Direct Production of Reactive Oxygen Species from Aggregating Proteins and Peptides Implicated in the Pathogenesis of Neurodegenerative Diseases

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Abstract: The deposition of abnormal protein fibrils is a prominent pathological feature of many different 'protein conformational' diseases, including some important neurodegenerative diseases. Some of the fibril-forming proteins or peptides associated with these diseases have been shown to be toxic to cells in culture. A clear understanding of the molecular mechanisms responsible for this toxicity should shed light on the probable link between protein deposition and cell loss in these diseases. In the case of the -amyloid (A) peptide, which accumulates in the brain in Alzheimer's disease, there is good evidence that the toxic mechanism involves the production of reactive oxygen species (ROS). By means of an electron spin resonance (ESR) spin-trapping method, we have shown that solutions of A liberate hydroxyl radicals when incubated *in vitro*, upon the addition of small amounts of Fe(II). We have also obtained similar results with -synuclein, which accumulates in Lewy bodies in Parkinson's disease, and with the PrP (106-126) toxic fragment of the prion protein. It is becoming clear that some transition metal ions, especially Fe(III) and Cu(II), can bind to these aggregating peptides, and that some of them can reduce the oxidation state of Fe(III) and/or Cu(II). The data suggest that hydrogen peroxide accumulates during incubation of these various proteins and peptides, and is subsequently converted to hydroxyl radicals in the presence of redox-active transition metal ions. Consequently, a fundamental molecular mechanism underlying the pathogenesis of cell death in several different neurodegenerative diseases could be the direct production of ROS during formation of the abnormal protein aggregates.

THE ACCUMULATION OF EXTRACELLULAR OR INTRACELLULAR FIBRILLAR PROTEIN AGGREGATES IS THE CAUSE OF SEVERAL DIFFERENT NEURODEGENERATIVE DISEASES

The formation of fibrillar aggregates from a range of different proteins and peptides is a common feature of numerous different 'protein conformational' diseases. These diseases include the amyloidoses where normally soluble proteins, or their proteolytic fragments, are deposited extracellularly in the form of insoluble amyloid fibrils, approximately 10 nm in diameter, with a characteristic cross- -pleated sheet protein structural conformation [1]. In the systemic amyloidoses, these amyloid deposits are found, sometimes in very large quantities, in many different tissues and organs throughout the body, excluding the brain [1]. More localised amyloid deposits are found in some other diseases, such as late-onset diabetes, in this case involving only the pancreas.

Several important neurodegenerative diseases constitute a special type of localised amyloidosis, where the amyloid deposits are restricted to the central nervous system (CNS) [2]. A key example of this is Alzheimer's disease (AD), one of the hallmark features of which is the accumulation of amyloid fibrils, composed of the -amyloid peptide (A), extracellularly at the centre of senile plaques, and sometimes

in the walls of cerebral blood vessels [3]. In some of these neurodegenerative diseases, fibrillar protein deposits are found inside nerve cells or glial cells. Examples of such deposits are the neurofibrillary tangles found in AD, the Lewy bodies found in Parkinson's disease (PD), the glial fibrillary inclusions found in multiple system atrophy and the intranuclear inclusions found in Huntington's disease (HD). These intracellular fibrils are, strictly speaking, not defined as amyloid. However, the boundaries between extracellular and intracellular protein aggregation are not always clear. For example, there is some evidence that the early stages of A aggregation actually start to occur inside neuronal cells [4] even though the amyloid fibrils themselves are extracellular. Furthermore, it is clear that some important lessons that have been learned from the study of extracellular amyloid proteins can be applied to intracellular fibrillar inclusions. Table **1** shows a list of some neurodegenerative diseases associated with the accumulation of extracellular and/or intracellular protein fibrils, together with the aggregating proteins and CNS lesions concerned.

In the systemic amyloidoses, there is little doubt that the formation of the amyloid deposits is the direct cause of the disease in question. Often, the sheer quantity of the amyloid deposits formed must obviously result in organ dysfunction and, ultimately, in organ failure [1]. Molecular genetic studies of many different types of inherited amyloidosis also point to the primary role of amyloid deposition in disease pathogenesis. This is because, in many instances, a pathogenetic mutation in the gene encoding the actual fibril-forming protein has been shown to lead to an inherited form

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Table 1.   Aggregating Proteins and Peptides Involved in Neurodegenerative Diseases

Diseases	Aggregating polypeptide(s)	Lesions concerned
Alzheimer's disease, Down's syndrome	-amyloid (A), tau	senile plaques, neurofibrillary tangles
Parkinson's disease	-synuclein	Lewy bodies
Dementia with Lewy bodies	-amyloid, -synuclein	senile plaques, Lewy bodies
Multiple system atrophy	-synuclein	glial fibrillary inclusions
Prion diseases	prion protein (PrP)	amyloid plaques, prion rods
Motor neuron disease	SOD-1	SOD-1 inclusions
British familial dementia	ABri, tau	amyloid plaques, neurofibrillary tangles
Danish familial dementia	ADan, tau	amyloid plaques, neurofibrillary tangles
Tauopathies (e.g. Pick's disease)	tau	tau-derived inclusions
Huntington's disease	huntingtin	intranuclear inclusions
Spinocerebellar ataxias	ataxins	intranuclear inclusions

of a particular amyloid disease. The resulting mutant proteins often show an increased propensity to fold into -pleated sheets and to aggregate *in vitro* into fibrils with the character-istic ultrastructural features of amyloid [see, for example, refs. 5,6]. In these inherited diseases, it is clear that the mutant amyloid-forming proteins are the initial cause of the disease, and the effects of the mutations can be linked directly, and not surprisingly, to the formation of amyloid fibrils.

In the relevant neurodegenerative diseases, there has been considerable dispute regarding the causal role, or otherwise, of the abnormal protein fibrils. This is partly due to the fact that, historically, some of these diseases have been considered to be due to primary abnormalities in particular neurotransmitter systems (e.g. the dopamine system in PD and the acetylcholine system in AD). Moreover, the situation is confused by the fact that in many neurodegenerative diseases, two, or even more, different types of protein aggregate can be found in the same brain. Again, this is where molecular genetic studies have proved to be a very powerful and revealing tool. In the neurodegenerative diseases listed in Table **1**, pathogenetic mutations have *not* been found in genes concerned with neurotransmitter function. On the contrary, inherited forms of AD, PD, frontotemporal dementia, the transmissible spongiform encephalpothies (TSEs) and motor neuron disease, can all be caused by a mutation in the gene encoding an aggregating protein, or another protein involved in its metabolism [2]. Examples of the latter are the presenilin mutations responsible for early-onset, familial AD, and the parkin or ubiquitin C-terminal hydrolase (UCH-L1) mutations responsible for early-onset, familial PD. The presenilins are thought to be involved in proteolytic release of A from its precursor protein (APP) [7]. Parkin and UCH-L1 are components of the ubiquitin-proteasome system [8] which is involved in the degradation of misfolded proteins. This is likely to include -synuclein which fails to be degraded effectively and so accumulates in Lewy bodies. HD and the spinocerebellar ataxias are all due to abnormally long trinucleotide expansions, encoding the amino acid glutamate, in a particular gene. The resulting mutant proteins have an extended polyglutamine tract which causes them (or a proteolytic fragment derived from them) to aggregate and accumulate inside the nuclei of nerve cells [9,10]. The familial British and Danish dementias are due to stop codon mutations in the *BRI* gene, with the extended mutant proteins undergoing proteolytic cleavage and the resulting mutant peptide fragments (ABri and ADan) accumulating in the form of amyloid plaques in the brain [11].

This direct link between molecular genetics and protein aggregation in several different neurodegenerative diseases strongly suggests that the aggregating proteins concerned play an important and, probably, seminal role in the pathogenesis of all of them [2]. This conclusion has been supported by numerous transgenic mouse studies, where mice expressing pathogenetic mutant forms of the human genes encoding each of the aggregating proteins mentioned above show many of the histopathological, neurochemical and behavioural changes associated with the relevant human disease [see, for example, refs. 12-22]. Furthermore, it has been shown that many of the fibril-forming proteins or peptides associated with neurodegenerative diseases are toxic to cultured neuronal cells. This implies a direct link between protein aggregation and neurodegeneration and raises the possibility of potential common molecular mechanisms linking protein aggregation to cell death. The direct production of reactive oxygen species (ROS) such as hydrogen peroxide from the proteins, as they aggregate, is one such potential common mechanism, and is the main focus of this review article.

LINK BETWEEN PROTEIN AGGREGATION AND CELL DEATH

The cytotoxic effects of A have been studied more extensively than any of the other aggregating proteins mentioned in Table **1**. Cell death caused by exposure to A

appears to be due to calcium influx and the induction of oxidative damage [23]. There are several different hypotheses that have been put forward to explain this effect. These include: (i) the formation by A of calcium channels in cell membranes [24]; (ii) an interaction between A and a specific cell surface receptor, such as the RAGE (receptor for advanced glycation endproducts) or scavenger receptors [25-27]; (iii) an interaction between A and an intracellular target molecule such as ERAB (endoplasmic reticulum A binding protein) [28]; (iv) non-specific intercalation of aggregated forms of A into membranes [29]; (v) the spontaneous fragmentation of A to give highly reactive peptidyl radicals [30]; and (vi) the direct production of hydrogen peroxide from A [31]. It is now generally accepted that A needs to be in a partially aggregated state before it becomes toxic to cells, and there is increasing evidence that 'soluble oligomers' in the form of protofibrils, annular protofibrils (ring-shaped structures) or ADDLs (A -derived diffusible ligands) could be the primary toxic species [32-37]. These various observations and hypotheses are not mutually exclusive. For example, a specific form of aggregated A could be required for free radical or hydrogen peroxide formation, or to interact with cell-surface receptors. Recent data generated in our laboratory have cast doubt on the idea that A can spontaneously generate peptidyl radicals [38]. However, we have confirmed that A does appear to generate hydrogen peroxide (see below) [39]. Hydrogen peroxide is toxic to cells in its own right, but if formed in the vicinity of metal ions, such as Fe(II), would be converted *via* the Fenton reaction into the even more toxic and highly reactive hydroxyl radical. Exposure of cells to ROS, such as hydrogen peroxide and hydroxyl radicals, would result in oxidative stress, and it is well established that this can ultimately lead to cell death.

OXIDATIVE DAMAGE IN NEURODEGENERATIVE DISEASES

The evidence for the importance of oxidative damage to affected areas of the brain in a range of neurodegenerative diseases has been gathering strength for two decades. Just over a decade ago, protein oxidation in aging was carefully reviewed by Stadtman [40] who noted the site-specific nature of metal-catalysed reactions and the role of ROS generated from molecular oxygen, such as superoxide and the hydroxyl radical, and the formation of hydrogen peroxide. The damaging consequences of oxidative stress have been studied extensively in AD and include markers such as elevated levels of certain metal ions (including redox-active iron and copper), lipid peroxidation, DNA oxidation and protein oxidation [reviewed in refs. 23, 41-44]. Proteins are major targets for free radical attack. Especially damaging in this respect is the hydroxyl radical, which reacts with most biomacromolecules with a rate constant close to the diffusion-controlled limit. The most common free radical propagation reaction is displacement, which normally exhibits itself as hydrogen atom abstraction. Other common propagation reactions include addition, electron transfer, fragmentation and rearrangement. In the presence of oxygen, any of these reactions result in major alterations to protein and peptide molecules such as oxidation of side-chain groups, cross-linking between chains and backbone

cleavage. Markers of oxidative damage, such as the introduction of a carbonyl group into proteins, have been widely reviewed [see, for example, refs. 45-48]. In principle all amino acid residues are susceptible to radical attack, but Tyr, Phe, Trp, His, Met and Cys residues appear to be the most common targets [40, 49-51]. There is now substantial evidence implicating the importance of oxidative stress and ROS in the pathology of a wide range of neurodegenerative diseases. The evidence has been most widely reviewed in AD [23, 41, 43, 52] and PD [53, 54] but there is also substantial evidence for oxidative damage to the CNS in a range of other diseases, such as HD, motor neuron disease and the prion disorders [42, 55, 56].

AGGREGATING PROTEINS AND METAL IONS

It has been suspected for some time that metal ions might promote amyloidogenic protein aggregation. For example, aluminium, copper, iron and zinc ions have all been reported to induce the aggregation of A [57-60]. However, it is now becoming clear that one of the major factors involved appears to be the direct binding of certain metal ions to the aggregating protein [58, 60-62]. Whilst it may not be surprising to find that transition metal ions can bind to these protein molecules it is, perhaps, surprising to find that they can apparently do so with very high affinity. For example, Cu(II) has been reported to bind to A (1-42) with a dissociation constant of *ca.* 10^{-17} M (*i.e.*, attomolar affinity) [63] which is comparable with the very best metal ion chelators known. The dissociation constant for Cu(II) binding to A (1-40) is reported to be several orders of magnitude less [63], with Fe(III) binding less strongly than Cu(II) to both A (1-42) and A (1-40). Complete removal of Fe(III) from the cell culture medium blocks the cytotoxicity of A [64]. Likewise, complete removal of Cu(II) from laboratory buffers has been reported to abolish the aggregation of A (1-42) [63].

Raman spectroscopy indicates that the amino acid residues involved in both Cu(II) and Zn(II) binding to A are the histidine residues at positions 6, 13 and 14 [65]. Iron, however, binds primarily to the phenolic oxygen atom of the tyrosine residue at position 10, but also to the carboxylate groups of the glutamate and aspartate side chains. A recent nuclear magnetic resonance (NMR) and electron spin resonance (ESR) study of A (1-28) reports Cu(II) binding to the three histidine residues in this fragment and suggests that the tyrosine residue at position 10 might also be involved [66].

Metal ion-protein binding does not appear to be unique to A . A decade ago Sulkowski proposed that the conversion of PrP^c (cellular isoform of the prion protein) into PrP^{sc} (scrapie isoform) involved the co-ordination of transition metal ions [67]. Several studies indicate that up to as many as five Cu(II) ions can be bound to the octapeptide repeat region of PrP^c [68, 69]. At least one of these sites apparently binds to Cu(II) with femtomolar affinity [70]. More recently, two high-affinity binding sites have been identified within the human prion protein, one involving the binding of Cu(II) within the octapeptide repeat segment and the other to the region around histidines 96 and 111 [70]. The aggregation of -synuclein is also promoted by Cu(II) [71].

A critically important feature of Cu(II) and Fe(III) binding to A [31, 72] and of Cu(II) binding to human prion protein [73] is that the binding can be accompanied by a reduction in the oxidation state of the metal ion (see Fig. (**1**)). The reduction of Cu(II) and Fe(III) to Cu(I) and Fe(II) in the presence of hydrogen peroxide (see below) sets up the correct conditions for the Fenton reaction and the release of the highly reactive hydroxyl radical.
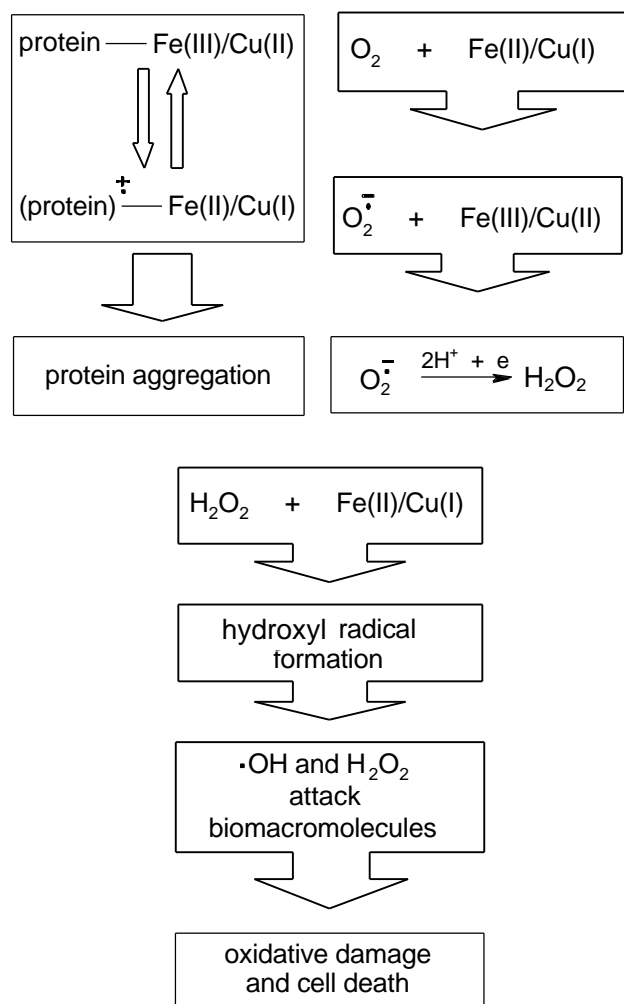
Fig. (1). A flow chart showing the cascade of events leading to the formation of ROS (superoxide, hydroxyl radicals and hydrogen peroxide), aggregation, oxidative damage and cell death.

A GENERATES HYDROGEN PEROXIDE

Not only does A bind to metal ions and reduce the oxidation state of Cu(II) and Fe(III), but it can also generate hydrogen peroxide. Behl *et al*. [74] were the first to conclude, based on cell toxicity data, that hydrogen peroxide was involved in the cellular toxicity induced by A . Although the measurement technique first employed was sensitive to the presence of any peroxide, the observation that catalase, which degrades hydrogen peroxide into water and oxygen, protected cells from A -induced toxicity indicated that hydrogen peroxide was the major peroxide

present. The direct formation of hydrogen peroxide from A (1-40) and A (1-42) was reported shortly afterwards [31, 72]. The possible generation of hydrogen peroxide by A is likely to be extremely important, not only because it is neurotoxic, and generated adjacent to the peptide, but also because it is freely permeable across membranes.

The ability of A to bind to metal ions, especially to Cu(II) and Fe(III), therefore appears to initiate a sequence of events which leads to aggregation, neurotoxicity and the formation of ROS (see Fig. (**1**)). The peptide reduces both Cu(II) and Fe(III), to Cu(I) and Fe(II), respectively, and generates neurotoxic hydrogen peroxide. The reduction of copper and iron ions must be a consequence of electron transfer from the A peptide. The methionine residue at position 35 of A could be involved here, since it is readily oxidised to methionine sulfoxide. However, other peptides which do not possess any methionine residues (e.g. the NAC peptide, see below) can also generate hydrogen peroxide, and so other amino acid residues, and possibly even the peptide backbone, must be able to participate in this process. Once present, both Cu(I) and Fe(II) can form the aggressive hydroxyl radical from hydrogen peroxide *via* Fenton chemistry. This reactive radical would then attack any biomacromolecules within its vicinity (see Fig. (**1**)), including A itself. This second phase of oxidative attack on A may, or may not, target the same amino acid residues. The exact mechanism by which hydrogen peroxide is generated by the A -metal ion complex remains to be established, but there are two feasible routes. One possibility, which is favoured by Bush and colleagues [31], is that the peroxide is generated *via* a one-step two-electron transfer process. The second possibility is *via* two sequential one-electron transfers in which the initial electron transfer to form superoxide (the oxygen radical anion, see Fig. (**1**)) could lead to hydrogen peroxide formation *via* a further one-electron transfer and protonation [75, 76].

DETECTION OF HYDROGEN PEROXIDE FORMA-TION FROM A BY ESR SPECTROSCOPY

We were intrigued by the possibility that protein aggregation in AD and other neurodegenerative diseases, such as PD, might be accompanied by hydrogen peroxide formation and so we sought a sensitive technique to test for the presence of hydrogen peroxide which was quick and required only small sample volumes. One technique meeting both of these requirements is ESR spectroscopy, which does not measure hydrogen peroxide (or hydroperoxides) directly but detects hydroxyl radicals liberated upon addition of Fe(II) (Fenton's reaction). Although the high reactivity of the hydroxyl radical prevents its direct detection, it can be readily stabilised employing spin-trapping. Unfortunately, one of the most common spin-traps available, *N*-tert-butyl- -phenylnitrone (PBN), is not suitable for this purpose as, in aqueous solution, its hydroxyl radical adduct immediately transforms into *tert*-butylhydroaminoxyl [77]. Since the ESR spectrum of this aminoxyl can also arise from hydrolysis and oxidation of PBN [38, 75, 76] it is an unreliable spin-trap for the detection of hydroxyl radicals. We, therefore, selected 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) whose hydroxyl radical adduct (DMPO-OH) is stable over the period of time required to record an ESR spectrum. In our experiments, the

spin-trap (DMPO) is added at the end of the required peptide incubation period along with diethylenetriaminepentaacetic acid (DETAPAC), as a metal ion sequester, and Fe(II) (to liberate the hydroxyl radical), thus giving a 'snapshot' of the hydrogen peroxide concentration at the time of these additions. The resulting ESR spectrum consists of four lines (of relative intensities *ca.* 1:2:2:1 and hyperfine parameters *a*(N) 1.50 and *a*(H) 1.46 mT) which are uniquely characteristic of the DMPO-OH adduct [39]. Although our measurement technique does not permit continuous monitoring of the hydrogen peroxide levels, it does have the advantage that the reagents required to make the measurement are not present during incubation. This avoids any complications that could arise if the spin-trap was involved in the redox chemistry of the protein-metal ion complex.

We employed the ESR technique, described above, to test for the generation of hydrogen peroxide during the incubation of A (1-40) in phosphate buffered saline (PBS) at 37°C [39]. The characteristic four-line DMPO-OH ESR spectrum was readily observed immediately upon addition of DMPO, DETAPAC and then, finally, Fe(II), after incubation periods as short as 0.5 to 1 hr (see Fig. (**2a**) and Table **2**) [39]. The same batch of A (1-40) formed fibrils and was found to be neurotoxic.

Table 2.   Relative ESR Spectrum Intensities of DMPO-OH (In Arbitrary Units) Obtained for Various A Peptides (100 μM) After Different Incubation Periods. ND = Not Determined

	Incubation Period/hr		
	1	6	48
A (1-40)	8,000	8,000	1,250
A (1-40) Met35Nle	0	0	0
A (1-42)	8,500	ND	8,000
A (25-35)	3,000	ND	4,500
A (40-1)	0	0	0

The characteristic four-line ESR spectrum was also observed when the same reagents were added to pre-incubated samples of A (1-42) and A (25-35) (Table **2**) [75] both of which are neurotoxic [see, for example, refs. 78, 79]. In complete contrast, however, no spectrum was observed with the AA (1-15) peptide (an N-terminal fragment of the amyloid A protein, associated with reactive systemic amyloidosis), which aggregates to give amyloid-like fibrils but is not cytotoxic [39, 75]. This result indicates that aggregation *per se* is not necessarily associated with neurotoxicity or with hydrogen peroxide generation. Control solutions, containing no peptide also failed to give a spectrum, as did A (40-1) (Table **2**) [80], the reverse sequence human peptide, which is not neurotoxic [81] and is not capable of reducing the oxidation state of Cu(II) [31]. When A (1-40), A (1-42) and A (25-35) were incubated in the presence of either catalase or DETAPAC, the DMPO-OH spectrum was completely abolished [76] thus supporting

earlier observations [74] and the concept of a metal-dependent pathway for hydrogen peroxide production.

Much of the interest in A (25-35) stems from the work of Yankner and colleagues who concluded that 25-35 is the minimum toxic domain of A (1-40) [78]. The fact that A (25-35) is neurotoxic, and gives a positive test for the generation of hydrogen peroxide, raises some important questions. This fragment does not, of course, have the histidine residues at positions 6, 13 and 14, or the tyrosine residue at position 10, which are considered to be important metal binding sites. Furthermore, A (25-35) has been reported previously not to generate hydrogen peroxide or to reduce Cu(II) *in vitro* [31, 82]. The reason for this apparent discrepancy is not clear at present. If metal binding is the key to hydrogen peroxide production, then there must be at least one suitable metal binding site on the A (25-35) peptide. One possibility is that the methionine residue at position 35 is involved in metal binding. Substitution of this residue in A (25-35) by either leucine, norleucine, lysine or tyrosine residues results in peptides which neither aggregate nor are neurotoxic. However, aggregation and toxicity are retained when this methionine is replaced by aspartate, serine or cysteine residues [83]. The replacement of the methionine at position 35 by norleucine also prevents the toxicity of full-length A (1-40) and A (1-42) [84, 85]. In accord with these observations, we found that the ESR spectrum of DMPO-OH was not generated from A (1-40) Met35Nle (Table **2**), indicating that the replacement of the methionine residue had blocked hydrogen peroxide generation [80].

GENERATION OF HYDROGEN PEROXIDE FROM -SYNUCLEIN

Following our findings for A , we were intrigued by the possibility that -synuclein, which is known to be toxic to cells [86], might also self-generate hydrogen peroxide and, consequently, we undertook a series of ESR experiments to investigate this. -Synuclein is a small protein (~14 kDa) that is expressed at high levels by neuronal cells [87]. The first indication of an involvement of -synuclein in the pathogenesis of neurodegenerative diseases came from the isolation of a peptide named non-A -component (NAC) from preparations of amyloid from the brains of patients with AD [88]. Amino acid sequencing revealed that NAC comprised 35 amino acids, corresponding to residues 61-95 of -synuclein [88]. Two mutations, which increase the propensity of -synuclein to form toxic oligomers [89, 90], have been identified in the gene encoding −synuclein [91, 92]. These two mutations are each associated with rare, inherited forms of early-onset PD. This led to the discovery that −synuclein is the main component of the intracellular aggregates found in Lewy bodies inside nerve cells in PD and related disorders (e.g. dementia with Lewy bodies) and in glial cytoplasmic inclusions found in multiple system atrophy [93-97]. Consequently, these diseases are collectively known as the 'synucleinopathies'. There is now a substantial amount of evidence to suggest that the conversion of −synuclein from soluble monomers to aggregated, insoluble forms in the brain is a key event in the pathogenesis of the synucleinopathies [97]. The function of −synuclein remains to be established; however it has been implicated in the regulation of synaptic plasticity [98],

neuronal differentiation [99, 100], as well as in regulation of dopamine synthesis [101], and also has chaperone-like activity [102]. Recent studies have shown that neuronal cells overexpressing wild-type –synuclein are more resistant to oxidative stress than untransfected cells [103, 104]. It has also been shown that -synuclein at low concentrations of non-aggregated protein protects neuronal cells against cellular stress conditions such as serum deprivation, oxidative stress, and excitotoxicity [104], whereas, pre-aggregated -synuclein was found to be toxic to neuronal cells [105].
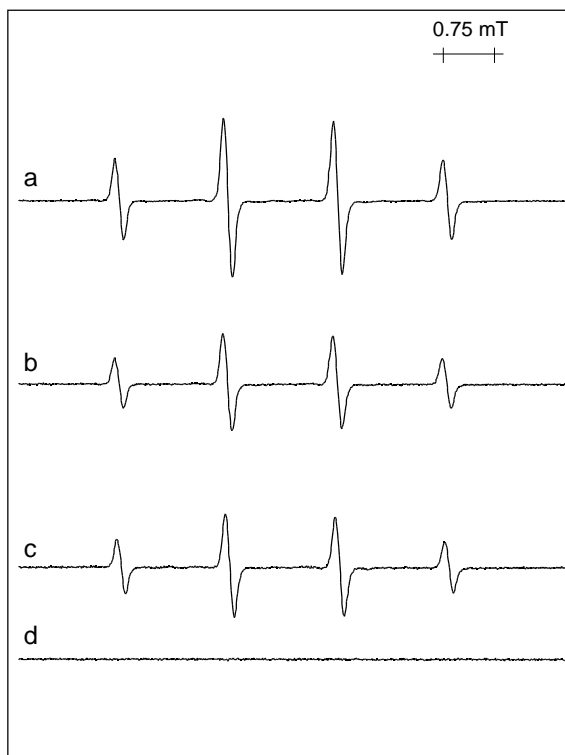
Fig. (2). ESR spectra recorded following the addition of Fe(II) (in the presence of DMPO and DETAPAC) to solutions of (a) A (1-40) (intensity/2), (b) -synuclein, (c) PrP (106-126) in the presence of 2 μM copper, and (d) a control (*i.e.*, no peptide), all pre-incubated at 100 μM for 1 hr in PBS. For details of experimental conditions and spectrometer settings see references 39 and 115.

When a sample of -synuclein was incubated, under our normal experimental conditions (*i.e.*, in PBS at 37°C), the characteristic four-line DMPO-OH ESR spectrum was readily observed after periods of between 0.5 and 48 hrs, see Fig. (**2b**) and Table **3** [39]. As with A , the formation of this spectrum was blocked when the incubation was undertaken in the presence of either catalase or DETAPAC.

We also investigated -synuclein (1-87), a carboxy-terminally truncated protein, which is reported to assemble into filaments more rapidly than full-length -synuclein under the same conditions [106]. This fragment also gave the characteristic four-line ESR spectrum of DMPO-OH, upon addition of Fe(II), as did -synuclein (1-80) and -synuclein (1-70), again over incubation periods of between 1 and 48 hrs (Table **3**).

Table 3. Relative ESR Spectrum Intensities of DMPO-OH (In Arbitrary Units) Obtained for Various Synucleins and NAC Fragments (100 μM) After Different Incubation Periods

	Incubation Period/hr	
	1	**48**
-synuclein	2,500	1,300
-synuclein	0	0
-synuclein	0	0
-synuclein (1-70)	0	2,500
-synuclein (1-80)	3,000	1,600
-synuclein (1-87)	1,400	3,250
NAC (1-35)	2,500	1,500
NAC (1-18)	3,500	0
NAC (19-35)	0	0
NAC (35-1)	0	0
NAC (18-1)	0	0

In contrast, no spectra were observed when the experiment was repeated with two related proteins, - or -synuclein, under identical conditions (Table **3**). These two synucleins fail to assemble into filaments under the same conditions and over the same time period as -synuclein [97] and, at present, are not known to be associated with any known neurodegenerative disorder.

The NAC fragment of -synuclein also aggregates into amyloid-like filaments and is known to be neurotoxic to cells [105]. Experiments on fragments of NAC have shown that the N-terminal region, *i.e.* residues 1 to 18, drives the aggregation of 1-35 [107]. NAC (1-18) both aggregates and is toxic to cells, whereas NAC (19-35) and the reverse sequence peptide, NAC (18-1), neither aggregate nor are neurotoxic [105]. These experiments involving NAC, and some of its fragments, raise the important question as to whether or not toxicity and aggregation are, again, accompanied by hydrogen peroxide generation. NAC (1-35), NAC (1-18), NAC (19-35), NAC (18-1) and NAC (35-1) were incubated for periods of up to 48 hrs, following our standard procedure. Of these fragments, the characteristic ESR spectrum of DMPO-OH was observed, upon addition of DMPO, DETAPAC and, finally, Fe(II), for NAC(1-35) and NAC(1-18) only (Table **3**) [80]. This result is in exact accord with expectations based on aggregation and toxicity data [97, 105, 107] and with the observations on the C-terminally truncated -synucleins noted above.

The self-generation of hydrogen peroxide by -synuclein, NAC (1-35) and NAC (1-18), but not by the non-toxic and non-filament forming - and -synucleins, nor by the non-aggregating NAC fragments [(19-35), (18-1) and (35-1)], supports the concept of hydrogen peroxide formation being responsible for the toxic properties of -

synuclein and its NAC fragments. The molecular mechanism responsible for the generation of hydrogen peroxide probably involves the binding of redox-active transition metal ions to -synuclein, with the self-generation of hydrogen peroxide accompanying protein aggregation, although further studies are required to establish this. The reduction of hydrogen peroxide to hydroxyl radicals gives rise to a major ROS which could explain much of the oxidative damage observed in the brain in PD.

PRIONS

The similarities between AD and the prion diseases (*i.e.* strong evidence for oxidative damage and the ability of PrP to bind to certain metal ions and reduce their oxidation state) are very striking. In order to gain an understanding of the neurotoxicity of PrP[Sc], many researchers have focussed on a small peptide, PrP (106-126), which behaves in some respects like PrP[Sc] itself (see, for example, refs. 108-114). PrP (106-126) readily aggregates into amyloid fibrils and is only toxic to cells expressing PrP[C]. What is particularly interesting, however, is the fact that the aggregation of PrP (106-126) has been reported to be critically dependent upon the presence of copper (or to a lesser extent zinc) ions, and is inhibited by bathocuproine (a copper ion chelator) [108, 112].

The above publications immediately suggested to us that conditions were again appropriate for the self-generation of hydrogen peroxide. We, therefore, undertook a series of experiments in which PrP (106-126) and a scrambled version of PrP (106-126) (as a negative control) were incubated, at 37°C, in the presence or absence of Cu(II). In solutions from which metal ions had been rigorously excluded, and from the PrP (106-126) scrambled peptide, the ESR spectrum of the DMPO-OH adduct was not detected upon addition of Fe(II) over incubation periods of up to 48 hrs [115]. However, in the presence of small concentrations of Cu(II) (such as 0.2 µM) the characteristic four-line DMPO-OH spectrum was observed when the incubated solution was monitored over the same time period, see Fig. (**2c**) and Table **4**. Importantly, this spectrum was abolished when the incubation was undertaken in the presence of either catalase or DETAPAC. Consequently, PrP (106-126) has exactly the same property as A and -synuclein (*i.e.*, the self-generation of hydrogen peroxide), but only in the presence of copper ions.

CONCLUSION

Based on our own data, and that of others, we propose that several neurodegenerative diseases actually share a common mechanism of neurodegeneration and cell death, as shown in Fig. (**3**). A and PrP both bind strongly to certain metal ions, and there is now substantial evidence that these metal ions play a crucially important role in aggregation and toxicity. The oxidation state of both Cu(II) and Fe(III) is reduced by A, and the oxidation state of Cu(II) is reduced by PrP. It is likely that −synuclein shares this same property. Crucially, the peptide-metal ion complex reduces oxygen to hydrogen peroxide (presumably via superoxide). Hydrogen peroxide is freely permeable across membranes and so readily inflicts oxidative damage to cells. Neuronal cells, particularly in the aging brain, are vulnerable to oxidative stress. Hydrogen peroxide is also readily reduced by Fe(II) and Cu(I) to liberate the highly reactive and unselective hydroxyl radical, which is capable of inflicting severe oxidative damage. This, in general outline, is what we suspect is the cause of neuronal cell death in several different neurodegenerative diseases. However, many unanswered questions remain. Precisely which forms of the aggregating proteins associated with these diseases (e.g. monomeric, oligomeric, protofibrils, mature amyloid fibrils) are responsible for the generation of ROS? Alternatively, is the generation of ROS actually a 'by-product' of the aggregation process itself? Do aggregating proteins other than A, -synuclein and PrP (including those found outside of the brain) also generate ROS? How can this hypothesis explain the selective vulnerability of different areas of the brain in different neurodegenerative diseases? We believe that further research into these various areas in the next few years is likely to be highly informative. Based on our hypothesis, we are also strongly supportive of the development of metal ion chelators and/or anti-oxidants for disease therapy.

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Table 4.   **Relative ESR Spectrum Intensities of DMPO-OH (In Arbitrary Units) Obtained for PrP (106-126) (100 µM) After Different Incubation Periods**

	Incubation Period/hr		
	1	**6**	**48**
PrP (106-126)	0	0	0
PrP (106-126) with 2 µM Cu(II)	800	1,200	800
PrP (106-126) with 0.2 µM Cu(II)	3,000	4,000	4,000
PrP (106-126) scrambled, with 2 µM Cu(II)	0	0	0
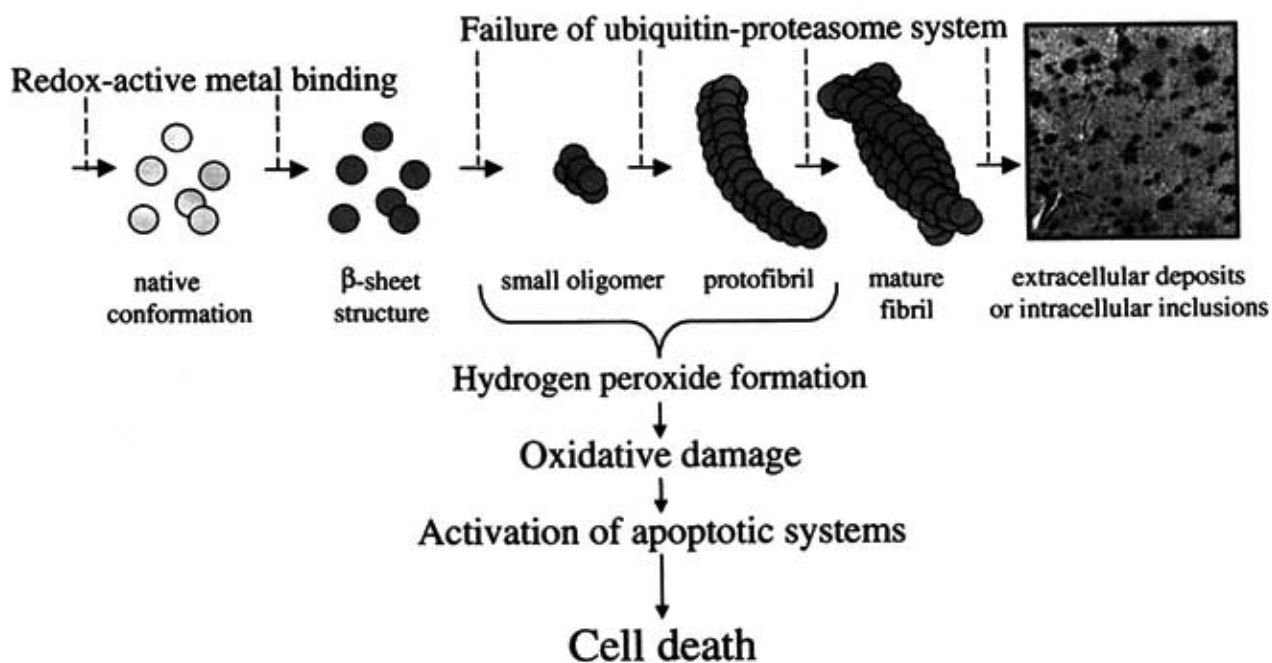
Fig. (3). A possible common mechanism of cell death in protein aggregation-dependent neurodegenerative diseases.

ABBREVIATIONS

ABri	=	familial British dementia peptide
A	=	-amyloid peptide
AD	=	Alzheimer's disease
ADan	=	Familial Danish dementia peptide
ADDLs	=	A -derived diffusible ligands
APP	=	-amyloid precursor protein
CNS	=	Central nervous system
DETAPAC	=	Diethylenetriaminepentaacetic acid
DMPO	=	5,5-dimethyl-1-pyrroline *N*-oxide
DMPO-OH	=	DMPO hydroxyl radical adduct
ERAB	=	Endoplasmic reticulum A binding protein
ESR	=	Electron spin resonance
HD	=	Huntington's disease
NAC	=	Non-A -component of -synuclein
NMR	=	Nuclear magnetic resonance
PBN	=	*N*-tert-butyl- -phenylnitrone
PBS	=	Phosphate buffered saline
PD	=	Parkinson's disease
PrP	=	Prion protein
PrP^C	=	Cellular isoform of PrP
PrP^{Sc}	=	Scrapie isoform of PrP
RAGE	=	Receptor for advanced glycation endproducts
ROS	=	Reactive oxygen species
TSEs	=	Transmissible spongiform encephalopathies
UCH-L1	=	Ubiquitin C-terminal hydrolase

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