doi:10.1016/j.jmb.2003.11.046 *J. Mol. Biol.* (2004) **335**, 1039–1049

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Two Types of Alzheimer's β-Amyloid (1–40) Peptide Membrane Interactions: Aggregation Preventing Transmembrane Anchoring *Versus* Accelerated Surface Fibril Formation

Marcus Bokvist[1], Fredrick Lindström[1], Anthony Watts[2] and Gerhard Gröbner[1]*

[1]*Department of Biophysical Chemistry, Umeå University 90187 Umeå, Sweden*

[2]*Biomembrane Structure Unit Department of Biochemistry University of Oxford South Parks Road Oxford OX1 3QU, UK*

**Corresponding author*

The 39–42 amino acid long, amphipathic amyloid-β peptide (Aβ) is one of the key components involved in Alzheimer's disease (AD). In the neuropathology of AD, Aβ presumably exerts its neurotoxic action *via* interactions with neuronal membranes. In our studies a combination of ^{31}P MAS NMR (magic angle spinning nuclear magnetic resonance) and CD (circular dichroism) spectroscopy suggest fundamental differences in the functional organization of supramolecular $A\beta_{1-40}$ membrane assemblies for two different scenarios with potential implication in AD: Aβ peptide can either be firmly anchored in a membrane upon proteolytic cleavage, thereby being prevented against release and aggregation, or it can have fundamentally adverse effects when bound to membrane surfaces by undergoing accelerated aggregation, causing neuronal apoptotic cell death. Acidic lipids can prevent release of membrane inserted $A\beta_{1-40}$ by stabilizing its hydrophobic transmembrane C-terminal part (residue 29–40) in an α-helical conformation *via* an electrostatic anchor between its basic Lys28 residue and the negatively charged membrane interface. However, if $A\beta_{1-40}$ is released as a soluble monomer, charged membranes act as two-dimensional aggregation-templates where an increasing amount of charged lipids (possible pathological degradation products) causes a dramatic accumulation of surface-associated $A\beta_{1-40}$ peptide followed by accelerated aggregation into toxic structures. These results suggest that two different molecular mechanisms of peptide–membrane assemblies are involved in Aβ's pathophysiology with the finely balanced type of Aβ–lipid interactions against release of Aβ from neuronal membranes being overcompensated by an Aβ–membrane assembly which causes toxic β-structured aggregates in AD. Therefore, pathological interactions of Aβ peptide with neuronal membranes might not only depend on the oligomerization state of the peptide, but also the type and nature of the supramolecular Aβ–membrane assemblies inherited from Aβ's origin.

Keywords: lipid-membrane; β-amyloid peptide; peptide insertion; circular dichroism; ^{31}P MAS NMR

Introduction

Alzheimer's disease (AD) is a chronic dementia, affecting an increasing number of people worldwide.[1–3] It belongs to the family of adult neurodegenerative disorders which includes Parkinson's, Huntington's, type II diabetes and senile systemic amyloidosis. These diseases share a common

Abbreviations used: AD, Alzheimer's disease; TFE, trifluoroethanol; APP, β-amyloid peptide precursor protein; MAS, magic angle spinning; NMR, nuclear magnetic resonance spectroscopy; Aβ, amyloid-β peptide; CD, circular dichroism; DMPC, L-α-dimyristoylphosphatidylcholine; DMPG, L-α-dimyristoylphosphatidylglycerol.

pathology characterized by the accumulation of proteinaceous aggregates as amyloid plaques or fibrils in various tissue types.[4,5] Amyloid plaques are mainly composed of specific fibrillar proteins which have been identified for each of these disorders.[4] The unifying theme for all of these proteins is the relationship between their toxicity and the pathological conversion or "misfolding" from their normal non-toxic globular or "natively unfolded" structure into toxic aggregates, a process not yet fully understood, despite recent progress.[3–6] In the case of AD, overwhelming evidence indicates as a key player the 39–43 amino acid long amyloid-β peptide (Aβ), the main constituent found in extracellar amyloid plaques localized in the brain of AD patients.[1–4] An important feature seems to be that Aβ peptide is released as a monomeric soluble peptide, but requires a minimal level of aggregation to exert its neurotoxic action.[3–8] In particular, oligomeric and protofibrillar Aβ structures are currently associated with the neuropathological events causing the deterious conditions of AD patients.[4,7–10] But, globular and non-fibrillar Aβs are continuously released during normal metabolism, and therefore the fundamental mechanisms behind AD still remain largely unknown due to the lack of detailed information about the pathway of Aβ production and the locus of its cytotoxic action.[10–13]

Recently, the discovery of various soluble amyloid oligomers having a common structure, independent of their location in extracellular or intracellular compartments, brought new insight into possible mechanisms of toxicity.[10] The inhibition of their toxicity by a common oligomer-specific antibody, clearly indicates a common mechanism of toxicity for these different oligomers. This clearly argues against a specific mechanism for one type of amyloid pool but favors a common mechanism in cell parts which are accessible by extra- and intracellular regions, such as cell membranes.[10,14–19] Various studies have already shown the enhancing effects of neuronal lipid membranes on Aβ-conversion into toxic oligomers,[14–36] and the amphipathic character of Aβ peptides, inherited from its precursor protein (APP), makes them ideal targets for membrane-associated toxic events. The predominantly found $A\beta_{1-40}$ and $A\beta_{1-40}$ fragments contain an hydrophilic, extracellular (residues 1–28) and an hydrophobic, transmembrane domain (residues 29–40/42) and are derived from its precursor protein APP, a highly conserved type I integral membrane protein with a single transmembane domain, which is found in many neuronal membranes.[14,15] Taken together, with the perturbation in lipid composition observed in many AD patients,[16–18] interactions between Aβ and cellular membranes might be crucial in the onset and development of AD.[19–36] Possible mechanism of Aβ action range from distortion of the physiological properties of membranes,[16,20,22,27,29,32] membrane-mediated triggering of neuronal apoptotic cell death,[24] lipid peroxidation,[26] to formation of calcium-permeable membrane ion-channels.[23,25] Also membrane components, such as anionic lipids, gangliosides or cholesterol were shown to be involved at various stages of Aβ aggregation,[27–36] and raft-like neuronal membranes seem to play a significant role in the regulation of Aβ production and its cytotoxic products.[14,15,31,34,35] Nevertheless, how Aβ peptides exert their toxic action is still an unsolved matter.

In healthy people the production of Aβ peptides from APP occurs without toxic effects, therefore the question arises, what changes occur in the production of Aβ and its membrane environment which turn this non-toxic peptide into a toxic agent associated with AD? Normally, a fraction of the total Aβ population is released as monomer in solution and is constantly removed in healthy persons, while another Aβ fraction is thought to remain in a membrane-bound form, prevented from aggregation by surrounding lipids.[24] Recently it has been proposed that soluble Aβ might reinsert into the membrane *via* its conversion from a toxic β-sheet structure to a non-toxic α-helical form, thus providing a possible mechanism to protect against aggregation.[16,36] However, how does the nature of interplay between Aβ peptides and membranes control the fate of lipid-bound Aβ, either to be protected by a lipid environment, or to accumulate into β-structured toxic fibrils and/or ion-channels?[23–25,27]

Here, we report, how the transmembrane location of the amphipathic $A\beta_{1-40}$ peptide, as one of the main major toxic AD components, but with a lower aggregation propensity than the $A\beta_{1-42}$ fragment,[9,10] prior to any release, might prevent its aggregation into toxic protofibrils, since lipids surround Aβ monomers. However, $A\beta_{1-40}$ once released as a soluble monomer has a drastically increased potency of accelerated aggregation into toxic aggregates upon contact with specific (possibly pathological altered) membrane surfaces. We have exploited ^{31}P MAS NMR and CD spectroscopy to ascertain how the initial form of non-aggregated $A\beta_{1-40}$ peptide, either free or membrane bound, effects the final fate of the peptide in the presence of membranes and discuss implications for development of AD. Our results demonstrate that acidic lipids can anchor $A\beta_{1-40}$ *via* its transmembrane C terminus in its presumably native helical structure in membranes, while the same lipid components induce accelerated aggregation of Aβ *via* direct membrane surface interactions. In both cases hydrophobic and electrostatic forces play important roles in the functional organization of these two different types of Aβ–membrane complexes involved in AD.

Results

^{31}P MAS NMR on membranes: $A\beta_{1-40}$ incorporation *versus* surface association

High resolution, non-perturbing solid state ^{31}P

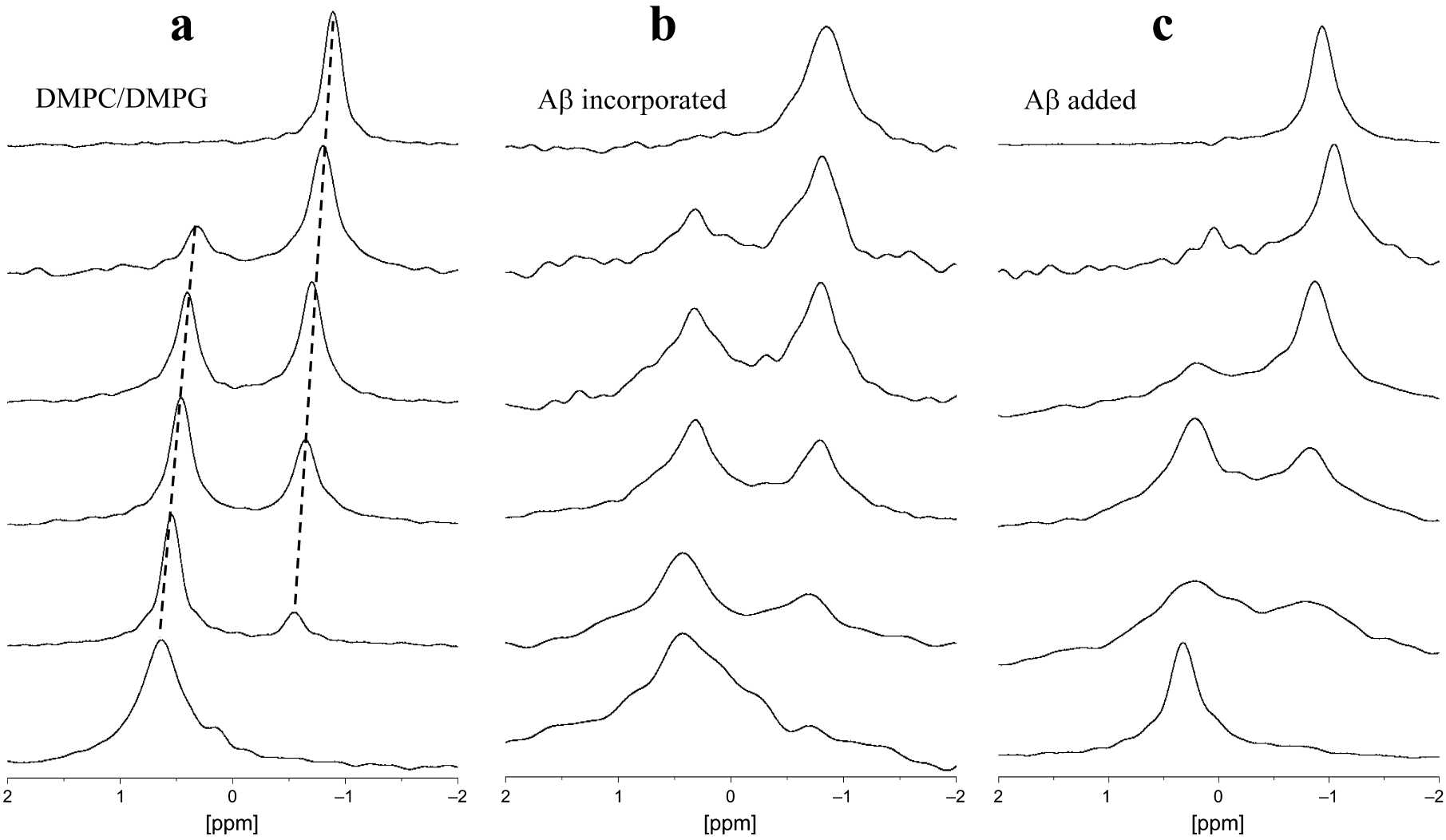

Figure 1. Phosphorus-31 MAS NMR spectra obtained at 308 K and 2 kHz spinning speed of multilamellar DMPC/DMPG vesicles before (a), upon incorporation (b) and after addition (c) of $A\beta_{1-40}$ peptide at 30:1 lipid/peptide molar ratio. The negative membrane surface charge was varied by progressively increasing the content of DMPG to DMPC bilayers in molar fraction steps of 0.2 starting with $x = 0$ (upper spectra) to $x = 1$ (lowest spectra).

MAS NMR spectroscopy was used to obtain, at membrane interfaces, molecular insight into nature and specificity of interactions of lipid membranes with $A\beta_{1-40}$ peptide. In general, phosphorous-31 NMR spectra can be interpreted to provide information regarding the local electrostatic environment for each lipid species at membrane interfaces.[37–40] In Figure 1(a) typical ^{31}P MAS NMR spectra are shown for multilamellar DMPC/DMPG vesicles with varying surface potentials. Interactions of these vesicles with $A\beta_{1-40}$ were studied by acquiring ^{31}P MAS NMR spectra as a function of phospholipid composition upon peptide incorporation (b) and upon surface binding (c). Under MAS conditions high resolution-like NMR spectra are obtained permitting the separate detection of both lipid components.[40] The narrow linewidths reflect the high fluidity of these membranes at 308 K in their liquid crystalline phase.[37] The relevant isotropic ^{31}P NMR chemical shift values are −0.9 ppm for pure DMPC bilayers and 0.62 ppm for pure DMPG membranes. As seen in the NMR spectra (Figure 1(a), broken lines) both DMPC and DMPG resonance lines move systematically downfield as the fraction of negatively charged lipid is increased. As visualized in Figure 2(a) and (b), where the chemical shift values for both lipids are plotted as a function of the fraction of charged DMPG lipids, there is a common response of the phospholipid head groups to the electrostatic potential, present at the membrane surface.

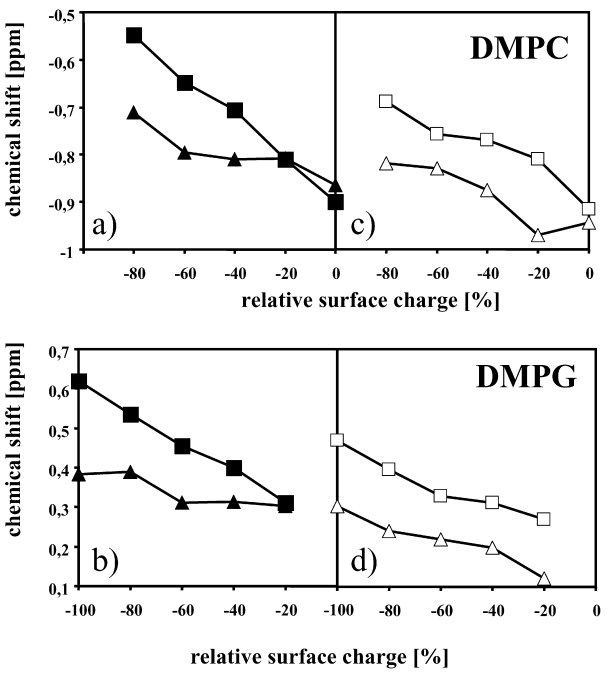

Figure 2. Isotropic ^{31}P chemical shift values (±0.05 ppm) obtained by ^{31}P MAS NMR at 308 K and 2 kHz spinning speed for DMPC (top) and DMPG (bottom) lipid component in DMPC/DMPG vesicles of different lipid molar ratios: before (filled squares) and after incorporation of $A\beta_{1-40}$ peptide (filled triangles) at 150 mM ionic strength: (a) and (b); and before (open squares) and upon binding of $A\beta_{1-40}$ peptide (open triangles) at 10 mM ionic strength (c) and (d). The lipid/peptide molar ratio was 30:1.

Incorporation of $A\beta_{1-40}$ peptide at a 30:1 lipid/peptide molar ratio into the various DMPC/DMPG vesicles resulted in no significant changes compared to the pure bilayer systems and no specific interactions between the lipid components and the peptide were observed (as seen in Figure 1(b)). The NMR linewidths are slightly increased, typical for broadening effects caused by a small restriction of motional freedom of the lipid head groups upon interaction with $A\beta_{1-40}$.[37] Both lipids show similar tendencies in their chemical shift variations (Figure 2(a) and (b)). However, the monitored changes are less pronounced as seen in the reduced slopes of both respective curves when compared to the values obtained for the peptide-free bilayers. In DMPC membranes containing none or only 20 mol% DMPG, the observed NMR chemical shift values are nearly unchanged. However, at increasing fractions of DMPG lipids (>20 mol%), the lipid head groups show a weaker response to an increase in fraction of negatively charged lipids upon $A\beta_{1-40}$ incorporation, as seen in the reduced slopes in Figure 2(a) and (b), when compared to peptide-free DMPC/DMPG systems.

To investigate the mainly electrostatic nature of the association of monomeric $A\beta_{1-40}$ with charged membrane surfaces, the peptide was added to the various DMPC/DMPG vesicles at a 30:1 lipid/peptide molar ratio, using a buffer of low ionic strength. The response of lipid head groups in pure DMPC/DMPG bilayers to variations in surface charge follows the same pattern as in the presence of high salt concentration, but is less pronounced.[38] The ^{31}P MAS NMR spectra obtained upon the binding of $A\beta_{1-40}$ peptide to the bilayers (Figure 1(c)) show two clear and distinct features. First, the chemical shift values for the lipid components change in the same relative way; both to each other and to the relative values obtained for the peptide-free systems (see Figure 2(c) and (d)), as the variation of chemical shift as a function of DMPG concentration is similar for all four curves. Second, upon association to the membrane surface, $A\beta_{1-40}$ induces a systematic upfield perturbation in chemical shift values for all lipid resonances in a way identical with a lowering of the amount of negatively charged lipid. This effect reflects a partial charge screening between negative lipid head groups and positive amino acids of the peptide, as shown previously for various systems.[38–42] The only exception are the NMR spectra of neutral DMPC lipids where no electrostatic binding (no chemical shift change) is observed upon addition of $A\beta_{1-40}$, in agreement with earlier studies.[22,27,28]

CD spectroscopy of $A\beta_{1-40}$ upon interaction with DMPC/DMPG vesicles

To correlate lipid–peptide interactions observed

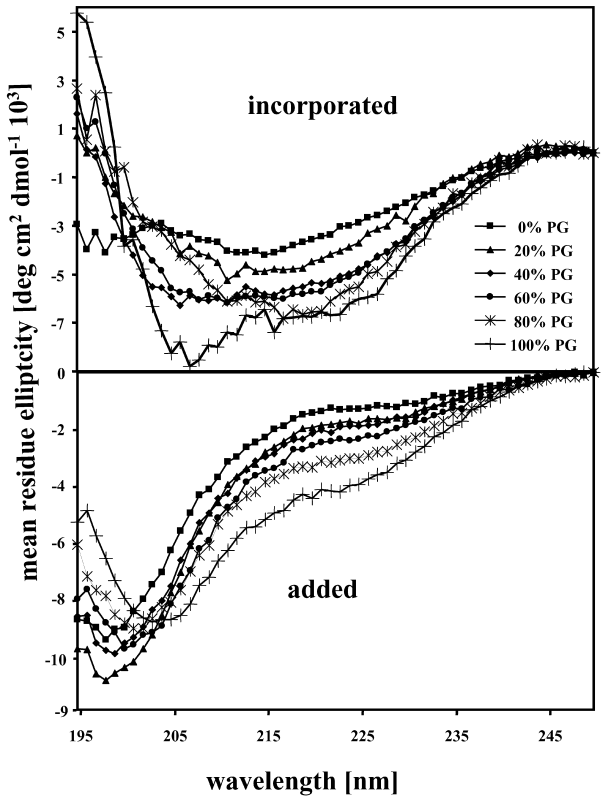

Figure 3. CD-spectra of $A\beta_{1-40}$ peptide interacting with vesicles made of different molar ratios of DMPC/DMPG lipids as indicated. Spectra were obtained at 298 K and correspond to a lipid/peptide molar ratio of 30:1. $A\beta_{1-40}$ incorporated into DMPC/DMPG bilayers as described (top). $A\beta_{1-40}$ added to DMPC/DMPG bilayers (bottom). See the text for details.

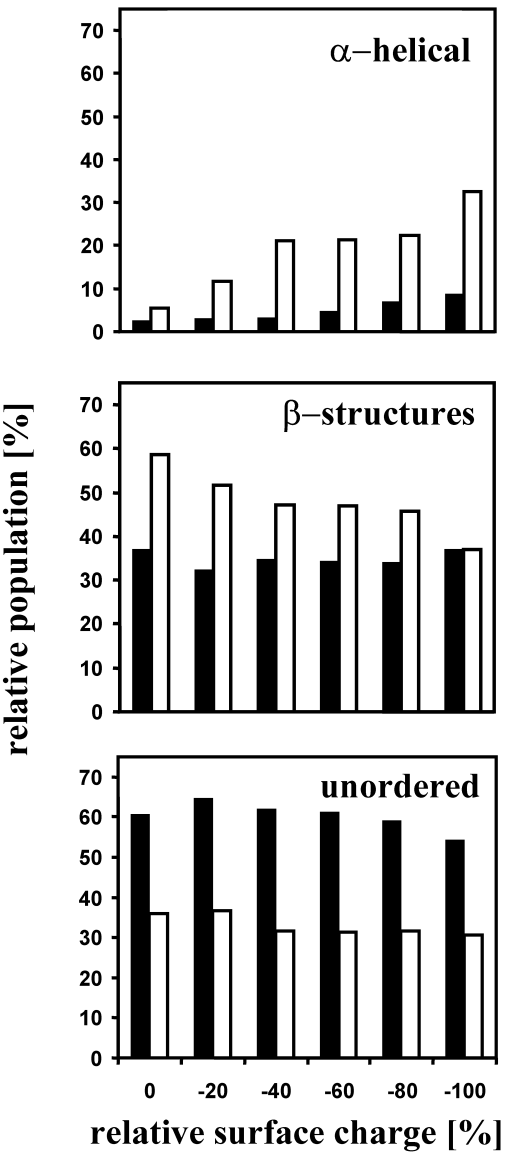

Figure 4. Lipid modulated conformational changes of $A\beta_{1-40}$ interacting with DMPC/DMPG vesicles of various composition (expressed as mol% of DMPG in DMPC bilayers). The relative percentage (±3%) of the various secondary structural elements are shown for peptide incorporated into membranes (open bars) and added to membrane surfaces (filled bars). CD spectra of Figure 3 were deconvoluted with CDPro (see the text for details).

by ^{31}P MAS NMR spectroscopy with the structural properties of $A\beta_{1-40}$ peptide, comparative CD experiments were carried out on samples as used in the NMR studies. $A\beta_{1-40}$ was either incorporated into (Figure 3, top) or added (Figure 3, bottom) to DMPC membranes with varying fraction of negatively charged DMPG. CD spectra are displayed in the spectral region between 195–250 nm. In this region the CD signal reflects the basic secondary structural features of peptides.[28,32,43,44] The spectra obtained for $A\beta_{1-40}$ incorporated into membranes reveal broad minima between 205 nm and 225 nm, and reflect major helical and β-sheet features in the peptide. The CD spectrum obtained for $A\beta_{1-40}$ embedded into neutral DMPC membranes, reveals a minimum around 215 nm indicating a higher β-sheet content. However, when gradually increasing the amount of negatively charged lipids, this minimum flattens out and the regions around 208 nm and 222 nm show more pronounced negative ellipticity values, typical for α-helix, this being most pronounced in the system containing 100% charged DMPG lipids, where a minimum around 205 nm can be detected. In general, a clear tendency can be seen in these CD spectra, where increasing the fraction of charged lipids in the bilayers is correlated with increased helical features, as confirmed by our analysis of the CD spectra using a suitable computer algorithm.[43,44] The results from these analyses are summarized in Figure 4 (open bars) and show a dramatic increase in the α-helical population from around 5% for neutral membranes to over 30% for negatively charged DMPG vesicles. The tendency for the population of β-structures reveals a reversed tendency, with an observed reduction from nearly 60% to around 37%.

For monomeric $A\beta_{1-40}$ peptide bound to the surface of vesicles of the same composition as before, a quite different picture is seen in the CD experiments, when compared to the reconstituted case. When monomeric $A\beta_{1-40}$ was added to a dispersion of pure DMPC vesicles (see Figure 3, bottom), the CD spectra of the peptide observes a minimum at 197 nm, typical of a disordered structure similar to the ones obtained for monomeric $A\beta_{1-40}$ freshly suspended in buffer solutions.[28,32] This is not surprising, since neutral membranes do not bind monomeric $A\beta_{1-40}$ under these conditions, as shown in various studies,[21,28,32] and confirmed by the unchanged ^{31}P chemical shift values for DMPC vesicles upon addition of peptide (see Figure 2(c)). However, two tendencies can be seen in these CD spectra upon increasing the concentration of negatively charged DMPG. Firstly, the spectral minimum observed around 197 nm for $A\beta_{1-40}$ bound to the vesicles moves gradually to higher wavelengths upon increasing the fraction of charged lipids. Secondly, the increase of negative surface charge density of the vesicles correlates with an increase in negative ellipticity in the region between 210 nm and 225 nm. This behavior indicates a growing presence of β-sheet/helical structures in $A\beta_{1-40}$ in contrast to the mainly random coil features observed for the non-vesicle associated state. There is a systematic change in the populations of the various secondary structures as a function of the membrane surface potential (see Figure 4, filled bars). The helical content increases steadily from below 5% in membranes containing DMPG lipids at 0.2 molar fraction, to nearly 10% for pure DMPG vesicles. The values for the β-sheet populations increase slightly from 32% to around 37%.

$A\beta_{1-40}$ assembly kinetics: incorporation *versus* surface association

Finally we performed temporal aggregation trials to address the question if transmembrane anchored Aβ-peptide is protected from aggregation, but surface-bound Aβ peptide not. We conducted for both different Aβ–lipid scenarios time-dependent CD studies to monitor the timescale of the conformational changes of Aβ-peptide into β-structured aggregates. For this purpose, $A\beta_{1-40}$ was therefore incorporated or added to DMPC/DMPG vesicles (2:1 molar ratio) at a 30:1 lipid/peptide ratio and the ongoing conformational changes monitored *via* CD spectroscopy over a period of several weeks. For comparison, $A\beta_{1-40}$ was also incorporated into SDS micelles, as used in solution NMR spectroscopy of the structure determination of Aβ peptides in membrane mimicking environments.[45,46] As seen in Figure 5, the CD spectra reveal no significant changes in the structural features of $A\beta_{1-40}$ upon incorporation over a period of several weeks. The same kinetic stability is also seen for $A\beta_{1-40}$ when embedded into SDS micelles. However, the secondary structure is moderately altered when compared to peptide inserted into lipid bilayers. While for incorporated $A\beta_{1-40}$ peptide into vesicles or SDS micelles, no major temporal structural rearrangements occur, dramatic structural changes are observed for surface-associated peptide (as shown in Figure 6). Immediately upon addition of $A\beta_{1-40}$ to vesicles, the peptide displays a mainly disordered structure. However, over a period of 18 days $A\beta_{1-40}$ exhibits major time-dependent conformational changes, resulting in a predominantly β-structural state following prolonged contact with charged lipid vesicles, where any effect through metal ions could be excluded due to the presence of EDTA. Analysis of the CD spectra by the CDPRO algorithm combined with a suitable reference set for disordered proteins, enabled us to monitor the time-course of all secondary structural elements simultaneously.[43,44] As seen in Figure 6 (bottom), following a typical lag-time of 13 days, major conformational changes occur, with the population of disordered structures drastically reduced (from 70% to less than 30%) while the amount of β-structures steeply increased, starting from 25% to over 60%. The α-helix content of the $A\beta_{1-40}$ peptide remains nearly unchanged, indicating a direct conversion from a random coil to a β-sheet conformation in the presence of a charged membrane surface. The fact, that $A\beta_{1-40}$ in solution displays a highly transient α-helical population as observed by Kirkitadze *et al.*,[9] suggests, that the peptide follows a different pathway in an potentially available three-dimensional aggregation space, compared to the situation on a two-dimensional membrane surface.

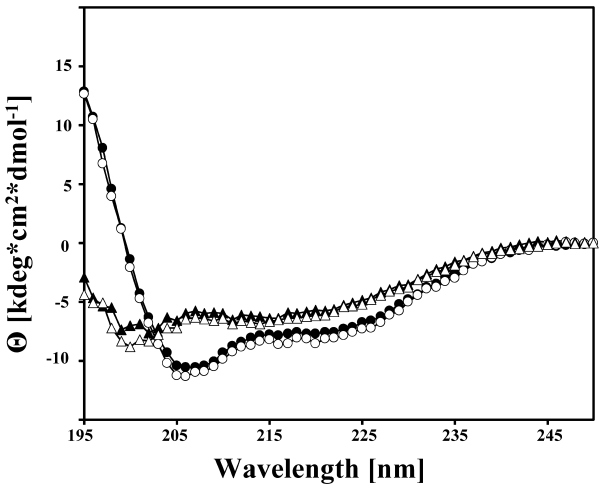

Figure 5. Time-dependence of the secondary structure of $A\beta_{1-40}$ peptide upon incorporation into DMPC/DMPG (2:1 molar ratio) bilayers at 30:1 lipid/peptide molar ratio (triangles), and solubilized into SDS micelles at 400:1 molar ratio (circles). CD spectra were obtained at 298 K after 0 day (filled) and after eight weeks (open). See the text for details.

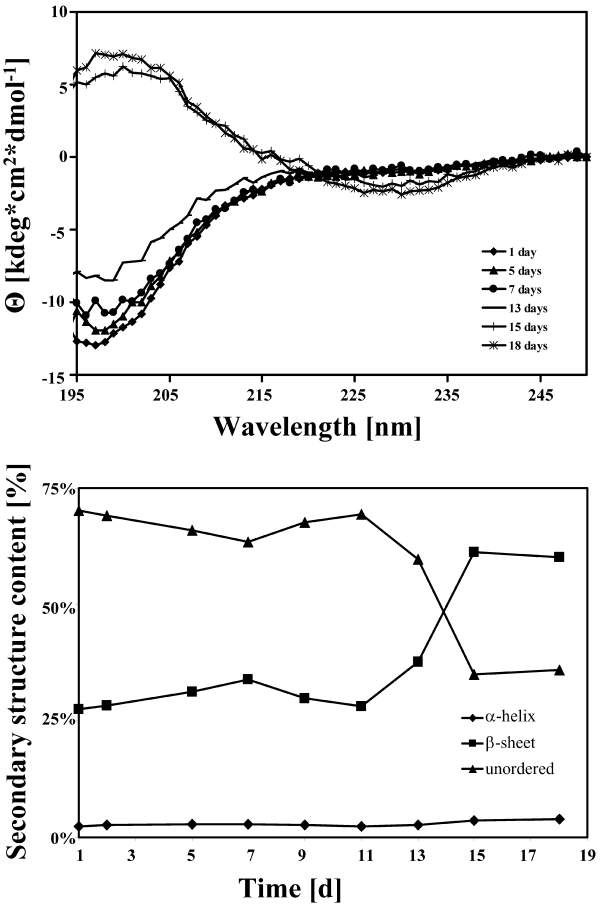

Figure 6. Time-dependent secondary structure changes of $A\beta_{1-40}$ peptide upon addition to small unilamellar vesicles composed of DMPC/DMPG (2:1) molar ratio. CD spectra were obtained at 298 K for a lipid/peptide ratio of 30:1 molar ratio (top). Spectra were acquired over several weeks (days of measurements are indicated). CD spectra were deconvoluted by the CDPro analysis package. The fractions of the various secondary structural populations of $A\beta_{1-40}$ are plotted as a function of time (bottom).

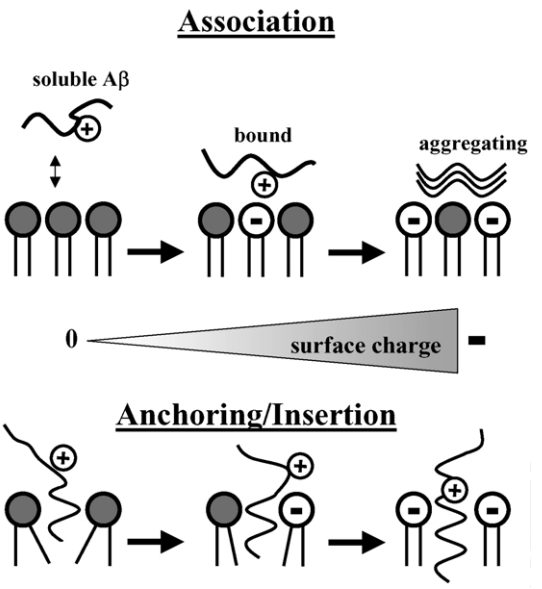

Figure 7. Schematic model for $A\beta_{1-40}$ peptide interactions with membranes. Top: Electrostatic adsorption to membrane surfaces. When the membrane exhibits a negative surface potential, $A\beta_{1-40}$ binds electrostatically. Increasing the presence of acidic lipid causes a significant increase in concentration of surface-associated peptide compared to bulk concentration, causing an accelerated conversion into β-sheet-like aggregates. Bottom: Insertion of $A\beta_{1-40}$ peptide into membranes. At neutral bilayers only a short part of the hydrophobic segment is inserted, since the charged hydrophilic part is stabilized in the bulk solution. Increasing the surface potential induces electrostatic anchoring of charged residues close to the surface thereby supporting an increased insertion of the hydrophobic segment into the membrane core in an α-helical conformation induced by the hydrophobic effect.

Discussion

In general, Aβ peptide (40 and 42 variant) is generated upon sequential action of β and γ-secretases on transmembrane APP, situated either at plasma or cellular membranes.[2,10,11] Once, Aβ is formed it can pursue several possible pathways. It can be released as soluble, non-toxic monomer into the aqueous environment by adopting a mainly random coil conformation, and then either be removed (most likely in healthy persons) or aggregate at higher concentrations in AD patients.[4–8] However, the amphipathic Aβ peptide in solution can also interact again with extra- or intracellular membranes,[8,27] and either aggregate on membrane surfaces,[19,28–31] or reinsert.[16,36] Finally, a fraction of Aβ peptide might even be retained in the membrane upon its formation.[19,23,24] This last scenario is likely, since the γ-secretase activity occurs within the membrane, and the C-terminal part of Aβ is very hydrophobic in its presumably native, transmembrane helical structure as shown here; ideally to promote interaction with the hydrophobic membrane core;[15,19–21,24,33–36] but which factors determine the fate of Aβ peptides upon generation?

As shown above, there are fundamentally different scenarios of Aβ-membrane assemblies: Aβ peptide can either be firmly anchored in a membrane, thereby hindering release and aggregation (causing potential ion-channel activity; see below), or it can have a fundamentally adverse effect when bound to membrane surfaces by undergoing accelerated aggregation followed by neuronal apoptotic cell death.[5,24] We elucidated on a molecular level the differences in Aβ–lipid interactions found in the transmembrane anchoring/insertion *versus* membrane surface association; both potentially significant in the pathophysiology of AD. Using a combination of ^{31}P MAS NMR to monitor the electrostatic environment at the lipid head group region, and CD spectroscopy to probe the secondary structure of the peptide we have been able to distinguish differences in the nature of the interactions between peptide and lipid surface

and understand how these interactions can alter the organization of the peptide in the presence of neuronal membranes, giving an insight into the pathological impacts on AD patients. Our proposed molecular interaction models for both assemblies are visualized in Figure 7: the transmembrane anchored Aβ-type (bottom) *versus* the surface bound Aβ-type (top).

Clearly the mechanism by which Aβ interacts with lipids is different in both scenarios, with hydrophobic and electrostatic forces contributing differently in each case. However, CD spectroscopy is not able to estimate the relative contributions of electrostatic and hydrophobic forces. Therefore, we used ^{31}P MAS NMR techniques to obtain on a molecular level an electrostatic description from the perspective of the membrane interface for each lipid component, due to the inherent sensitivity of the phosphorous nucleus to changes in its local electrostatic environment.[38–41] As seen in the experiments, where the amount of negative charged lipid compounds was systematically varied, the electrostatic potential present at membrane surfaces plays a crucial role for both types of Aβ–lipid assemblies. In aqueous solution, $A\beta_{1-40}$ can bind to negatively charged membrane surfaces *via* electrostatic attraction of its basic residues. Therefore, the $A\beta_{1-40}$ surface concentration is distinctively larger than in solution, as reflected in the relevant binding constants.[47] Surface associated Aβ assemblies already display a significant population of β-structures (Figure 4), and therefore are prone to accelerated aggregation due to the high local Aβ concentration resulting from the two-dimensional nature of their surface association (as demonstrated in Figure 6). If negative surface potential increases, more peptide can be bound to the surface electrostatically causing even faster aggregation, as observed.[15,22,27–32] The results of the ^{31}P MAS NMR experiments clearly support the importance of electrostatic interactions in this process. The isotropic ^{31}P chemical shift values as a function of membrane surface potential change upon binding of monomeric $A\beta_{1-40}$ (Figure 2(c) and (d)), but their slope is nearly unchanged, indicating a simple partial electrostatic compensation mechanism. At pH 7.8, only an arginine residue at position 5 and two lysine groups at position 16 and 28, remain positively charged and are able to exert electrostatic binding. This indicates a simple location of monomeric peptide with its charged residues above the membrane interface region, as suggested by various groups,[28,29,32] and seen for systems such as pentalysine,[38] myelin basic peptide,[48] or polymyxin.[49] The loss of electrostatic binding at high salt concentrations supports an initial electrostatic binding process.[28,50] CD and fluorescence studies using negatively charged gangliosides reveal major structural changes in $A\beta_{1-40}$ peptide at high salt concentrations, which does not contradict the present findings.[29–31] Our model lipid for inducing negative bilayer charges, DMPG, contains its negatively charged group between the electrostatic diffuse double layer (charge effect screenable by NaCl presence) and the lipid interior, while the charged parts of the ganglioside head groups reach far beyond this electrostatic boundary as shown by McLaughlin *et al*.[51]

For $A\beta_{1-40}$ incorporated into membranes a different situation occurs. Negatively charged lipids stabilize the transmembrane C-terminal part (residues 29–40) in an α-helical form, most likely inherited from it precursor APP, as hydrophobicity plots and insertion studies suggest.[16,33,36,45] Co-solubilization of $A\beta_{1-40}$ with lipids using organic solvents, followed by rehydration in the appropriate buffer ensured a most likely transmembrane, monomeric incorporation of Aβ into lipid bilayers in our studies.[33,49–50] CD studies revealed a conformational change into partially α-helical structures upon membrane incorporation (Figures 3 and 4). Increasing the amount of acidic lipids in the membrane matrix results in a deeper insertion of the transmembrane part of $A\beta_{1-40}$ into the hydrophobic core, correlated with an increasing α-helical fraction (see Figure 4), in its more stable helical conformation. This is also reflected in the observed ^{31}P chemical shift values in Figure 2(a) and (b), where the change in slope indicates the presence of hydrophobic interactions in this process. Since only a lower fraction of α-helical features for $A\beta_{1-40}$ in neutral or only 20% charged membranes was seen in the CD spectra, a partial burial of the hydrophobic part of $A\beta_{1-40}$ in membranes of low surface charge can be assumed. Then, Lys28 would be far away from the DMPG head group and its charge would be neutralized by the anions present in solution at high ionic strength (shown schematically in Figure 7), explaining the nearly unchanged ^{31}P chemical shift values seen in Figure 2(b). Only at a high surface charge density does the hydrophobic domain appear to be completely buried between the lipid acyl chains, reflected in a higher α-helical content; and significant changes in the ^{31}P chemical shift values which (presumably) arise from electrostatic coupling of Lys28 with the negatively charged lipid phosphate group (see Figure 7). This model of a molecular interaction mechanism developed by us, is also supported by other studies, ranging from NMR studies of membrane inserted $A\beta_{1-40}$, labeled in its transmembrane region,[33] Aβ/micelle systems,[45] or a membrane mimicking system.[46]

In general, Aβ aggregate formation might be induced by the high amyloidogenic potential of the hydrophobic C-terminal domain. This part is the driving force behind the transition from a nontoxic soluble random coil structured peptide to insoluble β-structured fibrils. The hydrophobicity of this C terminus should normally prevent insertion into a bilayer because of the repulsive forces present at the hydrophilic interface of membranes. But as various studies show, Aβ can reinsert into bilayers under the influence of pH, metal ion, membrane composition, conformational

state etc.[16,23–25,36] Therefore, there must be two completely different types of Aβ-membrane assemblies, transmembrane location *versus* surface location, to be involved in processes of Aβ-release from membranes or reinsertion. For both assemblies hydrophobic and electrostatic forces are important, but both contribute differently to both types of lipid–peptide complex, as shown here. For example, the presence of acidic lipid in our studies demonstrates that electrostatic forces can keep the peptide in a transmembrane position, since they couple hydrophilic extracellular part of Aβ to the membrane *via* Aβ's positively charged residues. Other studies used the effect of metal ions for a change in pH or an increase in membrane hydrophobicity to induce changes in the balance between hydrophobic/electrostatic properties, changes which enable Aβ to pass with its hydrophobic C-terminal part the hydrophilic membrane interface barrier during reinsertion.[16,36] Connected with this reinsertion process is a transition from a disordered or β-sheet structure into an α-helical form which allows hydrophobic interactions between the transmembrane, helical Aβ part and the lipid fatty acids. The conformational change probably occurs at the membrane interface to overcome this barrier during reinsertion, as we assume based on our studies here.

However, what happens to monomeric Aβ upon formation? Does it leave its native membrane matrix or does it reinsert again? If it leaves without any reinsertion, toxic aggregates can be produced at neuronal membrane surfaces. Alternatively, Aβ peptides can insert or remain in membranes and produce toxic ion-channels built of Aβ subunits, as indicated by recent atomic force microscopy studies.[23,25] Perhaps, an increased production of Aβ in AD patients might in combination with their altered lipid composition, be responsible for the presence of a significant fraction of these cation-active oligomeric channels, causing neuronal cell death or neuronal dysfunction;[10,23,25] but at low concentrations, as found in healthy people, Aβ does not form these channels, but instead it is catabolized before any toxic action can occur.

Materials and Methods

Materials

DMPC, DMPG were obtained from Sigma (UK). Amyloid-β_{1-40} peptide (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV) was synthesized by standard solid-phase FMOC chemistry, subsequently purified by HPLC and found to be over 90% pure by MALDI-TOF mass spectroscopy.[33] To obtain the monomeric form of the Aβ peptide, 10 mg were dissolved in 500 μl of trifluoroacetic acid (TFA) to break up any aggregates which remain after HPLC purification.[33,52] After removal of TFA under a stream of nitrogen gas the peptide film was resuspended twice in 5 ml of TFE (trifluoroethanol) and dried under high vacuum to remove trace solvent.

Sample preparation

For experiments where Aβ was added to lipid vesicles of various composition, the peptide film was resuspended under vigorous stirring for ten minutes in buffer A (10 mM Tris, 10 mM KCl, 0.5 mM EDTA (pH 7.8)) at a final concentration of 100 μM. Multilamellar vesicles of DMPC and DMPG were prepared by co-solubilizing the desired DMPC/DMPG lipid ratios in $CHCl_3/CH_3OH$ (3:1, v/v) followed by removal of the solvent under high vacuum for five hours. Buffer A was added to each lipid mixture (3 mM concentration), followed by resuspension of the film under extensive vortexing. Finally, the peptide solution was added to multilamellar vesicle suspensions to give a 30:1 lipid/peptide molar ratio. Following incubation for 30 minutes at 310 K under stirring, followed by three freeze–thaw cycles the samples were pelleted (20,000*g*; 45 minutes at 293 K) and kept frozen prior to NMR and CD experiments. Incorporation of Aβ into DMPC/DMPG bilayers at a lipid/peptide molar ratio of 30:1 was carried out as described before for amphipathic peptides.[33,52] Briefly, the Aβ peptide film was dissolved in TFA (0.5 ml) and added to lipid mixtures in $CHCl_3/CH_3OH$ (3:1, v/v), followed by rotary evaporation and drying overnight under high vacuum. Subsequently, the peptide/lipid films were resuspended in buffer B (buffer A plus 140 mM NaCl), followed by three freeze cycles and density gradient centrifugation to confirm peptide incorporation and sample homogeneity. The lipid/peptide molar ratio in all samples was determined as 30:1(±10)%. Finally, pelleted samples were kept frozen prior to NMR studies. For incorporation of $A\beta_{1-40}$ into SDS micelles, 25 μM peptide was solubilized in 100 mM SDS solution in buffer A.

Solid state NMR measurements

^{31}P MAS NMR experiments were carried out on an Infinity spectrometer (Chemagnetics, USA) at a proton frequency of 100 MHz using 7 mm double resonance MAS probe (Bruker, Germany). ^{31}P MAS NMR spectra were acquired under proton decoupling (40 kHz) using a single phosphorus-31 $\pi/2$ pulse with 5 μs duration. For static ^{31}P NMR measurements a Hahn echo pulse sequence was applied with an interpulse delay of 50 μs (spectra not shown, but used to determine the bilayer phase at 308 K). Between 1000 and 4000 transients were collected for each spectrum, with a repetition period of three seconds. All ^{31}P NMR spectra were referenced externally to 0 ppm for 10% H_3PO_4 at room temperature. Data were transformed with 2 Hz exponential multiplication.

Circular dichroism experiments and analysis

All samples were sonicated under cooling for five minutes using a probe-type sonicator and any metal debris removed by centrifugation (15,000*g* for ten minutes, room temperature). CD-spectra (Jasco J-720 spectropolarimeter, USA) were obtained between 190 nm and 250 nm using a 1 mm path-length quartz cell (Hellma, Germany). The number of scans varied between four and 32 for each sample, and time-dependent measurements were performed without additional sonication steps at later time-intervals. Finally, the CD spectra were corrected by substracting CD spectra obtained for sonicated lipid mixtures without peptide. To estimate the secondary structure contents of the

Aβ-peptide, an analysis of the relevant CD spectra was carried out using the CDPro software† developed by R. W. Woody and co-workers.[43,44] Due to the disordered structure of Aβ in aqueous solutions as indicated in the CD spectra, an extended reference set of 48 peptides was used, which included various spectra of denatured/disordered systems (basis set 7 in CDPro). The analysis was performed using three methods, CONTIN, CONTIN/LL, and SELCON 3. In general, CONTIN/LL, a self-consistent method with an incorporated variable selection procedure, produced the most reliable results.

Acknowledgements

P. T. F. Williamson, B. Bonev, C. Glaubitz, D. Murray, L. Johansson and G. Lindblom and his group are gratefully acknowledged for all their support. This work was supported by Knut and Alice Wallenberg Foundation, Swedish Research Council (621-2001-3185), Umeå University Biotechnology Fund and BBSRC (43/B11683). A.W. is recipient of a BBSRC Senior Fellowship (43/SF09211).

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Edited by A. R. Fersht

(Received 5 September 2003; received in revised form 22 October 2003; accepted 25 November 2003)