Generation of Hydrogen Peroxide from Mutant Forms of the Prion Protein Fragment PrP121−231[†]

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ABSTRACT: By means of electron spin resonance spectroscopy, in conjunction with the spin trapping technique, we have shown previously that Aβ and α-synuclein (aggregating proteins that accumulate in the brain in Alzheimer's disease, Parkinson's disease, and related disorders) both induce the formation of hydroxyl radicals following incubation in solution, upon addition of Fe(II). These hydroxyl radicals are apparently formed from hydrogen peroxide, via Fenton's reaction. An N-terminally truncated fragment of the mouse prion protein (termed PrP121−231) is toxic to cerebellar cells in culture, and certain human mutations, responsible for inherited prion disease, enhance this toxicity. Here we report that PrP121−231 containing three such mutations (E200K, D178N, and F198S) also generated hydroxyl radicals, upon addition of Fe(II). The formation of these radicals was blocked by catalase, or by metal chelators, each of which also reduced the toxicity of the PrP121−231 fragments to cultured normal mouse cerebellar cells. Wild-type PrP121−231, full-length cellular PrP, and its homologue doppel did not generate any detectable hydroxyl radicals. We conclude that the additional cytotoxic effects of the mutant forms of PrP121−231 could be due to their ability to generate hydrogen peroxide, by a metal-dependent mechanism. Thus, one effect of these (and possibly other) prion mutations could be production of a particularly toxic form of the prion protein, with an enhanced capacity to induce oxidative damage, neurodegeneration, and cell loss.

The transmissible spongiform encephalopathies (TSEs)[1] are a group of infectious neurodegenerative diseases that includes Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy in cattle. According to the "protein only" or "prion" hypothesis, the transmissible agent responsible for these diseases is a pathological form of a normal host sialoglycoprotein called the prion protein (PrP) (*1*−*3*). The normal cellular form of PrP (PrP^C) is noninfectious. However, this protein also exists in an infectious form (designated PrP^{Sc} or the scrapie isoform) which has a protein conformation markedly different from its normal cellular counterpart, with a much higher β-sheet content (*3*). It is thought that PrP^{Sc} acts as a template for the conversion of PrP^C into more PrP^{Sc} (*3*). The consequent accumulation of PrP^{Sc} in the brain is likely to be the cause of neurodegeneration in the TSEs, but the precise molecular mechanisms that lead to neuronal cell death have not been established.

In some of the TSEs, PrP accumulates in the cerebellar and sometimes the cerebral cortices of the brain in the form of extracellular amyloid plaques. Furthermore, the presence of amyloid-like "prion rods" in brain extracts is a pathognomonic feature of the TSEs (*4*). The accumulation of abnormal protein fibrils in the brain is a common theme in the pathogenesis of a range of other important (nontransmissible) neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and related disorders, the "tauopathies", and Huntington's disease (*5*). Inherited forms of all of these diseases can be caused by a mutation in the gene encoding an aggregating protein [β-amyloid (Aβ), α-synuclein, tau, and huntingtin, respectively] (*5*). This link between protein aggregation and molecular genetics also applies to various inherited human TSEs, namely, familial CJD, Gerstmann-Sträussler-Scheinker syndrome, and fatal familial insomnia, which are caused by mutations in the gene (*prnp*) encoding PrP^C on chromosome 20 (*6*). These genetic studies on the aforementioned nontransmissible and transmissible neurodegenerative diseases have been supported by a considerable amount of work with transgenic mouse models (*7*−*12*). Mice expressing pathogenic mutant forms of the human genes encoding each of the aggregating proteins noted above exhibit many of the histopathological and neurochemical changes associated with the relevant human disease. Taken together, all of these genetic and transgenic animal

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[1] Abbreviations: Aβ, β-amyloid peptide; AD, Alzheimer's disease; BCS, bathocuproine sulfonate; BPDA, bathophenanthrolinedisulfonic acid; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; CJD, Creutzfeldt-Jakob disease; DETAPAC, diethylenetriaminepentaacetic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMPO-OH, hydroxyl radical adduct of DMPO; ESR, electron spin resonance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PD, Parkinson's disease; PrP, prion protein; PrP^C, cellular isoform of PrP; PrP^{Sc}, scrapie isoform of PrP; TSE, transmissible spongiform encephalopathy.

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studies point to the central importance of protein aggregation in the pathogenesis of several different neurodegenerative diseases (*5*).

There is considerable evidence for oxidative damage to affected areas of the brain in many neurodegenerative diseases, including AD (*13*), PD (*14*), and the TSEs (*15*−*17*). Since it is well established that oxidative stress can lead to apoptosis and cell death, this could be one of the major causes of neuronal loss in these diseases. In AD, a fibrillar form of the *β*-amyloid peptide (A*β*) accumulates in the brain, at the center of senile plaques, and often in the walls of cerebral blood vessels (*18*). In PD, dementia with Lewy bodies, and multiple-system atrophy, fibrils composed of α-synuclein accumulate within nerve cells or glial cells to form Lewy bodies, Lewy neurites, or glial fibrillary inclusions (*19*−*21*). We have used the technique of electron spin resonance (ESR) spectroscopy, in conjunction with spin trapping, to demonstrate that A*β* and α-synuclein both liberate hydroxyl radicals when Fe(II) is added to the peptide or protein following its incubation and aggregation *in vitro* (*22*). The formation of these hydroxyl radicals was blocked by metal chelators, or by catalase, suggesting that hydrogen peroxide is generated from A*β* or α-synuclein by a metal-dependent mechanism. Others have shown that A*β* can bind to and reduce the oxidation state of both Fe(III) and Cu(II) and have suggested that hydrogen peroxide is generated from this peptide *via* electron transfer involving the metal ion (*23*). In our experiments (*22*), the addition of Fe(II) would convert any hydrogen peroxide generated during incubation to hydroxyl radicals, by Fenton's reaction.

More recently, we have extended our ESR experiments (*24*) to include the PrP fragment, PrP106−126, which has been widely used as a model for the toxicity induced by PrP^{Sc}. We found that this peptide generated hydroxyl radicals, upon addition of Fe(II), only when it was preincubated in the presence of small amounts of Cu(II) ions. This finding correlates well with the results of Jobling *et al*. (*25*), who have shown that the aggregation and toxicity of PrP106−126 are critically dependent on copper (and to a lesser extent zinc) binding. Strong interactions have been discovered recently between PrP^{C} and copper, with each molecule of PrP^{C} being able to bind up to five atoms of copper (*16*, *26*−*32*). Thus, the normal function of PrP^{C} could be to act as a copper transport protein or as an antioxidant enzyme such as superoxide dismutase (*16*, *33*). Our ESR data on A*β*, α-synuclein, and PrP106−126 suggest that at least some of the oxidative damage to the brain in AD, PD, and the TSEs could be due to a metal and protein aggregation-dependent oxidative stress pathway involving the generation of hydrogen peroxide and, subsequently, hydroxyl radicals (*34*, *35*).

Recently, a second neurotoxic domain has been identified in PrP, separate from the PrP106−126 region. This originates from work on PrP-null mice, in which overexpression of residues 121−231 or 134−231 of PrP, attached to the signal sequence for targeting to the endoplasmic reticulum, was found to cause severe neurological symptoms and degeneration of the granular layer of the cerebellum (*36*). Coexpression of full-length PrP^{C} with either of these two deletion mutants prevented this neurodegeneration (*36*). Subsequently, it was reported that an N-terminally truncated form of mouse PrP corresponding to residues 121−231 (PrP121−231) was toxic when added externally to cultured primary mouse cerebellar neurons (*37*). In contrast to the effects of PrP106−126, the toxic effects of PrP121−231 were more evident on cells from PrP-knockout mice. Furthermore, the human mutations E200K and F198S, which are the cause of inherited prion disease, were shown to enhance this toxicity (*37*). In this report, we have tested normal PrP121−231 and three different mutant forms of PrP121−231 (E200K, D178N, and F198S) for their ability to generate hydroxyl radicals, upon addition of Fe(II) to preincubated solutions. The data were compared with those obtained from normal, full-length PrP^{C} and also its homologue, doppel. Our results show clear differences between the normal and mutant forms of PrP and suggest that the additional cytotoxic effects of the mutant forms of PrP121−231 could be due to their ability to generate hydrogen peroxide *via* a mechanism which is dependent on the presence of metal ions. For this reason, we have also looked at the effects of catalase and various metal ion chelators on the viability of cells exposed to normal and mutant forms of PrP121−231.

MATERIALS AND METHODS

PrP Peptides and Protein. Mouse prion protein peptides (PrP121−231), both the wild type and those carrying human mutations, were generated as described by Liemann and Glockshuber (*38*). The wild-type peptide (PrP121−231) was mutated to carry amino acid residue substitutions equivalent to human mutations E200K, D178N, and F198S (*38*). The numbering refers to the human sequence, but the equivalent amino residue of the mouse sequence was altered (one codon proximal in each case). Purification of these mutants from inclusion bodies was as described previously (*38*) except that the bacteria were grown at 26 °C to avoid major degradation of the protein and no gradient was applied to the DE52/CM52 column during purification. Full-length protein (PrP23−231) and doppel protein were prepared as described previously (*33*, *39*).

ESR Spectroscopy. Solutions of the PrP121−231 peptides, full-length PrP^{C} or doppel [100 μM in 50 μL of phosphate-buffered saline (PBS)], were incubated in Eppendorf tubes at 37 °C, in complete darkness, for periods of 0, 1, 6, and 48 h. After incubation, solutions of Fe(II) sulfate (12.5 μL, 50 μM), diethylenetriaminepentaacetic acid (DETAPAC) (12.5 μL, 125 μM) (as a metal ion sequestrant), and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (12.5 μL, 50 mM) (as a spin trap) were added and the solutions were transferred, immediately after mixing, to ESR sample tubes. All spectra were recorded, at room temperature, on a Bruker EMX *X*-band spectrometer operating with 100 kHz magnetic field modulation. Spectra were recorded with a modulation amplitude of 0.05 mT, with a microwave power of 20 mW, and with spectrum accumulation over 25 scans. Relative spectral intensities were determined by double integration.

Neuronal Cell Culture. Preparation of cerebellar cells from 6-day-old normal mice (P6) was as described previously (*40*). Briefly, the cerebella were dissociated in Hank's Solution (Gibco) containing 0.5% trypsin (Sigma) and plated at 1−2 × 10^6 cells/cm^2 in 24-well trays (Falcon) coated with poly-D-lysine (50 μg/mL, Sigma). Cultures were maintained in Dulbecco's minimal essential medium (Gibco) supplemented with 10% fetal calf serum, 2 mM glutamine, and 1% antibiotics (penicillin, streptomycin, and fungizone) (Gibco).

Table 1: Relative ESR Signal Intensities (arbitrary units) for the Four-Line DMPO-OH Spectrum Obtained with Normal and Mutant Forms of Mouse PrP

	incubation for 1 h	incubation for 6 h	incubation for 48 h	familial prion disease
full-length PrP^C	0	0	0	
doppel	0	0	0	
PrP121−231	0	0	0	
PrP121−231 D178N[a]	4500	7000	3500	Creutzfeldt-Jakob disease (*52*)
PrP121−231 F198S[a]	0	8000	1000	Gerstmann-Sträussler syndrome (*53*)
PrP121−231 E200K[a]	600	7000	2000	fatal familial insomnia (*54*)

[a] Point mutations are numbered according to the human sequence, but the equivalent amino acid residues in mouse PrP121−231 were altered (*37*).

Cultures were maintained at 37 °C with 5% CO_2 for 10 days. The PrP121−231 peptides were added to cultures initially and on the third day. Serum-free cultures were grown in DMEM supplemented with "total serum replacement" (TCM) (ICN Biomedicals Inc., Irvine, CA). Cell survival was determined after 4 days. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma] was diluted to 200 μM in Hanks' solution (Gibco) and added to cultures for 1 h at 37 °C. The MTT formazan product was released from cells by addition of dimethyl sulfoxide (Sigma) and the amount measured at 570 nm in a Unicam Helios spectrophotometer (ATI Unicam). Relative survival in comparison to untreated control cultures could then be determined. Potential inhibitors of toxicity [catalase, Cu/Zn superoxide dismutase, and the metal chelators bathocuproine sulfonate (BCS), bathophenanthroline disulfonic acid (BPDA), and *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA), all from Sigma] were added to the cerebellar cultures from stock solutions in water, following dilution in serum-containing media to the indicated concentrations, and incubated for 4 days in the presence of the PrP121−231 fragments. After this time, the survival was assessed using an MTT assay. Cell culture toxicity data are from four independent experiments, each carried out in triplicate. Percentage inhibition of toxicity for each PrP121−231 fragment was calculated relative to untreated control cultures (100% inhibition) and to cells treated with the relevant PrP121−231 fragment in the absence of any potential inhibitor (0% inhibition).

RESULTS

We compared full-length PrP^C and its homologue doppel with normal and three different mutant forms of PrP121−231 (E200K, D178N, and F198S) for their ability to generate hydroxyl radicals, upon addition of Fe(II), following the preincubation of the protein or peptide solutions (100 μM in PBS) for up to 48 h (Table 1 and Figure 1).

Hydroxyl radicals, derived from hydrogen peroxide upon addition of Fe(II), were detected by employing the spin trap DMPO, the hydroxyl radical adduct of which (DMPO-OH) gives a characteristic four-line "signature" ESR spectrum with relative signal intensities at a 1:2:2:1 ratio and an a(N) of 1.50 mT and an a(H) of 1.46 mT (*22*). Full-length PrP^C, doppel, and wild-type PrP121−231 were completely inactive, but in clear contrast, all three mutants gave a clear four-line spectrum, which reached a maximum after preincubation of the peptide solutions for approximately 6 h, but could still be detected after preincubation for 48 h. The production of this spectrum from all three mutant forms of PrP121−231 was completely blocked when they were preincubated in the presence of the metal chelator, diethylenetriaminepentaacetic acid (DETAPAC), or the hydrogen peroxide degrading enzyme, catalase (Figure 1 shows data for E200K only). Thus, only the mutant forms of PrP121−231 appear to generate hydrogen peroxide, *via* a metal-dependent mechanism.

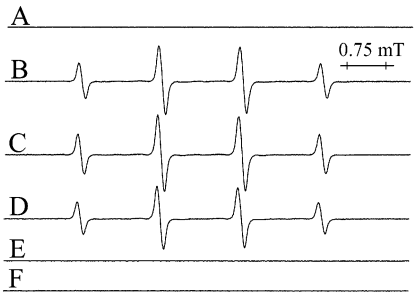

FIGURE 1: Generation of hydroxyl radicals from various forms of PrP121−231. ESR spectra were recorded following the addition of Fe(II) (in the presence of DMPO) to solutions of (A) PrP121−231, (B) PrP121−231 D178N, (C) PrP121−231 F198S, and (D) PrP121−231 E200K, all preincubated for 6 h, (E) PrP121−231 E200K in the presence of DETAPAC, and (F) PrP121−231 E200K in the presence of catalase, both preincubated for 1 h.

Data for the toxic effects of the same wild-type and mutant forms of PrP121−231 on normal primary mouse cerebellar (mainly neuronal) cultured cells, exposed for 4 days to the peptides (50 μM) in the presence or absence of serum, are shown in Figure 2. The culture medium, containing fresh peptide, was replenished on days 1 and 3. At the end of the incubation period (day 4), the viability of the cells was determined using an MTT assay. Under these conditions, all of the peptides showed some toxicity to the cerebellar cells, particularly when they were treated in the absence of serum. The rank order from the least to the most toxic was as follows: wild type < D178N < F198S < E200K. In the presence of serum, wild-type PrP121−231 was only marginally toxic (86% of the viability of nontreated controls). Compared to this, all three mutants showed significantly enhanced toxicity (Student's t test, $p < 0.05$) with only 35% cell viability for the most potent PrP121−231 mutant (E200K). It has been established previously that full-length wild-type PrP^C is not toxic when added externally to mouse cerebellar cultures (*37*).

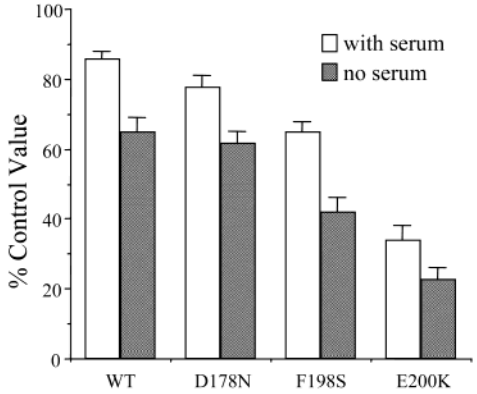

FIGURE 2: Toxic effects of the various forms of PrP121−231 on mouse cerebellar cells.

Because the ESR data suggested that the toxicity of (particularly the mutant forms of) PrP121−231 could be due to the generation of hydrogen peroxide, we looked at the effects of catalase in the cell viability assays. We have speculated previously that the generation of hydrogen peroxide from certain amyloidogenic proteins, complexed with suitable metal ions, could involve oxygen binding and the intermediate formation of superoxide (*34*, *35*). For this reason, we also looked at the effects of superoxide dismutase in the cell viability assays. Both catalase and superoxide dismutase gave a dose-dependent partial protection against the toxic effects of the normal and mutant forms of PrP121−231 (Figure 3). However, at the concentrations that were tested, the best protection was afforded by catalase, the highest dose of which (10 microunits) resulted in 50−90% inhibition of this toxicity. We also tested three different metal chelators (BCS, BPDA, and CDTA over a concentration range of 0.1−10 μM) in the cell viability assays (Figure 4). CDTA, a manganese chelator, did not show any significant protection against the toxicity of the peptides. In contrast, BCS and BPDA (with selectivity for chelation of copper and iron, respectively) exhibited dose-dependent protection against the toxic effects of all forms of PrP121−231.

DISCUSSION

There are currently more than 20 different mutations in the *prnp* gene that give rise to inherited TSEs. An obvious effect of these mutations would be to alter the conformation of the prion protein so that it is richer in β-sheet structure and consequently becomes more prone to aggregation, more resistant to proteolysis, and more toxic to cells and, importantly, behaves like an infectious protein. There is some limited experimental evidence to support this idea (*6*). Studies of the secondary structure of mutant forms of PrP have revealed some changes compared to the wild-type protein (*41*−*43*). The P102L mutation has been reported to enhance β-sheet fibril formation in synthetic peptide fragments of PrP (*44*). Mutant PrPs expressed in CHO cells do acquire some properties (detergent insolubility, resistance to proteolysis) that are characteristic of PrP^{Sc} (*45*), although their infectious nature remains to be established. Of particular relevance to this report, some fragments of PrP bearing point mutations, including those used here, are more toxic to cells than the corresponding wild-type fragments (*6*, *37*, *46*). One problem, however, is that so far these findings on PrP conformation, aggregation, and toxicity have not been consistent for all mutations (*6*). Our most striking observation was the ability, under the conditions of our experiments, of all three mutant forms of PrP121−231 (E200K, D178N, and F198S) to generate hydroxyl radicals *in vitro*, in complete contrast to wild-type PrP121−231 and full-length PrP^{C} which were inactive. This observation supports the idea that these particular prion mutations result in a form of PrP that is more toxic than the wild-type protein. Further studies will be required to determine if this is also the case for other PrP mutations. Moreover, our data immediately suggest a plausible mechanism for this enhanced toxicity, based on generation of hydrogen peroxide by the mutant peptides. Exposure of cells to hydrogen peroxide (and hydroxyl radicals derived from it in the presence of redox-active transition metal ions) would result in oxidative stress and cell death. The fact that wild-type PrP121−231 and PrP^{C} were both inactive suggests that the C-terminal fragments behave in the same way as the full-length protein. Even if this were not the case, there is good evidence that various N-terminally truncated fragments of PrP do exist in the brain in the TSEs (for example, PrP112−231 which is generated by normal proteolytic cleavage) (*47*), and so our data on the C-terminal fragments may be pathologically relevant.

The generation of hydrogen peroxide from the PrP121−231 mutants seems to be dependent on the presence of metal ions, since it was abolished by the metal chelator DETAPAC

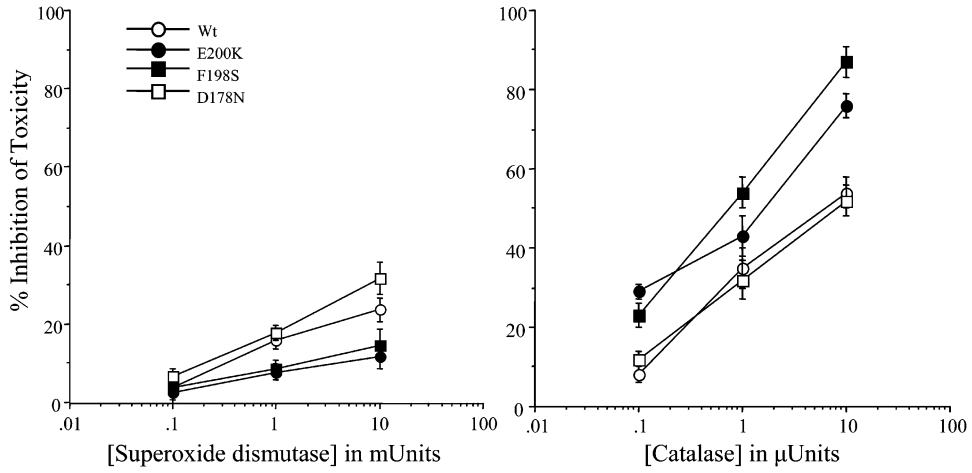

FIGURE 3: Effects of superoxide dismutase and catalase on PrP121−231 cytotoxicity.

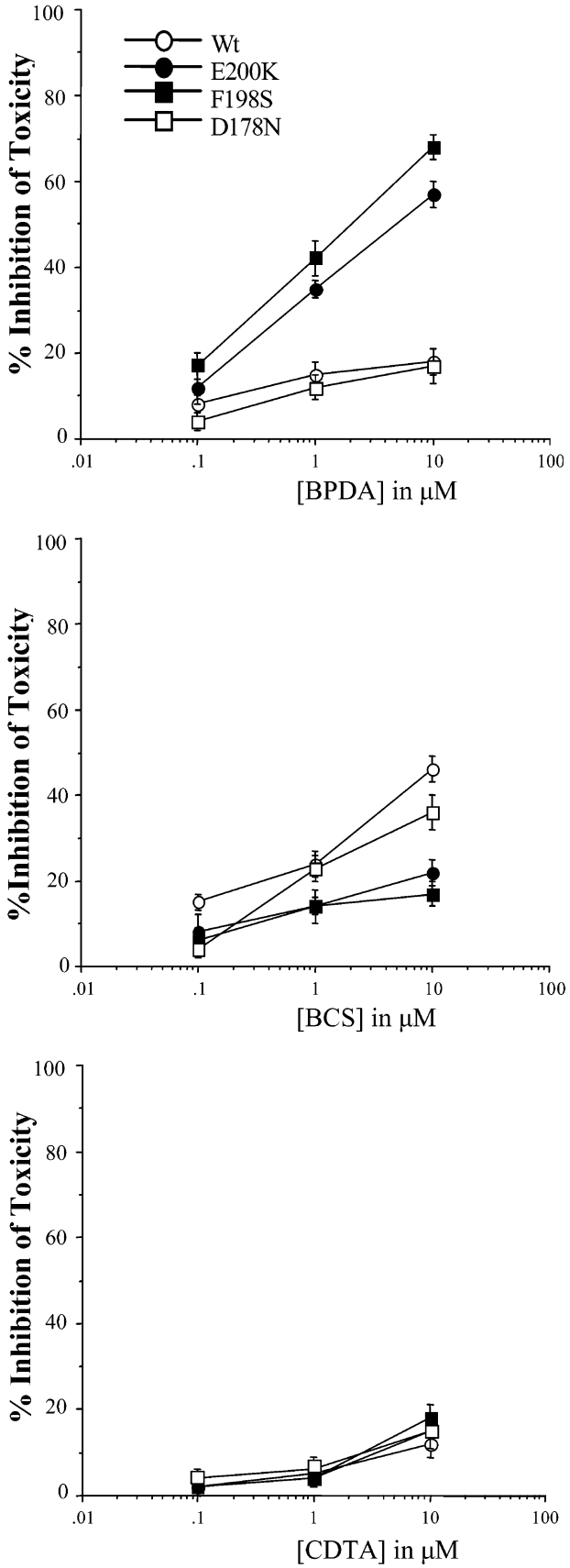

FIGURE 4: Effects of metal chelators on PrP121−231 cytotoxicity.

(Figure 1) and by other metal chelators (data not shown). There is a growing body of evidence which shows many of the aggregating proteins involved in neurodegenerative diseases can interact with redox-active metal ions and generate hydrogen peroxide (*34*, *35*). The ability of the PrP121−231 mutants to do this in the absence of added metal ions is similar to our previous observations with Aβ and α-synuclein, and is presumably due to low levels of metals unavoidably present in the buffer solutions or to metal already bound to protein (*22*). Although most of the known copper binding sites are in the N-terminal octapeptide repeat region of PrP, there is some recent evidence for metal binding to the C-terminal part of the molecule (*32*). A potential mechanism for the generation of hydrogen peroxide from an amyloidogenic protein, with its associated redox-active transition metal ion(s), is *via* the binding of molecular oxygen and the formation of superoxide (*34*, *35*). Our experience to date is that, in general, hydrogen peroxide formation is most prevalent during an active phase of amyloid aggregation and fibril growth. Others have reported a similar conclusion (*48*). Thus, our data on the generation of hydrogen peroxide from the PrP121−231 mutants could simply reflect the fact that their aggregation is more rapid than that of the wild-type protein. Previous studies by Liemann and Glockshuber (*38*) have shown that the PrP121−231 mutants D178N and F198S do have a lower thermodynamic stability and a greater tendency to form insoluble inclusion bodies (when expressed in *Escherichia coli*) than wild-type PrP121−231. However, this was not the case for the E200K mutant (*38*). Further detailed conformational and aggregational studies will be required to fully explain the effects of the mutations.

The cell toxicity data (particularly in the presence of serum) show that, as expected from the ESR results, the three PrP121−231 mutants were more toxic than the wild-type peptide, although the effects of the D178N mutation were small in magnitude. The wild-type peptide did not generate any ESR spectrum and yet did show some limited toxicity. This could be due to the fact that the "baseline" toxicity observed with the wild-type peptide is due to a mechanism other than hydrogen peroxide formation, or to the fact that small amounts of hydrogen peroxide are generated from the wild-type peptide when it is present for an extended time period in a cell culture environment. In support of the latter argument, we have found that the addition of small amounts of Cu(II) to PrP121−231 (which would be present in culture medium) does result in a very weak ESR spectrum (data not shown). The fact that catalase was particularly effective in protecting against the toxic effects of all forms of PrP121−231 clearly supports the idea that hydrogen peroxide formation is involved. Two of the three metal chelators that were tested (those with selectivity for iron or copper) showed protection in the cell toxicity assays, confirming that, as discussed above, metals also appear to be involved in the toxic mechanism. The most effective chelator was BPDA, which has selectivity for iron. Iron could be important for the generation of hydrogen peroxide, or for the conversion of hydrogen peroxide into the highly reactive and toxic hydroxyl radical. However, it should be noted that interpretation of the results with these metal chelators depends greatly on the relative affinities of the chelators compared to the PrP121−231 peptides for binding to the same metal ions. For example, copper could be essential for the generation of hydrogen peroxide from Prp121−231, but the production of hydrogen peroxide would only be partly inhibited by a copper chelator with a lower affinity for the metal than the peptide itself.

We have reported previously that PrP106-126 generates hydrogen peroxide in the presence of Cu(II) ions (*24*). Here we have shown that certain mutant forms of PrP121−231 can also generate hydrogen peroxide. These data suggest that oxidative damage, neurodegeneration, and cell loss in the TSEs could be due to a direct pro-oxidant effect of PrP^{Sc}. In support of this argument, increased levels of various markers for oxidative stress (e.g., lipid peroxidation, heme oxygenase-1, nitrotyrosine, and nucleic acid oxidation products) have been detected in the brains of animals or humans infected with prion disease (*16*, *49*), and there is also good evidence that cultured cells infected with PrP^{Sc} suffer from oxidative stress (*16*).

Finally, our data show some remarkable parallels to those already reported for Aβ and α-synuclein. In particular, both Aβ and α-synuclein have been shown to generate hydrogen peroxide (*22*, *23*), and the neurotoxicity of Aβ can be attenuated by catalase (*50*) and by metal ion chelators (*51*). Thus, the metal-dependent formation of hydrogen peroxide from an aggregating protein could be a common mechanism of neurotoxicity and cell death in a number of different neurodegenerative diseases (*34*, *35*).

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