

## Examining the zinc binding site of the amyloid- $\beta$ peptide

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The amyloid  $\beta$ -peptide (A $\beta$ ) is a principal component of insoluble amyloid plaques which are characteristic neuropathological features of Alzheimer's disease. A $\beta$  also exists as a normal soluble protein that undergoes a pathogenic transition to an aggregated, fibrous form. This transition can be affected by extraneous proteinaceous and nonproteinaceous elements, such as zinc ions, which may promote aggregation and/or stabilization of the fibrils. Protein chelation of zinc is typically mediated by histidines, cysteines and carboxylates. Previous studies have demonstrated that the A $\beta$ -Zn<sup>2+</sup> binding site is localized within residues 6–28 and that histidines may serve as the principal sites of interaction. To localize key residues within this region, a series of A $\beta$  peptides (residues 1–28) were synthesized that contained systematic His/Ala substitutions. Circular dichroism and electron microscopy were used to monitor the effects of Zn<sup>2+</sup> on the peptide  $\beta$ -sheet conformation and fibril aggregation. Our results indicate that substitution of either His13 or His14 but not His6 eliminates the zinc-mediated effects. These observations indicate a specific zinc binding site within A $\beta$  that involves these central histidine residues.

**Keywords:** amyloid- $\beta$ ; Alzheimer's disease; zinc; senile plaques; fibril formation.

Alzheimer's disease (AD) is the most common form of cerebral degeneration leading to dementia. A key neuropathological feature of AD is the deposition of amyloid fibrils within the neuropil as senile plaques and in the walls of cerebral and meningeal blood vessels [1,2]. The major component of the extracellular amyloid deposits is a 40–42 residue protein termed amyloid  $\beta$ -protein (A $\beta$ ) [3,4] which is derived from endoproteolysis of the integral membrane amyloid precursor protein (APP) [5]. A $\beta$  in the plaques is in the form of amyloid fibrils [6] that are insoluble under physiological conditions. Aggregation of A $\beta$  into cytotoxic amyloid fibres or protofibrils may be a factor in the AD-related neuronal loss [7–10].

Under normal physiological conditions, A $\beta$  is a soluble, cellular metabolite that is produced by a variety of cells and is found in the cerebrospinal fluid and plasma. With the exception of familial AD cases, A $\beta$  is present at comparable levels in both sporadic AD and unaffected individuals [11–14]. Understanding the polymerization mechanism of A $\beta$  transition from a monomeric to fibrous form and what role modulating factors play in this pathway has been the focus of a number of studies. These investigations have demonstrated that amyloid aggregation is a nucleation-dependent process [15,16] that is affected by several basic factors such as primary sequence [17], peptide concentration [18], pH [18,19], membrane lipids [20] and hydration forces [21]. It has also been established that amyloid associated elements are important modulators of A $\beta$  fibre formation and aggregation. These non-A $\beta$  elements include apolipoprotein E [22–24], proteoglycans [25–27],

$\alpha_1$ -antichymotrypsin [23,28,29] and metal ions such as Zn<sup>2+</sup> and Cu<sup>2+</sup> [30].

Several lines of evidence suggest that zinc may be a contributing factor to the pathogenesis of AD possibly through the promotion and stabilization of amyloid fibrils [31,32]. Although the degree to which zinc affects amyloid plaque formation has yet to be fully resolved *in vivo*, connections between Zn<sup>2+</sup> and A $\beta$  have been revealed by a series of *in vitro* studies. These have demonstrated, for example, that Zn<sup>2+</sup> will induce rapid and extensive aggregation of synthetic A $\beta$  [30,33–35]. Low concentrations of Zn<sup>2+</sup> promote the aggregation of endogenous A $\beta$  in canine cerebrospinal fluid which has an identical sequence to that of human A $\beta$  [36]. In support of such an association, higher levels of Zn<sup>2+</sup> have been found in the senile plaques of AD cases as compared to levels in the neuropil of AD and control subjects [37]. Solubilization of A $\beta$  from postmortem brain tissue of AD cases and controls is significantly enhanced by the presence of metal chelators which further supports a Zn<sup>2+</sup>-mediated stabilization of A $\beta$  amyloid fibrils [38]. Moreover, in A $\beta$  transgenic mice appreciable quantities of Zn<sup>2+</sup> are present in Congo red positive mature plaques but are absent in preamyloid, nonfibrillar deposits [39]. Interestingly, preamyloid A $\beta$  deposits fail to develop into mature plaques in cerebellum where synaptic vesicle Zn<sup>2+</sup> is deficient [39]. These supportive *in vitro* findings have nevertheless raised some questions as to the relative affinity of the A $\beta$ -Zn<sup>2+</sup> interaction, the concentrations required to elicit an aggregation response, as well as the amount of free zinc that is available in the extracellular environment. Obviously some debate remains on the precise role of zinc in amyloid fibrillogenesis but a number of observations have led to the suggestion that Zn<sup>2+</sup> may be a key factor in AD plaque formation and/or stabilization.

Protein chelation of zinc can be mediated by its histidine, cysteine and carboxylate residues. In the case of A $\beta$ , it has been proposed that the histidine residues constitute a primary site of interaction, possibly with some contribution from sidechain and C-terminal carboxylates. The objective of the current study is to

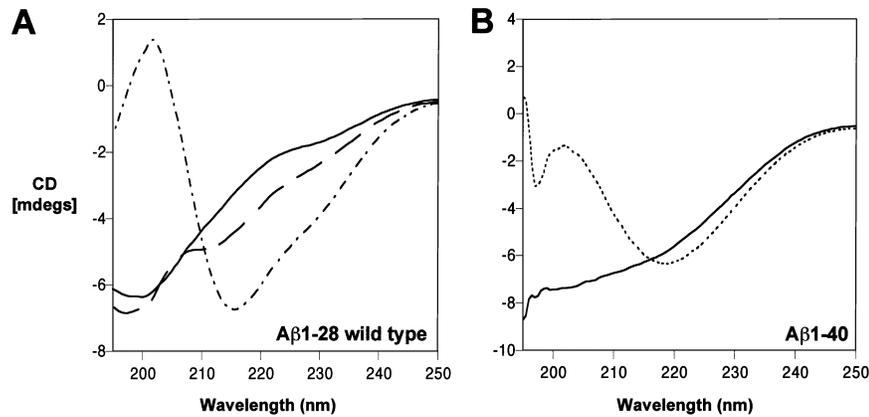
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Abbreviations: A $\beta$ , amyloid  $\beta$ -peptide; AD, Alzheimer's disease; APP, amyloid precursor protein.

(Received 26 June 2000, revised 7 September 2000, accepted 18 September 2000)



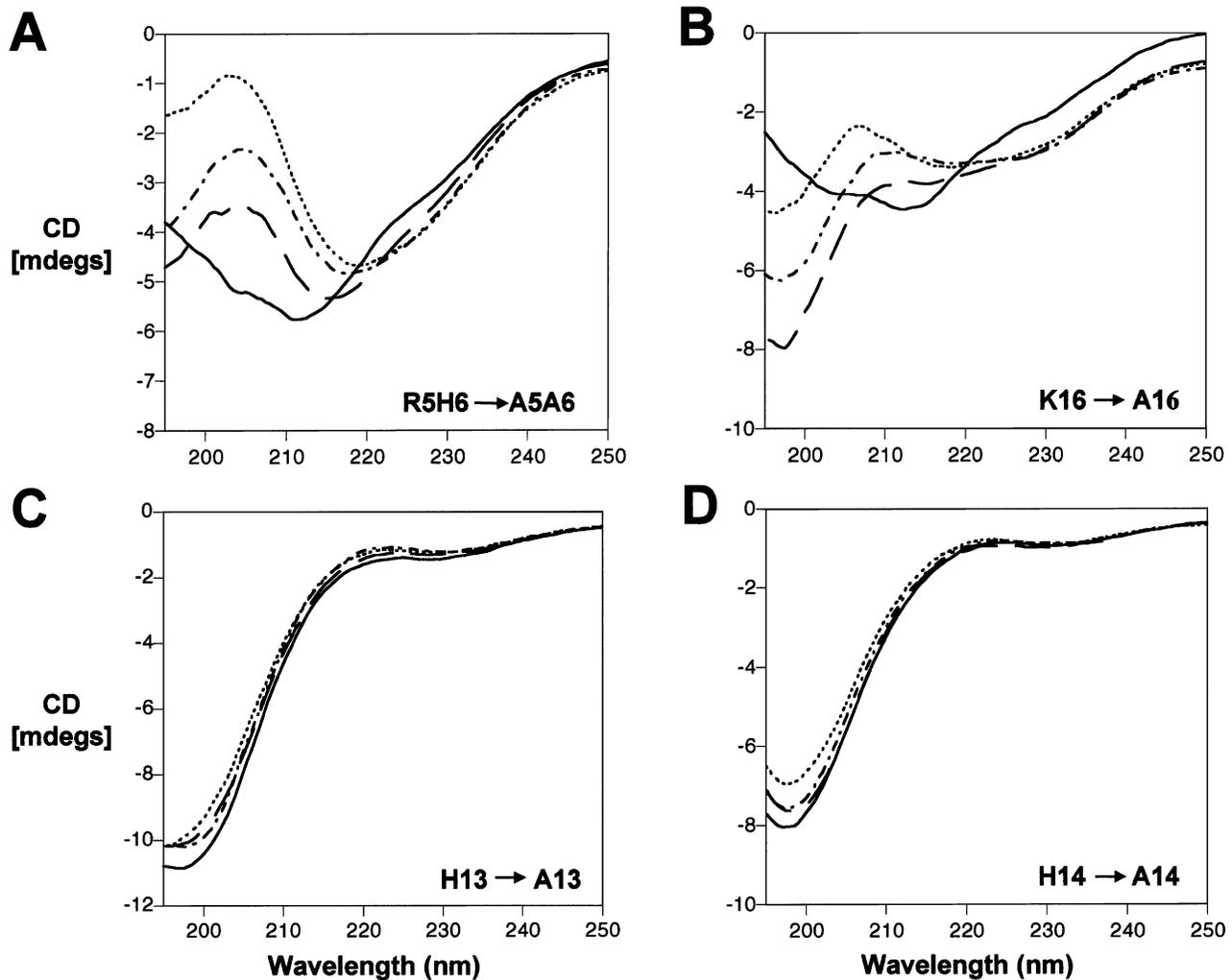


**Fig. 2. Secondary structure of A $\beta$  peptides.** CD spectra of wild type A $\beta$ 1–28 (A) and A $\beta$ 1–40 (B) demonstrating the random coil conformation of both peptides when dissolved in aqueous buffer (solid line) and the conversion to  $\beta$ -sheet conformation in the presence of Zn $^{2+}$ . Peptide concentrations were 55  $\mu$ M with the zinc concentrations varying from 110  $\mu$ M (---), 275  $\mu$ M (-----) in (A), and 275  $\mu$ M (...) in (B).

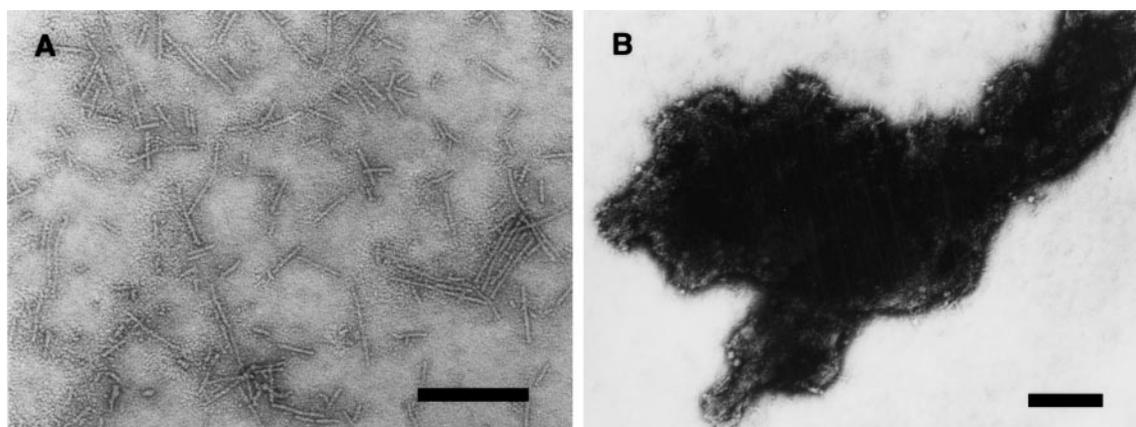
completely converted to a  $\beta$ -structure in the presence of Zn $^{2+}$  (Fig. 2B). These findings are consistent with previous aggregation studies of zinc with A $\beta$  peptide fragments. With the exception of the A $\beta$ 40/42 C-terminal carboxylate, the 1–28

fragment contains all the residues (carboxylates and histidines) which are potential zinc ligands.

We examined the effects of amino acid substitutions on the interactions of A $\beta$  with Zn $^{2+}$  to evaluate the contributions to



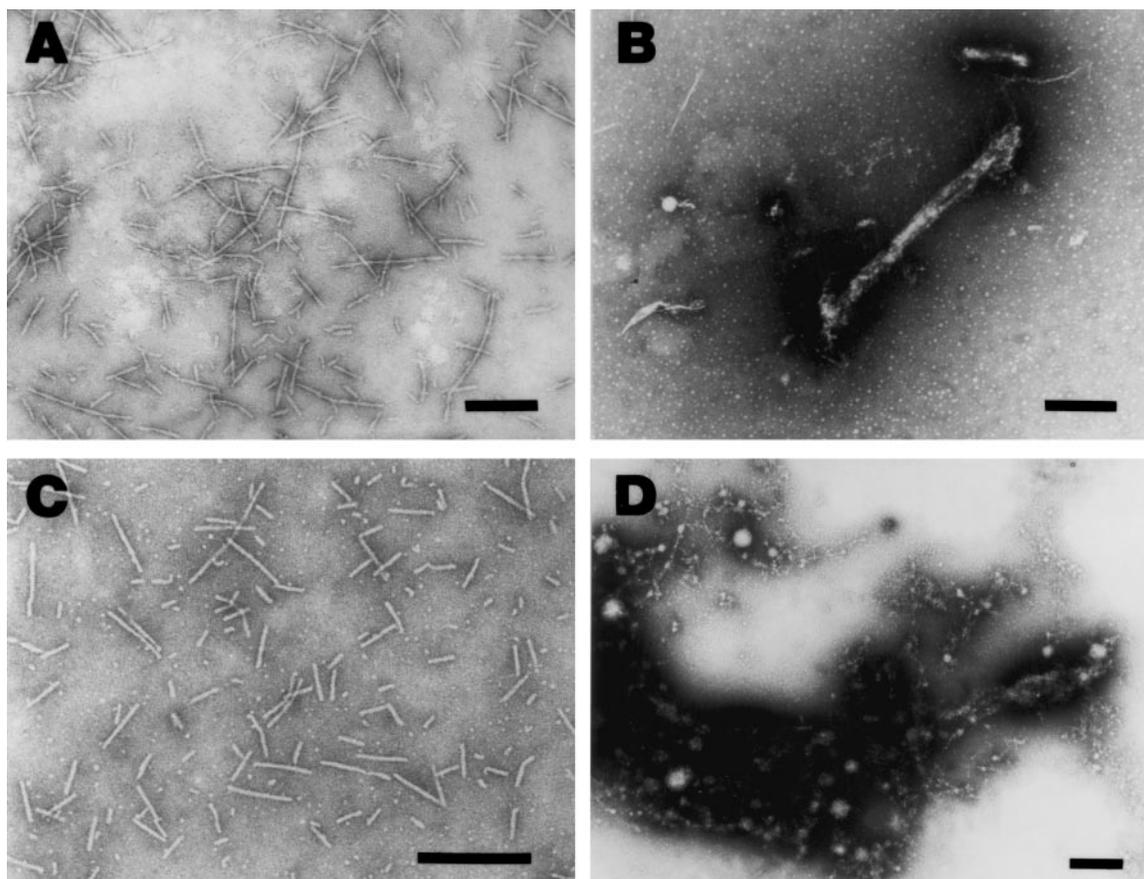
**Fig. 3. Zn $^{2+}$ -induced conformational changes.** CD spectra of alanine substituted peptides in the presence and absence of Zn $^{2+}$  demonstrating the conformational transitions from random coil to  $\beta$ -sheet structure for the R5H6/A5A6 and K16/A16 substituted peptides in (A) and (B), and the absence of any detectable change for the H13/A13 and H14/A14 peptides in the presence of Zn $^{2+}$  in (C) and (D). Spectra for control peptides (55  $\mu$ M) in buffer only are shown by solid lines and in combination with zinc at concentrations of 55  $\mu$ M (---), 275  $\mu$ M (-----) and 550  $\mu$ M (.....) in (A and D) and 55  $\mu$ M (---), 550  $\mu$ M (-----) and 1.1 mM (.....) in (B and C).



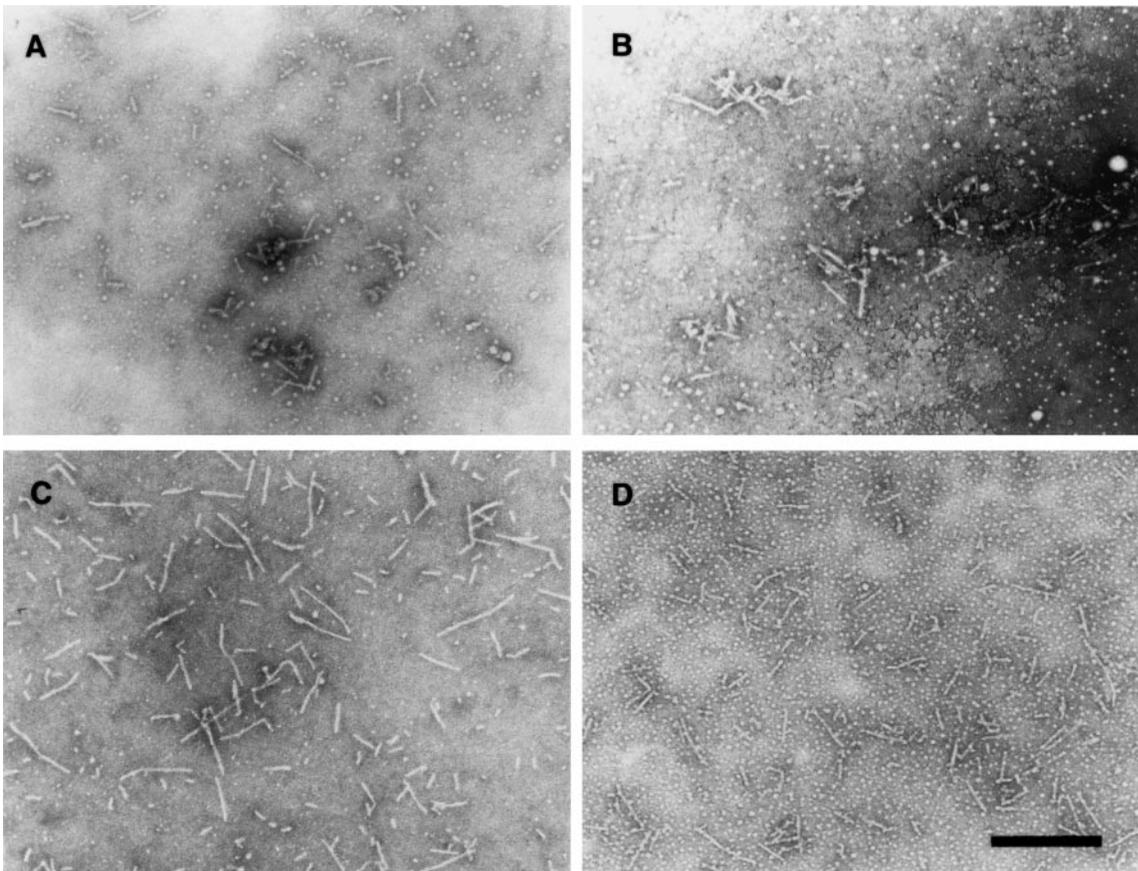
**Fig. 4. Fibril formation of wild type A $\beta$  1–28 peptide.** Electron micrographs of negatively stained preparations of wild type A $\beta$ 1–28 demonstrating the typical morphology of individual fibres 6–9 nm in diameter and of varying length (A). In the presence of Zn $^{2+}$  the transition to large aggregated structures resulting from lateral association of the individual short fibres (B). Scale bars are 200 nm.

this interaction by specific residues. In the absence of Zn $^{2+}$ , both the R5H6/A5A6 and K16/A16 substituted peptides exhibited spectra consistent with a predominately random coil conformation (Fig. 3A,B). However, they contained a small amount of  $\beta$ -sheet conformation and were therefore not a pure random coil as was seen with wild-type A $\beta$ 1–28 and A $\beta$ 1–40. This difference could reflect the presence of some preexisting aggregates or the result of some slight aggregation caused by

the increased hydrophobicity associated with the alanine substitution. However, as with the wild-type A $\beta$  the R5H6/A5A6 peptide and the negative control K16/A16 peptide were converted to a  $\beta$ -sheet conformation upon the addition of Zn $^{2+}$  (Figs 3A and 3B). Therefore Zn $^{2+}$  can still exhibit an effect on the folding and aggregation of A $\beta$  even with the loss of the N-terminal histidine suggesting it is unlikely to represent a critical site for metal binding.



**Fig. 5. Aggregation of A $\beta$  fibrils by Zn $^{2+}$ .** Electron micrographs of negatively stained preparations of the R5H6/A5A6 and K16/A16 substituted peptides. The R5H6/A5A6 formed short, twisted fibres when incubated in buffer alone (A) that underwent significant lateral aggregations in the presence of Zn $^{2+}$  (B). The K16/A16 peptide formed fibres similar to those of the wild type (C) and also displayed extensive aggregates when exposed to Zn $^{2+}$  (D). Scale bars are 200 nm.



**Fig. 6. A $\beta$  histidine residues and Zn<sup>2+</sup> binding.** Electron micrographs of negatively stained preparations of the H13/A13 and H14/A14 substituted peptides. The H13/A13 formed short fibres with reduced numbers when incubated in buffer only (A) and no detectable change in aggregation or morphology was observed upon addition of Zn<sup>2+</sup> (B). The H14/A14 substituted peptide formed fibres similar to those of the wild type in the control buffer (C) and no detectable change in aggregation or morphology was observed in the presence of Zn<sup>2+</sup> (D). Scale bar is 200 nm.

Substitution of the histidines at residues 13 or 14 (H13/H14) resulted in marked changes of the A $\beta$  folding pattern in response to Zn<sup>2+</sup>. CD spectra indicate that both the H13/A13 and H14/A14 substituted peptides adopt a random coil conformation when initially dissolved in aqueous buffer similar to that seen for wild type (Figs 3C and 3D). However, even at much higher concentrations (1.1 mM) zinc failed to induce any detectable  $\beta$ -sheet conformation (Figs 3C and 3D). These results demonstrate that substitutions at either of these sites eliminates the Zn<sup>2+</sup> effects most likely by a disruption of the binding site. These findings confirm a key role for His13 in the zinc interaction as reported by Liu and colleagues [45] in their investigation of the rodent sequence and the effects of the naturally occurring His/Arg substitution at residue 13. Our findings now indicate that both histidines, His13 and His14, in this region constitute binding sites. While these combined observations argue that the histidine residues are essential for binding, they may not constitute the entire binding site. As discussed above, carboxylates may be acting in tandem with the histidines but, in the absence of a high resolution molecular structure or similar substitution studies, it is unclear which Asp/Glu residues may be involved.

### Zn<sup>2+</sup> induced aggregation of amyloid- $\beta$ fibrils

Negative stain electron microscopy was used to investigate Zn<sup>2+</sup>-induced polymerization and/or fibril formation of the peptides and determine which residues were involved. In

control buffers the wild type A $\beta$ 1–28 formed individual short fibres of 6–9 nm in width with lengths varying from 40 to 500 nm with no readily apparent lateral aggregation (Fig. 4A). These are similar to those observed in previous studies [19,46]. Addition of Zn<sup>2+</sup> resulted in a dramatic lateral aggregation of the A $\beta$  fibrils into heavily stained masses (Fig. 4B). The aggregates were composed of numerous densely packed fibres but no significant elongation was observed. This suggests that the zinc binding site may, in part, be located on the surface of the fibrils which would account for the extensive side-to-side aggregation. This is in keeping with previous data indicating that the histidines (residues 13 and 14) may be located at the interface of the subunit protofilaments [47].

Peptides containing the R5H6/A5A6 substitution displayed a similar morphology as the wild-type with the exception of some helical twisting which appeared to be due to the lateral packing of two protofilaments (Fig. 5A). However, these changes did not affect the ability of the peptide to respond to Zn<sup>2+</sup>. This was evident by the extensive lateral aggregation of the individual fibres into large bundles (Fig. 5B). The K16/A16 substituted peptide formed fibres similar to those of the wild type A $\beta$ 1–28 (Fig. 5C). In the presence of Zn<sup>2+</sup>, lateral aggregation was observed but some differences in morphology were apparent. These zinc-induced aggregates were composed of short, thin filaments, globular particles of various sizes and amorphous structures (Fig. 5D). These results suggest that Zn<sup>2+</sup> may induce the underlying fibre subunits (e.g. protofilaments) to undergo a rearrangement in packing before the

formation of aggregates, a mechanism previously proposed [47].

The histidine-to-alanine substitution at position 13 (H13/A13) did not greatly influence the formation of short fibres although some minor reduction in the overall density and length of the filaments was observed (Fig. 6A). More noticeable was the striking contrast to the other alanine substituted peptides as, even at relatively high concentrations of Zn<sup>2+</sup>, the H13/A13 peptide showed no differences in fibril morphology or degree of lateral aggregation (Fig. 6B). This is consistent with the CD data where no conformational changes were observed upon addition of zinc. The H14/A14 substituted peptide assembled into fibres indistinguishable from those formed by the wild type A $\beta$  although some reduction in length was observed (Fig. 6C). Similar to the H13/A13, the introduction of Zn<sup>2+</sup> did not alter fibre morphology or aggregation (Fig. 6D). These results indicate that Zn<sup>2+</sup> does not promote assembly of the H13/A13 or H14/A14 into larger aggregates. This loss of zinc-induced aggregation is most likely due to a disruption of the normal binding site upon removal of these key histidines.

Our investigation examined the role of various residues within the A $\beta$  sequence that facilitates interactions with zinc ions. In addition to other similar metal ions, zinc has been shown to be a potentially important factor in amyloid fibril formation and stabilization of the plaques [30,41]. The mechanism of their action is not entirely clear but could be mediated by an enhanced aggregation and/or stabilization of the deposits leading to a protection against proteolytic degradation. Substitution of the potential histidine ligands in A $\beta$  suggests that residues 13 and 14 represent a critical domain for zinc interaction. These results indicate a mechanism for this event and may represent a means of both understanding and controlling any potential contribution of zinc (or possibly other ions) to the amyloidogenic pathway *in vivo*. Modulation or attenuation of the zinc–A $\beta$  interaction represents a potential therapeutic target and additional information about this process may allow us to design specific agents that may be used to control amyloid plaque deposition.

## ACKNOWLEDGEMENTS

This work was supported by the Ontario Mental Health Foundation (P. E. F.), Scottish Rite Charitable Foundation of Canada (P. E. F.) and the Alzheimer Society of Ontario (P. E. F., D. W., J. M.). Support from Neurochem. Inc. (Montreal, Canada) is also gratefully acknowledged (P. E. F., D. S. Y.). J. M. is the Kevin Burke Memorial Amyloid Research Associate.

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