb approximately 70% of ROS in cells while nucleic acids and lipids provide other important targets. Reaction with ROS can change the structure of proteins in a number of ways including backbone fragmentation, hydroxylation and carbonylation of amino acid residue side-chains, glutathionylation and effects on disulphide bridges. Thus, oxidation introduces considerable structural diversity into the proteome. Redox proteomics takes advantage of these structural changes to identify specific effects on individual proteins even where the absolute amount of the protein may be unchanged. The following section outlines some experimental strategies that have proven especially useful for exploring the complex interactions of ROS with proteins. These approaches offer potential to radiation biologists since it is known that exposure to radionuclides causes oxidative stress.

Probing the Proteome for Redox-Based Modification

Several workers have pioneered an approach to environmental proteomics involving making comparisons between matched control and test samples to discover up- or down-regulated proteins followed by spot identification by mass spectrometry methods (Shepard et al., 2000; Bradley et al., 2002; Romero-Ruiz et al., 2006). The protein expression signature acts as a "fingerprint" of the proteome in the given set of experimental circumstances. In practice, a very small number of proteins are found to change in most field or laboratory exposure studies. A limitation of this classical proteomics approach is that it can be difficult to identify proteins from organisms that are not well represented in sequence databases. This is especially true of sentinel species widely used in ecotoxicology such as mussels and clams. A possible strategy for overcoming this limitation in the context of redox proteomics is to use molecular probes capable of identifying biologically widespread chemical structures. Commercially-available antibodies provide a very convenient means of detecting features such as glutathionylation, ubiquitination and carbonylation in proteins separated by 2D SDS–PAGE. They are also useful in selecting sub-proteomes by immunoprecipitation. Alternatively, some stress-response proteins are sufficiently structurally conserved that they can be detected with antibodies raised to their mammalian orthologues. Good examples are the heat shock proteins, GSH transferases and ubiquitin. Redox-based lesions which we have found especially useful include carbonylation, glutathionylation, effects on disulphide bridges and ubiquitination.

CARBONYLATION

Many amino acid side-chains are readily converted to aldehyde or ketone groups on exposure to ROS, a process called carbonylation (Levine and Stadtman, 2001). This is an irreversible modification leading usually to inactivation, crosslinking and turnover of the damaged protein. Its irreversibility makes carbonylation an especially useful measure of redox-based damage because the lesion withstands extraction and proteomic separation (Fig. 2). Protein carbonyls react quantitatively with hydrazines to form hydrazones (Levine et al., 1990). After 2D SDS–PAGE, proteins can be transferred to nitrocellulose membranes and carbonylated proteins labelled with 2, 4-dinitrophenyl hydrazine (hydrazine-DNP) followed by detection with anti-DNP (England and Cotter, 2004). As mentioned earlier, proteins derivatized with hydrazine-DNP can be immunoprecipitated with anti-DNP prior to 2D SDS–PAGE (McDonagh et al., 2006). Carbonylation is mainly



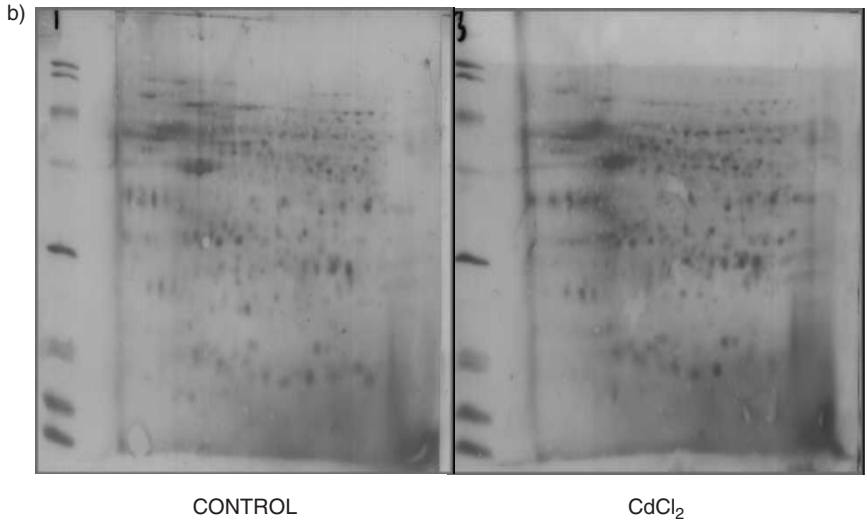


Fig. 2. Protein carbonyls. (a) Proteins can be carbonylated by oxidative stress. 2, 4-Dinitrophyl hydrazine (hydrazine-DNP) reacts with carbonyl groups to form hydrazone-DNP, which is detectable with anti-DNP. (b) 2D SDS–PAGE separation of *Mytilus edulis* extracts of gills dissected from animals exposed to $CdCl_2$ and stained with fast stain.

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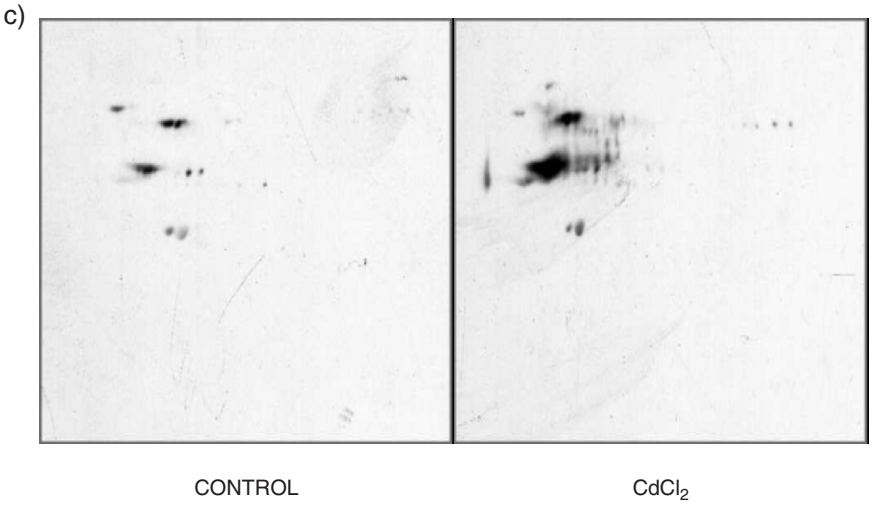

Fig. 2. (continued) (c) The same separation probed with anti-DNP. Note that only some proteins are carbonylated

caused by the HO· radical and this has been suggested to be rather non-specific (Levine and Stadtman, 2001). However, recent studies have found selective protein carbonylation. In mussels and clams we have found that exposure to pro-oxidants causes extensive carbonylation of specific proteins against a largely unchanged proteomic background (Dowling et al., 2006; McDonagh and Sheehan, 2006; McDonagh et al., 2006). Intriguingly, the effect does not depend on protein quantity in that some abundant proteins are not carbonylated at all while some low-abundance proteins are heavily carbonylated. We also find that the effect is surprisingly rapid (appearing within 2 hours of exposure to pro-oxidants) and shows both tissue- and treatment-specificity. Quantitative information can be obtained by measuring the total staining intensity of spots and comparing tests to controls. Alternatively, image analysis software such as PDQuest facilitates spot counting and matching.

GLUTATHIONYLATION

GSH can form mixed disulphides with sulphydryl groups of proteins that are key targets of oxidation (Dalle-Donne et al., 2001). Unlike most other lesions, this is a reversible modification. Using 1D SDS–PAGE followed by immunoblotting with anti-GSH, we have identified actin as a key target for glutathionylation (McDonagh et al., 2005). Normally, the second dimension of 2D SDS–PAGE is run under reducing conditions which would reduce

mixed disulphides thus losing the GSH from its target protein. This can be avoided by running the second dimension under reducing conditions or else by immunoprecipitating with anti-GSH before 2D SDS–PAGE and silver staining (McDonagh et al., 2006).

UBIQUITINATION

Ubiquitin is a highly conserved protein with an approximate mass of 8.5 kDa. It plays a key role in targeted turnover of short-lived proteins in cell (Fig. 3). Oxidatively damaged proteins have been reported as being cleared via ubiquitinylation followed by digestion in the 20S core of the 26S proteasome in the cytosol and nucleus (Davies, 2001; Marques et al., 2004; Petroski and Deshaies, 2005; Friguet, 2006) and by the Lon protease in the mitochondrial matrix (Bota et al., 2002). Commercially-available antibodies to ubiquitin and polyubiquitin facilitate identification of ubiquitinated proteins in blots of 2D SDS–PAGE separations (Fig. 3) (McDonagh and Sheehan, 2006). In mammalian systems, carbonylated proteins have been

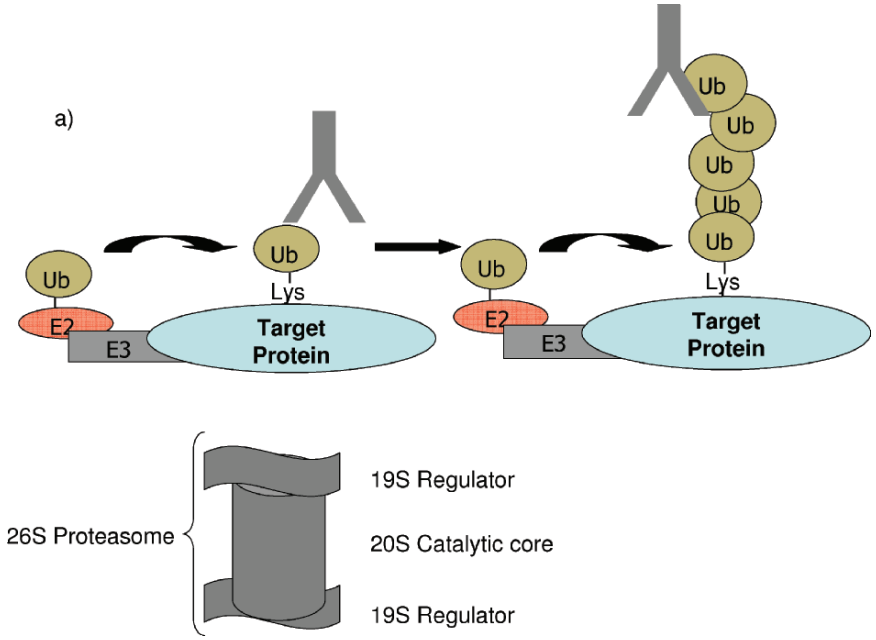

Fig. 3. Protein ubiquitinylation. (a) Proteins can be labelled with ubiquitin via the ubiquitin proteolytic pathway leading to polyubiquitination. Anti-ubiquitin reacts specifically with ubiquitinated proteins

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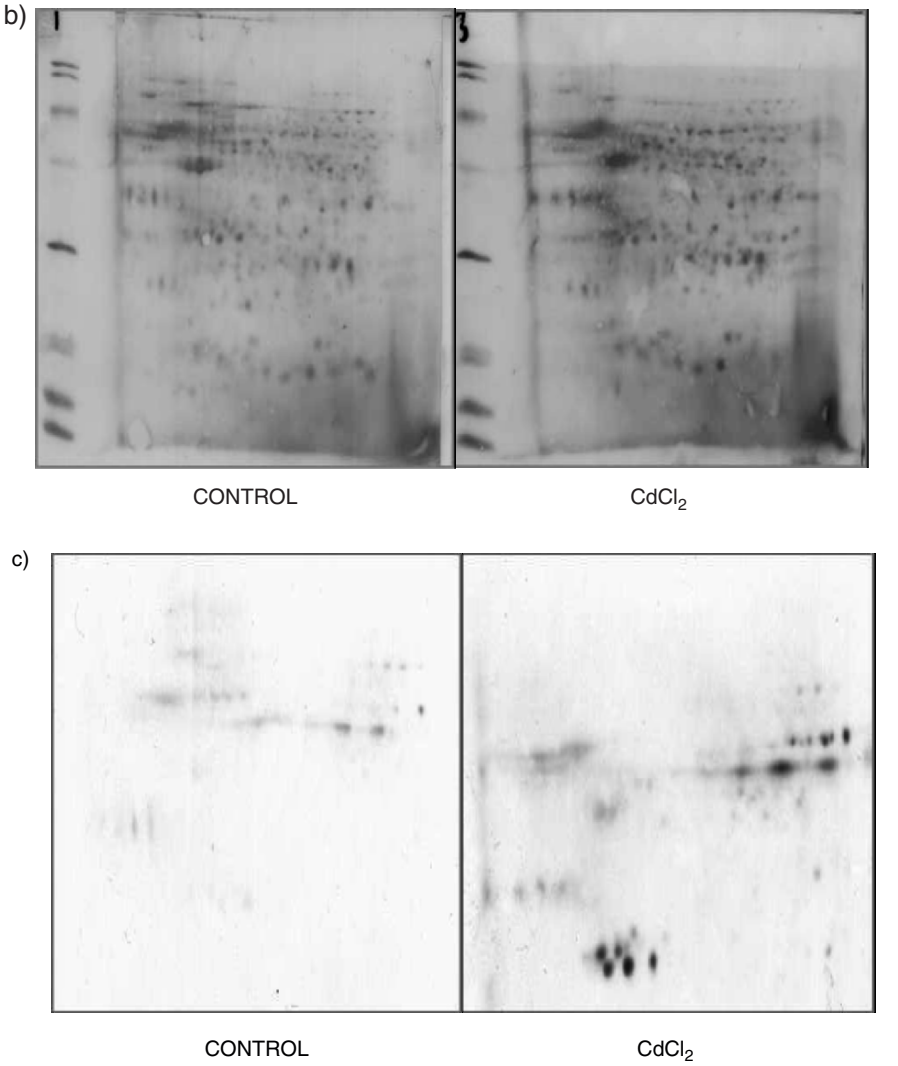

Fig. 3. (continued) (b) 2D SDS–PAGE separation of *Mytilus edulis* extracts of gills dissected from animals exposed to $CdCl_2$ and stained for total protein with fast stain. (c) The same separation probed with anti-ubiquitin. Note that ubiquitination increases with exposure to pro-oxidant but that the pattern is distinct from that for carbonylated proteins (Fig. 2)

reported as good substrates for the 20S proteasome core without necessarily passing through the ubiquitin proteasome pathway (Marques et al., 2004). Our recent work with oxidatively stressed molluscs suggests that there may not always be a correlation between carbonylation and ubiquitination (McDonagh and Sheehan, 2006).

PROTEIN THIOLS

Cysteines are key targets for ROS and it is now being increasingly realized that this is important in signal transduction in cells. Protein thiols do not react with oxidants at their biological concentrations (Eaton, 2006). However, thiol pKa values can be lowered by their surrounding environment which makes individual cysteines especially redox-sensitive. Thiol modifications include direct oxidation (to sulphenic, sulphinic and cysteic acids), formation of mixed disulphides (e.g. with GSH, cysteine and homocysteine) and formation of intra/intermolecular protein disulphides (Davies, 2005; Eaton, 2006). Oxidized variants of –SH generally do not react with thiol-specific reagents such as maleimides, iodoacetic acid, iodoacetamide and thiosulphates. Thus it is possible to compare samples using labels covalently attached to these chemical functionalities. Examples include biotin (selectable by binding to avidin) (Eaton et al., 2002), polyethylene glycol (increases Mr) (Marques et al., 2004), fluorescein (immunodetectable with anti-fluorescein) (Baty et al., 2002) and radionuclides (Brooker and Slayman, 1983). A rare reversible redox modification is represented by nitrosothiol (SNO) resulting from reaction of specific thiols with nitric oxide. This can be detected with the "biotin switch" assay which depends on the fact that ascorbate specifically reduces SNO but not disulphides (Jaffrey et al., 2001). Reaction with N-ethyl maleimide-biotin allows specific labelling of new –SH groups with subsequent selection on avidin columns and 2D SDS–PAGE (Fig. 4). Thiol-specific chemical functionalities can also be used to detect available –SH groups in proteins. Iodoacetamide-fluorescein labels available –SH (i.e. those not involved in disulphides) and these proteins can be identified by immunoblotting with anti-fluorescein. Since –SH groups are readily oxidized to species which do not react with iodoacetamide, comparisons of oxidized with control separations reveal proteins containing susceptible cysteines. A modification of this approach involves first treating the proteins with NEM followed by reduction with DTT and treatment with iodoacetamide-fluorescein which selects for cysteines involved in disulphide bridges. Another way of probing effects of oxidative stress on disulphide bridge patterns of proteins is to use diagonal gels (McDonagh and Sheehan, 2006). A 1D SDS–PAGE separation is performed first under non-reducing conditions (Fig. 5). The track of proteins is excised, reduced and run orthogonally on a reducing gel. Proteins with no disulphide bridges run along a diagonal. Proteins with intrachain disulphides run above the diagonal (since their structure is now more "open") while proteins with interchain disulphides run below the diagonal. Thus, a characteristic pattern of disulphide-bridged proteins is generated. An elaboration of this technique is to probe the separation for oxidative lesions such as protein carbonyls.

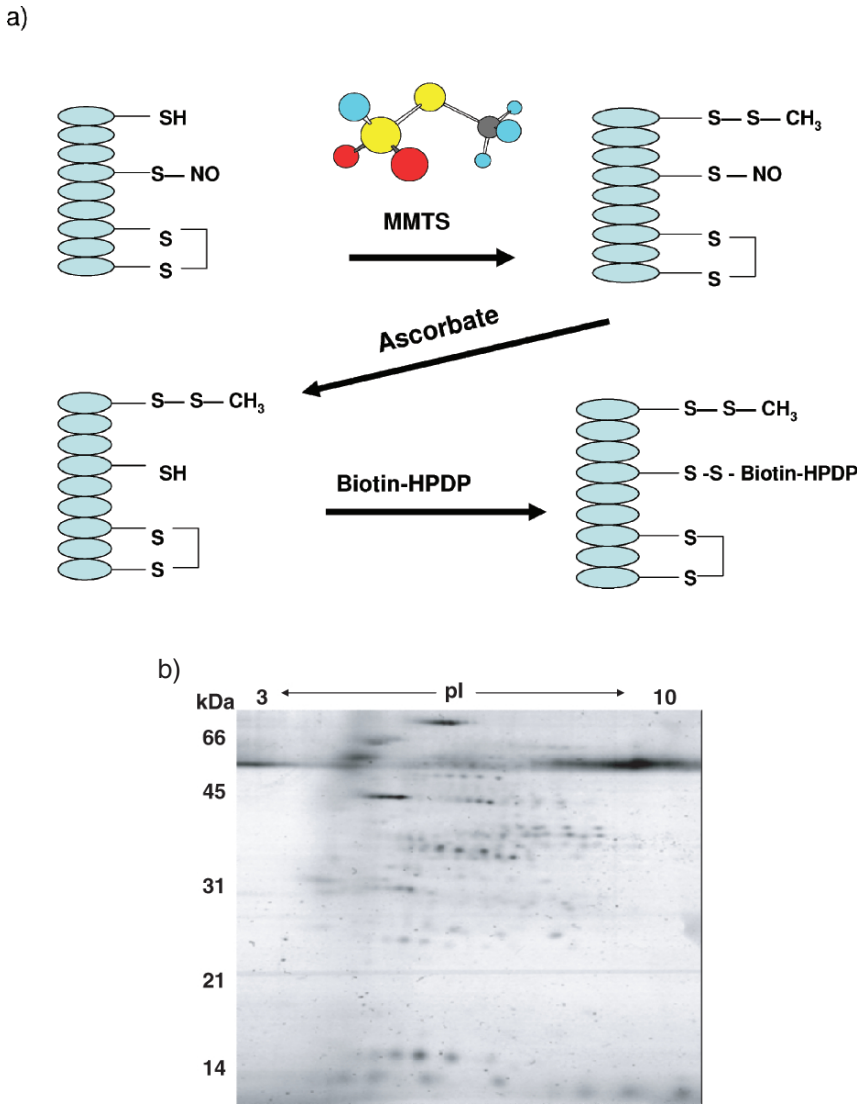

Fig. 4. The Biotin switch assay. (a) Ascorbate specifically reduces -SNO to SH without reducing disulphides. (b) Silver-stained 2D SDS–PAGE gel of streptavidin-agarose purified proteins from rat kidney medulla, following biotin switch assay

Future Perspectives

Several bottlenecks prevent full exploitation of redox proteomics in environmental science (Dowling and Sheehan, 2006). Chief amongst these is lack of sequence information to facilitate protein identification. As this

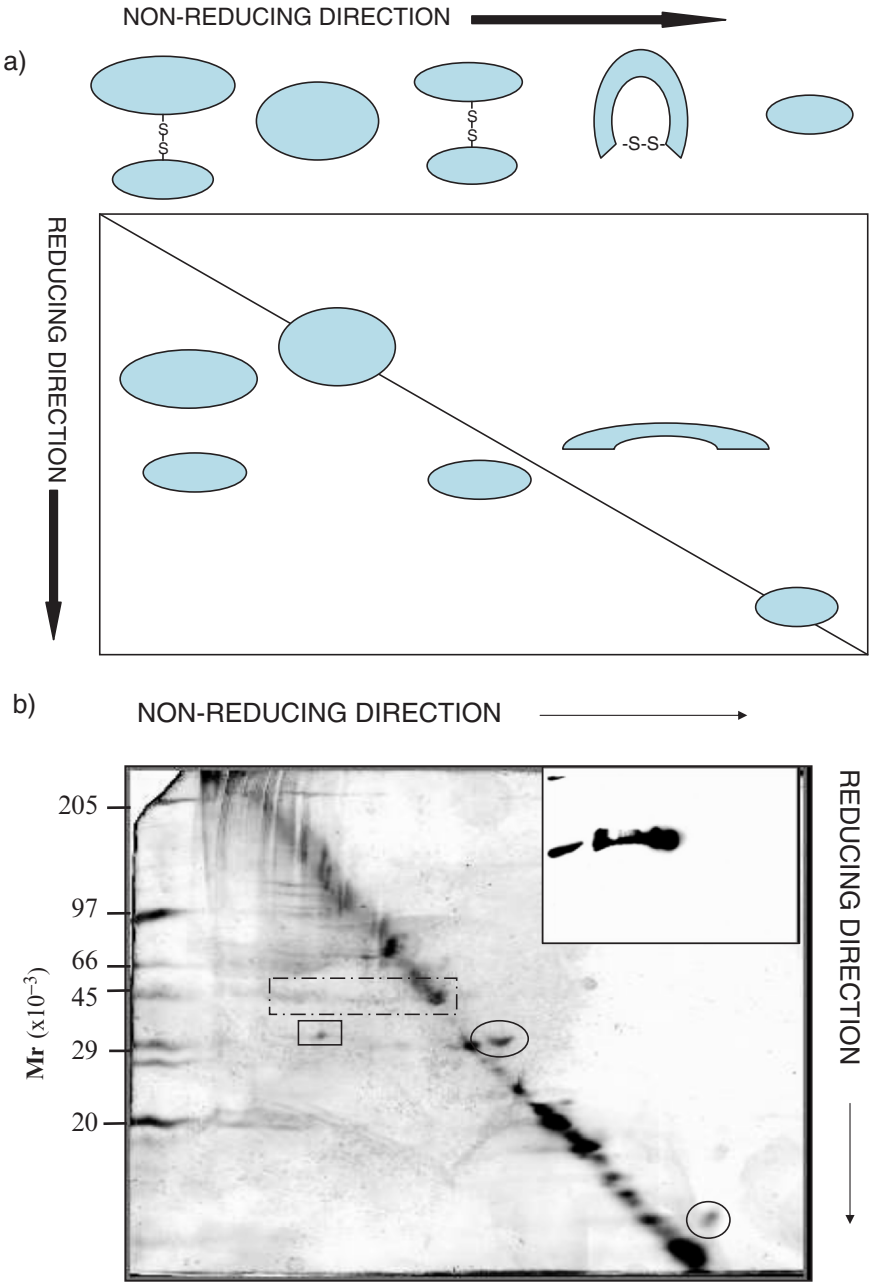

Fig. 5. Diagonal electrophoresis in study of protein disulphides. (a) A gel track from a non-reducing gel is excised, exposed to reducing buffer and laid orthogonally on a reducing SDS–PAGE gel. Proteins lacking disulphides migrate to a diagonal position as they electrophorese identically in reducing and non-reducing conditions. Proteins with interchain disulphides migrate as separate polypeptides below the diagonal while those with intrachain disulphides migrate more slowly under reducing conditions and thus appear above the diagonal. (b) Separation of extract of gill from *Mytilus edulis* stained with silver. Inset shows an immunoblot with anti-actin corresponding to dashed box

deficiency is unlikely to be remedied in the short term, we suggest that "species-independent" chemical-based approaches as outlined here may provide a route around this bottleneck. It can also be difficult to distinguish specific causes of effects observed. For example, environmental samples notoriously contain complex cocktails of pollutants. Thus, there is a continuing need for well-designed dose-response experiments under controlled laboratory conditions. Redox proteomics allows study at the level of organelle, cell, tissue and whole organism and has a dynamic range covering three orders of magnitude. It therefore represents a powerful set of techniques for the exploration of oxidative and other stress scenarios.

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