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Methionine-Oxidized Amyloid Fibrils Are Poor Substrates for Human Methionine Sulfoxide Reductases A and B2[†]

Katrina J. Binger,*[,‡] Michael D. W. Griffin,[‡] Stefan H. Heinemann,[§] and Geoffrey J. Howlett[‡]

[‡]*Department of Biochemistry and Molecular Biology, The University of Melbourne, Parkville, Victoria, Australia, and* [§]*Center for Molecular Biomedicine, Department of Biophysics, Friedrich Schiller University of Jena, Jena, Germany*

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ABSTRACT: A common feature of many amyloid diseases is the appearance of oxidized, aggregated proteins. Methionine is one of the most readily oxidized amino acids, and its oxidative state is regulated in vivo by the methionine sulfoxide reductases (Msr). Here, we have explored the basis by which methionine oxidation is linked to amyloid disease by comparing the reduction of oxidized amyloid fibrils and monomer. We show that oxidized amyloid fibrils are not as effectively reduced by the Msr enzymes as the monomer. This work suggests a mechanism by which oxidized proteins and aggregates can accumulate as a part of degenerative disease.

Oxidative damage to proteins, DNA, and lipids accumulates with age, and this damage can result in gross cellular dysfunction and degeneration (*1*, *2*). Sources of oxidants in vivo are varied; they can result as a byproduct of normal cellular functions, such as metabolism and the cytochrome P450 enzymes, and also as a result of infection and inflammatory processes (*3*). Several cellular systems are in place to prevent excessive oxidative damage. These include low-molecular weight and enzymatic antioxidants that reduce the amount of free oxidants and protein repair systems that reduce oxidized cysteine and methionine residues (*4*).

The methionine sulfoxide reductases (Msr[1]) are enzymes responsible for reducing oxidized methionines, one of the most readily oxidized amino acids (*5*). Oxidation of methionine to methionine sulfoxide (MetO) results in the formation of two diastereoisomers (*S* and *R*), which are specifically reduced by MsrA and MsrB, respectively. Thus, complete reduction of oxidized methionines is only accomplished with a combination of both MsrA and MsrB (*6*). In humans, there is one MsrA gene and there are three MsrB genes, which encode several proteins that differ in their cellular location (cytoplasm, mitochondria, or endoplasmic reticulum) but have essentially the same function (*7*, *8*). Although MsrA is structurally unrelated to MsrB, they have been shown to have a common catalytic mechanism; the enzymes reduce the target MetO at the expense of themselves becoming oxidized (*9*, *10*).

A decline in Msr activity has also been linked to human aging and degenerative diseases, including Alzheimer's disease (AD) (*11*). AD is an example of the so-called conformational diseases or amyloidoses, which also include diseases such as Parkinson's disease, type II diabetes, and the transmissible spongiform encephalopathies or prion diseases (*12*). These diseases are all characterized by the accumulation of misfolded proteins, which subsequently aggregate and deposit, either intra- or extracellularly, as amyloid fibrils (*12*). Deposition of amyloid fibrils in tissues is then thought to be causative for diseases such as AD; however, more recent evidence proposes that small prefibrillar oligomers may have more toxic properties (*13*). Methionine oxidation has been shown to have an impact on amyloid formation both in vitro (*14*−*18*) and in vivo (*19*). In addition, an MsrA knockout mouse was found to have an elevated level of deposition of Aβ and subsequent neurodegeneration (*20*). These studies all implicate methionine oxidation and Msr activity in the pathogenesis of amyloid diseases.

Apolipoprotein (apo) C-II is a member of a family of apolipoproteins with a propensity to assemble into amyloid fibrils (*21*). Under lipid free conditions, monomeric apoC-II rapidly misfolds and aggregates at physiological pH into twisted-ribbon-like fibrils (*22*). ApoC-II contains two methionine residues (Met-9 and Met-60), with Met-60 located within the core fibril structural region (*23*). We have previously shown that methionine oxidation weakens the ability of apoC-II fibrils to assemble in vitro and promotes their disassembly (*14*). To address the question of whether oxidized amyloid fibrils are substrates of the Msr system, the effect of MsrA and MsrB2 on methionine-oxidized apoC-II monomer and fibrils was explored.

Human recombinant MsrA and MsrB2 were expressed and purified according to the method of Jung et al. (*24*), with some modifications (see the Supporting Information). ApoC-II with both methionine residues oxidized (MetO-apoC-II) was prepared as previously described (*14*) (see the Supporting Information). To compare the ability of MsrA and MsrB2 to reduce MetO-apoC-II monomer and fibrils, HPLC was employed, to quantify the amount of unoxidized material. Monomeric MetO-apoC-II was incubated at a concentration of 112 μM at room temperature in refolding buffer [100 mM sodium phosphate (pH 7.4)] for 7 days. Under these conditions, MetO-apoC-II can be induced to form fibrils, after some delay (*14*). Aliquots of the MetO-apoC-II fibril sample (100 μL) were removed and incubated with MsrA and MsrB2, each enzyme at a final concentration ranging from 0.05 to 2 μM (*24*). A sample of the methionine-oxidized preparation was also freshly refolded (i.e., monomeric apoC-II) into refolding buffer and then immediately incubated with MsrA and MsrB2, as per the fibril sample. The HPLC traces of the fibril and monomer samples incubated with MsrA and MsrB2 are shown in Figure 1. Two peaks were visible in both the monomeric and fibril samples, eluting at ~35.3 and 36.5 min. These peaks were identified as apoC-II with Met-60 oxidized and reduced, respectively. For

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*To whom correspondence should be addressed. E-mail: katrinabinger@gmail.com. Phone: +61 3 8344 2274. Fax: +61 3 9348 1421.

[1]Abbreviations: apo, apolipoprotein; AD, Alzheimer's disease; MetO, methionine sulfoxide; MetO-apoC-II, methionine-oxidized apoC-II; Msr, methionine sulfoxide reductase(s); PrP, prion protein.

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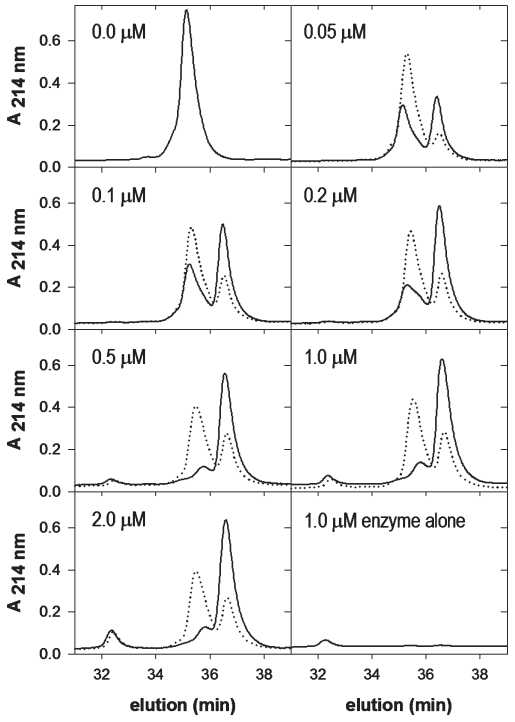

FIGURE 1: Comparison of the reduction of methionine-oxidized monomer and fibrillar apoC-II by MsrA and MsrB2 by HPLC. Methionine-oxidized monomer (solid lines) and fibrils (dotted lines) were incubated with the desired concentration of MsrA and MsrB2 and analyzed by HPLC. The enzyme concentration reflects the individual concentration of each enzyme; i.e., a concentration of 2 μM indicates 2 μM MsrA and 2 μM MsrB2.

ease, Met-60 oxidized and reduced apoC-II will be termed "oxidized" and "unoxidized" apoC-II, respectively. Full details of HPLC peak assignments are provided in the Supporting Information. A small peak was present at ~32.3 min, which was also present in a control solution of enzyme alone, indicating this was a low-molecular weight contaminant from the enzyme preparations.The relative amounts of MetO and unoxidized apoC-II were determined via analysis of the HPLC traces according to a multiple-Gaussian model (see the Supporting Information). The percentage of unoxidized protein was calculated by determining the difference in the total amount of the MetO-apoC-II peak (35.3 min) at each concentration of Msr, compared to the amount of the MetO-apoC-II peak in the sample with no Msr present (Figure 2). At Msr concentrations of $\geq 0.5\,\mu$M, approximately $90 \pm 9\%$ of monomeric MetO-apoC-II is reduced. In contrast, at 0.5 μM Msr, only $35 \pm 6\%$ of fibrillar MetO-apoC-II is reduced, which increased to only $37 \pm 8\%$ after incubation with a 4-fold higher enzyme concentration. One possibility is that a proportion of this reduced material is not fibrillar in nature, but instead in solution as a free monomeric pool (*25*). To address this, we prepared MetO-apoC-II fibrils as described above and then purified from free monomer by centrifugation. The fibrils (pellet) were then resuspended in refolding buffer, treated with MsrA and MsrB2, and analyzed by mass spectrometry. Monomeric MetO-apoC-II was also analyzed as a control. After incubation of monomeric MetO-apoC-II with both MsrA and MsrB2, a shift in the mass spectrum of −32 Da (from 8947 to 8915 Da) was visible, indicating the complete loss of two oxygen molecules (Figure 3). In contrast, fibrillar MetO-apoC-II had three main species, with masses of 8915, 8931, and 8947 Da. The large amount of singly oxidized

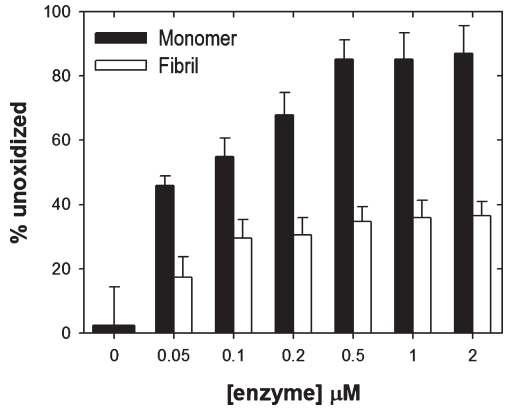

FIGURE 2: Extent of reduction of methionine-oxidized monomer (black) and fibrils (white) by treatment with increasing concentrations of MsrA and MsrB2. The HPLC profiles shown in Figure 1 were analyzed according to a multiple-Gaussian model to determine the percentage of unoxidized apoC-II.

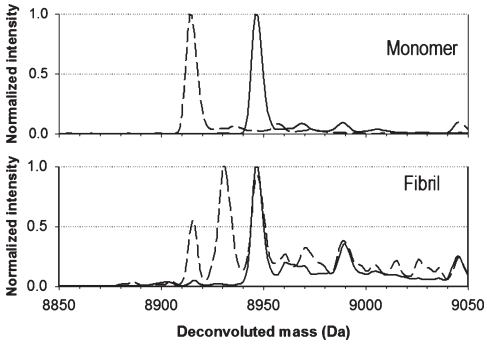

FIGURE 3: Reduction of methionine-oxidized monomeric and purified, fibrillar apoC-II by MsrA and B2. ApoC-II with both methionines oxidized (solid line) was incubated with 2 μM MsrA and 2 μM MsrB2 for 30 min at 37 °C in the presence of 15 mM DTT (dashed line). The deconvoluted mass spectrum is presented with a normalized signal intensity.

apoC-II in this fibril sample is most likely to be composed of Met-9 oxidized apoC-II. This is supported by the HPLC results (Figure 1), which did not show a significant amount of Met-60 oxidized apoC-II present. The identification of small amounts of singly oxidized and completely unoxidized apoC-II shows that the fibrils are able to be reduced; however, reduction is much less efficient for fibrils than for monomeric apoC-II.

In summary, we have shown that methionine-oxidized apoC-II amyloid fibrils are a poor substrate for MsrA and MsrB2. This work provides a simple explanation for why methionine-oxidized, aggregated proteins accumulate in amyloid diseases, compared with others who have proposed mechanisms such as concentrations of oxidized proteins being too high to be repaired, self-oxidation and inactivation of Msr, or a reduced level of protein expression (*11*, *18*, *26*). This work is particularly relevant for those amyloid proteins that polymerize intracellularly and are thus in the same location as the Msr. Several studies have identified amyloid aggregates intracellularly (*27*, *28*), and there is accumulating evidence that small oligomers assemble intracellularly prior to extracellular amyloid plaque deposition (*29*, *30*).

Preliminary evidence also suggests that oxidized amyloid aggregates are not able to be reduced in vivo. A recent study has examined the presence of prion protein (PrP) containing methionine sulfoxides, in the brains of animals infected with PrP^{Sc} (*31*). The authors show that MetO is present in the

amyloidogenic conformation (PrP^{Sc}), while the native protein, PrP^{c}, is not oxidized. The authors propose that PrP^{c} is readily reduced by the Msr while PrP^{Sc} is a poorer substrate for this system, and thus, MetO residues persist (*31*). As amyloid fibrils are structurally similar, it may hold true that methionine-oxidized amyloid fibrils formed from other amyloidogenic proteins, such as α-synuclein and the amyloid-β peptide, are also poor substrates for the Msr system.

Several studies have shown amyloid fibrils isolated ex vivo contain high levels of MetO (*32*−*34*). However, specific details of the mechanism by which MetO is involved in the pathogenesis of amyloidoses, and whether this oxidation occurs before or after amyloid formation, remain unknown. A recent study by our laboratory has shown that methionine oxidation induces the formation of apolipoprotein A-I amyloid fibrils in vitro (*35*), suggesting a role for MetO in the formation of amyloid. For other systems in which oxidation has been shown to inhibit or reduce fibril formation in vitro, the question of how MetO is involved in amyloidosis remains. It has been suggested that MetO may reduce the extent of fibril formation but promote the formation of prefibrillar oligomeric species (*15*, *19*), providing a mechanism by which methionine oxidation may specifically play a role in oligomer-mediated toxicity and amyloidosis. Combined with the results presented here, showing that aggregated species containing MetO are less effectively repaired by the Msr system, this may result in the persistence and self-perpetuation of these toxic oligomeric species.

SUPPORTING INFORMATION AVAILABLE

Details of experimental procedures and HPLC peak assignment. This material is available free of charge via the Internet at http://pubs.acs.org.

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