Amyloid fibril formation *in vitro* from halophilic metal binding protein: Its high solubility and reversibility minimized formation of amorphous protein aggregations

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Abstract: Halophilic proteins are characterized by high net negative charges and relatively small fraction of hydrophobic amino acids, rendering them aggregation resistant. These properties are also shared by histidine-rich metal binding protein (HP) from moderate halophile, *Chromohalobacter salexigens*, used in this study. Here, we examined how halophilic proteins form amyloid fibrils *in vitro*. His-tagged HP, incubated at pH 2.0 and 58°C, readily formed amyloid fibrils, as observed by thioflavin fluorescence, CD spectra, and transmission or atomic force microscopies. Under these low-pH harsh conditions, however, His-HP was promptly hydrolyzed to smaller peptides most likely responsible for rapid formation of amyloid fibril. Three major acid-hydrolyzed peptides were isolated from fibrils and turned out to readily form fibrils. The synthetic peptides predicted to form fibrils in these peptide sequences by Waltz software also formed fibrils. Amyloid fibril was also readily formed from full-length His-HP when incubated with 10–20% 2,2,2-trifluoroethanol at pH 7.8 and 25°C without peptide bond cleavage.

Keywords: amyloid fibril; halophilic; thioflavin; acid-hydrolysis; 2,2,2-trifluoroethanol; solubility

Abbreviations: AFM, atomic force microscope; BLA, β -lactamase; CD, circular dichroism; Gu, guanidine-HCl; HEW, hen egg white; HP, histidine-rich metal binding protein; PAGE, polyacrylamide gel electrophoresis; PN buffer, Na-phosphate buffer, pH 7.4, containing 0.15M NaCl; TEM, transmission electron microscope; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; Tht, thioflavin; TOF-MS, time-of-flight mass spectrometry

Additional Supporting Information may be found in the online version of this article.

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Introduction

Amyloid fibrils were originally observed to occur in certain proteins involved in amyloidosis, for example, Alzheimer's and Huntington diseases.^{1–3} However, it is becoming clear that many proteins and peptides unrelated to amyloid diseases can also form fibrils.^{4–7} It is now believed that amyloid fold is not just aberrant random aggregation but is associated with a functional structure, such as formation of bacterial biofilm matrix.^{8–10} Fibril formation occurs through partially unfolded structure, caused by high temperature or low pH *in vitro*.^{11–13} Schmittschmitt and Scholtz reported that the efficiency of amyloid

fibril formation of ribonuclease Sa, when induced by cosolvent, was highest at around *pI* of the protein, suggesting that fibril formation was maximum under conditions, at which protein solubility should be minimum.¹⁴ It is thus clear that both protein stability and solubility play a critical role in amyloid fibril formation.^{7,14}

Extremophiles, such as thermophilic and halophilic microbes, produce extremophilic proteins.^{15,16} Proteins from thermophiles generally show high stability against heat or other stresses. Nevertheless, they form fibrils under certain conditions.^{17,18} We have been studying proteins from both moderate and extreme halophilic microorganisms.^{19–21} Halophilic proteins that are potentially exposed to high salt concentration possess unique amino acid compositions, that is, a large excess of acidic amino acids and a relatively small fraction of hydrophobic amino acids.^{22–25} Namely, halophilic proteins are characterized as acidic, low-*pI* proteins. Abundant negative charges, combined with weak hydrophobicity, at neutral pH made halophilic proteins highly soluble even in unfolded structure caused by high temperature.^{21,26,27} Based on such high solubility, it may be anticipated that halophilic proteins show rather low tendency to form amyloid fibrils. Histidine-rich periplasmic metal binding protein (HP) from moderate halophile, *Chromohalobacter salexigens*, is a typical halophilic protein containing abundant acidic residues, Asp and Glu, throughout the sequence (10.0 and 9.1 mol % of Asp and Glu residues) and has a ratio of acidic/basic amino acid residues as 3.7 and a *pI* of 4.3.²⁸ It showed high solubility and structural reversibility after heat-denaturation upon cooling.²⁸

Here, we have examined acid-induced structure changes and amyloid formation of halophilic His-HP and then isolated peptides, generated upon exposure to acid, which also formed fibrils. We also demonstrated that His-HP readily formed fibrils in the presence of moderate concentration of cosolvent, 10–20% 2,2,2-trifluoroethanol (TFE), at pH 7.8 and 25°C.

Results

Amyloid fibril formation from halophilic metal-binding protein (HP) and β -lactamase (BLA)

We have been studying halophilic characteristics of HP and BLA,^{21,26,28} both of which could be highly expressed in and easily purified from *E. coli*: ~150 mg of highly homogeneous His-tagged HP (His-HP)/liter culture and ~50 mg of His-tagged BLA (His-BLA)/liter culture were purified by one-step Ni-column affinity chromatography. Both proteins are extremely acidic, as indicated by a high ratio of acidic/basic amino acid residues of 3.7 for HP and 2.1 for BLA. Thus, both proteins possess large net negative charges at neutral pH, which are considered to be associated with their high aqueous solubility in

both native and denatured states.^{21,26,28} These proteins are strongly resistant to aggregation, suggesting that they might not form fibril structures.

First, we examined the possibility of fibril formation from halophilic proteins under the most frequently used conditions, that is, acidic pH and high temperature (58°C). As shown in Figures 1–3, it is evident that these halophilic proteins do form amyloid fibrils rather easily. Figure 1 shows transmission electron microscopic (TEM) and atomic force microscopic (AFM) observations of fibrils formed from His-HP and His-BLA at pH 2.0 and at 58°C after 1-month incubation. His-HP formed a mixture of long fibrils and short fibrils, while His-BLA formed only short fibrils.

Figure 2 shows TEM observation of His-HP samples incubated at pH 2.0 and 58°C in the absence (panel A) and presence (panel B) of seeds. No apparent fibrils were observed immediately after incubation (A-1 and B-1). After 8-h incubation (A-2), many short fibrils were detected. After 24 h (A-3) and 48 h (A-4), fibril grew longer. Long amyloid fibrils were clearly observed at 96 h (A-5). When sonicated amyloid fibrils were added as seeds, fibril formation was faster [Fig. 2(B)]. Long amyloid fibrils were already detected at 24-h incubation (B-2). Further incubation to 48 h resulted in nearly entire population forming long amyloid fibrils. This seed-enhanced fibril formation is consistent with the general property of amyloid fibril formation.

We then measured time course of Thioflavin (ThT) fluorescence of His-HP samples: the fluorescence intensity of ThT is greatly enhanced upon binding to

Figure 1. Amyloid fibrils formed from halophilic histidine-rich metal binding protein (HP) and β -lactamase (BLA). Amyloid fibrils were observed by TEM and AFM. His-tagged HP and BLA were incubated at pH 2.0 and 58°C for 1 month. Black bar for TEM represents 0.5 μ m, and white bar for AFM represents 1 μ m. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.interscience.wiley.com).]

Figure 2. Time course of fibril formation from His-HP by TEM observation. His-HP was incubated in 50 mM Gly-HCl buffer, pH 2.0, at 58°C (3.2 mg/mL incubation mixture) and observed by TEM as described in Materials and Methods. (A) Without seeds; (B) 2% volume of sonicated preformed-fibril seeds were added at 0 time. Black bar shows 0.5 μ m.

fibrils and can be used to follow fibril formation.²⁹ As shown in Figure 3(A), fluorescence intensity of Tht sharply increased within the first 12-h incubation [inset in Fig. 3(A)] to 70% of the maximum value and reached a plateau after 4 days [Fig. 3(A), line HP]. Even after 1 h, Tht fluorescence showed about 30% intensity of the plateau level [Fig. 3(A), insert], indicating a fairly rapid fibril formation. The effects of seeds (2 and 4 vol %) on Tht fluorescence and hence fibril formation were examined. As seen in Figure 3(A), the addition of seeds increased the fluorescence intensity by 1.4-fold in both cases (line HP + seed), indicating that the seeds enhanced fibril formation. Figure 3(B) shows fluorescence spectra of His-HP samples in the presence of Tht with an emission maximum at 481 nm when excited at 440 nm. There is little fluorescence at time 0, that is, in the absence of fibrils, consistent with the notion that this dye does not bind to monomeric protein. No changes in spectral shape were observed with time, indicating that the way Tht binds is independent of the length of the fibrils. Fluorescence intensity gradually increased over 120-h incubation, an indication of time-dependent formation of fibrils.

As described above, halophilic proteins are in general highly acidic with high contents of acidic amino acids, Asp and Glu. It is also the case for His-HP, which contains 31 Asp and 28 Glu (in total 330 residues). Since COOH-side of peptide bond at Asp residue is known to be chemically labile to acid hydrolysis,^{12,30} we examined the possibility of degradation of His-HP protein under the acidic conditions

Figure 3. Characterization of fibril formation by thioflavin fluorescence, molecular mass, and CD spectra. Same sample as in Figure 1 was used for characterization of fibril formation. A: Time course of Tht fluorescence of His-HP samples is shown with (HP + 2% seed and HP + 4% seed) or without seeds (HP). Tht fluorescence intensity for first 12 h is shown in inserted figure. B: Time course (0–120 h) of Tht fluorescence emission spectra at 440 nm excitation is shown. C: Tricin-SDS-PAGE of His-HP incubated at pH 2.0 and 58°C. D-1: CD spectra of His-HP samples at pH 2.0 and pH 7.8 at 25°C. D-2: Time course of CD change for 0, 2, and 4 days incubation at pH 2.0 and 58°C.

used here. Figure 3(C) shows Tricin-SDS-PAGE of His-HP during incubation at pH 2.0 and at 58°C. His-HP rapidly degraded with time, perhaps due to acid hydrolysis of peptide bonds at the COOH-side of Asp residue.^{12,30} A majority of intact His-HP degraded at 8 h, at which time a few bands with apparent molecular masses of 26, 20, and 16 kDa were apparent. Several smaller polypeptides with the apparent masses of 20, 16, 15, 12, and 6.5 kDa were observed after 24 h. After 48 h, the bands at apparent molecular masses of 16, 15, 12, and 6.5 kDa were dominant. Half time ($T_{1/2}$) for the loss of intact His-HP was estimated to be $\sim$ 4 h from Figure 3(C). This hydrolysis rate of His-HP was much faster than that of hen egg white (HEW) lysozyme

(Sigma L6876) under the same conditions ($T_{1/2} = \sim 2$ days, data not shown). This rapid hydrolysis was also observed for other halophilic proteins: His-BLA ($T_{1/2} = \sim 8$ h), starch-binding domain of α -amylase and thioredoxin were also rapidly hydrolyzed under the same conditions (data not shown). At 58°C, $\sim 80\%$ of His-HP was hydrolyzed within a day even at pH 7.0 and 7.8 (data not shown).

Circular dichroism (CD) spectra of His-HP at pH 7.8 and 2.0 at 25°C (as a control before 58°C incubation) are shown in Figure 3(D). His-HP was confirmed to be full length protein under these conditions by SDS-PAGE analysis [data not shown and see control lane of Fig. 7(A)].

The CD spectrum of His-HP in 20 mM Tris-HCl buffer, pH 7.8, showed negative peak at 208 and 222 nm [Fig. 3(D),1], indicating helix-rich secondary structure (26% helix, 14% anti-parallel β , and 6% parallel β). CD spectrum at pH 2.0 showed a broad negative peak with a minimum at 216 nm (19% helix, 20% anti-parallel β , and 6% parallel β), an indication of more β -sheet type structure. Thus, it appears that the secondary structure of His-HP shifts from helix-rich to β -rich structure upon pH change from 7.8 to 2.0 at 25°C [Fig. 3(D),1]. The secondary structure at pH 2.0 further changed with incubation at 58°C for 2–4 days. As shown in Figure 3(D) (right panel), the gradual shift of spectra at both 208 and 216 nm toward more negative direction was detected [Fig. 3(D), 2]. The CD profile of amyloid fibrils precipitated by ultracentrifugation showed distinct β -structure-rich CD profile as shown in later section [see next section and Fig. 4(C)].

Precipitation of amyloid fibril by centrifugation and analysis of its peptide

His-HP preparation incubated at pH 2.0 and 58°C for 1 month was centrifuged at 12,500g (Kubota AF-2018 rotor at 14,000 rpm) or 150,000g (Beckman TLA-100.4 rotor at 60,000 rpm) for 1 h to precipitate amyloid fibrils. Transparent gel-like precipitate was obtained by both centrifugation conditions. As shown in Figure 4(A), total sample [Fig. 4(A-1) Lane 2 and (A-2), Lane 1] contained several protein bands: major bands of apparent size, 20, 16, 15, 12, 6.5, and 3.5 kDa, were observed. By 12,500g centrifugation (A-1, 14 k centri), only small portion of total sample with the molecular size of around 12–3.5 kDa and a major band of 6.5 kDa were precipitated (Lane 4). In contrast, a larger amount of transparent gel-like precipitate was obtained using 150,000g centrifugation. As seen in Figure 4 (A-2, 60k centri), almost protein bands present in the incubated samples were pelleted (Lane 3). Only ~ 55 kDa smear band remained in the supernatant (Lane 2). The mobility of this band was aberrant depending on the gel system used (not shown). TEM observation revealed that the low speed pellets mainly contained long

(A) Tricin-SDS-PAGE of centrifuged sample

(B) TEM of precipitated fraction

(C) CD spectra of 60 k centri. sample

Figure 4. Analysis of fibrils precipitated by centrifugations. A: His-HP (3.5 mg/mL) incubated at pH 2.0 and 58°C for 1 month was centrifuged at 14,000 rpm (A-1) and 60,000 rpm (A-2) for 1 h and subjected to Tricin-SDS-PAGE. (A-1) Lane 1, His-HP without incubation (HP); Lane 2, total sample before centrifugation (T); Lane 3, supernatant fraction (S); Lane 4, precipitated fraction (P). (A-2) Lane 1, total sample before centrifugation (T); Lane 2, supernatant fraction (S); Lane 3, precipitated fraction (P). M_w represents molecular weight standard (TEFCO, Tokyo). B: TEM observation of precipitated fractions. Precipitated fraction by 14,000 rpm (B-1) and 60,000 rpm centrifugation (B-2) are shown. Black bar shows 0.5 μ m. C: CD spectra of centrifuged fractions at 60,000 rpm. Total sample before centrifugation (T), supernatant (S), and precipitated (P) fractions are measured.

fibrils (B-1, 14 k centri. ppt.), while high speed pellets were made of both long and short fibrils (B-2, 60 k centri. ppt.).

To precipitate more fibrils, longer ultracentrifugation was carried out (5 h). The protein distribution after 5-h ultracentrifugation, determined by absorbance at 280 nm or bicinconic acid method, was $\sim 61\%$ in supernatant and $\sim 39\%$ in precipitated fraction. SDS-PAGE profile of 5-h ultracentrifuged

fractions (not shown) was the same as that shown in Figure 4(A-2) (60 k centri) for 1 h ultracentrifugation. With 5 h ultracentrifugation at 60,000 rpm (150,000g), a very small amount of short fibrils was detected in the supernatant with TEM observation (not shown). Although even 60% of the total protein amount were recovered in the supernatant fractions, most peptide bands of the total sample were detected in the precipitated (pellet) fraction; this fact suggests that the majority of peptides in the supernatant fraction must be so small that they were not detected by 16% acrylamide Tricin-PAGE [same as Fig. 4(A-2), Lane 2].

Figure 4(C) shows CD spectra of samples obtained after 60 k ultracentrifugation for 5 h. CD profile of the precipitated fraction [Fig. 4(C), P] revealed a typical β -structure rich shape, indicated by a valley at around 216 nm characteristics of amyloid fibrils. The β -structure was more apparent for the pellet than the total (T), as the supernatant fraction [Fig. 4(C), S] did not show distinct secondary structures.

The pellet fraction from 14 k centrifugation was further characterized by dissolving it in 8M guanidine-HCl (Gu) at pH 2.0 and subjected to octyl-80Ts reverse-phase HPLC analysis. Two major peaks were eluted at 44.2 and 46.0% acetonitrile (Peaks I and II in Supporting Information Fig. 1, left side chart). Tricine SDS-PAGE of Peak I showed two bands at the apparent molecular masses of 6.5 kDa (Peak A in lower right panel) and 3.5 kDa (Peak B in upper right panel), whereas Peak II (Peak C in upper right panel) contained other two bands, C1 and C2. These peptides were purified by the combination of reverse-phase HPLC and Ni-column chromatography, and characterized by determining the molecular masses and N-terminal sequences (Supporting Information Figs. 1 and 2 and Table 1). Four peptide sequences were identified in the pellet fraction of 14 k centrifugation as shown in Figure 5: that is, G2-D55 (line A in Fig. 5), L76-D109 (line B), A68-D109 (line C1), and A207-N235 (line C2). Note that peptide bonds at carboxy-side of Asp residues were not always hydrolyzed, probably depending on

the properties of X residue of Asp-X linkage. Similar observation was reported in the case of HEW lysozyme that only three of seven Asp-X bonds were hydrolyzed at pH 1.6–2.0 and 65°C.^{12,30}

Fibril formation from isolated peptide fractions described above and from synthetic peptides predicted to be core region of these isolated peptides by “Waltz” software (<http://waltz.switchlab.org/>) was examined. The G2-D55 fraction (Peak A) from reverse-phase chromatography was dried, dissolved in 50 mM Gly-HCl buffer, pH 2.0 and incubated at 58°C for 2 weeks at protein concentration of 3.5 mg/mL. Reconstitution of amyloid fibril was confirmed by TEM observation [Fig. 6(A), left panel]. We then analyzed the sequence for high propensity of fibril formation by software “Waltz” and found SVVASF-SILG sequence (thick line SVV10 peptide in Fig. 5). This short peptide was chemically synthesized and tested for fibril formation. It readily formed fibrils at pH 2.0 in 50 mM Gly-HCl buffer [Fig. 6(B), left panel]. This 5637 Da G2-D55 peptide was most abundant in precipitated amyloid fibrils, as obvious

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mgsshhhhhh ssglvprgsh mEGPMSVVAS FSILGDMVEE VGGEHVDVTT LVGPDGDAHV 60
A      SVV10
FSPSPDARA VGEADLFVVN GLHFEGWLDL LVEASGYEGP VVASRGIDA LSFDEEREHH 120
C1      EAD17
SSDHEGHDHA TGHDDHDHDD HDHDDHSEH AGHDHGPEDP HAWQDLQNGK QYVANIRDAL 180
VAADPEHAAD YRRNAEYVE AMTDLAEVH RRIGAIPEAN RVLITNHDAF GYFANAYGLD 240
C2      AFG10
VLSPVGLSTA AEPSAAGMAK LIEQIQARNV KALFLENMTS PALLEQLADE TGVITGGTLY 300
AGALAAEGEA STYLGMRHN VDTLTEALKD 330

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Figure 5. Amino acid sequence of His-HP and its peptides involved in fibril formation. Lines A, B, C-1, and C-2 show sequences of peptides detected in Peaks A–C (Supporting Information Fig. S1). Thick lines SVV10, EAD17, and AFG10 represent peptide sequences with high propensity of fibril formation predicted by Waltz software.

(A) TEM observation of reconstituted fibril

(B) TEM observation of fibril from synthetic peptides

(C) AFM observation of fibrils from synthetic peptides

Figure 6. TEM and AFM observations of fibrils formed from isolated or chemically synthesized peptides. A: TEM observation of fibrils reconstituted from isolated peptides from Peak A (Supporting Information Fig. S1) and Peak C (Supporting Information Fig. S1) fractions. B: TEM observation of fibrils formed from synthetic peptides, SVV10 (left panel), EAD17 (middle panel), and AFG10 (right panel) were shown. C: AFM observation of fibrils formed from synthetic peptides, EAD17 (left and middle panels) and AFG10 (right panel). White box in left panel was re-scanned with 4.5-fold magnification and the re-scanned image is shown in middle panel. Black bar in TEM panel and white bar in AFM panel (middle) represent 0.5 μ m. White bar in left and right panel of (C) represents 1.0 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 7. Fibril formation from full-length His-HP at pH 7.8 and 25°C in the presence of 2,2,2-trifluoroethanol. A: His-HP samples incubated in 50 mM Tris-HCl buffer, pH 7.8, with 0–40% TFE at 25°C for 8 days were applied to Tricine-SDS-PAGE. Samples with 0% (Lane 1), 10% (Lane 2), 20% (Lane 3), 30% (Lane 4), and 40% (Lane 5) of TFE are shown. M_w , molecular mass standard. B: Time course of ThT fluorescence of His-HP samples with 0–40% TFE. C: CD spectra of His-HP with 0–40% TFE for 0, 4, and 8 days incubation. D: His-HP sample with 20% TFE was centrifuged at 60,000 rpm for 5 h, and CD spectra of supernatant (S) and precipitated (P) fractions are measured.

by SDS-PAGE (Supporting Information Fig. 1). Peak C fraction, containing both A68-D109 (C1) and A207-N235 (C2) peptides, was dissolved in 50 mM Gly-HCl buffer, pH 2.0, and incubated at 58°C for 2 weeks at 2.1 mg/mL protein concentration: it clearly formed amyloid fibrils [Fig. 6(A), right panel]. We examined core region of these sequences by Walts and found peptide EAD17 starting E73 in C1 (thick line EAD17 in Fig. 5) and peptide AFG10 (thick line AFG10 in Fig. 5) starting A229 in C2 (C-terminal three amino acids of AFG10 peptide were outside the line C2). A synthetic EAD17 peptide also readily formed fibrils at pH 2.0 [Fig. 6(B), middle panel]. Synthetic peptide corresponding to AFG10 formed amyloid fibrils at slightly alkaline pH, pH 8.0: this peptide was insoluble in the buffer of acidic to neutral pH [Fig. 6(B), right panel]. Since the amount of purified peptide L76-D109 in Peak B fraction was low, reconstitution experiments were not carried out.

AFM observation of fibrils formed with synthetic peptides, EAD17 and AFG10, were shown in Figure 6(C). Fibrils from EAD17 [Fig. 6(C), left panel] are short, 0.5 to a few micrometers long. The area shown as box in left panel in Figure 6(C) for EAD17 was rescanned with a 4.5-fold magnification and is shown in middle panel of Figure 6(C): it was clear that thin fibril was twisted to form thicker fibrils. Fibrils formed by AFG10 are long, exceeding the panel size [Fig. 6(C), right panel].

Amyloid fibril formation from full-length His-HP in the presence of TFE at pH 7.8

It is not clear from the above data due to rapid degradation whether the intact His-HP forms amyloid fibrils. When incubation was done at 58°C at pH 2.0, 5.4, 6.8, and 8.5, the rate of degradation was reduced, but so did the fibril formation. Thus, we have used a different condition. Trifluoroethanol (TFE) has been used to induce amyloid fibril formation for various proteins, such as acylphosphatase,⁴ α -chymotrypsin,³¹ insulin,³² and β_2 -microglobulin.³³ His-HP (final protein concentration of 5 mg/mL) was incubated with 0, 10, 20, 30, and 40% TFE in 50 mM Tris-HCl buffer, pH 7.8 at 25°C for up to 8 days. As shown in Figure 7(A), only full-length His-HP was detected by Tricine-SDS-PAGE, indicating no degradation of His-HP under these conditions, consistent with the speculation that the observed degradation at low pH at 58°C is due to acid hydrolysis. As shown in Figure 7(B), the ThT fluorescence intensity in 10 and 20% TFE increased rapidly to almost plateau level at 4-day incubation. At 30% TFE, fluorescence intensity only slightly increased during 8-day incubation and no fluorescence increase was observed at 40% and 0% TFE [Fig. 7(B)]. Namely, fibril formation occurred only at intermediate TFE concentrations. By TEM observation, the short fibrils were formed at 10% TFE and longer thread-like fibrils and fibril aggregates were detected at 20% TFE [Fig. 8(A)]. Consistent with ThT fluorescence [Fig. 7(B)], no fibrils were detected in 0, 30, and 40% TFE. CD spectra of His-HP in the presence of 0, 10, 20, 30, and 40% TFE were shown in Figure 7(C) as a function of incubation time. CD profile of control His-HP (0% TFE, 0 day) showed double negative peaks at 208 and 222 nm, suggesting helix-rich structure. The addition of 10% TFE resulted in decrease in 208 nm signal (toward more negative value) and increase in 222 nm signal, clearly indicating structure changes. Increasing TFE concentration in 10% increment resulted in downward shift in the spectra, indicating further structure alterations. Interestingly, while structure changes occurred in monotone with TFE concentration, fibril formation was observed only at 10 and 20% TFE. Either the structures at 10 and 20% are critical for fibril formation or higher TFE concentrations are too strong for

(A) His-HP incubated with 0–40% TFE in 50 mM Tris-HCl buffer, pH 7.8, at 25 °C

(B) Effects of 50 and 100 mM NaCl on fibril formation of His-HP incubated with 20% TFE

Figure 8. TEM observation of His-HP samples incubated with 0–40% TFE and effects of NaCl on fibril formation. A: TEM observation of samples incubated with 0–40% TFE at 25°C for 8 days. B: TEM observation of samples incubated with 20% TFE and 50 mM or 100 mM NaCl. Left panel shows addition of 50 mM NaCl, and middle and right panels show addition of 100 mM NaCl. Middle and right panel images were taken from the same grid; most area shows image like middle panel (a), while some place shows image like right panel (b). Black bar shows 0.5 μ m.

solubilizing the His-HP. CD profile at 4 days was qualitatively similar to that of 0 day except for 20% TFE: the signals at both 208 and 222 nm were less negative than those at 0 day. At 8 days, the CD profile of His-HP in the presence of 20% TFE showed a distinct change from 0 and 4 days: that is, the spectrum was broader centered at around 216 nm, suggesting increased content of β structure. This appears to reflect the observed fibril formation. CD profile of His-HP in the presence of 40% TFE showed double negative peaks at 208 and 222 nm up to 8 days, indicating helix-rich secondary structure, consistent with no fibril formation.

His-HP incubated with 20% TFE for 10 days was ultracentrifuged at 150,000g for 5 h, resulting in transparent gel-like precipitate. The distribution of protein was determined to be $\sim$ 55% in supernatant and $\sim$ 45% in precipitated fraction estimated by SDS-PAGE and protein measurements. Figure 7(D) shows CD profile of supernatant and precipitated fractions. The spectrum of supernatant [Fig. 7(D), S] was similar to that of His-HP without TFE [Fig. 7(C), 8 days]. The spectrum of the precipitate showed a negative peak at around 216 nm, indicating high content of β -structure. However, comparison of this precipitate

CD [Fig. 7(D), P] with the acid structure shown in Figure 4(C-P) revealed that the spectrum was more flat, indicating the presence of α -helix in the TFE precipitate. We finally examined the effects of NaCl on fibril formation of His-HP at 20% TFE. His-HP was incubated with 20% TFE at 25°C for 8 days in the absence and presence of 50 or 100 mM NaCl. The ThT fluorescence intensity revealed that addition of salt enhanced ThT fluorescence by $\sim$ 2-fold (data not shown), suggesting salt enhanced amyloid fibril formation. Figure 8(B) shows TEM observation of His-HP sample in 20% TFE with addition of 50 or 100 mM NaCl. The fibril in the presence of 50 mM NaCl [Fig. 8(B), +50 mM NaCl] was observed to be slightly longer than in the absence of NaCl [Fig. 8(A), 20% TFE panel]. Both middle (+100 mM NaCl-a) and right (+100 mM NaCl-b) panels in Figure 8(B) were taken from the same grid: the image in middle panel was mostly composed of thread-like fibrils, while the right panel showed long fibrils. Most area showed an image like middle panel (+100 mM NaCl-a), while some place showed an image like right panel (+100 mM NaCl-b), indicating a mixed population of major short and minor long fibrils under these conditions.

All these data shown in Figures 7 and 8 indicate that TFE facilitated fibril formation composed of full length His-HP at an optimum TFE concentration of $\sim$ 20%. His-BLA (final protein concentration of 10 mg/mL) was found to form amyloid fibrils without hydrolysis of peptide bond under the same conditions, that is, 25°C incubation at pH 7.8 with 20% TFE (data not shown).

Discussion

For *in vitro* fibril formation of His-HP, we usually made a stock solution of 25–80 mg/mL His-HP at pH 7.8 and pH 2.0 at 4°C. Its solubility was greatly reduced at around pH 4–5, that is, at or near the *pI*, leading to rapid formation of white amorphous precipitates without fibrillation at 4–25°C, confirmed by TEM observations (data not shown). This amorphous precipitate was readily solubilized by changing the pH to 7.8 or 2.0 with recovery of respective secondary structures of His-HP by CD measurements before *pI* precipitations (data not shown). These data clearly demonstrate that His-HP is highly resistant to irreversible formation of amorphous aggregates at wide range of pH, as has been observed upon thermally denaturation.²⁸

His-HP contains a large amount of net negative charges, and consequently retains high solubility at pH 7.8. Although these properties may inhibit fibril formation of intact His-HP at neutral pH, the addition of 10–20% TFE clearly resulted in amyloid fibrils. The effects of TFE could be due to at least two mechanisms. First is the structure changes. It is interesting that only intermediate structures at 10–20% TFE resulted in fibril formation. The second

effect may be due to the effect of TFE on the solubility of His-HP. There should be strong repulsion between charged His-HP and nonpolar TFE, leading to entropically unfavorable system and consequent phase separation in the form of amyloid fibrils. The addition of salt augmented TFE-induced fibril formation, which may be due to salting-out effects of NaCl.

We have been studying the mechanism of amyloid fibril formation using HEW lysozyme and have observed that it formed not only amyloid fibrils but also a large fraction of irregular aggregates *in vitro*^{34,35} under partially unfolding conditions, especially with cleavage of intramolecular disulfide bonds. This is most likely due to aggregation propensity of partially unfolded structures of the lysozyme, which are stabilized by forming either soluble aggregates or regularly structured amyloid fibrils. On the contrary, it was observed here that HP formed a relatively homogeneous amyloid structure without forming aggregates. This may be due to weaker aggregation propensity of His-HP or its peptide fragments, leading to destabilization of irregular aggregates and thereby stabilization of either monomers or amyloid fibrils.

In the fibrillation of His-HP, there appears to be no stable intermediate aggregates that normally occur with other proteins, such as HEW lysozyme described above. This implies that amyloid formation can occur independent of the stable intermediate aggregates, raising a potential of using His-HP or other halophilic proteins in studying the mechanism of amyloid formation and structure analysis of amyloids: namely, such analysis may not be hampered by irregular amorphous aggregates using halophilic proteins. Recently, Yoshimura *et al.* discussed the difference between crystal-like amyloid fibrils and glass-like amorphous aggregates of β_2 -microglobulin.³⁶ Supersaturation of soluble unfolded protein resulted in formation of amyloid fibrils by the addition of seeds, nuclei for ordered assembly of unfolded proteins, or by spontaneous nucleation in more labile states. On the other hand, amorphous aggregation occurred in association with excess nuclei and excessive exclusion of denatured protein from water. In this context, halophilic proteins should not cause formation of such excess nuclei because of their aggregation-resistant characteristics due to strong charge repulsions and high solubility. Stable nuclei of His-HP might be readily formed in the presence of 20% TFE. To investigate this point in more detail, we are now progressing comparison of behavior of halophilic and nonhalophilic protein homologues in the formation of amyloid fibrils and amorphous aggregates.

Materials and Methods

Bacterial strains and media

The gene for HP (Csal_0220) was amplified by PCR from a chromosomal DNA of *Chromohalobacter*

saalexigens DSM3043 and cloned to *E. coli* vector pET15b (Novagen) to construct pET15b-HP.²⁸ *E. coli* BL21(DE3) was used for expression of protein. LB-ampicillin (100 μ g/mL) medium with or without 0.4% glucose was used for *E. coli*. *C. saalexigens* was grown in Nutrient broth containing 2M NaCl.

Expression and purification of halophilic His-HP and His-BLA

E. coli BL21(DE3) cells harboring pET15b-HP was grown in 1 L of LB-ampicillin (100 μ g/mL) medium containing 0.1 mM isopropyl-1-thio- β -galactopyranoside at 18°C overnight. Harvested cells were sonicated in 100 mL of 50 mM Na-phosphate buffer, pH 7.4, containing 0.15M NaCl (PN buffer), 20 mM imidazole and protease inhibitor cocktail (Nakarai Chem, 25955-11 or Roche, 11873580001). After centrifugation at 16,000g for 20 min, the supernatant that has been diluted 2-fold with PN buffer was applied to His Trap FF column (GE Healthcare, 5 mL). The bound proteins were eluted with 20, 50, 100, 200, and 300 mM imidazole in PN buffer in stepwise manner. His-HP eluted at 100–200 mM imidazole was homogeneous and pooled for further experiments. Halophilic β -lactamase (BLA) was expressed and purified with the same method as described above from *E. coli* BL21(DE3) cells harboring pET-bla as described previously.²¹

In vitro experimental conditions for amyloid fibril formation

His-HP or His-BLA was dialyzed against 50 mM Gly-HCl buffer, pH 2.0, and aliquot of proteins (0.1–1 mL of 2–5 mg/mL) was incubated at 58°C. His-HP was also dialyzed against 5 mM Tris-HCl buffer, pH 7.8, adjusted to 5 mg/mL in the final incubation mixture and incubated in 50 mM Tris-HCl buffer, pH 7.8, containing 0–40% TFE at 25°C. All synthetic peptides were dissolved in dimethyl sulfoxide (10 mg peptide/0.1 mL) and then incubated in 50 mM glycine-HCl (pH 2.0) or Tris-HCl (pH 8.0) at 37°C (peptide concentration at 2 mg/mL in the final incubation mixture).

Determination of thioflavin fluorescence intensity

Ten microliter aliquots of the above incubated samples were mixed with 250 μ L of 10 μ M Tht dissolved in 50 mM Na-phosphate buffer, pH 6.7. Fluorescence intensity due to thioflavin bound to fibrils were measured at 485 nm with excitation at 435 nm. The fluorescence spectra excited at 440 nm was also measured with Corona microplate reader fluorescence spectrophotometer (Hitachi Japan).

CD measurement

Far UV CD spectra in the range of 200–260 nm were determined on a Jasco J-820 spectropolarimeter at room temperature. The samples were diluted

with 20 mM buffer to a final protein concentration of 0.1 mg/mL for CD measurement. The protein concentration was spectrophotometrically determined at 280 nm using the absorbance value of 0.64 for 1 mg/mL His-HP solution. Four scans were accumulated at scan rate of 10 nm/min and at response of 4 s. The solvent spectrum was subtracted from the sample spectrum and the subtracted spectrum was converted to the mean residue ellipticity using the path-length of the cell (0.1 cm), the protein concentration (0.1 mg/mL) and the mean residue weight (108). The α -helical content was estimated according to Greenfield and Fasman.³⁷

TEM measurements

TEM measurements were performed on a 7000 electron microscope (Hitachi Japan) at 80 kV and JEOL JEM-3010 (Nihon Denshi) at 100 kV. His-HP fibril samples (10 μ L), diluted 20-fold with Milli Q water, were placed on copper grid (200 mesh) covered with carbon coated collodion film (Nisshin EM collodion film #6511 and excel support film #649). After 1 min, 5 μ L of 1% phosphotungstic acid (pH 7.0) was supplemented and incubated for 30 s. The solution was removed carefully with a filter paper, followed by air-drying prior to examination. The observation magnification was usually 20,000-fold.

AFM measurements

Ten microliter of samples, diluted 40-fold with Milli Q pure water, were placed on freshly cleaved mica and dried in a vacuum desiccators for 5 min. Samples in atmosphere were imaged with a JEOL JSPM-5200 (Nihon Denshi) AFM. Cantilevers with etched silicon tips (Olympus) having a resonance frequency of 300 kHz were used with tapping mode. The scan rate was 0.5–1 Hz with a pixel number of 512×512 .

Others

SDS-PAGE was carried out according to Laemmli³⁸ and Schagger and Jagow.³⁹ Protein amount was determined by A_{280} measurement or bicinonic acid method.⁴⁰

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