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Wanted and wanting: Antibody against methionine sulfoxide

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ABSTRACT

Methionine residues in protein can be oxidized by reactive oxygen or nitrogen species to generate methionine sulfoxide. This covalent modification has been implicated in processes ranging from normal cell signaling to neurodegenerative diseases. A general method for detecting methionine sulfoxide in proteins would be of great value in studying these processes, but development of a chemical or immunochemical technique has been elusive. Recently, an antiserum raised against an oxidized corn protein, DZS18, was reported to be specific for methionine sulfoxide in proteins (*Arch. Biochem. Biophys.* **485**:35–40; 2009). However, data included in that report indicate that the antiserum is not specific. Utilizing well-characterized native and methionine-oxidized glutamine synthetase and aprotinin, we confirm that the antiserum does not possess specificity for methionine sulfoxide.

Methionine and cysteine are the two commonly occurring sulfur-containing amino acids, and both can undergo reversible oxidations—cysteine to cystine and methionine to methionine sulfoxide. Sensitive and specific analytical methods for detection and quantitation of disulfide-containing proteins are readily available. That is not the case for methionine sulfoxide, and this analytical void greatly hinders the study of methionine oxidation and reduction in proteins. The importance of the methionine redox cycle is highlighted by the existence of several methionine sulfoxide reductases in almost all organisms exposed to oxygen, from bacteria to mammals. Reducing or knocking out methionine sulfoxide reductase A in cultured cells and several lower organisms caused increased susceptibility to oxidative stress [1–5]. Mice lacking methionine sulfoxide reductase A are also more susceptible to oxidative challenge, although their life span is not altered [6]. Methionine sulfoxide reductase A is down regulated in human breast cancer cells, and this down regulation causes a more aggressive phenotype primarily due to increased oxidative stress [7]. Conversely, overexpression of methionine sulfoxide reductase A increases resistance to oxidative stress [4,8–11]. Overexpression also reduces accumulation of oxidatively damaged proteins [11] which has been hypothesized to be an important mechanism of the aging process [12]. It is thus notable that overexpression of methionine sulfoxide reductase A in *Drosophila* doubled the life span of the flies [4].

Because of the evident importance of methionine sulfoxide in proteins, many laboratories have made numerous attempts to raise an antibody which is specific for methionine sulfoxide. Each of these attempts was unsuccessful, but as is often the case with unsuccessful outcomes, none has been published. The situation for methionine sulfoxide appears to be the same as that for phosphoserine and phosphothreonine: One can raise antibodies which are specific for a peptide that contains methionine sulfoxide [13] or phosphoserine or phosphothreonine, but one cannot obtain an antibody which reacts specifically with the residue itself. Our efforts as well as those of others with whom we have communicated have included the screening of large phage display libraries [18] for specific methionine sulfoxide antibodies. In our case, we screened 15 billion clones 3 separate times and found not a single antibody which was specific for methionine sulfoxide.

Thus the paper by Oien, Moskovitz, and colleagues [17] reporting a methionine sulfoxide specific antiserum was of considerable interest. They exposed a methionine-rich corn protein, DZS18, to hydrogen peroxide to convert its methionine residues to methionine sulfoxide. Oxidized DZS18 was utilized as the immunogen to produce antiserum in a rabbit. A patent application has been submitted for this process [19], and the antibody or more precisely, the antiserum, is currently sold by at least 7 companies. Several papers utilizing the antiserum have been published [14–17]. The developers of the antiserum state that it is specific for methionine sulfoxide in proteins, a claim which is surprising in view of the inability of other investigators to produce such antibodies. This prompted us to examine the specificity of the antiserum.

Materials and methods

Preparation of oxidatively modified test proteins

Recombinant *Escherichia coli* glutamine synthetase is routinely prepared in this laboratory with the construct *YMC* 10/*pgln*6 and

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purified by the zinc-induced aggregation procedure as described [20]. It is stored at 4 °C in 50 mM Hepes, 100 mM KCl, 1 mM $MnCl_2$, pH 7.0. A 4 mg/ml glutamine synthetase solution was oxidized [21,22] with 250 mM hydrogen peroxide for 1 h at 37 °C in a buffer composed of 71 mM KH_2PO_4, 13 mM Hepes, 26 mM KCl, and 0.3 mM $MnCl_2$, with a final pH of 5.7. Methionine-oxidized glutamine synthetase was dialyzed against 50 mM Hepes, 100 mM KCl, 1 mM $MnCl_2$, pH 7.0 in CelluSep T4 with a 12,000–14,000 Da nominal cutoff (1430-10, Membrane Filtration Products, Inc., San Antonio, TX USA). A 4 mg/ml solution of aprotinin (A-1153, Sigma, St. Louis, MO, USA) was oxidized with 250 mM hydrogen peroxide for 1 h at 37 °C in 97 mM KH_2PO_4, pH 5.5. Methionine-oxidized aprotinin and its native control were separately dialyzed against four changes of water over 16 h in Spectra/Por Biotech CE with a nominal cutoff of 500–1000 Da (131084, Spectrum Laboratories, Rancho Dominguez, CA, USA). Proteins were analyzed for methionine oxidation by mass spectroscopy using an Agilent 6520 QTOF LC/MS (Agilent, Santa Clara, CA, USA) and by amino acid analysis (Eclipse, Agilent) [23] after cyanogen bromide oxidation of methionine to homoserine [24,25].

Western blots on nitrocellulose and PVDF

Antiserum raised against oxidized DZS18 was purchased from two suppliers (600161, Cayman Chemical, Ann Arbor, MI; MS01, Oxford Biomedical Research, Rochester Hills, MI). Western blots of native and methionine-oxidized glutamine synthetases and aprotinins on nitrocellulose (Invitrogen LC2001, Life Technologies, Grand Island, NY, USA) and PVDF (IPFL00010, Immobilon-FL PVDF, Millipore, Billerica, MA, USA) were prepared from identical Tris glycine nonreducing gels (Invitrogen EC61355), each with test proteins loaded at 1 μg protein per lane. A third gel was prepared and stained with Coomassie blue to confirm the protein load [26]. Membranes were blocked 7 h in 0.5% nonfat dry milk (170–6404 Bio-Rad, Hercules, CA, USA) in 137 mM NaCl, 25 mM Tris, 2.7 mM KCl, pH 7.4 (TBS), washed 5 min three times in TBS with 0.1% Tween 20, and incubated 16 h at 4 °C with a 1:250 dilution of DZS18 antiserum (Cayman) in TBS with 0.5% dried milk. The secondary antibody incubation was for 1 h with 1:10,000 dilution of goat anti-rabbit antibody tagged with DyLight 800-labeled fluorescent dye (072-07-15-06, KPL, Gaithersburg, MD) diluted in Odyssey blocking buffer (927–40000, LI-COR, Lincoln, NE, USA) with 0.1% Tween 20. Washes after antibody applications were done in triplicate for 5 min in TBS, 0.1% Tween 20. Membranes were rinsed twice briefly in water and scanned in the 800 channel of an Odyssey imager (LI-COR). After detection and quantitation of bound antibody from the DZS18 antiserum, glutamine synthetase on the blots was quantitated using a 1:75,000 dilution of a polyclonal antibody prepared in our laboratory against bacterial glutamine synthetase. Aprotinin was detected by staining the membranes with FCF Fast Green [26].

Dot blots

Antiserum was also evaluated with dot blots on nitrocellulose. In addition to the anti-DZS18 antiserum, we used the same dilutions of a nonimmune rabbit serum, a gift from Geumsoo Kim of this laboratory. Native and methionine-oxidized glutamine synthetases and aprotinins were diluted in 10 mM Hepes, 1 mM $MgCl_2$, pH 7.2, to give solutions containing 0.5, 0.25, and 0.05 μg/μl. Two microliters of each solution was applied in duplicate onto five replicate dot blots on 0.45 μm nitrocellulose membranes (Invitrogen LC2001). One blot were stained with Fast Green for quantitation of protein binding. Four blots were detected using a general protocol suggested by Oxford in which membranes were blocked with 5% nonfat dry milk in 75 mM Na_2HPO_4, 68 mM NaCl, pH 7.2 (PBS) overnight at 4 °C. The membranes were incubated with 1:500 and 1:2000 dilutions of either the Oxford DZS18 antiserum or nonimmune rabbit serum in PBS with 1% dried milk and 0.05% Tween 20 for 6 h at room temperature. The secondary antibody, goat anti-rabbit tagged with DyLight 800-labeled fluorescent dye (072-07-15-06 KPL) diluted 1:10,000 in Odyssey blocking buffer with 0.1% Tween 20, was added for 1 h at room temperature. Blots were washed three times for 5 min each with PBS containing 0.1% Tween 20 after each antibody incubation. Blots were washed twice briefly with water and read in the 800 nm channel of an Odyssey Imager.

Results and discussion

Reactivity toward native and methionine-oxidized glutamine synthetase

In Fig. 1 of their paper, Oien and colleagues show a Western blot against native and methionine-oxidized glutamine synthetase [17]. While one would expect no staining of the native protein and strong reactivity of the methionine-oxidized form, both forms are equally reactive with the antiserum. The authors explain this surprising result by stating that glutamine synthetase "is naturally oxidized under its recommended storage conditions at 4 °C." This unreferenced claim is incorrect. The glutamine synthetase used in their studies was produced and given to them by our laboratory which has studied the enzyme for decades. We did demonstrate in the early 1980s that the protein undergoes a site-specific oxidative modification under the storage conditions utilized at the time [27]. However, we convincingly demonstrated that the oxidation was of a specific histidine residue located at the active site metal-binding site. We isolated a peptide containing the oxidized histidine [28] with the sequence $Met_{268}His_{269}Cys_{270}His_{271}Met_{272}$. We pointed out the remarkable specificity of the oxidation in that only His_{269} was oxidized. Neither the other histidine nor either methionine nor the cysteine was modified, a finding which was explained when the crystal structure was solved, and His_{269} was shown to be liganded to the divalent cation required for catalytic activity [29]. In the course of those early studies we also demonstrated that manganese prevented the oxidation [30]. As a consequence of this observation, we changed the storage buffer for glutamine synthetase from one containing 10 mM $MgCl_2$ to one containing 1 mM $MnCl_2$. We have used the manganese-containing

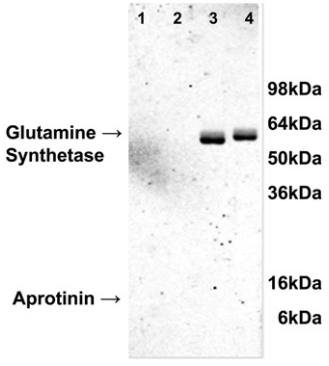

Fig. 1. Western blot of methionine-oxidized and native proteins probed with DZS18 antiserum (Cayman, 600161). Blots were prepared on nitrocellulose with 1 μg of each protein. Lane 1, native aprotinin; Lane 2, methionine-oxidized aprotinin; Lane 3, native glutamine synthetase; Lane 4, methionine-oxidized glutamine synthetase. The glutamine synthetase band intensities were quantitated by the Odyssey Imager and were 3.1 for native and 2.4 for methionine-oxidized glutamine synthetase. Aprotinin signals were not detectable.