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# Oxidation of methionine residues in proteins of activated human neutrophils

(methionine sulfoxide assay/respiratory burst/nascent polypeptide chain oxidation)

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**ABSTRACT** **A simple assay for the detection of $^{35}S$-labeled methionine sulfoxide residues in proteins is described. The assay, which is based on the ability of CNBr to react with methionine but not with methionine sulfoxide, requires the prelabeling of cellular proteins with [$^{35}S$]methionine. The assay was used to study the extent of methionine oxidation in newly synthesized proteins of both activated and quiescent human neutrophils. In cells undergoing a phorbol 12-myristate 13-acetate-induced respiratory burst, about 66% of all methionine residues in newly synthesized proteins were oxidized to the sulfoxide derivative, as compared with 9% in cells not treated with the phorbol ester. In contrast, quantitation of methionine sulfoxide content in the total cellular protein by means of amino acid analysis showed that only 22% of all methionine residues were oxidized in activated cells as compared with 9% in quiescent cells. It is proposed that methionine residues in nascent polypeptide chains are more susceptible to oxidation than those in completed proteins.**

Methionine residues in proteins can readily be oxidized to the sulfoxide derivative in the presence of suitable oxidizing reagents, a reaction that can result in the inactivation of certain proteins (for reviews, see refs. 1 and 2). Despite indications that sulfoxidation of methionine may occur frequently under physiological conditions, the importance of this reaction remains largely unrecognized (3). The absence of a rapid facile assay for the detection of methionine sulfoxide [Met(O)] residues in proteins has hindered studies in this area. Because Met(O) is unstable to acid hydrolysis (4), long and tedious methods, involving alkaline hydrolysis or alkylation of methionine, are normally used (5). The present report describes a simple assay for the detection of Met(O) residues in proteins labeled with [$^{35}S$]-methionine. The assay, which is based on the ability of CNBr to react with methionine but not with methionine sulfoxide (6), was used to examine the oxidation of methionine residues in human neutrophils.

Neutrophils were chosen for these studies because of their ability to produce copious quantities of oxidizing reagents on activation. As part of their microbicidal response, neutrophils can be activated by a variety of agents such as chemotactic peptides, complement, insoluble particles, and others (7). The activated cells undergo a respiratory burst during which they produce oxidizing reagents such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), hydroxyl radicals, and hypochlorite ions (8–10), which are highly toxic to extracellular microorganisms and tissues (9, 11–13). These reagents can also inactivate chemotactic peptides and other polypeptides through selective oxidation of methionine residues (14–18). Recently, it has become apparent that the indiscriminate release of these oxy-radicals can damage the neutrophils themselves. Several studies have shown that the respiratory burst can lead to a decrease in neutrophil function through autooxidation (19–22) and to possible inactivation of intracellular proteins (23). In view of the high sensitivity of protein-bound methionine residues to these oxidizing reagents and the ability of added free methionine to diminish this oxidative damage (21), it was of interest to examine the level of Met(O) in proteins from neutrophils treated with phorbol 12-myristate 13-acetate (PMA), a potent inducer of the respiratory burst (24–26). The present report shows that methionine residues are oxidized in proteins of neutrophils activated with this phorbol ester.

## MATERIALS AND METHODS

**Materials.** L-[$^{35}S$]Methionine (1,230 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham; *N*-[$^{14}C$]acetylmethionine and its sulfoxide derivative were synthesized by R. Muccino of Hoffmann–La Roche (Nutley, NJ). CNBr was procured from Aldrich. PMA (P-L Biochemicals) was dissolved in a small volume of dimethyl sulfoxide, brought to a concentration of 1 mg/ml with water, and stored in liquid nitrogen. Ficoll was purchased from Sigma, and sodium diatrizoate, from Winthrop Laboratories (New York). Cultures of HL-60 cells were provided by P. Familletti of Hoffmann–La Roche.

**Isolation of Neutrophils.** Heparinized (10 units/ml) venous blood was obtained from healthy donors. The isolation and subsequent incubation of neutrophils were carried out in polypropylene tubes (Corning). Neutrophils were isolated by centrifugation through discontinuous gradients of Ficoll and sodium diatrizoate (27). Contaminating erythrocytes were lysed by diluting cell suspensions in phosphate-buffered saline ($P_i$/NaCl) 1:4 with distilled water. Isotonicity was restored after 45 sec by the addition of 4 M NaCl.

**Preparation of $^{35}S$-Labeled Proteins from Neutrophils.** In a typical reaction, 2.5 μl of PMA (50 ng) and 5 μl of [$^{35}S$]methionine (55 μCi) were added simultaneously to neutrophils (0.5 ml, $7 \times 10^6$ cells) in $P_i$/NaCl/2 mM glucose. An aliquot (50 μl) was removed immediately for determination of superoxide anion production (see below), and the remainder of the suspension was incubated at 37°C with gentle shaking for 30 min. Cell suspensions without PMA served as controls. Neutrophil proteins were prepared for analysis in one of two ways. (*i*) The neutrophil suspension was treated with 1 ml of 10% trichloroacetic acid/0.2 M methionine and heated in a boiling water bath for 10 min. The protein was pelleted and washed twice with 1 ml of 10% trichloroacetic acid. (*ii*) The cell suspension was frozen (5 min in a dry ice/acetone bath) and thawed three times to lyse the cells and then centrifuged (15 min, 12,000 × *g*) to pellet the cellular debris. The supernatant was adjusted to pH 12 with 1 M NaOH and incubated at 37°C for 15 min to deacylate any

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Abbreviations: Met(O), methionine sulfoxide; PMA, phorbol 12-myristate 13-acetate; $P_i$/NaCl, phosphate-buffered saline.

aminoacyl tRNA. The solution was then dialyzed for 8 hr at 4°C against several changes of water.

**CNBr Assay for [$^{35}$S]Met(O) in Proteins.** This assay was routinely performed in 1.5-ml tightly capped polypropylene centrifuge tubes. Aliquots of protein preparations labeled with [$^{35}$S]-methionine (generally, 5–20 μg of protein, 1–5 × $10^5$ cpm) were treated with CNBr (final concentration, 6.5 mg/ml) at 23°C for 20 hr, with continuous stirring, in a total vol of 800 μl containing 70% formic acid (6). The tubes were uncapped in a fume hood and an aliquot was removed and assayed for radioactivity in a liquid scintillation spectrometer. The remainder of the reaction mixture was taken to dryness in a fume hood under a stream of nitrogen gas to evaporate the volatile methyl[$^{35}$S]-thiocyanate. The residue was dissolved in 0.1 M NaOH and an aliquot was assayed for radioactivity in a liquid scintillation spectrometer. The radioactivity retained in the dry residue, which represents [$^{35}$S]Met(O) residues, is expressed as a percentage of the total initial radioactivity in the sample ([$^{35}$S]Met(O) + [$^{35}$S]Met). Identical values were obtained with protein samples prepared by either of the two methods described above. Under conditions in which the cells are labeled for brief periods, the [$^{35}$S]methionine-labeled proteins represent nascent chains and newly synthesized proteins. In control experiments using free [$^{35}$S]Met, it was found, by means of thin layer chromatography, that >98% of the methionine reacted with CNBr under the conditions of the assay.

**Quantitation of Met(O) in Proteins by Amino Acid Analysis.** Protein samples, prepared by method *ii*, as described above, were treated with CNBr and taken to dryness (see above). Aliquots of the resuspended residue were hydrolyzed for 20 hr at 110°C in 6 M HCl/1 mM dithioerythritol. Under these hydrolysis conditions, Met(O) is converted to methionine (28). The methionine recovered from these hydrolysates represents, therefore, the Met(O) (both labeled and unlabeled) originally present in the preparations and is expressed as percentage of the total methionine recovered from hydrolysates of identical protein samples that had not been treated with CNBr. Amino acid analysis of hydrolyzed samples was carried out using a Glenco MM-70 amino acid analyzer adapted to measure fluorescence after reaction with *o*-phthaldehyde (29). Cysteine could not be detected with this reagent.

**Superoxide Production.** The generation of superoxide anions by neutrophils was determined using ferricytochrome *c* (30). PMA-activated neutrophils consistently showed high production of superoxide (generally, 10 $A_{550}$ units per 5 × $10^6$ cells per 20 min).

**Preparation and Chemical Oxidation of Proteins Uniformly Labeled with [$^{35}$S]Methionine.** [$^{35}$S]Methionine (440 μCi) was added to HL-60 cells (50 ml, in RPMI 1640 medium/10% fetal calf serum, 1 × $10^5$ cells per ml), and the suspension was incubated at 37°C for 60 hr to ensure uniform labeling of cellular proteins. The cells (5 × $10^5$/ml) were pelleted, washed three times with cold $P_i$/NaCl, and suspended in 0.5 ml of $P_i$/NaCl. A cell lysate was prepared by three cycles of freezing and thawing as described above. An aliquot (300 μl) of the lysate was brought to pH 3 with perchloric acid and was treated with 15 μl of 30% $H_2O_2$ for 30 min at 37°C to oxidize the methionine residues in the proteins (31). Catalase (Sigma; 30 μl of 0.06% solution) was added to stop the reaction. Deacylation of tRNA was carried out at pH 12 as described above, and the lysate was dialyzed for 8 hr at 4°C against several changes of 1 mM HCl. HL-60 cell lysates not treated with $H_2O_2$ were prepared in an identical fashion.

**Preparation of [$^{35}$S]Met(O).** [$^{35}$S]Methionine (27 μCi) was incubated with 2.5 μl of 0.5 M HCl and 3 μl of 0.6% $H_2O_2$ for 60 min at 23°C in a final volume of 30 μl. [$^{35}$S]Met(O) was purified by thin layer chromatography as described (32).

**Enzyme Assays.** Triton X-100 (Rohm and Haas) (final concentration, 0.2%) was added directly (33) to suspensions of neutrophils that had been incubated in the presence or absence of PMA, as described above. The preparations were centrifuged at 12,000 × *g* for 15 min, and the supernatants were assayed for enzymatic activity. Met(O)-peptide reductase activity was determined as described (32). Lysozyme and lactate dehydrogenase activities were assayed using established procedures (34, 35).

## RESULTS

**CNBr Assay for [$^{35}$S]Met(O) in [$^{35}$S]Methionine-Labeled Proteins.** A rapid assay for the presence of [$^{35}$S]Met(O) residues in labeled proteins was developed. This assay takes advantage of the fact that [$^{35}$S]methionine residues in proteins are cleaved by CNBr to give homoserine lactone and methyl[$^{35}$S]thiocyanate, which is volatile. Because [$^{35}$S]Met(O) residues are stable to CNBr, any nonvolatile $^{35}$S remaining after CNBr treatment should represent oxidized [$^{35}$S]methionine residues. The validity of the assay was established by testing preparations of proteins that had been uniformly labeled with [$^{35}$S]methionine and oxidized with $H_2O_2$. The results are shown in Table 1. The amount of [$^{35}$S]Met(O) present in these proteins was determined by the above method, whereas the total amount of Met(O) was determined by amino acid analysis of acid hydrolysates. The results show excellent agreement in the data obtained by the two methods.

The amino acid analyses also showed that, with the possible exception of tryptophan and cysteine, which could not be quantitated by this assay, no amino acid aside from methionine was oxidized in neutrophils treated with PMA (data not shown).

**Effect of Neutrophil Respiratory Burst on Methionine Oxidation in Proteins.** PMA-activated neutrophils release high concentrations of oxidizing reagents, as shown by the oxidation of extracellular free methionine and *N*-acetylmethionine to their respective sulfoxides (Table 2). Inactivation of three cellular enzymes was also observed in neutrophils that had undergone a respiratory burst. Thus, in activated cells, lysozyme, lactate dehydrogenase, and Met(O)-peptide reductase showed decreases in specific activities of 37%, 63%, and 44%, respectively, as compared with the activities of these enzymes in neutrophils not treated with PMA (data not shown). The possibility that the inactivation of these, and possibly other, proteins may be due to oxidation of methionine residues prompted investigation into the extent of oxidation of this amino acid in neutrophil proteins.

The CNBr assay was to determine the extent of methionine oxidation in $^{35}$S-labeled proteins of isolated neutrophils under a variety of conditions. Because the neutrophils were incubated with [$^{35}$S]methionine for only 30 min, the estimates of [$^{35}$S]-Met(O) in these proteins represent the extent of methionine oxidation in only the newly synthesized proteins. In most experiments, it was observed that the percentage of [$^{35}$S]Met(O)

Table 1. Chemical oxidation of methionine in proteins previously labeled uniformly with [$^{35}$S]methionine

| Condition | CNBr assay, % [$^{35}$S]Met(O) | Amino acid analysis, % Met(O) |
|---|---|---|
| Without $H_2O_2$ | 15 | 16 |
| With $H_2O_2$ | 86 | 87 |

HL-60 cellular proteins were labeled uniformly with [$^{35}$S]methionine and then oxidized with $H_2O_2$.

Table 2. Oxidation of extracellular methionine and *N*-acetylmethionine

| Substrate | % oxidized | | |
|---|---|---|---|
| | | With neutrophils | |
| | Without neutrophils | Without PMA | With PMA |
| [$^{35}$S]Methionine | 4 | 22 | 88 |
| *N*-[$^{14}$C]Acetylmethionine | 13 | 19 | 75 |

Neutrophils ($1.5 \times 10^7$ cells/ml in $P_i$/NaCl) were incubated at 37°C for 30 min in the presence of PMA (100 ng/ml) and either 50 μM [$^{35}$S]methionine (2.2 mCi/μmol) or 100 μM *N*-[$^{14}$C]acetylmethionine (26.5 mCi/mmol). The neutrophils were pelleted, and aliquots of the supernatant were analyzed for [$^{35}$S]Met(O) or *N*-[$^{14}$C]acetyl-Met(O), as well as unreacted substrate, by thin layer chromatography as described (32). The amount of radioactivity in the product is expressed as a percentage of the total radioactivity recovered in both the product and unreacted substrate. Incubation mixtures from which neutrophils were omitted served as controls.

in the proteins of neutrophils not treated with PMA averaged about 10%, but occasionally values as high as 25% were obtained (see *Discussion*). In the experiment shown in Table 3, 9% of the [$^{35}$S]methionine residues in protein was oxidized in the absence of PMA. However, as shown in Table 3, when PMA and [$^{35}$S]methionine were added simultaneously to identical preparations of neutrophils, the level of [$^{35}$S]Met(O) in proteins increased to 66% as determined by the CNBr assay. Amino acid analyses of the same samples, which determined the level of Met(O) in the total cellular proteins, confirmed the presence of 9% oxidized methionine in the proteins of cells not treated with PMA. However, the analyses showed that the Met(O) concentration (both labeled and nonlabeled) in proteins increased to only 22% in the cells treated with PMA. In control experiments, HeLa cells, which do not undergo a respiratory burst in the presence of PMA, did not show an increase in the concentration of [$^{35}$S]Met(O) in protein in response to this phorbol ester (Table 3), showing that PMA itself was incapable of oxidizing methionine.

The discrepancy between the level of radiolabeled Met(O) in the PMA-activated cells, as shown by the CNBr assay, and the level of total cellular Met(O), as determined by acid hydrolysis, indicated that, under the experimental conditions used, newly synthesized proteins contained a higher concentration of oxidized methionine than that found in total cellular protein. This, in turn, suggested that methionine residues in nascent polypeptide chains may be more susceptible to oxidation than those located within the tertiary structure of completed proteins. To test this hypothesis, neutrophils were incubated for various lengths of time with [$^{35}$S]methionine before initiating the respiratory burst with PMA. The prediction was that, if the respiratory burst was initiated at the same time that [$^{35}$S]methionine was added to the cells, the amount of [$^{35}$S]Met(O) in proteins would be higher than if the respiratory burst was initiated at a later time. The results of such an experiment are shown in Fig. 1. When PMA was added at the same time as [$^{35}$S]Met (0 time, Fig. 1), ≈60% of the incorporated radioactivity was in the form of Met(O). However, when PMA was added at various times after the addition of [$^{35}$S]Met, the percentage of [$^{35}$S]Met(O) in the proteins decreased. In the absence of PMA, the percentage of [$^{35}$S]Met oxidized remained constant at ≈23% (Fig. 1). Quantitation of the Met(O) concentrations by amino acid analysis in the total cellular protein of identical neutrophil suspensions showed that, in PMA-treated cells, the level of Met(O) remained constant at ≈22% regardless of the time of addition of PMA (data not shown). In cells not treated with PMA, the concentration of Met(O) remained constant at 9%. These data support the view that methionine residues in nascent polypeptide chains are more sensitive to oxidation and provide an explanation for the difference in the results obtained by the two assays (Table 3).

When neutrophils were exposed continuously to [$^{35}$S]Met and PMA, the incorporation of [$^{35}$S]Met was linear for 1 hr and the amount of [$^{35}$S]Met(O) in proteins remained at 60–65% for up to 2 hr. These results indicate that the activated neutrophils maintained a high level of oxy-radical production for at least 60 min after the introduction of PMA.

The effect of PMA concentration on the oxidation of methionine residues in proteins was also determined. As shown in Table 4, the concentration of PMA used in these studies (100

Table 3. Oxidation of methionine in intracellular proteins

| Cells | PMA | CNBr assay, % [$^{35}$S]Met(O) | Amino acid analysis, % Met(O) |
|---|---|---|---|
| Neutrophils | No | 9 | 9 |
| | Yes | 66 | 22 |
| HeLa | No | 6.5 | ND |
| | Yes | 6.5 | ND |

Neutrophils were incubated with [$^{35}$S]methionine in the presence and absence of PMA, and protein preparations were analyzed for [$^{35}$S]Met(O) by the CNBr method and for total Met(O) by amino acid analysis. HeLa cells were treated with [$^{35}$S]methionine and PMA in a fashion identical to that used with neutrophils and analyzed by the CNBr assay. ND, not determined.

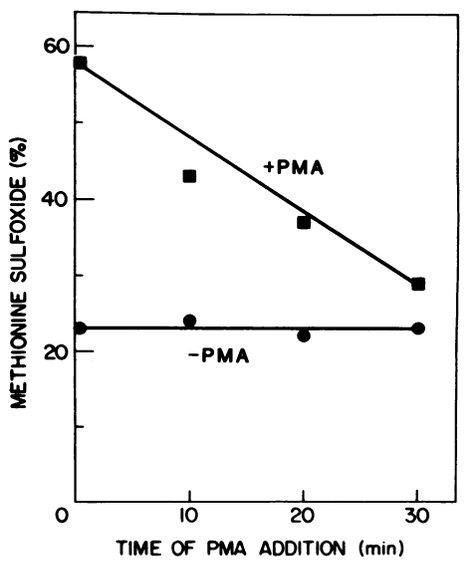


FIG. 1. Oxidation of [$^{35}$S]methionine in neutrophil proteins as a function of time of PMA addition. [$^{35}$S]Methionine (55 μCi) was added to tubes containing neutrophils (0.5 ml, $7 \times 10^6$ cells) in $P_i$/NaCl/2 mM glucose, and the cells were incubated at 37°C with shaking. PMA (final concentration, 100 ng/ml) was added to different tubes at various times after the introduction of [$^{35}$S]methionine. Thirty min after the addition of [$^{35}$S]methionine, the cells in all tubes were pelleted. The supernatants, containing proteins released through degranulation, were retained. The cell pellets were washed once with 0.5 ml of $P_i$/NaCl and then suspended in $P_i$/NaCl/2 mM glucose/PMA (100 ng/ml) and further incubated so that each cell suspension was exposed to PMA for a total of 30 min. The cell suspensions were then combined with the respective retained supernatants, protein preparations were obtained from each tube by procedure *ii* as described in *Materials and Methods*, and [$^{35}$S]Met(O) content was determined by the CNBr assay. The respiratory burst of the resuspended cells, as indicated by superoxide production, was measured and found to be equivalent to that of unwashed cells.

Table 4. Effect of PMA concentration on the oxidation of methionine in proteins

| PMA, ng/ml | $O_2^-$ generated, $\Delta A_{550}$ | [$^{35}$S]Met(O), % |
|---|---|---|
| 0 | 0.1 | 9 |
| 2 | 0.3 | 30 |
| 10 | 5.5 | 69 |
| 100 | 9.7 | 66 |

Neutrophils were incubated with [$^{35}$S]methionine and various concentrations of PMA. The amount of superoxide anion produced is expressed as $\Delta A_{550}$ units per $5 \times 10^6$ cells per 20 min. The concentration of [$^{35}$S]Met(O) was determined by the CNBr assay.

ng/ml) was in excess of the level needed to obtain maximal oxidation of methionine. Significant oxidation of this amino acid in proteins could be obtained with PMA at 2 ng/ml.

## DISCUSSION

The assay used in these studies to detect Met(O) in proteins is based on the known ability of CNBr to react exclusively with methionine residues, with the production of homoserine lactone and methyl thiocyanate, which is volatile (6). A requirement of the assay is that the protein must first be labeled with [$^{35}$S]methionine. Thus, the reaction of CNBr with [$^{35}$S]methionine in proteins, followed by evaporation of the methyl[$^{35}$S]-thiocyanate, results in the loss of radioactivity. Any retained radioactivity is associated solely with Met(O). As the data in Table 1 indicate, this assay is valid. The estimation of the concentration of Met(O) in proteins that had been uniformly labeled with [$^{35}$S]methionine, by either amino acid analysis or the CNBr assay, yielded similar results.

The present studies show that the oxidizing reagents produced by neutrophils during PMA-induced respiratory bursts can oxidize intracellular protein-bound methionine residues. Although the possible oxidation of tryptophan and cysteine residues cannot be excluded by these studies, no other amino acid appeared to react under the conditions used. These data are in agreement with the results of other studies, which have shown that, under certain conditions, methionine residues may be more readily oxidized than other amino acids such as tryptophan, tyrosine, and histidine (3, 31). It is, therefore, possible that the interaction of the oxidizing reagents with methionine residues in proteins may be a significant cause of the dysfunctions observed in neutrophils that have undergone a respiratory burst (19–23, 36, 37).

Although the oxidation of methionine residues does not always result in protein inactivation (3, 38), significant reduction in biological activity has been observed with numerous proteins (1, 2). In these studies, three enzymes, lysozyme, lactate dehydrogenase, and Met(O)-peptide reductase, were partially inactivated in PMA-treated neutrophils. Although this decrease in activity cannot be attributed, as yet, to methionine oxidation, another study has shown that the oxidation of methionine residues in lysozyme results in the inhibition of enzymatic activity (39). It should be noted that, because PMA induces degranulation and partial cell lysis in neutrophils, some of the observed inactivation of these enzymes may have occurred in the extracellular medium (23, 40–42).

Because the CNBr assay requires [$^{35}$S]methionine-labeled proteins it permits the quantitation of Met(O) in selected groups of proteins within the total population of cellular proteins. In these studies, brief incubation of neutrophils with [$^{35}$S]methionine permitted the labeling of only the newly synthesized proteins. Subsequent analysis of the total cellular protein by amino acid analysis, and of the newly synthesized proteins by the CNBr assay, showed that, in neutrophils treated with PMA, the concentrations of Met(O) in the newly synthesized proteins greatly exceeded those found in the preexisting proteins (Table 3). The elevated level of Met(O) in the newly synthesized proteins suggests that methionine residues in nascent polypeptide chains are more susceptible to oxidation than are those in intact proteins. It is known that the reactivity of methionine residues with oxidizing reagents is dependent on their location in the protein (43–47). Because methionine residues are usually located within the hydrophobic interior of proteins (31), they may, therefore, be more resistant to oxidation than methionine residues in nascent polypeptide chains. Further evidence for the high sensitivity of methionine residues in nascent polypeptide chains is provided by the observation that high concentrations of Met(O) (up to 25%) were found occasionally in the newly synthesized proteins but not in the preexisting proteins of neutrophils not treated with PMA. Freshly isolated neutrophils were frequently activated to a low degree (evidently by the isolation procedure) and produced small, yet detectable, amounts of $O_2^-$. These data suggest that low concentrations of oxidizing reagents may cause significant oxidation of methionine in proteins. Although it is likely, therefore, that, under conditions of a physiological respiratory burst, newly synthesized proteins would be inactivated to a greater extent than preexisting proteins, it is not clear what effect this would have on the viability of neutrophils and whether this damage can be repaired by endogenous Met(O)-peptide reductase (48, 49).

In general, the assay described here should prove useful in the study of methionine oxidation in tissues and cell cultures in which adequate radiolabeling can be achieved. Moreover, because only radiolabeled methionine residues are subject to analysis with this assay, pulse–chase experiments should facilitate the examination of methionine oxidation under a variety of metabolic conditions or at different stages of the cell cycle.

**Note Added in Proof.** Recent experiments have shown that the CNBr assay is complete after 1 hr at 60°C.


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