Thiol modifications in a snapshot

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The oxidation state of proteins in cells can be analyzed with a simple labeling method.

Taking a picture of the oxidation state of proteins *in vivo* involves substantial technical challenges that are similar to what photographers call the red-eye problem. Just as it is difficult to photograph someone without distorting their image—as seen in eyes glowing red from the light of a flash—sample processing can easily cause artifactual oxidation or reduction of proteins in cells. Moreover, modifying a protein with a thiol-specific probe changes its behavior, complicating the task of determining what fraction of a given protein is modified. In a recent issue of *PLoS Biology*, Leichert and Jakob circumvent these difficulties with a general method for visualizing thiol modifications that allows for the accurate and simultaneous quantification of the *in vivo* thiol-disulfide status of hundreds of cellular proteins[1].

Cysteines are both exceptionally useful to cells and exceptionally vulnerable. The reactivity of free thiol groups makes them useful in the active site of enzymes and allows proteins to coordinate metals. When two cysteines in a protein meet, a stabilizing disulfide bond is often the result. Yet the reactivity of cysteines often makes them vulnerable to oxidative damage[2]. Reactive oxygen species produced by environmental toxins or by normal metabolism can act to either destroy or regulate the function of cysteine-containing proteins.

Knowledge of the thiol status of proteins *in vivo* would allow one to identify which proteins contain structural disulfides, which cysteines are oxidized as part of their catalytic mechanism and which cysteines are vulnerable to oxidative damage. Analyses of this kind could, for instance, lead to insights into the cellular targets of the oxidative aging process[2].

Determining the thiol-disulfide status of a single purified protein can be done relatively easily by reacting the protein with a thiol-specific probe. The probe changes the properties of the protein, allowing the modified and unmodified proteins to be separated and the extent of the modification to be determined. But the analogous experiment for a protein *in vivo* is much more difficult. To be certain that the observed oxidation status corresponds to what is actually present *in vivo*, one must take careful steps to stop thiol-disulfide exchange reactions during sample processing[3].

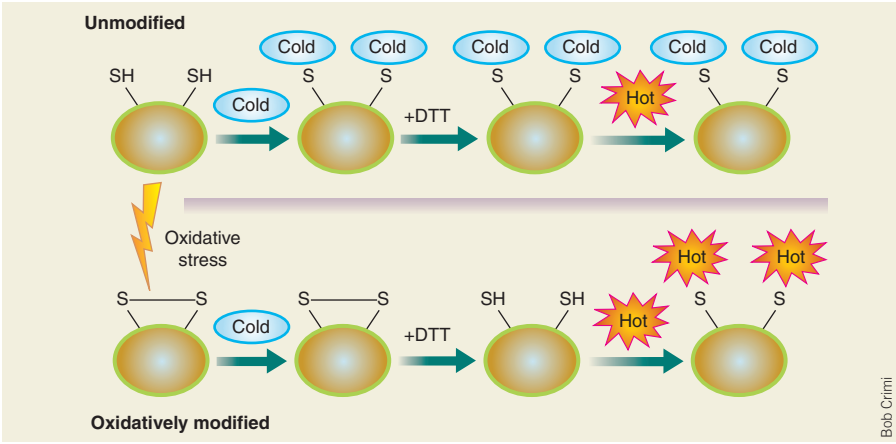

Figure 1 A method to detect oxidatively modified proteins. Cells are lysed using trichloroacetic acid, which quenches thiol exchange and denatures proteins (orange ovals). The proteins are treated with non-radioactive iodoacetamide (blue ovals), which specifically and irreversibly carbamidomethylates all accessible cysteine residues. Cysteines that were found in the cell in an oxidized state (lower panel) do not react with iodoacetamide. In a second step, the cellular lysate is treated with the reductant dithiothreitol to reduce oxidized cysteine residues, making them vulnerable to a second round of iodoacetamide modification. The carbamidomethyl group added to the free cysteines in the second round is radioactive (orange stars) but otherwise chemically identical. Radioactivity is therefore specifically incorporated into oxidatively modified proteins. Since the cold and hot proteins are chemically identical, they perfectly comigrate on 2D gels. Proteins that are oxidatively modified can thus be identified in whole cells, and the amount of radioactivity provides information on the extent of the modification.

The proteins whose *in vivo* oxidation status we want to determine most are often proteins that contain catalytic cysteine residues or are redox regulated. These oxidation-sensitive proteins are exactly those that are most likely to undergo artifactual rapid thiol-disulfide exchange during the cell lysis procedure. Even if one could find a thiol-specific probe so rapid in its action that it effectively quenched all thiol exchange reactions, one is faced with an even more vexing problem. The very act of modifying proteins with thiol-specific probes changes their properties. This causes the modified proteins to behave differently from their unmodified counterparts, making it very difficult to determine the proportion of each protein that is modified.

Leichert and Jakob have found a clever way around these problems. They describe a simple lysis procedure that quenches disulfide exchange followed by a labeling method that uses two variants of the thiol-modifying reagent iodoacetamide. This allows thiol-modified and nonmodified proteins to be differentially labeled without affecting their migration properties on two-dimensional (2D) gels (**Fig. 1**). With this method, Leichert and Jakob determined the cellular redox potential of protein thiols, identified proteins that undergo oxidative modifications during oxidative stress *in vivo* and determined the substrate proteins of cellular thiol-disulfide oxidoreductases.

The method is so simple one wonders why no one thought of it before. Cells are lysed and disulfide exchange is stopped by trichloroacetic acid precipitation. This acid-quenching step protonates free thiol groups, destroying their ability to participate in thiol-exchange reactions. Acid has a number of advantages. It is the most rapid method of quenching thiol-disulfide exchange known, occurring with a rate constant of greater than 10^9 $s^{-1}M^{-1}$, and

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it is effective even when thiol groups are relatively inaccessible[3]. It also denatures proteins, which destroys any special reactivity of oxidation-sensitive cysteines.

The acid quench is followed by a novel two-step procedure that first modifies the reduced cysteines with the thiol-specific reagent iodoacetamide in a non-radioactive form and then modifies the oxidized cysteines with radioactive iodoacetamide (**Fig. 1**). Since the modifying agents differ only in their radioactive properties, the oxidized and reduced proteins end up perfectly comigrating on 2D gels. Information about the *in vivo* oxidation status of each protein is thus obtained simply by measuring its degree of radioactivity. This allows the simultaneous determination of the *in vivo* degree of oxidation of all proteins that can be separated on 2D gels.

The use of this method in various mutant backgrounds allowed Leichert and Jakob to identify the *in vivo* substrates of cellular oxidoreductase systems such as the DsbA-DsbB machinery, which catalyzes disulfide bond formation in the periplasm of prokaryotes[4]. As expected, the majority of oxidized proteins present in wild-type strains were found in oxidizing compartments, presumably because these proteins contain structural disulfides. The method was even rapid and sensitive enough to detect cytoplasmic enzymes, such as lipoamide dehydrogenase, that transiently form disulfide bond intermediates as part of their catalytic cycle. Significantly more oxidized cytoplasmic proteins were identified following exposure of cells to oxidative stress or in strains that lack thiol protective systems such as thioredoxin.

This method also seems able to detect redox-regulated proteins. These are a new class of proteins that use reactive oxygen species to regulate their activity[5]. One of the canonical members of this class is the chaperone Hsp33. Under normal cellular conditions, Hsp33 is reduced and inactive. After exposure to oxidative stress, the four conserved cysteines in Hsp33 are rapidly oxidized. This turns on Hsp33 chaperone's activity, allowing it to deal rapidly with the damaging effects of oxidants on proteins.

Remarkably, a number of the cytoplasmic proteins that were identified as oxidized after exposure to oxidative stress appear to be redox regulated as well. One enzyme that was found to be very sensitive to oxidative stress was the GTP cyclohydrolase FolE, which is required for tetrahydrofolate synthesis. This makes a lot of sense, given that tetrahydrofolate is extremely sensitive to oxidation and its synthesis under oxidative-stress conditions would be wasteful. This result implies that the method will be useful for discovering additional proteins whose thiol modifications play regulatory roles.

This exciting new method appears to be very general—applicable to any cellular compartment, to numerous cell types including bacteria, yeast and tissue culture cells, and to any tissue that can be homogenized into a highly acidic solution. It should allow scientists to explore the cellular redox consequences of aging and of a wide variety of chemical treatments, mutant backgrounds and disease states. It will also lead to the discovery of additional proteins that use thiols as redox switches.

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