



Effects of pH on aggregation kinetics of the repeat domain of a functional amyloid, Pmel17

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Pmel17 is a functional amyloidogenic protein whose fibrils act as scaffolds for pigment deposition in human skin and eyes. We have used the repeat domain (RPT, residues 315–444), an essential luminal polypeptide region of Pmel17, as a model system to study conformational changes from soluble unstructured monomers to β -sheet-containing fibrils. Specifically, we report on the effects of solution pH (4–7) mimicking pH conditions of melanosomes, acidic organelles where Pmel17 fibrils are formed. Local, secondary, and fibril structure were monitored via intrinsic Trp fluorescence, circular dichroism spectroscopy, and transmission electron microscopy, respectively. We find that W423 is a highly sensitive probe of amyloid assembly with spectral features reflecting local conformational and fibril morphological changes. A critical pH regime (5 ± 0.5) was identified for fibril formation suggesting the involvement of at least three carboxylic acids in the structural rearrangement necessary for aggregation. Moreover, we demonstrate that RPT fibril morphology can be transformed directly by changing solution pH. Based on these results, we propose that intramelanosomal pH regulates Pmel17 amyloid formation and its subsequent dissolution *in vivo*.

fibril | fluorescence | melanosome | tryptophan | melanin

Though amyloid structure is mostly recognized for its involvement in human diseases such as Alzheimer's and Parkinson's disease, it is striking to find that amyloid fibrils can also serve essential biological roles (1–5). A central question is why functional amyloids are benign whereas their disease-related counterparts are harmful. Specifically, it is not clear if functional amyloidogenic proteins are not cytotoxic because they circumvent misfolded fibrillar precursors such as spheres and annuli (6), or because their amyloid formation and degradation are efficiently regulated by the cell. Thus, detailed studies on amyloid formation kinetics as well as the solution conditions and biomolecules that impact this assembly are required. In this work, we examined aggregation kinetics and fibrillar structures of a crucial polypeptide fragment, the repeat domain (RPT) of the functional amyloid, Pmel17, whose fibrils serve as the structural scaffolding required for melanin deposition in human skin and eyes (7). In particular, we sought to determine how solution pH mediates fibril formation because melanosomes, organelles where Pmel17 fibrils are formed, are acidic compartments that change pH during maturation (8).

Melanin is synthesized in melanosomes, organelles related to both endosomes and lysosomes, and stored in melanocytes, cells responsible for pigmentation (7). The rate-limiting step in melanin synthesis is catalyzed by tyrosinase. In this reaction, tyrosine is converted to dihydroxyphenylalanine (DOPA) which is then transformed to dopaquinone and readily oxidized to 5,6-dihydroxyindole followed by indole 5,6-quinone: melanin is the polymerized product. Importantly, Pmel17 fibrils have been proposed to sequester and detoxify these potent cytotoxic precursors (9).

The melanosome maturation process involves four distinct stages that have been characterized at the ultrastructural level by transmission electron microscopy (TEM) (10). Fibrous structures begin to form in stage I with aggregation completed by stage

II (7). Melanin is produced and deposited during stages, III and IV (7). While melanosome ultrastructure is known, the molecular nature of Pmel17 fibrils and how intramelanosomal solution conditions mediate fibril formation remain to be elucidated. Interestingly, it is known that solution pH changes with melanosome maturation with stages I and II being the most acidic and later stages III and IV reaching near neutral pH (11, 12). Tyrosinase activity is also pH dependent *in vitro* (13), with the final step in melanin polymerization slowed under acidic conditions (14) suggesting that melanin synthesis indeed is more likely to occur in the mature melanosomes near neutral pH. Because pH mediates melanin synthesis and has been shown to affect the aggregation of disease-related amyloidogenic polypeptides *in vitro* (1), we suggest that solution pH also could influence melanosomal Pmel17 fibril formation.

The Pmel17 gene was first discovered in mice in 1930 (15); although, it was not until 1991 when it was named and mapped to the *silver* locus (16). Because its overexpression in nonpigmented cells produces late endosomes exhibiting indistinguishable fibrous striations from those found in melanosomes (17), Pmel17 was implicated as the main protein component in melanosomal fibrils. Furthermore, when fibrils are not present, mice present a silver genotype, hence corroborating the biological function of Pmel17 in melanin production (18). The 668-residue polypeptide, Pmel17, is composed of a large luminal domain containing several subdomains with short C-terminal transmembrane (35 aa) and cytoplasmic segments (30 aa) (18). Posttranslational proteolytic processing of Pmel17 is critical for function producing respective C- and N-terminal fragments, M β and M α (19), the latter reported to form amyloid *in vitro* (9). *In vivo*, subsequent cleavage of M α results in M α N and M α C, where the C-terminal fragment, M α C is believed to contain the fibril-forming region; however, neither the precise sequence of M α N or M α C is fully identified (20–23). Though there is no current consensus as to which polypeptide domain solely or partly constitutes the Pmel17 amyloid core, recent results showed that both isolated luminal regions, RPT, residues 315–444 (Fig. 1) (24) and the polycystic kidney disease 1-like domain, residues 201–314 (25) are sufficient for amyloid formation *in vitro*. While both domains are important for fibril formation *in vivo* (26), we choose RPT as a model system to study aggregation kinetics because prior observation revealed that RPT fibril stability is particularly pH sensitive (24). Further, the inability of a closely related, melanosomal glycoprotein, non-metastatic melanoma protein b, which lacks a RPT domain, to form fibers also points to the role of this sequence in amyloid formation (27).

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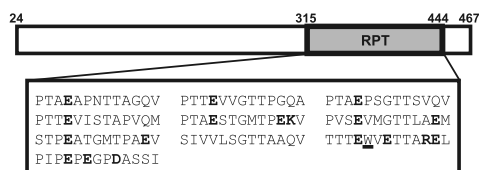


Fig. 1. Schematic representation of the N terminal domain (Ma) of Pmel17 with the repeat domain (RPT, residues 315–444) highlighted. The RPT primary sequence is shown with ionizable side chains in bold. The native fluorophore, W423, utilized in this study is underlined.

Here, we study the RPT conformational change from soluble and unstructured monomers to aggregated, β sheet containing fibrils. To mimic the changing acidic pH conditions of the maturing melanosome (8, 11, 12), we measured RPT amyloid formation kinetics and the conversion of fibril morphology as a function of solution pH (4–7). Because tryptophan emission is highly sensitive to solvent polarity, local conformational changes, and protein protein interactions (28, 29), we exploited the only intrinsic tryptophan (W423) as a site specific fluorescent probe of amyloid structure and aggregation kinetics. We find that W423 is exquisitely sensitive to soluble and fibrillar RPT conformation with spectral properties exhibiting distinct temporal changes under the various solution conditions examined. Complementary techniques, TEM and circular dichroism (CD) spectroscopy, also were employed to characterize fibrillar ultrastructures and secondary structural content, respectively.

Results and Discussion

RPT Amyloid Structure and Aggregation Kinetics. The RPT primary amino acid sequence is comprised of 10 imperfect 13 residue repeats that are rich in Pro, Ser, Thr, and Glu (Fig. 1). RPT contains 16 carboxylates underscoring its propensity to undergo pH induced conformational changes. Because pH and protein structure likely are linked in vivo (*vide supra*), we employed TEM, static light scattering, and a thioflavin T (ThT) fluorescence assay (30) to determine the effects of pH on RPT amyloid formation (Fig. 2). Fibril formation processes generally exhibit sigmoidal kinetic behavior characterized by a lag, growth, and stationary phase (31) and during each of these phases, we made frequent light scattering measurements. TEM was used as a probe of macroscopic morphology before and during the initial growth

phase, and then again after aggregation was completed. As it is typical for amyloid formation kinetics, we observed sample to sample variations in the lag phase, during which time little or no apparent changes in fibril concentration can be detected (32). However, despite greater uncertainties in the absolute lag times, we ascertained that the pH dependent trends (relative rates and respective spectroscopic data) and corresponding fibril morphologies were comparable and reproducible. Specifically, aggregation kinetics are accelerated from pH 5.5 (20–30 h) to 4.0 (<1 h). We find that fibrils are formed only below pH 6.0, consistent with previous observation that RPT fibrils will dissolve in near neutral and basic solutions (24).

At pH 5.5, aged RPT forms long and homogenous fibrils that have moderate ThT fluorescence and light scattering signals (Fig. 2). At the beginning of the fibril growth phase (after 20–30 h of incubation at 37 °C and shaking at 600 rpm), a few large laterally associated small fibrillar species (~70 nm in average length) appear. After growth is complete (~60–100 h), we find primarily straight and long fibrils (>1 μ m in length, ~18 nm in width) reflecting significant restructuring between early growth and stationary phases.

In slightly more acidic environments (pH 5.0–4.5), differences in aggregation kinetics and fibril morphologies are revealed. Greater light scattering and ThT signals are observed as well as increased fibril heterogeneity (Fig. 2 and Fig. S1). By the beginning of the growth phase (~10–15 h) a heterogeneous species appears, containing both fibrillar and amorphous structures of varying sizes and shapes. These early aggregates finally give rise to primarily long (>1 μ m) and short (~125 nm) straight fibers as well as sharply curved fibrous structures (Fig. 2, Right inset). At this intermediate pH, RPT is likely sampling multiple conformations resulting in several distinct intermediates leading to polymorphic fibrils.

In contrast, at pH 4.0 RPT rapidly aggregates (<1 h) with large preformed oligomers observed at initial measurement. While the aggregates form immediately, our TEM images show that there is no conversion to the longer fibrils over time (Fig. 2). These data indicate that RPT adopts a conformation leading to alternative oligomeric structures that preclude fibril elongation. We note that the initial aggregates exhibit a low ThT signal that increases modestly upon aging. This increase suggests that while there are no long fibrils, the mature aggregates do contain some amyloid like features. While CD data confirm that all aged samples

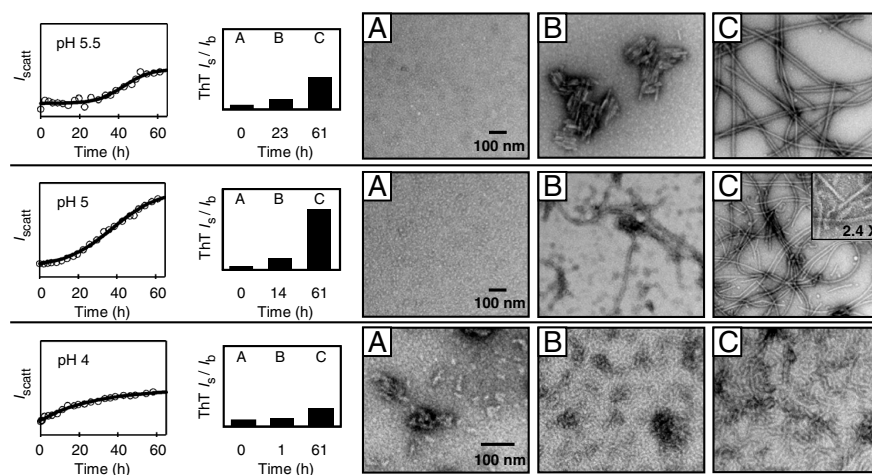


Fig. 2. pH dependent RPT aggregation kinetics and amyloid structures. Light scattering (I_{scatt}) (Left), thioflavin T assay (Center), and TEM images (Right) of RPT (30 μ M) as a function of incubation time at 37 °C in pH 5.5 (Top), 5.0 (Center), and 4.0 (Bottom) buffers (20 mM sodium acetate and 100 mM NaCl). Integrated intensity of ThT emission of RPT sample and buffer alone are denoted as I_s and I_b , respectively. Labels, A, B, and C, denote times of measurement specified in the respective ThT data (Center).

below pH 6.0 exhibit β sheet structure (Fig. S2), pH 4.0 samples have a slightly reduced intensity below 217 nm, reflecting in creased random coil content.

Critical Concentration for RPT Aggregation at pH 5.0. To determine the critical RPT concentration (concentration below which aggregation will not occur) we examined aggregation kinetics at pH 5 as a function of protein concentration. We find that amyloid can form at concentrations as low as 2 μ M. This value likely represents an upper limit given that we are at the current detection limit of our light scattering apparatus. Interestingly, we find that for all protein concentrations examined (2–60 μ M) aggregation kinetics exhibited similar lag times with variable growth rates (Fig. S2). These results suggest that RPT aggregation may not proceed according to a nucleation/polymerization mechanism (33) that is characteristic for disease related amyloid formation processes (34). Alternatively, the lack of lag time reduction as a function of protein concentration could imply that the critical RPT concentration is extremely low.

W423, a Site-Specific Probe of RPT Conformation. We sought to determine how pH influences local protein conformation by fluorescence measurements of the only intrinsic tryptophan in RPT, W423, as a function of solution pH. W423 serves as an excellent structural probe as there are two nearby carboxylates and $\sim 40\%$ of the total carboxylates residing in the last two repeat domains (Fig. 1). Prior to thermal incubation ($T = 0$), we observe variations in the steady state W423 emission as the solution pH is lowered ($7 \rightarrow 4$) (Fig. 3). These changes demonstrate that protonation of C terminal carboxylates indeed affect local protein conformation.

At neutral pH, W423 emission indicates a solvent exposed indole with a mean wavelength ($\langle \lambda \rangle$) of 362 nm comparable to a model complex, N acetyl tryptophanamide (NATA) ($\langle \lambda \rangle = 365$ nm). In more acidic environments, W423 emission blue shifts (Table S1), with an apparent midpoint transition at pH 4.5. As evidenced by TEM (Fig. 2), spectral blue shifts reflect a more hydrophobic environment for W423 as protein protein contacts ensue at pH 4.0.

W423 also exhibits pH dependent changes in integrated emission intensity, I , with a small decrease from pH 7 to 5.5 and a substantial increase from pH 5.5 to 4.0 (Fig. 3). Trp fluorescence quantum yields can be modulated in the presence of charged residues, with proximity to positive (negative) charges corresponding to a quantum yield increase (decrease) (35). It is plausible that observed I changes could reflect nearby carboxylate protonation states as well as intra- and/or interprotein conformational changes. To shed light on these possibilities, we used dynamic light scattering measurements to assess the molecular dimensions of RPT as a function of pH prior to thermal incubation.

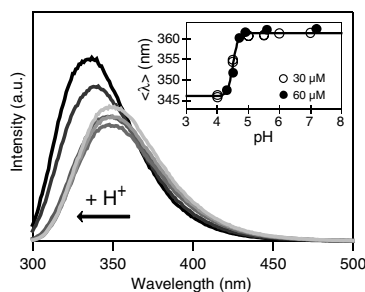


Fig. 3. W423 emission spectra for RPT (60 μ M) in pH 4.0–7.3 (black to gray, respectively) buffers (pH 4.0–5.6: 20 mM sodium acetate, 100 mM NaCl; pH 6.0: 20 mM MES, 100 mM NaCl and pH 7.0–7.3: 20 mM MOPS, 100 mM NaCl). Inset: W423 mean wavelength, $\langle \lambda \rangle$, as a function of solution pH ($[RPT] = 30$ and 60 μ M). Fit is shown as a solid line.

We find that there are pH dependent global structural changes consistent with the presence of larger oligomers at pH 4 [correlation time (τ_c) pH 4.0 > 5.0 $\sim$ 6.0] (Fig. S3). These results support that W423 spectroscopic changes are sensitive to overall protein conformation and its molecular environment.

Using the Henderson Hasselbalch equation, we obtain a $pK_a \sim 4.5$ (Fig. 3, Inset). We find the involvement of at least three protons (n) in this process ($n = 5$ yielded the best fit; but, adequate fits can be obtained for $n \geq 3$), which is reasonable given the close proximity of W423 to several carboxylates (6 total in repeat 9 and 10). CD data confirm that for all solution conditions examined, RPT is unstructured with only minor differences below 230 nm (Fig. S2) demonstrating the utility of W423 to detect even small changes in solution conformations.

W423, a Site-Specific Probe of RPT Amyloid Formation. Subsequent conformational changes and protein protein interactions during aggregation also were monitored by W423 as a function of incubation time (37°C, 600 rpm). The temporal evolution (0–90 h) of W423 steady state fluorescence for RPT is shown in Fig. 4. At and above pH 6.0, W423 emission is unchanged for the duration of the experiment demonstrating that RPT remains unstructured and monomeric. However, for all acidic conditions (<6.0), distinct and time dependent W423 changes in integrated intensity and spectral shifts are observed. Initially, W423 emission for pH 5.5 is nearly identical to that observed for pH 6.0; however, during the growth phase (after ~ 40 –50 h) I drops dramatically and a spectral blue shift is observed [$\Delta \langle \lambda \rangle = 10$ nm; 362 $\rightarrow$ 352 nm]. As the pH is lowered to 5.0, an increased blue shift [$\Delta \langle \lambda \rangle = 14$ nm; 362 $\rightarrow$ 348 nm] as well as a moderate decrease in I is observed by ~ 20 –30 h (during growth phase). Indeed, time resolved anisotropy (Fig. S4) and acrylamide quenching (Fig. S3) data confirm that at pH 5.0, W423 in the fibrillar state exhibits reduced local mobility and greater solvent protection [$K_{sv}(\text{pH } 5.0) = 10(1) \text{ M}^{-1}$; $K_{sv}(\text{NATA}) = 34(5) \text{ M}^{-1}$] as compared to initial measurement [$K_{sv}(\text{pH } 5.0) = 22(2) \text{ M}^{-1}$]. Re

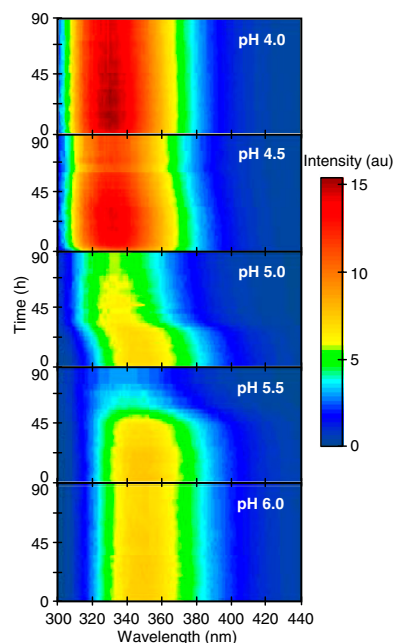


Fig. 4. W423 emission intensity surfaces as a function of pH (4 to 6, Top to Bottom). RPT (30 μ M) emission surfaces are plotted as a function of the aggregation time (0–90 h) and wavelength (300–440 nm). W423 emission intensity is in arbitrary units (blue to red) normalized to the highest intensity (pH 4.0).

flecting faster kinetics, W423 in pH 4.5 solution exhibits a blue shift comparable to that observed at higher pH [$\Delta\langle\lambda\rangle = 13$ nm; 355 $\rightarrow$ 342 nm] within the first 10 h of incubation. In contrast to other conditions, at pH 4.5, W423 exhibits a significant I increase with only a small decrease after ~ 40 h of incubation. At the lowest pH, we observed rapid RPT oligomerization with a high I and blue shifted spectrum at our earliest measurement ($t = 15$ min). While W423 emission continues to blue shift and increase in I , spectral changes are completed within 5 h.

Tryptophan Fluorescence Reflects Fibril Morphologies. We found correlations between W423 spectral changes and amyloid morphology. In all cases for which a W423 decrease in I is observed (pH 4.5–5.5), distinct fibrillar species are also present. Measurements of W423 excited state decays are consistent with steady state data showing that the average fluorescence lifetime is decreased in the amyloid form for pH 5 (Fig. S3). Specifically, decreases in I are most pronounced at pH 5.0–5.5 and commence during the growth phase when fibrils first appear. At pH 4.5 long fibrils are only present after the small decrease in I commences (~ 40 h). Contrastingly, we find an increase rather than a reduction in W423 I at pH 4.0 and accordingly no long fibrillar species are present. We suggest that this fluorescence signature (I decrease) could be attributable to Trp Trp stacking, possibly resulting from a parallel in register β sheet conformation. We propose this fibrillar structure, where corresponding residues of adjacent polypeptides (W423–W423) are aligned in individual β strands, because this is a common feature of several amyloidogenic proteins (36, 37). While Trp Trp stacking would explain the observed decreases in W423 intensity upon fibril formation, it is also reasonable that local molecular interactions and/or changes in environment could facilitate other quenching mechanisms.

We attribute the spectral blue shifts and increased I at low pH (< 4.5) to reflect an increased hydrophobic local environment upon protein oligomerization. Bimolecular acrylamide quenching data ($K_{sv} = 10(1)$ M $^{-1}$, pH 4.0) are consistent with a polypeptide structural rearrangement in which W423 is sequestered from the aqueous environment. Additionally, the I increases in the oligomeric state could reflect intra- or interprotein contacts that promote W423 interaction with positively charged residues (K378 and R429, Fig. 1).

Regardless of the exact nature of these distinguishing spectroscopic features, it is clear that W423 is an exquisite probe of RPT amyloid formation and has provided kinetic information that is not available from other measurements. We note that while Trp fluorescence has been used to report on the amyloid formation process for the disease related proteins α synuclein (38, 39) and $A\beta_{1-40}$ (40), large changes in $\langle\lambda\rangle$ and I are not always observed and are highly site dependent. Due to the high sensitivity of W423 emission upon aggregation, we propose that the C terminal repeats may play a key role in RPT fibril assembly; however, we do not preclude the possibility of involvement of other polypeptide regions. Supplementary evidence also support our hypothesis that the RPT amyloid core likely involves the C terminal repeats: (i) amyloid structure destabilizing Pro residues are found primarily between repeats 1 to 7 and repeat 10 (41); (ii) in vivo, repeats 2–3 are heavily *O* glycosylated with highly charged sialylated glycans which would add steric constraints within the N terminal domain and therefore less likely to contribute to amyloid assembly (20, 42); and (iii) the amyloid sequence predictor Waltz identified $^{403}\text{AEVSIVV}^{409}$, located within repeats 7 and 8, as the most amyloidogenic sequence (43). Future studies investigating the role of specific residues would be necessary to delineate the vital polypeptide regions for amyloid formation.

Implications of pH Dependent Amyloid Formation. Our data clearly show that solution pH affects both RPT aggregation kinetics and fibril morphology with W423 reporting on the local conformational

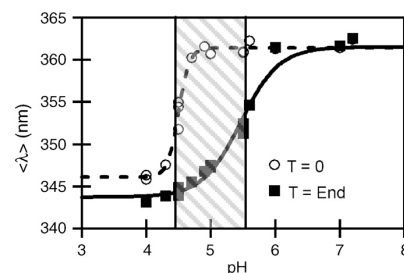


Fig. 5. Comparison of critical pH regimes for soluble and fibrillar RPT. W423 mean wavelength, $\langle\lambda\rangle$, of RPT samples at the beginning ($T = 0$) and end ($T = \text{End}$) of aggregation in different buffers (pH 4.0–5.6: 20 mM sodium acetate, 100 mM NaCl; pH 6.0: 20 mM MES, 100 mM NaCl; and pH 7.0–7.3: 20 mM MOPS, 100 mM NaCl). Solid lines are fits. Critical amyloid formation pH is identified to be pH 4.5–5.5.

changes corresponding to observed ultrastructures. By comparison of pH dependent W423 data we find that the pK_a of aggregated samples is shifted from 4.5 to 5.5 (Fig. 5). This shift is reasonable given that it would be more favorable to have neutral rather than charged groups buried within the hydrophobic amyloid core. Therefore, protonation is facilitated upon aggregation. In contrast to protonation of the soluble protein, this process in the fibril involves only one ionizable group rather than multiple ones, suggesting that a specific carboxylate is responsible for fibril formation/stability.

Because pH is critical in mediating RPT conformation and aggregation, we hypothesize that the changes in pH that occur during melanosome maturation provide one potential natural regulator of Pmel17 amyloid formation. To test this hypothesis, we monitored fibril morphological and local polypeptide conformational changes upon pH titration of aggregated RPT from pH 4 $\rightarrow$ 5 $\rightarrow$ 7 through TEM and W423 fluorescence (Fig. 6). In the preformed aggregate at pH 4 ($[RPT] = 30$ μM for 72 h at 37°C, 600 rpm), the Trp indole is mostly solvent protected. Upon sequential solution neutralization, spectral red shifts (343 $\rightarrow$ 350 $\rightarrow$ 360 nm) and integrated intensity decreases are observed from pH 4 $\rightarrow$ 5 $\rightarrow$ 7, similar to that of the RPT samples individually prepared and aged (Fig. 4). Interestingly, the observed fibril morphological changes from small, indistinct to long, striated fibrils are rapid (< 10 min, 25°C) and are reminiscent of those observed in stage I (pH 3–4) and II (pH 4.6–5) melanosomes (7, 10). Further neutralization to pH 7 results in almost complete dissolution of fibrillar RPT, supporting a highly reversible aggregation disassembly process. It is this feature that makes RPT

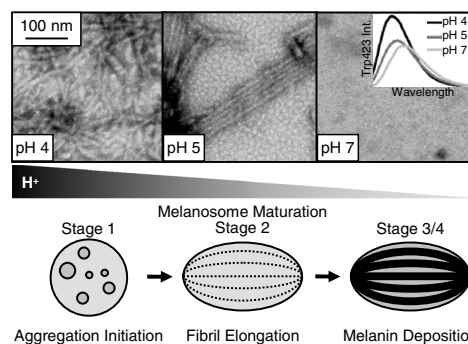


Fig. 6. pH induced RPT fibril morphological conversion and the melanosome maturation process. TEM images (Top) of aggregated RPT at pH 4 (20 mM sodium acetate, 100 mM NaCl, Left), after adjusting the pH to 5 (after 10 min, Center), and to 7 (after 10 min, Right). Inset: Corresponding W423 steady state emission spectra. Schematic representation (Bottom) of melanosome maturation: stage I, protein aggregation is initiated; stage II, fibrils elongate; stages III/IV, melanin deposition occurs.

amyloid unique as disease related fibrils are extremely difficult to dissolve even upon enzymatic digestion and detergent denaturation (44).

Based on our results, we propose in the highly acidic melanosomes (stage I), protein aggregation is initiated with fibril elongation occurring only after the compartment solution reaches an optimized pH ~5 (stage II). Upon protonation of specific carboxylates, the electrostatic charge repulsion within the polypeptide chain reduces, thereby leading to formation of compact structures that promote key interactions required for fibril formation. In addition, our observation that RPT will readily aggregate ($\leq 2 \mu\text{M}$) at the optimized pH (5.0) could suggest that only fibrils are stabilized in lieu of potentially toxic oligomers (9). While our data show that fibrils would dissolve in the near neutral conditions (pH 6 and above) found in stage III and IV melanosomes, it is unclear whether upon melanin deposition, the polymeric material could sequester the fibrils from solution and hence protect them from dissolution. Nevertheless, if released and exposed to the neutral environments outside the melanosome, fibrils will readily disintegrate and thus maintain their benign nature in the body.

Materials and Methods

Protein Expression and Purification. RPT was expressed and purified as previously described with the following modification (24). Lysis buffer contained 8 M guanidinium hydrochloride, 100 mM NaCl, 100 mM K_2HPO_4 , pH 7.5, and 10 mM imidazole. Purified RPT was stored at 4 °C until use.

Fibril Formation Kinetics and pH Titration. Purified RPT was filtered through Microcon YM 100 filter units (molecular weight cutoff 100 kD, Millipore) and exchanged into the appropriate aggregation buffer (pH 4.0 5.6: 20 mM sodium acetate, 100 mM NaCl, pH 6.0: 20 mM 2 (N-morpholino)ethanesulfonic acid (MES), 100 mM NaCl and pH 7.0 7.3: 20 mM 3 (N-morpholino)propanesulfonic acid (MOPS), 100 mM NaCl) using PD10 (GE Healthcare) desalting columns. Integrity of protein samples were assessed by SDS PAGE on a Pharmacia Phastsystem (Amersham Biosciences) visualized by silver staining methods. All buffers were filtered (0.2 μm). Final protein solutions (2–60 μM , 1.5–2.0 mL) were added to and sealed in screw cap, quartz cuvettes (10 $\times$ 10 mm). To initiate RPT aggregation, the cuvettes were put into an incubating microplate shaker (VWR International, LLC) at 37 °C and 600 rpm.

At various aging times, protein samples (cuvettes) were removed from the incubator for measurements of absorption, fluorescence, and laser light scattering. Absorption and fluorescence [λ_{ex} = 280 nm, λ_{obs} = 300–500 nm, 0.3 s integration time, 1 nm bandwidths, 25 °C, a long pass filter (290 nm) was used to minimize scattering background] spectra were obtained on a CARY 300 Bio spectrophotometer (Varian) and a Fluorolog 3 fluorimeter (Horiba Jobin Yvon), respectively. To verify minimal thermal and/or photo damage ($\leq 5\%$ over the course of 1 w, 29 time points) and to correct for any emission intensity variations (lamp power) all steady state emission spectra were normalized to the emission of a model fluorophore, N-acetyl tryptophanamide incubated at 37 °C. Laser light scattering experiments were carried out using a home built apparatus consisting of a CW laser (λ_{ex} = 450 nm, CrystaLaser) as the excitation source and a photodiode as the detector at a 90° geometry. Scattering intensities (mV) were recorded

using a data recorder and averaged over a 30 s period while stirring at ambient temperature. Intensities obtained for a standard scattering sample (polystyrene beads) as well as from a reference photodiode monitoring laser power were used for normalization.

For pH titration, increments of 1 M sodium hydroxide (5–20 μL) were added to preaggregated RPT samples at pH 4.0 [1.5 mL, $[\text{RPT}] = 30 \mu\text{M}$ for 72 h at 37 °C, 600 rpm] to raise pH from 4 $\rightarrow$ 5 and then from pH 5 $\rightarrow$ 7. At each pH, emission spectrum (uncorrected, dilution effects were negligible <2%) was collected and a TEM grid was prepared after equilibrium was reached as evidenced by stabilization of the W423 emission spectrum (within 10 min after NaOH addition, 25 °C, stirring).

Fibril Characterization. At various aging times, cuvettes containing protein were opened to extract samples for ThT assays (133 μL) and TEM (10 μL). For ThT experiments, protein samples were diluted (6.7 μM final) with freshly prepared ThT (20 μM) in appropriate aggregation buffer (*vide supra*).

ThT samples were equilibrated at room temperature and fluorescence measured on a Fluorolog 3 fluorimeter (Horiba Jobin Yvon) (λ_{ex} = 400 nm, λ_{obs} = 450–700 nm, 0.25 s integration time, 2 nm bandwidths, 25 °C). TEM was performed using a JEOL JEM 1200EX transmission electron microscope (accelerating voltage 80 keV) equipped with an AMT XR 60 digital camera [Electron Microscopy Core; National Heart, Lung, and Blood Institute (NHLBI)]. Sample grids (400 mesh formvar and carbon coated copper, Electron Microscopy Sciences) were glow discharged for 30 s using an EMScope TB500 (Emscope Laboratories) to increase surface hydrophilicity prior to deposition of protein sample (5 μL). After 1.5 min, excess sample was absorbed with filter paper and stained by two application/wick cycles (30 and 10 s) of 0.5% w/v aqueous uranyl acetate solution. Before and after incubation, circular dichroism (CD) spectroscopy was performed to verify secondary structural transitions. CD spectra were collected on a Jasco J 715 spectropolarimeter (205–260 nm, 1 nm steps, 1 nm bandwidth, 0.5 s integration time, and 50 nm/min, 25 °C).

Data Analysis. Mean wavelength (λ) was determined according to the equation $\langle \lambda \rangle = \frac{\sum I_i \lambda_i}{\sum I_i}$ where I_i and λ_i are the emission intensity and wavelength, respectively, for $i = 300–500$ nm. Integrated intensity (I) of W423 emission was determined according to trapezoidal integration. Using (λ) data, apparent pK_a and the number of ionization events involved in the process, n , were extracted from fits to the Henderson-Hasselbalch equation:

$$\langle \lambda \rangle = \frac{(\langle \lambda \rangle_{\text{neutral}} + \langle \lambda \rangle_{\text{acidic}} 10^{n(pK_a - \text{pH})})}{1 + 10^{n(pK_a - \text{pH})}},$$

where $\langle \lambda \rangle_{\text{neutral}}$ and $\langle \lambda \rangle_{\text{acidic}}$ are the W423 fluorescence mean wavelengths at neutral (≥ 6.0) and acidic (≤ 4.0) pH respectively. Data fitting and trapezoidal integration was performed using Igor Pro (WaveMetrics).

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