Early Synergy between Aβ42 and Oxidatively Damaged Membranes in Promoting Amyloid Fibril Formation by Aβ40

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Oxidative lipid membrane damage is known to promote the misfolding of Aβ42 into pathological β structure. In fully developed senile plaques of Alzheimer's disease, however, it is the shorter and more soluble amyloid β protein, Aβ40, that predominates. To investigate the role of oxidative membrane damage in the misfolding of Aβ40, we have examined its interaction with supported lipid monolayer membranes using internal reflection infrared spectroscopy. Oxidatively damaged lipids modestly increased Aβ40 accumulation, with adsorption kinetics and a conformation that are distinct from that of Aβ42. In stark contrast, pretreatment of oxidatively damaged monolayer membranes with Aβ42 vigorously promoted Aβ40 accumulation and misfolding. Pretreatment of saturated or undamaged membranes with Aβ42 had no such effect. Parallel studies of lipid bilayer vesicles using a dye binding assay to detect fibril formation and electron microscopy to examine morphology demonstrated that Aβ42 pretreatment of oxidatively damaged membranes promoted the formation of mature Aβ40 amyloid fibrils. We conclude that oxidative membrane damage and Aβ42 act synergistically at an early stage to promote fibril formation by Aβ40. This synergy could be detected within minutes using internal reflection spectroscopy, whereas a dye-binding assay required several days and much higher protein concentrations to demonstrate this synergy.

Extracellular amyloid deposits known as senile or neuritic amyloid plaques are one of the defining histopathological features of Alzheimer's disease (AD). The core of a neuritic plaque is a compact heterogeneous meshwork in which fibrillar forms of amyloid β (Aβ) proteins predominate. The best characterized Aβ proteins are 40 and 42 residues in length, varying at their COOH terminus. The 40-residue Aβ protein (Aβ40) and various amino- and carboxyl-terminal derivatives appear to be the predominant Aβ protein in the amyloid plaques of AD (1), in cerebrovascular amyloid deposits (2), and in at least some forms of hereditary amyloidosis (3). The 42-residue Aβ protein (Aβ42) appears to associate with GM1 ganglioside and is the predominant Aβ species in diffuse plaques (2, 4–7). Because of its prevalence in early appearing lesions, its lower solubility, and its tendency to form and stimulate the formation of fibrillar aggregates, it has been proposed that Aβ42 serves to nucleate amyloid plaque formation (8–11).

A model of this process appears to occur in solution, wherein a nucleation-dependent step in the process of fibril formation from Aβ proteins has been observed (8, 10). This prompts one to look for conditions that promote nucleation by serving as “surrogate nuclei” or “folding templates.” Strategies to design synthetic templates for β sheet formation have been explored (12, 13). Such templates need not be large, inasmuch as some rather small ligands are known to promote fibril formation (14).

The possibility that lipid membranes might serve as templates or nucleation sites for Aβ proteins was suggested nearly a decade ago (8), and an abundance of circumstantial evidence has appeared in the interim suggesting that lipid membranes may play an important role in the pathogenesis of AD. For example, the Aβ proteins that aggregate into fibrils, toxic or otherwise, are derived from amyloid precursor protein (APP), a membrane-anchored protein. Following its cleavage, Aβ proteins are associated with detergent-resistant lipid membrane domains in the brain (15), specific lipid components (5), or even its membrane-anchored parent, APP (16). Lipid membranes of varying composition strongly influence the rate at which Aβ proteins form fibrils (17–20). Ultrastructural studies suggest that fibril formation tends to occur first in portions of diffusible deposits that are closest to membranes (21–23), and that intermediate forms give rise to pore-like assemblies within membranes (24).

We have previously observed that oxidatively damaged lipid membranes are much more effective than ordinary membranes at adsorbing Aβ proteins, and at misfolding them into pathological β structure (25). This observation brings to mind the associations previously noted between oxidative stress, metal ions, and the toxicity of Aβ or APP (26, 27), and indirect measures of lipid peroxidation that have more recently showed that oxidative stress correlates with amyloid plaque formation in animal models (28), and with disease severity in humans (29). It is also noteworthy that vitamin E, a lipophilic antioxidant, is relatively deficient in the post-mortem brain tissue of patients of Alzheimer’s disease (30), and its administration in pharmacological doses appears to slow the clinical progression of disease (31).

We have now employed attenuated total internal reflection-Fourier transform infrared (ATIR-FTIR) spectroscopy to inves-
tigate further into the role of membranes and oxidative damage in the pathological misfolding of Aβ proteins. ATIR-FTIR is a powerful and increasingly popular technique for studying proteins, lipid membranes, and protein-lipid interactions (32–36). It is an especially powerful technique for the study of polypeptides that form β structure because they exhibit a distinctive “splitting” of their amide I absorption. It may not be clear whether this β structure is parallel or antiparallel in mature amyloid fibrils (37), however, this question does not bear on the present analysis. ATIR-FTIR spectra of membrane-bound proteins are collected under conditions in which membrane lipids are highly ordered (as in ordinary lipid bilayers), and in which variables such as lipid composition, buffer composition, surface pressure, and temperature may be controlled. It yields quantitative information about the concentration, conformation, and orientation of various chemical groups on microgram samples. This degree of sensitivity enables the collection of complete spectra several times per minute for the assessment of adsorption kinetics.

These studies reveal much earlier events in fibrillogenesis than do measures of fibril formation based on dye binding, and demonstrate that oxidatively damaged lipid membranes and Aβ42 are potently synergistic in promoting fibril formation by Aβ40.

**EXPERIMENTAL PROCEDURES**

**Materials**—1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phospho-r-serine, and 1-stearoyl-2-arachidonyl-sn-glycero-3-phosphocholine (SAPC) were obtained from Avanti Polar Lipids (Alabaster, AL). All three lipid species were ordered specially packaged in 5–15-mg quantities, under argon, in sealed glass ampules, and packaged in 5–15-mg quantities, under argon, in sealed glass ampules, and packaged in 5–15-mg quantities, under argon, in sealed glass ampules, and packaged in 5–15-mg quantities, under argon, in sealed glass ampules. Aqueous suspensions of pure SAPC in 50 mM Tris buffer, pH 7.4, by bath sonication, and extruded at 25 °C through 100-nm polycarbonate filters to produce unilamellar vesicles. Aliquots of stock Aβ protein solutions in hexafluoro-2-propanol were evaporated and redissolved in phosphate-buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4) to yield concentrations of 0.16 mg/ml for Aβ42 and 0.5 mg/ml for Aβ40. The final concentrations of lipid, Aβ42, and Aβ40 in the aggregation mixture were adjusted to 10 μM, 12 μg/ml, and 48 μg/ml, respectively, and incubated at room temperature with continuous gentle agitation for multiday intervals during which aggregation occurred. 30-μl aliquots of these mixtures were added to 110 μl of 10 μM Congo Red in phosphate-buffered saline and incubated at room temperature for 30 min. Fibril formation was assayed by spectrophotometric measurements of Congo Red binding (48, 49).

**Negative Staining Electron Microscopy**—Fibrillized Aβ40 was adsorbed onto 300-mesh carbon-coated copper grids, stained with 1% aqueous uranyl acetate, and visualized with a JOEL 100CX transmission electron microscope (EM) (Peabody, MA). EM images were captured with a Hamamatsu digital camera (Bridgewater, MA) using AMT software (Danvers, MA).

**RESULTS**

**Individual Proteins on Lipid Monolayer Membranes**—Monolayer membranes of three types were prepared for examination with ATIR-FTIR: saturated (100% DMPC), unoxidized (20% SAPC and 80% DMPC), and oxidized (20% oxidized SAPC and 80% DMPC). The monolayers were formed in a Langmuir trough and applied onto germanium crystals as previously described (46). 8-μg injections of Aβ proteins were made into 6 ml of subphase buffer under the crystal yielding subphase concentrations of ~0.3 μM. Protein accumulation was measured by calculating the total amide I band area in spectra collected over 15-s intervals (a prime mark is added to amide I references when exchangeable hydrogens have been replaced by deuterium).

Aβ40 accumulates to a significantly greater degree on oxidized membranes than on saturated or unoxidized membranes, similar in some respects to the behavior reported previously for Aβ42 (25). However, the accumulation of Aβ40 on oxidized membranes differed from that of Aβ42 in two significant ways. First, Aβ40 accumulation reached a plateau within 15 min and remained at this level for the remainder of the initial 60-min “seeding” period. In contrast, Aβ42 accumulation on the oxidized membrane continued over the initial 60-min seeding period without reaching a plateau (Fig. 1). Second, the ATIR-FTIR spectrum of membrane-adsorbed Aβ40 exhibited a prominent band at 1628 cm−1 but minimal absorption between 1680 and 1690 cm−1. In contrast, the spectrum of membrane-adsorbed Aβ42 exhibited the characteristic split amide I band (Fig. 2, a and b).

**Mixed Proteins on Oxidized Lipid Monolayer Membranes**—After oxidized membranes were exposed to subphase buffers containing either 8 μg of Aβ40 or 8 μg of Aβ42 for 1 h, a series of co-added interferograms using a Bio-Rad FTS-60A spectrometer, a liquid nitrogen-cooled MCT detector, a resolution of 2 cm−1, scanning speed of 20 MHz, triangular apodization, and one level of zero filling. An enclosure around the Langmuir trough is filled with argon to avoid spontaneous air oxidation of lipids at the air-water interface. As is typical for this instrument, non-level baseline correction, water vapor subtraction, and smoothing manipulations were not necessary and spectra are reported “raw” with only flat and level baseline correction. Spectra were fitted using IRfit, a procedure that fits a limited set of component bands simultaneously to several spectra (45). The aim of this analysis is to describe a set of related spectra with a minimum number of adjustable parameters. For the collection of spectra in kinetics mode, the resolution was set to 4 cm−1, the scanning speed was set to 0.1 kHz, and a complete spectrum was recorded every 15 s by coadding 78 interferograms. All spectroscopic studies were performed at 27 °C, i.e. slightly above the phase transition temperature of pure DMPC.

**Vesicle Extrusion and Fibril Assay**—Lipid mixtures consisting of 90% DMPC and 10% of either pure SAPC or oxidized SAPC in chloroform were evaporated, suspended in 30 mM HEPES, pH 7.4, by bath sonication, and extruded at 25 °C through 100-nm polycarbonate filters to produce unilamellar vesicles. Aliquots of stock Aβ protein solutions in hexafluoro-2-propanol were evaporated and redissolved in phosphate-buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4) to yield concentrations of 0.16 mg/ml for Aβ42 and 0.5 mg/ml for Aβ40. The final concentrations of lipid, Aβ42, and Aβ40 in the aggregation mixture were adjusted to 10 μM, 12 μg/ml, and 48 μg/ml, respectively, and incubated at room temperature with continuous gentle agitation for multiday intervals during which aggregation occurred. 30-μl aliquots of these mixtures were added to 110 μl of 10 μM Congo Red in phosphate-buffered saline and incubated at room temperature for 30 min. Fibril formation was assayed by spectrophotometric measurements of Congo Red binding (48, 49).

**Phospholipid Oxidation**—Aqueous suspensions of pure SAPC in 50 mM Tris buffer, pH 7.5, were extruded at 25 °C through 100-nm polycarbonate filters to produce unilamellar vesicles. A 100 μM suspension of these vesicles was oxidized with H2O2 (2 mM) and CuSO4 (200 μM) in 50 mM Tris buffer, pH 7.5, that had been presaturated with nitrogen gas. Peroxidation was monitored by following conjugated diene formation with UV absorption spectrometry at 234 nm (39). Oxidation was terminated by withdrawing aliquots at the prescribed times, adding 75 μl BHT and 75 μl EDTA, followed immediately by vigorous extraction with two volumes of 2:1 (v/v) chloroform/methanol and storage at −80 °C under argon until used. Phospholipid concentrations were determined by phosphate assay (38, 40). Ultraviolet and mass spectrometric characterization of lipids oxidized in this manner has been published elsewhere (41).

**Infrared Spectroscopy of Monolayers**—Lipid monolayers were prepared in a Langmuir trough by applying lipids dissolved in hexane: ethanol to the surface of a buffer containing 30 mM HEPES in D2O at pH 7.5. The lipids applied in this manner consisted of 100% DMPC, a mixture of 20 mol % SAPC (unoxidized), 80 mol % DMPC, and 1 mol % BHT; or a mixture of 20 mol % SAPC (oxidized), 80 mol % DMPC, and 1 mol % BHT. The monolayer formed in the trough at the air-water interface was compressed to a surface pressure of 20 dynes/cm and applied onto a silane-treated germanium crystal as previously described (25, 34, 42–47). The protein was introduced in 8-μg aliquots to a continuously stirred subphase compartment containing 6 ml of buffer. ATIR-FTIR spectra were collected in rapid-scanning mode as 1024 co-added interferograms using a Bio-Rad FTS-60A spectrometer, a liquid nitrogen-cooled MCT detector, a resolution of 2 cm−1, scanning speed of 20 MHz, triangular apodization, and one level of zero filling. An enclosure around the Langmuir trough is filled with argon to avoid spontaneous air oxidation of lipids at the air-water interface. As is typical for this instrument, non-level baseline correction, water vapor subtraction, and smoothing manipulations were not necessary and spectra are reported “raw” with only flat and level baseline correction. Spectra were fitted using IRfit, a procedure that fits a limited set of component bands simultaneously to several spectra (45). The aim of this analysis is to describe a set of related spectra with a minimum number of adjustable parameters. For the collection of spectra in kinetics mode, the resolution was set to 4 cm−1, the scanning speed was set to 0.1 kHz, and a complete spectrum was recorded every 15 s by coadding 78 interferograms. All spectroscopic studies were performed at 27 °C, i.e. slightly above the phase transition temperature of pure DMPC.
injections containing 8 μg of Aβ40 were made into the subphase. In preparations initially exposed only to Aβ40, subsequent injections of Aβ40 up to a total of 40 μg had little effect on the total amount of protein that accumulated (Fig. 1), or on the amide I band shape. Similarly, injections of Aβ42 under oxidized membranes initially seeded with Aβ40 did not result in significant protein accumulation. In preparations initially seeded with Aβ42, however, injections of Aβ40 resulted in markedly increased protein accumulations. Moreover, each injection of Aβ40 raised the integrated absorbance to a new plateau, indicating that membrane binding sites were not saturated, and suggesting that each plateaux represented depletion of protein from the subphase by adsorption to the supported membrane. Protein could not be detected in the subphase with bichinonic acid reagent at these times, however, the starting concentrations of protein were only barely within detection limits.

Control experiments with saturated and unsaturated membranes seeded with Aβ42 yielded results that were indistinguishable from those of the unseeded preparations (data not shown). Other control experiments demonstrate that Aβ40 does not effectively seed Aβ42 accumulation, suggesting that the Aβ40 that does accumulate interferes with the ability of Aβ42 to promote protein accumulation. It should be noted that 1 mol % BHT was present in both the oxidized and oxidized lipid samples and thus, cannot account for the behavior of Aβ40 on oxidatively damaged membranes. As reported previously (25), BHT is added to all samples of unsaturated lipid because the effects of oxidative damage disappear after prolonged exposure of unsaturated monolayers to atmospheric oxygen. We conclude from these observations that oxidative damage and Aβ42 work synergistically to cause the accumulation and misfolding of Aβ40.

The data in our previous report describing the interaction of Aβ42 with oxidatively damaged lipid membranes spanned only 60 min, and could not be extended because of environmental and instrumentation instabilities. The results in this report span continuous 4-h intervals. These much longer observation times were made possible by technical improvements that enhance signal stability and reduce noise, including tightly controlled ambient temperatures in the lab environment, higher output purge gas generators, and an enclosure around the instrumentation that helps stabilize temperatures and exclude water vapor by retaining exhausted purge gas around the outside of the spectrometer.

**Mixed Proteins on Oxidized Lipid Vesicles**—To determine whether the synergy between oxidatively damaged monolayer membranes and Aβ42 could be demonstrated in bilayer membranes, and by more established means, we performed analogous experiments with unilamellar lipid vesicles using a Congo Red binding assay to detect fibril formation. The results shown in Fig. 3 compare unoxidized and oxidized lipid vesicles that were treated either with Aβ40 alone or pre-treated with Aβ42 followed by Aβ40.

When 10 μM Aβ40 was mixed with 10 μM unoxidized lipid in the form of vesicles, there was a marginally detectable amount of Congo Red binding to fibrils after 3 days. There was no significant binding detected when vesicles were composed of oxidized lipid, or when vesicles were not present (data not shown). With unoxidized lipid, pretreatment of lipid vesicles with 2.5 μM Aβ42 for 1 day before adding 10 μM Aβ40 also caused only a marginally significant increase in Congo Red binding after 2 days. We conclude that the conditions of these experiments give rise to minimal, if any, spontaneous fibrillogenesis over a 3-day incubation. These results are consistent with prior reports indicating that the concentration of Aβ40 in our vesicle experiments is only slightly above its solubility limit, whereas the concentration of Aβ42 is well below its solubility limit (9).

In contrast, the use of oxidized lipid and pretreatment with Aβ42...
in this type of experiment caused a marked increase in Congo Red binding after 2 days of incubation (Fig. 3). Examination of this preparation by electron microscopy after 3 days incubation showed that fibrils, 10 nm in diameter and 500–1000 nm long, had formed (Fig. 4).

Vesicles used in these experiments contained 10% unoxidized or oxidized lipid, whereas the monolayer experiments were performed with 20% unoxidized or oxidized lipid. The use of 20% oxidized SAPC was problematic in vesicle experiments because it enhanced fibril formation to such a degree that Aβ40 fibrils formed after 3 days with or without seeding by Aβ42. This suggests that oxidized lipids can promote Aβ40 misfolding in the absence of Aβ42. However, this observation was made at an Aβ40 concentration of 10 μM, i.e. slightly above its thermodynamic solubility limit in pure solution of 9 μM (9). It was not detected in our ATIR-FTIR experiments where the Aβ40 concentration was only 0.3 μM in the subphase buffer. Reducing the unoxidized and oxidized lipid concentrations to 10% virtually eliminated fibril formation by Aβ40 alone over the 3-day period of observation. These observations suggest that fibril formation by Aβ40 is promoted by synergistic effects of oxidative membrane damage and pre-treatment with Aβ42, and that the effect of oxidized membranes is dose-dependent.

Amide I’ Band Shape Analysis—Amide I’ bands from the infrared spectra of Aβ40 and Aβ42 are illustrated in Fig. 2. The amide I’ band shape of Aβ42 adsorbed onto oxidatively damaged lipid membranes from D₂O is dominated by a low frequency component at 1622 cm⁻¹, but a smaller high frequency component is evident at 1684.0 cm⁻¹ as well as a broad absorption centered between 1640 and 1670 cm⁻¹ (Fig. 2a). This band shape closely resembles that of dry fibrillized Aβ protein segments (from H₂O) previously reported (9).

The amide I’ band shape of unseeded Aβ40 on SAPCOX is also dominated by a low frequency component at 1629.1 cm⁻¹, and it has a broad absorption between 1640 and 1670 cm⁻¹, but the high frequency component at 1685.9 is only barely detectable (Fig. 2b). The amide I’ band shape of Aβ42-seeded Aβ40 is likewise dominated by a low frequency component at 1627.8 cm⁻¹ (Fig. 2c), but it differs from that of unseeded Aβ40 in that there is substantially more absorption between 1680 and 1630 cm⁻¹ and the high frequency component at 1685.9 cm⁻¹ is more prominent. The spectra in Fig. 2, b and c, correspond to time = 4 h in Fig. 1.

A 100 μM solution of Aβ40 at pH 7.4 was allowed to fibrillize for 10 days incubation at room temperature, after which time there is no detectable protein by assay in the supernatant of a centrifuged sample. This solution was then applied to an internal reflection crystal and the solvent was allowed to evaporate. The ostensibly “mature” fibrils in this preparation exhibit a prominent amide I’ component at 1622.7 cm⁻¹, in addition to some minor components (Fig. 2d). The amide I’ band shape obtained from unfibrillized material (examined prior to the 10-day incubation) is dominated by a component at 1656.8 cm⁻¹, in addition to some minor components (Fig. 2e). It should be noted that the 1656.8 cm⁻¹ component of this unfibrillized Aβ40 cannot be detected in fibrillized Aβ40 or in Aβ proteins that had adsorbed onto oxidatively damaged membranes. Thus, the 1656.8 cm⁻¹ component appears to be characteristic of dry unfibrillized Aβ40.

Quantitative analyses of these spectra were performed using IRfit (45), an approach designed to describe a set of related spectra under varying conditions with a minimum of fitting parameters. This approach is advantageous because it tends to identify components common to each spectrum with the same frequency, width, and shape. The results of this analysis are

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**Fig. 2. Amide I’ bands in the ATIR-FTIR spectra of Aβ proteins.** Experimental data are represented by discrete symbols; individual component bands obtained from IRfit are shown in thin lines, and the sum of the fit components is represented by a thick solid line overlying most of the data points. The experimental data were derived from 1024 co-added interferograms recorded at 2 cm⁻¹ resolution, and reproduced without smoothing, water vapor subtraction, deconvolution, or other manipulations to enhance resolution or apparent signal to noise ratio. A flat level baseline correction has been applied. a, Aβ42 absorbed to an monolayer containing oxidized lipids (SAPCOx). b, spectrum of unseeded Aβ40 on SAPCOX, corresponding to time = 4 h in Fig. 1. The absorption maximum is 0.0011. c, Aβ42-seeded Aβ40 on SAPCOX at time = 4 h in Fig. 1. The absorption maximum is 0.0032, and the integrated area of the amide I’ band is about 5-fold larger that that shown in panel b. d, dry Aβ40 fibrils, formed after 10 days incubation in dilute HEPES buffer and evaporated onto an internal reflection crystal. e, dry Aβ40 in dilute HEPES buffer immediately evaporated onto an internal reflection crystal.
listed in Table I, and several observations should be noted. First, the dominant low frequency components in each of the fibrillized A\textsubscript{B}/H\textsubscript{9252} proteins (Fig. 2, \textit{a}–\textit{d}) all lie between 1620 and 1630 cm\textsuperscript{-1}. The amplitude of this component is 3-fold greater when the membrane is seeded with A\textsubscript{B}/H\textsubscript{9252} \textit{A42} (compare Fig. 2, \textit{b} and \textit{c}). Because each component has the same width and shape, these amplitudes are proportional to their area. Second, each of the fibrillized A\textsubscript{B}/H\textsubscript{9252} proteins has two components at 1644.1 and 1669.7 cm\textsuperscript{-1} with the same position, width, and shape, but none bear evidence of the component at 1656.8 cm\textsuperscript{-1} that is characteristic of un fibrillized protein. Third, the spectrum from un fibrillized A\textsubscript{B}40 requires a fitting component at 1656.8 cm\textsuperscript{-1} and it cannot be fitted with the components used to fit fibrillized A\textsubscript{B} proteins. Attempts to include a 1656.8 cm\textsuperscript{-1} component when fitting the spectra of fibrillized proteins yielded a component with zero amplitude, and this component could not supplant the components at 1644.1 or 1669.7 cm\textsuperscript{-1}. Interpretation of these amide I band shapes is offered below.
DISCUSSION

These results demonstrate that oxidative lipid damage is a promoter of Aβ40 misfolding, aggregation, and fibril formation when pretreated with Aβ42. Although seeding of Aβ40 aggregation by Aβ42 was observed a decade ago (9), the experiments described herein were performed below the concentration threshold where this occurs, and they constitute the first report that oxidatively damaged membranes work synergistically with Aβ42 to promote Aβ40 aggregation. This is a significant step forward in the development of an in vitro model of amyloid plaque formation because it provides an experimental system in which mechanistic details about the possible pathogenic relationships between oxidative stress, metal ions, Aβ40/Aβ42 deposition patterns, and Aβ/APP toxicity may be explored (26–29).

It is also significant that this link between oxidative damage and pathological misfolding of Aβ40 was demonstrated using two independent techniques. Whereas it was possible to demonstrate this link using lipid vesicles and a Congo Red binding assay, it was much more quickly and clearly detected using supported lipid membranes and a specialized form of ATIR-FTIR spectroscopy. The latter approach offers at least three significant advantages over dye binding assays. First, speed, reporting on events that occur over minutes rather than days. Second, sensitivity, with clear results from 0.3 μM solutions of each protein, compared with 2.5 μM Aβ42 and 10 μM Aβ40 for dye binding. Assays using thioflavin T fluorescence (50) are more sensitive than Congo Red, but still not nearly as sensitive as ATIR-FTIR and they do not offer a speed advantage. Third, additional information, about adsorption kinetics, secondary structure, and the organizational state of membranes lipids. The vesicle experiments confirm the ATIR-FTIR results, and are key control experiments showing that negative results in our ATIR-FTIR experiments are not merely because of proteins leaving the membrane surface and becoming undetectable by ATIR-FTIR.

Jarret et al. (9) demonstrated the potential of Aβ42 (below its solubility limit) to seed fibril formation by Aβ40 (above its solubility limit) over the course of several days. The current data expand on these much earlier results by reporting the existence of an interaction within minutes of mixing, by using concentrations of both proteins well below their solubility limits, and by demonstrating that oxidative damage works synergistically with Aβ42 to promote Aβ40 misfolding.

Insofar as the internal reflection technique reports on adsorption and folding events that occur within minutes of exposing proteins to oxidatively damaged membranes, we do not know whether the proteins being detected have formed short segments of true fibrils, protofibrillar intermediates, interme-

diates with helical structure (51), or another as yet undescribed stage of fibril formation. In any case, it is clear that the results from internal reflection predict and correlate to the formation of classic amyloid fibrils that bind Congo Red and can be visualized by electron microscopy. At this point, technical obstacles preclude the examination of membrane-adsorbed Aβ40 by electron microscopy or atomic force microscopy.

It has been suggested that the Aβ42 used to seed membranes in these experiments may have formed small seed fibrils even before it encountered the membrane. We cannot rule out this possibility, but the proteins are treated with hexafluoro-2-propanol immediately prior to use, and after redissolving the proteins in buffer, they are ultrafiltered. This reportedly yields a structurally homogenous non-fibrillar protein preparation (52). In any case, and irrespective of its aggregation state, Aβ42 seeding was only effective at promoting Aβ40 misfolding on oxidatively damaged membranes: Aβ42 did not adsorb or misfold on undamaged membranes, and it did not promote this behavior by Aβ40 on undamaged membranes. Thus, the presence of seed fibrils in our Aβ42 preparations would not diminish the significance of oxidative damage to the process of fibril formation.

The interpretation of amide I’ band shapes in terms of conformational assignment is fraught with pitfalls, especially when there are differences in sample preparation. One must be circumspect when comparing the spectra of membrane-adsorbed spectra (Fig. 2, a–c) to those of dried protein (Fig. 2, d and e). For example, the 1656.8 cm⁻¹ component of dry unfrizzilized Aβ40 (Fig. 2e) does not correlate well to a secondary structure category. One would expect the protein to be random coil under these conditions (53), but random coil polypeptides only absorb at this frequency in H₂O. All exchangeable hydrogen atoms in the protein samples subjected to infrared analysis in this work have been thoroughly replaced with deuterium, as evidenced by the lack of any residual amide II band between 1600 and 1500 cm⁻¹. Under these conditions, the characteristic absorption band of random coil protein shifts to 1643 cm⁻¹ (54). The value of this spectrum is not its interpretation in terms of secondary structure, but its distinctiveness from that of Fig. 2, a–d; it demonstrates that unfrizzilized Aβ protein cannot be detected in fibrillized Aβ40 under the same conditions, or in Aβ proteins when they are membrane-adsorbed.

The value of the spectrum from dry fibrillized Aβ40, on the other hand, is its similarity to the spectra from membrane-adsorbed Aβ proteins, especially Aβ42 and Aβ42-seeded Aβ40. Despite differences in preparation (adsorbed at the water-membrane interface versus dried), the spectra in Fig. 2, a, c, and d, both have dominant low frequency components at 1622–1627 cm⁻¹ and clearly discernable high frequency components.

### TABLE I

| Frequency | Widthᵃ | Shapeᵇ | Aβ42 on SAPCₘₐ | Unseeded Aβ40 on SAPCₘₐ | Seeded Aβ40 on SAPCₘₐ | Dry Aβ40 fibrils | Unfrizzilized Aβ40 |
|-----------|--------|--------|----------------|-------------------------|-----------------------|-----------------|---------------------|
| cm⁻¹      | cm⁻¹   |        |                |                         |                       |                 |                     |
| 1685.9    | 9.6    | 100.0  | 1%             |                         |                       |                 |                     |
| 1684.9    | 9.6    | 100.0  | 5%             |                         |                       |                 |                     |
| 1675.2    | 42.2   | 60.0   | 21%            |                         |                       |                 |                     |
| 1669.7    | 27.6   | 100.0  | 34%            |                         |                       |                 |                     |
| 1656.8    | 29.7   | 3.6    | 50%            |                         |                       |                 |                     |
| 1644.1    | 33.3   | 100.0  | 37%            |                         |                       |                 |                     |
| 1629.1    | 19.2   | 35.1   | 28%            |                         |                       |                 |                     |
| 1627.8    | 19.2   | 35.1   | 7%             |                         |                       |                 |                     |
| 1624.0    | 8.9    | 76.7   | 1%             |                         |                       |                 |                     |
| 1622.7    | 19.2   | 35.1   | 5%             |                         |                       |                 |                     |
| 1622.2    | 19.2   | 35.1   | 41%            |                         |                       |                 |                     |
| 1621.5    | 36.1   | 53.6   | 4%             |                         |                       |                 |                     |

ᵃ Half-maximum.
b % Gaussian shape, remainder is Lorentzian.
at 1684–1686 cm$^{-1}$. This pair of components has been experimentally and theoretically correlated with extended multiple-stranded $\beta$-sheet structures formed in other proteins (55, 56). Unseeded A$\beta$40 is distinguished from A$\beta$42-seeded A$\beta$40 by having only a trace of the high frequency component, and an overall absorption intensity that is only one-third as large after 4 h of accumulation. Nonetheless, A$\beta$42-seeded A$\beta$40 is distinct from mature A$\beta$40 fibrils by the blue-shift of its low frequency to 1627.8 cm$^{-1}$ and the prominence of a mid-amide I’ component at 1641.1 cm$^{-1}$. This suggests that the A$\beta$40 that has adsorbed onto an oxidatively damaged membrane is immature with respect to folding, and that after 4 h it has still not fully adopted the conformation it will eventually adopt in a mature fibril.

The amide I’ components at 1644.1 cm$^{-1}$ cannot be interpreted definitively. This is a relatively broad component that may well have irrevocable subcomponents. Furthermore, secondary structure assignments for amide I’ components between 1680 and 1630 cm$^{-1}$ are imprecise. Bands at 1648–1655 cm$^{-1}$ are generally associated with $\alpha$-helical conformation, whereas bands at 1643 cm$^{-1}$ are traditionally associated with unordered structure (54, 57). This interpretation would suggest that A$\beta$42-seeded A$\beta$40 adsorbs onto the membrane in a random configuration and that it subsequently adopts a less non-random fold as it matures into an amyloid fibril. More recent studies, however, have demonstrated amide I’ bands in helical proteins far outside this range, even as low as 1632 cm$^{-1}$ (58). Thus, one cannot rule out the possibility that a portion of the 1644.1 cm$^{-1}$ component in A$\beta$42-seeded A$\beta$40 represents a helical structure, possibly even the oligomeric helical intermediate state detected in circular dichroism studies (51, 53).

A preliminary characterization of the oxidatively damaged lipids used in this work has been published showing that the preparation contains hydroperoxides, conjugated dienes, and isoprostanes (41). This analysis also showed that the protocol employed in this work actually damages only a very small percentage of the SAPC used in forming supported membranes, that have yet to be developed. Thus, further details about the chemical nature of the active lipid species in these experiments are not available at this time.

We have observed that oxidatively damaged lipids promote A$\beta$42 misfolding when monolayers are compressed to 20 mN/m before their application to the germanium internal reflection crystal support, but this does not occur if they are compressed to 36 mN/m. Because the higher monolayer pressure corresponds more closely to widely accepted values for the bilayer-equivalent lateral pressure of a monolayer (59), one possible interpretation of these observations is that oxidized lipids may not promote A$\beta$ accumulation and misfolding in the bilayer membranes of cells. However, our experiments were not conducted on monolayers at an air-water interface, but rather on monolayers supported by a firm hydrophobic alkyl-silane surface. The pressure to which a monolayer must be compressed to yield a bilayer-equivalent lateral pressure when supported on such a surface is not clear. This uncertainty underscores the importance of our experiments performed with bilayer vesicles: they corroborate the monolayer experiments performed at 20 mN/m by demonstrating that oxidized lipids in bilayers promote true amyloid fibril formation, and by establishing that the ability to synergize with A$\beta$42 in promoting A$\beta$40 aggregation is a bilayer-equivalent property of supported monolayers.

We have demonstrated the synergistic effects of oxidative damage and A$\beta$42 in vitro on synthetic membranes, whereas membranes in the brain are vastly more complex mixtures. Therefore, one must keep in mind that it is the more complex membrane, the one containing myriad oxidation products, that accelerates the accumulation and folding of A$\beta$ in these experiments. We have confirmed the presence of the isoprostane 8,12-iso-PF$_{2\alpha}$-VI in our lipid preparations (41), and the other 31 isoprostanes are likely to be present as well. In addition, we can safely assume that a diverse assortment of conjugated diene species, oxygen-addition products, and various partially degraded lipid forms will also be present. Hence, the membranes promoting amyloidogenesis in this work are complex and heterogeneous. It is the simple and homogeneous membranes that do not accelerate the accumulation and misfolding of A$\beta$.

It is not yet known how oxidative damaged membranes promote the misfolding of A$\beta$ proteins, but one can readily imagine them acting kinetically as “seeds” or thermodynamically as “templates.” Either way, they become sites at which there is a locally high concentration of the pathological form. It is also not known at this point whether membranes, damaged or otherwise, promote the pathological folding of A$\beta$ in vitro. In pursuing answers to these questions, one must keep in mind that seeds and templates would not need to persist for long periods of time, nor would they need to involve large areas of membrane, to promote pathological conformational changes. The transient formation of a small amyloidogenic patch of membrane may be sufficient to create a nucleus of misfolded A$\beta$ proteins that promotes the misfolding of additional protein with no need to maintain the amyloidogenic patch.

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Early Synergy between Aβ42 and Oxidatively Damaged Membranes in Promoting Amyloid Fibril Formation by A β40

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