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REVIEW

How is protein aggregation in amyloidogenic diseases modulated by biological membranes?

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Abstract The fate of proteins with amyloidogenic properties depends critically on their immediate biochemical environment. However, the role of biological interfaces such as membrane surfaces, as promoters of pathological aggregation of amyloidogenic proteins, is rarely studied and only established for the amyloid-β protein (Aβ) involved in Alzheimer's disease, and α-synuclein in Parkinsonism. The occurrence of binding and misfolding of these proteins on membrane surfaces, is poorly understood, not at least due to the two-dimensional character of this event. Clearly, the nature of the folding pathway for Aβ protein adsorbed upon two-dimensional aggregation templates, must be fundamentally different from the three-dimensional situation in solution. Here, we summarize the current research and focus on the function of membrane interfaces as aggregation templates for amyloidogenic proteins (and even prionic ones). One major aspect will be the relationship between membrane properties and protein association and the consequences for amyloidogenic products. The other focus will be on a general understanding of protein folding pathways on two-dimensional templates on a molecular level. Finally, we will demonstrate the potential importance of membrane-mediated aggregation for non-amphiphatic soluble amyloidogenic proteins, by using the SOD1 protein involved in the amyotrophic lateral sclerosis syndrome.

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Amyloidogenic diseases: the role of membrane interfaces

The pathological self-assembly of proteins into toxic structures plays a key role in amyloidogenic diseases (Masters et al. 1985; Haas and Selkoe 1993; Iversen et al. 1995; Lansbury 1999; Rochet and Lansbury 2000; Bucciantini et al. 2002; Glabe 2006; Kayed et al. 2003). These diseases give rise to neurodegenerative, metabolic and systemic symptoms, and share a common pathology in the form of aberrant protein folding, leading to the accumulation of proteinaceous aggregates in various tissue types. For all these proteins, the lethal action is linked to a pathological conversion or "misfolding" of their native non-toxic (globular or "natively unfolded") structure into toxic aggregates. Evidence suggests that the formation of toxic Aβ assemblies is an intrinsic property of the protein's primary sequence, without any requirement of post-translational modification or specific enzyme activities (Walsh et al. 2000; Rochet and Lansbury 2000; Bucciantini et al. 2002; Glabe 2006). As has recently been established, mature amyloid fibrils are not the most toxic forms of amyloidogenic proteins, but early oligomers being formed during aggregation process are very toxic (Walsh et al. 1999; Rochet and Lansbury 2000; Klein et al. 2004; Haass and Selkoe 2007). The discovery that various amyloid oligomers have a common structure, brought new insight into possible toxicological pathways (Glabe 2006; Kayed et al. 2003; Bucciantini et al. 2002). The inhibition of oligomeric toxicity by a common antibody, independent of the location of the oligomers in extracellular or intracellular compartments, clearly argues against a specific mechanism for one type of amyloid pool and instead favours a common mechanism in areas of the cell which are accessible via extra- and intracellular regions,

such as cell membranes (Glabe 2006; Kayed et al. 2003; Demmester et al. 2000; Kawara et al. 2000; Lin et al. 2001).

In Alzheimer's disease, Aβ's actual folding fate in situ critically depends not only on its concentration but also on its immediate biochemical or possible pathologically altered environment (Walsh et al. 2000; Zhao et al. 2004; Grimm et al. 2005; Selkoe 2004; Fernandez and Berry 2003; McLaurin et al. 2000; Curtain et al. 2003; Rochet et al. 2004). Biological membranes have been shown to modulate the pathological conversion of structurally and functionally non-related proteins into amyloidogenic assemblies, as has been established for Aβ and α-synuclein proteins which are involved in Alzheimer's and Parkinson's disease (Selkoe 2004; Haas and Selkoe et al. 2007).

The amphiphatic character of the Aβ-protein, inherited from its precursor protein (APP), makes it an ideal candidate for membrane-associated toxicity inducing events. The APP protein itself is a type-I transmembrane protein whose dimerization in membranes might play a pivotal role in its breakdown into Aβ (Scheuermann et al. 2001; Haass and Selkoe 2007). Various studies have reported enhancing effects of neuronal lipid membranes on Aβ-conversion into toxic oligomers: a potential key process in Alzheimer's disease (Klein et al. 2004; Walsh et al. 2000; McLaurin et al. 2000; Curtain et al. 2003; Terzi et al. 1997; Simons et al. 1998). Target membranes containing charged lipid components were shown to induce a dramatic electrostatically driven surface accumulation of Aβ-protein, followed by accelerated misfolding into toxic Aβ-aggregates at rates much higher than those in a membrane-free environment (Zhao et al. 2004; Lindström et al. 2002; Bokvist et al. 2004; Kakio et al. 2002; Waschuk et al. 2001; Gibson Wood et al. 2003; Walsh et al. 2000; McLaurin et al. 2000; Curtain et al. 2003; Terzi et al. 1997; Simons et al. 1998; Devanathan et al. 2006). But which general rules govern the misfolding behaviour of amyloidogenic proteins under the reduced dimensionality of membrane interfaces?

Membranes as two-dimensional folding templates

Giacomelli and Norde (2005) showed that the time-dependent conformational changes in surface-adsorbed Aβ-protein are crucially dependent on the properties of the applied surface material. By using Teflon as a hydrophobic surface and silica wafers as hydrophilic one, they could reveal an initial association of Aβ via either its hydrophobic C-terminal (29–40 aa) or its hydrophilic N-terminal part (1–28 aa) (Fig. 1). Due to the different anchoring conditions at the beginning of the experiments, the protein folding had to follow different pathways depending on the part of the protein which was still free to move. Clearly, the adsorption of

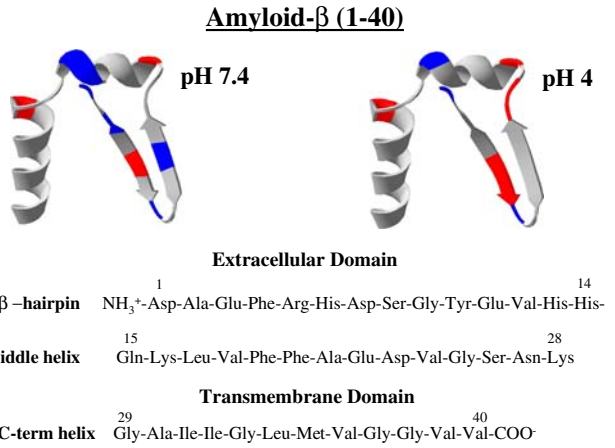

Fig. 1 Amyloid-β (1–40) protein: membrane-inserted monomeric structural model (Durell et al. 1994) and primary sequence. Dependence of Aβ's overall charge on pH value; shown for pH 7.4 and 4; *red* basic aa, *blue* acidic aa

this amphiphatic protein to a surface brought residues spatially together, which would otherwise be far away from each other in the polypeptide chain. The nature of the interface—either hydrophobic or hydrophilic—obviously brings different groups or residues together.

How does the misfolding of proteins occur on a two-dimensional surface compared to a three-dimensional folding space for non-associated proteins in the presence of potential target lipid membrane systems in vivo? Clearly, surface adsorption will put selective pressure onto the folding of the involved protein, thereby forcing it down a specific folding pathway and its respective kinetics. These changes might then result in a pathologically aggregated conformation instead of a non-toxic functional one, which would be the case in a interface-free solution. Based on the work of many colleagues and of our own work (Lindström et al. 2002; Bokvist et al. 2004; Lindström et al. 2005), we have developed a molecular concept, which describes the origin of the association–aggregation process occurring upon two-dimensional membrane surfaces, as well as the factors which control the two-dimensional folding pathways of membrane adsorbed proteins. This model contains two main molecular effects, which together lead towards an increased population of destabilized proteins, resulting in a dramatic increase in amyloidogenic products (schematic concept shown in Fig. 2):

- Membrane association of proteins induces a surface crowding effect (Minton 1999). Two factors, electrostatics and hydrophobicity, are the major determinants of non-specific membrane binding and protein aggregation.
- Membrane surfaces act as aggregation templates: association of proteins to specific membrane interfaces might reduce the stability of the proteins' native state. As a consequence, above a critical surface concentration

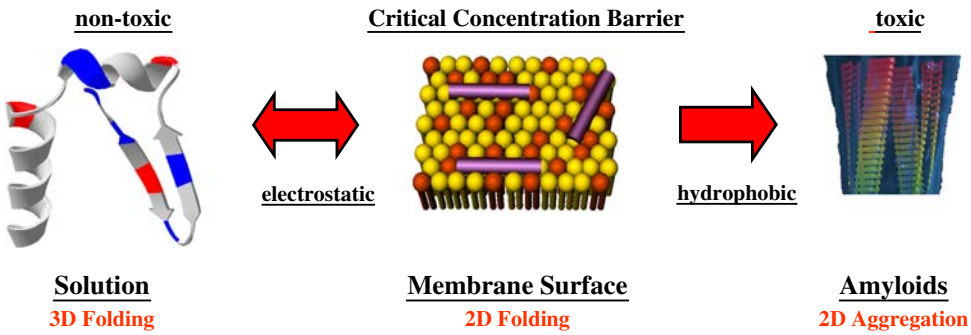

Fig. 2 General description of association and aggregation of amyloidogenic proteins on 2D surfaces. Aβ monomer (Durell et al. 1994) binds electrostatically to a membrane displaying a negative surface potential. The significantly increased concentration of surface associated protein compared to bulk concentration results in hydrophobically driven accelerated conversion into β-sheet-like amyloids (adapted Jimenez et al. 1999) occuring on the 2D membrane interface acting as an aggregation template

barrier, accelerated transformation of a protein from a monomeric state into toxic aggregates can occur despite a much lower bulk concentration.

Reduction from 3D to 2D: implications for protein interactions?

The membrane surface is in many aspects a very special environment for a protein. The geometric restriction from a 3D space in bulk solution (e.g. cytosol) to the 2D space of the membrane surface has pronounced consequences for membrane interactions. The average distance between proteins is an important factor for protein–protein interactions. In a 3D space the average distance between molecules is related to the cube root of the total amount of proteins, whereas in a 2D space the average distance is related to the square root of the total amount of proteins (Fig. 3).

In order to illustrate this very basic but abstract property we may consider the example of brain tissue. Human brain tissue consists of about 1 g of lipids/100 g of dry substance and the water content is in the range of 70% (Balakrishnan et al. 1961). Considering the rather rough estimation of 1,000 g/l density and an average molar mass of 800 g/mol for a lipid, the concentration of lipids is in the range of 3.75 mM. With an average area of one lipid being 68Å^2 (Kuchinka and Seelig 1989), one obtains the rather high membrane surface area of 1,500 m^2/l. Figure 3c expresses the average distance for a random distribution in solution (dashed line) and for a random distribution on a surface of 1,500 m^2/l (solid line). The effect of membrane adsorption on the average distance between the protein molecules is more pronounced for higher protein concentrations. In other words, an increased concentration of a protein lowers the average distance more for a membrane protein than for a free cytosolic protein. Notably, the intersection of both lines in Fig. 3c is in the range of µm; this is a reasonable value for the distance between lipid bilayers in some tissues.

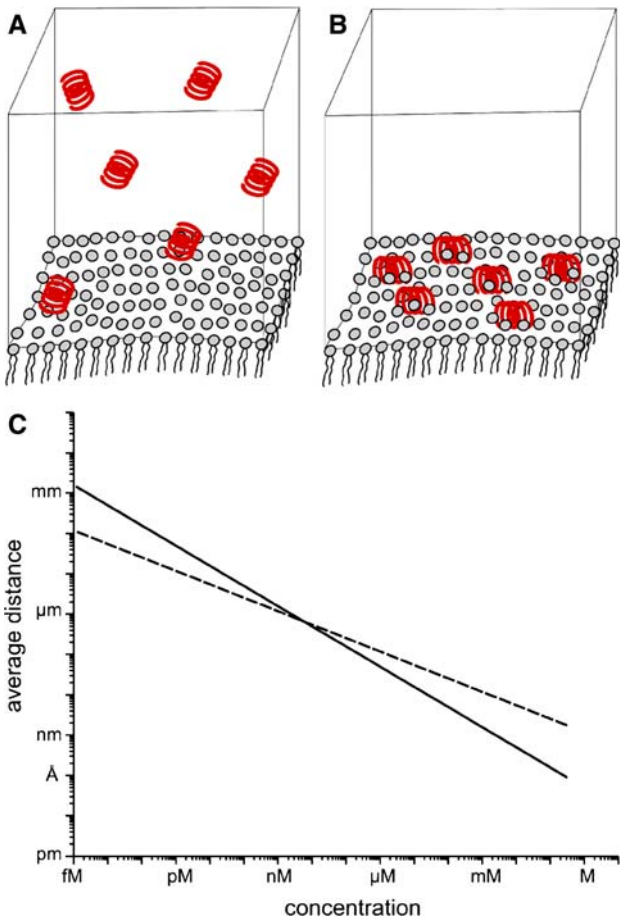

Fig. 3 **a** Distribution of molecules in a 3D space. **b** Distribution of molecules in the 2D space of the membrane surface. **c** Average distance between molecules at given concentration in solution (*dashed line*) and adsorbed on a surface of 1,500 m^2/l (*solid line*). Both axes are shown in logarithmic scale

These principal geometric properties will of course interfere with the more specific effects of the membrane. There exists, for example, a modification for the protein diffusional behavior, and the interaction sides of the protein might be either protected (e.g. if the hydrophobic sides of a

protein are buried in the membrane; Fernandez et al. 2003) or exposed, in the membrane-adsorbed form of the protein.

Interaction of amyloidogenic proteins with membrane surfaces

The interaction of peripheral proteins with lipid membranes plays a key role in many cellular processes. For amyloidogenic proteins such as Aβ or α-synuclein, the initial binding to lipid membranes is, in principle, driven by electrostatic forces. These are present between protein domains rich in positive (basic) amino acid residues and negatively charged (acidic) lipid membranes (Fig. 1). Subsequently, additional hydrophobic interactions can induce amyloidogenic structures (Fig. 2). The binding behaviour of these proteins is similar to the one found for membrane-perturbing toxins and antimicrobial peptides, which also use positively charged amphiphatic segments for initial nonspecific binding to negatively charged target lipid membranes prior to inducing membrane poration and lysis (Bonev et al. 2000; Wieprecht et al. 2000).

The process of an electrostatically driven association of Aβ to membranes has been described by various groups (Zhao et al. 2004; Lindström et al. 2002; Bokvist et al. 2004; Kakio et al. 2002; Waschuk et al. 2001; Gibson Wood et al. 2003; Walsh et al. 2000; McLaurin et al. 2000; Curtain et al. 2003; Terzi et al. 1997; Simons et al. 1998; Ege et al. 2005; Lau et al. 2006). They all found that the presence of neutral lipids such as phosphatidylcholine had no effect on the time-dependence of β-sheet formation in Aβ. In the opposite case, the presence of vesicles containing acidic lipids (mostly phosphatidylglycerol or phosphatidylserine) accelerated the protein aggregation, often dramatically (for review: McLaurin et al. 2000, Gibson Wood et al. 2003). Early isothermal calorimetry studies by Seelig's group (Terzi et al. 1995) clearly indicated the electrostatic nature of an Aβ_{1-40}-membrane association, which could be quantified by a concentration dependent binding constant ranging between 700 and 2,100 mol^{-1} for a POPC/POPG (75/25 mol/mol) membrane system. In the presence of high salt concentrations or by the use of neutral vesicles, no Aβ-membrane binding was detected; which is another observation confirming the electrostatic nature of this first assembly step. Even membrane binding of α-synuclein seems to be an important factor in the pathogenesis of Parkinson's disease (Review: Selkoe 2004).

Example of membrane-mediated Aβ folding

How are temporal conformational changes of Aβ-protein affected by the process of 2D crowding at membrane surfaces? The main factor involved is the electrostatic potential present at the membrane and the protein surface, which controls the affinity of the protein towards the membrane. To visualize the role of these parameters, temporal CD aggregation trials were carried out and plotted in the spectral region between 195–250 nm (Fig. 4), where the CD signal reflects the basic secondary structural features of proteins. For this purpose 50 µM Aβ_{1-40} at a 60:1 lipid/protein molar ratio was added at 300 K and at a pH 4 to either buffer (Fig. 4a) or to small unilamellar vesicles of DMPC. A negative surface potential was introduced by the incorporation of 33 mol% of acidic DMPG lipids (Fig. 4b), and a positive potential by the presence of positively charged DDAB at the same fraction (Fig. 4c). At this low pH, Aβ possesses six basic and three acidic residues distributed in a non-homogenous way along the hydrophilic part of the protein (Fig. 1). The protein (Fig. 4a) underwent gradual conformational changes over a couple of days, as expected in a membrane free buffer solution. However, in the presence of vesicles containing acidic lipids (Fig. 4b), the protein aggregated at a highly accelerated rate into a main β-sheet population. In the opposite case, the presence of positively charged vesicles, significantly slowed down the transition from random coil features (minimum at 195 nm) to β-like structures (minimum at 218 nm). The protein does not seem to experience a significant crowding effect, most likely due to its many positive charges. Surprisingly, at pH 7.4, the protein experiences accelerated aggregation on both, positive and negative membrane surfaces, as seen by us and others (Lindström et al. 2002; Ege et al. 2005). At this pH the protein has an overall negative charge but the basic residues seem to be sufficiently separated from the acidic ones, in order to enable the protein to still bind to negatively charged vesicles (presumably Lys_{28} is one of the main players there).

NMR insight

In general, NMR measurements permit a molecular picture of ongoing peptide/protein–lipid interactions. In order to address the question, whether these proteins bind unspecifically to a negatively charged membrane or specifically to the anionic phospholipid headgroup, wideline ^{2}H NMR, using specifically headgroup deuterated phospholipids was often used (Terzi et al. 1997). However in the case of the amyloid-β protein, this method could not reveal any effect of the electrostatic interactions on the phospolipid headgroup, probably due to the location of Aβ outside the lipid headgroup region. Also, the wideline ^{31}P NMR exploiting the 100% naturally abundant phosphorus-31 nucleus present in all phospholipid constituents of the lipid membranes, failed to show lipid specific changes induced by the presence of protein due to complex NMR spectra. Multicomponent phospholipid systems usually produce wideline ^{31}P

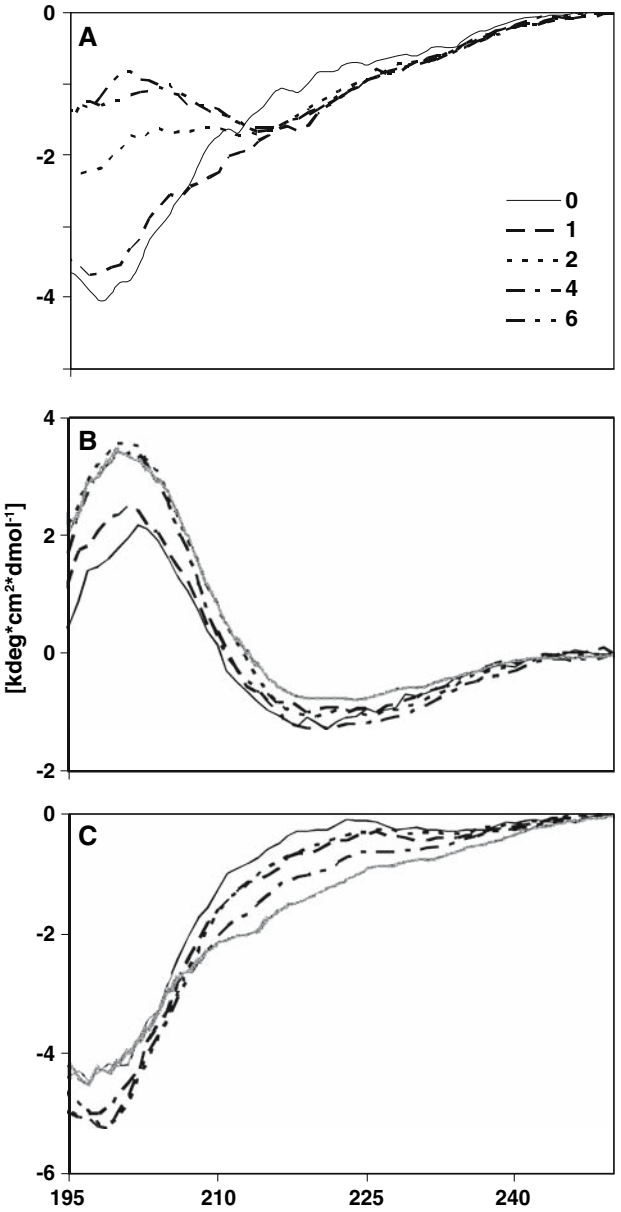

Fig. 4 Temporal CD spectra of 50 μM Aβ_{1-40} protein at 300 K and pH 4: **a** in membrane-free buffer (10 mM Tris, 10 mM KCl, 0.5 mM EDTA); **b** upon addition to anionic vesicles composed of DMPC/DMPG (2:1 molar ratio) at a 60:1 L/P ratio; and **c** as in **b** but cationic vesicles of DMPC/DDAB (2:1 molar ratio) used. CD spectra were acquired over several days as indicated

NMR spectra consisting of overlapping intensity distributions, arising from the individual lipid components, which therefore cannot be resolved (Seelig 1978). However, these individual membrane phospholipid constituents can be observed simultaneously by high resolution natural abundance ^{31}P MAS NMR (Pinheiro and Watts 1994; Carbone and Macdonald 1996; Lindström et al. 2002; Lau et al. 2006), where magic angle spinning (MAS) is used to average, in part or completely, the effective CSA (chemical shift anisotropy) of the lipid phosphates. In this way it is possible to obtain a molecular view of the protein–lipid interaction for each lipid component separately, and most interestingly, any changes in the membrane surface potential upon protein association. The isotropic chemical shifts for both phosphates of mixed lipid bilayers of various PC and PG content showed systematic changes upon the presence of Aβ (Bokvist et al. 2004). For pure PC vesicles no changes were detected. As seen in Fig. 5 both lipids react to the presence of surface associated Aβ in the same way, excluding a specific protein–lipid interaction. Both isotropic NMR resonances move upfield (reduced negative surface potential) upon increasing the amounts of present Aβ protein. As shown in combined ^{14}N and ^{31}P NMR studies (Lindström et al. 2002, 2005), these systematic changes can be analysed semi-quantitatively in order to provide the size of the occurring electric field change at the membrane interface, accompanied by a specific reorientation of the PC headgroup dipoles.

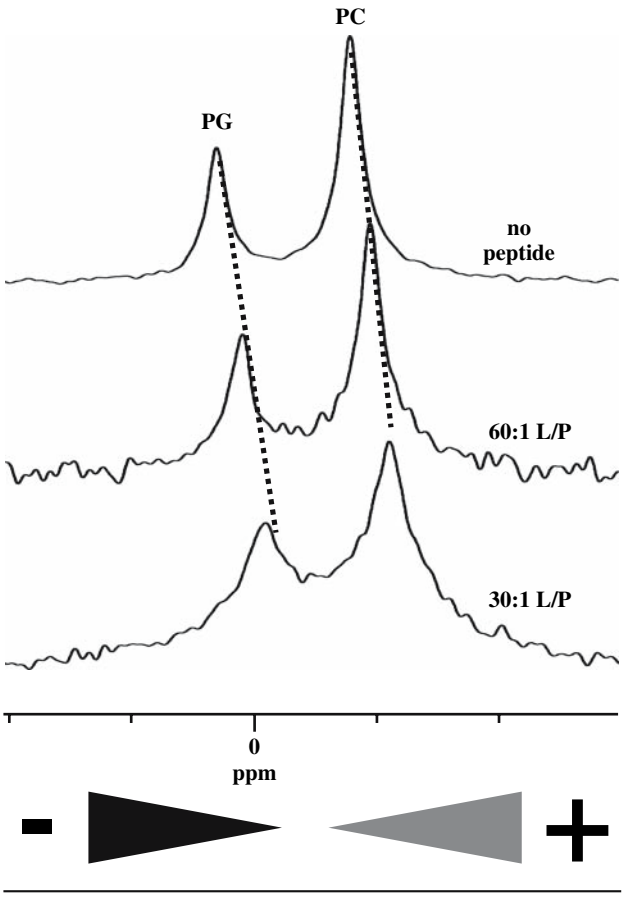

Fig. 5 ^{31}P CP MAS NMR spectra obtained at 308 K, 6 kHz spinning speed and 20 ms contact time of multilamellar DMPC/DMPG vesicles before and after the addition of increasing amounts of Aβ_{1-40} protein (as indicated). Isotropic ^{31}P chemical shift values move upfield as expected upon partial membrane surface charge compensation induced by basic residues of Aβ_{1-40}

The role of metal ions

The amyloidogenic plaques found in the brain of AD patients contain high concentrations of transition metals including copper (around 400 μM), zinc (ca. 1 mM) and iron (ca. 1 mM). As a result there has been a long ongoing debate about the involvement of metal ions in AD. Special focus in recent years has been on Cu as a pathological risk factor, since its redox potential (Cu^{2+}/Cu^{+}) provides an easy way to induce oxidative stress in AD patients. Numerous studies revealed the ability of Cu−Aβ complexes to produce reactive oxygen species (Barnham et al. 2004; Smith et al. 2006, 2007; Dai et al. 2006). Treatment of human amyloid precursor protein expressing transgenic mice with a copper attenuating compound led to an improvement in their general health and brain amyloid plaque deposition (Cherny et al. 2001). This finding was followed by an encouraging small phase 2 clinical trial with the same compound (Ritchie et al. 2003). The coordination of Cu^{2+} to Aβ and even APP was revealed by EPR, NMR, and Raman and was shown to be formed by three histidines and presumably tyrosine (Smith et al. 2006, 2007; Kong et al. 2007). Recently, an NMR study found a similar coordination for Zn^{2+} with three histidines and the N-terminus of Aβ (Danielsson et al. 2007). Even a second weaker binding site involving the residues Asp_{23} and Lys_{28} was described, which does not seem to exist for Cu^{2+}.

Curtain and collegues (Curtain et al. 2003) revealed quite early the influence of Zn^{2+} and Cu^{2+} ions on the insertion ability of Aβ proteins into membraneous systems by using EPR and CD spectroscopy. For the $A\beta_{1-40}$ species, a pH dependence was found if Cu^{2+} ions were present, suggesting the necessity of a specific coordination sphere to trigger protein insertion. Barnham and collegues (Smith et al. 2006) could correlate copper mediated Aβ toxicity to the formation of intermolecular histidine bridged dimers. The same authors could show that in the presence of lipid membranes, the toxicity was related to lipid peroxidation and dityrosine formation (Barnham et al. 2003; Smith et al. 2006). In a later study the same group found that the process of oxidative modification in Aβ, occurring at high Cu^{2+}/protein molar ratios, could be traced back to the formation of dityrosine crosslinking, presumably a consequence of tyrosine radicals formed under oxidative stress (Smith et al. 2006, 2007). In the context of the potential role of copper and zinc in AD, various studies have focused on the influence of these ions on the association of Aβ with membranes and the correlated toxic behaviour. A recent biophysical study showed the different effect of Zn^{2+} and Cu^{2+} on charged lipid membranes (Lau et al. 2006). $A\beta_{1-42}$ was still able to bind to membranes in the presence of elevated levels of ions, despite the change in protein charge due to coordinated ions. The group concluded that the aggregation could be initiated by metal triggered abnormal lipid−protein interactions.

Non-amphiphatic amyloidogenic proteins: the potential action of membranes

Membrane modulated folding behaviour seems even to be inherent in soluble, but amyloidogenic proteins without amphiphatic properties. As first discovered on cellular prion proteins, missing H-bond protection in the native structures makes these proteins prone to membrane association and structural instability (Fernandez et al. 2003; Fernandez and Berry 2003). Fernandez et al. (2003) and Fernandez and Berry (2003) established a model correlating an increase in missing H-bond protection to an increase in membrane-association and amyloidogenic propensity when looking into the general features of amyloidogenic proteins; a behaviour he could verify experimentally for a wide range of amyloidogenic proteins (Figs. 2, 3 in Fernandez et al. 2003). As recently reported, these soluble proteins can in the presence of membranes, produce mature fibrils containing acidic lipid components (Zhao et al. 2004, Sparr et al. 2004). Whether these lipid containing fibrils or their oligomeric precursors are more toxic than lipid-free ones is still unknown.

As an example of a non-fibrillar soluble protein with amyloidogenic properties we used superoxide dismutase 1 (SOD1) to study any occurring membrane interaction which might be of potential interest in the pathology of the closely related amyotrophic lateral sclerosis (ALS). There is strong evidence that ALS is induced by misfolded toxic species of SOD (Bruijn et al. 1998; Zetterström et al. 2007). This model is supported by the good correlation of clinical data with the stability of the native structure of different SOD mutants (Lindberg et al. 2005). However, the aggregation process seems to be more complex, since in vitro aggregation assays of different mutants do not correlate well with clinical data, especially for the frequent mutation A4V. This leads to the idea that lipid membranes might possibly be involved in the (mis-)folding process.

We therefore characterized the interaction of SOD with lipid vesicles. Figure 6 shows the circular dichroism spectra of the SOD1 A4V mutant (Lindberg et al. 2005) in the presence of small unilamellar lipid vesicles. All spectra were recorded for the APO form of the protein under reducing condition. DOPC vesicles represent a neutral lipid surface, whereas DOPC/DOPG possesses a negatively charged surface. The circular dichroism spectra clearly indicate the interaction of SODA4V with negatively charged lipid vesicles (Fig. 6a). This interaction is correlated with a clear modification of the secondary structure of the protein. The

charge of the lipid surface seems to be crucial for the interaction, since the presence of neutrally charged lipid vesicles has no effect on the secondary structure of the protein (Fig. 6b).

The interaction of SOD1 with charged lipid membranes is of special interest, since SOD1 is translocated into the inter-membrane space of mitochondria via the TOM mechanism (Liu et al. 2004). The highly charged lipid membranes of the mitochondria might be important to the refolding of the protein in the inter-membrane space after translocation.

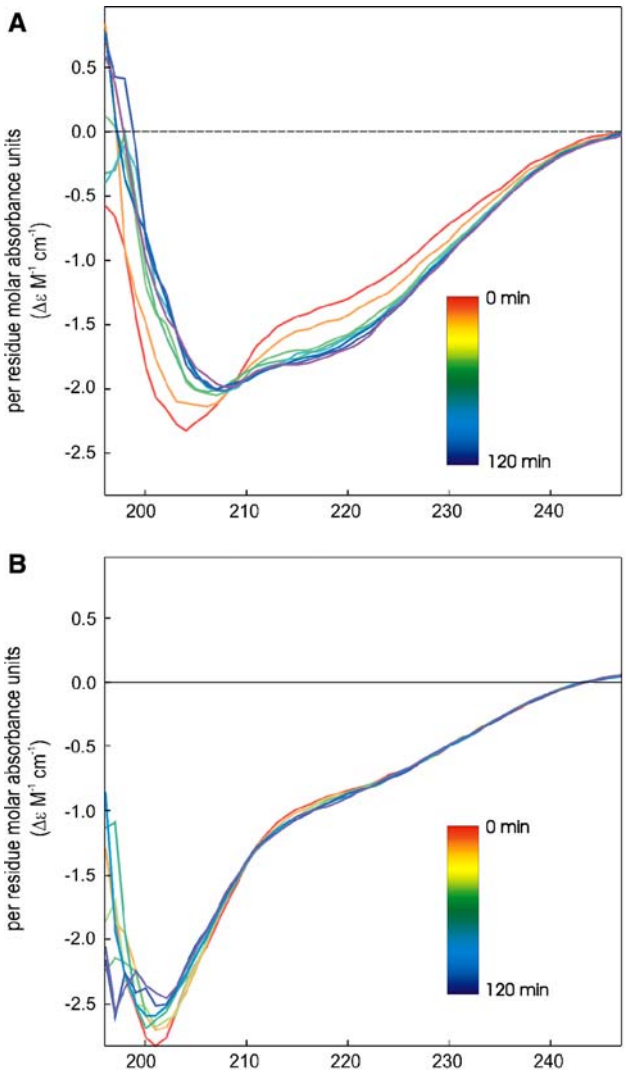

Fig. 6 **a** 15 μM of SOD A4V in the presence of 50 μM DOPC/DOPG 1/1 and **b** DOPC vesicles immediately after addition of the lipid vesicles (*red curve*) up to 120 min after addition of the lipid vesicles (*dark blue curve*)

Outlook amyloidogenic diseases: a common denominator?

One of the most important and challenging aspects of science in the area of neurodegenerative diseases at the present time is the frantic search for a common molecular mechanism (Haass and Selkoe 2007; Barnham et al. 2006; Glabe 2006). Based on the observation that different proteins/peptides, including Aβ, can form toxic oligomers, all of them exhibiting a similar topography (sensitive to the same antibody), these soluble protein oligomers seem to be the main toxic species (Snyder et al. 2005). Already in 2003 Glabe's group (Kayed et al. 2003) had suggested a potential role for cellular membranes in the neurotoxic action of these oligomeric structures. These oligomers can bind to synaptic membranes as recently shown (Review: Haass and Selkoe 2007). But the biochemical mechanism by which soluble oligomers associate with membranes and tamper with different signaling pathways is unclear. However, recent work suggests an effect on NMDA or AMPA receptors, residing at synaptic plasma membranes (Haass and Selkoe 2007; Snyder et al. 2005; Shankar et al. 2007).

How amyloidogenic structures induce cell death in neurodegenerative diseases is still a big mystery in cell biology. However, information about a potentially unique common mechanism is gradually becoming clear, providing the indispensable input for the design of a single therapeutic strategy against these non-curable diseases, which cost millions of lives each year worldwide.

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