

Evidence that Perutz's double- β -stranded subunit structure for β -amyloids also applies to their channel-forming structures in membranes

S. Jonathan Singer^{*†} and Nazneen N. Dewji^{*‡§}

^{*}Division of Biological Sciences, [†]Department of Medicine, and [§]Stein Institute for Research on Aging, School of Medicine, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093

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Although there is a growing body of evidence that different amyloidoses may have a similar molecular mechanism in common, the many details of this mechanism are not understood. In this study, we propose that there is a common molecular structure of the primary agents of these diseases, namely a small oligomer of Perutz's cylindrical double- β -stranded subunit for polyglutamine and that this structure, which contains a central water-filled core, can spontaneously integrate into the bilayers of membranes to form aqueous pores. We suggest that this ability to produce permeable channels in appropriate neuronal membranes is a key element in the toxicity of the β -amyloids. One strong criterion for the stability of the Perutz structure for an amyloid is that it contain ≈ 40 or more amino acid residues. We show here that the neurotoxic A β amyloids 1-40 and 1-42, related to Alzheimer's disease, spontaneously enter the membranes of intact erythrocytes and cause their lysis but that A β 1-38 and A β 1-35, which are not neurotoxic, have no observable effects on erythrocytes, supporting our proposal. Other aspects of the proposed mechanism of cytotoxicity of the β -amyloids are explored.

Alzheimer's disease | neurodegeneration | Parkinson's disease

A large and continually increasing number of diseases, including the neurodegenerative syndromes Alzheimer's disease (AD), Parkinson's disease, and Huntington's disease as well as the spongiform encephalopathies (prion diseases) such as mad cow disease are all associated with the formation of characteristic proteinaceous components that can form aggregates and/or fibrils under appropriate conditions in water. These components are collectively called β -amyloids. In these fibrils and aggregates, the β -amyloids are all largely in the β -conformation and can be stained with Congo red, which reveals that they contain a hydrophobic environment that bonds the dye. These extensively aggregated β -amyloid structures are present at equilibrium with small water-soluble species containing from one to a dozen or so of the repeating subunits of the β -amyloid, each bonded non-covalently to its neighboring subunits. It is widely accepted that some form of the particular β -amyloid is critically involved in the neurotoxicity of its associated disease, but the detailed mechanisms for this involvement have not been elucidated. In previous years, it was vigorously disputed whether the larger aggregates or one or more of the smaller soluble species of β -amyloids are the primary toxic components, but in the last few years, several studies (see refs. 1–4) have clearly implicated the smaller soluble species in the toxic process. The fibrils and aggregates may serve as storage depots of longer β -amyloid chains rather than of the shorter soluble forms.

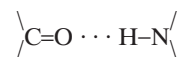
Close Similarities in the Structures and Functions of Different β -Amyloids

One of the most remarkable of these recent studies is that of Kaye *et al.* (3). Working with A β 1-40, which is a β -amyloid associated with AD, Kaye *et al.* reported the production of a MAb that binds to, and inhibits the toxicity of, water-soluble

intermediate-sized oligomers of A β 1-40, but the MAb does not bind to the water-soluble monomer, the large-molecular-weight oligomers, or to A β 1-40 fibrils, none of the last three being neurotoxic. In addition, with five other β -amyloids of different amino acid sequences and associated with a wide range of different neurodegenerative diseases, the same MAb that binds specifically to the intermediate-sized water-soluble oligomer of A β 1-40, also reacts with similar intermediate-sized water-soluble oligomers of each of the other five amyloids but not with their monomers, larger oligomers, or their fibrils or aggregates. Although the antigenic epitope specific to this MAb is not known, these results strongly suggest that the different β -amyloids, despite their considerable differences in amino acid composition and in mechanisms of production, form intermediate-sized oligomers in the water-soluble state that have closely similar structures that all generate this epitope and, further, suggest that the mechanisms by which the different β -amyloids produce their particular neurotoxicity are closely similar.

The X-Ray Diffraction Structure of Polyglutamine Fibrils Cast from Water Solution

Perutz *et al.* (5), in 2002, published a subunit (monomer) structure for polyglutamine (poly Q) derived from the x-ray diffraction patterns of fibers of D₂ Q₁₅ K₂ that had been cast from water solution. Poly Q β -amyloids are involved in Huntington's disease and several other neurodegenerative diseases (6). The subunit structure consists of two turns of a cylindrically wound single β -chain (Fig. 1B) that surround a water-filled channel; the Q side chains extend alternately on either side of the main chain, which makes each turn of the β -chain 5 Å long and the two complete turns 10 Å long, parallel to the chain axis. The two turns are strongly bound to one another by successive main-chain



hydrogen bonds on the side of the β -chain facing the bilayer, and, on the side of the β -chains facing the channel, the C—O and N—H dipoles form hydrogen bonds with each other or with H₂O molecules. The Q residues are hydrogen-bonded to their neighboring Qs on both sides of the β -sheet (Fig. 1D). Thus, all of the groups capable of forming hydrogen bonds do so, which greatly stabilizes the double-stranded structure in water (and even more so for those hydrogen bonds facing a lipid bilayer, see below). The aqueous central core is 15 Å in diameter, large enough to form an ion-conductance channel. Each subunit contains 40

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Abbreviation: AD, Alzheimer's disease.

[†]To whom correspondence should be addressed. E-mail: ssinger@ucsd.edu.

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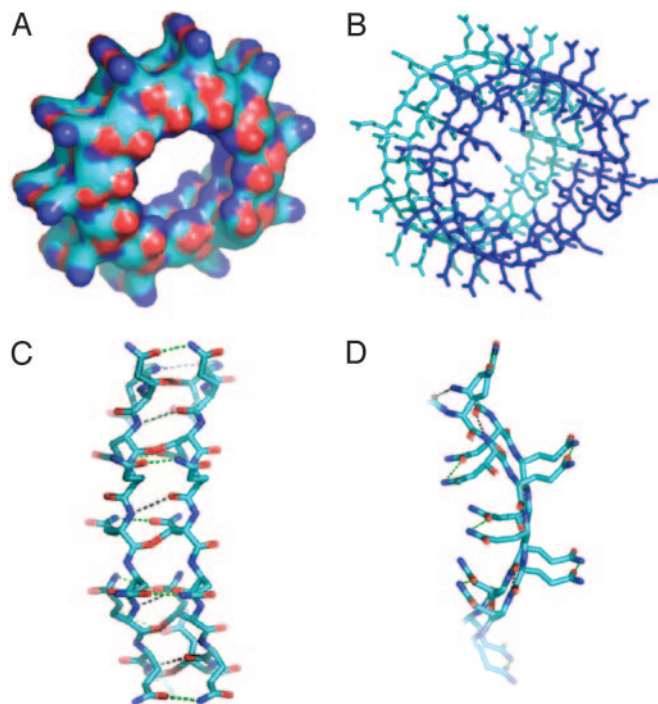
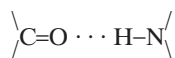


Fig. 1. Computer renderings of the Perutz *et al.* (5) structure of poly Q. (A) Space-filling model of one subunit of 42 Q residues with two turns of β -strands, showing the central pore, here tilted, that is perpendicular to the plane of the membrane. Note the outer convolutions of the surface of the subunit, by the outward-facing Q residues. These might serve to make gear-like associations between β -amyloid subunits in the plane of the membrane; others might serve as regions of attachment of specific integral membrane proteins to the membrane-interior regions of the poly Q amyloid, such as we propose in the text. Red balls are O atoms, and blue ones are N atoms. (B) Stick model of two stacked subunits of poly Q, each subunit containing 42 Q residues, one subunit rendered in dark blue, the other in light blue. (C) Stick model of a short segment of one subunit (enlarged) that faces a region of bonding between one turn and another of the subunit, showing the hydrogen bonds (strings of dots) formed between backbone $\text{C}=\text{O}$ (red) and $\text{H}-\text{N}$ (dark blue) groups. (D) Stick model of a small segment of one subunit, showing (by strings of dots) the hydrogen bonds formed by (i) inter- β -strand bonds between the backbone $\text{C}=\text{O} \cdots \text{H}-\text{N}$ groups, (the O in red and the N in dark blue) and (ii) $\text{C}=\text{O} \cdots \text{H}-\text{N}$ of the alternate Q residues extending from one or the other surface of the two-turn helix sheet. This view is parallel to the axis that runs down the pore of the subunit, and perpendicular to the plane of the membrane bilayer.

amino acids, 20 in each turn. As Perutz *et al.* (5) emphasized, β -chains <40 amino acids, for thermodynamic reasons, would not be able to form stable cylindrical fibrils of repeating subunits in water. It is clear from Fig. 1 that, for example, one turn of a subunit containing 20 residues could not form all of the



main-chain hydrogen bonds with a second turn that had, say, only 16 residues. This would increase the free-energy change accompanying the formation of that two-turn subunit, making its solubility less likely. It is of great interest, therefore, that poly Q repeats with <37 Q are generally not neurotoxic, whereas all those that have 40 or more repeats invariably are (6). In a closely related context, the neurotoxic forms of the $\text{A}\beta$ amyloid of AD that are produced *in vivo* are either 40 or 42 amino acids long (see below). These considerations are of particular relevance to the experimental results obtained with $\text{A}\beta$ in this article.

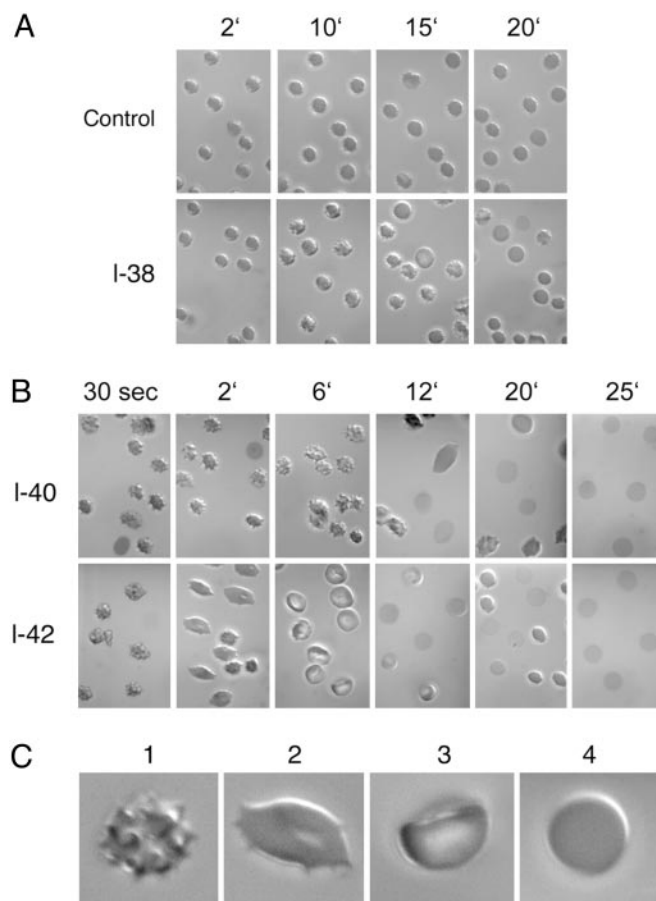


Fig. 2. Bilayer couple experiments with intact erythrocytes treated with a total of $20 \mu\text{M}$ of one $\text{A}\beta$ oligomer of the size indicated at the left margins of A and B as a function of time after mixing (numbers in min above each row of figures in A and B). Pictures were taken with Nomarski optics in light microscopy. (C) Enlargements of a succession of the major cell forms observed with time (1, crenated; 2, flattened; 3, cup-shaped; and 4, near-spherical). The photographs indicate that $\text{A}\beta$ 1-40 and 1-42 (B) show a rapid succession of shapes of the intact erythrocyte (details in text), more rapidly for $\text{A}\beta$ 1-42 than for $\text{A}\beta$ 1-40. [Control cells (A Upper) without added $\text{A}\beta$ exhibit no significant shape changes over the same time interval.] By 25 min, all of the erythrocytes with added $\text{A}\beta$ 1-40 or $\text{A}\beta$ 1-42 are lysed, leaving membrane ghosts behind. The effects of $\text{A}\beta$ 1-38 on erythrocyte shape, by contrast to $\text{A}\beta$ 1-40 and $\text{A}\beta$ 1-42, are not distinguishable from the unchanged controls (A).

More recently, a dispute has arisen about the question whether the x-ray data analyzed in Perutz *et al.* (5) lead exclusively to the model of the poly Q subunit that they developed or equally well to other models of quite different structure (see ref. 7). In the remainder of this article, therefore, we provisionally regard the Perutz *et al.* model as a plausible and attractive, but not proven, structure for the poly Q subunit, irrespective of the present state and relevance of the Perutz x-ray data. We present different kinds of data in support of the Perutz *et al.* structure dissolved in membrane bilayers.

β -Amyloids Form Conducting Pores in Black Lipid Bilayer Films and in Intact Cell Membranes

In early 1994, we became interested in the question of whether $\text{A}\beta$ 1-40 might spontaneously solubilize in cell membranes and whether it could cause cytolysis of intact cells. We carried out experiments (unpublished work) with human erythrocytes and $\text{A}\beta$ 1-40 and other $\text{A}\beta$ -related compounds that were closely similar to experiments that we have performed more recently,

some of which are shown in Fig. 2. The results of the two sets of experiments were essentially the same. A β 1-40 entered the membrane of the intact erythrocyte spontaneously, and, in \approx 25 min, all of the cells had lysed. We never published the earlier results because we could not think of a membrane-permeating structure for A β that explained thermodynamically its solubility in membrane bilayers as well as its ability to lyse the cell. Since then, many related experiments have been carried out by others with a number of β -amyloids, usually in conjunction with black lipid bilayers and occasionally with live cells (for reviews, see refs. 8 and 9). These studies yielded generally similar results, including those with poly Q (10, 11); as measured by the onset of conductance, the black lipid bilayers doped with any of a variety of β -amyloids developed ionic (generally cationic) conductances. These data indicated that the amyloids had penetrated the bilayers and created pores across them. However, no thermodynamically satisfactory detailed integral membrane structures were advanced for the β -amyloids to account for their solubility in a bilayer or for their pore formation, nor was a detailed mechanism proposed for the connection between these findings and the neuronal target-specificity of different toxic β -amyloids.

In this article, we propose that the structure of neurotoxic β -amyloids in cell membranes are often, perhaps generally, that of the intermediate aggregates [of the order of trimers (30 Å) or tetramers (40 Å)] of the pore-forming cylindrical double- β -stranded subunit that Perutz *et al.* (5) ascribed to the oligomeric state of poly Q in water solution or suspension (Fig. 1). In addition, we provide experimental evidence with A β oligopeptides and intact erythrocytes that is consistent with this structural proposal for bilayer-bound A β subunits.

Results

The Detection of Oligopeptide Solubility and of Pore Formation in the Membranes of Intact Erythrocytes. The entry of a small molecule into, and its passage through, the erythrocyte membrane bilayer can be followed with Nomarski optics on a light microscope by using the “bilayer couple” description of the erythrocyte bilayer (12, 13). If the penetration of a small-molecule substance from the outside medium into the bilayer is sufficiently large and slow enough, its entry, first into the outer half of the lipid bilayer, can be detected in the light microscope by the transient formation of crenated intact cells (e.g., see Fig. 2C1) because of the differential expansion of the total area occupied by the outer half layer relative to the inner half. If the substance then continues to penetrate the bilayer further, into the inner half bilayer, the intact cell flattens and elongates slightly when the total areas occupied by the two half layers are about equal (e.g., Fig. 2C2). If further penetration of the substance occurs, and its concentration in the inner half layer increases, the intact cell is induced to adopt a “cup” morphology (Fig. 2C3), as the area occupied by the inner half of the bilayer exceeds that of the outer half. (Other more irregular shapes are also observed.) In this condition, if the penetrating small molecules now span the thickness of the bilayer and if the substance has a structure that forms a water-filled, ion-conducting channel traversing the bilayer, osmotic equilibration will cause the cell to lyse, releasing the hemoglobin and leaving behind the membrane “ghost.”

The Behavior of Intact Erythrocytes Toward Several A β Oligopeptides.

As described in the previous section, intact erythrocytes can undergo transient shape changes, detectable in the light microscope, that reflect several stages of insertion of small molecules from the aqueous medium into the erythrocyte bilayer. For example, as shown in Fig. 2B, by 30 sec after immersion in an aqueous medium of 20 μ M total concentration of A β 1-40, most of the erythrocytes have acquired a crenated shape (as shown enlarged in Fig. 2C1), indicative of entry of the oligopeptide into

the outer half of the membrane bilayer, which persists until about 12 min. At this time, many of the cells have become flattened and elongated (as shown enlarged in Fig. 2C2), reflecting diffusion of A β 1-40 from the outer to the inner half layer, with similar concentrations in each half layer. By 25 min, most of the erythrocytes have lysed to form ghosts, reflecting the formation of pores in the membrane created by A β 1-40 molecules, with the cores spanning the bilayer.

With A β 1-42 at the same total concentration (Fig. 2B), a similar progression of shape changes and lysis occurs as with A β 1-40 only faster. At 6 min, many of the erythrocytes already exhibit cup shapes (shown enlarged in Fig. 2C3), demonstrating that a higher concentration of the amyloid is present in the inner half of the bilayer than in the outer half. By 12 min, significant cell lysis has already occurred and, by 25 min, is complete.

Although we do not know the concentrations of the various oligomers in the A β 1-40 and A β 1-42 solutions at 20 μ M total concentration of the two A β s, the more rapid membrane entry and formation of membrane pores by A β 1-42 than by A β 1-40 is correlated with the generally accepted view that A β 1-42 is more neurotoxic in AD than is A β 1-40, which, in turn, is consistent with the suggestion that pore formation in neuronal membranes by A β -amyloids is a critical step in A β -induced neurotoxicity.

As discussed in the introduction, the stability of the cylindrically wound double-stranded subunit, either in water or in a membrane bilayer, is expected to undergo a sharp transition as the length of the oligopeptide increases beyond \approx 39 amino acids. We therefore synthesized A β 1-38 and A β 1-35 and examined their effects compared with the erythrocyte shape changes produced by A β 1-40 and A β 1-42. As shown in Fig. 2A, 20 μ M A β 1-38 in aqueous solution produced no significant changes in erythrocyte shape at 20 min compared with a control, by which time the A β 1-40- and A β 1-42-treated erythrocytes were already undergoing lysis. The results obtained with erythrocytes treated with 20 μ M A β 1-35 and A β 1-28 were indistinguishable from those with A β 1-38 (data not shown). Because A β 1-38 has a smaller stretch of hydrophobic amino acids than either A β 1-40 or A β 1-42, this result cannot be attributed to a lower solubility of the small oligomers of A β 1-38 than those of A β 1-40 and A β 1-42 in water solutions. Instead, the result is that predicted by Perutz *et al.* (5) for the instability in water of the β -stranded subunit structure for β -amyloids of lengths $<$ 40 residues. Their reasoning about an unfavorable shortage of hydrogen bonding with A β s $<$ 40 residues in water is even more cogent inside a membrane bilayer.

We have also carried out experiments with these several A β oligopeptides acting on neurons. In these studies, mouse hippocampal and cortical neurons in culture were treated with 20 μ M A β 1-40, A β 1-42, or A β 1-38. After 2 h, the neurons treated with A β 1-40 and A β 1-42 had undergone marked degeneration, but those treated with A β 1-38 for 2 or 24 h were indistinguishable from untreated neurons.

Discussion

The Structure of β -Amyloids in Membranes. Our proposal is that many, if not most, neurotoxic β -amyloids exhibit the same or closely similar cylindrically wound double- β -stranded-subunit structure in cell membranes that Perutz *et al.* (5) proposed for the structure of poly Q in water (Fig. 1) and that this similar subunit structure accounts, in large part, for the striking similarities of the diverse neurotoxicities of the β -amyloids (3). The two turns of a single β -chain of each subunit surround a water-filled channel of \approx 15 Å in diameter, large enough to pass small ions. Because each subunit with its Q residues is \approx 10 Å in length along its axis, three or perhaps four subunits linked linearly (A–B \cdots A–B \cdots A–B) would be required to span a membrane bilayer. The membrane-spanning oligomer could be

example, each cell type may have in its plasma membrane one or more specific integral proteins that specifically bind to the membrane-intercalated Perutz *et al.* structure of the particular β -amyloid to which it is vulnerable. Not only would such a specific protein-amyloid interaction discriminate the particular cell target for the β -amyloid, but it would serve to intercalate the toxic amyloid oligomer into the specific membrane at much lower ambient aqueous concentrations than are required for its intercalation into nonspecific bilayers.

If, furthermore, such hypothetical integral membrane proteins specific for the AD-susceptible neurons were localized within the synapses of these neurons (17, 18), and the toxic $A\beta$ 1-42 oligomer with its aqueous pore would become bound and concentrated there, it could account for observations that, in AD, synapses often appear to disintegrate as an early consequence of the disease (19–22). Another problem often encountered with neurodegenerative diseases is their late onset in life. If the β -amyloid involved is produced throughout life, what is responsible for late onset? One among many possible explanations is that the β -amyloid-specific integral proteins that we propose exist may not be expressed in the target cell plasma membrane until late in life, thereby limiting the disease to old age.

Materials and Methods

Human Erythrocytes. Normal human blood was collected into EDTA-coated Vacutainers. The cells were separated by centrifugation and washed several times with freshly prepared 150 mM

NaCl. The erythrocytes were stored in citrate-phosphate-dextran (CPD, Sigma) (1.4 volumes CPD per 10 volumes erythrocytes) and washed with 150 mM NaCl before the addition of the $A\beta$ oligopeptides. Erythrocytes were used within 48 h of drawing.

$A\beta$ -Related Oligopeptides. The $A\beta$ oligopeptides 1-28, 1-38, 1-40, and 1-42 were obtained from Bachem (Torrance, CA), and 1-35 was synthesized by Biopeptide (San Diego). These oligopeptides were solubilized according to the manufacturer's instructions as follows: The oligopeptides 1-28 and 1-35 were solubilized in water at 6 mg/ml. To the oligopeptides 1-38 and 1-40 was added dilute acetic acid to help solubilize them. The oligopeptide solutions were then diluted to 1 mg/ml with phosphate-buffered saline without Ca^{+2} (PBS, pH 7.4) and incubated at 37°C for 3–5 days before use. Oligopeptide 1-42 (at 6 mg/ml in H_2O) was diluted to 1 mg/ml with 0.1% NH_4OH ; this solution was then diluted to 100 μ g/ml with PBS and incubated at 37°C for 3–5 days.

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