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The amyloid architecture provides a scaffold for enzyme like catalysts

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Natural biological enzymes possess catalytic sites that are generally surrounded by a large three dimensional scaffold. However, the proportion of the protein molecule that participates in the catalytic reaction is relatively small. The generation of artificial or miniature enzymes has long been a focus of research because enzyme mimetics can be produced with high activity at low cost. These enzymes aim to mimic the active sites without the additional architecture contributed by the protein chain. Previous work has shown that amyloidogenic peptides are able to self assemble to create an active site that is capable of binding zinc and catalysing an esterase reaction. Here, we describe the structural characterisation of a set of designed peptides that form an amyloid like architecture and reveal that their capability to mimic carbonic anhydrase and serve as enzyme like catalysts is related to their ability to self assemble. These amyloid fibril structures can bind the metal ion Zn^{2+} via a three dimensional arrangement of His residues created by the amyloid architecture. Our results suggest that the catalytic efficiency of amyloid like assembly is not only zinc dependent but also depends on an active centre created by the peptides which is, in turn, dependent on the ordered architecture. These fibrils have good esterase activity, and they may serve as good models for the evolution of modern day enzymes. Furthermore, they may be useful in designing self assembling fibrils for applications as metal ion catalysts. This study also demonstrates that the ligands surrounding the catalytic site affect the affinity of the zinc binding site to bind the substrate contributing to the enzymatic activity of the assembled peptides.

Introduction

Many peptide sequences are capable of self assembling to form amyloid like fibrils ¹. Amyloid fibrils are characterised by their regular cross β architecture ² and are associated with diseases such as Alzheimer's, transmissible spongiform encephalopathies, Parkinson's and Diabetes type 2 ³. The classical cross β structure for amyloid has been interpreted from X-ray fibre diffraction providing a characteristic ~ 4.7 to 4.8 Å reflection on the meridian arising from the hydrogen bonded β strands that run perpendicular to the fibre axis and a 10 to 12 Å signal on the equator, arising from the spacing between the β sheets ⁴.

Amyloid fibrils assemble into highly ordered supramolecular structures via hydrogen bonding, and hydrophobic and electrostatic interactions, leading to the formation of extremely stable fibres ^{1,5,6}. Therefore, they are useful for a wide range of applications in biotechnology and material sciences such as hydrogels for tissue engineering ⁷, nanostructured protein films,

nanowires and silica nanotubes ⁸⁻¹². Furthermore, a huge variety of architectures is possible whilst maintaining a core cross- β structure ^{6,13-15}. Self-assembled amyloid-like fibrils have been utilised as functional scaffolds to form active antimicrobial nanobiomaterials ¹⁶, and nano-fibres of amphiphilic peptides were described as a model of esterase hydrolysis activity ¹⁷.

Recently, short synthetic peptide sequences were demonstrated to form amyloid fibrils with esterase catalytic activity upon binding to zinc or cobalt, showing the ability to catalyse *p*-nitrophenyl acetate (*p*-NPA) hydrolysis ¹⁸. The substrate *p*-NPA is a simple chromogenic compound and can be hydrolysed by many hydrolytic enzymes including 3-phosphoglycerdehyde dehydrogenase ¹⁹, pancreatic lipase ²⁰, carbonic anhydrase ²¹, and esterase enzymes ²².

It is well known that the metal ions play a central role in the metal-dependent hydrolase function of many enzymes like carboxyl peptidase, natural proteases, and carbonic anhydrase ²³. The zinc ion is stable in a biological environment and does not contribute to redox reactions ²⁴. It is an ideal cofactor for a Lewis acid-model catalyst such as carbonic anhydrase ²⁵ and has also been suggested to positively affect the protein folding and stability ²⁶. Finally, Zn^{2+} is able to rapidly exchange ligands, which is important to dissociate the intermediate rapidly into the product, and it provides high catalytic efficiency ²⁷. These properties make Zn^{2+} ideal as a catalytic cofactor for native enzymes.

Carbonic anhydrase (CA) is a highly efficient hydrolase enzyme used for the biological confinement of CO_2 ²⁸. The active site of the enzyme CA-II contains one Zn^{2+} with three histidine residues ²⁹. Mechanistically, Zn^{2+} coordinates with a water molecule to

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reversibly form zinc hydroxide and binds two histidine side chains at positions i and $i+2$ from a β strand (His 92 and His 94) and a third histidine from an amino acid residue on a neighbouring strand (His 119). This binding lowers the pK_a of a bound water molecule, stabilising and orienting the hydroxide group for nucleophilic attack on the incoming substrate, CO_2 . The hydrolysis regenerates the enzyme catalyst and liberates bicarbonate. Acidic and basic groups in neighbouring side chains can serve as secondary ligands and assist in protonation/deprotonation steps during these hydrolysis events.

Several studies have explored the ability of selfo assembled peptides to act as catalysts ^{22, 30}. Alternatively, a catalytically active surface has been created by attaching metal ions such as copper(II) ³¹, cobalt (II), nickel(II) ³², and zinc(II) ³³. However, the relationship between the selfo assembled architectures and the catalytic activity remains unclear. Further information may give rise to the ability to design novel catalysts and improve understanding of catalyst activity and the evolution of modern day enzymes.

Here, we report the design, structural characterisation and enzymatic activity of six peptides with seven or eleven residues that have an alternating sequence of hydrophobic isoleucine (I) and hydrophilic histidine (H) amino acids at position i and $i+2$, which upon selfo assembly, create a catalytic binding site. These peptides are amphiphilic and are able to form β sheet structures via hydrophobic interactions. The designs were based on known amyloidogenic sequences ¹⁸. However, the peptide sequences were modified to include a tyrosine (Y) residue which has the potential to form photoo induced cross links via dityrosine ³⁴ and tyrosine may increase the binding affinity to Zn^{2+} . We also designed a longer sequence (11mer) with duplication of the histidine motif to provide the potential for two catalytic binding sites. The selfo assembly and amyloidogenicity of the peptides with and without Zn^{2+} was monitored using transmission electron microscopy (TEM), thioflavine T fluorescence (ThT), tyrosine fluorescence, Fourier transform infrared (FTIR) spectroscopy and circular dichroism (CD). X-ray fibre diffraction (X-ray RFD) patterns from each of the fibril samples were analysed to provide structural information regarding the arrangement of the peptides within the fibrils and to reveal how the threeo dimensional architecture may generate the catalytic site. Enzyme kinetics measurements were collected to correlate sequence, structure and assembly to catalytic activity, providing insights into how the architecture influenced Zn^{2+} binding and how catalytic amino acids are organised with the overall amyloid scaffold.

Experimental

Materials: All $ZnCl_2$, *po* NPA, Fmoc amino acids, resin (Chem Matrix), DIPEA, HBTU, TIPS, TFA and piperidine (99%) were purchased from Sigma Aldrich. DMF and CH_2Cl_2 were purchased from Fisher Chemical and ether (diethyl ether) and Tris base from Fisher Scientific. TEM grids were purchased from Agar Scientific. 0.2 μM filtered Millio Q water was used for all experiments and solutions.

Synthesis of peptides

The peptides were synthesised using microwaveo assisted solid

phase peptide synthesis (CEM Liberty Blue synthesizer), using standard Fmoc solid-phase peptide methodology. For the capped peptides, preloaded Fmoc-protected ChemMatrix® resin (loading of 0.49 mmol/g) was first washed with CH_2Cl_2 -DMF (1:1 v/v). A solution of piperidine (20%) in DMF was used to cleave the Fmoc group. Single coupling of the acids was achieved at 75 °C (25 W initial power) and for 770 s. Activation and coupling were performed using (1*H*-benzotriazol-1-yloxy)(dimethylamino)-*N,N*-dimethylmethaniminium hexafluorophosphate (HBTU) with DIPEA as the base in DMF solvent with standard Fmoc protected α -amino acids. The deprotection was achieved at 75 °C (100 W initial power) with a hold time of around 180 s. Capping of the *N*-terminus and amidation of the *C*-terminus was achieved by treating the peptide-resin with a mixture of acetic anhydride and pyridine in DMF (0.25:0.3:5.0 v/v) with gentle shaking for 30 min. The peptide was cleaved from the resin by treatment of the resin with a mixture of TFA- H_2O -triisopropylsilane (TIPS) (9.5:0.25:0.25 v/v) (10 mL) via the Liberty Blue cleavage system for 40 min under microwave irradiation. After cooling, the mixture was concentrated via a stream of nitrogen and the crude peptides were precipitated by the addition of cold ether, washed with ether and lyophilised.

Peptides with free *N*- and *C*-termini (uncapped) were synthesised in a similar manner using high swelling, low loading Wang resin (loading 0.24 mmol/g). The resin was first swelled in DMF (10 mL) for 15 min, drained and submitted to standard solid phase procedures. The cleavage procedure was then followed as above.

Fmoc-histidine was double-coupled to the growing resin-bound sequence at 2* (75 °C, 25 W, 600 s) to avoid racemic mixture formation by increasing the rate of coupling. Other residues in the sequences were single coupled to the growing chain.

Peptide characterization

a) HPLC

Peptides were injected onto reversed phase HPLC (JASCO) C18 column (details in Supplementary methods).

b) Mass spectrometric analysis

Peptide fractions were analysed by direct infusion mass spectrometry on an Orbitrap-XL instrument (details in Supplementary methods).

Peptide stock and working solution

Peptides were dissolved in 10 mM HCl to produce a 1.1 mM stock solution, which was stable for one week. A peptide solution with or without Zn^{2+} was freshly prepared by mixing the stock solution (180 μL) with 2-propanol (20 μL) and Tris base buffer (25 mM, pH 8) with 1 mM $ZnCl_2$ (1.8 mL) as previously described by others ¹⁸.

Transmission electron microscopy (TEM)

The peptide solution (99 μM , 4 μL) was incubated for 2 min on a grid (400 mesh copper grid with Carbon/Formvar film from Agar Scientific), washed with filtered distilled water and then negatively stained using 2% uranyl acetate (4 μL) for 2 min.

Excess liquid was blotted using filter paper after each incubation. This experiment was repeated over a time course of 10 days. Grids were examined using a Hitachi 7100 electron microscope operated at 80 kV and images were collected on a CCD detector immediately after dissolution (zero time) and after 10 days incubation.

Circular Dichroism (CD)

The peptide buffer solution (99 μM), with and without Zn^{2+} , was monitored after 10 days incubation at 38 °C, using a Jasco J-715 spectropolarimeter with a Peltier temperature control system at 20 °C. The parameters were set as the following: the wavelength was between 180–320 nm, a pitch of 0.1 nm, a scan speed 50 nm/min, response time 4 s, slit widths 1 nm and with standard sensitivity. Each set of data was collected in triplicate. Tris base buffer with and without Zn^{2+} was subtracted from the readings. Spectra were converted to molar ellipticity per residue (MER).

Fourier Transform Infrared spectroscopy (FTIR)

Lyophilised powder peptide samples were placed into the diamond sample holder of the ATR/FTIR instrument, inserted into the photoacoustic cell, and then sealed for IR measurement. Air was used as a control.

Tyrosine Fluorescence Emission

Fluorescence data for peptide samples (99 μM) with and without Zn^{2+} were collected with excitation of λ 280 nm and emission of λ 305 nm over time. Excitation and emission slits were set to 5 nm with a scan rate 600 nm/min at 1 nm data intervals, an average time of 0.1 s and high voltage of photomultiplier (800 V) at 20 °C. Cuvettes were washed before and after use with 2% Hellmanex, water and ethanol, and then airtight dried. All fluorescence measurements were carried out on a Varian Cary Eclipse fluorimeter (Varian Ltd., Oxford, UK) using a 1 cm path length quartz cuvette (Starna, Essex, UK) in triplicate.

Dityrosine Fluorescence emission

Dityrosine fluorescence was monitored using an excitation of λ 320 nm, and emission was at λ 340 and 500 nm, with maximum fluorescence intensity around λ 400–420 nm at a controlled temperature of 20 °C. Excitation and emission slits were both set to 10 nm, and the scan rate was set to 300 nm/min with 2.5 nm data intervals and an averaging time of 0.5 s. The photomultiplier tube detector voltage was set at 500 V. Fluorescence measurements were carried out on a Varian Cary Eclipse fluorimeter (Varian Ltd., Oxford, UK) using a 1 cm path length quartz cuvette (Starna, Essex, UK) in triplicate.

Thioflavin T fluorescence assay (ThT)

A stock ThT solution (1.5 mM in water) was filtered using 0.22 μm Millipore filter and added to the peptide solutions (99 μM) (with and without Zn^{2+}) to a final concentration of 30 μM , gently stirred and incubated for about 5 min to enable binding before reading. The excitation was λ 450 nm and emission intensities at λ 485 nm were plotted against time. Excitation and emission slits were set to 5 and 10 nm, respectively; the scan rate was 600 nm/min with 0.1 s as an average time and 1 nm intervals. ThT was also added to Tris base buffers with and without Zn^{2+} and subtracted from readings as blank solutions.

X-Ray Fibre Diffraction (X-RFD)

Each peptide was allowed to assemble over 3 days at 10 mg/mL with or without Zn^{2+} , and a 10 μL droplet of fibril suspension was placed between two wax-tipped glass capillaries and allowed to align at room temperature in a parafilm sealed petri dish. Partially aligned fibre samples were placed onto goniometer head and data were collected using a Rigaku rotating anode source ($\text{CuK}\alpha$) and Saturn CCD detector with a specimen to detector distances of 50 mm or 100 mm and exposure times of 30 s and 60 s respectively. To ensure fibre alignment, each sample was prepared separately at least twice. The diffraction patterns were examined using Mosflm³⁵ and CLEARER³⁶.

Enzyme Kinetics Reactions

All kinetic measurements were collected using a plate reader (PHERA star F5, BMG LABTECH Spectrum) to obtain the absorbance of the product (*p*-nitrophenol) at λ 348 and 408 nm, at 25 °C in 96-well clear plates.

Effect of substrate concentration [S]: To determine the effect of changing the concentration of substrate [S], a stock of substrate (*p*-NPA) solution in MeCN (0.1 M) was used to prepare fresh working solutions of [S] in 25 mM Tris base, pH 8 with 1 mM ZnCl_2 ¹⁸. The concentration series was between 0.04–1.5 mM for uncapped peptides and 0.1–1.5 mM for capped peptides, in which the final content of MeCN was 2% (v/v) in all reaction mixtures. Similarly, a series of [S] were prepared without Zn^{2+} to test the catalytic efficiency without Zn^{2+} . A volume (50 μL) of the working peptide solution was added to 150 μL of the working [S] solutions (the final peptide concentration was 24 μM), and the product *p*-nitrophenol was monitored at 0.816 s intervals for 30 min against a blank solution containing 150 μL of 1 mM [S] solution and 50 μL buffer (with or without Zn^{2+} , depending on the experiment). The enzyme kinetic measurements were collected following incubation times of 0, 3 and 10 days. Enzyme kinetics of CA were conducted using the procedure described previously³⁷. Briefly, 100 μL of [S] solution was added to 200 μL of the buffered enzyme (prepared in 0.1 M phosphate buffer, pH 7), mixed rapidly and then the reaction was followed using a plate reader against a blank buffer solution for 5 min.

Effect of the zinc concentration [Zn^{2+}]: To test the effect of the ionic strength on the catalytic activity of the peptides, a series of Zn^{2+} concentrations between 0.005 to 0.15 mM (in the well) were used. 100 μL Tris base buffer solution, pH 8 (without Zn^{2+}) was mixed with 50 μL of each Zn^{2+} solution, and then 50 μL peptide solution (24 μM) (aged for 3 days without Zn^{2+} at room temperature) was added. The product was monitored every 17 s for 30 min. [S]=0.4 mM or 0.7 mM for peptide **III** and **IV** respectively.

Effect of the peptide concentration: To analyse the effect of increasing the concentration of peptides on the velocity of the reaction, a series of concentrations (24 to 200 μM in the well) of peptides **III** and **IV** (aged for 10 days with 1 mM Zn^{2+} at 37 °C) were used. 150 μL Tris base buffer solution, pH 8 (with 1 mM Zn^{2+}) was mixed with 50 μL of each peptide solution. The product was monitored every 17 s for 30 min at room temperature. [S]=0.4 mM or 0.7 mM for peptide **III** and **IV** respectively.

All peptides were incubated at 37 °C except where noted. At least three independent runs were performed for each condition with triplicate readings. PRISM software was used to find kinetic parameters (k_{cat} and K_m) by fitting the data to the linear and non-linear regression of the Michaelis–Menten plot.

Table 1. Peptide designs.	
Key	Sequence
I	H- IHIHIYI -OH
II	H- IHIHIYIHIHI -OH
III	Ac- IHIHIYI -NH ₂
IV	Ac- IHIHIYIHIHI -NH ₂
V	Ac- IHIHDYI -NH ₂
VI	Ac- IHIHGYGHIHI -NH ₂

Results

Peptide designs are shown in Table 1. Peptides **I-II** have free *N*- and *C*-termini (uncapped), and **III-VI** peptides (capped) were acetylated at the *N*-terminus and amidated at the *C*-terminus. Capped and uncapped peptides were compared to examine the effect of capping on assembly and influence on the catalytic activity. To explore the effect of peptide length, sequence and structure of assembly and catalytic ability, peptides **I** (H-**IHIHIYI**-OH) and **III** (Ac-**IHIHIYI**-NH₂) with seven residues were compared to **II** (H-**IHIHIYIHIHI**-OH) and **IV** (Ac-**IHIHIYIHIHI**-NH₂) which have 11 residues and a repeat of the IHIHI motif. Peptides **II** and **IV** have the potential for two zinc binding sites. Two further peptides (**V** and **VI**) were designed to examine the impact of the sequence on enzymatic activity and structure. Isoleucine was substituted for aspartic acid at position 5 (**V**), or for glycine at position 5 and 7 (**VI**).

Figure 1. Electron micrographs of self-assembled peptides **I-VI** with and without Zn²⁺.a) at zero time and (b) after 10 days incubation. Peptides (99 μM) were grown in 25 mM Tris base/HCl buffer, pH 8, with and without 1 mM ZnCl₂. Scale bars are 0.2 μm, and 50 nm for enlarged images.

The sequence and composition of the peptides shown in Table 1 were confirmed using reverse phase-semi-preparative HPLC at λ 280 nm and 210 nm and direct infusion MS spectrometry (Figures S1 and S2). Purity is shown in Table S1.

Assembly of designed peptides

All peptides formed narrow fibrils with and without Zn^{2+} after 10 days incubation (Figure 1). TEM showed the appearance of small assemblies immediately after dissolution. In the presence of Zn^{2+} , the uncapped peptide fibrils (**I**, **II**) were generally longer than those of the capped peptides **III** (~40-155 nm), and **IV** (~53-87 nm) after 10 days incubation. Capped peptides (**III**, **IV**) formed very thin fibrils in dense networks (~8 and 5 nm, respectively). Peptide **V** showed no fibrils at time zero (0t) incubation, but some fibrils were observed after 10 days (Figure 1). Peptide **VI** showed many, well-ordered fibrils immediately, and after 10 days incubation (Figure 1).

Circular dichroism (CD) spectroscopy was used to examine the β -sheet content of peptide solutions. All the CD spectra from the assembled peptides showed minima indicating a strong signal for β -sheet structure around λ 218 nm with different intensities. Figure 2 shows CD signals of peptides after 10 days incubation at 37 °C. Incubation with Zn^{2+} increased the intensity at 218 nm for capped peptides (**III** and **IV**) but did not have a significant effect on the signal intensity for uncapped peptides (**I** and **II**) (Figure 2a and b).

Figure 2. CD spectra from peptides after 10 days incubation with Tris base/HCl, pH 8 incubated 37 °C, in the absence (a) and presence of Zn^{2+} (b). Spectra for peptides grown with Zn^{2+} have a more intense β -sheet signal (λ 218 nm) than those grown in the absence of Zn^{2+} . The ellipticity was calculated as molar ellipticity per residue (MER) ($\text{deg.cm}^2.\text{dmol}^{-1}$).

FTIR was used to further confirm the presence of β -sheets. All peptide samples showed a strong Amide I band near ν 1627-1630 cm^{-1} , which indicates β -sheet structures³⁸ (Figure 3). Furthermore, weak bands were observed closer to ν 1668-1660 cm^{-1} from peptides **I**, **II**, **IV** and **VI**, which may be attributed to the presence of antiparallel β -sheets³⁹. The shorter peptides, **III** and **V**, did not clearly show this band, which may indicate parallel β -sheets. Amide II bands were observed between ν 1548-1516 cm^{-1} for all peptides. A broad absorption/transition was observed at ν 3700–2400 cm^{-1} , which could be attributed to –OH and –NH groups engaged in hydrogen bond stretching⁴⁰. All peptide designs showed transition peaks of around ν 32740

3267 cm^{-1} which could be attributed to the expected hydrogen bonding involved in the fibril structure (Figure S3, Table S2). CD and FTIR spectroscopic analysis thus confirmed that all peptides formed a β -sheet structure in the presence or absence of Zn^{2+} .

Figure 3. Fourier transform infrared spectra of peptides **I-VI** show the amide absorption region indicating the presence of a β -sheet structure with minor bands that can be attributed to anti-parallel β -sheets. Dotted lines are shown to highlight differences between peptide spectra.

Figure 4. Fluorescence emission intensities over 25 days in the absence (top) and the presence of Zn^{2+} (bottom). a) Tyrosine fluorescence for peptide **III**, b) dityrosine fluorescence for each peptide, c) ThT fluorescence for each peptide over incubation time of 25 days.

Tyrosine fluorescence (excitation at λ 280 nm, emission at λ 305 nm) was measured to monitor the change in the environment of the tyrosine during peptide assembly. However, very little change was observed over time for these peptides and this was likely to be due to the rapid assembly of the peptides at time zero (Figure 1). Only peptide **III** showed a decreasing signal for tyrosine at λ 305 nm (Figure 4a) which may indicate some structural rearrangement took place during maturation of the fibrils. The intensity at 305 nm was stronger in the presence of Zn^{2+} (Figure 4a) which could imply the formation of dityrosine crosslinks. The presence of dityrosine cross-links was detected using an excitation wavelength of λ 320 nm and the dityrosine signal appeared at λ 400-410 nm (Figure 4b) only in the absence of Zn^{2+} and increased over time. In the presence of Zn^{2+} , this signal was not observed for any of the peptides suggesting that the tyrosine may prefer water-bridge binding with Zn^{2+} rather than crosslinking. The strongest dityrosine signal was observed

for peptides **I** and **IV**, weaker for **II** and much weaker signals were observed for peptides, **III**, **V** and **VI**. Thioflavin T (ThT) fluorescence is used to follow the assembly of amyloidogenic peptides over time⁴¹. All peptides showed a positive intensity at λ 483-485 nm consistent with the presence of amyloid fibrils. In the presence of Zn^{2+} , the ThT fluorescence did not show any significant increase over time (Figure 4c), despite clear evidence of fibril formation from TEM images. This may be due to rapid fibril formation or alternatively due to the ability of Zn^{2+} to coordinate with the anilino group of the ThT, which could quench the emission signal. In the absence of Zn^{2+} , peptides **II** and **VI** appear to have very high-intensity signals over time, peptide **I** and **III** have high-intensity signals at the earliest time point, whilst peptides **IV** and **V** show a steady but slow increase over 25 days incubation, which could be due to the rapid fibrillation rate, consistent with the observations from TEM.

around 3.8 Å which is generally thought to arise from the distance between αC in the β -pleated sheet². Differences in the degree of the alignment of the fibril samples gave rise to some differences in the number of the reflections observed (Figure 5). However, comparison of the reflection positions for the peptides incubated in the presence and absence of Zn^{2+} reveals no significant differences, supporting the view that although Zn^{2+} plays an important role in the assembly process and catalytic activity, it does not have a significant effect on the architecture of the fibrils. All the fibrils gave rise to a diffraction signal on the equator at 22-23 Å which is surprising when comparing the seven residue and 11 residue peptides since we might expect these to differ in chain length (Figure 5, Table S3). The diffraction signal that could be attributed to the β -sheet spacing was also similar between peptides at 11-12 Å. Only peptide **I** in the absence of Zn^{2+} showed a narrower spacing at 10 Å. Further

Figure 5. X-Ray fibre diffraction patterns from partially aligned fibrils formed by each peptide after three days incubation (10 mg/mL in water), with and without Zn^{2+} . a) Peptide **I**, b) peptide **II**, c) peptide **III**, d) peptide **IV**, e) peptide **V**, f) peptide **VI**. The meridional reflections are marked with an M which is parallel to the fibre axis, and the equatorial direction which is perpendicular to the fiber axis, is marked by E.

To further explore the structures of the amyloid fibrils formed by the different peptides grown in the presence and absence of Zn^{2+} , diffraction data were collected and analysed using CLEARER³⁶. This was used to examine whether there were any structural differences between fibrils formed by the different catalytic peptides sequences. The diffraction patterns and their diffraction signals are shown in Figure 5, Table S3.

A diffraction signal was observed at around 4.7 Å on the meridional axis of each diffraction pattern and arises from the hydrogen bonding distance between β -strands that run perpendicular to the fibre axis. Equatorial diffraction signals were observed at 10-11 Å and 22-23 Å and correspond to the sheet spacing, and chain length perpendicular to the fibre axis, respectively (Figure 5, Table S3). The peptides gave a similarly strong signal from the H-bonding distance between strands at approximately 4.70 to 4.72 Å. Off meridionals were observed at

reflections were observed on the equator at 8 Å and 6 Å.

Enzyme activity of the peptides

The enzyme activity for each peptide was determined in the presence of Zn^{2+} by measuring the hydrolysis of *p*-NPA to give *p*-nitrophenol and acetic acid. The rate of the hydrolysis of *p*-NPA by the uncapped peptides **I**, **II** was extremely slow after fibrillogenesis for time zero and 10 days (Figure S4a-b), despite the formation of fibrils by peptides **I** and **II** (Figure 1). Peptide **V** did not form well-defined fibrils at time zero but fibrils were evident by day 10 (Figure 1). However despite this, the fibrils formed by peptide **V** were not able to catalyse the hydrolysis reaction (Figure S4c). Therefore, peptides **I**, **II** and **V** appear to be unable to hydrolyse *p*-NPA under the conditions used.

The enzyme kinetics revealed that the capped peptides (**III**, **IV** and **VI**) followed Michaelis-Menten kinetics in the presence of Zn^{2+} and peptide **III** showed the highest activity (Table 2). In the absence of Zn^{2+} , peptide **III** showed virtually no activity, (Figure S4d). Error bars (Figure 6) are sometimes quite large and this variation between experiments may be due to difficulties in ensuring even distribution of fibrillar material. However, analysis of the data available for peptide **III** suggested a turnover number (k_{cat}) $8.04 \times 10^3 \pm 0.0004 \text{ s}^{-1}$, and the rate of interaction of S and the active site (the catalytic efficiency $k_{\text{cat}}/K_{\text{m}}$) was $\sim 138 \pm 4.0 \text{ M}^{-1} \text{ s}^{-1}$ at time zero peptide incubation. The efficiency of the catalytic reaction appeared to increase after incubating the peptide for 10 days to $355.0 \pm 15.00 \text{ M}^{-1} \text{ s}^{-1}$, with a higher affinity toward the active centre revealing that the catalytic activity increased by about 6.5 fold over time. This may accompany increased assembly and ordering of the fibrils derived from peptide **III** and this would be consistent with the results from tyrosine fluorescence which showed structural rearrangement in the tyrosine environment with time (Figure 4a). In contrast, peptide **IV** showed decreased catalytic efficiency over the incubation period which decreased slightly to $49.00 \pm 8.00 \text{ M}^{-1} \text{ s}^{-1}$ after 10 days (Figure 6b, Table 2). The efficiency/mole of enzyme of peptide **III** was calculated from the data to be higher than that of $66 \mu\text{M}$ CA of human erythrocytes (experimental value), which catalyses the hydrolysis of *p*-NPA with a catalytic efficiency of $604.88 \pm 1.838 \text{ M}^{-1} \text{ s}^{-1}$ when compared on a molar basis (Peptide **III** 29.58×10^4 compared to 4.83×10^4 for CA) (Figure 6d and e, Table 2). No catalytic activities were observed for any

Figure 6. Catalytic experiments using peptides **III**, **IV** and **VI** over 10 days. a) peptide **III**, b) peptide **IV**, c) peptide **VI**. All peptides were used at $24 \mu\text{M}$ concentration in the well. d) Carbonic anhydrase (CA), e) the catalytic efficiency/mole of peptides versus that of CA. All peptides were incubated in 25 mM Tris-base/HCl, pH 8 with 1 mM ZnCl_2 at 37°C . Peptides **III-VI** represent Michael-Menten equation fitting, and steady-state kinetics. Error bars refer to the standard deviation of at least three runs, each in triplicate.

In order to investigate the dependence of catalytic activity on Zn^{2+} binding, titration of the Zn^{2+} was performed for peptides **III** and **IV**. The activity increased linearly until the stoichiometry of binding to peptide **III** reached $\sim 1:1$ (peptide: Zn^{2+}) and to peptide **IV** was $\sim 1:2$ (peptide: Zn^{2+}). After that, no increase was observed (Figure 7a). This finding supports the design

Figure 7. Dependence of the hydrolysis rate of *p*-NPA on the concentration of ZnCl_2 and the concentration of peptides **III** and **IV**. a) Peptide **III** ($24 \mu\text{M}$) shows 1:1 peptide: Zn^{2+} stoichiometry, while peptide stoichiometry of **IV** ($24 \mu\text{M}$) is 1:2 (peptide: Zn^{2+}). b) The dependence of the catalytic activities of **III** and **IV** on the precursor peptide concentration grown at 37°C for 10 days. Error bars refer to the standard deviation of at least two runs, in triplicate readings.

hypothesis that peptide **III** binds one Zn ion the active site, while peptide **IV** has the potential for two structurally distinct sites within a pair of peptide molecules. Both peptides **III** and **IV** displayed a linear increase in the catalytic rate as peptide concentration increased, showing the expected concentration dependence for an enzyme (Figure 7b).

Discussion

The design of seven-residue peptides that assemble to form an active site to coordinate Zn^{2+} , and catalyse an esterase reaction was previously reported¹⁸. We have built upon these designs to add new features and to conduct an in-depth study of the structures that accompany high catalytic ability. Short 7mer peptides (**I**, **III**, **V**) were designed as model peptides to investigate the binding to Zn^{2+} , self-assembled structure and hydrolysis activity compared to longer 11mer peptides (**II**, **IV**, **VI**). The eleven residue peptides **II**, and **IV** were rationally designed to have the potential to bind two Zn^{2+} atoms and potentially have two active sites. It was predicted that if this was the case, the catalytic activity of longer peptides may be higher than the seven residue peptides. The non-polar isoleucine residues were engaged in the sequence to drive the assembly through hydrophobic interactions⁴². In addition, we would predict that peptide self-assembly would be enhanced by the alternating incorporation of polar, charged histidine residues⁴³. Furthermore, histidine residues are well known to coordinate metals and promote Zn^{2+} binding. Zn^{2+} would then act as a co-factor to participate in catalysis^{18, 33, 44, 45}. Tyrosine was introduced into our peptides at position six in the sequence as it is well known to promote amyloid assembly^{1, 15, 46} and also to have the ability to form covalent dityrosine cross-links in fibrillar structures^{47, 48}, thus further stabilizing the fibrils. Dityrosine cross-links may be promoted in presence of metals such as copper, but may also form spontaneously by photo-oxidation³⁴.

All the designed peptides (Table 1) had the ability to self-assemble to form amyloid fibrils and they shared similar morphologies upon analysis by electron microscopy (Figure 1). Their β -sheet structure was confirmed by CD and FTIR spectroscopic analyses (Figure 2 & 3). The amyloid-like

structure of the fibrils was confirmed by positive intensity in ThT binding experiments although only peptide **III** showed self assembly over time due to the very rapid nature of fibrillogenesis by the peptides at time zero (Figure 1 & 4). All fibrils showed the crosso β Xo RFD pattern expected for amyloid fibrils ² (Figure 5). Surprisingly, the presence or absence of zinc appeared to have no significant effect on the morphology of the fibrils or their architecture by Xo RFD, although there was some indication from TEM that the fibrils were slightly longer in the absence of Zn^{2+} (Figure 1). CD data revealed a stronger β sheet signal at λ 218 nm for those peptides incubated with Zn^{2+} . Fibrils formed in the absence of Zn^{2+} were able to form dityrosine crosso links, which were not detected in those that were formed with Zn^{2+} (Figure 4).

Xo RFD diffraction data revealed that all the peptides self assembled to form crosso β amyloid fibrils and these shared a significant number of reflections. In particular, all the peptide fibrils showed the characteristic diffraction signals at 4.7 Å on the meridian and at 11o 12 Å on the equator (Figure 5). All the peptides shared the lowero angle reflection on the equator at 22o 23 Å which was surprising since we would expect that the chain length reflection would vary between the 7mer and 11mer peptides. This suggests that the peptides fold and pack into very similar architectures. Furthermore, we did not observe any significant differences between the architectures of capped and uncapped peptides, which suggests that capped peptides do not form a different packing arrangement from uncapped precursors (Table 4). Zn^{2+} did not have any observable effect on diffraction signals from the fibril structures (Table S3). Diffraction data from peptide **III** fibrils were analysed and the data used to produce two possible models to highlight the catalytic and Zn^{2+} binding site, in which the peptides are arranged in two parallel β sheets, 12 Å apart. In the first model, the peptides are arranged antiparallel across the sheet and in the second, they are parallel. This generates two possible arrangements of the side chains in which the histidine residues can coordinate with a potential zinc ion (Figure 8). The models cannot be distinguished from one another using Xo ray data, but both fit the diffraction data well (see supplementary information and Figure S5). Further lateral assembly of the peptides would be expected to bury the hydrophobic surface of the peptides and to

Recently, solid state NMR (ssNMR) and structural bioinformatics studies have been combined to elucidate the geometry of Zn^{2+} coordinating to a catalytic amyloid peptide ⁴⁹. Results suggested that β -strands were arranged in a parallel fashion and assembled into bilayers with an alternating polar and apolar interface. The hydrated polar interface is mediated by Zn/water ligands to interact with the substrate. Zn^{2+} coordinated by three His from two adjacent strands leaving a free site on the Zn^{2+} for water activation. A His from one strand can bridge two Zn^{2+} (each half of the imidazole ring coordinates one Zn^{2+} with another His from the neighbouring strand). The β -sheets stack with anti-parallel orientation where the hydrophobic residues face one other to promote the 3D assembly. This ssNMR structure is consistent with the model presented here.

The catalytic activities were temperature, Zn-concentration, and substrate concentration-dependent and followed Michaelis-Menten kinetics. Peptides assembled in the absence of Zn^{2+} and the uncapped peptides showed no significant catalytic activity (Figure S4). Aspartic acid in peptide **V** disrupted the fibrillar catalytic activity possibly due to altered binding to Zn^{2+} and/or substrate. In contrast, capped peptides demonstrated notable significant catalytic activity and peptide **III** displayed catalytic activity, which increased with self-assembly incubation time. This supports the view that the activity is closely linked to the self-assembly ability and the production of an active site within the three-dimensional fibrillar structure. Acetylation of the *N*-termini and amidation of the *C*-termini may stabilize the structure and prevents the charge-charge interactions between the peptide termini and also perhaps with Zn^{2+} . This result is in good agreement with previous studies ¹⁸.

Overall, the neutralisation of the *N*- and *C*-termini with the sequence of alternating isoleucine and histidine promotes the formation of an active site and a binding site for Zn^{2+} within the self-assembled fibrillar structure. We compared the catalytic activity of a 7mer and 11mer peptide but revealed that the 7mer peptide **III** possessed a higher activity than the longer peptide. This finding leads us to suggest two potential models for Zn-binding for folded peptide **IV**. It may form an anti-parallel β -hairpin structure, or remain as an extended β -strand that hydrogen bonds to an adjacent peptide. The two peptides may differ in their binding to Zn^{2+} within the active site and this is reinforced by the results of the zinc titration study. Studying the influence of Zn^{2+} on the catalytic activity of peptide **III** revealed a stoichiometry of 1:1 (Zn^{2+} :peptide) at which point the peptide showed no further increase in velocity of the reaction. Results from peptide **IV** showed a stoichiometry of 2:1 (Zn^{2+} : peptide) (Figure 7). This finding suggests that one peptides of **III** bind to one Zn^{2+} , whilst one peptide of **IV** coordinates two Zn^{2+} . If Zn^{2+} is able to bind in two positions within the 11mer peptide, this may cause disruption of the active site and reduction of activity. Furthermore, in molar basis, fibrillar peptide **III** activity appeared to be higher than CA activity (Figure 6e and Table 2). Based on data from X-RFD and Zn titration data, we were able to model the architecture (Figure 8)

Within the fibrillar structure formed by the peptide **III**, it is reasonable that an imidazole-zinc species is activated through a water molecule to enable quaternary coordination geometry of Zn (His)₃-OH which directly reacts with the acetate group of *p*-NPA, to form a complex of acylium-Zn-imidazole. In the presence of a water molecule, this complex will dissociate to

Figure 8. The proposed models of peptides **III** in the presence of zinc built from X-RFD data. View down the fibre axis (into page). Both models show two parallel β -sheets. Left hand panel shows β -sheets in an antiparallel orientation and right hand panel shows β -sheets in a parallel orientation. Models were created using Pymol (1.7.4.4).

regenerate the imidazole-zinc and liberate acetic acid (see Scheme 1). It has been suggested that an anionic ligand is required to activate the imidazole-zinc species of the active centre of CA⁵⁰, therefore Tyr engaged at position 6 in the peptide design could aid protonation. Moreover, inspired by the active site of CA where histidine 64 acts a shuttle group to aid the deprotonation step⁴⁴, this role could be fulfilled by the third histidine in the peptide design that is coordinated with Zn²⁺. Zinc acts as a powerful electrophilic catalyst since it activates the water molecule for nucleophilic attack, lowers the energy of the C=O π^* orbital adjacent to the scissile bond and stabilizes the negative charge in the transition state^{51,52}.

Peptide **V** was not able to act as a catalyst and showed no activity over the course of the study under the conditions used (Figure S4c). Peptide **V** has an aspartic acid residue at position 5

Cabr

Scheme 1. The proposed mechanism of hydrolysis for the substrate p-NPA by fibrils of peptide **III**

Conclusions

This study aimed to explore the fibrillogenesis, fibre structure, zinc binding and catalytic activity of designed seven-residue or eleven-residue peptides with or without capped ends. We have shown that all peptides in the presence and absence of Zn²⁺ are able to self-assemble to form amyloid fibrils and that these show similar architectures. However, the binding of Zn²⁺ is critical to the catalytic ability and only those peptides with capped ends were able to catalyze an esterase reaction. We have shown that one of the seven residue designed peptides forms highly ordered fibrils over time that are able to catalyze the reaction with very high efficiency. The three-dimensional organization of the active site is provided by the self-assembled, amyloid-like architecture.

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in place of isoleucine and the introduction of a large acidic side chain in place of an aliphatic group may disrupt the formation of the catalytic site or may compromise the binding of the Zn²⁺ ion with the substrate. Interestingly, the peptide is still able to assemble despite the interruption of the alternating charged-hydrophobic motif. The optimal coordination of Zn²⁺ appears to be critical since the longer peptides **IV** and **VI** resulted in lower catalytic efficiency than peptide **III** (Table 2). The catalytic efficiency of the fibrils was also peptide concentration-dependent. The velocities increased linearly as concentration increased (24-200 mM) to give first-order concentration dependencies for both peptides, Figure 7b.

regarding enzyme kinetics measurements. Thanks to Dr Michael Paradowski and Dr Marco Derudas (Sussex Drug Discovery Centre) for useful discussion regarding HPLC. We are very grateful to Dr Natasha Burgess, Dr Drew Thomson and Professor Dek Woolfson from the Chemistry Department, University of Bristol for help with peptide synthesis and Dr Julian Thorpe, University of Sussex for help with TEM.

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Table 2. The catalytic activity of peptides grown in 25 mM Tris-base/HCl at pH 8 in the presence of 1 mM ZnCl₂, incubated for 10 days at 37 °C. The shaded number refers to the highest catalytic efficiency, lower K_m and higher activity per each mole of peptide. The catalytic efficiency (k_{cat}/K_m) indicates the interaction of S with the active centre of E. To clearly compare the catalytic efficiencies of peptides with that of CA, the catalytic efficiency calculated per a mole is shown in the last column. *Carbonic anhydrase.

Peptide	Conc. μM	Time/days	$k_{cat} \cdot 10^{-3}$ (s ⁻¹)	K _m (mM)	Catalytic efficiency (k_{cat}/K_m) (M ⁻¹ s ⁻¹)	Catalytic efficiency/ mole peptide x 10 ⁴
(I)	24	No reaction				-
(II)	24	No reaction				-
(III)	24	0	8.04 ± 0.00038	0.13 ± 0.02	138.50 ± 4.00	11.54
		3	6.44 ± 0.0005	0.05 ± 0.02	168.00 ± 18.51	14.00
		10	8.26 ± 0.0005	0.02 ± 0.001	355.00 ± 15.00	29.58
(IV)	24	0	22.81 ± 0.0030	0.39 ± 0.14	68.00 ± 18.00	5.66
		3	2.72 ± 0.0019	0.36 ± 0.08	89.00 ± 64.00	7.41
		10	16.90 ± 0.0011	0.35 ± 0.17	49.00 ± 8.00	4.83
(V)	24	No reaction				-
(VI)	24	10	55.94 ± 0.0009	0.05 ± 0.05	62.00 ± 20.00	5.16
CA*	66	0	37.97 ± 0.0029	0.12 ± 0.043	604.88 ± 1.84	4.58