Targeted Control of Kinetics of β-Amyloid Self-association by Surface Tension-modifying Peptides*

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Brain tissue from Alzheimer’s patients contains extracellular senile plaques composed primarily of deposits of fibrillar aggregates of β-amyloid peptide. β-Amyloid aggregation is postulated to be a major factor in the onset of this neurodegenerative disease. Recently proposed is the hypothesis that oligomeric intermediates, rather than fully formed insoluble fibrils, are cytotoxic. Previously, we reported the discovery of peptides that accelerate β-amyloid aggregation yet inhibit toxicity in vitro, in support of this hypothesis. These peptides contain two domains: a recognition element designed to bind to β-amyloid and a disrupting element that alters β-amyloid aggregation kinetics. Here we show that the aggregation rate-enhancing activity of the disrupting element correlates strongly with its ability to increase surface tension of aqueous solutions. Using the Hofmeister series as a guide, we designed a novel peptide with terminal side-chain trimethylammonium groups in the disrupting domain. The derivatized peptide greatly increased solvent surface tension and accelerated β-amyloid aggregation kinetics by severalfold. Equivalent increases in surface tension in the absence of a recognition domain had no effect on β-amyloid aggregation. These results suggest a novel strategy for targeting localized changes in interfacial energy to specific proteins, as a way to selectively alter protein folding, stability, and aggregation.

β-Amyloid (Aβ) is a 40–42-amino-acid fragment cleaved from membrane-bound amyloid precursor protein, containing sequences from both extracellular and transmembrane regions of the parent protein. Postmortem analysis of Alzheimer’s diseased brains reveals the presence of extracellular senile plaques composed primarily of deposits of Aβ fibrillar aggregates. The “amyloid hypothesis,” that Aβ amyloid deposition is a causative factor in the onset of Alzheimer’s disease, is supported by biochemical, genetic, and animal studies (1). Aβ self-association proceeds from the random coil monomer, through β-sheet structure formation and oligomerization, filament (or protofibril) initiation and growth, and then fibril assembly, growth, and deposition (2). The hypothesis, that Aβ is toxic only when aggregated into fibrils, is supported by a substantial body of data (3–5). Recently, an alternative hypothesis has been put forth: specifically, that a soluble intermediate in the fibrillogensis pathway, rather than the fully formed fibrillar end product, is the most cytotoxic form of Aβ (2, 6–10). The issue of the conformation and aggregation status of the toxic Aβ species remains controversial.

Several groups have reported the synthesis of compounds that interfere with Aβ aggregation and inhibit toxicity (11–20). Our group chose a strategy employing hybrid peptides as inhibitors; these peptides contain a recognition domain, designed to bind specifically to Aβ, and a disrupting domain, designed to interfere with normal Aβ aggregation (21). As the recognition domain, we chose residues 16–20 (KLVFF) of full-length Aβ; this region was identified as critical for Aβ self-association (22, 23). Hybrid peptides with the strongest affinity for binding to Aβ were the most effective at protecting against Aβ toxicity (24). We identified hybrid peptides that, when mixed with Aβ, inhibit Aβ toxicity while promoting more rapid formation of larger Aβ aggregates (21, 25, 26). If it proves to be true that intermediate oligomeric species in the Aβ aggregation pathway are the toxic species, the cytoprotection afforded by these compounds might result from their ability to reduce the concentration of toxic intermediate species.

The objective of the work reported here is to identify a plausible physicochemical basis for the action of hybrid peptides in accelerating Aβ aggregation. We demonstrate a strong positive correlation between the surface tension of aqueous solutions of active compounds and the ability of these compounds to increase the rate of Aβ aggregation. This concept is used to rationally design a modified peptide with markedly enhanced activity.

EXPERIMENTAL PROCEDURES

Peptides—Aβ-(1–40) was purchased from AnaSpec, Inc. (San Jose, CA). Protected amino acids, resin, and HBTU were purchased from Novabiochem. Betaine, ethyl acetate, and piperdine were purchased from Sigma. Diisopropylethylamine was purchased from Advanced Chemtech (Louisville, KY). KKKK was purchased from Bachem, Inc. (King of Prussia, PA). All other materials were purchased from Fisher Scientific or Sigma. KLVFFKKKKKK was synthesized on a Wang resin but with Mtt protection on the 6 C-terminal lysines. While still on the resin, the free acid form of double Boc-protected lysine was converted to the free acid form using sulfuric acid and ethyl acetate extraction. To make the betaine-modified peptide, LVFFFKKKKKK was synthesized on a Wang resin but with Mtt protection on the 6 C-terminal lysines. While still on the resin, the free acid form of double Boc-protected lysine was coupled at the N terminus in the presence of HBTU and diisopropylthylamine activators. Mtt-protecting groups were cleaved using 1% trifluoroacetic acid followed by addition of piperdine. Betaine was coupled to the free amines to form an amide linkage in the presence of HBTU and diisopropylthylamine. 95% trifluoroacetic acid was added to cleave the resulting peptide from the resin and remove the Boc-protecting groups. The cleaved peptides...
were purified by reverse-phase HPLC (C4 column) using an acetonitrile/water gradient. Fractions were collected and analyzed by MALDI mass spectrometry. Purified peptides were stored as lyophilized powders at −70 °C.

Surface Tension—The equilibrium surface tension of peptide solutions was measured using an FTÅ200 pendant drop tensiometer (First Ten Angstroms, Portsmouth, VA). A droplet of inhibitor solution was formed at the end of a blunt, 22-gauge stainless steel needle, and the shape of the droplet was imaged. The surface tension was measured by fitting the Young-Laplace equation to the contour of drop shape once equilibrium was reached.

Light Scattering—Peptide or betaine solutions were prepared by dissolving the compounds in double-filtered (0.22 μm) PBSA. Aβ was dissolved in double-filtered (0.22 μm) 8 mM urea at a concentration of 2.8 mM for 10 min and then diluted into double-filtered PBSA or PBSA containing test compound, to 140 μM Aβ. All samples were at pH 7.4 and contained 0.4 mM urea. Samples were quickly filtered through 0.45-μm filters directly into clean light scattering cuvettes. Dynamic light scattering data as well as average scattered intensity at 90° scattering angle were collected using a Coherent argon ion laser at 488 nm and a Malvern 4700 system, as described in more detail elsewhere (26).

RESULTS

Previously, we proposed a strategy for generating hybrid peptide compounds that modulate Aβ aggregation and inhibit Aβ toxicity (21). Effective hybrid peptides contain an N-terminal recognition domain, KLVFF, homologous to residues 16–20 of Aβ, and a C-terminal disrupting domain, a repeat sequence of non-homologous amino acids. We observed that KLFFKKKKKK and KLFFEEEEE, protected cells from Aβ toxicity (25, 26). Interestingly, protection from toxicity was accompanied invariably with an increase in the rate of aggregation of Aβ.

Given that both cationic and anionic, but not polar uncharged, disrupting domains were capable of accelerating Aβ aggregation, we hypothesized that the disrupting domain acted by altering physical properties of the solvent. To test this, the surface tension of aqueous solutions of hybrid peptides (with- or without Aβ) was measured using a pendant drop method. Compounds with charged disrupting domains increased surface tension in a concentration-dependent manner, but compounds with polar uncharged disrupting domains, or the recognition element alone with no disrupting domain, had little effect on surface tension (Table I). Increased surface tension of aqueous solutions of the hybrid peptide correlated strongly with an increased rate of aggregation of mixtures of Aβ + hybrid peptide (Fig. 1).

We next tested whether the disrupting domain alone was sufficient to accelerate Aβ aggregation. KKKK at 140 and 280 μM increased solvent surface tension to 53.9 ± 0.8 and 57 ± 1 dyne/cm, respectively, similar to that of KLFFKKKKKK at
the same concentrations (Table I), but even 420 μM KKKK had no effect on Aβ aggregation, whereas 140 μM KLVFFKKKKKK greatly accelerated Aβ aggregation (Fig. 2). These results indicate that modest increases in surface tension alone are insufficient to cause acceleration of aggregation and provide further support for the notion that the Aβ binding ability of the KLVFF recognition domain is required for activity.

We reasoned that even greater acceleration of Aβ aggregation could be obtained with a KLVFF recognition element coupled to a disrupting domain that produced a greater solvent surface tension effect. To identify an appropriate candidate, we turned to the Hofmeister series, which lists ions in the order of their ability to stabilize protein folded structure (27). Protein structure stabilization by co-solutes correlates strongly with the ability of the co-solute to increase the surface tension of water (28). The Hofmeister cation series is N(CH₃)₄⁺ > NH₂(CH₃)₂⁺ > NH₄⁺ > K⁺ > Na⁺ > Cs⁺ > Li⁺ > Mg²⁺ > Ca²⁺ > Ba²⁺, with the cations on the left classified as kosmotropes (protein structure-stabilizing, or “salting-out”) and those on the right as chaotropes (protein structure-destabilizing, or “salting-in”) (27).

The lysine hexamer disrupting domain of our most effective hybrid peptide reported to date, KLVFFKKKKKK, contains terminal amines that are protonated at neutral pH (-(CH₂)₄-NH₃⁺). We reasoned by analogy to the Hofmeister series that a side chain with a methyl-substituted terminal amine group might have enhanced activity as compared with lysine. Betaine ((CH₃)₃NCH₂COO⁻) is a naturally occurring compound that contains the requisite methyl-substituted amino group and also contains a free carboxyl group allowing facile coupling to a lysine side chain. We modified the lysine side chains in the disrupting domain using the following strategy. KLVFFKKKKKK was synthesized using standard Fmoc solid-phase synthesis techniques but with Mtt protection of the 6 C-terminal lysines and Boc protection of the N-terminal lysine. While maintaining the peptide on the resin, the Mtt groups were cleaved, and betaine was coupled to the lysine side chains via an amide linkage (Fig. 3). Cleavage from the resin produced a mixture of KLVFFKKKKKK derivatized with four, five, or six betaines, as confirmed by mass spectroscopy analysis (Fig. 3). A fraction highly enriched in the fully derivatized peptide, compound 1, was isolated by reverse-phase HPLC for further study (Fig. 3).

Compound 1 was extremely water-soluble. Size-exclusion chromatographic analysis confirmed that it was monomeric in PBSA (data not shown). Pendant drop measurements of aqueous solutions of the purified compound demonstrated greatly increased surface tension as compared with KLVFFKKKKKK (Table II).

The effect of compound 1 on Aβ aggregation was remarkable (Fig. 4). After 4 h of aggregation, the hydrodynamic diameter of Aβ in the presence of compound 1 was more than 100-fold greater than Aβ alone (~2300 versus ~20 nm) and nearly 50 times greater than Aβ with KLVFFKKKKKK, which was our
**Fig. 3.** Synthesis of surface tension-modifying peptide. *A*, schematic showing protection/deprotection strategy for linking betaine to 6 C-terminal lysines. *B*, structure of synthesized peptide. *C*, reverse-phase HPLC trace of cleavage product showing three major products. The molar mass of each fraction as determined by MALDI mass spectroscopy is indicated. The molar masses correspond closely to calculated molar masses for KLVFFKKKKKK with four, five, or six betaine additions. Compound 1 was purified and used in subsequent studies.
most active compound prior to the discovery of compound 1. Betaine at 1700 μM increased the surface tension of an aqueous solution to 61.4 ± 0.9 dyne/cm, equivalent to 140 μM compound 1, but betaine at 3400 μM had no measurable effect on Aβ aggregation kinetics (Fig. 4). These results show that surface tension can be used as a design strategy for improving activity of the disrupting domain of hybrid peptides and confirm that a specific recognition domain is required for activity.

**DISCUSSION**

Self-association of protein into large aggregates of β-sheet structure and fibrillar morphology is a feature of a number of diseases, including the primary amyloidoses and neurodegenerative diseases such as Alzheimer’s. Many, perhaps even most, proteins and peptides can be coaxed into forming β-sheet fibrils under appropriate conditions of solvent, pH, and temperature (29). Alarmingly, fibril-forming peptides and proteins appear to be toxic to a wide number of cell types (29). The end product of aggregation, the fully formed amyloid fibril, may be the primary toxic species, but recent evidence suggests that instead, it is a structured kinetic intermediate that is killing cells (2–10). Indeed, our earliest studies provided the first published data, to our knowledge, linking accelerated aggregation with inhibition of toxicity (21).

Our observations that hybrid peptides with either anionic or cationic disrupting domains accelerate Aβ aggregation (26) led to the hypothesis that disrupting domains act by affecting solvent properties. In particular, an increase in surface tension of aqueous solutions by addition of co-solutes is strongly linked to changes in protein stability and protein aggregation (28). Indeed, we observed that those hybrid peptides that accelerated Aβ aggregation also measurably increased the surface tension of the solvent. This observation prompted us to test the hypothesis that disrupting domains act by affecting solvent properties. In particular, an increase in surface tension of aqueous solutions by addition of co-solutes is strongly linked to changes in protein stability and protein aggregation (28).

**TABLE II**

| Compound       | Concentration (μM) | Surface Tension (dyne/cm) | Increase* (× 10^4 dyne/cm/μM) |
|----------------|--------------------|---------------------------|-------------------------------|
| KLVFFKKKKKK    | 70 μM              | 57 ± 2                    | 54.0 ± 0.2                   |
|                | 140 μM             | ND                        | ND                            |
|                | 180 μM             | 59 ± 1                    | 61 ± 1                        |
|                | 280 μM             | ND                        | ND                            |
|                | 420 μM             | ND                        | ND                            |

* The increase in surface tension was determined from the slope of the linear fit to the data.

**Fig. 4. Effect of compound 1 on Aβ aggregation.** Aβ was dissolved in 8 M urea and then diluted 20-fold into PBSA (□), PBSA with compound 1 at 1:1 molar ratio (△), or PBSA with 24-fold molar excess of betaine (indicated by ×). The final Aβ concentration was 140 μM. Growth was followed by laser light scattering. A, average hydrodynamic diameter versus time, as determined by least-squares fit of autocorrelation data. B, average scattered intensity at 90° scattering angle.
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tension of aqueous solutions (Fig. 1). The increase in surface tension was mediated solely through the disrupting domain (Table I). We tested whether the disrupting domain alone was capable of affecting solvent properties and/or ζ aggregation. Although a lysine tetramer at 420 µm increased the surface tension, it failed to alter ζ aggregation kinetics. This is not surprising, given that molar concentrations (>0.1–1 τt or higher) of co-solute are generally required for a sufficient change in solvent properties to produce measurable changes in protein folding and aggregation (27, 30, 31).

These encouraging results served as a basis for rational design of novel compounds with greater efficacy. We used the Hofmeister series as a guide toward selecting functional groups with strong surface tension activity. The Hofmeister series has proven to be a reasonably reliable predictor of co-solute effects on protein structure, aggregation, and activity (e.g. Ref. 32). The strongest salting-out (kosmotropic) cations in the Hofmeister series are methylimmonium ions (27). Several related compounds, such as betaine (CH₃)H₂N⁺CH₂COO⁻, sarcosine (CH₃)H₂N⁺CH₂COO⁻, and trimethylamine N-oxide ((CH₃)₂NO), are naturally occurring intracellular solutes that regulate osmotic pressure and modulate protein folding and enzyme function (e.g. Ref. 33–35). These compounds also affect protein aggregation. For example, sarcosine stabilized the native conformation of the serpin α₁-antitrypsin and protected against thermal inactivation and aggregation (30). Similarly, betaine partially inhibited light chain amyloid fibril formation from immunoglobulin light chain (29). In contrast, trimethylamine N-oxide accelerated fibril assembly from ζ aggregation (36). This apparent contradiction can be resolved by noting that kosmotropes drive the system toward more compact protein structure. For α₁-antitrypsin and immunoglobulin light chain, the most compact structure is the natively folded monomer, but since ζ monomer is random coil, its most compact and folded structure is the β-sheet fibril.

Given these clues, we developed a method for synthesizing hybrid peptides with terminal trimethylammonium groups in the disrupting domain. The betaine-derivatized KLVFFKKKKKK was remarkably active at increasing surface tension and was dramatically more effective at accelerating ζ aggregation than KLVFFKKKKKK. Although betaine alone increased surface tension of aqueous solutions, the osmolyte did not measurably affect ζ aggregation. These results indicate the specificity of the action of compound 1.

It is well established that co-solutes that increase solvent surface tension favor compact folded and/or aggregated protein structures. What is unique about the compounds described here is that the increase in surface tension is apparently localized via the recognition element to the solvent near the aggregating peptides. We hypothesize that this leads to a much greater localized change in solvent surface tension than that experienced in the bulk solvent or in the absence of the recognition element. This localized change in solvent properties then drives further growth of highly aggregated species. It is interesting to speculate that similar strategies could be used as novel mechanism of targeting interfacial changes to individual proteins to influence protein folding, stability, and aggregation in a highly specific manner.

REFERENCES

1. Hardy, J., and Selkoe, D. J. (2002) Science 297, 353–356
2. Pallitto, M. M., and Murphy, R. M. (2001) Biophys. J. 81, 1805–1822
3. Pike, C. J., Burdick, D., Wang, J. T., Glabe, C. G., and Cotman, C. W. (1993) J. Neurosci. 13, 1676–1687
4. Simmons, L. K., May, P. C., Tomaselli, K. J., Rydel, R. E., Fussen, K. S., Brigham, E. F., Wright, S., Lieberburg, I., Becker, G. W., Brems, D. N., and Wu, W. (1994) Mol. Pharmacol. 45, 373–379
5. Seilheimer, B., Bohrmann, B., Bondolfi, L., Muller, F., Stuhler, D., and Dobeli, H. (1997) J. Struct. Biol. 119, 59–71
6. Roher, A. E., Chang, M. O., Kuo, Y. M., Webster, S. D., Stine, W. B., Haverkamp, L. J., Woods, A. S., Cotter, R. J., Tuohy, J. M., Krafft, G. A., Bonnell, B. S., and Emmerling, M. R. (1996) J. Biol. Chem. 271, 20631–20635
7. Lambert, M. P., Barlow, A. K., Chrzymy, B. A., Edwards, C., Freed, R., Liosatos, M., Morgan, T. E., Rozovskiy, I., Trenner, B., Biola, K. L., Pals, P., Zhang, C., Finch, C. E., Kraft, G. A., and Klein, W. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 95, 6444–6453
8. Hartley, D. M., Walsh, D. M., Ye C. P., Diehl, T., Vasquez, S., Vassiliev, P. M., Teplow, D. B., and Selkoe, D. S. (1999) J. Neurosci. 19, 8876–8884
9. Ward, R. V., Jennings, R. K., Jeppra, R., Neville, W., Owen, D. E., Hawkins, J., Christie, G., Davis, J. B., George, A., Karran, E. H., and Howlett, D. R. (2000) Biochem. J. 348, 137–144
10. Yazed, R., Head, E., Mcintyre, T. M., Milton, S. C., Cotman, C. W., and Glabe, C. G. (2003) Science 300, 466–489
11. Klunk, W. E., Debnath, M. A., Korsos, A. M., and Pettgrew, J. W. (1998) Life Sci. 63, 1807–1814
12. Soto, C., Sigurdsson, E. M., Morelli, L., Kumar, R. A., Castano, E. M., and Ratnaparkhi, G. S., and Varadarajan, R. (2001) Biochemistry 40, 8237–8245
13. Nakagami, Y., Nishikuma, S., Muraeagi, T., Kanoeko, J., Meguro, M., Marumoto, S., Kogen, H., Koyama, K., and Oda, T. (2002) Br. J. Pharmacol. 137, 676–682
14. Deo, A. J., Hughes, E., Burke, R. M., Su, T. J., Heenan, R. K., and Lu, J. (2002) Biochem. Soc. Trans. 30, 537–542
15. Gordon, D. J., and Meredith, S. C. (2001) Biochemistry 40, 7892–7899
16. Lowe, T. L., Strzelec, A., Kiessling, L. L., and Murphy, R. M. (2001) Biochemistry 40, 8782–8789
17. Cacace, M. G., Landsu, E. M., and Ramadan, J. M. (1997) Q. Rev. Biophys. 30, 241–277
18. Lin, T.-Y., and Timasheff, S. N. (1996) Protein Sci. 5, 372–381
19. Cecuttiini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zardo, J., Taddei, N., Ramponi, G., Dobson, C. M., and Stefani, M. (2002) Nature 416, 507–511
20. Ke, Y.-S., Cope, S. P., Chi, E., Raff, R., Wilkins-Stevens, P., Stevens, F. E., Manning, M. C., Randolph, T. W., Solomon, A., and Carpenter, J. F. (2001) J. Biol. Chem. 276, 1626–1633
21. Chow, M. K. M., Devlin, G. L., and Bottomley, S. P. (2001) Biochemistry 39, 1593–1599
22. Ahmad, A., Akhtar, M. S., and Bhakuni, V. (2001) Biochemistry 40, 1945–1955
23. Wang, A., and Bolen, D. W. (1997) Biochemistry 36, 9101–9108
24. Ratnaparkhi, G. S., and Varadarajan, R. (2001) J. Biol. Chem. 276, 28789–28798
25. Bourou, S., Sire, O., Trastewetter, A., Touze, T., Wu, L. F., Blanco, C., and Emmerling, M. R. (2000) J. Biol. Chem. 275, 1505–1506
26. Yang, D.-S., Yip, C. M., Huang, T. H. J., Chakrabarty, A., and Fraser, P. E. (1999) J. Biol. Chem. 274, 32970–32974