Technetium Complexes for the Quantitation of Brain Amyloid

Hogyu Han, Cheon-Gyu Cho,† and Peter T. Lansbury, Jr.*,†

Department of Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

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The early descriptions of Alzheimer's disease (AD) were based, in part, on the detection of neuritic amyloid plaques in the brains of demented patients by staining tissue sections with the dye Congo Red (CR, **1**).[1,2] Although a definitive diagnosis of AD still depends on the observation of congophilic amyloid fibrils[2] in the brain at autopsy, the critical question of whether amyloid deposition is a cause or effect of AD neurodegeneration remains unresolved.[3] The brains of nondemented elderly individuals contain markedly reduced *numbers* of amyloid plaques relative to age-matched AD patients, but among AD patients, a correlation of plaque number with the degree of dementia at time of death has been difficult to establish.[3,4] Increased plaque *size* is characteristic of the early-onset form of AD associated with Down syndrome, but not late onset AD.[4c,5] We sought a more precise, chemical descriptor of AD neuropathology, based on quantitation of amyloid, which may be a more accurate indicator of disease progression. We report herein the synthesis of two ^{99}Tc complexes which bind *in vitro* to amyloid fibrils comprising a major AD amyloid protein, β1-40, as well as to fibrils comprising a minor constituent, NAC, with high affinity. The analogous γ-emitting ^{99m}Tc complexes could be quantitated by SPECT (single-photon emission computerized tomography) imaging.

CR **1** stains native amyloid fibrils in tissue and fibrils formed *in vitro* from synthetic β proteins in a birefringent manner, suggesting an ordered association.[1] Since the fibril structure is unknown, the molecular basis of the affinity of **1** is unclear.[6−9]

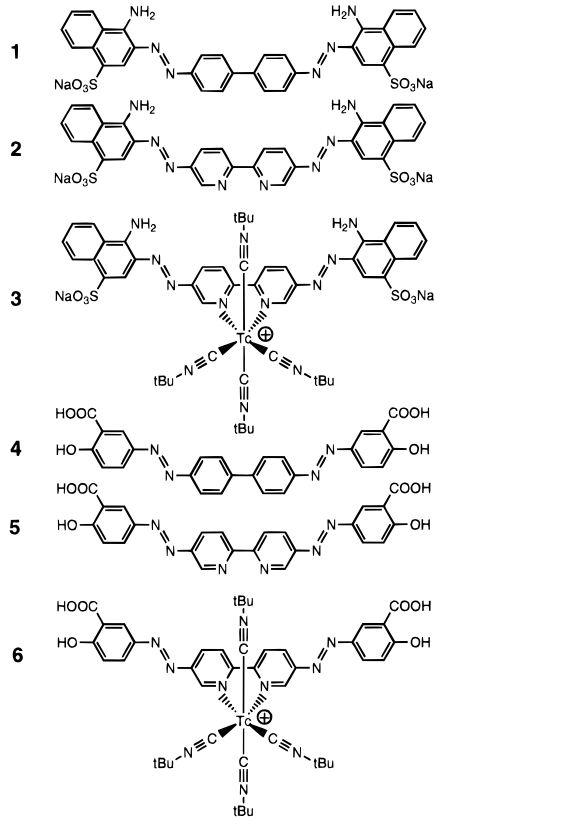

Figure 1. The structures of the amyloid fibril-binding compounds and the two amyloid peptides discussed herein.

However, other biphenyl-linked aromatic azo dyes, such as chrysamine G (CG, **4**), have been shown to have comparable affinity for model amyloid fibrils.[8] We have shown that modifications of the biphenyl moiety of **1** do not necessarily affect its affinity for β34-42 amyloid fibrils.[9] Our strategy was to replace the biphenyl moiety which is shared by **1** and **4** with a bipyridyl moiety which is capable of binding a reporter technetium ion (Figure 1).[10]

Both bipyridyl-containing compounds were synthesized from 5,5′-diamino-2,2′-bipyridine.[11] Diazotization of 5,5′-diamino-2,2′-bipyridine, followed by coupling with 4-amino-1-naphthalenesulfonic acid, provided bipyridyl-CR **2** in 58% yield.[11] Coupling of the same bis-diazo compound with salicylic acid provided bipyridyl-CG **5** in 73% yield.[11] Technetium (^{99}Tc) complexes of **2** (**3**) and **5** (**6**) were formed in 28% and 29%

† Address as of July 1, 1996: Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, 221 Longwood Ave., LMRC, Boston, MA 02115.

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(2) Neuritic, or senile, amyloid plaques consist of an insoluble fibrous amyloid core surrounded by dystrophic neurites. Amyloid fibrils are proteinaceous and, by definition, congophilic. AD amyloid fibrils comprise primarily variants of the 4 kD β protein, β1-40 and β1-42 being the most abundant.[3,15]

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(5) Plaques are counted by microscopic examination of chemically or immunochemically stained tissue.[4] The latter method is complicated by the fact that antibodies directed against the major amyloid proteins β1-40 and β1-42 cross-react with nonfibrous β protein deposits ("diffuse plaques") which are not specific for AD brain.[3]

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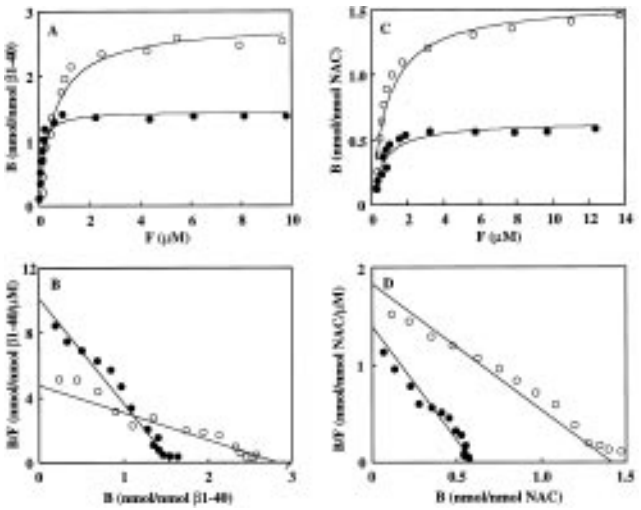

Figure 2. Saturation experiments for binding of **3** (○) and **6** (●) to β1-40 fibrils (A, 5 μM total protein) and NAC fibrils (C, 15 μM total protein). Scatchard analyses of the binding data from A and C are shown below, in B and D, respectively.[19] The labeling of the *x*-axes in B and D is based on total protein concentration. Data shown is from one representative experiment.

yield, respectively.[12] The ^{99}Tc:bipyridyl ligand stoichiometries of the resultant complexes **3** and **6** were determined to be 1:1 by mass spectrometry, which showed parent ions for both complexes, and by ^{1}H NMR, which showed two distinct resonances, corresponding to two equatorial and two axial *tert*-butyl isocyanide ligands.[12a,13] The anticipated chelation of Tc by the bipyridyl group[12] was confirmed by ^{1}H NMR, which showed a retention of ligand symmetry and a downfield shift of the protons adjacent to the bipyridyl nitrogens in the complexes **3** (δ = 9.53) and **6** (δ = 9.52) relative to the uncomplexed ligands **2** (δ = 8.82) and **5** (δ = 9.16). A 2:1 complex of ligand **2** and Zn(II) was also prepared.[14]

The affinity of the radioactive complexes **3** and **6** for β1-40 (Figure 1) amyloid fibrils was determined. Bound and free ligand were separated by centrifugation. In both cases, saturable binding was observed (Figure 2). The apparent K_d's of **3** and **6** were 630 and 160 nM, respectively (Table 1). These values were comparable to the reported K_d's of **4** to β10-43 fibrils[8a] and of **1** to insulin amyloid fibrils.[1g] The relative affinities (apparent K_i's) of the free bipyridyl ligands **2** and **5** were determined by displacement of complexes **3** or **6** (Table 1). Comparable values were obtained for displacement of either complex, suggesting shared binding sites, which may be hydrophobic pockets spaced at regular intervals along the fibril surface. The ordered nature of these sites is suggested by the observed birefringent staining by **1**[1] and the fact that the Zn-(**2**)$_2$ complex,[14] in which the two bipyridyl CR ligands are likely to be orthogonal, does not bind more tightly than the 1:1 Tc complexes (Table 1). The stoichiometry of saturation, as estimated by Scatchard analysis of the binding curves, differed slightly, with 2.7 mol of **3** and 1.6 mol of **6** bound per mole of β1-40 (Figure 2). The molecular basis for this difference is unclear.

Table 1. Dissociation Constants (K_d, μM)[19] and Inhibition Constants (K_i, μM)[20] for β1-40 Fibrils and NAC Fibrils at 25 °C and pH 7.4[a]

amyloid	Congo Red analogs	K_d	K_i[b]	K_i[c]
β1-40	[Tc(CN*t*Bu)$_4$(bpcr)]$^+$ (**3**)	0.63 (0.06)		
	[Tc(CN*t*Bu)$_4$(bpcg)]$^+$ (**6**)	0.16 (0.05)		
	Congo Red (**1**)		0.46 (0.03)	0.56 (0.14)
	Bipyridyl-CR (**2**)		0.51 (0.11)	0.48 (0.12)
	Bipyridyl-CG (**5**)		0.76 (0.15)	0.42 (0.10)
	Zn(**2**)$_2$[9,14]		1.52 (0.23)	
NAC[16]	[Tc(CN*t*Bu)$_4$(bpcr)]$^+$ (**3**)	0.77 (0.14)		
	[Tc(CN*t*Bu)$_4$(bpcg)]$^+$ (**6**)	0.43 (0.15)		

[a] Data shown are an average of at least three separate experiments and are expressed as mean (±SD). [b] This value was obtained using [Tc(CN*t*Bu)$_4$(bpcr)]$^+$ as the labeled ligand. [c] This value was obtained using [Tc(CN*t*Bu)$_4$(bpcg)]$^+$ as the labeled ligand.

Binding of complexes **3** and **6** to amyloid fibrils comprising the minor brain amyloid peptide NAC[15] (Figure 1) showed features similar to the binding of β1-40 fibrils (Table 1). Again, the CR complex **3** bound less avidly than the CG complex **6**; dissociation constants of 770 and 430 nM, respectively, were measured. Analogous to the case with β1-40 fibrils, the stoichiometry of saturation with **3** (1.4 mol **3**/mol NAC) was slightly higher than that for **6** (0.6). The NAC amyloid fibrils and the β1-40 fibrils may share a general feature, suggested by their local sequence homology,[15] which is recognized by Congo Red, **3**, and **6**.[16]

Since the ^{99m}Tc analogs of **3** and **6** are charged, they may require the assistance of a nonspecific blood−brain barrier opener[17] in order to be useful for noninvasive imaging of brain amyloid. The establishment of a timecourse of amyloid deposition could resolve the continuing debate as to whether amyloid is a cause or result of AD neurodegeneration and symptoms.[3] If amyloid is found to cause AD, its imaging would allow the early diagnosis and the monitoring of chemotherapeutic strategies aimed at preventing or reversing amyloid formation.[3c,18]

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Supporting Information Available: Synthesis and analysis of compounds **2**, **3**, **5**, and **6** and the procedure for the amyloid binding assay (4 pages). Ordering information is given on any current masthead page.

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(14) Upon addition of $ZnCl_2$ to **2**, the absorptions at 344 and 514 nm disappeared and were replaced by new bands at *ca*. 390 nm and *ca*. 610 nm (similar shifts were seen for the Cd(II), Ni(II), and Cu(II) complexes).[9] Titration of **2** with $ZnCl_2$ indicated that a 2:1 **2**:Zn complex was formed.

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(16) Although complex **6** binds to both β1-40 and NAC amyloid fibrils 2- to 4-fold more avidly than does **3**, other studies in our laboratory demonstrate that the relative affinity of two amyloid probes is not constant for a series of amyloid fibrils comprising different proteins.[9] Thus, it may be possible to develop protein-specific amyloid probes.

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