Human Pancreatitis-associated Protein Forms Fibrillar Aggregates with a Native-like Conformation*

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Human pancreatitis-associated protein was identified in pathognomonic lesions of Alzheimer disease, a disease characterized by the presence of filamentous protein aggregates. Here, we showed that at physiological pH, human pancreatitis-associated protein forms non-Congo Red-binding, proteinase K-resistant fibrillar aggregates with diameters from 6 up to as large as 68 nm. Interestingly, circular dichroism and Fourier transform infrared spectra showed that, unlike typical amyloid fibrils, which have a cross-β-sheet structure, these aggregates have a very similar secondary structure to that of the native protein, which is composed of two α-helices and eight β-strands, as determined by NMR techniques. Surface structure analysis showed that the positively charged and negatively charged residues were clustered on opposite sides, and strong electrostatic interactions between molecules were therefore very likely, which was confirmed by cross-linking experiments. In addition, several hydrophobic residues were found to constitute a continuous hydrophobic surface. These results and protein aggregation prediction using the TANGO algorithm led us to synthesize peptide Thr84 to Ser116, which, very interestingly, was found to form amyloid-like fibrils with a cross-β structure. Thus, our data suggested that human pancreatitis-associated protein fibrillization is initiated by protein aggregation primarily because of electrostatic interactions, and the loop from residues 84 to 116 may play an important role in the formation of fibrillar aggregates with a native-like conformation.

Many neurodegenerative diseases, such as Alzheimer disease (AD), Huntington’s disease, and the transmissible prion diseases, are characterized by fibrillar deposits of proteins or peptides in the brain (1). Although these amyloidogenic precursor proteins have different amino acid sequences and native folds, the resulting amyloid fibrils are similar in several aspects. They have histochemical staining properties similar to those of starch (e.g. apple-green birefringence of Congo Red (CR)) and are therefore called amyloid fibrils (2) and are proteinase-resistant, thermally stable, typically rigid, straight, unbranched, and 5–13 nm in diameter (3). In addition, the x-ray diffraction pattern suggests that amyloid fibrils are composed of a cross-β structure (4), and this is supported by CD and Fourier transform infrared (FTIR) spectroscopy (5). However, the ability to form amyloid fibrils is not restricted to amyloidogenic precursor proteins. Many other proteins not associated with known diseases also form fibrils with the cross-β-patterns characteristic of amyloid fibrils (6, 7). In addition, many short peptides from proteins, either alone or when bound to globular proteins, form amyloid-like fibrils (8–10). De novo designed peptides have also been shown to form amyloid-like fibrils (11). Mutational studies have revealed a simple correlation between aggregation and physicochemical properties, such as β-sheet propensity, hydrophobicity, and charge (12–14). It is therefore believed that there are certain sequence motifs that are more prone to fibrillize when they are cleaved from the whole protein or once they move from a buried position in the native protein to an exposed position. Several computer algorithms, e.g. the TANGO algorithm of Serrano and co-workers (15) and the Zyggregator algorithm of Dobson and co-workers (16), were therefore developed to identify aggregation-prone regions in the amino acid sequence of a protein. The three-dimensional profile method recently developed by Eisenberg and co-workers (17) can be further used to identify sequences that form amyloid-like fibrils. The establishment of rules that link sequence and amyloid features is critical for the understanding of amyloid diseases and therefore has significant applications.

Pancreatitis-associated protein (PAP), also known as p23, hepatocarcinoma-intestine-pancreas protein, or Reg3-encoded protein (the third member of the Reg family) (18–20), a secretory stress protein synthesized by pancreatic acinar cells during the acute phase of pancreatitis, has been found in several vertebrates, such as the rat and human (21, 22). Human PAP (hPAP) has been detected in several cancer tissues, such as liver and stomach (19, 23), and has been identified as a biomarker for pancreatic ductal adenocarcinoma (24). Several biological functions of PAP have been proposed, including induction of bacterial aggregation (21), stimulation of cell proliferation (20), inhibition of apoptosis (25), and anti-inflammatory properties (26); however, its actual function is not known. PAP shows high...
sequence homology with lithostathine (LIT, also known as pancreatic stone protein or Reg1-encoded protein), another stress protein found in pancreatic juice (27). Both PAP and LIT contain a carbohydrate recognition domain linked to a signal peptide and belong to group VII of the C-type lectin family (28). Rat PAP I and III (an isoform of rat PAP) and LIT can form highly organized fibrillar structures following trypsin cleavage, which removes the N-terminal propeptide and converts the 16-kDa proteins into the 14-kDa mature isofoms (29). However, it is difficult to understand how precipitation properties could serve a useful function in pancreatic physiology. Furthermore, human LIT (hLIT) and hPAP have been identified in the pathognomonic lesions of AD (the extracellular senile plaques and intracellular neurofibrillary tangles) and are highly expressed even during the very early stages of AD before clinical signs appear, showing that they may be involved in AD (30). Thus, hPAP is an important protein for exploring the relationship between neurodegenerative diseases and protein deposition. Here, we used electron microscopy (EM), atomic force microscopy (AFM), glutaraldehyde cross-linking, optical absorbance, CD, fluorescence, FTIR, and NMR spectroscopy to investigate the structural properties of recombinant hPAP lacking the N-terminal propeptide, both in the soluble form and as fibrillar aggregates. Based on the solution structure analysis and the TANGO prediction, peptide hPAP84–116 was synthesized and found to form fibrils. The mechanism of fibril formation by hPAP is proposed and compared with that of hLIT.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant hPAP**—Recombinant hPAP was prepared as described previously (31). Briefly, the DNA sequence encoding hPAP in which the N-terminal 12 residues are replaced by the sequence MKHHHHHHQ was obtained by growing the cells in M9 minimum medium containing the desired15N-laabeled amino acid (0.1 g/liter) and deuterated hPAP were obtained from the resin obtained by growing the cells in M9 minimum medium containing the desired15N-laabeled amino acid (0.1 g/liter), and deuterated hPAP were obtained from the resin. 100 μM NaHPO4, 50 mM NaCl, pH 7.4 as described previously (32). hPAP aggregates (100 μg/ml) or hPAP84–116 aggregates (100 μg/ml) were mixed for 30 min at room temperature with CR (15 μg/ml) and then the absorption was corrected by subtracting the spec-

**Preparation of Peptide hPAP84–116**—Peptide hPAP84–116 was synthesized in automated mode on a solid phase synthesizer (model 431 A; Applied Biosystems), cleaved from the resin with trifluoroacetic acid, and purified by reverse phase high pressure liquid chromatography. The authenticity of the peptide was determined by electron spray/mass spectrometry.

**Preparation of Fibrils**—hPAP fibrillar aggregates were prepared by incubating hPAP (10 μM) in 20 mM NaHPO4, 50 mM NaCl, pH 7.5, for 1 month. To check the proteinase K resistance, hPAP fibrils were mixed with proteinase K (2.5 ng/ml) for 6 h at room temperature. hPAP84–116 fibrils were prepared by incubating the peptide (10 μM) in 20 mM NaHPO4, 50 mM NaCl, pH 4.0, for 2 weeks. All of the fibrillar aggregates were collected by centrifugation for 60 min at 13,000 × g and resuspended in double distilled H2O. This procedure was repeated three times, and the collected aggregates were stored at −20 °C until needed.

**Electron Microscopy and Atomic Force Microscopy**—Samples of hPAP (10 μM) in 20 mM NaHPO4, 50 mM NaCl at different pH values (pH 4–7) were incubated for 1 month with gentle shaking, and the aggregates were collected as described above. For EM, the samples were applied to Formvar-coated copper grids (200 and 400 mesh) for 5 min, negatively stained with 2% phosphotungstic acid (w/v) for 3 min, and the images were viewed in a JEOL JEM 1200EX transmission electron microscope operating at 80 kV. For AFM, the samples were applied onto a freshly cleaved mica surface for 10 min, washed three times with double distilled H2O, and air-dried at room temperature, and the bound material was imaged with the SPM-A-100 AFM (AngsNanoTek Co., Ltd., Taipei, Taiwan) using Tapping Mode in air. The images were collected at an optimized scan rate corresponding to 0.7–1 Hz and were flattened and presented in height mode using SPM-A-100 Imaging Process software (AngsNanoTek Co., Ltd.).

**Fourier Transform Infrared Spectroscopy**—To compare soluble and fibrillar hPAP, thin film attenuated total reflectance FTIR spectra were recorded (33). 50 μl of 1 mg/ml solution of soluble hPAP freshly prepared at pH 7 and suspension of fibrillar hPAP incubated at pH 7 for 1 month were deposited on ZnSe ATR crystal plates (50 × 10 × 2 mm; Harrick, Ossining, NY) and air-dried to form a hydrated thin film. It had been shown that the formation of a hydrated thin film does not disrupt the native structure of proteins (34, 35). FTIR spectra were recorded on a Nicolet Nexus 470 spectrometer continuously purged with dried nitrogen. The attenuated total reflection mode was selected with a spectral resolution of 2 cm−1. Resid-

**Circular Dichroism Spectra**—Soluble hPAP and hPAP84–116 were diluted to a final concentration of 20 μM in 20 mM NaHPO4, 50 mM NaCl at the desired pH. Fibrillar hPAP and hPAP84–116 were prepared by incubating the samples at desired pH for a period that is sufficient for fibril formation and col-
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lected by centrifugation as described above. The fibrillar samples were resuspended in double-distilled H₂O before acquiring the spectra. All of the CD spectra were collected on an Aviv CD 202 spectrometer (Lakewood, NJ) at 25 °C. For far-UV CD spectra, a 1-mm-path length cuvette was used, and the spectra were recorded three times from 190 to 260 nm with a wavelength step of 0.5 nm. For near-UV experiments, a 10-mm-path length cuvette was used, and the spectra were recorded three times from 320 to 250 nm with a wavelength step of 0.5 nm. Thermal denaturation experiments were carried out by monitoring the changes in ellipticity at 220 nm from 15 to 95 °C with a 2 °C interval and 2 min for equilibrium. The curves were fitted and analyzed using SigmaPlot 8.02 (SPSS Inc.).

Fluorescence Spectra—hPAP was diluted to a final concentration of 10 μM in 20 mM NaH₂PO₄, 50 mM NaCl at the desired pH and mixed with 1 μM 1-anilinonaphthalene-8-sulfonic acid (ANS). The fluorescence spectra were acquired using a 10-mm quartz cell at 25 °C on an LS55 spectrofluorometer (PerkinElmer Life Sciences). The excitation wavelength was 365 nm, and the emission spectra were recorded from 440 to 600 nm with a slit width of 5 nm.

Glutaraldehyde Cross-linking Assay—hPAP was diluted to a concentration of 0.1 mg/ml in 20 mM NaH₂PO₄, 50 mM NaCl at pH 4, 5, 6, or 7. Two more samples at pH 7 were prepared at higher concentrations of NaCl (150 mM and 500 mM). All of the samples were then mixed for 5 min at room temperature with 0.25% (w/v) glutaraldehyde (Sigma) and then analyzed by 15% SDS-PAGE.

NMR Spectroscopy and Resonance Assignments—NMR experiments on ¹⁵N,¹³C-labeled and ²H,¹⁵N,¹³C-labeled hPAP in 50 mM NaH₂PO₄, 100 mM NaCl, pH 4.0, were carried out at 298 K on Bruker AVANCE 600 and 800 NMR spectrometers (Bruker, Karlsruhe, Germany) equipped with a triple (¹H,¹³C, and ¹⁵N) resonance cryoprobe including a shielded z-gradient. The NMR spectra of, and sequential assignment for, hPAP were carried out as described previously (36). In brief, the triple-resonance experiments (HNCOC, HN(CA)CO, CBCA(CO)NH, and HNCACB) were used for backbone resonance assignment and the aliphatic ¹³C and ¹H side chain resonances were determined from the three-dimensional C(CO)NH, HBBH(CO)NH, and HCCH-TOCSY spectra. Aromatic resonances were assigned as described (37). All of the NMR spectra were processed using the NMRPipe package (38) and analyzed using NMRView 5.0 (39).

Structure Determination of hPAP—The dihedral angle information was predicted using the program TALOS (40). The hydrogen bonding information was obtained from D₂O exchange monitored by the ¹H-¹⁵N HSQC spectra. The nuclear Overhauser effect restraints from the ¹H-¹⁵N NOESY-HSQC and ²H-¹³C NOESY-HSQC spectra were automatically assigned by the CANDID module of CYANA (41) and checked manually. NMR structures were calculated from all experimental restraints by simulated annealing using the program Xplor-NIH. The final 20 structures with no distance restraint violation greater than 0.3 Å, and no dihedral angle restraint violations larger than 3° were chosen on the basis of total energy. The quality of structures was analyzed using the program PROCHECK-NMR (42).

Data Bank Accession Number—The chemical shifts of recombinant hPAP at pH 4.0 and 298 K have been deposited in the BioMagResBank under accession number BMRB-6231. The best 20 structures, together with the complete list of restraints, have been deposited in the Brookhaven Protein Data Bank under accession number 2GOO.

RESULTS

hPAP Forms Non-Congo Red-binding, Proteinase K-resistant Fibrillar Aggregates at Physiological pH—Fibril formation by hPAP at different pH values was examined using EM and AFM. The hPAP samples were incubated for 1 month in buffers from pH 4.0 to 8.0. EM showed that, at pH 4.0, hPAP only formed circular aggregates (Fig. 1A), which had diameters ranging from 6 to 30 nm and were prone to clustering. At pH 5.0 (Fig. 1B), short protofibrillar aggregates were also observed, with diameters of around 6 nm, i.e. smaller than most of the circular aggregates. At pH 6.0 (Fig. 1C), abundant long, straight, unbranched protofibrils, as well as circular aggregates, were observed. At pH 7.0, these protofibrils assembled with each other to form thicker fibrils, as shown by EM (Fig. 1, D–F) and AFM (Fig. 1G). These thicker fibrils had different shapes and thicknesses; the thickest having a diameter of 68 nm (Fig. 1F), which is rare in typical amyloid fibrils. At pH 8, the EM images of fibrillar aggregates were similar with those observed at pH 6 (data not shown). We then examined whether the aggregates formed by hPAP have the characteristics of amyloid fibrils.

Because amyloid fibrils deposited in tissue as a result of disease are highly protease-resistant (1), the protease resistance of the hPAP fibrillar aggregates was tested. hPAP incubated at pH 7.0 for 1 week was exposed for different periods of time to proteinase K, a broad spectrum protease that is highly active under these conditions, and the extent of digestion was monitored by SDS-PAGE (data not shown) and EM. As shown in Fig. 1H, after 6 h of digestion, protofibrillar aggregates could still be seen, whereas circular aggregates were virtually absent, demonstrating that the fibrillar aggregates of hPAP are compactly assembled and highly proteinase K-resistant, whereas the circular aggregates are accessible to proteinase K.

Congo Red is widely used as a histological indicator of amyloid fibril deposition. The binding of CR to amyloid fibrils has been characterized and shown to depend on the presence of extensive arrays of β-pleated sheet (2). For example, after CR binds to the cross-β structure of Aβ fibrils, its absorbance peak shifts from 485 nm to 512 and 541 nm (32). However, in our study, the absorbance spectrum of CR when mixed with hPAP fibrillar aggregates was similar to that of CR alone (Fig. 2A), indicating that CR does not bind to the aggregates, suggesting that they may not have a typical β-pleated sheet structure.

The Secondary Structure of hPAP Fibrillar Aggregates Resembles That of the Native Protein—The structural properties of hPAP fibrillar aggregates were studied by FTIR and CD spectroscopy, both of which have been widely used in structural studies of protein aggregates, for example, to examine the structural rearrangement of prion protein HET-s from a soluble α-helical protein to β-sheet fibril aggregates (5). FTIR and far-UV CD spectra give information on the average secondary structure of the peptide chain, whereas the near-UV CD spectra
can monitor the environment of aromatic side chains, especially Trp. The hPAP fibrillar aggregates had almost the same FTIR spectrum as soluble hPAP at pH 7 (Fig. 2B). Deconvolution of the amide I region showed that both spectra had two major peaks at 1658 and 1639 cm\(^{-1}\) (Fig. 2B, inset), corresponding, respectively, to \(\alpha\)-helix and \(\beta\)-sheet. Multiple linear regression analysis showed that the hPAP fibrillar aggregates contained 19% \(\alpha\)-helix and 34% \(\beta\)-sheet, whereas soluble hPAP contained 20% \(\alpha\)-helix and 36% \(\beta\)-sheet. The far-UV CD spectra of soluble and aggregated hPAP were also basically similar (Fig. 3A), the only difference being the absorption at 230 nm, which may be associated with Trp side chains (43). Secondary structures were estimated using CDPro software (44) and gave values of 19 ± 2% \(\alpha\)-helix and 37 ± 5% \(\beta\)-sheet for hPAP aggre-
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Figure 3. Circular dichroism and ANS fluorescence spectra of soluble and fibrillar hPAP. A, far-UV CD spectra of soluble hPAP (20 μM) at pH 4.0 (black), 5.0 (red), 6.0 (green), 7.0 (yellow), and 8.0 (blue). The dashed line shows the signal for hPAP fibrillar aggregates (20 μM) at pH 7.0. B, near-UV CD spectra of the same samples shown in A. C, temperature denaturation experiments on soluble hPAP followed by CD at 216 nm for the same samples show in A. D, ANS fluorescence spectrum of soluble hPAP mixed with 1 μM ANS. All of the samples were freshly prepared, and the fluorescence spectra were recorded using excitation at 365 nm.

Gates and 17 ± 1% α-helix and 41 ± 5% β-sheet for soluble hPAP at pH 7. The near-UV CD spectra of fibrillar aggregates and soluble protein had similar minima at 297, 289, and 283 nm (Fig. 3B). However, the extra minimum seen at 275 nm in the aggregates indicated that the orientation of one or more Trp side chains were different. In conclusion, the FTIR and CD spectra suggest that hPAP retains a native-like secondary structure when assembled into fibrillar aggregates and that assembly perturbs the side chain orientation of one or more Trp residues.

Soluble hPAP Protein Has a Similar Conformation over the pH range of 4–8—The conformational features of soluble hPAP and soluble protein had similar minima at 297, 289, and 283 nm (Fig. 3B). However, the extra minimum seen at 275 nm in the aggregates indicated that the orientation of one or more Trp side chains were different. In conclusion, the FTIR and CD spectra suggest that hPAP retains a native-like secondary structure when assembled into fibrillar aggregates and that assembly perturbs the side chain orientation of one or more Trp residues.

Soluble hPAP forms oligomers at physiological pH—The oligomerization of soluble hPAP was investigated by glutaraldehyde cross-linking test. Glutaraldehyde reacts with the amino group of the Lys side chain and has been extensively used to cross-link oligomeric proteins (45). Soluble hPAP at different pH values was incubated for 5 min with 0.25% glutaraldehyde, and then the oligomeric state was analyzed by SDS-PAGE. As shown in Fig. 4, hPAP clearly formed oligomers at physiological pH. Furthermore, increasing the NaCl concentration lowered the degree of oligomerization, indicating that electrostatic interaction is an important factor for the formation of hPAP oligomers. These oligomers may then undergo conformational rearrangement to form fibrillar aggregates.

Solution Structure of hPAP—As described previously, hPAP has similar structural features at pH 4.0 and 7.0 but tends to
aggregate at pH 7.0. We therefore decided to solve the solution structure of hPAP at pH 4.0. Using 70% deuterated hPAP (Fig. 5A) and hPAP 15N-labeled with specific amino acids (Fig. 5B), we overcame the difficulty of NMR analysis arising from protein aggregation and completed most of the resonance assignment, which has been deposited in the BioMagResBank under accession number BMRB-6231 (31).

NMR solution structures for hPAP were calculated using 1708 NMR experimental restraints including 1493 nuclear Overhauser effect interproton distances, 52 hydrogen bonds, and 163 backbone \( \phi \) and \( \psi \) dihedral angles. An ensemble of 20 NMR structures was selected on the basis of the overall energy, and the structure possessing the lowest energy was chosen as the representative structure. The root mean square deviation of the 20 NMR structures superimposed on the averaged structure was 0.49 ± 0.08 Å for backbone atoms and 1.21 ± 0.14 Å for all heavy atoms (Fig. 6A). The structural statistics for the NMR structures are listed in Table 1.

hPAP folds into an \( \alpha + \beta \) structure and consists of two \( \alpha \)-helices and eight \( \beta \)-strands cross-linked by three disulfide bridges packed into a heart-like shape, similar to that of the C-type lectin domain (Fig. 6B). However, hPAP was found to have a unique distribution of charged residues on the surface. Nine of the eleven acidic residues (Asp37, Asp39, Glu58, Asp82, Glu88, Glu92, Glu95, Asp100, and Glu149) were found to be clustered on one side, whereas twelve of the thirteen basic residues (Arg13, Lys16, Lys19, Lys33, Lys44, Arg45, Lys67, Arg109, Arg125, Arg131, Lys133, and Arg140) were located on the other side (Fig. 7B). This surface charge distribution results in a highly polarized protein molecule, suggesting strong electrostatic interactions between molecules when incubated at physiological pH, consistent with the glutaraldehyde cross-linking results. In addition, several hydrophobic residues, such as residues Leu28–Pro32 and Val101–Trp107, were found to be highly exposed and to constitute a continuous hydrophobic surface (Fig. 7C). The hydrophobic loop Val101–Trp107 is located between the negatively charged and positively charged surfaces and is especially interesting, because the TANGO program predicts that this region in hPAP shows high propensity for \( \beta \)-aggregation. Because hPAP forms fibrils with a native-like structure, it is possible that some conformational rearrangements in the loop regions that do not perturb the native secondary structure may drive the assembly of protein into fibril.

**Aggregation Behavior of hPAP84–116** —The peptide hPAP84–116 (residues Thr84–Ser116), which contains two long loops of hPAP, was synthesized and tested for fibril formation. As shown in Fig. 8A, the CD spectra indicated that hPAP84–116 was initially unstructured when dissolved in buffers at pH 4.0 and 7.0 but that, after 2 weeks the secondary structure of hPAP84–116 incubated at pH 4.0 transformed from random coil to \( \beta \)-structure, whereas the peptide incubated at pH 7 remained unfolded.

**FIGURE 5.** NMR spectra of labeled hPAP. A, one-dimensional spectra of 15N,13C-labeled (black) or 2H,15N,13C-labeled hPAP (red). The deuteration was about 70%, as calculated from the intensity of the aliphatic signals. B, 1H,15N-HSQC spectra of uniformly 15N labeled (black), 15N-Leu labeled (blue), 15N-Val labeled (red), or 15N-Tyr labeled (green) hPAP. Several small peaks of metabolic side products were also observed.
hPAP$_{84-116}$ incubated at pH 4.0 for 2 weeks also showed an increase in the absorption signal of CR at 512 and 541 nm (Fig. 8B), which is characteristic of the binding of CR to the cross-$\beta$ structure of amyloid fibrils (32). EM showed that hPAP$_{84-116}$ formed fibrils after incubation at pH 4.0 for 2 weeks (Fig. 8, C and D). In summary, hPAP$_{84-116}$ forms amyloid-like fibrils after incubation at acidic pH for 2 weeks.

### DISCUSSION

A large number of neurodegenerative diseases, such as AD, are associated with slow onset amyloid fibril deposition in the brain. In the present work, we showed that hPAP, an AD-related protein, formed fibrillar aggregates with a native-like structure at neutral pH and that these aggregates had several unique characteristics distinct from those of amyloid fibrils. We also showed that the peptide hPAP$_{84-116}$, which covers an exposed hydrophobic loop of hPAP, formed amyloid-like fibrils at pH 4.0, a pH at which hPAP remained soluble for a long time. These unique characteristics and the possible mechanisms by which hPAP forms fibrillar aggregates are discussed below.

The detailed pathways followed by a protein or peptide going from its soluble state to amyloid fibrils have remained elusive because of the complex nature of the process. However, many studies have suggested some general principles governing protein aggregation. For example, Aβ, tau, and some natively nonfolded peptides start to form fibrils when they gain a critical ordered $\beta$-structure, whereas globular proteins, such as lysozyme, transform into $\beta$-rich fibrils when their secondary structures are destabilized (46, 47). Although these amyloidogenic precursor peptides or proteins have different amino acid sequences and native folds, the resulting amyloid fibrils are composed of a cross-$\beta$ structure. However, in some cases, the proteins were found to form fibrils with a native-like structure (48–52) and even native-like activity (53, 54). Several studies indicated that the short segment of the protein that becomes exposed and has a tendency to form $\beta$-structure can stack on top of a cross-$\beta$ spine and the fibril grows with the stacking of this short segment (10, 54–56). For example, the structure model of the yeast prion Ure2p filament showed that the N-terminal segment of the protein forms a parallel superpleated $\beta$-structure backbone and that the linker region connects the surrounding globular C-terminal domains to the N-terminal fibril backbone (56). There is also convincing evidence that a cross-$\beta$ spine with three-dimensional domain swapping is present in the fibrils of a designed RNase A (54). In addition to the models of cross-$\beta$ spine, the direct stacking model is also proposed for fibril formation (48, 52). The solvent accessibility of transthyretin amyloid probed by solution NMR in combination with hydrogen/deuterium exchange suggested that strands C and D are dislocated from their native edge region and become a solvent-exposed loop, leaving a new interface involving rest of the native $\beta$-strands for intermolecular interactions.

### TABLE 1

| Constraints used                  | Number of structures in the final set | X-PLOR energy (kcal.mol$^{-1}$) |
|-----------------------------------|--------------------------------------|---------------------------------|
| Nuclear Overhauser effect distance restraints | 20                                   | $E_{\text{NOE}} \pm 2.4$       |
| Sequential $| \pm 1 | 460                                   | $E_{\text{Dih}} \pm 0.5$       |
| Medium range $| \pm 5 | 427                                   | $E_{\text{Long}} \pm 3.6$      |
| Total nuclear Overhauser effect distance restraints | 1493                                  | $E_{\text{Ang}} \pm 2.4$       |
| Hydrogen bonds                    | 52 × 2                                | $E_{\text{Low}} \pm 4.3$       |
| Dihedral angles                   | 163                                   |                                 |

### Statistics for the final X-PLOR structures

- Number of structures in the final set: 20
- X-PLOR energy (kcal.mol$^{-1}$): $E_{\text{NOE}} = 62.7 \pm 2.4$, $E_{\text{Dih}} = 6.9 \pm 0.5$, $E_{\text{Long}} = 155.5 \pm 3.6$, $E_{\text{Ang}} = 126.5 \pm 4.3$
- Mean global root mean square deviation (Å): 0.49 ± 0.08 for all backbone atoms, 1.21 ± 0.14 for all heavy atoms
- Ramachandran plot statistics:
  - Residues in most favored regions (%): 69.9
  - Residues in allowed regions (%): 26.2
  - Residues in generously allowed regions (%): 3.2
  - Residues in disallowed regions (%): 0.7
interactions (48). However, these fibrils with native-like structures are formed by either direct stacking or stacking with a cross-β spine; they do bind to Congo Red (54, 57–59).

In our study, formation of hPAP fibrillar aggregates was seen at pH levels above 5.0 and was maximal at pH 7.0. Increasing the salt concentration decreased the level of oligomerization and thus hindered the formation of fibrils. The secondary and tertiary structures of hPAP were not pH-sensitive in the range 4.0 to 8.0. Furthermore, the far-UV CD and FTIR spectra suggested that the hPAP fibrillar aggregates had a similar secondary structure to that of native protein, and the near-UV CD spectra showed that the orientation of Trp residues was different in the fibril-form and soluble hPAP. Finally, we showed that peptide hPAP84–116 formed amyloid-like fibrils at acidic pH. All of these results demonstrate that hPAP forms fibrillar aggregates in the absence of a partially folded or destabilized intermediate. Instead, it assembled into fibrils with a native-like conformation. hPAP oligomerization may be initiated by electrostatic interactions between charged residues and further stabilized by the interactions of hydrophobic residues on the surface. Two kinds of stacking modes are possible. First, some residues from Thr84 to Ser116 may transform into a short β-strand, and hPAP fibril with a native-like conformation grows by the stacking of this short β-strand on top of the cross-β spine. This β-strand may be too short to increase the total percentage of β structure. It may also be surrounded by native structure from the binding of Congo Red. However, it is also possible that hPAPs stack directly with each other to form

**FIGURE 7. Comparison of hPAP and hLIT. A**, sequence alignment of hPAP and hLIT (identity, 47%). The secondary structures of hPAP are shown, and the trypsin cleavage site is indicated by a red arrow. Residue numbers for hPAP are shown above the sequences, and those for hLIT are below the sequences. The electrostatic potentials of the molecule surface of hPAP (B) and hLIT (D) are shown with a similar orientation. Negative potentials are colored red, and positive potentials are blue. The hydrophobic surfaces of hPAP (C) and hLIT (E) are also shown with a similar orientation. The protein surface is shown in green, and exposed hydrophobic residues (Ala, Val, Leu, Ile, Pro, Phe, Tyr, and Trp) are in yellow.
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There is also a discrepancy between the conditions in which hPAP and hPAP^84–116 form fibrils. hPAP formed fibrillar aggregates at neutral pH, whereas hPAP^84–116 formed amyloid-like fibrils at acidic pH. Studies on the aggregation behavior of unfolded peptides have revealed that an increase in hydrophobicity can accelerate aggregation and that the introduction of charged residues can reduce aggregation (12–14). The pI value for hPAP^84–116 is about 4.3. At neutral pH, the huge repulsive electrostatic forces between unfolded hPAP^84–116 will be very effective at opposing aggregation. However, the negative charges of this loop in hPAP can be neutralized by the positive charges on another hPAP molecule at neutral pH, and the electrostatic interaction may draw protein molecules close, facilitating the intermolecular interactions for fibril formation.

hLIT is a homologue of hPAP. They show 47% sequence identity (Fig. 7A) and high structural similarity (the root mean square deviation between the two structures is 0.91 Å for backbone atoms in the secondary structure region). hLIT forms non-Congo Red-binding, proteinase K-resistant fibrillar aggregates with native-like structure after removal of the N-terminal propeptide (60, 61). Based on the x-ray structure, on the high resolution electron microscopic images of protofibrils, and on other biochemical studies, a structural model of the hLIT protofibril, called the quadruple-helical filament (QHF-litho), has been proposed (60). In this model, hLIT forms tetramers by intermolecular interactions between the N-terminal residues (Ile^12–Ser^23) and between the hydrophobic residues (Tyr^24–Phe^28 and Phe^136–Phe^142), and the elongation of the stacked tetramer involves longitudinal electrostatic interactions between acidic residues (Glu^30–Glu^33 and Asp^72–Asp^73) and basic residues (His^81–His^90 and Lys^100). The model consists of seven repeated monomers with a pitch of 18 nm and a diameter of 10 nm. In our studies of hPAP, EM showed that the diameter of the hPAP protofibril was about 6 nm, suggesting the formation of dimers. The N-terminal region of hPAP contains three positively charged residues (Arg^13, Lys^16, and Lys^19), which would cause strong electrostatic repulsion between hPAP molecules if they were stacked into a tetramer, as in hLIT. In addition, although both hLIT and hPAP have a highly polarized surface structure, their surface charge distributions are different (Fig. 7B and D). The acidic region (residues 30–33 and 72–73) of hLIT is replaced by a hydrophobic region (Leu^30, Pro^32, and Tyr^73) in hPAP, whereas the basic region of residues 81–90 in hLIT is replaced by a longer and acidic loop (His^81–Glu^95) in hPAP (Fig. 7A). Thus, the longitudinal electrostatic interactions between hPAP molecules would not be strong if hPAP assembled into fibrils with a similar orientation to that in other biochemical studies.

![FIGURE 8. Formation of amyloid-like fibrils by hPAP^84–116. A, far-UV CD spectra of hPAP^84–116 (20 μM) at pH 7.0 (solid line) and 4.0 (dashed line). The spectra of freshly prepared samples are shown in gray, and that of samples incubated for 2 weeks are shown in black. B, absorption spectra of CR (solid line), CR mixed with hPAP^84–116 previously incubated for 2 weeks at pH 7 (dotted line), or CR mixed with hPAP^84–116 previously incubated at pH 4 for 2 weeks (dashed line). The concentrations of CR and peptide were 15 and 10 μM, respectively. C and D, EM images of the amyloid-like fibrils formed by hPAP^84–116. The obvious helical twist can be observed in D. The scale bars represent 200 nm in C and 50 nm in D.](http://example.com/fig8.png)
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the QHF-litho model. In addition, in contrast to that for hPAP^{14–116}, the TANGO-predicted aggregation score for the corresponding sequence in hLT is zero. This may due to the replacement of Phe^{65} in hPAP by Lys^{100} in hLT. Thus, although hPAP and hLT share high sequence homology and similar properties of their fibrillar aggregates, they may not have a similar mechanism of fibril formation.

The detailed model of hPAP fibrillar aggregates requires further investigation. Hydrogen/deuterium exchange combined with two-dimensional NMR is a powerful technique for mapping the core of the amyloid fibril (62,63), and we are currently applying it to hPAP fibrils. The hPAP protofibrils can form super-complex fibers at physiological pH, and the diameter of these fibers can be increased if incubation is continued for a longer time. This property may make the hPAP fiber a good target for x-ray diffraction studies if it can be grown to a size in the micrometer range, like the microcrystal formed by the amyloid peptide GNNQNY (10) or the partially aligned fibrous crystal formed by a designed amyloid peptide (64). In addition, the super-complex aggregates may have applications in bio-nanotechnology. For example, the amyloid fibrils of the yeast prion protein, Sup35, can produce long, stable conducting nanowires (65). The review article by Waterhouse and Gerrard (66) indicates that one of the challenges in the production of amyloid-based nanomaterials is the macroscopic assembly of the basic fibril form into complex, higher ordered structures. The hPAP fibrillar aggregate provides a good target for the investigation of the process by which protofibrils form a higher ordered super-complex.

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