Methionine oxidation inhibits fibrillation of human α-synuclein in vitro

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Received 31 January 2002; revised 15 March 2002; accepted 15 March 2002

First published online 4 April 2002

Edited by Thomas L. James

Abstract We examined the effect of methionine oxidation of human recombinant α-synuclein on its structural properties and propensity to fibrillate. Both oxidized and non-oxidized α-synucleins were natively unfolded under conditions of neutral pH, with the oxidized protein being slightly more disordered. Both proteins adopted identical partially folded conformations under conditions of acidic pH. The fibrillation of α-synuclein at neutral pH was completely inhibited by methionine oxidation. This inhibitory effect was eliminated at low pH. The addition of oxidized α-synuclein to the unoxidized form led to a substantial inhibition of α-synuclein fibrillation.

Key words: Parkinson's disease; α-Synuclein; Oxidative stress; Methionine oxidation; Fibrillation; Inhibition of fibrillation; Partially folded intermediate

1. Introduction

Although oxygen is important for life, under certain conditions it can give rise to a number of highly reactive and, thus, potentially toxic species. Under normal conditions, cells are able to eliminate reactive oxygen species (ROS) by antioxidants, metal chelators or enzymatic reduction. However, when the level of ROS exceeds its defense mechanisms, the cell may suffer from the oxidative stress. Organisms have evolved complex antioxidant defenses to minimize oxidative damage to proteins and other macromolecules. They also possess repair systems for reversing some oxidative modifications and disposal systems for removing modified macromolecules, which are not repaired.

All amino acids are susceptible to oxidation, although their susceptibilities vary greatly [1]. It is known that methionine is one of the most readily oxidized amino acid constituents of proteins. It is easily oxidized to methionine sulfoxide, MetO, by H_2O_2, hydroxyl radicals, hypochlorite, chloramines, and peroxynitrite; all these oxidants are produced in biological systems [2]. However, this modification can be repaired by MetO reductase (MSR), which catalyzes the thioredoxin-dependent reduction of MetO back to methionine [3,4]. Based on these observations it is assumed that oxidation of surface-exposed methionines may serve to protect other functionally essential residues from oxidative damage [5].

Oxidation-modified proteins accumulate during normal aging [6–8], and oxidative injury is implicated in the pathogenesis of several disorders including Alzheimer's disease (AD) [9], Parkinson's disease (PD) [10], dementia with Lewy bodies (LBs) [11], amyotrophic lateral sclerosis [12], Huntington's disease [13], inflammatory diseases [14], cataractogenesis [14], atherosclerosis [15], brain ischemia and carcinogenesis [16].

PD is the second most common neurodegenerative disorder (after AD) that affects approximately 1% of the population over age 60. PD symptoms are attributed to the progressive loss of dopaminergic neurons from the substantia nigra region of brain. Some surviving nigral dopaminergic neurons contain cytosolic filamentous inclusions known as LBs and Lewy neurites (LNs) [17,18]. Several observations have implicated the presynaptic protein α-synuclein in the pathogenesis of PD. α-Synuclein was shown to be a major fibrillar component of LBs and LNs [19], and the missense mutations A53T and A30P in α-synuclein have been identified in a small number of kindreds with autosomal-dominantly inherited, early-onset PD [20,21]. Finally, the production of α-synuclein in transgenic mice [22] or in transgenic flies [23] leads to the motor deficits and neuronal inclusions reminiscent of PD.

α-Synuclein is a small (14 kDa), highly conserved presynaptic protein that is abundant in various regions of the brain [24]. It has four methionines, Met1, Met5, Met116 and Met127, located outside the repeat-containing region. Structurally, purified α-synuclein belongs to the rapidly growing family of natively unfolded proteins [25–35], which have little or no ordered structure under physiological conditions, due to a unique combination of low overall hydrophobicity and large net charge [36]. In vitro, the protein forms fibrils with morphologies and staining characteristics similar to those extracted from disease-affected brain [37–42].

Oxidative injury has been implicated in the pathogenesis of PD [10,43]. The existence of extensive and widespread accumulation of nitrated α-synuclein (i.e. protein containing the product of the tyrosine oxidation, 3-nitrotyrosine) in the signature inclusions of PD brains has been demonstrated [10,43]. Accumulation of another product of tyrosine oxidation, dityrosine, has been detected in vitro during experiments on the aggregation of α-synuclein in the presence of copper and H_2O_2 [44] or catecholamines [45]. Despite these reports, noth-

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0014-5793/02/$22.00
PII: S0014-5793(02)02638-8

ing is currently known about the effect of methionine oxidation on the structural and fibrillation properties of α-synuclein. In this report, we describe the effect of methionine oxidation to the sulfoxide on the structural and fibrillation properties of human recombinant α-synuclein in vitro.

2. Materials and methods

2.1. Expression and purification of human α-synuclein

Human wild-type (WT) α-synuclein was expressed in the *Escherichia coli* BL21(DE3) cell line transfected with pRK172/α-synuclein WT plasmid (kind gift of M. Goedert, MRC Cambridge) and purified by a procedure described by Conway et al. [37].

2.2. Supplies and chemicals

Thioflavine T (TFT) was obtained from Sigma, St. Louis, MO, USA. All other chemicals were of analytical grade from Fisher Chemicals.

2.3. Oxidation of α-synuclein by hydrogen peroxide

Oxidation of α-synuclein was induced by the incubation of protein solution (3.12 mg/ml in 25 mM sodium phosphate buffer, 100 mM NaCl, pH 8.0) in the presence of 4% H_2O_2 for 20 min. Non-reacted peroxide was removed from the protein solution by ultrafiltration (10 000 molecular weight cutoff, Microcon (Amicon)) at 7000 rpm for 2 h. Success of oxidation was confirmed by mass spectrometry (MS) analysis (MicroMass Quattro II). Samples for MS analysis were prepared by diluting 2 μl of protein solution in 200 μl of 50% acetonitrile/50% pH 2.0 HCl mixture.

2.4. Circular dichroism (CD) measurements

CD spectra were obtained on an AVIV 60DS spectrophotometer (Lakewood, NJ, USA) using α-synuclein concentrations of 1.0 mg/ml. Spectra were recorded in 0.01 cm cells from 250 to 190 nm with a step size of 1.0 nm, with a bandwidth of 1.5 nm, and an averaging time of 10 s. For all spectra, an average of five scans was obtained. CD spectra of the appropriate buffers were recorded and subtracted from the protein spectra.

2.5. Fourier transform infrared (FTIR) spectra

Data were collected on a Nicolet 800SX FTIR spectrometer equipped with an MCT detector. The IRE (72×10×6 mm, 45° germanium trapezoid) was held in a modified SPECAC out-of-compartment ATR apparatus. The hydrated thin films were prepared and analyzed as described previously [46].

2.6. Fibril formation assay

Fibril formation of oxidized and non-oxidized α-synuclein at pH 3.0 and 7.5 (20 mM Na-phosphate buffer, 100 mM NaCl) was monitored in a fluorescence plate reader (Fluoroskan Ascent). Protein solutions contained 20 μM TFT and protein at a concentration of 1.0 mg/ml (70 μM) in the corresponding fibrillation buffer. A volume of 145 μl of the mixture was pipetted into a well of a 96-well plate (white plastic, clear bottom) and a 1/8 inch diameter Teflon sphere (McMaster-Carr, Los Angeles, CA, USA) was added. Each sample was run in triplicate or quadruplicate. The plate was loaded into a fluorescence plate reader and incubated at 37°C with shaking at 150 rpm with a shaking diameter of 20 mm. The fluorescence was measured at 30 min intervals with excitation at 450 nm and emission at 485 nm, and curve fit as described in [47].

3. Results and discussion

3.1. In vitro oxidative modification of human α-synuclein

MS analysis of α-synuclein prior to the oxidative modification revealed one major peak, whose molecular mass was centered at 14 460 ± 3 Da, which coincides with the molecular mass of human α-synuclein calculated from its amino acid sequence (14 460.09 Da). The conditions used for the in vitro oxidative modification of α-synuclein result in oxidation of all four methionines, as it was evidenced by the MS major peak positioned at 14 524 ± 3 Da that corresponds to the mass of human α-synuclein plus four oxygens (i.e. 14 460.09 Da+16.00×4 Da = 14 524.09 Da).

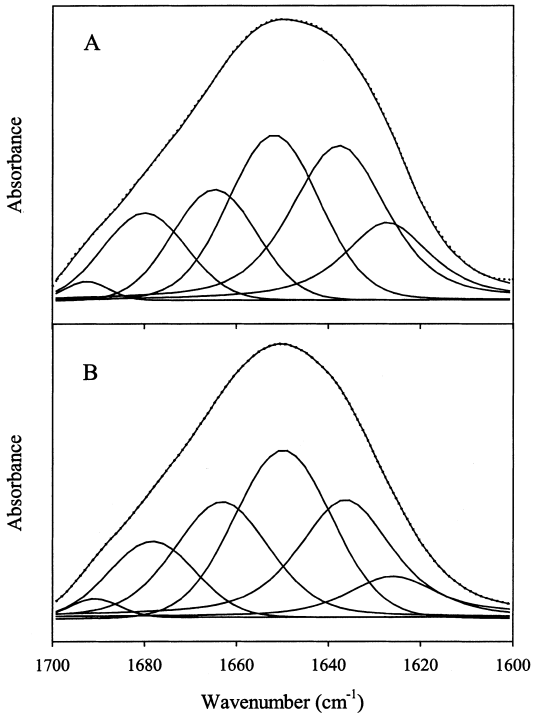

Fig. 1. Secondary structure analysis of non-oxidized (A) and oxidized α-synuclein (B) by FTIR. FTIR spectra of the amide I region were measured at pH 7.5 (solid line). Curve fit spectra are present by dotted lines.

3.2. Effect of methionine oxidation on secondary structure of human α-synuclein

Fig. 1 shows the FTIR (amide I region) spectra measured for non-oxidized (A) and oxidized forms (B) of human recombinant α-synuclein at pH 7.5. The FTIR spectra of both proteins are typical of a substantially unfolded polypeptide chain. The results of the deconvolution (FSD and second derivative) of the FTIR spectra are summarized in Table 1 and show that α-synuclein-MetO contains smaller amounts of β-structure under these conditions. We attribute the increased degree of unfolding of the oxidized protein to the decreased hydrophobicity of oxidized methionine [2] leading to a decrease in the overall hydrophobicity of the protein. In fact, in contrast to the cysteine oxidation, which will change the charge on the protein, the conversion of Met to MetO is expected to modify the polarity and hydrophobicity of polypeptide chain, but not its charge [2]. Moreover, the formation of MetO was even regarded as a chemical 'mutagenesis', which substitutes the hydrophobic and flexible side chain of methionine with MetO, a larger, more polar and less flexible side chain [48]. Interestingly, it has recently been reported that another member of the synuclein family, β-synuclein, which lacks the 11 residues of the central hydrophobic region of α-synuclein, also has the properties expected of a random coil, whereas some residual structure was detected in α-synuclein [49]. Thus, a decrease in the overall hydrophobicity in both β-synuclein and α-synuclein-MetO leads to a more disordered conformation, compared to α-synuclein.

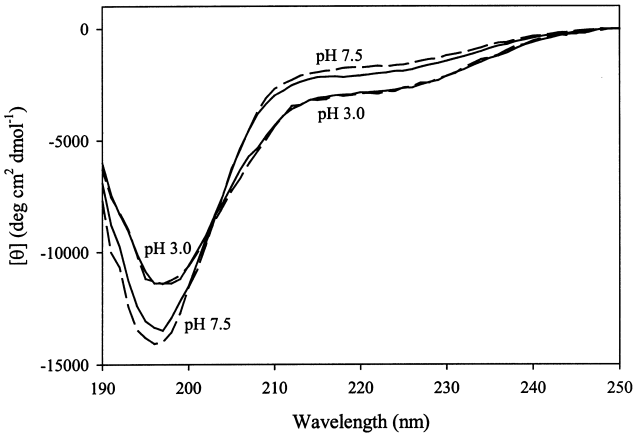

Fig. 2. Comparison of the effect of pH on far-UV CD spectra of non-oxidized (solid lines) and oxidized α-synuclein (dashed lines). Far-UV CD spectra were measured at pH 7.5 and pH 3.0 (20 mM Na-phosphate buffer, 100 mM NaCl). For far-UV CD measurements the cell pathlength was 0.1 mm. Measurements were carried out at 23°C. Protein concentration was 0.5 mg/ml.

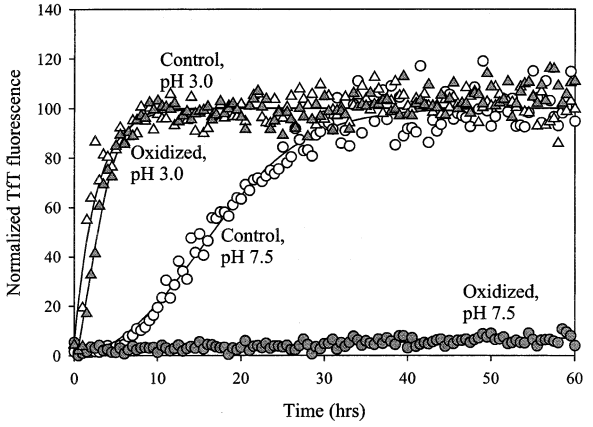

Fig. 3. Kinetics of fibrillation of non-oxidized (open symbols) and oxidized (gray symbols) α-synuclein monitored by the enhancement of TFT fluorescence intensity. Measurements were performed at 37°C and pH 7.5 (circles) or pH 3.0 (triangles) in 20 mM Na-phosphate buffer containing 100 mM NaCl. ThT fluorescence was excited at 450 nm, and the emission wavelength was 482 nm.

In agreement with FTIR data, the far-UV CD spectrum of α-synuclein-MetO measured at pH 7.5 shows a slightly increased degree of disorder in comparison with the non-oxidized protein (Fig. 2). This is manifested by a small increase in negative ellipticity in the vicinity of 196 nm and somewhat lower intensity in the vicinity of 222 nm. As the pH is decreased, a decrease in the minimum at 196 nm is accompanied by an increase in negative intensity around 222 nm, reflecting pH-induced formation of secondary structure (Fig. 2). Importantly, non-oxidized and oxidized proteins possess almost identical far-UV CD spectra at acidic pH. Moreover, the pH-induced changes in the far-UV CD spectra of both forms of α-synuclein were completely reversible. Previously we have shown that the pH-induced increase in structure in members of the synuclein family represents an intramolecular process involving the formation of a partially folded intermediate, and not self-association [26,40,49]. Thus, this also appears to be the situation for the MetO form of human α-synuclein.

3.3. The effect of methionine oxidation on the fibrillation of α-synuclein

TFT is a fluorescent dye that interacts with amyloid fibrils leading to an increase in the fluorescence intensity in the vicinity of 480 nm [50]. Fig. 3 compares fibrillation patterns of non-oxidized and oxidized α-synuclein monitored by TFT fluorescence. Fibril formation for the non-oxidized α-synuclein at neutral pH is characterized by a typical sigmoidal curve. In contrast, there was no evidence of fibril formation by methionine-oxidized α-synuclein at neutral pH on the timescale shown. In fact, fibrils were not formed even after incubation of α-synuclein-MetO for 300 h.

In agreement with earlier studies [26,49], acidification of non-oxidized α-synuclein solution leads to significant acceleration of the fibril formation process (Fig. 3). This is attributed to the pH-induced stabilization of a partially folded intermediate, which represents a crucial primary step in the α-synuclein fibrillation pathway [26]. Fig. 3 shows that the non-oxidized and oxidized forms of α-synuclein fibrillate similarly under conditions of low pH. This means that the inhibitory effect of methionine oxidation is eliminated under conditions of low pH, which favor formation of a partially folded intermediate in both forms of the protein.

3.4. Aggregation of non-oxidized and oxidized α-synucleins

In the presence of methanol, α-synuclein undergoes a conformational change and self-associates to form soluble oligomers, enriched with β-structure, which aggregate and form insoluble precipitates at higher protein and methanol concentrations [40]. Consequently, neutral pH methanol solutions represent suitable conditions to study the propensity of α-synuclein to aggregate. We have established that in contrast to the non-oxidized protein, α-synuclein-MetO does not form insoluble aggregates in 80% methanol and high protein concentrations. In fact, there was no light scattering in the solution of the α-synuclein-MetO even when the protein concentration was as high as 3 mg/ml (data not shown). Thus, our

Table 1
Secondary structure analysis of non-oxidized and oxidized forms of human α-synuclein, determined by FTIR

Structural assignment	α-Synuclein, non-oxidized		α-Synuclein, oxidized	
	Wavenumber (cm^{-1})	%	Wavenumber (cm^{-1})	%
Turn or β-sheet	1692	1.4 ± 0.1	1691	1.6 ± 0.1
Turn	1680	12.7 ± 0.9	1678	12.4 ± 0.9
Loops	1664	16.4 ± 1.1	1663	22.1 ± 1.5
Disordered	1651	27.1 ± 1.9	1649	31.5 ± 2.2
β-Sheet	1637	28.7 ± 2.0	1636	26.1 ± 1.9
β-Sheet	1627	13.8 ± 1.0	1625	6.3 ± 0.5

The estimated error in the frequencies is ± 1.5 cm^{-1}.

data are consistent with the conclusion that the propensity of α-synuclein to aggregate is significantly diminished by the methionine oxidation. Similar MetO-induced inhibition of aggregation has been reported earlier for Aβ(1–40) [51].

3.5. α-Synuclein-MetO inhibits fibrillation of the non-oxidized protein

Fig. 4 represents an intriguing set of data on the effect of oxidized α-synuclein on the fibrillation kinetics of the non-oxidized protein. The addition of oxidized protein in a two-fold molar excess to 70 μM solution of non-oxidized α-synuclein substantially increased the duration of the lag time, but had little effect on the rate constant for fibril growth (Fig. 4). Further increase in the relative concentration of the oxidized protein (4:1 molar excess of α-synuclein-MetO over the non-oxidized α-synuclein) led to almost complete inhibition of the non-oxidized protein fibrillation (Fig. 4). These results suggest that a non-productive interaction between oxidized and non-oxidized forms of human α-synuclein inhibits the formation of nuclei, rather than growth of nascent fibrils. Similar mutual inhibition with respect to nucleation, but not to fibril elongation, has been reported for several other pairs of homologous proteins. Examples include non-fibrillogenic γ-chain/fibrillogenic mutant β-chain of hemoglobin in sickle cell disease [52]; Aβ40/Aβ42 in Alzheimer's β-amyloid fibril formation [53]; mouse/WT human and mouse/A53T human α-synucleins [54]; human β/α- and γ/α-synucleins [49]. Importantly, it has been reported that inhibition of mouse α-synuclein fibrillation by human WT and A53T proteins leads to the accumulation of non-fibrillar spherical oligomers [54]. We assume that the inhibition of fibril formation by non-oxidized α-synuclein in the presence of oxidized protein also originates from the effective formation of soluble hetero-oligomers, which are located off the fibrillation pathway.

Models for the aggregation of non-oxidized (A) and oxidized (B) α-synuclein at neutral pH are shown in Fig. 5. The model for aggregation of both forms of the protein at acidic pH is also shown for comparison (Fig. 5C). In the model ***O***, ***F*** and ***A*** represent soluble oligomers, fibrils and amorphous aggregates, respectively; $\boldsymbol{U_N}$ and $\boldsymbol{U_{N*}}$ are the natively unfolded states of non-oxidized and oxidized α-synuclein, respectively; and ***I*** represents the partially folded intermediate. The Roman numerals indicate the major stages of the aggregation process. Taking into account the observations presented in the current study, we propose that methionine oxidation leads to a decrease in α-synuclein residual structure ($\boldsymbol{U_{N*}}$ vs. $\boldsymbol{U_N}$), due to the oxidation-induced increase in the overall polarity of the protein. This leads to a shift in the equilibrium in stage I for the oxidized protein, $\boldsymbol{U_{N*} \leftrightarrow I}$, in favor of the unfolded conformation $\boldsymbol{U_{N*}}$. Finally, stages II, III and V are arrested (or at least strongly inhibited) by the oxidation at neutral pH, but formation of soluble oligomers still occurs, stage IV (Fig. 5B). Both forms of α-synuclein show similar aggregation behavior at acidic pH (Fig. 5C). Here non-oxidized and oxidized forms of α-synuclein are more prone to form the partially folded conformation (thicker arrow at stage I) and, as a consequence, show a faster rate of aggregation (stages II–V).

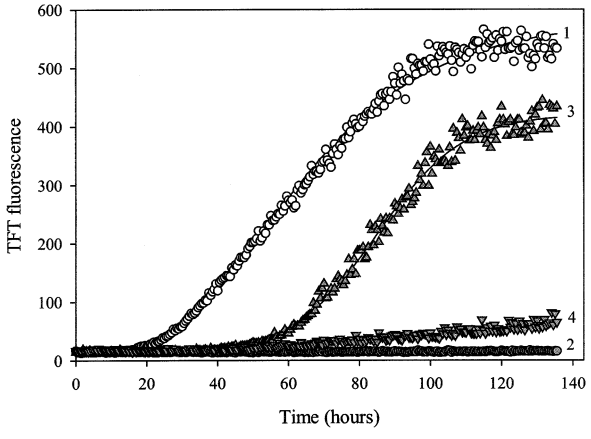

Fig. 4. Inhibition of fibrillation of non-oxidized α-synuclein in the presence of oxidized α-synuclein. The fibrillation kinetics were studied for 70 μM non-oxidized α-synuclein solution in 20 mM Na-phosphate buffer, 100 mM NaCl, pH 7.5 in the absence (1) or presence of oxidized protein in a 1:2 (3), and 1:4 molar ratio (4). Fibrillation kinetics of the oxidized proteins are shown for comparison (2).

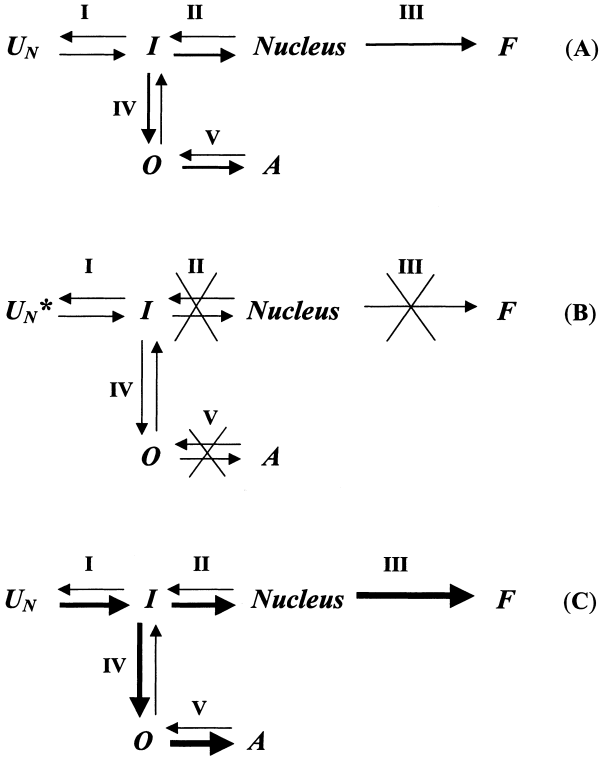

Fig. 5. Models of non-oxidized and oxidized α-synuclein aggregation under the different experimental conditions (see the text for explanations). A: Aggregation of the non-oxidized protein at neutral pH. B: Aggregation of the oxidized protein at neutral pH. C: Aggregation of non-oxidized and oxidized proteins at acidic pH.

A model for the inhibitory effect of oxidized α-synuclein on the non-oxidized protein fibrillation is shown in Fig. 6. In the mixture of non-oxidized and oxidized proteins the conformational equilibrium is shifted toward the formation of soluble hetero-oligomers.

Giving the prevailing view [10,43] that oxidative stress is a key factor in the etiology of PD, our data on the effect of methionine oxidation are interesting. We have shown that methionine-oxidized α-synuclein, which is expected to represent one of the most common products of oxidative damage to α-synuclein, fails to form fibrils at neutral pH. Moreover, fibrillation of non-oxidized α-synuclein is inhibited in the presence of an excess of methionine-oxidized α-synuclein. As

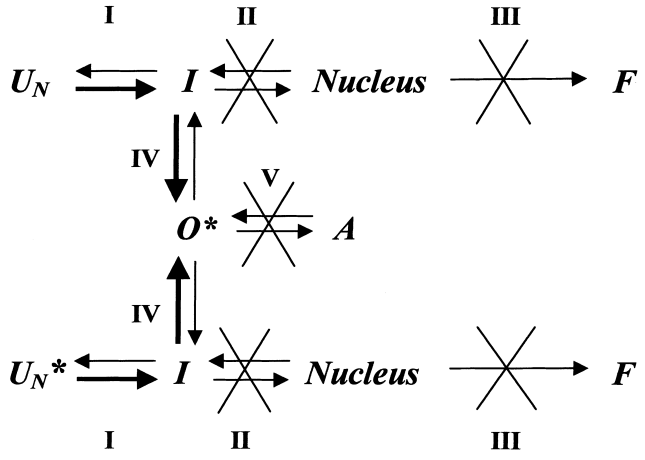

Fig. 6. Model of the inhibition of non-oxidized α-synuclein fibrillation in the presence of oxidized protein (see the text for explanations).

noted, oxidized methionine can be repaired by MSR, which catalyzes the thioredoxin-dependent reduction of MetO back to methionine [3–5]. Based on this observation it was assumed that reversible oxidation of methionines might protect other functionally essential residues from oxidative damage [5]. The efficiency of such protection in case of α-synuclein may be significant, since the concentration of α-synuclein is high (this protein has been estimated to account for as much as 1% of the total protein in soluble cytosolic brain fractions [55]). Moreover, it has been shown recently that exposure of mice to the herbicide paraquat causes upregulation of α-synuclein, giving rise to the idea that increased expression of α-synuclein is a part of a neuronal response to toxic insult [56]. This observation, together with the potential protective role of methionine, leads to an intriguing hypothesis that α-synuclein may be used by the cells as a natural scavenger of ROS. This may keep α-synuclein from irreversible over-oxidation, but at the same time may protect any oxidizable proteins present in the α-synuclein microenvironment, and may also protect against lipid oxidation. Most importantly, the proposed α-synuclein scavenging system, which may protect α-synuclein from over-oxidation, is dependent upon the effectiveness of the MSR system to continually recycle the oxidized methionine residues. We assume that the efficiency of the MSR system in the substantia nigra of healthy vs. Parkinsonian brains may differ. Of potential relevance in this regard is a recent report indicating a decline in the activity levels of MSR in various regions of the brains of Alzheimer's patients [57]. Thus, impaired MSR function may represent an additional risk factor favoring the development of PD.

Acknowledgements: This research was supported by grants from the National Institutes of Health.

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