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An electrophoretic mobility shift assay for methionine sulfoxide in proteins

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Abstract

Study of the post-translational modification of methionine to its sulfoxide has been receiving increasing attention because of its implication in regulation of activity, but techniques for the detection of this modification remain limited. In particular, there has been no method to detect the oxidation of methionine on polyacrylamide gels. We demonstrate that alkylation of methionine introduces a charge change that shifts the mobility of the protein on an acidic gel relative to the alkylation resistant sulfoxide form.

Keywords

acid PAGE gel; methionine sulfonium; methionine alkylation; methionine oxidation

All of the amino acids that make up proteins are susceptible to oxidation by reactive oxygen species (ROS), with methionine residues being among the most susceptible to this oxidation, typically to the sulfoxide form [1]. Enzymatic systems for the reduction of methionine sulfoxide are ubiquitous [2] and methionine oxidation has been suggested as an important agent in the process of ageing [3], a cause of disease [4], and a normal method for regulation of protein activity [5; 6]. However, the biochemical toolset to detect this post-translational modification is still fairly limited.

It has previously been shown that at acidic pH the alkylation of methionine proceeds readily, while other nucleophiles in proteins become protonated and their alkylation is suppressed [7; 8]. While not readily protonated, disulfides react ten to a hundred times more slowly than thioethers [9] and the product is not stable in water [10]. The positively charged sulfonium that results from the alkylation of a thioether is reasonably stable, as the example of S-adenosyl methionine illustrates. However, methionine sulfoxide is much more resistant to alkylation. This difference in properties of the two versions of methionine forms the basis of a method for an electrophoretic mobility shift assay that we demonstrate here.

Using this difference in the reactivity of methionine and methionine sulfoxide is not new. In 1959, it was pointed out that iodoacetate would react with methionine to form a carboxy-methylsulfonium derivative [7]. In 1960, Vithayathil and Richards [11] showed that the methionine of ribonuclease S-peptide could be alkylated by iodoacetic acid and iodoacetamide. At that time, it had recently been recognized that methionine sulfoxide was

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reduced back to methionine in the standard conditions of peptide hydrolysis [12]. Vithayathil and Richards showed that, after alkylation of any normal methionine in a mixture of peptides, sulfoxides could be further oxidized with performic acid to the sulfone, which is resistant to reduction during acid hydrolysis. The sulfonium was resistant to oxidation, so sulfone could be attributed to any methionine sulfoxide which had been in the original samples. In 1962, Stein and Moore's group further developed this as an indirect method for determining the amount of methionine sulfoxide in a protein [13]. Although appearing in *Methods in Enzymology* twice [14; 15], it has not been used much. It not only requires substantial amounts of pure protein, it requires alkylation, performate oxidation, hydrolysis, and then amino acid analysis; a great deal of work.

We recognized that alkylation creates a sulfonium [16], which has a positive charge. This charge addition should cause a mobility shift in native gel electrophoresis. The sulfoxide form of methionine is neutral and resistant to alkylation. This basic premise is laid out in Figure 1.

In order to successfully detect change of the protein electrophoretic mobility a gel system that would work at low pH values is desirable for several reasons. First, under acidic conditions any alkylation reaction is more specific for methionine. Second, the sulfonium derivatives of S-alkylated methionine have been shown to be more stable at low pH [17]. Third, low pH helps to denature proteins, which is important to ensure uniform reaction of methionines with alkylating agent [18]. Fourth, low pH leads to very predictable electrophoretic behavior. At low pH any ionizable residues either carry no charge or a positive charge. The addition of a charged sulfonium upon alkylation of methionine should result in an increase in electrophoretic mobility relative to the unalkylated and uncharged methionine sulfoxide. Therefore, a system that is around pH 2.0–3.0 seemed optimal.

Most polyacrylamide gels use the standard ammonium persulfate and N, N, N', N'-tetramethylethylenediamine (TEMED) system as the catalyst for polymerization. It is well known that as pH is lowered, the efficiency for this catalyst system decreases, due to the protonation of TEMED, and, eventually, no gel is formed. This system also uses a significant amount of an oxidative species (the peroxy sulfate) which can introduce artifactual methionine oxidation [19].

Lyubimova *et al.* [20] describe a catalyst system of photoexcited methylene blue (a source of radicals) and sodium toluenesulfinate/diphenyliodonium chloride (redox couple) for the polymerization. Gels readily polymerize at low pH values using visible light and do not use or create oxidative species (provided the gel mixtures are degassed prior to polymerization). Acetic acid/urea gels have previously been established as a useful system for the study of basic proteins [21]. 6M urea may also be used in the gel and buffers to aid in denaturing the proteins. In our hands, 0.9M acetic acid kept the pH at the appropriate level and gave sharper bands than mineral acids. The recipes for the gel components are summarized in Table 1. Both stacking and separating gels were prepared using a 30% (30%T, 2.67%C) acrylamide stock solution.

The electrophoresis was performed with the electrodes reversed at the power source as the charge migration is opposite of what one would use in a standard SDS-PAGE experiment. A constant voltage of 100 V was used for 4–6 hours in a mini-gel. Migration through the gel is slower than a typical gel system, presumably because the charge on each protein is less than in the case of an SDS gel. The gels were stained and destained using established Coomassie Blue staining protocols.

Rather than the iodoacetate derivatives as an alkylating agent we chose to use methyl methanesulfonate. This reagent has been shown to modify proteins *in vivo* [22] and *in vitro*

[23] and has several advantages over iodoacetic acid and iodoacetamide. Most importantly, it is a more potent alkylating agent. Reaction of methionine with excess iodoacetamide at room temperature is only 93% complete after 48 hours. Alkylation is faster at higher temperature, but heating leads to peptide cleavage at the modified methionine [24]. Incomplete alkylation has been seen in proteins as well [8; 25]. Further, the iodide resulting from alkylation with iodoacetate derivatives can cause side reaction in the presence of peroxide, as will be discussed in a future communication.

Staphylococcal nuclease was chosen as a model protein because of its highly basic character and the ready availability of methionine mutants [26]. Nuclease is 149 amino acids and 16.8 kDa. In terms of potential nucleophiles, the wild-type protein has four methionines, four histidines, one tryptophan, 23 lysines, and no cysteines. As would be expected for a protein that interacts with nucleic acids, it is a very basic protein with a charge of +13 at pH 7. At the pH of the gels here the net charge of wild-type nuclease is approximately +17. This worst case scenario of high charge in a small protein means addition of a single charge results in a relatively small mobility increase and, thus, if charge changes can be detected in nuclease, it will be possible to do it in other proteins.

Samples of protein were alkylated with 100 mM methanesulfonic acid methyl ester in a 6 M urea and 0.9 M acetic acid solution. Protein concentrations were typically 0.1 mg/ml. The alkylation reactions were generally allowed to proceed overnight with constant shaking at room temperature. Times as short as 3 hours were shown to be sufficient for complete alkylation but no negative effects of extending the incubation were observed. For samples requiring oxidation, 1% H₂O₂ was used. In those samples that were oxidized prior to alkylation, the oxidation was allowed to proceed for 30 minutes before the addition of the methyl methanesulfonate.

The results of this protocol are shown in Figure 2. Wild-type staphylococcal nuclease whose methionine residues have been oxidized to methionine sulfoxide shows no shift in mobility when exposed to an alkylating reagent compared to the unmodified protein. The protein with methionine residues in their normal oxidation state has its mobility shifted notably by the four additional charges that result upon alkylation. We used a mutant protein, M65F, to demonstrate that a protein with just one less methionine is readily distinguishable as well.

There are some caveats that should be borne in mind. If a cysteine is oxidized to sulfonic acid at the same protein, this negative charge would cancel the positive charge of an alkylated methionine. Phosphorylation will have a similar effect, although proteins can be dephosphorylated if need be. Alkylated methionines can cleave, so samples should be used shortly after alkylation and not subjected to heating.

This work demonstrates two important concepts. First we have demonstrated a novel use for an acid-PAGE system in separating proteins based on methionine sulfoxide content. Secondly we have revisited the idea that methionine residues are efficiently alkylated under acidic conditions while not affecting methionine sulfoxide residues, but with a new class of alkylating reagents. Alkylation with reagents that contain other positive charges (*e.g.* [27]) is possible, to introduce even greater electrophoretic mobility change. As a further development, alkylation with a spectroscopic, isotopic, or affinity tag can also be imagined.

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Figure 1.
Reaction of methionine residues compared to that of methionine sulfoxide residues.

Figure 2. Acid-PAGE of staphylococcal nuclease. From right to left: Wild-type staphylococcal nuclease with no chemical modification; wild-type staphylococcal nuclease oxidized with H₂O₂ prior to alkylation with methyl methanesulfonate; wild-type staphylococcal nuclease alkylated with methyl methanesulfonate without oxidation; the mutant M65F alkylated with methyl methanesulfonate without oxidation.

Table 1

Summary of gel components.

Separating Gel	Stacking Gel	Running Buffer	Loading Buffer
15% acrylamide	4% acrylamide	0.9 M acetic acid	0.9 M acetic acid
0.9 M acetic acid	0.9 M acetic acid	0.1 M glycine	6 M urea
6 M urea	6 M urea		1% methyl green
1 mM STS ^a	1 mM STS		
50 μ M DPIC ^b	50 μ M DPIC		
100 μ M MB ^c	100 μ M MB		
	50 mM CH ₃ COONa		

^a sodium tolunesulfinate^b diphenyliodonium chloride^c methylene blue