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Original Contribution

Redox activity of α-synuclein–Cu is silenced by Zn_7-metallothionein-3

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ABSTRACT

The aggregation of α-synuclein (α-Syn), the major component of intracellular Lewy body inclusions in dopaminergic neurons of the substantia nigra, plays a critical role in the etiology of Parkinson disease (PD). Long-term effects of redox-active transition metals (Cu, Fe) and oxidative chemical imbalance underlie the disease progression and neuronal death. In this work, we provide evidence that a brain metalloprotein, Zn_7-metallothionein-3 (Zn_7MT-3), possesses a dynamic role in controlling aberrant protein–copper interactions in PD. We examined the properties of the α-Syn–Cu(II) complex with regard to molecular oxygen, the biological reducing agent ascorbate, and the neurotransmitter dopamine. The results revealed that under aerobic conditions α-Syn–Cu(II) possesses catalytic oxidase activity. The observed metal-centered redox chemistry significantly promotes the production of hydroxyl radicals and α-Syn oxidation and oligomerization, processes considered critical for cellular toxicity. Moreover, we show that Zn_7MT-3, through Cu(II) removal from the α-Syn–Cu(II) complex, efficiently prevents its deleterious redox activity. We demonstrate that the Cu(II) reduction by thiolate ligands of Zn_7MT-3 and the formation of $Cu(I)_4Zn_4$MT-3, in which an unusual oxygen-stable $Cu(I)_4$–thiolate cluster is present, comprise the underlying molecular mechanism by which α-Syn and dopamine oxidation, α-Syn oligomerization, and ROS production are abolished. These studies provide new insights into the bioinorganic chemistry of PD.

Parkinson disease (PD) is the most common neurodegenerative movement disorder, characterized by a selective loss of dopaminergic neurons in the substantia nigra pars compacta and by the presence of intraneuronal inclusions, named Lewy bodies, of amyloid fibrils largely constituted by the small (~14 kDa) protein α-synuclein (α-Syn) [1,2]. A large amount of extracellular α-Syn has also been found in the cerebrospinal fluid of PD patients. Long-term effects of environmental factors that promote oxidative stress and the aberrant binding of the redox-active transition metals iron and copper to α-Syn have been related to the disease progression and neuronal death. Accelerating effects of metals (Al (III), Fe(III), Cu(II)) on α-Syn fibrillation in vitro have been shown [3]. Initial work showed that Cu(II) is the most effective ion at enhancing the α-Syn oligomerization [4]. There is increasing evidence that altered metal homeostasis may be involved in the progression of neurodegenerative diseases [5,6]. In this regard, aberrant metal–protein interactions involving the amyloidogenic proteins amyloid-β (Aβ), in Alzheimer disease (AD); α-Syn, in PD (synucleinopathies); and prion protein (spongiform encephalopathies) are critical for the progression of these neurodegenerative diseases. Copper as a cofactor in several enzymes can activate molecular oxygen (O_2) to perform controlled catalytic oxidation of substrates. However, redox cycling of aberrantly bound copper may catalyze the generation of reactive oxygen species (ROS) through Fenton-type reactions [7]. Aberrant copper binding to α-Syn and oxidative stress seem to contribute to the degeneration of dopaminergic neurons through the abnormal oligomerization and subsequent aggregation of this protein [8]. α-Syn binds Cu(II) ions with a high affinity (nanomolar range) at the N-terminus. At this site, Cu(II) is anchored through the N-terminal amino group of Met_1 (NH_2) and the deprotonated backbone amide (N^-), carboxylate of Asp_2, and water in a square planar or distorted tetragonal geometry [9,10]. A recent EPR study suggests the presence of two binding modes, which are interrelated and share three of the same ligands, whereas the fourth is interchangeable between a nitrogen and an oxygen ligand [11].

Previous studies of individuals affected by PD and hemiparkinsonian rats indicate that the expression levels and radical-

Abbreviations: Aβ, amyloid-β; AD, Alzheimer disease; α-Syn, α-synuclein; CC, cluster centered; 3-CCA, coumarin 3-carboxylic acid; CD, circular dichroism; CysS, cysteine thiolate; DEAE, diethylaminoethyl; EPR, electron paramagnetic resonance; ESI-Q-TOF MS, electrospray ionization quadrupole time-of flight mass spectrometry; MBTH, 3-methyl-2-benzothiazolinone hydrazone; MT-3, metallothionein-3; NMR, nuclear magnetic resonance; $OH^{\bullet}$, hydroxyl radical; $O_2^{\bullet-}$, superoxide radical; 7-OH-CCA, 7-hydroxycoumarin 3-carboxylic acid; PD, Parkinson disease; ROS, reactive oxygen species; SEC, size-exclusion chromatography; THB, 1,2,3-trihydroxylbenzene; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; UV–Vis spectroscopy, ultraviolet–visible electronic absorption spectroscopy.

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scavenging potency of metallothionein-3 (Zn_7MT-3) are reduced, thereby accelerating the disease progression [12,13]. Zn_7MT-3, a small (~7 kDa) metal- and cysteine-rich protein, is highly expressed in the normal human brain, where it plays an important role in the homeostasis of the essential metal ions Cu(I) and Zn(II) and in the regulation of neuronal outgrowth [14,15]. Structural studies revealed that Zn_7MT-3, like other mammalian metallothioneins, contains two metal–thiolate clusters localized in independent protein domains, i.e., the $Zn_3(CysS)_9$ cluster in the N-terminal β-domain and the $Zn_4(CysS)_{11}$ cluster in the C-terminal α-domain [16–18]. In the brain, the protein is present in neurons, astrocytes, and the extracellular space in comparable amounts [19]. Furthermore, Zn_7MT-3 rescues neuronal cells from the toxicity of Aβ peptide [20]. In our recent work, aimed at the understanding of this protective effect, we showed that efficient scavenging of Cu(II) bound to Aβ by Zn_7MT-3 is the underlying mechanism by which the ROS production and related cellular toxicity of Aβ–Cu(II) are abolished. In this process, a metal swap between Zn_7MT-3 and Aβ–Cu(II) concomitant with Cu(II) reduction and binding to MT-3 occurs [21]. However, at present a potential protective effect of Zn_7MT-3 in PD and the neurochemistry of the α-Syn–Cu(II) complex remain unexplored.

In this work, we address the question whether Zn_7MT-3 through Cu(II) removal can modulate the α-Syn–Cu(II) reactivity and whether a redox reaction is involved in this process. The studies were carried out using α-Syn-to-Cu(II) binding stoichiometries of ≤ 1, to ascertain the metal occupancy of the physiologically relevant high-affinity binding site. The results revealed that the α-Syn–Cu(II) complex possesses catalytic oxidase activity and that this reactivity is modulated by Zn_7MT-3.

Material and methods

Expression and purification of recombinant human α-synuclein

A pET-3d plasmid (Novagen) encoding the human α-Syn sequence was generated by Genescript (Piscataway, NJ, USA) and used for recombinant protein expression. α-Syn was expressed in *Escherichia coli* strain BL21(DE3) and purified as described previously [22]. The last purification step was performed on DEAE Sepharose Fast Flow (25 ml) anion-exchange column and the protein eluted with a linear salt gradient of 20 mM Tris–HCl, 1 M NaCl, pH 8.0. The correctness of the protein expression was verified by ESI MS (Supplementary Fig. S1).

Expression, purification, and metal reconstitution of recombinant human metallothionein-3

A pET-3d plasmid (Novagen) encoding the human MT-3 sequence was used for recombinant protein expression. The protein was expressed in *E. coli* strain BL21(DE3)pLys and purified as described previously [17]. Correctness of the expressed protein was confirmed by ESI MS (Supplementary Fig. S2). The metal-free protein (apoprotein) was generated using the method of Vašák [23]. The fully Zn^{2+}-loaded form was prepared by metal reconstitution [23]. Zinc-to-protein ratios were determined by measuring protein concentration photometrically in 0.1 M HCl ($\varepsilon_{220} = 53{,}000\ M^{-1}\ cm^{-1}$) and metal content was measured by flame atomic absorption spectroscopy (SpectrAA–110; Varian, Inc.). Cysteine-to-protein ratios were determined via photometric quantification of sulfhydryl groups (CysSH) upon their reaction with 2,2′-dithiopyridine in 0.2 M sodium acetate, 1 mM EDTA (pH 4), using $\varepsilon_{343} = 7600\ M^{-1}\ cm^{-1}$ [24] and those of zinc by atomic absorption. In all cases a zinc-to-protein ratio of 7.0 ± 0.3 and a CysSH-to-protein ratio of 20 ± 2 were obtained.

Measurements of dopamine oxidation using 3-methyl-2-benzothiazolinone hydrazone

The dopamine oxidation was determined by the spectrometric quantification of the quinone using 3-methyl-2-benzothiazolinone hydrazone (MBTH). Various dopamine concentrations (0.125–4 mM) were mixed with 1 mM MBTH in 20 mM 4-ethylmorpholine, 100 mM NaCl, pH 7.4, at 37 °C, in the presence of 10 μM α-Syn–Cu(II). This was generated by mixing α-Syn (10 μM) with $CuCl_2$ (10 μM). The formation of the red *ortho*-quinone product was monitored by electronic absorption spectroscopy over a period of 100 min by recording absorption spectra every 5 min. The autoxidation of dopamine was determined under the same conditions in the absence of α-Syn–Cu(II). The dopamine oxidation of 10 μM α-Syn–Cu(II) in the presence of 0.25 mol equivalent of Zn_7MT-3 was determined after 60 min incubation followed by the addition of MBTH and dopamine.

Determination of copper-catalyzed hydroxyl radical production

The ascorbate-driven copper-catalyzed hydroxyl radical production was monitored by fluorescence spectroscopy using the hydroxyl radical-scavenging compound 3-coumarin carboxylic acid (3-CCA; Sigma–Aldrich). The formation of the product 7-OH-CCA was followed by fluorescence spectroscopy (excitation at 395 nm; emission at 450 nm; SPEX fluorolog) over 15 min [25]. The hydroxyl radical production was measured upon the addition of ascorbate (300 μM) or dopamine (1 mM) to the samples containing (i) $CuCl_2$ (10 μM), (ii) α-Syn–Cu(II) generated upon the reaction of α-Syn (12.5 μM) with $CuCl_2$ (10 μM), or (iii) α-Syn–Cu(II) to which 2.5 μM Zn_7MT-3 was added. All measurements were carried out in 20 mM phosphate buffer, pH 7.4, containing 200 μM 3-CCA.

ESI-Q-TOF MS measurements of the oxidation products of α-synuclein

α-Syn–Cu(II) samples (60 μM) were incubated in 20 mM 4-ethylmorpholine, pH 7.4, for 6 h at 37 °C under agitation (300 rpm) in the absence or presence of 1 mM dopamine or 1 mM ascorbate. After incubation, the reducing agents were removed by three washing cycles on a Microcon ultrafiltration device using a 10-kDa molecular weight cut-off membrane. Similar experiments were conducted upon the reaction of α-Syn–Cu(II) with 0.25 mol equivalent of Zn_7MT-3. The samples were subsequently diluted into 50% acetonitrile, 0.2% formic acid, pH 2 and immediately analyzed by mass spectrometry. Mass spectra were obtained on a nano-ESI-Q-TOF MS Ultima API mass spectrometer (Micromass, UK). The mass spectra were deconvoluted using the MaxEnt1 program.

Fluorescence detection of dityrosine

α-Syn–Cu(II) samples (10 μM) containing 1 mM ascorbate were incubated in 20 mM phosphate buffer, pH 7.4, at 37 °C. Similar measurements were carried out upon the reaction of α-Syn–Cu(II) with 0.25 mol equivalent of Zn_7MT-3. Emission spectra between 375 and 550 nm were recorded at 37 °C on a SPEX fluorolog spectrofluorimeter using an excitation wavelength of 325 nm. Kinetics of dityrosine formation was monitored at the wavelength of 418 nm over a period of 1 h.

Characterization of α-Syn–Cu(II) oligomerization process by size-exclusion chromatography

α-Syn–Cu(II) samples (30 μM) were incubated in 20 mM 4-ethylmorpholine, pH 7.4, for 12 h at 37 °C under agitation (700 rpm) in the absence and presence of 1 mM dopamine. Aliquots of the samples were removed every 90 min and characterized by size-exclusion chromatography (SEC). Similar experiments were carried