FEBS Letters 511 (2002) 118–122 FEBS 25704

Carbonyl formation on a copper-bound prion protein fragment, PrP_{23-98}, associated with its dopamine oxidase activity

Noriyuki Shiraishi, Morimitsu Nishikimi*

Department of Biochemistry, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-0012, Japan

Received 17 November 2001; revised 18 December 2001; accepted 18 December 2001

First published online 7 January 2002

Edited by Barry Halliwell

Abstract The amino-terminal part of prion protein (PrP), containing a series of octapeptide repeats with the consensus sequence PHGGGWGQ, has been implicated in the binding of copper ion. This region possesses amino acid residues susceptible to oxidation, such as histidine, lysine, arginine and proline. In this study, we have investigated copper-catalyzed oxidation of an N-terminal part of human PrP, PrP_{23-98}, that was prepared by the recombinant DNA technique. Carbonyl formations on copper-bound PrP_{23-98} induced by dopamine and L-ascorbate were analyzed kinetically, and it was found that the redox cycling of PrP_{23-98}-bound copper, especially induced by dopamine, was coupled to the formation of carbonyls on the protein. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Prion protein; Copper; Carbonyl; Dopamine; L-Ascorbate

1. Introduction

Multiple lines of evidence have suggested that the most important mechanism of oxidative damage to proteins involves catalysis by transition metals bound to them. This process consists of reduction of Fe^{3+} or Cu^{2+} by electron donors, such as O_2^-, catecholamines, L-ascorbate and mercaptanes, and generation of $^{\bullet}OH$ radical through reduction of H_2O_2 by the reduced metals. This highly reactive free radical attacks neighboring amino acid residues, producing carbonyl-containing derivatives. In addition, carbonyls are introduced to protein as a consequence of oxidative cleavage of the peptide backbone. The carbonyls thus produced serve as an important marker of oxidative protein damage. The carbonyl formation is known to cause structural alterations and loss of biological functions of proteins (for reviews, see [1–3]).

Previous work has established that the proline- and histidine-rich octapeptide repeat region of the prion protein (PrP) constitutes a copper-binding site [4–10]. Under physiological conditions, this region binds four copper ions and is suspected to be a target of copper-catalyzed oxidation. We studied the pathophysiological aspect of the copper–PrP complex by using a fusion of glutathione *S*-transferase with an N-terminal 76-amino acid region of human PrP, designated PrP_{23-98}. Carbonyl formation on the protein induced by Cu^{2+} plus L-ascorbate was marginal at a copper/protein molar ratio of 1, but became obviously detectable at a molar ratio of 4 [11], in accordance with the previous result obtained with a synthetic peptide of the octapeptide repeat [9]. We also observed that an appreciable amount of carbonyl was produced on PrP_{23-98} fusion protein purified from *Escherichia coli* that had been treated with a high concentration of copper [11]. More recently, Requena et al. [12] have investigated metal-catalyzed oxidation of the Syrian hamster recombinant SHa(29–231) PrP in vitro and shown that incubation of this protein with Cu^{2+} and L-ascorbate causes a marked decrease in histidine content concomitantly with an extensive structural transition leading to aggregation.

Autoxidizable substances, such as L-ascorbate, dopamine, 3,4-dihydroxyphenylalanine, and noradrenaline, react with O_2 to generate O_2^- and H_2O_2 through catalysis by metal ion [13,14]. Dopamine is present in various anatomical regions of the brain, its extraordinarily high concentrations being found in the striatum (37 and 53 μM in the caudate nucleus and putamen of human, respectively) [15]. The L-ascorbate concentration in the cerebrospinal fluid is 150 μM [14], and the L-ascorbate concentration in the brain is in millimolar levels [16]. With these backgrounds in mind, we have investigated copper-catalyzed oxidation of PrP_{23-98}. Studies on the effect of the interaction of copper and PrP have been done in two ways. One is to use a mere mixture of the two, and the other is to use copper-containing PrP previously prepared from recombinant PrP. Choosing the latter way, we have here studied carbonyl formation on copper-bound PrP_{23-98} induced by dopamine in comparison with that induced by L-ascorbate, and found that the redox cycling of PrP_{23-98}-bound copper, especially induced by dopamine, was associated with formation of carbonyls on the protein.

2. Materials and methods

2.1. Preparation of copper-loaded PrP

A fusion protein of glutathione *S*-transferase with the human PrP_{23-98} was expressed in *E. coli* [9]. Triton X-100 and sarcosyl were added to a cell suspension in phosphate-buffered saline (pH 7.4) to a final concentration of 2% and 4%, respectively [17]. The suspension was sonicated three times for 10 s with a microtip sonicator. The crude lysate was applied to a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) that had been equilibrated with phosphate-buffered saline (pH 7.4) containing 2% Triton X-100 and 4% sarcosyl. The column was washed with the same buffer, and then with factor Xa cleavage buffer (100 mM NaCl, 2 mM $CaCl_2$ and 20 mM Tris–HCl, pH 8.0). Re-

*Corresponding author. Fax: (81)-73-441 0628.
E-mail address: nishikim@wakayama-med.ac.jp (M. Nishikimi).

Abbreviations: PrP, prion protein; PrP_{23-98}, recombinant human PrP residues 23–98; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; BCS, bathocuproinedisulfonic acid

0014-5793/02/$22.00 © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0014-5793(01)03324-5

combinant PrP_{23-98} was obtained by cleavage with factor Xa followed by dialysis against 50 mM Mes buffer (pH 7.5) at 4°C, and stored at −80°C until use. The concentration of PrP_{23-98} was determined spectrophotometrically by using a molar extinction coefficient at 280 nm of 36 334 M^{-1} cm^{-1}, which was deduced from the contents of tryptophan and tyrosine (molar extinction coefficient 5609 M^{-1} cm^{-1} for tryptophan and 1340 M^{-1} cm^{-1} for tyrosine). For preparation of copper-loaded PrP_{23-98}, mixtures (2 ml) containing 50 mM Mes buffer (pH 7.5), 15 μM PrP_{23-98}, and varying concentrations (10–110 μM) of glycine–copper were incubated at 37°C for 10 min and dialyzed at 4°C overnight against three changes of 2 l of 50 mM Mes buffer (pH 7.5). The copper content of the resulting copper-loaded PrP_{23-98} was measured by the flameless mode with a Shimadzu atomic absorption spectrophotometer AA-6800 (Shimadzu, Kyoto, Japan).

2.2. Western blotting

Mixtures (1 ml) containing 50 mM Mes buffer (pH 7.5), 2 μM copper-loaded PrP_{23-98}, and 100 μM L-ascorbate or 100 μM dopamine were incubated at 37°C for 30 min, then 100 μl of 5 mM diethylenetriamine-*N*,*N*,*N′*,*N′*,*N″*-pentaacetic acid (DTPA) was added to stop the reaction. After lyophilization, the samples were dissolved in distilled water, and carbonyl contents were determined by SDS–polyacrylamide gel electrophoresis followed by Western blot analysis as described [18]. The membranes were scanned on a scanner (EPSON GT-9500), and relative intensities of the stained bands were determined using ATTO Densitograph Software Library, Version 4.0 (ATTO Co. Ltd., Tokyo, Japan).

2.3. Amino acid analysis

Control and oxidized copper-loaded PrP_{23-98} were hydrolyzed in 6 M HCl/1% phenol at 150°C for 1 h. The hydrolysates were dried in a vacuum centrifuge and dissolved in deionized water. Amino acid analysis was performed with a Hitachi L-8500 amino acid analyzer (Hitachi Ltd., Tokyo, Japan).

2.4. Metal analysis

The above reaction mixtures used for Western blotting were also used for the measurement of copper released from the protein upon incubation. Aliquots of the reaction mixtures (400 μl) were dispensed into cups of Ultrafree-MC centrifugal filter units (Biomax-5, Millipore Corp., MA, USA) and centrifuged at 6700×*g* at 10°C for 3 min, and the copper concentration of the resulting filtrates was measured by atomic absorption spectrophotometry.

2.5. Oxidation of reducing substances

Oxidation of L-ascorbate was traced spectrophotometrically by the decrease in absorbance at 265 nm [19] at 37°C with a Shimadzu UV-visible recording spectrophotometer UV-2500 (Shimadzu, Kyoto, Japan), and oxidation of dopamine was followed spectrophotometrically by the increase in absorbance at 490 nm (dopachrome formation) [20].

3. Results

Our present study aimed to examine the redox activity of copper that was bound to human PrP_{23-98}. Since a preliminary ultrafiltration experiment indicated that a substantial amount of copper was filterable from a mixture of the protein and an equimolar amount of copper–glycine, we prepared copper-loaded PrP_{23-98} containing varying amounts of copper, by incubation of mixtures of PrP_{23-98} and copper–glycine at 37°C for 10 min followed by extensive dialysis and used for the test of carbonyl formation by incubation with dopamine and L-ascorbate. The copper-loaded PrP_{23-98} was found to produce carbonyls by incubation with dopamine or L-ascorbate, whereas in the absence of the reducing substances, no significant increase in carbonyl was observed with all the PrP_{23-98} containing different amounts of copper (Fig. 1). It is noted that in the incubation with dopamine, the largest amount of carbonyl was detected on PrP_{23-98} containing 0.44 mol of copper per mol of protein. The carbonyl formation tended to decrease with an increase in the copper content of the protein. On the other hand, in the incubation with L-ascorbate, only a marginal amount of carbonyl was detected on PrP_{23-98} containing 0.44 mol of copper per mol of protein; the amount of carbonyl increased with an increase in the bound copper. Table 1 summarizes the result of amino acid analysis of copper-loaded PrP_{23-98} oxidized by incubation with dopamine. A decrease in histidine content (17–37%) was observed dependently on the copper content of the protein. While the losses of other amino acid residues were not evident. Thus it is clear that histidine residues are most susceptible to protein oxidation as is the case with the Syrian hamster recombinant SHa(29–231) PrP oxidized by copper ion plus L-ascorbate [12]. SDS–polyacrylamide gel electrophoresis under non-reducing conditions showed no obvious formation of polymeric forms of the protein, although the incubation with L-ascorbate gave faint dimer bands for PrP_{23-98} samples with high copper contents (data not shown).

To ascertain the participation of copper in the formation of carbonyls on copper-loaded PrP_{23-98}, the inhibitory effect of copper chelators was tested (Fig. 2). The inhibition was com-

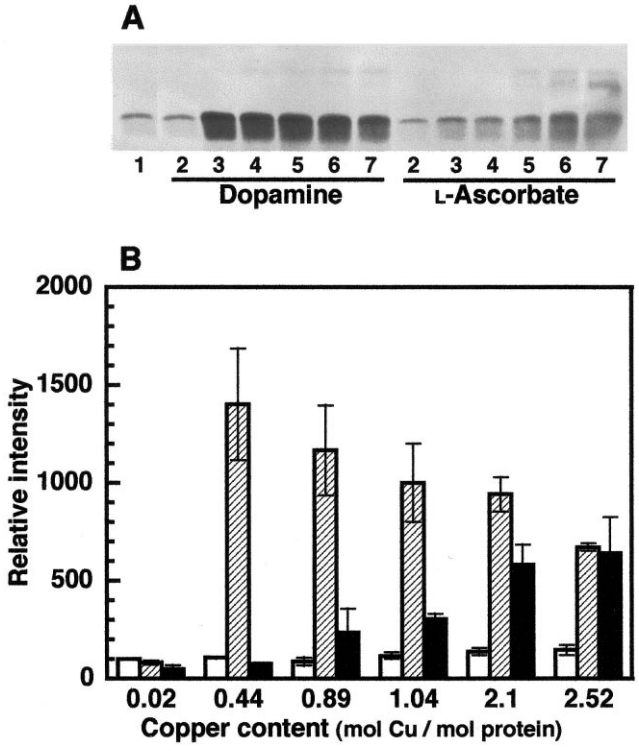

Fig. 1. Western blot immunoassay for carbonyls formed on copper-loaded PrP_{23-98}. A: Western blot analysis for protein-bound carbonyls. Purified PrP_{23-98} and copper-loaded PrP_{23-98} containing varying contents of copper were incubated at 2 μM for 30 min at 37°C, in the absence (lane 1 and open bars) and presence of 100 μM dopamine (lanes 2–7 and hatched bars) or 100 μM L-ascorbate (lanes 2–7 and closed bars). Western blot analysis was carried out essentially as described [18]. The copper contents (in mol of copper/mol of protein) of the PrP_{23-98} samples were: lanes 1 and 2, 0.02; lane 3, 0.44; lane 4, 0.89; lane 5, 1.04; lane 6, 2.10; and lane 7, 2.52. B: Quantification of the Western blot data. Signals of specific bands were quantified by densitometry and plotted as a percentage of the control (PrP_{23-98} incubated without reducing substances). Data were means ± S.D. ($n = 3$).

Table 1
Relative amino acid composition of control and oxidized copper-loaded PrP_{23-98}

Amino acid	Oxidized/control (molar ratio)			
	Copper content (mol Cu/mol protein)			
	0.03	0.59	1.46	2.11
Arginine	0.89	0.95	0.93	0.95
Aspartate	0.98	1.00	1.01	1.02
Glutamate	1.03	1.00	1.00	1.00
Glycine	1.00	1.00	1.00	1.00
Histidine	0.98	0.83	0.69	0.63
Lysine	0.92	0.97	0.92	0.89
Proline	1.01	0.93	0.97	0.99
Serine	1.09	1.00	0.96	0.96
Threonine	0.96	1.00	0.95	0.96
Tyrosine	0.96	0.98	1.01	0.99

PrP_{23-98} and copper-loaded PrP_{23-98} (2 μM) were incubated with dopamine (100 μM) at 37°C for 30 min, and after acid hydrolysis of the proteins, the resulting samples were subjected to amino acid analysis. Each protein preparation incubated without dopamine and carried out through the same procedure was taken as control, and ratios of the amount of each amino acid of oxidized protein to that of control were calculated for the respective preparations by normalization with the amount of glycine. This normalization should be reasonable because this amino acid is not readily oxidized [12,25] and contained most abundantly (40%) in the protein.

plete with the Cu^{1+}-specific chelator bathocuproinedisulfonic acid (BCS), indicating that copper was removed from the PrP_{23-98} and formed Cu^{1+}–BCS complex, rendering the copper not readily oxidizable by O_2. Although DTPA and EDTA inhibited the carbonyl formation, the inhibition was not complete. The bound copper seems to be in the Cu^{1+} state, and these Cu^{2+}-specific chelators cannot effectively remove the bound copper.

Next, we examined whether the addition of dopamine and L-ascorbate affects the binding of copper to PrP_{23-98} because

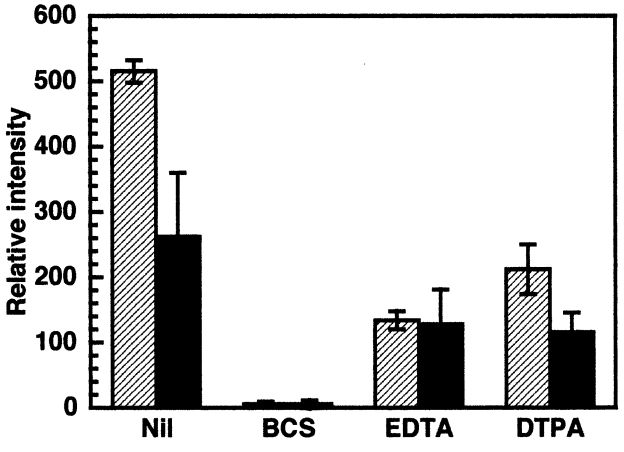

Fig. 2. Inhibitory effect of metal chelators on carbonyl formation on copper-loaded PrP_{23-98}. Copper-loaded PrP_{23-98} (containing 1.04 mol of copper/mol of protein, 2 μM) and metal chelator (100 μM) were incubated for 30 min at 37°C, with 100 μM dopamine (hatched bars) or 100 μM L-ascorbate (closed bars). Carbonyl contents were determined as specified in Fig. 1. Signals of specific bands were quantified by densitometry and plotted as a percentage of the control value (copper-loaded PrP_{23-98} incubated without reducing substances). Data were means ± S.D. ($n = 3$).

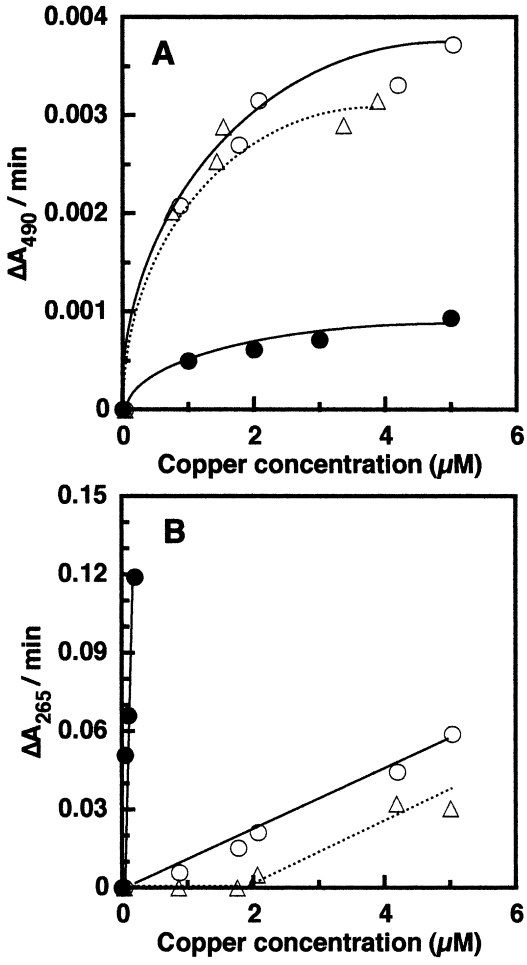

Fig. 3. Redox cycling of the copper bound to PrP_{23-98} in the presence of dopamine and L-ascorbate. Mixtures (1 ml) containing 50 mM Mes buffer (pH 7.5), copper-loaded PrP_{23-98} (open circles), and 100 μM L-ascorbate (A) or 100 μM dopamine (B) were incubated at 37°C, and oxidations of dopamine and L-ascorbate were traced spectrophotometrically at 490 nm and 265 nm, respectively. The same measurements were carried out with the same mixture containing copper–glycine (solid circles) in place of copper-loaded PrP_{23-98}. Initial rates of their oxidations catalyzed solely by protein-bound copper (open triangles) were obtained by subtraction of the initial rates of the reactions to be catalyzed by free copper (copper released in the experiment of Table 2) from those experimentally observed with copper-loaded PrP_{23-98}.

Table 2
Release of copper from copper-loaded PrP_{23-98} by dopamine and L-ascorbate

Copper content (mol Cu/mol protein)	Copper released[a] (μM)	
	Dopamine	L-Ascorbate
0.02	ND	ND
0.44	0.123 ± 0.037	0.016 ± 0.017
0.89	0.336 ± 0.029	0.018 ± 0.012
1.04	0.538 ± 0.051	0.016 ± 0.005
2.10	0.826 ± 0.063	0.012 ± 0.023
2.52	1.147 ± 0.079	0.028 ± 0.023

[a]PrP_{23-98} or copper-loaded PrP_{23-98} was mixed at 2 μM with dopamine or L-ascorbate, mixtures were immediately centrifuged in an ultrafiltration cup, and copper concentrations were measured as detailed under Section 2. The measured concentrations were corrected for the copper concentration of the buffer used. Values are means ± S.D. of three independent experiments. ND, not detected.

these compounds are known to interact with copper. Dopamine or L-ascorbate (100 μM, the final concentration) was added to solutions of varying copper-loaded PrP_{23-98}, and the amount of copper filterable by ultrafiltration was measured immediately after mixing. In the experiment with dopamine, large amounts (14–26%) of filterable copper were detected (Table 2), indicating that the bound copper was released to some degree and formed a complex with dopamine. On the other hand, in the experiment with L-ascorbate, the amount of copper released was marginal (less than 1.8%) (Table 2).

To see how readily the PrP_{23-98}-bound copper redox cycles, we did a kinetic study on oxidations of dopamine and L-ascorbate in their incubation with copper-loaded PrP_{23-98}. The initial rate of oxidation of dopamine observed as dopachrome formation increased dependently on the copper concentration (Fig. 3A). Since substantial amounts of the bound copper are released by the interaction with dopamine (Fig. 3A), the rate of reaction to be catalyzed by the released copper was subtracted from the observed rate to estimate the initial rate of reaction catalyzed solely by the PrP_{23-98}-bound copper. The catalysis by the protein-bound copper was approximately three times faster than that by free copper (Fig. 3A). In contrast, the oxidation of L-ascorbate was found to be markedly suppressed compared to the reaction catalyzed by free copper. Amounts of released copper were very low (< 0.02 μM) (Table 2), but the catalysis by free copper is very powerful. The protein-bound copper was redox inactive below a copper concentration of 2 μM; L-ascorbate oxidation by the protein-bound copper started to increase above 2 μM.

4. Discussion

In the present study, we observed the carbonyl formation on copper-loaded PrP_{23-98} by its incubation with dopamine and L-ascorbate. It was noted that the PrP_{23-98}-bound copper was less catalytically active than free copper when L-ascorbate was used as a reductant. Conversely, the protein-bound copper catalyzed the oxidation of dopamine approximately three times as rapid as free copper. Thus the copper-bound PrP_{23-98} appears to act as dopamine oxidase, and this dopamine oxidation process is coupled to carbonyl formation on the protein. The mechanism of the dopamine-induced carbonyl formation may be delineated as follows. The copper catalysis of the oxidation of catechol to *o*-quinone is mediated through the formation of a Cu^{2+}–catechol complex, which favors the transfer of electrons from catechol to Cu^{2+} and the formation of CuO_2^+ from the resulting Cu^{1+} and O_2 [21,22]. This CuO_2^+ species effectively oxidizes catechol or is cleaved to O_2^- and Cu^{2+}. Generation of O_2^- always leads to formation of H_2O_2, which is reduced by Cu^{1+} to form $^{\bullet}OH$ radical. This highly reactive radical directly attacks the protein backbone and side chains of amino acid residues. Dopamine exists vastly in the brain with fairly high concentrations in certain regions as mentioned in Section 1, and high concentrations of copper (100–250 μM) are released into the synaptic cleft [23]. Also, it is reported that PrP is located on the synaptic membrane [24]. Therefore, combination of these three components is feasible at the synapse of neurons and possibly causes oxidative damage to PrP, which might be associated with the pathology of prion disease. In relation to pathogenesis of other neurodegenerative diseases, the pro-oxidant effect of dopamine in combination with copper was previously reported for oxidative DNA damage [13].

The protein aggregation is observed in several brain neurodegenerative disorders, such as prion disease and Alzheimer's disease [3]. Recently, Requena et al. [12] have indicated that copper-catalyzed oxidation induces extensive aggregation and precipitation of the Syrian hamster recombinant SHa(29–231) PrP. They hypothesized that the ensuing damage might cause structural alterations that could be relevant to the conversion of the soluble cellular isoform to the pathogenic and infectious scrapie isoform. In our study, copper-loaded PrP_{23-98} migrated mainly as a single band with a molecular mass of monomer (8.2 kDa) upon non-reducing SDS–polyacrylamide gel electrophoresis. The discrepancy between the result of Rquena et al. [12] and ours may be interpreted as indicating that the aggregation requires the C-terminal globular portion of the protein.

Acknowledgements: We thank Dr. Y. Konishi and Dr. H. Kinebuchi for performing amino acid analysis.

References

[1] Berlett, B.S. and Stadtman, E.R. (1997) J. Biol. Chem. 272, 20313–20316.
[2] Stadtman, E.R. and Levine, R.L. (2000) Ann. N.Y. Acad. Sci. 899, 191–208.
[3] Butterfield, D.A. and Kanski, J. (2001) Mech. Ageing Dev. 122, 945–962.
[4] Hornshaw, M.P., McDermott, J.R., Candy, J.M. and Lakey, J.H. (1995) Biochem. Biophys. Res. Commun. 214, 993–999.
[5] Brown, D.R., Qin, K., Herms, J.W., Madlung, A., Manson, J., Strome, R., Fraser, P.E., Kruck, T., von Bohlen, A., Schulz-Schaeffer, W., Giese, A., Westaway, D. and Kretzschmar, H. (1997) Nature 390, 684–687.
[6] Stöckel, J., Safar, J., Wallace, A.C., Cohen, F.E. and Prusiner, S.B. (1998) Biochemistry 37, 7185–7193.
[7] Viles, J.H., Cohen, F.E., Prusiner, S.B., Goodin, D.B., Wright, P.E. and Dyson, H.J. (1999) Proc. Natl. Acad. Sci. USA 96, 2042–2047.
[8] Miura, T., Hori-I, A., Mototani, H. and Takeuchi, H. (1999) Biochemistry 38, 11560–11569.
[9] Shiraishi, N., Ohta, Y. and Nishikimi, M. (2000) Biochem. Biophys. Res. Commun. 267, 398–402.
[10] Aronoff-Spencer, E., Burns, C.S., Avdievich, N.I., Gerfen, G.J., Peisach, J., Antholine, W.E., Ball, H.L., Cohen, F.E., Prusiner, S.B. and Millhauser, G.L. (2000) Biochemistry 39, 13760–13771.
[11] Shiraishi, N., Ohta, Y. and Nishikimi, M. (2000) in: 10th Biennial Meeting of the Society for Free Radical Research International, p. 127, OICA International, Kyoto.
[12] Requena, J.R., Groth, D., Legname, G., Stadtman, E.R., Prusiner, S.B. and Levine, R.L. (2001) Proc. Natl. Acad. Sci. USA 98, 7170–7175.
[13] Spencer, J.P.E., Jenner, A., Aruoma, O.I., Evans, P.J., Kaur, H., Dexter, D.T., Jenner, P., Lees, A.J., Marsden, D.C. and Halliwell, B. (1994) FEBS Lett. 353, 246–250.
[14] Halliwell, B. and Gutteridge, J.M.C. (1999) in: Free Radicals in Biology and Medicine, Oxford University Press, New York.
[15] Sano, I., Gamo, T., Kakimoto, Y., Taniguchi, K., Takesada, M. and Nishinuma, K. (1959) Biochim. Biophys. Acta 32, 586–587.
[16] Carr, R.S., Bally, M.B., Thomas, P. and Neff, J.M. (1983) Anal. Chem. 55, 1229–1232.
[17] Weiss, S., Famulok, M., Edenhofer, F., Wang, Y.H., Jones, I.M., Groschup, M. and Winnacker, E.L. (1995) J. Virol. 69, 4776–4783.
[18] Levine, R.L., Williams, J.A., Stadtman, E.R. and Shacter, E. (1994) Methods Enzymol. 233, 346–357.

[19] Nishikawa, T., Lee, I.S., Shiraishi, N., Ishikawa, T., Ohta, Y. and Nishikimi, M. (1997) J. Biol. Chem. 272, 23037–23041.
[20] Walaas, E., Walaas, O. and Haavaldsen, S. (1963) Arch. Biochem. Biophys. 100, 97–109.
[21] Balla, J., Kiss, T. and Jameson, R.F. (1992) Inorg. Chem. 31, 58–62.
[22] Bindoli, A., Rigobello, M.P. and Deeble, D.J. (1992) Free Radic. Biol. Med. 13, 391–405.
[23] Kardos, J., Kovács, I., Hajós, F., Kálmán, M. and Simonyi, M. (1989) Neurosci. Lett. 103, 139–144.
[24] Brown, D.R. (2001) Trends Neurosci. 24, 85–90.
[25] Uchida, K. and Kawakishi, S. (1994) J. Biol. Chem. 269, 2405–2410.