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AGGREGATION STATE-DEPENDENT BINDING OF β-AMYLOID PEPTIDE TO PROTEIN AND LIPID COMPONENTS OF RAT CORTICAL HOMOGENATES

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SUMMARY: β-amyloid peptide (Aβ) is the primary protein component of senile plaques in Alzheimer's disease. Aβ is toxic to neuronal cell cultures, although the mechanism of neurotoxicity is unknown. Neurotoxicity has been correlated to the aggregation state of the peptide. In this work, the synthetic β-amyloid peptide Aβ(1-39) was radioiodinated and fractionated into samples containing varying degrees of aggregated material. Binding of the peptide to rat cortical homogenates (containing both lipids and membrane-associated protein) and to artificial neuronal membrane (containing only lipids) was measured. Binding increased with increasing percent aggregated peptide in the solutions. Aggregated peptide bound to both cortical homogenate and membrane, whereas monomeric peptide bound to homogenate only. These results may help discriminate among alternative mechanisms of neurotoxicity of Aβ.

β-amyloid (Aβ) is a 39-43 residue peptide which is the primary protein component of senile plaques in Alzheimer's disease (1). Aβ spontaneously aggregates into amyloid fibrils in vitro (2), and is toxic to cultured cortical cells (3-6). Neuronal responses to Aβ that have been postulated to cause neurotoxicity include membrane depolarization (7,8), loss of calcium homeostasis (5), increased vulnerability to excitotoxins (4), and decreased membrane integrity (9). It has been suggested that toxicity is positively correlated with the degree of peptide aggregation (10-12).

The component(s) of cortical tissue with which Aβ interacts to exert neurotoxic effects remain(s) uncertain. One possibility is that Aβ interacts directly with the plasma membrane. Incorporation of Aβ(1-40) into a synthetic lipid bilayer resulted in the formation of cation channels (13). There is evidence that Aβ may form a choline carrier (14). Alternatively, Aβ may act via specific protein interactions. Aβ activity has been linked directly or indirectly to several receptors, including the serpin enzyme complex (SEC) receptor (15), tachykinin receptors (3, 16), and acetylcholine receptors (17). Association of Aβ with heparan sulfate proteoglycan has been reported (18). Aβ activity has also been linked to ion channels, including the NMDA receptor channel (19), voltage-gated calcium channels (20), and potassium channels (21).

This investigation was carried out to determine (a) if aggregated and monomeric forms of Aβ differed in their binding to rat cortical homogenates, and (b) if association of Aβ with cortical homogenates was due to a peptide-lipid interaction, a peptide-protein interaction, or both. Binding

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of Aβ(1-39) to rat cortical homogenate and artificial neuronal membranes was measured as a function of the aggregation state of the peptide. We have demonstrated that aggregated peptide binds to cortical homogenate to a greater extent than monomeric peptide, and that aggregated peptide binds to both lipid and protein components of cortical homogenates, whereas monomeric peptide binds primarily to non-lipid components.

MATERIALS AND METHODS

Materials: Aβ(1-39) was synthesized and purified by Nuros, Inc. (San Jose, CA). The sequence is DAEFRHDSGYEVHHQKLVFFADVGSNKGAIIGLMVGGV. Molecular mass and purity were confirmed by MS and reverse phase HPLC (M = 4232, >95 % purity). All other chemicals, unless otherwise specified, were obtained from Sigma (St. Louis, MO).

Radiolabeling of Aβ: Aβ(1-39) was radioiodinated via a modified Bolton Hunter method. This method of iodination was chosen to preferentially label at the N-terminus of the peptide. Labeling at other primary amines was inhibited by maintaining the pH below the pKa of those residues. 300 pmol of sulfo-SHPP (Pierce, Rockford, IL) was iodinated with 400 μCi of $Na^{125}I$ (Dupont NEN, Boston, MA) using IodoBeads (Pierce). The reaction was carried out for 15 minutes in pH 8 borate buffer in a volume of 140 μL. The catalyst was removed and 40 pmol (3 μL) of freshly prepared Aβ(1-39) (in water) was added to the iodinated sulfo-SHPP. The reaction was allowed to proceed for 3 hours at 4 C. The resulting condensation product was separated from free $Na^{125}I$ using a G-5 desalting column (Pierce) which also fractionated the peptide. The peptide was eluted from the column with phosphate buffered saline (0.01 M KH_2PO_4/K_2HPO_4, 0.14 M NaCl, 0.02% NaN_3, pH 7.4) and stored at 4 C until use, typically within one month of preparation.

The free activity associated with ^{125}I-labelled Aβ(1-39) was determined by precipitation of the peptide with 5 wt% phosphotungstic acid in the presence of 5 mg/ml bovine serum albumin. Precipitable activity was 75 to 80%, and was constant within the uncertainty of measurement for at least 3 weeks after preparation.

Peptide concentration was determined from the activity of ^{125}I-labelled Aβ(1-39) by assuming that 50% of the peptide was recovered from the desalting column using one column volume of the eluting buffer and that the peptide concentration was proportional to the precipitable activity of that fraction. This method of estimation of peptide concentration yielded consistent results for different batches of radiolabeled material. Based on these assumptions, 5 to 10% of the peptide was labeled. No attempt was made to separate unlabeled from labeled peptide.

Characterization of extent of peptide aggregation: Native PAGE was used to determine the size of Aβ(1-39) aggregates. Stock solutions of ^{125}I-labelled Aβ(1-39) were separated on 20% polyacrylamide gels using the Pharmacia (Uppsala, Sweden) pHastgel system. The gels were cut at approximate molecular weights of 11 and 28 kDa, and activities of gel pieces were measured in a gamma counter.

Preparation of cortical homogenates: Frozen rat cortices (Zivic Miller Labs, Zelienople, PA) were homogenized in 20 volumes of phosphate buffer (0.01 M KH_2PO_4/K_2HPO_4, pH 7.2) using several strokes of a Potter Elvian (Kontes, Vineland, NJ) homogenizer. The homogenate was centrifuged at 2500 g for 40 minutes at 4 C. The pellet was rehomogenized in 20 volumes of buffer, centrifuged, and washed three more times. Homogenized tissue was stored at -70 C until use. Upon thawing, the homogenate was washed and centrifuged, and then resuspended in 5 volumes of buffer. Protein content of the homogenized tissue was determined on the day of use by Lowry's method (22). The dry weight of the homogenate was determined by washing a fixed volume of homogenate suspension in MilliQ water, and drying the pellet overnight at 90 C. The lipid content of the homogenate was assumed to be the difference between the total dry weight of the homogenate per ml of suspension and the protein content of the homogenate. All determinations were made in triplicate. The mass ratio of lipid to protein in homogenate was 0.9±0.1.

Phospholipids from cortical membranes were obtained by Folch Extraction (23). One g of tissue was homogenized and centrifuged as described above. Washed homogenate (pellet only) was then suspended in 19 ml of chloroform/methanol (2:1), sealed under nitrogen, and allowed to sit for 4 hours at room temperature to extract lipids. After the addition of 4 ml saline (0.14 M NaCl) to the extract, the mixture was shaken vigorously to partition lipid into the chloroform phase and proteins into the methanol/water phase. The mixture was centrifuged for 15 minutes at 2500 g to separate phases. The protein phase was discarded. The lipid/chloroform phase was dried under

nitrogen to allow determination of the mass of lipid collected. Vesicles were prepared by the same method as described for pure lipids.

Preparation of artificial neuronal membranes: Artificial neuronal membranes consisted of 36 wt% dipalmytoyl phosphatidyl choline, 36 wt% dipalmytoyl phosphatidyl ethanolamine, 10 wt% dioeyl phosphatidyl serine, and 18 wt% cholesterol (Avanti Polar Lipids, Alabaster, AL). The phospholipid composition approximated that of neuronal membranes (24,25). Lipids dissolved in chloroform were dispersed on the inside of a flask by evaporating off the solvent under nitrogen. Phosphate buffer was added and lipid was suspended in the buffer. The suspension of artificial membranes was sonicated for 5 minutes, then frozen and thawed several times to obtain a more homogeneous distribution of vesicle sizes. Artificial membranes were stored at -70 C until used.

Equilibrium binding experiments: Homogenate containing approximately 0.5 mg/ml protein was incubated with ^{125}I-labelled Aβ(1-39) at a constant temperature for 2 hours. Preliminary experiments indicated that the fraction bound after 30 min, 1 hr, or 2 hr was identical, within experimental error. Bovine serum albumin was added at a final concentration of 1 mg/ml to inhibit nonspecific binding. The homogenate suspension was centrifuged for 5 minutes at 12000 g to separate free from bound peptide. The activity of a fixed volume of supernatant was measured to determine the concentration of free peptide. The pellet was washed twice with cold buffer containing bovine serum albumin and 0.05% Tween 20, then the activity of the pellet was counted to determine bound peptide. Analogous experiments were performed using artificial membranes and membranes prepared from lipids extracted from cortical homogenate as binding substrates.

RESULTS

Distribution of peptide aggregation states

Solutions of ^{125}I-labelled Aβ(1-39) were analyzed by native PAGE to determine the distribution of oligomers. Results for some representative solutions are listed in Table 1. β(1-39) was present primarily in the <11 kDa (1-2 monomer units) fraction and in the >28 kDa (> 6 monomer units) fraction. Relatively little material migrated at intermediate (11-28 kDa) molecular weights. Peptide solutions were stable with respect to aggregation, within the uncertainty of the measurement, for at least 3 weeks. The species migrating with molecular weight <11 kDa was classified as monomeric (although it is recognized that the material could be dimer) and the species migrating with molecular weight >28 kDa was classified as aggregated.

Effect of peptide aggregation state on binding

Representative binding data are shown in Figure 1 for monomeric Aβ(1-39) and for a solution containing 66 ± 5 % aggregate. Aggregated peptide bound to a greater extent than did

Table 1. Oligomer distribution of representative Aβ solutions. Aβ was radioiodinated and fractionated as described in Materials and Methods. Fractions were analyzed by native PAGE followed by gel slicing and counting. The data shown are representative of all the samples used in the binding experiments.

	Mass Fraction of Oligomeric Species		
Molecular Weight (kDa)	Sample A	Sample B	Sample C
< 11	0.99 ± 0.01	0.61 ± 0.03	0.29 ± 0.02
11 - 28	0.00 ± 0.01	0.00 ± 0.01	0.05 ± 0.01
> 28	0.01 ± 0.01	0.39 ± 0.02	0.66 ± 0.05

monomeric peptide to rat cortical homogenate. Binding was not saturable in the concentration range tested. Binding could not be inhibited by the addition of 100-fold excess unlabeled Aβ(1-39). Less than 0.1% of the peptide was precipitated in the absence of homogenate.

Similar binding experiments were performed for several other solutions of Aβ(1-39) containing varying fractions of aggregated peptide. The slopes of binding data (pmol bound peptide/mg protein in homogenate/nM peptide in solution) were determined by least-squares regression and plotted against the mass fraction of aggregates in solution. As shown in Figure 2, binding increases linearly with increasing mass fraction of aggregates in solution.

Binding of Aβ(1-39) to artificial neuronal membranes was measured for peptide solutions containing varying mass fractions of aggregated peptide. The ratio of bound peptide to free was plotted versus the mass fraction of aggregate in solution (Figure 2). Binding increased linearly with mass fraction of aggregate, with a slope roughly comparable to that for binding to cortical homogenate but with a near-zero intercept. Binding to membranes prepared from lipids extracted from rat cortical homogenate was essentially the same as binding to artificial membranes (data not shown).

Effect of temperature and salt on peptide binding

Binding of Aβ(1-39) to cortical homogenate as a function of temperature was measured. Binding of aggregated (66 ± 5% aggregates) β(1-39) decreased with increasing temperature while binding of monomeric peptide was unaffected (Figure 3). Binding of aggregated and monomeric Aβ(1-39) to artificial membranes did not change significantly with temperature (data not shown).

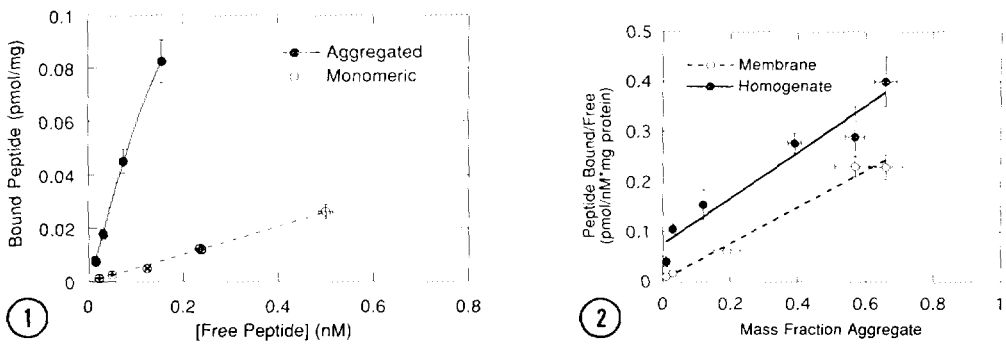

Figure 1. Equilibrium binding of Aβ to cortical homogenate. Representative binding curves for a monomeric and an aggregated (66 ± 5%) solution of Aβ. The incubation temperature was 32°C and the buffer was PBSA at pH 7.3. Concentrations of peptide are reported as equivalent moles of monomer.

Figure 2. Binding of Aβ to cortical homogenate or artificial membrane as a function of the mass fraction of aggregate. Experiments were conducted at 25°C in PBSA at pH 7.3. For binding to cortical homogenates, initial slopes of binding curves were determined for peptide solutions containing different aggregate mass fractions from data similar to that shown in Figure 1. Each binding curve contained at least five data points and each slope was determined in at least two separate experiments. Binding of Aβ to artificial neuronal membrane at low concentrations (0.02 nM - 0.2 nM) of free Aβ was measured for peptide solutions containing different fractions of aggregated peptide. For ease of comparison, the bound/free ratio for binding to membrane was determined as pmol/mg lipid-nM and converted to pmol/mg protein-nM by multiplying by 0.9, the mass ratio of lipid to protein in the homogenate. All data points are an average of at least two experiments, each of which was done in triplicate.

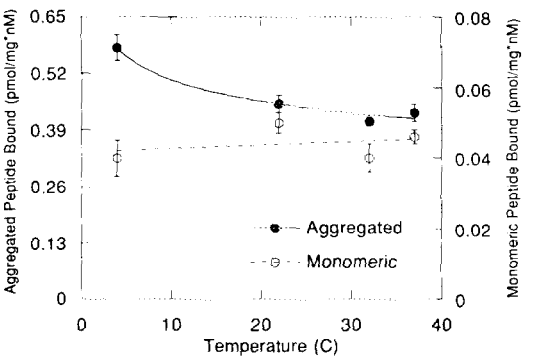

Figure 3. Binding of Aβ to cortical homogenate as a function of temperature. Initial slopes of binding curves were determined at several different incubation temperatures for both a monomeric and an aggregated (66 ± 5%) sample of Aβ. Experiments were conducted in PBSA at pH 7.3. Each binding curve contained at least five data points and each slope was determined in at least two separate experiments.

Binding of Aβ(1-39) to cortical homogenate and to artificial membranes as a function of salt concentration was measured. Binding of both aggregated (66 ± 5% aggregates) and monomeric Aβ(1-39) to cortical homogenate was greater at physiological than at low salt concentrations (Table 2). Salt concentration had little effect on binding to membrane for either monomeric or aggregated peptide. At low salt concentration, binding of aggregated peptide to homogenate was equal to binding to membrane, whereas at physiological salt concentration aggregated peptide bound more strongly to homogenate than to membrane. Monomeric peptide bound more strongly to cortical homogenate than to artificial membrane at both low and physiological salt concentration, but the difference was greater at the higher ionic strength. Binding at intermediate saline concentration (0.07 and 0.1 M NaCl) was similar to that at physiological saline concentration in all cases (data not shown).

Table 2. Effect of salt on binding of Aβ to cortical homogenates and artificial neuronal membranes. Binding of monomeric and aggregated (66 ± 5% aggregated) Aβ to cortical homogenate or artificial membrane at low concentration (0.02 - 0.2 nM) of Aβ was measured at low (0.035 M) and physiological (0.14 M) NaCl concentrations in 0.01 M potassium phosphate buffer. For ease of comparison, the bound/free ratio for binding to membrane was determined as pmol/mg lipid-nM and converted to pmol/mg protein-nM by multiplying by 0.9, the mass ratio of lipid to protein in the homogenate. Experiments were conducted at 32°C and pH 7.3. All data points are an average of at least two experiments, each of which was done in triplicate.

	Peptide Bound/Free (pmol/mg protein-nM)			
	Low Salt		Physiological Salt	
Aggregation State	Homogenate	Membrane	Homogenate	Membrane
Monomeric	0.021± 0.001	0.009± 0.001	0.040± 0.004	0.010± 0.001
Aggregated	0.20 ± 0.02	0.27 ± 0.03	0.41 ± 0.04	0.19 ± 0.03

DISCUSSION

Results reported here demonstrate that binding of Aβ to rat cortical homogenates is a strong function of peptide aggregation. Only aggregated peptide binds to a measurable degree to lipid components, whereas both aggregated and monomeric peptide bind to protein components. At low salt concentrations, virtually all of the binding of aggregated peptide to homogenate can be accounted for by binding to lipid. At physiological salt concentrations, binding of aggregated peptide is roughly equally distributed between lipid and protein components. The effect of aggregation is weak for peptide-protein interactions and stronger for peptide-lipid interactions: most of the increase in the peptide bound/free ratio with increasing mass fraction aggregate could be accounted for by an increase in association with lipids.

Binding of aggregates to cortical homogenate showed a temperature dependence whereas binding of monomer to cortical homogenate, or binding of either aggregate or monomer to artificial membrane, did not. Qualitative van't Hoff analysis of the temperature-dependence data shows that for all four cases ΔH^0, the enthalpy change due to association, is slightly negative or zero, whereas ΔS^0, the entropy change due to association, is positive. Therefore, favorable entropy changes are the primary driving force for both peptide-protein and peptide-lipid interactions. Combined with the data on salt effects, these results suggest that the peptide-protein association is primarily hydrophobic. The nature of peptide-lipid association is more difficult to assess. The combined data on salt and temperature effects suggest that the peptide-lipid interaction involves charged groups on the membrane surface; this does not preclude interaction with nonpolar internal groups as well.

Binding was not saturable under our experimental conditions. This could be due to the relatively low concentrations of peptide used in our experiments (1 nM or less). Concentrations were kept low, in part, to ensure that some solutions contained a significant fraction of unaggregated peptide, and to inhibit additional aggregation of the sample over the course of the experiments. Binding could not be competed off by excess unlabelled peptide. This may be due to peptide aggregation at the surface and incorporation of unlabelled peptide into the aggregate. Alternatively, the conformation or aggregation state of the peptide may influence its interaction with the homogenate. If so, then the unlabelled peptide may be in a different conformation or aggregation state than the labelled peptide and therefore unable to compete.

Maggio et al. (26) reported that Aβ(1-40) bound specifically to brain tissue from Alzheimer disease victims but not to normal human or rat cortical tissue. Specific binding was defined as that which was displaceable by excess unlabelled Aβ. Our data are not inconsistent with theirs, since we were unable to displace labelled with unlabelled Aβ. As explained above, this may be due to incorporation of unlabelled peptide into Aβ aggregates. In their study, the peptide concentration was low (10 pM to 1 nM) and was likely to be unaggregated. In addition, the peptide was iodinated at the tyrosine. In preliminary experiments, we found that peptide iodinated at the tyrosine residue displayed a reduced binding to cortical homogenate, and was less stable with respect to aggregation state, compared to peptide iodinated at the N-terminus.

These results should be helpful in discriminating among alternative mechanisms of Aβ-mediated toxicity. If aggregation is necessary for toxicity, as has been suggested by others, then our results imply that peptide-lipid interactions may play a significant role in mediating that toxicity.

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