You cannot oxidize what you cannot reach: Oxidative susceptibility of buried methionine residues

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Oxidation of protein methionines to methionine sulfoxides can result in protein structural alterations with a wide variety of biological implications. Factors that determine susceptibility to oxidation are not well understood. The recent JBC Editors Pick by Walker *et al.* applied proteomic methodologies to show that the oxidative susceptibility of buried methionine residues is strongly correlated with folding stability of the contextual peptide. Proteome-wide analysis of oxidation-susceptible methionines promises to answer open questions about the biological functions of reversible methionine oxidation.

Along with cysteine, methionine is one of two sulfur-containing amino acids found in proteins. The cysteine side chain is a reactive thiol that is susceptible to oxidation, producing a disulfide that can be enzymatically reduced. This oxidation/reduction interchangeability of cysteine is known to be involved in a variety of functions, including catalysis, protein structure, redox sensing, and enzyme regulation. Methionine residues in proteins also undergo enzymatically reversible oxidation, but the implications of these modifications have not been as extensively studied as for cysteine (1).

The thioether side chain of methionine is susceptible to oxidation, producing methionine sulfoxide (MetO) in two diastereomeric forms, S-MetO and R-MetO. These isomers are reduced by different methionine sulfoxide reductases. Formation of MetO converts the hydrophobic side chain to a polar one, which can alter protein structure and inactivate enzymes. The susceptibility of methionine residues to oxidation varies widely for different methionines within a single protein. Solvent-exposed methionines are more prone to oxidation than their buried counterparts. In addition, neighboring amino acids influence oxidative propensity of methionines. In the Editors Pick by Walker *et al.* (2), proteomic methodologies were applied to this emergent field to elucidate the protein structural factors that determine the susceptibility of methionine residues to oxidation.

Walker *et al.* studied proteome-wide methionine oxidation rates by probing *Escherichia coli* folded peptides, unfolded peptides, and intact native proteins with $H_2{}^{18}O_2$. Unfolded proteins were fragmented with a protease and denatured prior to oxidative treatment, thereby tagging *in vitro*-generated MetO with the ^{18}O-label, compared with oxidations present in the starting sample, which would be tagged with ^{16}O. The susceptibility of individual methionines to oxidation was determined by LC–MS/MS using a protocol that the group had previously developed to measure methionine oxidation of the human proteome (3). The results showed that the rate constants measured for 3900 methionine-containing unstructured peptides varied by 10-fold, values comparable to the oxidation rate of free methionine. Analysis of the amino acid sequence of these peptides revealed several primary sequence factors that influenced oxidation rate, including a more rapid oxidation rate for methionines located at the amino terminus of peptides compared with internal methionines. The authors also identified several neighboring residue effects, including a slower oxidation rate for methionines with proline at the +1 position and glycine at the −1 position and a faster oxidation rate for methionines with arginine at the +1 position. In summation, methionine oxidation rates in unstructured peptides were found to be within an order of magnitude of the oxidation rate of free methionine, with primary sequence context producing minor differences. These results confirmed prior work. By contrast, the result obtained with intact proteins was markedly different. For analysis of intact proteins, the protocol was changed by treating samples with H_2O_2 prior to protease fragmentation and LC–MS/MS analysis, as opposed to the unfolded peptides, which were treated with H_2O_2 after denaturation. In this way, methionines located within the folded structure of proteins were probed for susceptibility to oxidation. Analysis of 1778 different methionine-containing peptides revealed differences in methionine oxidation rate spanning 1000-fold. As denatured peptides showed only 10-fold difference in methionine oxidation rate, these results suggested that in the context of intact proteins, some methionine residues are markedly more protected against oxidation.

Postulating that these "protected" methionines are buried within the protein structure, Walker *et al.* next examined the solvent accessibility of the peptides derived from oxidation of intact proteins. Solvent accessibility information was derived from information in the Protein Data Bank or predicted using Alphafold2 (EMBL's European Bioinformatics Institute; https://alphafold.ebi.ac.uk/). They found a statistically significant correlation between lower solvent accessibility and increased protection of methionine against oxidation. The

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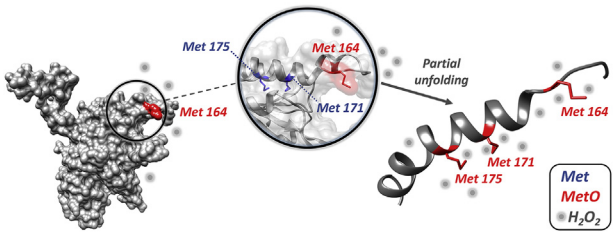

Figure 1. Model depicting the procedure used by Walker *et al.* to identify H_2O_2-accessible and buried methionines. Oxidation of methionine 164 (Met 164) of *Escherichia coli* RecA is known to result in the loss of the recombination activity, which is reversed by reduction (5). We propose that further study of the methionine oxidation rates in RecA using the methods developed by Walker *et al.* would enhance our understanding of its activity in DNA recombination.

methionines with the slowest oxidation rates were postulated to be the most deeply buried within protein structure. The authors then used a recently developed proteomic methodology called SPROX (4), *S*tability of *P*roteins from *R*ates of *Ox*idation, to analyze thermodynamic protein stability based on susceptibility of methionines to oxidation at different degrees of unfolding generated by varying concentrations of denaturant. The results revealed a statistically significant correlation between thermodynamic stability and the degree of protection from oxidation, demonstrating that folding stability plays a major role in protecting buried methionines from oxidation. This correlation was tested by analyzing the highly stable proteome of a thermophile, *Thermus thermophilus*, compared with *E. coli*, the mesophile used in prior experiments. The *T. thermophilus* proteome was found to be significantly more stable than the proteome of *E. coli*. In addition, when measured over a range of temperatures, the *T. thermophilus* proteome was found to be significantly more resistant to methionine oxidation than *E. coli* at 25 and 37 °C. Interestingly, the methionines in the *T. thermophilus* proteome showed similar solvent accessibility to those of *E. coli*, revealing that the greater protection of methionines in the *T. thermophilus* proteome is the outcome of increased folding stability, rather than increased embedding. The *T. thermophilus* proteome at 50 °C was found to be less stable than at 25 °C and as stable as the *E. coli* proteome at 25 °C. The authors noted that *T. thermophilus* is nonviable at 25 °C, suggesting that proteome overstabilization may be harmful.

This report by Walker *et al.* is the first to use proteome-wide analysis to define the determinants that influence susceptibility of protein methionines to oxidation. The results provide evidence showing that solvent-accessible methionines are most susceptible to oxidation, with small-scale differences attributed to position and neighboring amino acid effects, supporting prior knowledge. Some embedded methionine residues become exposed to oxidation during transient unfolded states, and these show intermediate oxidation rates. The susceptibility of deeply embedded methionines to oxidation is dependent on thermodynamic stability of the contextual region. The methodology used in this study will likely prove useful in identifying regulation systems based on reversible methionine oxidation and examining the conditions under which methionine positions that serve a regulatory function become modified, for example, cytoskeletal remodeling based on oxidation of methionine in actin by the MICAL oxidoreductase (5). Also, the methodology will prove useful in cataloging the proteome-wide targets of methionine sulfoxide reductase and the individual methionine residues these reductases act on. One additional example of an enzyme that should be characterized is the *E. coli* RecA protein shown in Figure 1, whose activity has recently been reported to be regulated *via* redox-dependent modulation of recombination activity at a methionine residue (6). We anticipate that proteome-wide analysis of oxidation-susceptible methionine residues will answer many open questions relating to the biological functions of methionine oxidation.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviation—The abbreviations used are: MetO, methionine sulfoxide.

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