Metal-Catalyzed Oxidation of Protein-Bound Dopamine†

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ABSTRACT: Dopamine (DA) is an unstable neurotransmitter that readily oxidizes to the DA quinone and forms reactive oxygen species, such as superoxide and hydrogen peroxide. The oxidized dopamine also forms thiol conjugates with sulfhydryl groups on cysteine, glutathione, and proteins. In the present study, we determined the redox potential of the protein-bound DA and established a novel mechanism for the oxidative modification of the protein, in which the DA-cysteine adduct generated in the DA-modified protein causes oxidative modification of the DA-bound protein in the presence of Cu^{2+}. Exposure of a sulfhydryl enzyme, glyceraldehyde-3-phosphate dehydrogenase, to DA resulted in a significant loss of sulfhydryl groups and the formation of the DA-cysteine adduct. When the DA-modified protein was incubated with Cu^{2+}, we observed aggregation and degradation of the DA-bound protein and concomitant formation of a protein carbonyl, a marker of an oxidatively modified protein. Furthermore, we analyzed the carbonyl products generated during the Cu^{2+}-catalyzed oxidation of the DA-modified protein and revealed the production of glutamic and aminoadipic semialdehydes, consisting of the protein carbonyls generated. The cysteinyl-DA residue generated in the DA-modified protein was suggested to represent a redox-active adduct, based on the observations that the cysteinyl-DA adduct, 5-*S*-cysteinyldopamine, produced by the reaction of cysteine with DA, gave rise to the oxidative modification of bovine serum albumin in the presence of Cu^{2+}. These data suggest that the DA-modified protein may be involved in redox alteration under oxidative stress, whereby DA covalently binds to cysteine residues, generating the redox-active cysteinyl-DA adduct that causes the metal-catalyzed oxidation of protein.

The major pathologic change of idiopathic Parkinson's disease (PD)[1] is the neuronal degeneration of melanized neurons in the substantia nigra. The exact cause of the melanized neuronal death in PD is still unknown; however, oxidative stress has been implicated as one of the most important contributors of nigral cell death in PD (*1*). Evidence to indicate the presence of oxidative stress in PD includes increase in iron content in the substantia nigra, reduced glutathione, increased Mn superoxide dismutase (Mn-SOD) activity, and mitochondrial respiratory failure. Free radicals appear to contribute to the pathogenesis of PD. Nigral dopaminergic neurons are particularly exposed to oxidative stress since hydrogen peroxide may be produced in the metabolism of dopamine (DA), which is high in the substantia nigra; more toxic hydroxyl radicals may be formed from hydrogen peroxide in the presence of metal ions (*2*, *3*). Previous studies have demonstrated the selective and highly significant elevation of metal ion levels in the substantia nigra of patients with PD (*4*, *5*). An increased level of metal ions in the substantia nigra may cause excessive formation of oxygen radicals, which may play a role in the nigral degeneration. This hypothesis can be supported by the observations that lipid peroxidation, as monitored by the accumulations of malondialdehyde (*6*) and protein-bound 4-hydroxy-2-nonenal (*7*), was significantly enhanced in the nigral neurons of PD patients.

One of the key contributors to oxidative stress in PD may be DA. DA has been shown to contribute to various toxic phenomena. For example, striatal damage produced by either an ischemic event or methamphetamine exposure was attenuated by pretreatment with the DA synthesis inhibitor α-methyl-*p*-tyrosine (*8*, *9*). The oxidative metabolism of DA proceeds via both enzymatic and nonenzymatic pathways (*10*, *11*). Alteration of the normal metabolism of DA might lead to elevated concentrations of this neurotransmitter, thereby accelerating its autoxidation in the cellular compartments in

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[1] Abbreviations: AAS, aminoadipic semialdehyde; ABA, *p*-aminobenzoic acid; BSA, bovine serum albumin; DA, dopamine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTPA, diethylenetriaminepentaacetic acid; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FTSC, fluorescein thiosemicarbazide; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GS, glutamic semialdehyde; IAB, iodoacetyl-LC-biotin; LC-MS/MS, liquid chromatography−tandem mass spectrometry; MES, 2-(*N*-morpholino)ethanesulfonic acid; NBT, nitro blue tetrazolium; PD, Parkinson's disease.

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which its concentration has increased (*11*). The autoxidation of DA in vivo produces DA quinones, superoxide radicals, hydroxyl radicals, and hydrogen peroxide, all of which generate oxidative stress contributing to the cytotoxicity of DA (*12*). DA quinones are electron-deficient species that can covalently bind to cellular nucleophiles, such as sulfhydryl groups, on free cysteine, reduced glutathione (GSH), and cysteinyl residues in proteins. This autoxidation mechanism is facilitated by the presence of catalysts, such as transition metal ions (*13*). Indeed, it has been shown that trace metal ions, such as Cu^{2+}, can accerelate the autoxidation of hydroquinones (*14*).

In the present study, we found that the protein-bound DA is not the end product but the reducing agent that possesses a strong reducing potential. Moreover, we established a novel mechanism for the oxidative modification of protein, in which the DA-cysteine adduct generated in the DA-modified protein causes oxidative modification of the DA-bound protein in the presence of Cu^{2+}. In addition to the autoxidation mechanism of free DA, our present findings suggest an alternative mechanism that utilizes the protein-bound DA as a potential electron donor and a source of reactive oxygen species.

MATERIALS AND METHODS

Materials. Dopamine hydrochloride was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Glyceraldehyde-3-phosphate dehydrogenase was from Roche (Indianapolis, IN). Iodoacetyl-LC-biotin (IAB) and horseradish peroxidase (HRP) conjugated NeutrAvidin were from Pierce. Enhanced chemiluminescence (ECL) Western blotting detection reagents were obtained from Amersham Biosciences. SYPRO Ruby protein gel stain was from Molecular Probes, Inc. The protein concentration was measured using the BCA protein assay reagent obtained from Pierce. All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell Culture. SH-SY5Y cells were grown in Cosmedium-001 (Cosmo-Bio, Tokyo, Japan) containing 5% Nakashibetsu precolostrum newborn calf serum, 100 μg/mL penicillin, and 100 unit/mL streptomycin. Cells were seeded in plates coated with polylysine and cultured at 37 °C.

Cell Viability. Cell viability was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells incubated with DA (0−400 μM) were treated with 10 mL of MTT solution (5 mg/mL) for 4 h. The cells were then lysed with 0.04 N HCl in 2-propanol, and the absorbance was read at 570 nm.

Assay of GAPDH Catalytic Activity. The GAPDH solution (2 mg/mL) was prepared by dilution of the enzyme suspension with 0.1 M sodium phosphate buffer (pH 7.2). An aliquot (0.5 mL) of the enzyme solution was mixed with an equal volume of DA solution and incubated at 37 °C. The GAPDH activity was assayed as described by Ishii et al. (*15*). A 10 μL aliquot of the reaction mixture was assayed in 3 mL of 15 mM sodium pyrophosphate and 30 mM sodium arsenate buffer (pH 8.5). The reaction of GAPDH was initiated by the addition of 100 μL of 7.5 mM NAD, 100 μL of 0.1 mM 1,4-dithiothreitol (DTT), and 100 μL of 15 mM DL-glyceraldehyde-3-phosphate. The mixture was incubated at room temperature for 5 min, and the absorbance at 340 nm was measured.

SDS−Polyacrylamide Gel Electrophoresis and Redox-Cycling Staining. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to the method of Laemmli (*16*). The protein was stained with SYPRO Ruby. A gel was transblotted onto a nitrocellulose membrane (Hybond ECL, Amersham Biosciences). The quinoproteins were detected by staining blots with nitro blue tetrazolium (NBT, 0.24 mM in 2 M potassium glycinate, pH 10) as described by Paz et al. (*17*). The blue-purple-stained quinoproteins were first photographed, the blots were then stained for total protein with Ponceau S (0.1% in 5% acetic acid), and the red-stained protein bands were rephotographed.

Thiol Assay. The protein thiols were analyzed by modification of the published method of Rabinovic and Hastings (*18*). An aliquot (0.3 mL) of the protein samples (1 mg/mL) incubated in the absence or presence of DA was mixed with an equal volume of 20% trichloroacetic acid (TCA, w/v, final concentration) and centrifuged at 35000*g* for 15 min at 4 °C. The pellet was washed twice with ethanol−ethyl acetate (1:1 v/v), and the pellet was then dissolved with 0.3 mL of 8 M guanidine hydrochloride, 13 mM ethylenediaminetetraacetic acid (EDTA), and 133 mM Tris solution (pH 7.6). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB, 10 μL of 10 mM solution) was then added, and the color was allowed to develop for 20 min. The absorbance was spectrophotometrically measured at 412 nm. The values were calculated by comparison with the identically treated *N*-acetylcysteine standards (10−200 μM).

Labeling with IAB. GAPDH (1 mg/mL) was exposed to DA (0−1000 μM) for 1 h. Aliquots (20 μL) of the protein samples were treated with 4 μL of DTT (100 mM) and incubated for 30 min at room temperature. These mixtures were then treated with 6 μL of 5 mM IAB for 60 min in the dark. The protein samples were boiled in the Laemmli sample buffer for 5 min, and the biotinylated proteins were then subjected to immunoblot and detection with HRP-conjugated NeutrAvidin and ECL.

Detection of Cysteinyl-DA by Liquid Choromatography−Tandem Mass Spectrometry (LC-MS/MS) Analysis. GAPDH (0.3 mg) was exposed to DA (1 mM) for 1 h. The reactions were arrested with an equivalent volume of 20% TCA followed by incubation at 4 °C for 2 h. The resulting precipitates were collected by centrifugation and washed three times with ice-cold 10% TCA. The final pellet was suspended in 1 mL of 6 M HCl, transferred to a hydrolysis vessel, and extensively purged with nitrogen before sealing. The protein samples were heated to 110 °C for 16 h. The hydrolyzed samples were dissolved in 0.2 mL of the HPLC mobile phase (see below) and analyzed by LC-MS/MS analysis. The cysteinyl-DA was identified by comparison with the cysteinyl-DA standard that has been previously synthesized and isolated (*19*).

The LC-MS/MS analyses were carried out on the API 2000 triple quadrupole mass spectrometer (Applied Biosystems) through a TurboIonSpray source. Chromatography was carried out on a Develosil ODS-HG-3 column (2.0 × 50 mm) using an Agilent 1100 HPLC system. The gradient programs (solvent A, 100% H_2O containing 0.1% formic acid; solvent B, 100% CH_3CN containing 0.1% formic acid) were as follows: 0−5 min, 0% B; 5−10 min, linear gradient to 50% B; 10−11 min, linear gradient to 100% B; 11−15 min, 100% B; 15−16 min, linear gradient to 0% B; 16−25

min, 0% B; flow rate = 0.1 mL/min. The instrument response was optimized by infusion experiments of the standard compounds using a syringe pump at a flow rate of 5 μL/min. The 5-*S*-cysteinyl-DAs were detected using electrospray ionization tandem mass spectrometry in the multiple reaction monitoring mode. Specific transitions used to detect products in the negative ionization mode were those between the molecular anion of the products and the characteristic daughter ion.

Oxidative Modification of Protein by a Metal Ion/DA System. GAPDH (0.3 mg) was incubated with DA (0.1 mM) in the absence and presence of a metal ion (Cu^{2+} or Fe^{3+}) in 0.1 M sodium phosphate buffer (pH 7.2) at 37 °C for 1 h. The samples were boiled with the Laemmli sample buffer and analyzed by SDS−PAGE.

Oxidative Modification of DA-Modified Protein by a Metal Ion. GAPDH (0.3 mg) was incubated with DA (0.1 mM) in 0.1 M sodium phosphate buffer (pH 7.2) at 37 °C for 1 h. The reaction was terminated by centrifugal filtration (Microcon 30, molecular weight cutoff of 30000) to remove the low-molecular-weight reactants. The DA-modified GAPDH (10 μg) was then incubated in the absence and presence of a metal ion (Cu^{2+} or Fe^{3+}) in 0.1 M sodium phosphate buffer (pH 7.2) at 37 °C for 1 h. The samples were boiled with the Laemmli sample buffer and analyzed by SDS−PAGE.

Protein Carbonyl. An aliquot (50 μg) of the protein sample was mixed with 5 μL of 0.1% fluorescein thiosemicarbazide (FTSC, dissolved in DMSO) and 30 μL of 1 M potassium acetate buffer (pH 4.6). The total volume was adjusted to 100 μL with H_2O and incubated in the dark for 2 h at room temperature. An equal volume of 20% TCA was added to the mixture and centrifuged at 13000*g* for 10 min. The precipitate was rinsed with 200 μL of 20% TCA, 10% TCA, and ethanol−ethyl acetate (1:1 v/v). The protein samples were then boiled with the Laemmli sample buffer and analyzed by SDS−PAGE.

Detection of Carbonyl Amino Acids. Carbonyl amino acids were determined by our previous method (*20*, *21*) as follows. The protein samples were dialyzed by ultrafiltration with Ultrafree-MC centrifugal filter units (10000 Da NMWL; Millipore, Billerica, MA). The dialysis was continued at 4 °C with three changes of the phosphate-buffered saline (PBS) containing 1 mM diethylenetriaminepentaacetic acid (DTPA) and a final change of 0.25 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) containing 1% SDS and 1 mM DTPA. A 250 μL aliquot of the protein solution was then transferred to a vial, and 500 μL of 50 mM *p*-aminobenzoic acid (ABA) in 0.25 M MES buffer (pH 6.0) was added. The reaction was started by the addition of 250 μL of freshly prepared 100 mM $NaCNBH_3$ in 0.25 M MES buffer (pH 6.0), and the mixture was allowed to react at 37 °C for 90 min with shaking in the dark. After the reaction, the protein was precipitated by the addition of 500 μL of cold 50% TCA. After being allowed to stand for 10 min in an ice bath, the mixture was centrifuged at 17000*g* for 10 min at 4 °C, and the pellet of the precipitated protein was separated. The pellet was washed twice with 1.0 mL of cold 10% TCA and 1.0 mL of cold ethanol. The resulting protein was then hydrolyzed for 24 h at 110 °C with 1.5 mL of 6 M HCl. The hydrolysate was evaporated to dryness in vacuo followed by reconstitution in 200 μL of 50 mM sodium acetate buffer (pH 5.4). After filtration using a PVDF syringe filter (0.45 μm pore size; Whatman, Clifton, NJ), the hydrolysate was analyzed by HPLC. The sample (20 μL) was injected into an HPLC instrument equipped with a C-18 reversed-phase column (COSMOSIL $5C_{18}$-AR-II, 250 × 4.6 mm; Nacalai Tesque) eluted by 50 mM sodium acetate buffer (pH 5.4). The HPLC instrument (Hitachi, Tokyo, Japan) consisted of an L-6020 pump and an L-7485 fluorescence detector in a D-2500 data station. The column oven (L-7300, Hitachi) was maintained at 40 °C, and the flow rate was 1.5 mL/min. The eluate was monitored with excitation and emission wavelengths set at 283 and 350 nm, respectively. The calibration curves were obtained for the authentic standards by plotting the peak areas. Correlation coefficients greater than 0.999 were obtained.

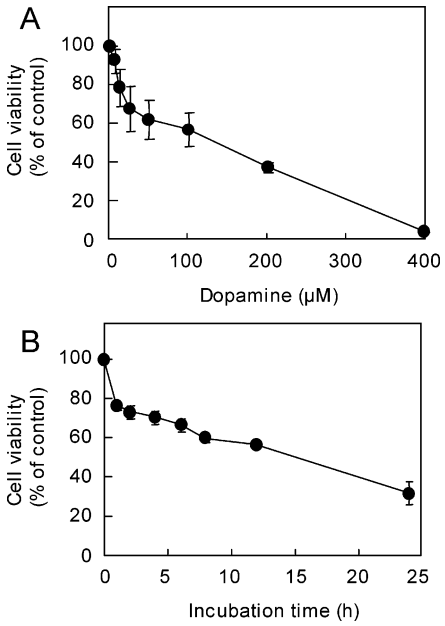

FIGURE 1: Viability of SH-SY5Y cells exposed to DA. (A) Dose-dependent reduction of cell viability induced by DA. Cells were exposed to DA (0−0.4 mM) for 12 h. (B) Time-dependent reduction of cell viability induced by DA. Cells were exposed to 0.1 mM DA for 0−24 h. The cell viability was measured by the MTT assay. Data are expressed as percent of control culture conditions.

RESULTS

Cytotoxicity of DA. We first examined the cytotoxicity of DA by MTT assay. As shown in Figure 1, in vitro exposure of human nueroblastoma SH-SY5Y cells to DA led to a dose- and time-dependent decrease in the number of viable cells. Even 0.1 mM DA did cause a 60% decrease in the MTT reduction level after 24 h of incubation. We also observed the production of reactive oxygen species in the cells exposed to DA. The level of ROS in the cells exposed to 1 mM DA for 60 min was approximately 2-fold higher than that of the control (data not shown). These data suggest that the DA cytotoxicity is associated with the production of intracellular reactive oxygen species.

Covalent Binding of DA to Sulfhydryl Groups of Protein. A previous study has shown that the DA treatment of PC12 cells results in a decrease in the sulfhydryl groups of cellular proteins, including GAPDH (*22*). In view of this effect of DA on GAPDH, we hypothesized that GAPDH could represent a target for DA modification. Hence, we examined the potential reactivity of DA toward GAPDH. As shown in

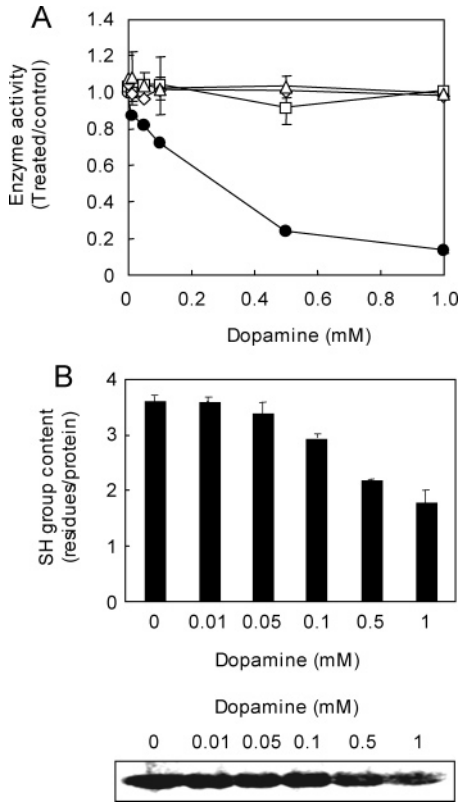

FIGURE 2: Covalent binding of DA to sulfhydryl groups of protein. GAPDH (1 mg/mL) was incubated with DA (0−1 mM) in 0.1 M sodium phosphate buffer (pH 7.2) for 30 min at 37 °C. (A) Changes in the GAPDH activity and effect of sulfhydryl compounds. GAPDH was incubated with DA for 30 min. Symbols: DA (□); DTT (●); DA + DTT (◇); DA + GSH (△). (B) Loss of sulfhydryl groups. Upper: DTNB assay. Lower: NeutrAvidin blot analysis using iodoacetyl-LC-biotin. GAPDH (1 mg/mL) was incubated with DA (0−1 mM) in 0.1 M sodium phosphate buffer (pH 7.2) for 30 min at 37 °C.

Figure 2A, when GAPDH (0.1 mg/mL) was incubated with DA (0−1 mM) in 0.1 M sodium phosphate buffer (pH 7.2) for 30 min at 37 °C, DA showed a significant inhibitory effect on the GAPDH activity ($IC_{50} = 285\ \mu M$). In addition, the inhibitory effect of DA on the enzyme activity was significantly retarded by the addition of sulfhydryl compounds, such as GSH or DTT. In accordance with the previous findings (*23*−*25*), the loss of enzyme activity by DA was associated with the decrease in the amount of the cysteine residues (sulfhydryl groups) (Figure 2B, upper). Sulfhydryl modification by DA was also confirmed by a NeutrAvidin blot analysis using iodoacetyl-LC-biotin (Figure 2B, lower).

Formation of 5-S-Cysteinyl-DA in DA-Modified GAPDH. DA has been shown to primarily react with the sulfhydryl groups of proteins, generating 5-*S*-cysteinyl-DA as the major product (Figure 3A) (*23*−*25*). To document the presence of the DA-cysteine adduct within GAPDH, the protein (0.3 mg) was treated with DA (1 mM), hydrolyzed, and analyzed by LC-MS/MS. As shown in Figure 3B, the authentic 5-cysteinyl-DA gave a single peak (upper). In addition, the same peak was detected in the hydrolysate of DA-treated GAPDH (middle) but not in the hydrolysate of native GAPDH (bottom). HPLC analysis with fluorescence detection also revealed the formation of this adduct as the major product (data not shown). Thus, the 5-cysteinyl-DA was identified as the major adduct generated in the DA-treated GAPDH. These data suggest that modification of the sulfhydryl groups underlies the modulation of the catalytic function of GAPDH by DA.

Redox-Cycling Potential of DA-Modified Proteins. Quinones and related catechols can catalyze redox cycling at an alkaline pH in the presence of excess glycine (*17*, *26*). The superoxide released in the redox cycling reduces NBT to its formazan, allowing the detection of quinones and related catechols. Because the major DA adduct, 5-*S*-cysteinyl-DA, possesses a catechol moiety, it is anticipated that this adduct is more readily oxidized to quinone than dopamine itself (*27*, *28*) and therefore has the redox-cycling potential. To examine whether the conjugate indeed possesses this function, GAPDH was treated with various concentrations (0−1 mM) of DA in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h at 37 °C and analyzed by SDS−PAGE/Western blot followed by redox-cycling staining with NBT/glycinate. As shown in Figure 4A, in accordance with the generation of the cysteinyl-DA adduct (Figure 3), DA modification of GAPDH resulted in concentration-dependent increases in the redox-cycling staining. Moreover, utilizing this unique redox property of protein-bound DA, we examined whether this conjugate was generated in the DA-treated cells. As shown in Figure 4B, the exposure to DA (0−0.5 mM) for 4 h indeed resulted in the increase in the redox-cycling staining positive proteins. Thus, the catechol ring of the cysteinyl-DA can undergo oxidation by one or two electron transfers, a process that is accelerated by the presence of transition metals such as iron, manganese, and copper (*12*, *29*). It is generally accepted that catechols in the presence of metal ions can produce reactive oxygen species through its reaction with oxygen, which gives rise to the oxidative modification of proteins. Hence, the effect of the addition of transition metal ions, such as Cu^{2+} and Fe^{3+}, on the DA modification of GAPDH was examined. As shown in Figure 4C, the incubation of GAPDH with DA in the presence of Cu^{2+} or Fe^{3+} indeed resulted in the significant decrease in the protein band, suggesting that the DA-modified protein possesses an activity comparable to that of other reducing agents known to cause a metal-catalyzed oxidative modification of proteins.

The Cysteinyl-DA Contains the Active Site of the Cu^{2+}-Catalyzed Oxidation of the DA-Modified GAPDH. To further characterize the decrease in the protein band during the incubation of GAPDH with DA in the presence of Cu^{2+}, the DA-modified protein was prepared by incubating GAPDH with DA for 1 h and subjected to dialysis to remove the unreacted DA. The DA-modified GAPDH was then incubated with Cu^{2+} or Fe^{3+} for 1 h and analyzed by SDS−PAGE. As shown in Figure 5A, Cu^{2+} markedly enhanced the loss of the protein band, whereas Fe^{3+} was less effective than Cu^{2+}. The loss of the DA-modified protein appeared to be Cu^{2+}-dependent on the basis of observations that a metal chelator, EDTA, completely inhibited the Cu^{2+}-catalyzed loss of the protein. In addition, when the DA-modified GAPDH (0−0.1 mg/mL) was incubated with native BSA (0.01 mg/mL) in the presence of Cu^{2+} for 24 h at 37 °C and analyzed by SDS−PAGE, we observed a significant loss of the BSA protein bands (Figure 5B), due probably to the oxidative cleavage of the polypeptide chain. More strikingly, this result was reproduced by the incubation of BSA with 5-*S*-cysteinyl-DA in the presence of Cu^{2+} (Figure 5C), suggesting that the

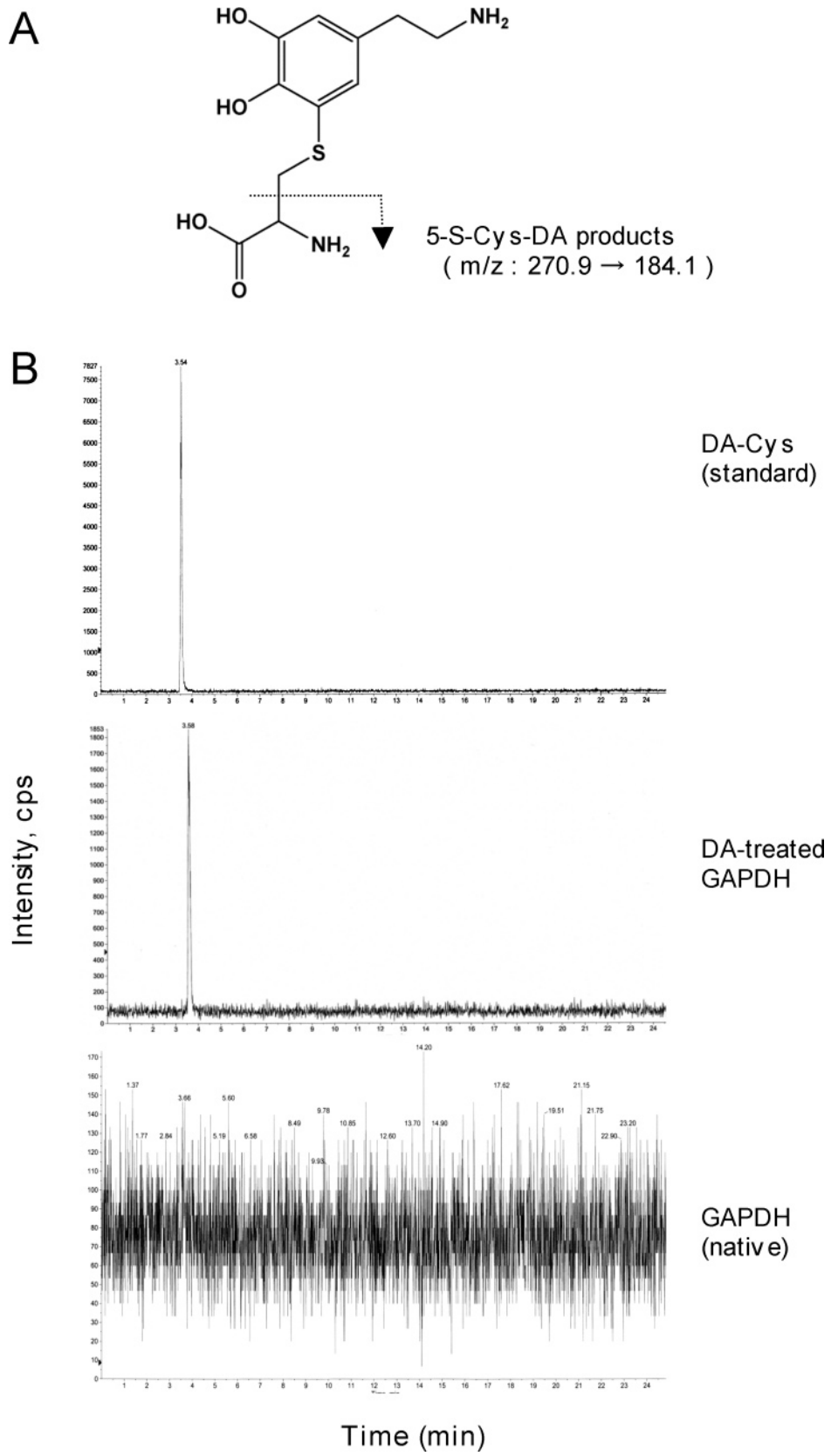

FIGURE 3: Formation of 5-*S*-cysteinyl-DA in the DA-treated GAPDH. (A) Chemical structure of 5-*S*-cysteinyl-DA. (B) LC-MS/MS analysis of 5-*S*-cysteinyl-DA generated in the DA-treated GAPDH. GAPDH (0.3 mg) was treated with DA (1 mM) in 0.1 M sodium phosphate buffer (pH 7.2) for 1 h at 37 °C, hydrolyzed, and analyzed by LC-MS/MS. Upper: the authentic 5-*S*-cysteinyl-DA. Middle: the hydrolysate of DA-treated GAPDH. Bottom: the hydrolysate of native GAPDH.

cysteinyl-DA adduct generated in the DA-modified protein comprises the active site and reductively activates Cu^{2+}, which may facilitate the oxidative modification of proteins. Alternatively, since these processes involve the generation of superoxide and H_2O_2 (*12*), these reactive oxygen species may also play a role as the reducing agents of Cu^{2+}. This

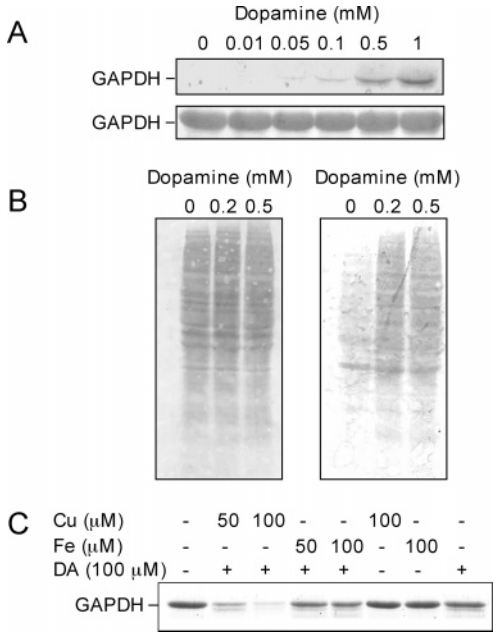

FIGURE 4: Redox-cycling potential of DA-modified proteins. (A) Redox-cycling potential of the DA-modified GAPDH. Upper: redox-cycling staining with NBT/glycinate. Lower: total protein staining with Ponceau S. GAPDH was treated with DA (0−1 mM) in 0.1 M sodium phosphate buffer (pH 7.2) for 1 h at 37 °C. (B) Redox-cycling staining of cellular proteins in the DA-treated SH-SY5Y cells. Cells were exposed to DA (0−0.5 mM) for 4 h. Left: total protein staining with Ponceau S. Right: redox-cycling staining with NBT/glycinate. (C) Metal-catalyzed oxidation of GAPDH by DA. GAPDH (0.3 mg) was incubated with DA (0.1 mM) in the absence and presence of a metal ion (Cu^{2+} or Fe^{3+}) in 0.1 M sodium phosphate buffer (pH 7.2) at 37 °C for 1 h. The samples were boiled with the Laemmli sample buffer and analyzed by SDS−PAGE.

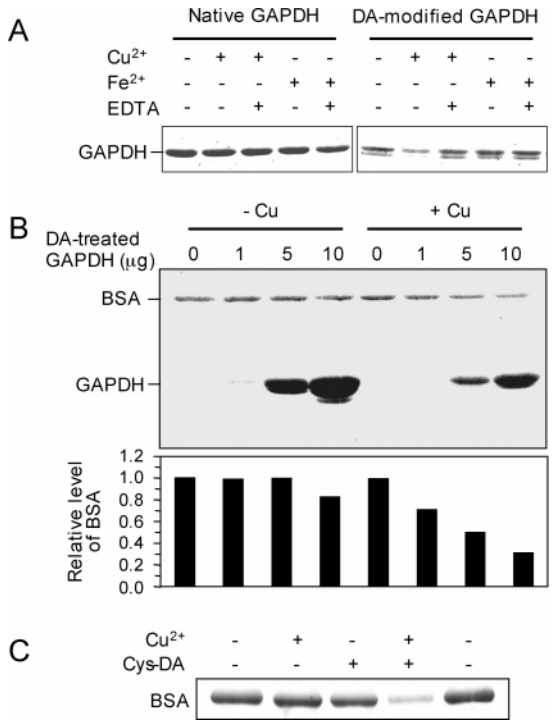

FIGURE 5: Cysteinyl-DA is the active site of Cu^{2+}-catalyzed oxidation of the DA-modified GAPDH. (A) Effects of EDTA on the metal-catalyzed oxidation of the DA-modified GAPDH. GAPDH (0.3 mg) was incubated with DA (0.1 mM) in 0.1 M sodium phosphate buffer (pH 7.2) at 37 °C for 1 h. The reaction was terminated by centrifugal filtration (Microcon 30, molecular weight cutoff of 30000) to remove the low-molecular-weight reactants. The DA-modified GAPDH (0.01 mg) was then incubated with 100 μM metal ion (Cu^{2+} or Fe^{3+}) in 0.1 M sodium phosphate buffer (pH 7.2) at 37 °C for 1 h. The samples were boiled with the Laemmli sample buffer and analyzed by SDS−PAGE. (B) Cu^{2+}-catalyzed oxidation of BSA by the DA-modified GAPDH. BSA (0.1 mg/mL) was incubated with the DA-modified GAPDH (0−10 μg/mL) in the presence of 100 μM Cu^{2+} for 24 h at 37 °C and analyzed by SDS−PAGE. The relative level of BSA was measured by quantification of the intensity of each BSA band using the Image program (NIH). (C) Cu^{2+}-catalyzed oxidation of BSA by 5-*S*-cysteinyl-DA. BSA (0.1 mg/mL) was incubated with 5-*S*-cysteinyl-DA (0.1 mg/mL) in the presence and absence of 100 μM Cu^{2+} for 12 h at 37 °C and analyzed by SDS−PAGE.

hypothesis was supported by the observation that, in addition to the metal chelator, the sulfhydryl antioxidants, such as *N*-acetylcysteine and glutathione, completely inhibited the protein modification (data not shown).

Generation of Carbonyl Amino Acids upon Cu^{2+}-Catalyzed Oxidation of the DA-Modified GAPDH. To obtain direct evidence that Cu^{2+} facilitates the metal-catalyzed oxidation of the DA-modified protein, we examined the generation of the protein-linked carbonyl groups on the protein molecule using FTSC as the molecular probe (Figure 6A). The DA-modified GAPDH was incubated with Cu^{2+}, labeled with FTSC, and analyzed by SDS−PAGE. As shown in Figure 6B, treatment of the DA-modified GAPDH with Cu^{2+} resulted in a significant generation of protein carbonyls on the protein molecule. It should be noted that although the Cu^{2+}-catalyzed oxidation of DA-modified GAPDH mainly resulted in the oxidative modification including oxidative cleavage of the chain, leading to the loss of the native molecular weight (Figure 5), only the protein bands which were not cleaved to lower molecular weight protein species were stained by the carbonyl reagent. It is curious that the carbonyl staining increased with an increase in the concentration of Cu^{2+} from 10 to 100 μM, since one might have guessed that the increase in carbonyl content would be offset by larger decreases in native molecular weight protein content at the higher concentration of Cu^{2+}.

On the basis of the fact that glutamic semialdehyde (GS) and aminoadipic semialdehyde (AAS) are the most predominant forms of carbonyl amino acids (*21, 30*), we examined whether these oxidized amino acids are generated during the Cu^{2+}-catalyzed oxidation of the DA-modified protein. Because GS and AAS present in proteins are destroyed by acid hydrolysis, we established the procedure for the detection of these products in the oxidized protein, in which carbonyl amino acids were stabilized by the ABA derivatization followed by acid hydrolysis and reversed-phase HPLC analysis (*20, 21*). The HPLC analysis of the authentic ABA derivatives of GS and AAS (Figure 7A) showed chromatograms containing basically one single peak in each case (data not shown). As shown in Figure 7B, two new peaks appeared at 7 and 20 min in the Cu^{2+}-oxidized DA−GAPDH. They were inseparable from the ABA derivatives of GS and AAS, respectively. The formation of these oxidized amino acids was dependent on the DA modification and Cu^{2+} treatment, because only trace amounts of GS and AAS were detected in the Cu^{2+}- or DA-treated protein (Figure 7C).

DISCUSSION

A variety of enzymes have been shown to be susceptible to inactivation by DA. These include the glutamate transport

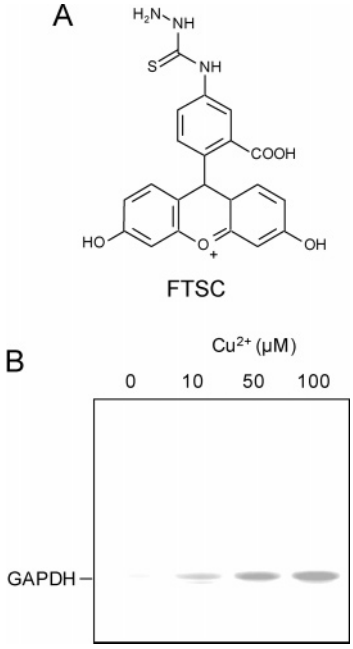

FIGURE 6: Protein carbonyl formation in the DA-modified GAPDH incubated with Cu^{2+}. (A) Chemical structure of the carbonyl reagent FTSC. (B) Introduction of carbonyl groups into the DA-modified GAPDH incubated with Cu^{2+}. GAPDH (0.3 mg) was incubated with DA (0.1 mM) in 0.1 M sodium phosphate buffer (pH 7.2) at 37 °C for 1 h. The reaction was terminated by centrifugal filtration (Microcon 30, molecular weight cutoff of 30000) to remove the low-molecular-weight reactants. The DA-modified GAPDH (0.01 mg) was then incubated with Cu^{2+} (0−100 μM) in 0.1 M sodium phosphate buffer (pH 7.2) at 37 °C for 1 h. The protein carbonyl content was determined by the procedure using FTSC.

(*31*), human DA transporter (*32*), and brain mitochondrial respiration (*33*). In the present study, we investigated the covalent binding of DA to protein using GAPDH. GAPDH, an important enzyme which catalyzes the oxidation and phosphorylation of D-glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate, is a tetrameric enzyme consisting of four identical catalytically active subunits. Each subunit has four cysteine residues of which Cys-149 is at the active site. This cysteine is involved in the catalysis and is highly reactive (*34*). We observed that, upon incubation of GAPDH (1 mg/mL) with 0−1 mM DA for 30 min, the enzyme activity declined to 17% of the initial value (Figure 2A) and that the loss in activity was accompanied by the loss of cysteine (sulfhydryl groups) residues (Figure 2B,C). In addition, covalent binding of DA to the cysteine residues was verified by NeutrAvidin blot analysis using iodoacetyl-LC-biotin (Figure 2C). Additional substantiation of the cysteinyl modification within GAPDH by DA was also provided by the results of the acid hydrolysis of DA-treated GAPDH (Figure 3). It is clear that the extent of inactivation of GAPDH by DA is related to the extent of the sulfhydryl group modification. These observations and the result that the sulfhydryl compounds, GSH and DTT, protected GAPDH from inactivation by DA (Figure 2A) strongly suggest that DA was covalently bound to cysteine residues of the protein. On the other hand, we demonstrated that DA caused a concentration-dependent increase in the redox cycling on the GAPDH molecule, shown by the ability of the DA-modified GAPDH to mediate the oxidation/reduction of NBT in the presence of glycinate (Figure 4A). Furthermore, we identified 5-*S*-cysteinyl-DA in DA-treated GAPDH by LC-MS/MS.

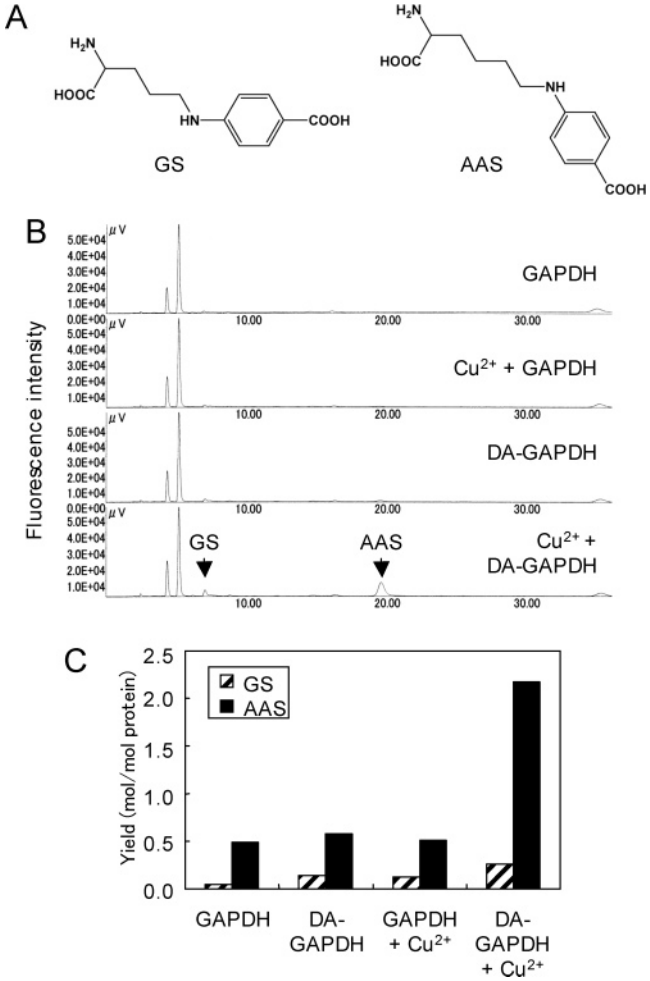

FIGURE 7: Generation of the carbonyl amino acids upon Cu^{2+}-catalyzed oxidation of DA-modified GAPDH. (A) Chemical structures of glutamic semialdehyde (GS) and aminoadipic semialdehyde (AAS). (B) Detection of GS and AAS generated during the Cu^{2+}-catalyzed oxidation of the DA-modified GAPDH. (C) Yields of GS and AAS generated during the Cu^{2+}-catalyzed oxidation of the DA-modified GAPDH.

These results indicated that the DA-modified proteins possess reducing property.

This study has established a novel mechanism for the oxidative modification of protein, in which the cysteinyl-DA generated in the DA-modified protein causes oxidative modification of DA-bound protein in the presence of a metal ion (Cu^{2+}). The cysteinyl-DA is known to play a role in the production of melanin in the mammalian brain (*35*). During the progression of PD, increased ratios of cysteinyl-DA over DA have been observed in the substantia nigra (*36*). Therefore, it has been postulated that, rather than being involved in the melanin production, the formation of cysteinyl-DA diverts production. In fact, with the increasing molar excess of cysteine, the formation of melanin in vitro is decreased and ultimately stopped (*37*). The oxidation of catecholamines to quinones in the brain occurs to a much greater extent than previously suggested (*38*). Indeed, intrastriatal injections of DA in neurotoxic doses are associated with the increased formation of cysteinyl-DA adducts in proteins (*18*, *24*), and postmortem samples of the substantia nigra from patients with PD contain significantly increased levels of the cysteinyl adducts of 3,4-dihydroxyphenylalanine (DOPA) and DA (*39*). The present findings suggest that

although the cysteinyl-DA has been reported to have no excitotoxicity (*40*), DA and its protein conjugate show their cytotoxicity through induction of oxidative stress. Further studies will be needed to understand the remarkable function of protein-bound DA in terms of the neuronal cell loss in neurodegenerative disorders.

Quinones are electron-deficient compounds with a high reactivity toward low-molecular-weight thiols, and the protectants could also exert their effects, in essence, by competing with the protein sulfhydryls for the quinones (*41*). It has been suggested that although free quinones are short-lived in vivo (*42*), the binding of quinones to a protein may dramatically extend the half-life of these reactive species (*17*, *42*). Neurotoxic doses of methamphetamine have been shown to increase the striatal content of the protein-bound DA quinones (*43*). Moreover, the DA quinones have been suggested to be involved in the damaging effects of methamphetamine and 3,4-methylenedioxymethamphetamine on DA and serotonin neurons (*44*).

Several lines of evidence indicate that the oxidative modification of proteins and the subsequent accumulation of the modified proteins have been found in cells during aging and oxidative stress and in various pathological states including premature diseases, muscular dystrophy, rheumatoid arthritis, and atherosclerosis (*45*−*48*). There is ample evidence to support the notion that the most important mechanism of oxidative damage to proteins is the metal-catalyzed oxidation (*49*). The oxidation can lead to oxidative modifications of amino acid side chains, to cleavage of the polypeptide chain, and to formation of cross-linked protein aggregates (*45*). This process involves the generation of H_2O_2 and reduction of Fe^{3+} or Cu^{2+} by a suitable electron donor such as NADH, NADPH, ascorbate, and mercaptans; Fe^{2+} and Cu^{+} ions bind to specific metal binding sites on proteins and react with H_2O_2 to generate the hydroxyl radical (·OH). This highly reactive free radical then attacks neighboring amino acid residues. The metal-catalyzed oxidation of proteins has been modeled in vitro by using a variety of electron donors and often results in a loss of enzymatic activity and alteration of the protein structure (*50*, *51*). In this study, we observed a significant decrease in protein bands in the incubation with DA-modified GAPDH and Cu^{2+} (Figure 5). This result suggests that the protein fragmentation and/or protein−protein cross-linking were caused by the metal-catalyzed oxidation of DA-modified GAPDH. We also showed that Cu^{2+} accelerated the production of protein carbonyl in the DA-modified GAPDH (Figure 6). Furthermore, using a method for the quantification of oxidized amino acids after their derivatization by reductive amination with ABA and $NaCNBH_3$, we revealed that the Cu^{2+}-catalyzed oxidation of the DA-modified protein resulted in the generation of GS and AAS as the major products (Figure 7). Since GS and AAS are the most predominant forms of the carbonyl amino acids (*21*, *30*), it is very likely that Cu^{2+} facilitates the metal-catalyzed oxidation of the DA-modified protein. Protein carbonyls represent a putative marker of oxidatively modified proteins and can be conveniently measured by sensitive methods, particularly those using DNPH, which reacts with carbonyl groups to generate dinitrophenylhydrazones with characteristic absorbance maxima at 360−390 nm (*52*). It has been established that protein carbonyls accumulate on tissue proteins during aging (*53*) and disease development. Increased levels of protein carbonyls are associated with Alzheimer's disease (*54*), progeria and Werner's syndrome (*55*), amyotrophic lateral sclerosis (*56*), and respiratory distress syndrome (*57*), among others. Although the experimental evidence is so far mostly correlative, it lends strong support to the hypothesis that the protein carbonyl content of tissues reflects the fraction of oxidatively damaged proteins with impaired functions and might therefore be at the root of disease and age-related functional losses (*58*).

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