Bicarbonate enhances α-synuclein oligomerization and nitration: intermediacy of carbonate radical anion and nitrogen dioxide radical

Christopher ANDREKOPOULOS, Hao ZHANG, Joy JOSEPH, Shasi KALIVENDI and B. KALYANARAMAN[1]

Department of Biophysics, Free Radical Research Center, Medical College of Wisconsin, 8701 Watertown Plank Road, P.O. Box 26509, Milwaukee, WI 53226, U.S.A.

α-Synuclein, a neuronal presynaptic protein, has been reported to undergo oligomerization to form toxic Lewy bodies in neurodegenerative disorders. One of the proposed mechanisms for aggregation of α-synuclein involves oxidative and nitrative modifications. In the present study, we show that addition of 3-morpholino-sydnonimine chloride (SIN-1) or slow infusion of pre-formed peroxynitrite ($ONOO^-$) to mixtures containing α-synuclein and HCO_3^- markedly enhanced both nitration and aggregation of α-synuclein through dityrosine formation. Bicarbonate-dependent peroxidase activity of Cu,Zn-superoxide dismutase (SOD1) also induced covalent aggregation of α-synuclein via a $CO_3^{\bullet -}$-dependent mechanism. Nitrone spin traps completely inhibited $CO_3^{\bullet -}$-mediated oxidation/nitration and aggregation of α-synuclein. Conversely, α-synuclein inhibited $CO_3^{\bullet -}$-induced spin adduct formation. Independent evidence for $CO_3^{\bullet -}$-mediated oxidation and dimerization of α-synuclein was obtained from UV photolysis of $[(NH_3)_5CoCO_3]^+$, which generates authentic $CO_3^{\bullet -}$. Irradiation of $[(NH_3)_5CoCO_3]^+$ and NO_2^- in the presence of α-synuclein yielded nitration and aggregation products that were similar to those obtained from a SIN-1 (or slowly infused $ONOO^-$) and HCO_3^- or a myeloperoxidase/H_2O_2/NO_2^- system. Hydrophobic membranes greatly influenced α-synuclein aggregation and nitration in these systems. We conclude that both $CO_3^{\bullet -}$ and $NO_2^{\bullet}$ could play a major role in the nitration/aggregation of α-synuclein.

Key words: Cu,Zn-superoxide dismutase (SOD1), electron spin resonance (ESR), neurodegenerative disease, Parkinson's disease, spin trapping, α-synuclein.

INTRODUCTION

α-Synuclein is a major component of Lewy bodies, the diagnostic brain lesions present at post-mortem in patients afflicted with Parkinson's disease [1]. Typically, α-synuclein exists in an aggregated and nitrated form in Parkinson's disease brain tissues [2]. Inflammatory reactive nitrogen species (RNS), such as peroxynitrite ($ONOO^-$), a potent oxidant formed from the reaction between $^{\bullet}NO$ and $O_2^{\bullet -}$ or $NO_2^{\bullet}$ [an oxidizing and nitrating intermediate generated from one-electron oxidation of NO_2^- by myeloperoxidase (MPO) and H_2O_2], were shown to induce nitration and oxidative aggregation of α-synuclein [3,4]. The peroxidase activity of bovine Cu,Zn-superoxide dismutase (SOD1) also promoted the aggregation of α-synuclein [5]. As these oxidation studies were performed in the presence of HCO_3^- [5], we decided to investigate more thoroughly the effect of HCO_3^- on oxidative aggregation and nitration of α-synuclein.

The bicarbonate anion is present in millimolar concentrations in cells and tissues. Under physiological conditions, $ONOO^-$ reacts rapidly with the CO_2 that is in equilibrium with HCO_3^- to form a nitrosoperoxycarbonate ($ONOOCO_2^-$) intermediate. This intermediate decomposes to $NO_2^{\bullet}$ and $CO_3^{\bullet -}$, potent nitrating and oxidizing species respectively [6,7]. The peroxidase activity of SOD1 is stimulated by HCO_3^- via a mechanism involving $CO_3^{\bullet -}$ [8,9]. However, the role of HCO_3^- in α-synuclein aggregation induced by SOD1 and H_2O_2 was not taken into consideration in the previous study [5]. In the present study, we clearly demonstrate that HCO_3^- plays a critical role in oxidation, nitration and aggregation of α-synuclein induced by $ONOO^-$ and the SOD1/H_2O_2 system.

We postulate that both $CO_3^{\bullet -}$ and $NO_2^{\bullet}$ are responsible for inducing the aggregation of α-synuclein via a radical mechanism involving tyrosine and methionine oxidation. $^{\bullet}NO$ released slowly from $^{\bullet}NO$ donors inhibited α-synuclein aggregation and nitration in these systems. Results from the present study also indicate that the hydrophobic lipid phase significantly alters α-synuclein oxidation, nitration and aggregation.

MATERIALS AND METHODS

α-Synuclein purification

Recombinant human α-synuclein was cloned and purified as follows. The full-length α-synuclein gene was generated using the forward (5′-CCCGGGCATGGATGTATTCATGAAAGGA-CTTTCA-3′) and reverse (5′-CTCGAGAGATATTTCTTAGG-CTTCAGGTTCGTAGT-3′) primers containing *Sma*I and *Xho*I restriction sites (underlined). The PCR product (approx. 440 bp) was purified and digested with *Sma*I and *Xho*I, and this fragment was ligated into pGEX 4T-1 vector (Amersham Biosciences, Little Chalfont, Bucks., U.K.) that was pre-digested with the same restriction enzymes using T4 DNA ligase from Invitrogen (Carlsbad, CA, U.S.A.). The ligated product (pGEX 4T-1$^{\alpha\text{-Syn}}$) was transformed in *Escherichia coli* DH5α cells, grown overnight at 37 °C and the plasmid DNA was purified using the Bio-Rad Maxiprep kit (Hercules, CA, U.S.A.). The DNA sequence was confirmed by sequencing the vector with both T7 and SP6 sequencing primers from Operon Technologies (Sunnyvale, CA, U.S.A.). α-Synuclein was expressed and purified from BL21 cells using glutathione–agarose beads according to a previously

Abbreviations used: DLPC, 1,2-dilauryl-*sn*-glycero-3-phosphatidylcholine; DMPO, 5,5′-dimethyl-1-pyrroline *N*-oxide; DTPA, diethylenetriaminepenta-acetic acid; ECL®, enhanced chemiluminescence; MPO, myeloperoxidase; PBN, *N*-tert-butyl-α-phenylnitrone; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; RNS, reactive nitrogen species; SIN-1, 3-morpholino-sydnonimine chloride; SNN, spermine NONOate {(*Z*)-1-(*N*-[aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino)-diazen-1-ium-1,2-diolate}; SOD1, Cu,Zn-superoxide dismutase; TBS, Tris-buffered saline; TBST, Tris-buffered saline with Tween 20; Y-4 peptide, acetyl-NH-KKAYALALALALALALALALAKK-CONH$_2$.

[1] To whom correspondence should be addressed (e-mail balarama@mcw.edu).

© 2004 Biochemical Society

published procedure [10]. The concentration of the purified α-synuclein was determined using a bicinchonic acid (BCA) assay kit from Pierce Chemical Company (Milwaukee, WI, U.S.A.). Purified α-synuclein was stored at − 80 °C.

Materials

SOD1 was obtained from Roche Diagnostics (Mannheim, Germany) and used as received. A $CO_3^{\bullet -}$-generating pentamine carbonato complex of Co(III) $\{[(NH_3)_5CoCO_3]^+\}$ was synthesized according to published procedures [11]. Briefly, 30 g of $Co(NO_3)_2 \cdot 6H_2O$ in 15 ml of water was added to 45 g of ammonium carbonate dissolved in 45 ml of water, followed by the addition of 75 ml of concentrated ammonia. Air was bubbled through the solution for 24 h. The resulting solution was cooled in an ice bath and the solid product was recrystallized by dissolving in 55 ml of water at 90 °C and then slowly cooling the solution in an ice bath. Pure crystals were isolated and used in the experiments. $ONOO^-$ was synthesized according to a previously published procedure [12], and the concentration was determined using the molar absorption coefficient ($\varepsilon_{max} = 1670\ M^{-1} \cdot cm^{-1}$ at 302 nm) [13]. 1,2-Dilauryl-*sn*-glycero-3-phosphatidylcholine (DLPC) and 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC) were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Spin-trapping agents, 5,5′-dimethyl-1-pyrroline *N*-oxide (DMPO) and *N*-tert-butyl-α-phenylnitrone (PBN) were purchased from Sigma (St. Louis, MO, U.S.A.) along with sodium bicarbonate, hydrogen peroxide, tyrosine, Trizma base and BSA. SIN-1 (3-morpholino-sydnonimine chloride), spermine NONOate {(*Z*)-1-(*N*-[aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino)-diazen-1-ium-1,2-diolate; SNN} and anti-nitrotyrosine antibody were from Cayman Chemical (Ann Arbor, MI, U.S.A.). Anti-α-synuclein antibody was acquired from BD Transduction Laboratories (Lexington, KY, U.S.A.). Horseradish-peroxidase-conjugated rabbit anti-mouse IgG was obtained from Pierce Chemical Company. Sodium nitrite was from Mallinckrodt (St. Louis, MO, U.S.A.). MPO was purchased from Calbiochem (San Diego, CA, U.S.A.). Apparent molecular masses of proteins were determined using a pre-stained broad range molecular mass marker from Invitrogen. Enhanced chemiluminescence (ECL®) Western blotting reagents and Kodak X-OMAT film were from Amersham Biosciences. Water used in all buffers and samples was purified using a Milli-Q A10 synthesis water purification system from Millipore (Bedford, MA, U.S.A.).

Aggregation of α-synuclein induced by $CO_3^{\bullet -}$

A typical reaction mixture (10 μl) containing α-synuclein (0.25 mg/ml), SOD1 (0.3 mg/ml), $NaHCO_3$ (25 mM) and H_2O_2 (1 mM) in a phosphate buffer (100 mM, pH 7.4) with diethylenetriaminepenta-acetic acid (DTPA; 100 μM) and other indicated reagents was incubated at 37 °C for 2 h. Alternatively, a 10 μl sample containing α-synuclein (0.25 mg/ml) and cobalt complex (4 mM) in a phosphate buffer (100 mM, pH 7.4) with DTPA (100 μM) was UV-photolysed for 30 s. Photolysis was performed using a 300 W EiMac VIX 300 UV 300 X Xenon arc source from ILC Technology INC (Fremont, CA, U.S.A.). The samples were then mixed with an equal volume of Laemmli sample buffer (5 % 2-mercaptoethanol) and boiled for 6 min. The samples were separated at 120 V for 1.5 h on SDS/PAGE (10 % gel, pH 8.8).

Aggregation of α-synuclein induced by $ONOO^-$

Slow infusion of $ONOO^-$ was performed using an infusion/withdraw pump from Harvard Apparatus (Model 966; Southnatick, MA, U.S.A.) under constant stirring of α-synuclein solution. $ONOO^-$ (15 mM diluted in 0.25 M NaOH) was infused at a constant rate (0.82 ± 0.05 μl per 10 min) into 0.25 ml of α-synuclein (0.25 mg/ml) in a phosphate buffer (100 mM, pH 7.4) with DTPA (100 μM) until the final concentration of $ONOO^-$ was 100 μM. The infusion rate for $ONOO^-$ was 5.0 μM/min. The maximum pH shift was less than 0.1 unit after infusion. Aggregation of α-synuclein induced by co-generation of $^{\bullet}NO$ and $O_2^{\bullet -}$ was performed by incubating α-synuclein (0.25 mg/ml) with SIN-1 (0.25 or 1 mM) in a phosphate buffer (100 mM, pH 7.4) with DTPA (100 μM) at room temperature (23 °C) for 16 h.

Aggregation of α-synuclein induced by the $MPO/H_2O_2/NO_2^-$ system

α-Synuclein (0.25 mg/ml) was incubated with $NaNO_2$ (0.5 mM), H_2O_2 (0.1 or 0.2 mM) and MPO (100 nM) in a phosphate buffer (0.1 M, pH 7.4) containing DTPA (100 μM) at 37 °C for 1 h. Samples were then separated by SDS/PAGE (10 % gel) and analysed by immunoblotting.

Immunoblot analysis

Following SDS/PAGE (10 % gel), proteins were transferred overnight at 30 V on to a 0.2 μm nitrocellulose membrane. The membrane was briefly washed in Tris (50 mM)-buffered saline with NaCl (140 mM, pH 7.2) (TBS) containing 0.1 % (v/v) Tween 20 (TBST) before blocking in TBST containing 5 % (w/v) BSA for 1 h. After five washes (5 min each) in TBST, the membrane was incubated for 2 h at room temperature with the primary antibody (1:5000) in TBST with 1 % (w/v) BSA. The membrane was then washed with TBST several times before a 1 h incubation with the secondary antibody (conjugated to horseradish peroxidase; 1:5000) in TBST with 1 % (w/v) BSA. The blot was washed an additional five times with TBST and then once for 5 min in TBS. The membrane was then immersed in ECL® solution for 1 min before being exposed to Kodak X-OMAT film. The images were documented using a Multimage Light Cabinet (Alpha Innotech Corporation, San Leandro, CA, U.S.A.).

Effect of liposomes on aggregation of α-synuclein induced by the $MPO/H_2O_2/NO_2^-$ system

Liposomes were prepared according to published procedures [14]. A methanolic solution of DLPC or PLPC was dried under a stream of N_2 gas and kept in a vacuum desiccator overnight. The dried lipid was thoroughly mixed in a phosphate buffer (100 mM, pH 7.4) containing DTPA (100 μM) and the liposome was freeze–thawed for five cycles using liquid nitrogen. α-Synuclein (0.25 mg/ml) was incubated with liposomes [30 mM DLPC, 3 mM Y-4 peptide (acetyl-NH-KKAYALALALALALALALA-LAKK-$CONH_2$) or 30 mM PLPC] in a phosphate buffer (67 mM, pH 7.4) with DTPA (67 μM) at 37 °C for 1 h. After incubation, the liposome was separated by centrifugation at 15 200 ***g*** for 2 h, and both aqueous and liposome phases were analysed by SDS/PAGE (10 % gel) and immunoblotting. The densitometric data are means of the density of the α-synuclein dimers from two separate images.

Fluorescence analysis of α-synuclein aggregation products

α-Synuclein (0.25 mg/ml) or tyrosine (100 μM) mixed with cobalt complex (4 mM) in a phosphate buffer (100 mM, pH 7.4) with DTPA (100 μM) was UV photolysed (1 min at 240 nm; bandwidth, 20 nm). The fluorescence spectra were recorded by a Shimadzu Fluorometer (bandwidth, 5 nm). Alternatively, the aggregates of α-synuclein induced by $CO_3^{\bullet -}$, $ONOO^-$ or $MPO/H_2O_2/NO_2^-$ were separated by SDS/PAGE (10 % gel) and scanned using a PerkinElmer LS55 Luminescence Spectrometer with a TLC Scan Accessory. The cobalt-complex-induced dimer of

© 2004 Biochemical Society

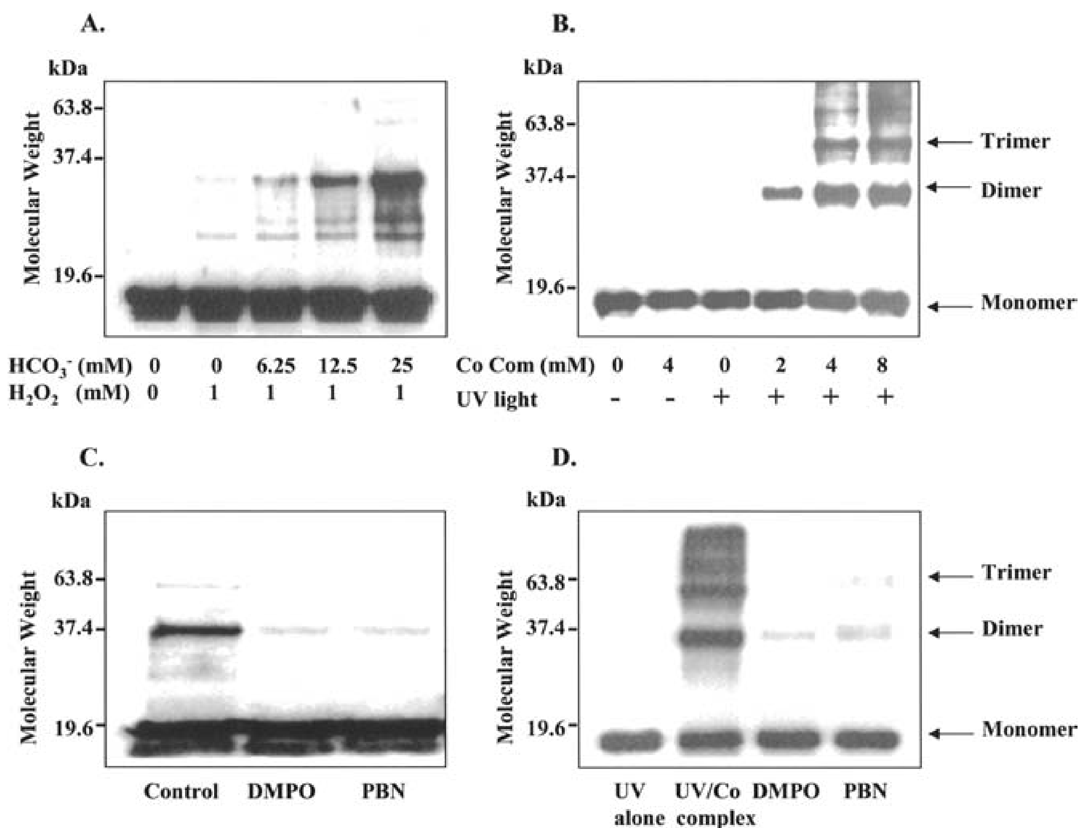

Figure 1 Aggregation of α-synuclein induced by HCO_3^--dependent SOD1-peroxidase activity

(**A**) Reaction mixtures contained α-synuclein (0.25 mg/ml), SOD1 (0.3 mg/ml), H_2O_2 (1 mM) and various concentrations of $NaHCO_3$ in a phosphate buffer (100 mM, pH 7.4) with DTPA (100 μM) were incubated for 2 h at 37 °C. Aggregates of α-synuclein were separated by SDS/PAGE (10 % gel) and immunoblotted with anti-α-synuclein antibody. (**B**) Reaction mixture contained α-synuclein (0.25 mg/ml) and various amounts of cobalt complex (Co Com) in a phosphate buffer (100 mM, pH 7.4) with DTPA (100 μM). Samples were UV-photolysed for 30 s before being separated by SDS/PAGE (10 % gel) and probed with anti-α-synuclein antibody as described previously. (**C**) α-Synuclein (0.25 mg/ml) was incubated with SOD1 (0.3 mg/ml), H_2O_2 (1 mM) and $NaHCO_3$ (25 mM) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (100 μM) with 25 mM DMPO or PBN as indicated. The control sample is the incubation mixture without spin traps. (**D**) Reaction mixtures containing α-synuclein (0.25 mg/ml), cobalt complex (4 mM) and DMPO or PBN (25 mM) were UV-photolysed and probed with anti-α-synuclein antibody as described.

α-synuclein was extracted from the gel using the syringe maceration extraction method [15]. A dityrosine emission spectrum was obtained from the dimer using a Shimadzu Fluorometer (excitation, 310 nm; bandwidth, 20 nm).

ESR measurements

ESR spectra were recorded at room temperature on a Bruker ER 200 D-SRC spectrometer operating at 9.8 GHz, with a cavity equipped with a Bruker AquaX liquid sample cell. Typical spectrometer parameters were: scan range, 10 mT; field set, 351 mT; time constant, 0.64 ms; scan time, 20 s; modulation amplitude, 0.1 mT; modulation frequency, 100 kHz; receiver gain, 2×10^5; microwave power, 20 mW. Reaction mixtures consisting of α-synuclein (0.25 mg/ml), SOD1 (1 mg/ml), $NaHCO_3$ (25 mM) and DMPO or PBN (25 mM) in a phosphate buffer (100 mM, pH 7.4) with DTPA (100 μM) were rapidly mixed with H_2O_2 (1 mM). Samples were subsequently transferred to a 100 μl capillary tube for ESR measurements.

RESULTS

HCO_3^- stimulates α-synuclein aggregation in a SOD1/H_2O_2 system

Figure 1(A) shows that the addition of HCO_3^- to a mixture of α-synuclein (0.25 mg/ml), SOD1 (0.3 mg/ml) and H_2O_2 (1 mM) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (100 μM) caused a dose-dependent increase in α-synuclein dimer and trimer formation. The lack of effect of SDS and 2-mercaptoethanol on dimer and trimer formation suggests the involvement of a covalent aggregation reaction. Note that in the absence of HCO_3^-, α-synuclein dimer formation was negligible. We suggest that the $CO_3^{\bullet-}$ formed in a SOD1/H_2O_2/HCO_3^- system is responsible for oxidative covalent aggregation of α-synuclein. Further support for this proposal came from photolysis of α-synuclein in the presence of a pentamine carbonato cobalt complex, $[(NH_3)_5CoCO_3]^+$. UV photolysis of the cobalt complex has previously been shown to generate the $CO_3^{\bullet-}$ radical [16]. Figure 1(B) shows a pronounced aggregation of α-synuclein in the presence of a cobalt complex and UV light. In the absence of UV light and the cobalt complex, there was negligible formation of α-synuclein dimers and trimers (Figure 1B). UV light alone did not induce α-synuclein dimer formation (Figure 1D, lane 1). Nitrone spin traps (DMPO and PBN) inhibited both SOD1/H_2O_2/HCO_3^-- and cobalt complex/UV light-induced aggregation of α-synuclein (Figures 1C and 1D). Nitrone spin traps have previously been shown to react with $CO_3^{\bullet-}$, forming the corresponding hydroxyl adducts [17]. These findings demonstrate that HCO_3^--dependent SOD1-peroxidase activity is able to generate a diffusible oxidant, such as $CO_3^{\bullet-}$, that can cause oxidative covalent aggregation reactions in a neighbouring protein molecule.

© 2004 Biochemical Society

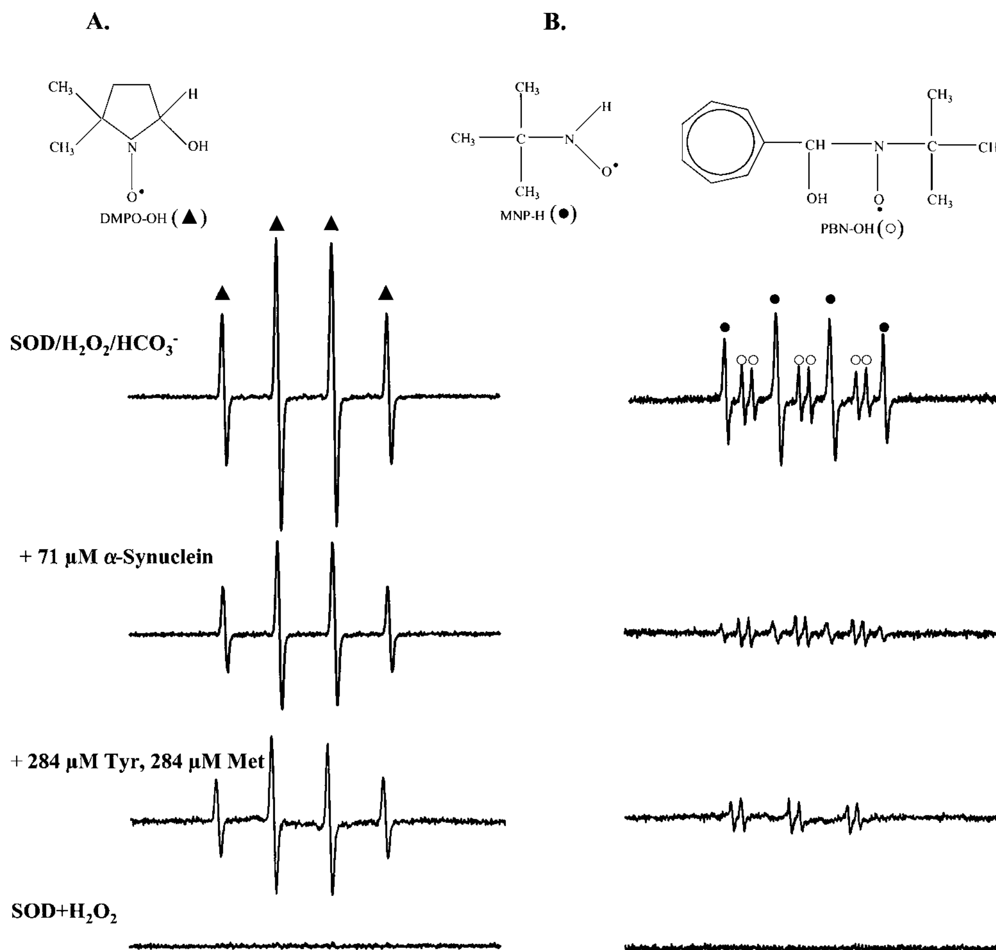

Figure 2 α-Synuclein inhibits SOD1/H_2O_2/HCO_3^--dependent hydroxylation of nitrone traps

(**A**) Incubations contained SOD1 (1 mg/ml), $NaHCO_3$ (25 mM), H_2O_2 (1 mM), DTPA (100 μM) and DMPO (25 mM) in a phosphate buffer (100 mM, pH 7.4). Samples were transferred to a 100 μl capillary tube and ESR spectra were recorded immediately. α-Synuclein (70 μM) or tyrosine (284 μM) and methionine (284 μM) were included in the reaction as indicated. (**B**) Same as (**A**), except that PBN (25 mM) was substituted for DMPO.

α-Synuclein inhibits SOD1/H_2O_2/HCO_3^--induced hydroxylation of nitrone spin traps

It has been previously shown that HCO_3^--dependent peroxidase activity of SOD1 can be measured by monitoring the hydroxylated products of nitrones by ESR [17]. As shown in Figure 2(A), addition of H_2O_2 (1 mM) to an incubation mixture containing SOD1 (1 mg/ml), DMPO (25 mM) and DTPA (100 μM) did not produce any ESR spectrum in the absence of HCO_3^-. However, in the presence of 25 mM HCO_3^-, an intense four-line signal with an intensity proportion of 1:2:2:1 (marked ▲) was obtained and attributable to the DMPO-OH adduct ($\alpha_N = 1.49$ mT, $\alpha_H = 1.49$ mT). Previously, this adduct formation in the SOD1/H_2O_2/HCO_3^- system was ascribed to an oxidative hydrolysis reaction between $CO_3^{\bullet -}$ and DMPO [21]. In the presence of 71 μM α-synuclein, the intensity of DMPO-OH was diminished, possibly due to scavenging of $CO_3^{\bullet -}$ by α-synuclein. α-Synuclein contains four tyrosine and four methionine residues [18]. The $CO_3^{\bullet -}$ radical reacts rapidly with tyrosine and methionine ($k \approx 10^7\ M^{-1} \cdot s^{-1}$) [19]. In the presence of a 284 μM concentration of tyrosine and methionine (approx. 4 times the α-synuclein concentration), the DMPO-OH signal intensity was inhibited to the same extent as occurred in the presence of α-synuclein (Figure 2A). Similar results were obtained with PBN as a SOD1-peroxidase probe (Figure 2B). As shown in Figure 2(B), the ESR spectra due to PBN-OH (marked ○) and 2-methyl-2-nitrosopropane hydronitroxide (marked ●) were inhibited to a similar extent in the presence of α-synuclein (71 μM) and in the presence of a mixture of tyrosine and methionine (284 μM). We conclude that the $CO_3^{\bullet -}$ radical generated in the SOD1/H_2O_2/HCO_3^- system reacts with the tyrosine and methionine residues in α-synuclein. However, under these conditions, we could not detect the corresponding radical derived from one-electron oxidation of α-synuclein associated with tyrosine and methionine.

Effects of HCO_3^- on $ONOO^-$- and $NO_2^\bullet$-induced aggregation and nitration of α-synuclein

Immunoblotting with anti-α-synuclein antibody showed α-synuclein dimer formation from incubations containing α-synuclein, SIN-1 and HCO_3^- (Figure 3A). In the presence of oxygen, SIN-1 generated $ONOO^-$ *in situ* by releasing $O_2^{\bullet -}$ and $^\bullet NO$ simultaneously [20]. In the presence of HCO_3^-, which is in equilibrium with CO_2 at pH 7.4, $ONOO^-$ forms a transient complex, nitrosoperoxocarbonate ($ONOOCO_2^-$), which rapidly decomposes to $NO_2^\bullet$ and $CO_3^{\bullet -}$ [6,7]. The $CO_3^{\bullet -}$, a potent one-electron oxidant, has been shown to oxidize tyrosine, forming

© 2004 Biochemical Society

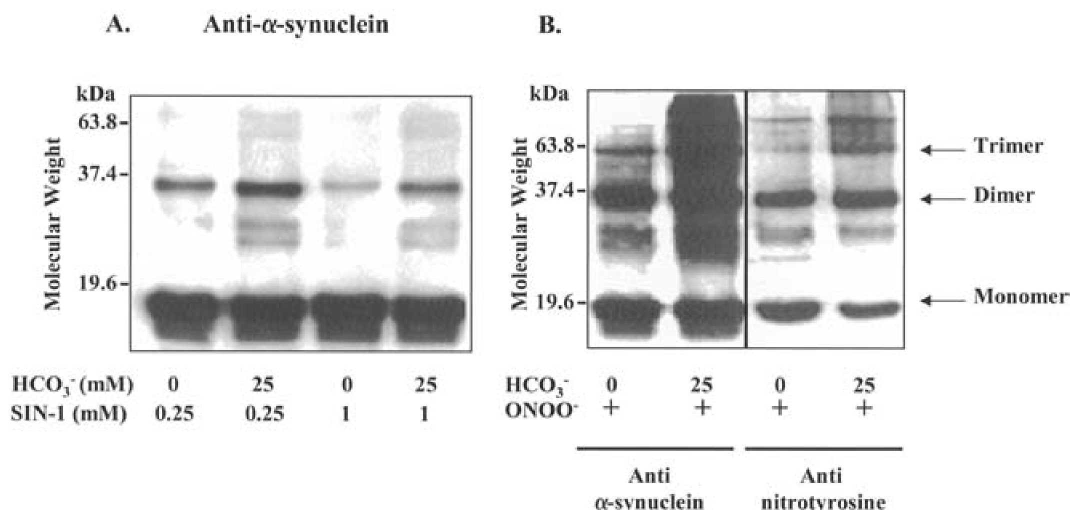

Figure 3 HCO_3^- enhances aggregation of α-synuclein induced by $ONOO^-$

(**A**) α-Synuclein (0.25 mg/ml) was incubated with SIN-1 (0.25 or 1 mM) with or without $NaHCO_3$ (25 mM) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (100 μM) at room temperature for 16 h. The aggregation of α-synuclein was revealed by immunoblotting with anti-α-synuclein antibody. (**B**) $ONOO^-$ was slowly infused (5 μM/min for 20 min) into vials containing α-synuclein (0.25 mg/ml) with or without $NaHCO_3$ (25 mM) in a phosphate buffer (100 mM, pH 7.4) with DTPA (100 μM). The Figure represents results from three separate incubations. Protein was detected with an anti-α-synuclein antibody (lanes 1 and 2) or anti-nitrotyrosine antibody (lanes 3 and 4).

dityrosine, trityrosine and tetratyrosine [21]. The amount of α-synuclein aggregation was decreased in the absence of HCO_3^- (Figures 3A and 3B, lanes 1 and 2). Slow infusion of $ONOO^-$ (100 μM) at a rate of 5 μM/min into solutions containing α-synuclein (0.25 mg/ml) and DTPA (100 μM) in a phosphate buffer (100 mM, pH 7.4) induced a marginal increase in α-synuclein oligomerization, which was enhanced by HCO_3^-. Figure 3(B) (lanes 1 and 2) gives data from three experiments showing the stimulatory effect of HCO_3^- on α-synuclein aggregation in the presence of slowly infused $ONOO^-$. Under these conditions, immunoblotting with anti-nitrotyrosine antibody showed that HCO_3^- significantly enhanced nitration of tyrosine residues present in α-synuclein (Figure 3B, lanes 3 and 4).

Figure 4(A) shows that the addition of MPO to an incubation mixture containing α-synuclein, H_2O_2 and NO_2^- in a phosphate buffer induced formation of dimeric and higher-molecular-mass complexes of α-synuclein, as detected by immunoblotting with anti-α-synuclein antibody. In the absence of added NO_2^-, MPO/H_2O_2 did not cause α-synuclein dimerization or oligomerization. Under conditions of maximal aggregation (Figure 4A, lane 4), extensive nitration of α-synuclein was detected when probed with anti-nitrotyrosine antibody (Figure 4B, lane 4). Based on published data, we attribute the oxidation and nitration of α-synuclein by MPO/H_2O_2/NO_2^- to the formation of $NO_2^\bullet$ by the one-electron oxidation of NO_2^- by the MPO-compound 1 [22,23]. In contrast with that due to $ONOO^-$, MPO/H_2O_2/NO_2^--mediated aggregation of α-synuclein was not affected by HCO_3^-. As shown in Figures 4(C) and 4(D), the addition of 25 mM HCO_3^- to incubations containing α-synuclein (0.25 mg/ml), MPO, H_2O_2 and NO_2^- (0.05, 0.1 and 0.2 mM) did not significantly affect the formation of dimeric and oligomeric products of α-synuclein (Figures 4C and 4D).

Effects of liposomes on MPO/H_2O_2/NO_2^-- and SOD1/H_2O_2/HCO_3^--induced aggregation and nitration of α-synuclein

Membrane-bound α-synuclein exists as a stable α-helical conformer, whereas cytosolic α-synuclein is disordered [24,25]. Lipid binding enhanced the α-helicity of α-synuclein [24,26]. We tested the effect of DLPC liposomes on MPO/H_2O_2/NO_2^--dependent aggregation and nitration of α-synuclein (Figures 5A and 5B). The addition of DLPC liposomes (30 mM) to incubations containing α-synuclein (0.25 mg/ml), MPO (100 nM), H_2O_2 (0.2 mM) and NO_2^- (0.2 mM) did not cause a significant difference to the amount of aggregated and nitrated α-synuclein in the aqueous phase. However, when the DLPC lipid phase was examined under these conditions, there was an increase in aggregated and nitrated α-synuclein in the lipid phase (results not shown). It is likely that the hydrophobic $NO_2^\bullet$ reacts with membrane-bound α-synuclein much more efficiently, resulting in enhanced formation of aggregated and nitrated α-synuclein. Hydrophobic membranes represent a focal point of α-synuclein aggregation and nitration.

To investigate the effect of unsaturated fatty acids on nitration and aggregation of α-synuclein, we used PLPC as a model liposomal system. As shown in Figure 5(A), MPO/H_2O_2/NO_2^--induced aggregation of α-synuclein was unaffected by fully saturated DLPC, whereas the addition of MPO/H_2O_2/NO_2^- to α-synuclein in the presence of PLPC totally inhibited α-synuclein aggregation (Figure 5A, lane 4). Concomitantly, α-synuclein nitration was determined by probing with anti-nitrotyrosine antibody (Figure 5B, lane 4). As shown in Figure 5(B), PLPC abrogated α-synuclein nitration induced by MPO/H_2O_2/NO_2^-. These findings suggest that the $NO_2^\bullet$ radical generated from MPO/H_2O_2/NO_2^- is scavenged by PLPC, resulting in decreased aggregation and nitration of α-synuclein. Under these conditions, we have previously reported an increase in malondialdehyde, an oxidation product of polyunsaturated fatty acids [14]. Nitration of α-synuclein occurred in the presence of DLPC; however, the yield of nitrated products was slightly less than in the control sample containing phosphate buffer.

To demonstrate further that hydrophobic membranes containing the $NO_2^\bullet$ trap will significantly affect the MPO/H_2O_2/NO_2^--induced aggregation and nitration of α-synuclein, we used DLPC liposomes containing the tyrosyl peptide, Y-4. We have previously reported that tyrosyl peptides anchored in the lipid bilayer effectively trap RNS formed from MPO/H_2O_2/NO_2^- and $ONOO^-$ [14]. Figures 5(C) and 5(D) (lanes 4 and 5) show that α-synuclein aggregation and nitration induced by MPO/H_2O_2/NO_2^-

© 2004 Biochemical Society

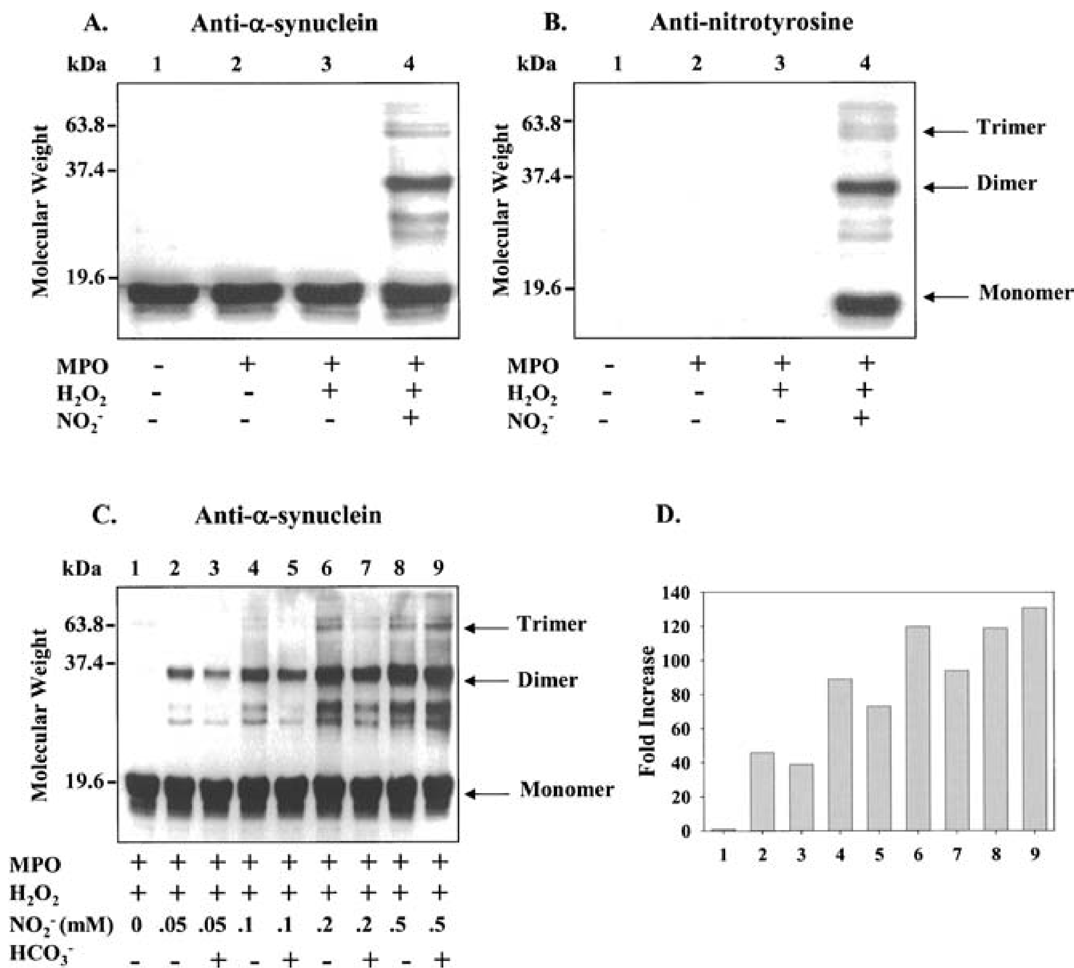

Figure 4 Aggregation of α-synuclein induced by the MPO/H_2O_2/NO_2^- system

(**A**) α-Synuclein (0.25 mg/ml) was incubated with $NaNO_2$ (0.5 mM), H_2O_2 (0.2 mM) and MPO (100 nM) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (100 μM) at 37 °C for 1 h. Samples were then separated by SDS/PAGE (10 % gel) and immunoblotted with an anti-α-synuclein antibody, or (**B**) probed with anti-nitrotyrosine antibody under otherwise similar conditions to those used in (**A**). (**C**) Effect of $NaHCO_3$ (25 mM) on α-synuclein aggregation induced by MPO/H_2O_2 at different concentrations of NO_2^-. Incubation conditions were identical with those described in (**A**). (**D**) Densitometric analysis of the data obtained under conditions shown in (**C**).

are potently inhibited in the presence of DLPC liposomes containing the Y-4 peptide.

Next, we sought to investigate the effects of differing concentrations of NO_2^- (50, 100 and 200 μM) on MPO/H_2O_2-induced aggregation and nitration of α-synuclein in the presence or absence of DLPC liposomes. Increasing the amount of NO_2^- was found to enhance both aggregation and nitration. However, incubation in the presence of DLPC did not significantly alter aggregation or nitration patterns of α-synuclein (results not shown).

In contrast with hydrophobic RNS-mediated aggregation and nitration of α-synuclein, $CO_3^{\bullet -}$-induced aggregation of α-synuclein was unaffected in the presence of PLPC liposomes or DLPC liposomes containing Y-4 peptide. Incubation mixtures were similar to those described in Figure 1, except that they included either PLPC or DLPC containing Y-4 (results not shown). These results are consistent with the fact that $CO_3^{\bullet -}$, being membrane-impermeant, is not scavenged by membrane-bound tyrosyl peptides or by PLPC.

Although unsaturated fatty acid inhibits α-synuclein aggregation and nitration mediated by $NO_2^\bullet$, previous data suggest that the oxidative modification of phospholipids greatly accelerates the aggregation of membrane-bound α-synuclein [27]. α-Synuclein is rich in lysine residues that are known to undergo oxidative modification during lipid-peroxidative damage. Upon prolonged incubation of α-synuclein in the presence of unsaturated fatty acid, α-synuclein aggregation was shown to be enhanced (results not shown); the unsaturated fatty acids promoted α-synuclein aggregation via a mechanism that did not involve tyrosine residues [27]. Thus α-synuclein aggregation, under the present experimental conditions, is presumably mediated by tyrosine oxidation [28].

$^\bullet$NO inhibits MPO/H_2O_2/NO_2^--induced aggregation of α-synuclein

As $NO_2^\bullet$ reacts rapidly with $^\bullet$NO at a diffusion-controlled rate, we investigated the effect of $^\bullet$NO released slowly from SNN (2 mol of $^\bullet$NO/mol of parent compound at pH 7.4; half-life, 39 min) on oxidation and nitration of α-synuclein by immunoblotting with anti-α-synuclein and anti-nitrotyrosine antibodies. As shown in Figures 6(A) and 6(B), the addition of SNN at different concentrations inhibited MPO/H_2O_2/NO_2^--dependent aggregation and nitration of α-synuclein. Because $^\bullet$NO and $^\bullet$NO-derived RNS are lipophilic [29], we investigated the effect of $^\bullet$NO on α-synuclein aggregation in the presence of membranes.

© 2004 Biochemical Society

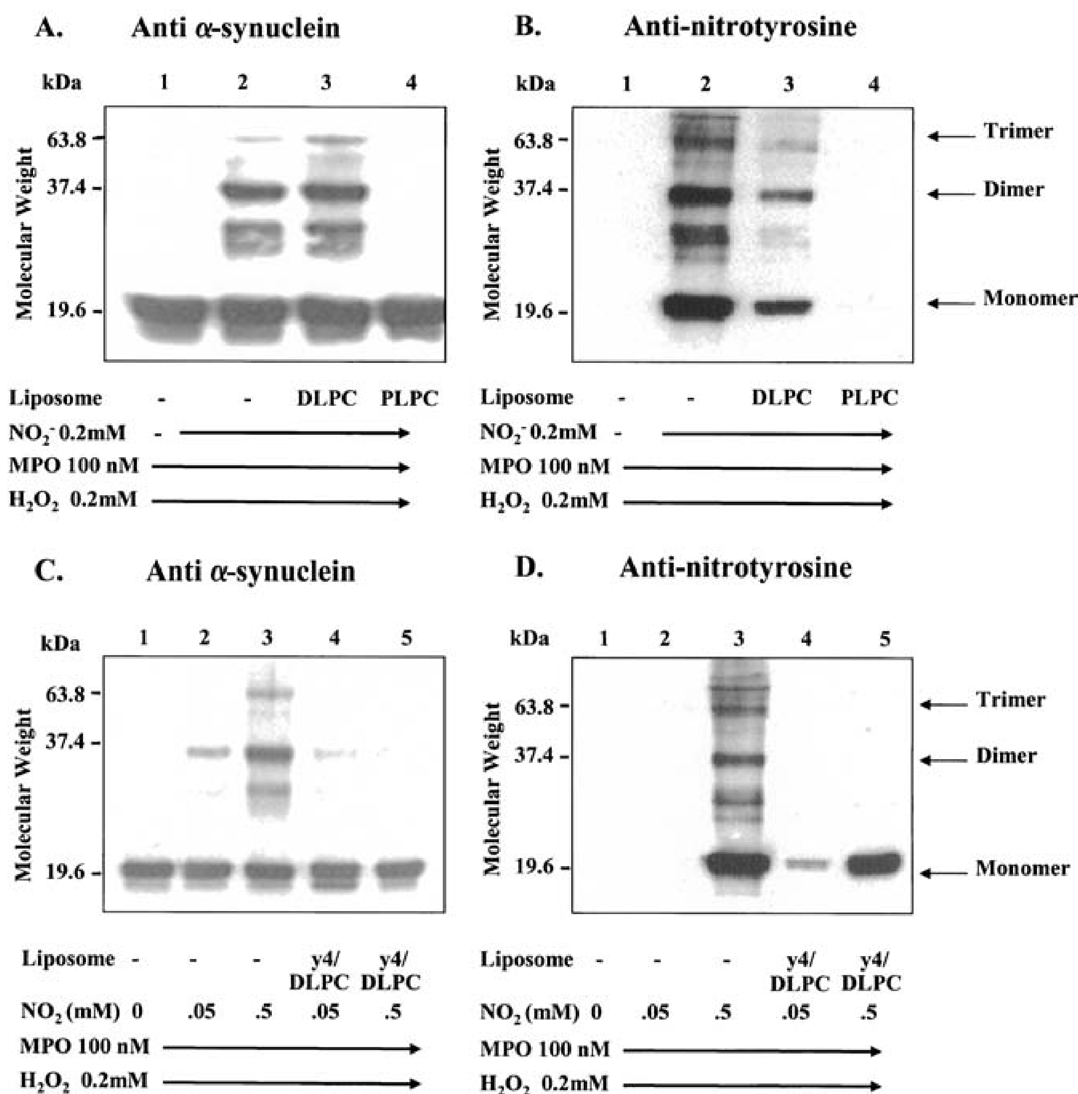

Figure 5 Effects of unsaturated liposomes and transmembrane tyrosyl peptide on aggregation and nitration of α-synuclein induced by MPO/H_2O_2/NO_2^-

(**A**) and (**B**) Incubation mixtures contained α-synuclein (0.25 mg/ml), MPO (100 nM), H_2O_2 (0.2 mM), NO_2^- (0.2 or 0.5 mM) and either DLPC (30 mM) or PLPC (30 mM) in a phosphate buffer (67 mM, pH 7.4) with DTPA (67 μM). α-Synuclein aggregation (**A**) and nitration (**B**) were probed in the aqueous phase. (**C**) and (**D**) Effect of the DLPC liposome containing the Y-4 peptide on aggregation and nitration of α-synuclein.

Figures 6(C) and 6(D) show that α-synuclein bound to lipid membrane underwent aggregation and nitration in the presence of MPO/H_2O_2/NO_2^-. The addition of SNN to this system greatly inhibited oxidation and nitration of α-synuclein bound to membranes. Thus MPO/H_2O_2/NO_2^- is unable to induce oxidative modification or nitration of membrane-bound α-synuclein under conditions when $^{\bullet}$NO is generated continuously.

Effects of $^{\bullet}$NO on SOD1/H_2O_2/HCO_3^--dependent oxidative modification and nitration of α-synuclein

As $^{\bullet}$NO has been shown to react rapidly with the $CO_3^{\bullet -}$ radical ($k = 3.5 \times 10^9\ M^{-1} \cdot s^{-1}$) [30], we determined the effect of SNN on SOD1/H_2O_2/HCO_3^--dependent oxidative modification of α-synuclein (Figure 7A). Immunoblotting experiments with anti-α-synuclein antibody showed that the addition of SNN to incubations containing α-synuclein (0.25 mg/ml), bovine SOD1 (0.3 mg/ml), H_2O_2 (1 mM), DTPA (100 μM) and HCO_3^- (25 mM) in a phosphate buffer (100 mM, pH 7.4) caused a dose-dependent decrease in the formation of α-synuclein dimer. Sodium nitrite did not have an appreciable effect on SOD1/H_2O_2/HCO_3^--induced aggregation and nitration of α-synuclein (Figure 7A, lane 5). Our interpretation of these findings is that $^{\bullet}$NO rapidly scavenges the $CO_3^{\bullet -}$ radical anion generated by the HCO_3^--dependent peroxidase activity of SOD1. The reaction between $CO_3^{\bullet -}$ radical and NO_2^- generates $NO_2^{\bullet}$ that stimulates α-synuclein oxidation, so the addition of NO_2^- does not have an effect on α-synuclein dimer formation in the SOD1/H_2O_2/HCO_3^- system (Figure 7A, lane 5). Concomitantly, an increase in α-synuclein nitration was observed (Figure 7B, lane 5).

The ESR spin-trapping data shown in Figure 7(C) provide further support for the rapid reaction between $^{\bullet}$NO and $CO_3^{\bullet -}$ radicals. In the absence of SNN, the incubation mixtures containing SOD1 (0.25 mg/ml), H_2O_2 (1 mM), HCO_3^- (25 mM), DMPO (25 mM) and DTPA (100 μM) in a phosphate buffer (100 mM, pH 7.4) yielded the DMPO-OH adduct, consistent with oxidative hydrolysis of DMPO by the $CO_3^{\bullet -}$ radical (Figure 7C). In the presence of SNN, a dose-dependent inhibition

© 2004 Biochemical Society

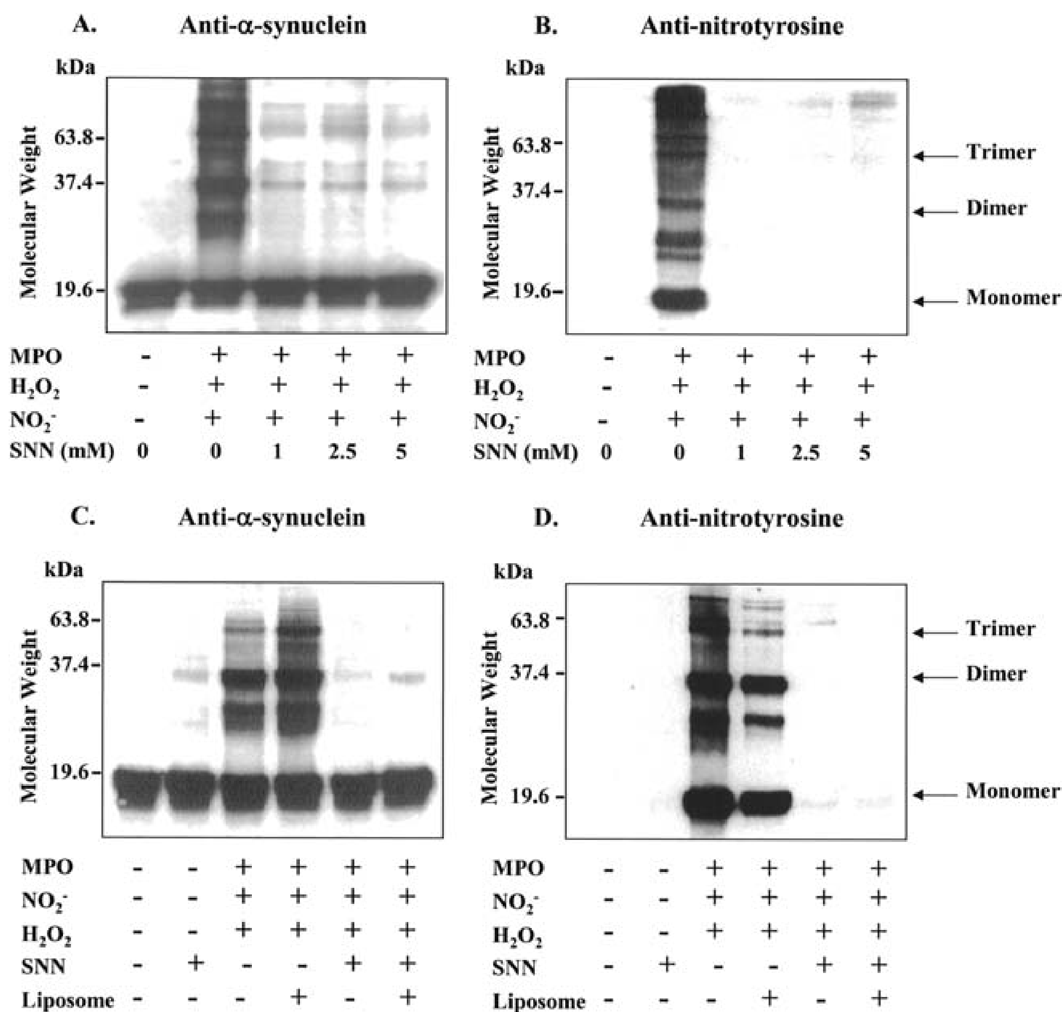

Figure 6 Effect of •NO on the α-synuclein aggregation induced by the $MPO/H_2O_2/NO_2^-$ system

(**A**) α-Synuclein (0.25 mg/ml) was mixed with H_2O_2 (0.2 mM), NO_2^- (0.5 mM) and MPO (100 nM) in the presence of a phosphate buffer (100 mM, pH 7.4) with DTPA (100 μM). Various amounts of •NO donor, SNN, were added as indicated. Samples were incubated for 1 h at 37 °C before separation by SDS/PAGE (10 %) and probed with anti-α-synuclein antibody or (**B**) anti-nitrotyrosine antibody. The effect of treatment with DLPC liposomes (30 mM) on this system is represented in (**C**) and (**D**).

of DMPO-OH adduct formation was observed (Figure 7C). Figure 7(D) shows the inhibitory effect of SNN on the formation of PBN-OH and its decomposition product (i.e. 2-methyl-2-nitrosopropane hydronitroxide), that are formed from $CO_3^{\bullet-}$-induced hydroxylation and oxidation of PBN. These findings support the hypothesis that •NO inhibits $SOD1/H_2O_2/HCO_3^-$-dependent aggregation of α-synuclein by reacting with the $CO_3^{\bullet-}$ radical.

$CO_3^{\bullet-}$- and $NO_2^{\bullet}$-mediated dimerization of tyrosine residues in α-synuclein

Previously, we showed that $CO_3^{\bullet-}$ could oxidize tyrosine to form the tyrosyl radicals that form dityrosine via radical–radical dimerization [21]. As shown in Figure 8(A), $CO_3^{\bullet-}$ generated from a cobalt complex in the presence of UV light reacts with tyrosine to form dityrosine, which exhibits a characteristic fluorescence spectrum (excitation, 294 nm; emission, 410 nm). This fluorescence spectrum is similar to that of authentic dityrosine (Figure 8B). In the presence of a cobalt complex and UV light, α-synuclein formed a dimeric product whose fluorescence spectrum (excitation, 294 nm; emission, 410 nm) is identical with that of dityrosine (Figure 8C). In the absence of UV light or a cobalt complex, no α-synuclein tyrosyl dimer was detected (Figures 8C and 8D).

Figure 9 shows the fluorescence spectra detected from scanning the dimer band of the oxidized α-synuclein. α-Synuclein (1 mg/ml) was treated with different oxidizing systems (cobalt complex and UV light, $MPO/H_2O_2/NO_2^-$ and slow infusion of $ONOO^-$ in the presence of HCO_3^-). In the absence of an oxidizing agent, the fluorescence scanning of the monomer band of α-synuclein corresponding to the excitation and emission wavelengths for dityrosine did not show any peak (Figure 9A). In contrast, the fluorescence scanning of the dimer band obtained from α-synuclein protein and cobalt complex irradiated with UV light (i.e. under conditions that generate $CO_3^{\bullet-}$) showed a characteristic fluorescence spectrum attributed to the dityrosyl moiety (Figure 9B). Dityrosine fluorescence was detected from scanning the dimer bands of α-synuclein following oxidations with $MPO/H_2O_2/NO_2^-$ ($NO_2^{\bullet}$ generation) (Figure 9C) and a slow infusion of $ONOO^-$ and HCO_3^- (Figure 9D). These results suggest that both $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ induce a covalent aggregation of α-synuclein through oxidation of the tyrosine residue to the dityrosyl residue in α-synuclein. Furthermore, the gel-extracted dimer of α-synuclein

© 2004 Biochemical Society

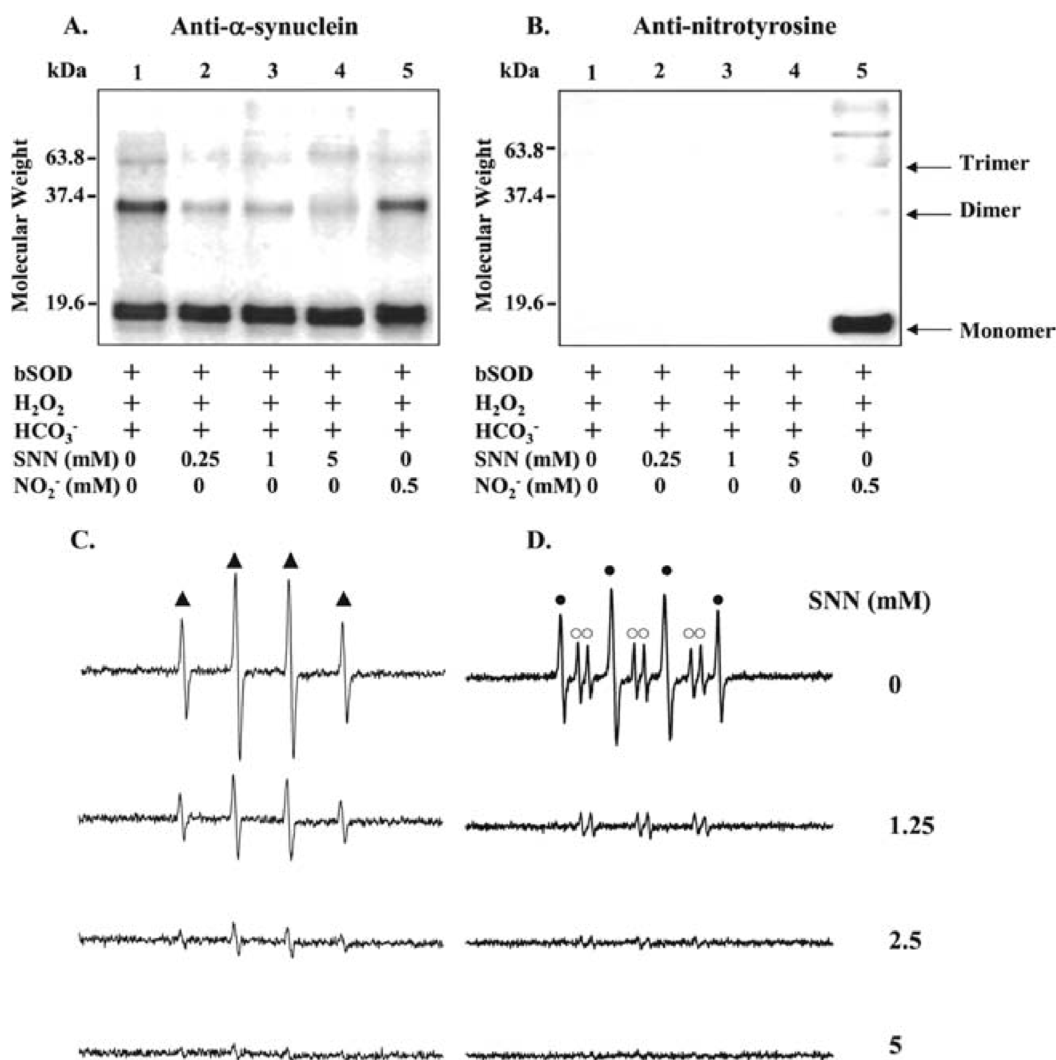

Figure 7 Effects of $^{\bullet}$NO and NO_2^- on α-synuclein aggregation induced by HCO_3^--dependent SOD1-peroxidase activity

(**A**) Samples containing α-synuclein (0.25 mg/ml), SOD1 (0.3 mg/ml), H_2O_2 (1 mM), $NaHCO_3$ (25 mM) and various concentrations of SNN or $NaNO_2$ (0.5 mM) in a phosphate buffer (100 mM, pH 7.4) with DTPA (100 μM) were incubated for 2 h at 37 °C. Following incubation, samples were separated by SDS/PAGE (10 % gel) and probed with anti-α-synuclein antibody or (**B**) anti-nitrotyrosine antibody. (**C**) Effect of $^{\bullet}$NO donor on SOD1/H_2O_2/HCO_3^--dependent hydroxylation of spin traps (DMPO and PBN). SOD1 (1 mg/ml) was mixed with 1 mM H_2O_2, 25 mM DMPO, 25 mM $NaHCO_3$ and various amounts of SNN at room temperature. Samples were subsequently transferred to a 100 μl capillary tube and ESR spectra were recorded. (**D**) Same as (**C**), except that 25 mM PBN was used in place of DMPO.

induced by UV photolysis of the cobalt complex exhibited the characteristics of dityrosine emission spectrum (Figure 9E).

DISCUSSION

A major finding of the present study is that HCO_3^- stimulates both SOD1/H_2O_2- and $ONOO^-$-induced aggregation and nitration of α-synuclein. $CO_3^{\bullet-}$ formed in these systems is responsible for oxidative covalent aggregation of α-synuclein. $NO_2^{\bullet}$ radical formed from the decomposition of nitrosoperoxycarbonate and from MPO/H_2O_2-catalysed oxidation of NO_2^- is responsible for nitration of α-synuclein. $^{\bullet}$NO released slowly from NO donor molecules inhibited $CO_3^{\bullet-}$- and $NO_2^{\bullet}$-induced nitration and aggregation of α-synuclein. This is attributed to the rapid reaction between $^{\bullet}$NO and $NO_2^{\bullet}$ and $CO_3^{\bullet-}$. In the presence of hydrophobic membranes consisting of unsaturated double bonds or tyrosyl peptides, MPO/H_2O_2/NO_2^--mediated nitration and aggregation of α-synuclein were inhibited due to trapping of $NO_2^{\bullet}$.

Intermediacy of HCO_3^- and $NO_2^{\bullet}$ in α-synuclein aggregation and nitration

The role of HCO_3^- in biological oxidations has largely been underappreciated, despite the fact that HCO_3^--dependent enhancement in luminal oxidation and SOD1-peroxidase activity were reported several decades ago [31–33]. Recent reports reveal a new perspective on the role of HCO_3^- in SOD1-catalysed peroxidative reactions [8,21]. In these reports, the enhanced peroxidase activity of SOD1 was attributed to the formation of $CO_3^{\bullet-}$. The X-ray structure of SOD1 indicates that access to the active site of copper occurs via a narrow channel that restricts the entry of large molecules [8,32,33]. However, a relatively small and physiologically abundant anion, such as HCO_3^-, could reach the active site of SOD1 and act as the peroxidase substrate [32,33]. The copper-bound oxidant of SOD1 (SOD-Cu^{2+}-$^{\bullet}$OH) formed in the presence of H_2O_2 could oxidize HCO_3^- to $CO_3^{\bullet-}$, a potent one-electron oxidant that can diffuse out of the active site and cause substrate oxidations [8,21]. The fact that $CO_3^{\bullet-}$ (and not $^{\bullet}$OH) is the

© 2004 Biochemical Society

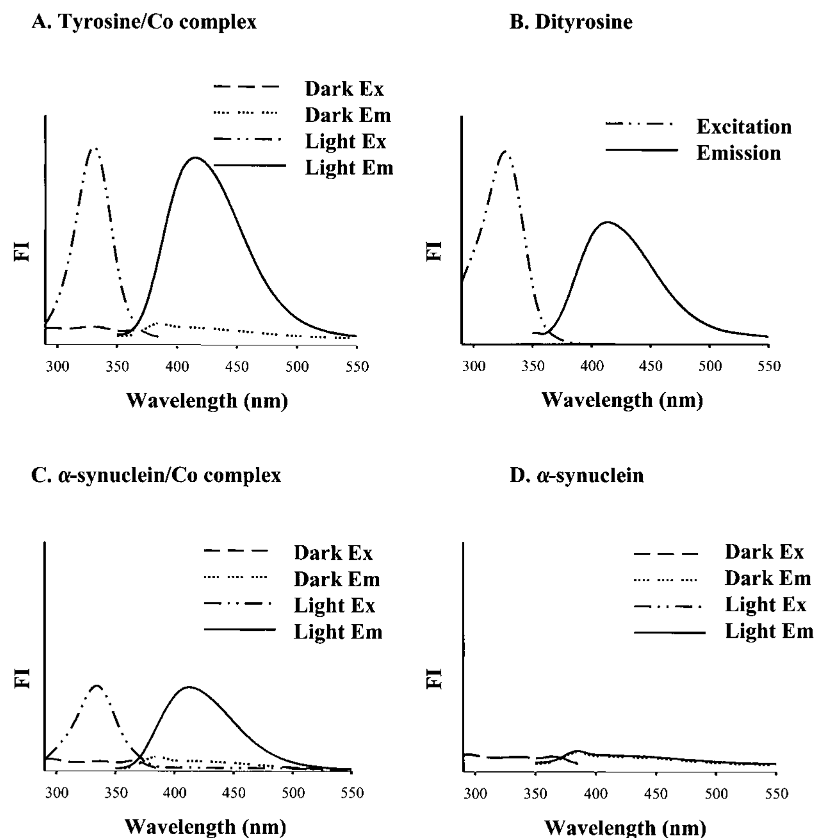

Figure 8 Fluorescence analysis of α-synuclein aggregation induced by $CO_3^{\bullet -}$

(**A**) Tyrosine (100 μM) was mixed with cobalt complex (4 mM) in a phosphate buffer (100 mM, pH 7.4) with 100 μM DTPA, irradiated with UV light (240 nm) for 4 min and fluorescence spectra were obtained at 330 nm for emission spectra and 410 nm for excitation spectra. Dark control spectra were measured before UV irradiation. (**B**) Fluorescence spectrum of an authentic dityrosine (2 μM) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (100 μM). (**C**) α-Synuclein (0.25 mg/ml) was mixed with cobalt complex (4 mM) in a phosphate buffer (100 mM, pH 7.4) with DTPA (100 μM) and irradiated with UV light under the same conditions as (**A**) and fluorescence spectra (excitation, 294 nm; emission, 410 nm) were obtained. Dark control spectra (-----) were measured before irradiation with UV light. (**D**) α-Synuclein (0.25 mg/ml) in a phosphate buffer (100 mM, pH 7.4) with DTPA (100 μM) and without cobalt complex, were irradiated with UV light. FI, fluorescence intensity.

primary oxidant generated by SOD1-peroxidase activity is physiologically significant, because $CO_3^{\bullet -}$ has a much longer half-life (than the $^{\bullet}$OH radical) [rate constants were obtained from the online publication of the Radiation Chemistry Data Center at the Notre Dame Radiation Laboratory (available at http://allen.rad.nd.edu/RCDC/RCDC.html)] and can, therefore, diffuse away from the active site and oxidatively modify neighbouring protein molecules. We have shown that HCO_3^--dependent SOD1-peroxidase activity can be conveniently measured by monitoring the oxidation of dichlorodihydrofluorescein to dichlorofluorescein [17].

In contrast with our interpretations, Kim et al. [5] proposed that elevated levels of $^{\bullet}$OH are responsible for the enhanced aggregation of α-synuclein observed in the presence of SOD1 and H_2O_2. In their study, although experiments were performed in HCO_3^- buffers, the role of $CO_3^{\bullet -}$ in α-synuclein aggregation was not considered [5]. Inclusion of copper-chelating thiols {DDT [1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane], penicillamine, etc.} and nitrone radical traps (PBN, DMPO) inhibited α-synuclein aggregation induced by SOD1/H_2O_2 [5]. Based on these results, the investigators concluded that $^{\bullet}$OH and copper ions released from the active site of SOD1 were responsible for α-synuclein aggregation.

The present data clearly show that, in the absence of HCO_3^-, no oxidation and aggregation of α-synuclein can be observed (Figure 1A). Previously, we have shown that $CO_3^{\bullet -}$ reacts with nitrone spin traps and causes hydroxylation and oxidation of nitrone traps. In addition, the present data show that inclusion of nitrone traps inhibited $CO_3^{\bullet -}$-induced aggregation of α-synuclein (Figures 1C and 1D). Kim et al. [5] reported that high concentrations of thiols (approx. 10 mM) inhibit SOD1/H_2O_2-induced aggregation of α-synuclein [5]. $CO_3^{\bullet -}$ has been reported to react rapidly with thiols [19]. Thus it seems reasonable to conclude that the inhibitory effects of thiols noted in that study [5] are indeed due to scavenging of $CO_3^{\bullet -}$ rather than to copper chelation during SOD1/H_2O_2/HCO_3^--catalysed oxidation and aggregation of α-synuclein.

α-Synuclein has four tyrosine residues, Tyr^{139}, Tyr^{125}, Tyr^{133} and Tyr^{136} [18]. The $CO_3^{\bullet -}$ will oxidize tyrosine present in α-synuclein to the tyrosyl radical, which undergoes radical–radical recombination to form dityrosine. The characteristic fluorescence spectrum corresponding to dityrosine was detected from incubations containing SOD1, H_2O_2, HCO_3^- and α-synuclein, and from mixtures containing α-synuclein and the cobalt carbonate complex that were irradiated with UV light (Figure 8C). Our results further support the conclusion that the α-synuclein dimer band is associated with dityrosine formation [3] (Figure 9B).

HCO_3^- influenced the oxidation, aggregation and nitration of α-synuclein induced by $ONOO^-$. It is known that the reactivity of $ONOO^-$ is heavily influenced by the presence of CO_2 in solution [28]. In the presence of HCO_3^-, which is in equilibrium with CO_2 at physiological pH, $ONOO^-$forms a transient complex,

© 2004 Biochemical Society

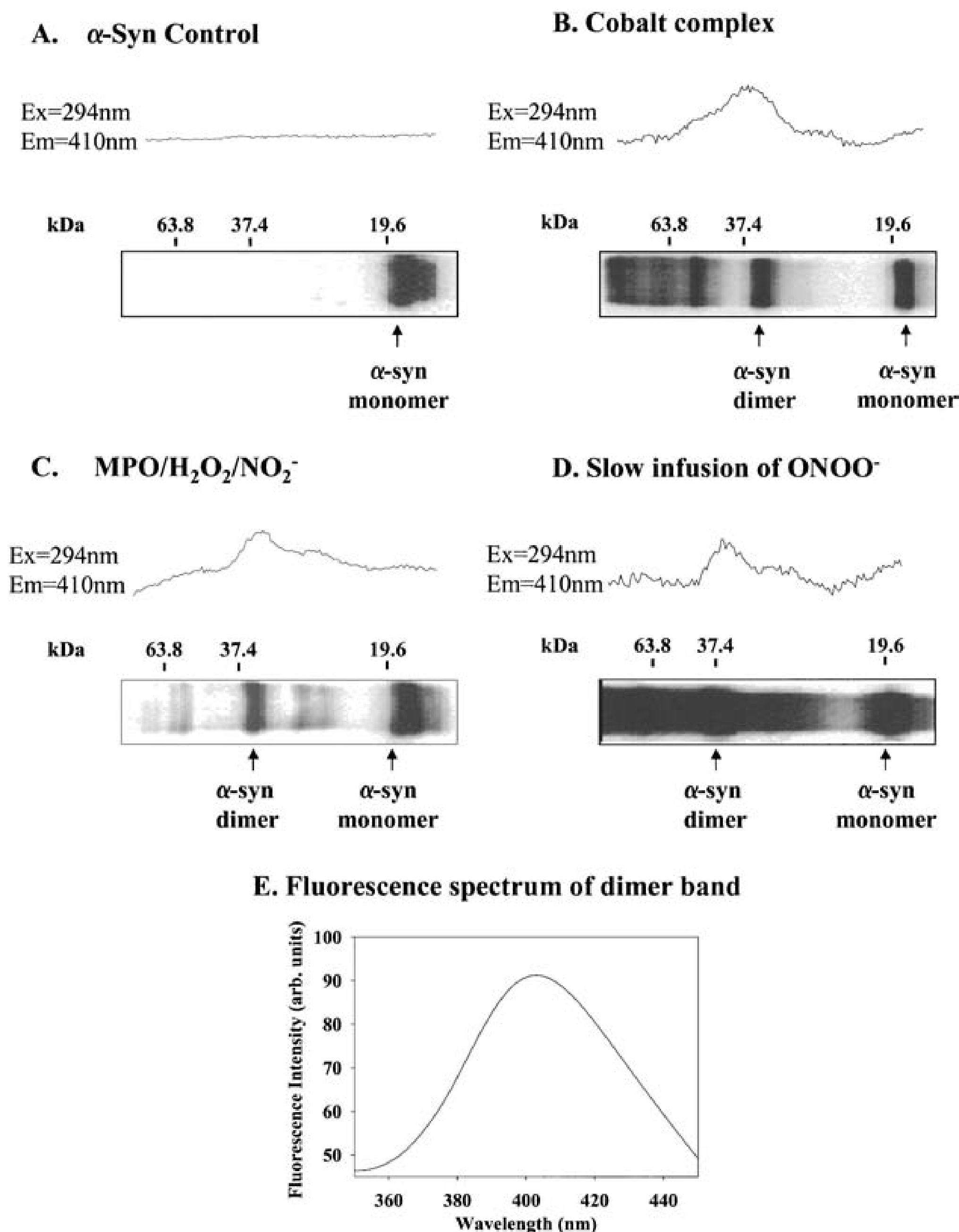

Figure 9 Fluorescence SDS/PAGE analysis of α-synuclein (α-syn) aggregation induced by various oxidants

(**A**) Incubation mixtures contained α-synuclein (10 μg) in a phosphate buffer (100 mM, pH 7.4) containing DTPA (100 μM). The unmodified α-synuclein monomer was separated by SDS/PAGE (10 % gel) and a fluorescence gel for dityrosine in the monomer was obtained. (**B**) Fluorescence gel scan for dityrosine in the dimer of α-synuclein was obtained from the UV-photolysis of the mixture in (**A**), but containing a cobalt complex. (**C**) α-Synuclein was treated with MPO/H_2O_2/NO_2^- using the same conditions as described in Figure 4(A) and a fluorescence gel scan for dityrosine in the dimer band was obtained. (**D**) Fluorescence gel scan for dityrosine in the dimer of α-synuclein was performed from an incubation mixture described in Figure 3(B) that was treated with slow infusion of $ONOO^-$. Products were separated by SDS/PAGE (10 % gel). (**E**) Fluorescence emission spectrum of α-synuclein dimer that was extracted from SDS/PAGE. The sample was prepared as described in (**B**).

$ONOOCO_2^-$, which rapidly decomposes to $NO_2^\bullet$ and $CO_3^{\bullet -}$. Consistent with this mechanism, both oxidation and nitration of α-synuclein increased.

The leucocytic peroxidase MPO, a ubiquitous cellular constituent of inflammatory cells, reacts with H_2O_2 to form higher oxidants (compounds I and II) that are capable of oxidizing a variety of inorganic anions including NO_2^- [22,23]. The NO_2^- anion is oxidized by MPO compound I to $NO_2^\bullet$ radical. In the absence of NO_2^-, there was no oxidation/aggregation of α-synuclein, suggesting that tyrosine residues present in α-synuclein are not accessible to MPO compound I. As shown in Figures 4(A) and 4(B), the MPO/H_2O_2/NO_2^- system enhanced both α-synuclein aggregation and nitration, and the α-synuclein dimer band was associated with dityrosine formation (Figure 9B). As $NO_2^\bullet$ is the major nitrating species at pH 7.4 in the MPO/H_2O_2/NO_2^- system, α-synuclein oxidation and nitration are presumably mediated by

© 2004 Biochemical Society

the abstraction of a hydrogen atom present in α-synuclein tyrosine residues by $NO_2^•$ followed by the reaction between $NO_2^•$ and α-synuclein tyrosyl radical. A major reason for the increased nitration/oxidation efficiency of α-synuclein has been attributed to the absence of cysteine residues and to the presence of several negatively charged glutamate residues proximal to the tyrosines in α-synuclein [35]. At pH 7.4, $MPO/H_2O_2/NO_2^-$ does not generate $ONOO^-$ [36] and, accordingly, we did not observe any stimulation of α-synuclein aggregation or nitration in the presence of HCO_3^-.

Effects of $^•NO$ on $MPO/H_2O_2/NO_2^-$- and $ONOO^-$-induced aggregation and nitration of α-synuclein

$NO_2^•$ is rapidly scavenged by $^•NO$ to form N_2O_3, which is hydrolysed to form NO_2^- [37]. $ONOO^-$ also reacts with $^•NO$ to form N_2O_3 [38]. As RNS can readily diffuse into the membrane [39], this radical–radical recombination reaction should occur rapidly in the hydrophobic domain of membranes. Figure 6 shows that $^•NO$ dramatically inhibits α-synuclein aggregation and nitration. We investigated the effect of $^•NO$ released slowly from SNN on α-synuclein aggregation and nitration induced by $MPO/H_2O_2/NO_2^-$ (Figure 6). The present study shows that $^•NO$ donors inhibit both MPO- and $ONOO^-/HCO_3^-$-induced aggregation and nitration of α-synuclein. $^•NO$ could potentially switch nitration to a nitrosation reaction that is primarily dominated by N_2O_3 in the hydrophobic membrane [14,39,40].

$^•NO$ released from SNN inhibits HCO_3^--stimulated aggregation and nitration of α-synuclein in the presence of slowly generated $ONOO^-$ or SIN-1. The addition of SNN totally inhibits the formation of α-synuclein dimers and trimers and their nitrated products (results not shown). This is attributed to a rapid oxidation of $^•NO$ by $CO_3^{•-}$ to NO_2^- and to the radical–radical recombination reaction between $^•NO$ and $NO_2^•$.

Implications in Parkinson's disease

The aggregation of α-synuclein appears to be a common pathological feature of many neurodegenerative diseases, including Parkinson's disease [1]. Selective nitration of α-synuclein has also been detected in Parkinson's disease [2]. Recent data show that, under nitrative and oxidative stress, α-synuclein undergoes very efficient nitration and oligomerization via dityrosyl formation [28]. Biochemical measurements of α-synuclein isolated from the brains of patients with Parkinson's disease reveal the presence of covalently linked α-synuclein oligomers [3]. The present results indicate that both $CO_3^{•-}$ and $NO_2^•$ are capable of forming cross-linked dityrosyl moieties in α-synuclein. Both tyrosine nitration and dityrosine cross-linking have been proposed to be critical for α-synuclein fibril formation and stabilization [41]. A major finding in the present study is that $^•NO$ dramatically inhibits both $NO_2^•$- and $ONOO^-/HCO_3^-$-induced oxidative and nitrative modification of α-synuclein. Parkinson's disease is characterized by a tetrahydrobiopterin deficiency that may lead to increased $O_2^{•-}$ and decreased $^•NO$ formation from NO synthase [42]. These conditions are likely to enhance formation of RNS, leading to oxidative and nitrative modification of α-synuclein.

This research was supported by grants from the National Institutes of Health (HL63119 and NS40494). Additionally, we thank Dr Harry Ischiropoulos for initially providing us with the α-synuclein plasmid and for technical advice during the course of this work.

REFERENCES

1 Spillantini, M. G., Schmidt, M. L., Lee, V. M. Y., Trojanowski, J. Q., Jakes, R. and Goedert, M. (1997) α-Synuclein in Lewy bodies. Nature (London) **388**, 839–840

2 Giasson, B. I., Duda, J. E., Murray, I. V. J., Chen, Q., Souza, J. M., Hurtig, H. I., Ischiropoulos, H., Trojanowski, J. Q. and Lee, V. M. Y. (2000) Oxidative damage linked to neurodegeneration by selective α-synuclein nitration in synucleinopathy lesions. Science **290**, 985–989

3 Souza, J. M., Giasson, B. I., Chen, Q., Lee, V. M. Y. and Ischiropoulos, H. (2000) Dityrosine cross-linking promotes formation of stable α-synuclein polymers: implication of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. J. Biol. Chem. **275**, 18344–18349

4 Takahashi, T., Yamashita, H., Nakamura, T., Nagano, Y. and Nakamura, S. (2002) Tyrosine 125 of α-synuclein plays a critical role for dimerization following nitrative stress. Brain Res. **938**, 73–80

5 Kim, K. S., Choi, S. Y., Kwon, H. Y., Won, M. H., Kang, T. and Kang, J. H. (2002) Aggregation of α-synuclein induced by the Cu,Zn-superoxide dismutase and hydrogen peroxide system. Free Radical Biol. Med. **32**, 544–550

6 Lymar, S. V. and Hurst, J. K. (1996) Carbon dioxide: physiological catalyst for peroxynitrite-mediated cellular damage or cellular protectant? Chem. Res. Toxicol. **9**, 845–850

7 Squadrito, G. L. and Pryor, W. A. (1998) Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide. Free Radical Biol. Med. **25**, 392–403

8 Liochev, S. I. and Fridovich, I. (1999) On the role of bicarbonate in peroxidations catalyzed by Cu,Zn superoxide dismutase. Free Radical Biol. Med. **27**, 1444–1447

9 Goss, S. P., Singh, R. J. and Kalyanaraman, B. (1999) Bicarbonate enhances the peroxidase activity of Cu,Zn-superoxide dismutase: role of carbonate anion radical. J. Biol. Chem. **274**, 28233–28239

10 Choi, J. Y., Sung, Y. M., Park, H. J., Hur, E. H., Lee, S. J., Hahn, C., Min, B. R., Kim, I. K., Kang, S. and Rhim, H. (2002) Rapid purification and analysis of α-synuclein proteins: C-terminal truncation promotes the conversion of α-synuclein into a protease-sensitive form in *Escherichia coli*. Biotechnol. Appl. Biochem. **36**, 33–40

11 Basolo, F. and Murmann, R. K. (1953) Synthesis of cobalt (III) carbonato complex for $CO_3^{•-}$ radical studies. Inorg. Synth. **4**, 171–172

12 Beckman, J. S., Chen, J., Ischiropoulos, H. and Crow, J. P. (1994) Oxidative chemistry of peroxynitrite. Methods Enzymol. **233**, 229–240

13 Hughes, M. N. and Nicklin, H. G. (1968) The chemistry of pernitrites. Part I: kinetics of decomposition of pernitrous acid. J. Chem. Soc., 450–452

14 Zhang, H., Bhargava, K., Keszler, A., Feix, J., Hogg, N., Joseph, J. and Kalyanaraman, B. (2003) Transmembrane nitration of hydrophobic tyrosyl peptides: localization, characterization, mechanism of nitration, and biological implications. J. Biol. Chem. **278**, 8969–8978

15 Scheer, J. M. and Ryan, C. A. (2001) A method for quantitative recovery of proteins from polyacrylamide gels. Anal. Biochem. **298**, 130–132

16 Cope, V. W., Chen, S. and Hoffman, M. Z. (1973) Intermediates in the photochemistry of carbonato-amine complexes of cobalt (III): $CO_3^{•-}$ radicals and the aquacarbonato complex. J. Am. Chem. Soc. **95**, 3116–3121

17 Zhang, H., Joseph, J., Gurney, M., Becker, D. and Kalyanaraman, B. (2002) Bicarbonate enhances peroxidase activity of Cu,Zn-superoxide dismutase: role of carbonate anion radical and scavenging of carbonate anion radical by metalloporphyrin antioxidant enzyme mimetics. J. Biol. Chem. **277**, 1013–1020

18 Jakes, R., Spillantini, M. G. and Goedert, M. (1994) Identification of two distinct synucleins from human brain. FEBS Lett. **345**, 27–32

19 Chen, S. N. and Hoffman, M. Z. (1973) Rate constants for the reaction of the carbonate radical with compounds of biochemical interest in neutral aqueous solution. Radiat. Res. **56**, 40–47

20 Hogg, N., Darley-Usmar, V. M., Wilson, M. T. and Moncada, S. (1992) Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. Biochem. J. **281**, 419–424

21 Zhang, H., Joseph, J., Felix, C. and Kalyanaraman, B. (2000) Bicarbonate enhances the hydroxylation, nitration, and peroxidation reactions catalyzed by copper, zinc superoxide dismutase: intermediacy of carbonate anion radical. J. Biol. Chem. **275**, 14038–14045

22 Eiserich, J. P., Cross, C. E., Jones, D. A., Halliwell, B. and van der Vliet, A. (1996) Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid: a novel mechanism for nitric oxide-mediated protein modification. J. Biol. Chem. **271**, 19199–19208

23 Rosen, H., Crowley, J. R. and Heinecke, J. W. (2002) Human neutrophils use the myeloperoxidase–hydrogen peroxide–chloride system to chlorinate but not nitrate bacterial proteins during phagocytosis. J. Biol. Chem. **277**, 30463–30468

24 Cole, N. B., Murphy, D. D., Grider, T., Rueter, S., Brasaemle, D. and Nussbaum, R. L. (2002) Lipid droplet binding and oligomerization properties of the Parkinson's disease protein α-synuclein. J. Biol. Chem. **277**, 6344–6352

25 Weinreb, P. H., Zhen, W. G., Poon, A. W., Conway, K. A. and Lansbury, Jr, P. T. (1996) NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded. Biochemistry **35**, 13709–13715

© 2004 Biochemical Society

26 Davidson, W. S., Jonas, A., Clayton, D. F. and George, J. M. (1998) Stabilization of α-synuclein secondary structure upon binding to synthetic membranes. J. Biol. Chem. **273**, 9443–9449
27 Perrin, R. J., Woods, W. S., Clayton, D. F. and George, J. M. (2001) Exposure to long chain polyunsaturated fatty acids triggers rapid multimerization of synucleins. J. Biol. Chem. **276**, 41958–41962
28 Norris, E. H., Giasson, B. I., Ischiropoulos, H. and Lee, V. M. Y. (2003) Effects of oxidative and nitrative challenges on α-synuclein fibrillogenesis involve distinct mechanisms of protein modifications. J. Biol. Chem. **278**, 27230–27240
29 Zhang, H., Joseph, J., Feix, J., Hogg, N. and Kalyanaraman, B. (2001) Nitration and oxidation of a hydrophobic tyrosine probe by peroxynitrite in membranes: comparison with nitration and oxidation of tyrosine by peroxynitrite in aqueous solution. Biochemistry **40**, 7675–7686
30 Czapski, G., Holcman, J. and Bielski, B. H. J. (1994) Reactivity of nitric oxide with simple short lived radical in aqueous solution. J. Am. Chem. Soc. **116**, 11465–11469
31 Hodgson, E. K. and Fridovich, I. (1976) The mechanism of the activity-dependent luminescence of xanthine oxidase. Arch. Biochem. Biophys. **356**, 202–205
32 Hodgson, E. K. and Fridovich, I. (1975) The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: inactivation of the enzyme. Biochemistry **14**, 5294–5299
33 Hodgson, E. K. and Fridovich, I. (1975) The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: chemiluminescence and peroxidation. Biochemistry **14**, 5299–5303
34 Reference deleted
35 Ischiropoulos, H. (1998) Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. Arch. Biochem. Biophys. **356**, 1–11
36 Brennan, M. L., Wu, W., Fu, X., Shen, Z., Song, W., Frost, H., Vadseth, C., Narine, L., Lenkiewicz, E., Borchers, M. T. et al. (2002) A tale of two controversies: defining both the role of peroxidases in nitrotyrosine formation *in vivo* using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. J. Biol. Chem. **277**, 17415–17427
37 Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E. and Chaudhuri, G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. U.S.A. **84**, 9265–9269
38 Jourd'heuil, D., Miranda, K. M., Kim, S. M., Espey, M. G., Vodovotz, Y., Laroux, S., Mai, C. T., Miles, A. M., Grisham, M. B. and Wink, D. A. (1999) The oxidative and nitrosative chemistry of the nitric oxide/superoxide reaction in the presence of bicarbonate. Arch. Biochem. Biophys. **365**, 92–100
39 Liu, X., Miller, M. J., Joshi, M. S., Thomas, D. D. and Lancaster, Jr, J. R. (1998) Accelerated reaction of nitric oxide with O_2 within the hydrophobic interior of biological membranes. Proc. Natl. Acad. Sci. U.S.A. **95**, 2175–2179
40 Espey, M. G., Thomas, D. D., Miranda, K. M. and Wink, D. A. (2002) Focusing of nitric oxide mediated nitrosation and oxidative nitrosylation as a consequence of reaction with superoxide. Proc. Natl. Acad. Sci. U.S.A. **99**, 11127–11132
41 Krishnan, S., Chi, E. Y., Wood, S. J., Kendrick, B. S., Li, C., Garzon-Rodriguez, W., Wypych, J., Randolph, T. W., Narhi, L. O., Biere, A. L. et al. (2003) Oxidative dimer formation is the critical rate-limiting step for Parkinson's disease α-synuclein fibrillogenesis. Biochemistry **42**, 829–837
42 Vasquez-Vivar, J., Hogg, N., Martasek, P., Karoui, H., Pritchard, K. A. and Kalyanaraman, B. (1999) Tetrahydrobiopterin-dependent inhibition of superoxide generation from neuronal nitric oxide synthase. J. Biol. Chem. **274**, 26736–26742

Received 24 September 2003/13 November 2003; accepted 26 November 2003
Published as BJ Immediate Publication 26 November 2003, DOI 10.1042/BJ20031466

© 2004 Biochemical Society