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Turnover of oxidatively modified proteins: the usage of in vitro and metabolic labeling

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ABSTRACT

Cellular reactions to oxidative stress always include a response in the protein turnover. Therefore, cellular handling of proteins is important to observe. In this method review, radioactive labeling of proteins in vitro and in intact cells is described. The use of techniques based on the radioactive quantification of amino acids is much more selective and reliable than other nonradioactive methods for studying the protein turnover of both long- and short-lived proteins. Variations of such measurements allow one to measure protein synthesis, protein degradation, formation of insoluble proteins, and, perhaps, the turnover of individual proteins.

Exposure of proteins to oxidants causes various changes in their structure. These oxidative modifications in protein structure may vary according to the kind of amino acid side chain and, in further steps, increases in surface hydrophobicity, and aggregation due to covalent cross-linking [1–4]. Susceptibility of proteins to proteolysis is increased as a result of unfolding and increased surface hydrophobicity [5,6]. Strong and perhaps chronic oxidation of proteins results in the formation of protein aggregates, known to be poor substrates for proteolysis, and may inhibit proteolysis [7]. Therefore the determination of protein aggregation and proteolytic degradation in cells provides valuable information with regard to oxidative modifications.

Early work done by Schimke et al. [8–12] used radioactively labeled or nonproteogenic amino acids. However, the most convenient way of measuring proteolysis of modified proteins in functionally intact cells is accepted to be the use of techniques based on the radioactive quantification of amino acids using liquid scintillation counting [13]. This method has been used in many studies to measure the changes in protein degradation [6,14–16] and protein aggregate formation [17,18] after various treatments.

Liquid scintillation counting is an analytical technique for measuring radiation from β-emitting nuclides such as ^{14}C, ^{3}H, ^{35}S, ^{32}P, and ^{63}Ni and is the most frequently used method to detect radioactivity in biologic samples labeled with isotopes [19,20]. Samples are dissolved or suspended in a cocktail containing an aromatic solvent and small amounts of other additives known as fluors. Sample preparation is critical for proper counting because the energy transfer process necessitates close contact between the sample and the fluor in the counting solution. β particles emitted from the sample transfer energy to the solvent molecules, which in turn transfer their energy to the fluors; the excited fluor molecules dissipate the energy by emitting light. In this way, each β emission results in a pulse of light. The primary and most extensively used scintillation cocktails contain 2,5-diphenyloxazole, toluene, xylene, and dioxane. During measurement, the samples are placed in small transparent or translucent (often glass or plastic) vials that are loaded into an instrument known as a liquid scintillation counter. The counter has two photomultiplier tubes connected in a coincidence circuit. The coincidence circuit ensures that genuine light pulses, which reach both photomultiplier tubes, are counted, whereas spurious pulses (due to noise, for example), which would affect only one of the tubes, are ignored [21].

In this review, we describe the proteolytic degradation and protein aggregate formation determinations using liquid scintillation counting under oxidative stress conditions.

Principles

Proteolysis determinations include either the degradation of metabolically radiolabeled cellular proteins or the degradation of radiolabeled "foreign" proteins or peptides during incubation with (unlabeled) cell lysates [15]. Additionally, with the metabolic labeling of intracellular proteins additional information can also be obtained, including the formation of insoluble protein complexes due to

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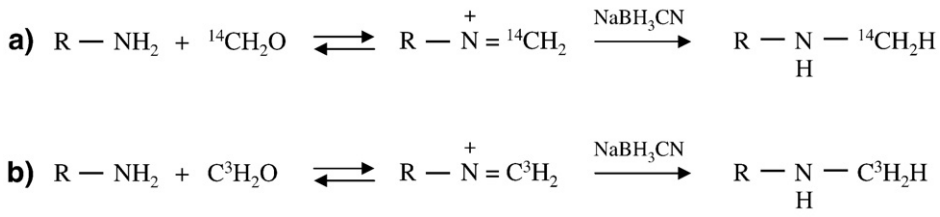

Scheme 1. Conversion of protein amino groups to mono- and dimethylamino groups, the sites of label incorporation. (a) Using ^{14}C and (b) using ^{3}H.

aggregation of unfolded, denatured proteins [18]; the turnover of single proteins; and more.

Radioactive labeling of proteins in vitro

^{125}I, ^{3}H, or [^{14}C]-containing groups are attached to the proteins. However, because ^{125}I irradiates high-energy irradiation, the use of this isotope is not recommended for testing the degradation of oxidized proteins [22,23]. Hence ^{3}H or ^{14}C labeling is used more often. Radioactively labeled (^{3}H or ^{14}C) formaldehyde is used and added to amino groups by reductive alkylation (Scheme 1). Sodium borohydride or sodium cyanoborohydride is used as reducing agent for Schiff bases [24,25].

Quantification of radiolabeled foreign protein degradation

The labeled proteins are incubated afterward with the cell lysate, protease mixture, etc., for a given amount of time under optimal conditions for the proteases. After incubation of the labeled protein with this biological sample, degradation is determined using liquid scintillation counting after trichloroacetic acid (TCA) precipitation [15,16]. If protein concentrations are very low, bovine serum albumin (BSA) has to be added as a precipitation carrier.

It is important to note that such methods reflect more the maximal capacity of a proteolytic system than the real capacity of this system in a living cell. Therefore, the degraded part of the protein substrate (% degradation) has to be small (not above 20%) to ensure the V_{max} of the proteolytic degradation of the substrate.

(*Note:* This is even more important in the case of oxidized proteins. In such a case it is not known how efficient the oxidation procedure is, how different the oxidation products are, or what their proportion of the total radioactivity is.)

Metabolic labeling of cellular proteins with amino acids

The most important step is the labeling of proteins in intact cells by incorporation of exogenously added radioactive amino acids. Metabolic labeling of cellular proteins is achieved by placing cells in a nutritional medium containing all components necessary for the growth of the cells in culture, except for one amino acid, which is substituted by its radiolabeled form. The radiolabeled amino acids are transported across the plasma membrane by carrier-mediated systems and, once in the cytosol, are loaded onto tRNA molecules before being incorporated into newly synthesized proteins [26].

Because metabolic labeling techniques use the metabolic machinery of the cell to incorporate radiolabeled amino acids, there are limitations on the type of radiolabeled amino acids that can be employed. The list of potential precursors is restricted to L-amino acids normally found in proteins, in which one or more atoms are substituted by a radioisotope [26] (Table 1).

Cells are able to synthesize nonessential amino acids from other compounds, and using nonessential amino acids in radiolabeling will cause a reduction in the specific activity by dilution with the endogenously synthesized amino acids. Therefore, essential amino acids should be favored for metabolic labeling [26]. Sulfuric amino acids, such as methionine and cysteine, are conveniently labeled with ^{35}S [27]. [^{35}S]Methionine/cysteine is the most frequently used radiolabeled amino acid because of its high specific activity (>800 Ci/mmol). For proteins that contain little or no methionine/cysteine, other amino acids labeled with ^{3}H and ^{14}C can also be obtained [22]. [^{3}H]Leucine, with activity of up to 190 Ci/mmol, is a good alternative to ^{35}S-labeled amino acids. But several problems can arise when using certain ^{3}H-labeled amino acids owing to their participation in metabolic pathways. When choosing the labeled amino acids, specific activities and half-lives have to be considered.

In general, the duration of labeling should be dictated by the half-life of the protein or protein fraction of interest. Short-time labeling of up to 2 h will result in radioactivity incorporation in "short-lived" proteins or proteins that are synthesized in large quantities. Longer incorporation times will lead to the detection of "long-lived" proteins. This protein fraction can be further defined if a 16-h labeling "pulse" is, for example, followed by a "cold chase" of 2 h (for degradation of short-lived proteins) before the start of the actual experiment [13].

In the case of cell lines it can be important to know the cell cycle duration of the cell line used. For example, a cell type that doubles

Table 1
Radiolabeled amino acids used in metabolic labeling of proteins [26]

Amino acid[a]	Freq (%)[b]	Radio-isotope	Sp act (Ci/mmol)	Organ or tissue[c]	Cells[c]
Leucine	10.4	^{3}H	5–190	Brain, heart	Ovary cells, neurons, pneumocytes
		^{14}C	0.22–0.3	Liver, muscle	Fat cells
Lysine	7.0	^{3}H	40–110	Kidney, brain, retina, thymus	Ovary cells
		^{14}C	0.22–0.26	Kidney, brain, liver, spleen	Nerve cells, blood cells
Valine	6.2	^{3}H	10–65	Liver, brain, heart, jejunum	Astroglial cells, hepatoma cells
		^{14}C	0.18–0.22	Liver, brain, kidney, skeletal muscle	Blastocysts
Threonine	5.6	^{3}H	5–25	Bronchial submucosal gland, colonic mucosa	
Histidine	2.5	^{3}H	30–70	Brain, intestine, nerve tissue	
Isoleucine	2.9	^{3}H	30–140	Brain, liver	Erythrocytes
		^{14}C	0.22–0.26	Brain, liver, white muscle	Fibroblasts
Cysteine	3.4	^{35}S	>800	Liver, brain, pancreas, kidney, heart	Leukocytes, fibroblasts, K562 cells, epithelial cells, etc.
Methionine	1.8	^{35}S	>800	Brain, epididymis	Lymphoblasts, reticulocytes, chromaffin cells, fibroblasts, ganglion cells, etc.

[a] All amino acids are in the L configuration.
[b] Frequency of amino acid residues in proteins.
[c] Organs, tissues, and cells that were used for radiolabeling with the corresponding amino acid.