Oligomer-dependent and -independent chaperone activity of sHsps in different stressed conditions

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A great number of studies have proven that sHsps protect cells by inhibiting protein aggregation under heat stress, while little is known about their function to protect cells under acid stress. In this work, we show that Hsp20.1 and Hsp14.1 oligomers dissociated to smaller oligomeric species or even dimer/monomer at low pH (pH 4.0 and pH 2.0), whereas no prominent quaternary structural changes were seen at 30 °C. Both oligomers and smaller oligomeric species exhibited abilities to suppress client aggregation at low pH and at 50 °C. These results suggest that sHsps may function in different modes in different stressed conditions.

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develops a sophisticated protective system including many proteins that are stable to low pH and high temperature in the extreme environment \cite{19,20}. Hsp20.1 and Hsp14.1 from \textit{S. solfataricus P2} are members of the family of stress-inducible molecular chaperone that can suppress protein aggregation and precipitation. Therefore, they are ideal candidates for probing the protection mechanism of sHsps in acid- and heat-stressed conditions.

In this report, we show that both Hsp20.1 and Hsp14.1 dissociate into smaller oligomeric species or even dimer/monomer at low pH (pH 2.0 and 4.0), but not at elevated temperature (50°C). Interestingly, Hsp20.1 exhibits strong ability to suppress T1 aggregation/precipitation at pH 4 and MDH aggregation/precipitation after neutralising the buffer from pH 2.0 to pH 7.0, while Hsp14.1 becomes unstable in acidic conditions and shows only limited activity to inhibit client aggregation/precipitation. We also find that Hsp20.1 and Hsp14.1 can effectively inhibit client aggregation at 50°C. These observations indicate that there may be different mechanisms for sHsps to protect clients in different stresses.

2. Materials and methods

2.1. Materials

Mitochondrial malate dehydrogenase (MDH) from porcine was purchased from Sigma. T1 lipase was expressed and purified as previously described \cite{21}. DNA polymerase and restriction enzymes were purchased from Takara.

2.2. Expression and purification of Hsp20.1 and Hsp14.1

The genes of Hsp20.1 and Hsp14.1 were amplified from the genome of \textit{S. solfataricus P2} by polymerase chain reaction (PCR). The purified PCR products of Hsp20.1 and Hsp14.1 were digested with NheI/XhoI and NdeI/XhoI, respectively, and inserted into pET-23a expression vector. Both sHsps were soluble when expressed in \textit{E. coli}. After breaking cells by sonication and clearing lysate by centrifugation (20,000g for 4°C for 30 min), the supernatant was first incubated at 75°C for 30 min to denature host proteins and then centrifuged at 20,000g for 4°C for 30 min to remove the denatured proteins. Finally, the sHsps were further purified by ion-exchange chromatography on a Resource Q 1/6 column (GE Healthcare Life Sciences) followed by size-exclusion chromatography on a Superdex 200 10/300 GL column (GE Healthcare Life Sciences). The proteins were concentrated to a final concentration of 20 mg/ml in 50 mM Tris–HCl buffer of pH 8.0 (with 150 mM NaCl and 1 mM Tris (2-carboxyethyl) phosphine (TCEP)).

2.3. Size exclusion chromatography assay

Size exclusion chromatography (SEC) was performed using a Superdex 200 10/300 GL column (GE Healthcare Life Sciences) at a flow rate of 0.5 ml/min with absorbance monitored at 280 nm. Acid induced oligomeric dissociations were examined by SEC in a 50 mM Tris–HCl buffer of pH 8.0 (with 150 mM NaCl), an HAc buffer of pH 4.0 (with 50 mM NaAc and 150 mM NaCl), or an HCl buffer of pH 2.0 (with 150 mM NaCl, adjusted to pH 2.0 with HCl, the final HCl concentration was 10 mM) at 25°C for 30 min, the samples were neutralized for 30 min by adding in 4 M sodium hydroxide and then centrifuged (10,000g, for 10 min) with the supernatants loaded on the column. For temperature induced quaternary structural changes, the sHsp proteins (1 mg) were incubated in a 50 mM Tris–HCl buffer of pH 8.0 (with 150 mM NaCl) at 25°C or 50°C for 30 min, the samples were then analyzed immediately by SEC in 50 mM Tris, 150 mM NaCl, pH 8.0.

2.4. Circular dichroism (CD) spectroscopy

CD spectra of sHsps were performed using a chirascan CD spectrometer (Applied photophysics Ltd, United Kingdom) in a range from 190 to 250 nm with a 1 mm quartz cuvette. For the secondary structure changes of sHsps in acid stress, the sHsp proteins (0.2 mg/ml) were incubated in a 50 mM Tris–HCl buffer of pH 8.0, an HAc buffer of pH 4.0 (with 50 mM NaAc), or an HCl buffer of pH 2.0 (adjusted to pH 2.0 with HCl, the final HCl concentration was 10 mM) at 25°C for 30 min before the recording. For the secondary structure changes of sHsps in acid stress and following neutralization (pH 8.0), the sHsp proteins (0.2 mg/ml) were first incubated in an HAc buffer of pH 4.0 (with 50 mM NaAc), or an HCl buffer of pH 2.0 (adjusted to pH 2.0 with HCl, the final HCl concentration was 10 mM) at 25°C for 30 min, the samples were then neutralized (pH 8.0) for 30 min by adding in 4 M sodium hydroxide before the recording.

For temperature induced secondary structure changes, the sHsp proteins (0.2 mg/ml) were incubated in a 50 mM Tris–HCl buffer of pH 8.0 at 25°C or 50°C before the recording.

2.5. Anti-aggregation activity assay by spectrophotometry

The chaperone activity of sHsps to inhibit client aggregation/precipitation in/following heat or acid treatment was assayed by monitoring turbidity change with a UV–755B spectrophotometer (Shanghai Metash Instruments Co., Ltd.).

For acid stress, T1 (6.5 μM) was incubated in HAc buffer of pH 4 (with 50 mM NaAc and 150 mM NaCl) in the presence (1.625 μM, 3.25 μM, 13 μM) or absence of sHsps at room temperature for 30 min. MDH (10 μM) was incubated in HCl buffer of pH 2.0 (with 150 mM NaCl and 150 mM ammonium sulfate, adjusted to pH 2.0 with HCl) in the presence (5 μM, 10 μM, 20 μM) or absence of sHsps at room temperature for 4 min. After 4 min, the samples were then neutralized for 6 min by adding in 4 M sodium hydroxide. Turbidity change was monitored at 320 nm during the whole process.

For temperature stress, MDH (4.5 μM) was incubated at 50°C in 50 mM Tris–HCl buffer of pH 8.0 (with 150 mM NaCl) in the presence (27 μM, 54 μM, 108 μM) or absence of sHsps. Absorbance of the samples at 360 nm was monitored in a time course manner during the whole process.

2.6. Anti-precipitation activity assay by SDS–PAGE

The chaperone activity of sHsps to inhibit client aggregation/precipitation in/following acid treatment was examined by SDS–PAGE. The client protein was treated with acidic buffer as described above and then neutralized (by adding in 4 M sodium hydroxide) and incubated at room temperature for 30 min. After these treatments, the sample was centrifuged (10,000g, for 10 min, at room temperature), and the pellet was re-suspended in the same buffer to a volume equal to that of the supernatant. Both supernatant and pellet were then analyzed by SDS–PAGE.
3. Results

3.1. Low pH induced dissociation of Hsp20.1 and Hsp14.1 oligomers

To investigate the effect of acid on oligomeric states of sHsps, we examined the apparent molecular size at varied pH by size exclusion chromatography (SEC). At pH 8.0, Hsp20.1 (Fig. 1A) and Hsp14.1 (Fig. 1B) both formed large oligomers with apparent molecular weight of about 480 kDa and 360 kDa, respectively, corresponding to oligomers consisting of 24 subunits. At pH 4.0, the 24-mer-like oligomeric peaks of Hsp20.1 and Hsp14.1 disappeared and were replaced by smaller oligomeric peaks, indicating the disruption of the large oligomers. Hsp20.1 showed up as two peaks with apparent molecular weight of about 38 kDa and 14 kDa, corresponding to dimer and monomer, respectively. However, only one symmetrical peak of about 28 kDa was observed for Hsp14.1, indicating the existence of Hsp14.1 dimer. As pH of the solution decreased to about 2.0, a single peak of Hsp14.1 corresponding to monomer molecular size of this protein was observed, indicating dissociation form dimer to monomer at this low pH. However, Hsp20.1 showed up as two peaks with apparent molecular weight roughly corresponding to oligomer of 6–10 subunits and dimer, respectively.

In order to detect whether the pH-induced disassembly is reversible, we then examined the oligomeric states of sHsps which were pre-incubated at low pH buffer and then returned to pH 8.0 buffer. When the pH value was raised from pH 4.0 to 8.0, both Hsp20.1 (Fig. 2A) and Hsp14.1 (Fig. 2B) showed the 24-mer-like oligomeric peaks, demonstrating that Hsp20.1 and Hsp14.1 small oligomeric species are able to reassociate to form 24-mers. However, except the 24-mer-like oligomeric peaks, some other heterogeneous oligomeric peaks were also observed, which suggested that the dissociation process of Hsp20.1 and Hsp14.1 was not completely reversible. Especially when the pH value was raised from pH 2.0 to 8.0, almost no 24-mer-like oligomeric peak of Hsp14.1 was observed, indicating that the dissociation process of Hsp14.1 from pH 8.0 to 2.0 was not reversible at all.

We also detected the effect of pH on the secondary structures of the sHsps. CD spectra indicated that Hsp20.1 have nearly identical secondary structural features at pH 4.0 and pH 8.0 (Fig. 3A and C).
However, the secondary structure was changed at pH 2.0 and following neutralization, revealing an acid-induced conformational transformation of Hsp20.1. Interestingly, Hsp14.1 kept the β-sheet rich conformation at acidic pH and neutralized pH (Fig. 3B and D). This result indicated that the acidic pH (2.0 and 4.0) had no strikingly effect on the secondary structure of Hsp14.1.

3.2. Chaperone activity of Hsp20.1 and Hsp14.1 at pH 4.0

To determine whether Hsp20.1 and Hsp14.1 can suppress client aggregation under acid stress, we first studied the interaction between these sHsps and the T1 lipase at pH 4.0. T1 lipase was sensitive to acid stress and underwent rapid aggregation at pH 4.0. As is shown in Fig. 4A, turbidity of the solution significantly increased over time when T1 was exposed in the acidic condition of pH 4.0 in the absence of Hsp20.1, indicating aggregation of this client protein, while in the presence of Hsp20.1, T1 aggregation was significantly reduced with increase amount of Hsp20.1. Suppression of aggregation reached the control level (Hsp20.1:T1 = 8:0, i.e. no T1 presented) when molar ratio of Hsp20.1:T1 increased to 8:4 (or 2:1), suggesting complete inhibition of T1 aggregation at this low molecular ratio. In agreement with the turbidity assay, SDS–PAGE showed that addition of Hsp20.1 significantly reduced the portion of insoluble T1 in this system, and that when the molecular ratio between Hsp20.1 and T1 reached 8:4, no precipitate of T1 was observed (Fig. 4C). Interestingly, below the 8:4 M ratio, small amount of Hsp20.1 also appeared in the precipitates. Since Hsp20.1 by itself did not aggregate and precipitate, this observation indicate that inadequate Hsp20.1 could not stop aggregation and precipitation, but still can bind to the denatured T1, thus was brought to precipitate by T1 in the assay.

More complicated phenomena were observed in the case of Hsp14.1. SDS–PAGE analysis indicated that adding in Hsp14.1 could help preventing precipitation of T1, but the ability of Hsp14.1 to protect T1 was weaker than that of Hsp20.1 (Fig. 4D). For example, even at 8:4 M ratio, Hsp14.1 still could not completely inhibit precipitation. What’s more, Hsp14.1 much more seriously co-precipitated with T1 in the acidic buffer than Hsp20.1 did. This observation was confirmed by the turbidity assay (Fig. 4B). Only at low molar ratio did Hsp14.1 seemed to inhibit aggregation. When the molar ratio increased, the observed turbidity increased accordingly. Taking together the SDS–PAGE data and the turbidity data, it becomes clear that the real reason that caused turbidity increasing was precipitation of Hsp14.1 in the presence of denatured T1. Although denatured T1 also co-precipitated with Hsp20.1 when there was not enough Hsp20.1, only a small portion of the total Hsp20.1 co-precipitated likely with the not fully protected T1 (Fig. 4A and C). In the case of Hsp14.1, the co-precipitation triggered by denatured T1 involved most of the Hsp14.1 protein. Actually, Hsp14.1 by itself was not stable in acidic buffer. A small portion of Hsp14.1 precipitated even in the absence of T1.

Taking together, these data indicate that although both Hsp20.1 and Hsp14.1 have chaperone activity to protect client protein (T1) in acidic condition, their potency is different. Hsp20.1 is a better protector than Hsp14.1 under acid stress, probably mainly because it is by itself more stable in this condition than does Hsp14.1.
3.3. Chaperone activities of Hsp20.1 and Hsp14.1 after neutralising the buffer from pH 2.0 to pH 7.0

It is helpful to analyze chaperone activity of sHsps both during and after acid stress, since in real life the stress may not last long. For this purpose, we studied aggregation/precipitation of porcine mitochondrial malate dehydrogenase (MDH) after neutralising the buffer from pH 2.0 to pH 7.0. MDH remained soluble at pH 2.0, but underwent aggregation after neutralization (Fig. 5), as was reported before [10]. The aggregation/precipitation was strongly suppressed by Hsp20.1, but not by Hsp14.1, which on the contrary, aggregated by itself at pH 2.0 and co-aggregated and precipitated with MDH after neutralization. These observations were coincide with the observed behavior of Hsp20.1 and Hsp14.1 on T1 at pH 4.0 (Fig. 4A and C), both suggesting the functional difference between the two sHsps under acid stress.

Additionally, there is a difference in peak volume in the SEC experiments. Both Hsp20.1 and Hsp14.1 showed different peak volumes in different pH (Fig. 1), which may indicate that some sample got lost (as insoluble aggregate) after incubation at low pH. Our SDS–PAGE results also indicate that a small portion of insoluble sHsps may be resulted by the rapid conformational changes induced by the low pH as the other chaperone HdeA. It is found that HdeA is only partially unfolded by acid treatment but retains significant secondary structure [22]. Our CD data also proved that sHsps can retain secondary structures in acid stress (Fig. 3). Conformation changes result the exposure of hydrophobic surfaces that may destabilize the sHsps structures.

3.4. Oligomeric status and chaperone activity of Hsp20.1 and Hsp14.1 at 50°C

Oligomeric status of Hsp20.1 and Hsp14.1 were studied by size exclusion chromatography (SEC) to investigate whether oligomers of these two proteins undergo dissociation at 50°C. Hsp20.1 showed up as one major peak appearing at potentially the same position after treatment at 25°C and 50°C (Fig. 6A), corresponding to 24-mer of this protein with about 480 kDa molecular weight. Similarly, Hsp14.1 also showed up as one major peak of about 360 kDa molecular weight after treatment at 25°C and 50°C (Fig. 6B), corresponding to 24-mer of this protein, too. Both Hsp20.1 and Hsp14.1 exhibited no changes in the circular dichroism (CD) spectra in the range from 25 to 50°C (Fig. 7), indicating that temperature (25°C and 50°C) had no effect on the secondary structures of Hsp20.1 and Hsp14.1.

We then studied the chaperone activity of Hsp20.1 and Hsp14.1 to suppress MDH aggregation at 50°C through turbidity assay. Both Hsp20.1 and Hsp14.1 showed strong capability to inhibit MDH aggregation (Fig. 6C and D), which is similar to the function of the previously reported Hsp14.0 [23]. Inhibition of MDH aggregation became stronger when the molar ratio between sHsp and MDH increased. Good inhibition behavior was observed when the molar ratio reached 24:1. Since Hsp20.1 and Hsp14.1 maintain the 24-mer-like oligomeric states and do not dissociate to smaller
oligomers at 50 °C, it may suggest that Hsp20.1 and Hsp14.1 use oligomers to bind MDH at 50 °C.

4. Discussion

Acid stress kills cells by causing unfolding of many cellular proteins that results in loss of their functions [22]. sHsps, as an important family of molecular chaperones, play important roles in maintaining of cellular protein homeostasis by preventing or reversing protein aggregation upon proteotoxic stresses such as heat or acid stresses [24]. However, although the function of sHsps to protect denatured clients under heat stress has been intensively studied, their behavior under acid stress remains poorly understood. In this work, we studied the behavior and activity of Hsp20.1 and Hsp14.1 in acidic conditions, and compared them with their behavior and activity under temperature stress.

sHsps were first discovered because of their expression can be strongly induced by heat shock [25,26]. Subsequently, several studies indicated that sHsps can be activated at high temperature with no or only limited changes in the quaternary structure [2,27]. Indeed, heating induced oligomer dissociation was observed only in few sHsps, such as pea Hsp18.1 and Schizosaccharomyces pombe Hsp16.0 [27]. Size exclusion chromatography (SEC) and cryo-electron microscopy (EM) researches indicated that Methanocaldococcus jannaschii Hsp16.5 existed as ordered 24-subunit oligomers and formed oligomeric complexes with client proteins at 37 °C [15]. Factors destabilizing the sHsp oligomers can impair the chaperone activity of sHsps, and disrupting the oligomer may result a total defect of the chaperone activity [28]. These data indicate that chaperone activity of some sHsps under heated condition may be oligomer-dependent. Our data also indicate that both sHsps work as oligomer at 50 °C.

Strikingly, acid induced dissociation of 24-subunit-like oligomer to smaller oligomer or even dimer/monomer was observed in our studies. Interestingly, these smaller oligomeric species maintain the capability to prevent T1 aggregation/precipitation at pH 4.0. Hsp20.1 can also prevent MDH aggregation/precipitation after neutralising the buffer from pH 2.0 to pH 7.0. These results may indicate that chaperone activity of Hsp20.1 and Hsp14.1 under acid stress may not be oligomer-dependent. It is then intriguing how sHsp capture denatured clients of various sizes and shapes at a low pH conditions.

Our data confirmed that Hsp20.1 works better than Hsp14.1 as holdase to prevent client aggregation under acid stress. Interestingly, both Hsp20.1 and Hsp14.1 can effectively inhibit client aggregation at 50 °C. These data along with the observation that both sHsps dissociate to smaller oligomer or dimer/monomer under acid stress but not under temperature stress indicate that they may interact with clients in different binding modes under acid and temperature stresses.

There is large amount of researches showing that sHsps use their N-terminus to bind substrate [29]. The N-terminus, which contains a lot of hydrophobic residues and is located on the inner surface of the oligomer, may undergo conformational change upon heat shock and act as a tentacle to bind the client protein [15]. The multi-tentacle architecture for sHsps to bind denatured clients under heat-stressed condition has been proposed by many studies and was indicated in our study, too. However, acid induced oligomer dissociation may expose a large number of hydrophobic surfaces that are normally hidden inside the oligomer, including not only the N-terminus, but also the dimer–dimer and even the monomer–monomer interfaces. These hydrophobic surfaces may all participate in interaction with unfolded substrates in acidic conditions. To this end, Hsp20.1 is similar to the E. coli proteins HdeA
and HdeB, which act as monomers with partially unfolded and highly flexible architecture at low pH and show high-affinity binding sites contribute to prevent protein aggregation in vitro [10]. However, the exact molecular mechanism underlying the chaperone activity of sHsps under acid stress needs to be further studied by means of biochemistry and structural biology.

Fig. 6. Oligomeric status and chaperone activity of Hsp20.1 and Hsp14.1 at 50 °C. Size-exclusion chromatography profiles of Hsp20.1 (A) and Hsp14.1 (B) after treating at 25 °C or 50 °C are shown. Apparent molecular weight corresponding to the peaks was evaluated by running molecular mass markers on the same column (ferritin, 440 kDa; BSA, 67 kDa; β-lactoglobulin, 35 kDa; ribonuclease A, 13.7 kDa). Inhibition of heating induced aggregation of MDH by Hsp20.1 (C) and Hsp14.1 (D) was shown by turbidity (light absorbance at 360 nm) change over time of the samples with increasing amount of sHsps.

Fig. 7. CD spectra of sHsps at different temperature. CD spectra of the Hsp20.1 (A) and Hsp14.1 (B) recorded at indicated temperature. Solid line and dot line indicate spectra at 25 °C and 50 °C respectively.
Author contributions

L.L. and J.-Y.C. designed experiments, conducted studies, analysed data and wrote the manuscript. B.Y. and Y.-H.W. instructed the project and analysed data.

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