The pH Sensitivity of Murine Heat Shock Protein 47 (HSP47) Binding to Collagen Is Affected by Mutations in the Breach Histidine Cluster*

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Background: HSP47 is a single-substrate molecular chaperone essential for collagen biosynthesis.

Results: H197A and H198A mutants show binding but no apparent pH switch, whereas H191A shows perturbed collagen release characteristics.

Conclusion: The residues participating actively in HSP47 pH switch mechanism localize to the breach cluster.

Significance: The data will be an important basis for future studies of HSP47 behavior at the cellular level, and on collagen binding by HSP47.

Heat shock protein 47 (HSP47) is a single-substrate molecular chaperone crucial for collagen biosynthesis. Although its function is well established, the molecular mechanisms that govern binding to procollagen peptides and triple helices in the endoplasmic reticulum (followed by controlled release in the Golgi) are unclear. HSP47 binds procollagen at a neutral pH but releases at a pH similar to the pKₐ of the imidazole side chain of histidine residues. It thus seems likely that these residues are involved in this pH-dependent mechanism. Murine HSP47 has 14 histidine residues grouped into three clusters, known as the breach, gate, and shutter. Here, we report the use of histidine mutagenesis to demonstrate the relative contribution of these three clusters to HSP47 structure and the “pH switch.” Many of the tested mutants are silent; however, breach mutants H197A and H198A show binding but no apparent pH switch and are unable to control release. Another breach mutant, H191A, shows perturbed collagen release characteristics, consistent with observed perturbations in pH-driven conformational changes. Thus, His-198, His-197 and His-191 are important (if not central) to HSP47 mechanism of binding/release to collagen. This is consistent with the breach cluster residues being well conserved across the HSP47 family.

In the event of cirrhosis (or other such pathological fibrosis), the liver may contain up to six times the normal amount of extracellular matrix. Among the most abundant components are collagen types I, II, and IV (1), which are critical to the specific mechanical and biochemical properties of tissues in multicellular animals (2).

Collagen contains bundles of triple helices, constructed from three individual polypeptide chains (3–5). Each polypeptide chain is characterized broadly by a (GXY)n triplet repeat amino acid residue sequence, wherein the first position is always a glycine residue and X and Y are usually proline or hydroxyproline residues (4). Polypeptide chains are synthesized initially in the endoplasmic reticulum (ER) of fibroblast cells, as procollagen polypeptides that possess additional N- and C-terminal propeptides. The interpolypeptide association of C-terminal propeptides (in particular in the ER) leads to the formation of trimeric procollagen polypeptide assemblies (6). Following association, interchain disulfide bridges are formed that initiate the generation of procollagen triple helices, propagated from C- to N-terminal. Such triple helices self-assemble into higher-order bundles with ease at body temperature; hence, procollagen/collagen-binding proteins are needed to control this and other processes in vivo, preventing premature associations (7, 8). Typically, bundle formation is initiated following transportation of triple helices from the ER to the Golgi apparatus. Thereafter, these are exported through the Golgi to the outside of cells (9) for further processing and assembly into collagen fibrils (10).

Throughout the synthesis and processing of procollagen, protein disulfide isomerase, prolyl 4-hydroxylase, and heat shock protein 47 (HSP47) (11, 12) all appear to play important roles in assisting and/or controlling the assembly of the triple helices. HSP47 (also known as colligin or J6 protein) is a single-
substrate molecular chaperone essential for collagen biosynthesis. Knocking out the hsp47 gene has been shown to lethally impair the development of mouse embryos, preventing the secretion of correctly folded triple helical molecules (13).

In our previous work, we described a comprehensive biophysical study of mature recombinant murine HSP47 (labeled here as wild-type: HSP47 WT), demonstrating its molecular chaperone characteristics for the first time (14). We showed that HSP47 WT binding is able to prevent collagen fibril formation at neutral pH in vitro, obviating normal uncontrolled aggregation under these conditions. HSP47 WT was also shown to bind to procollagen mimic peptides (Pro-Pro-Gly)_{10}, thereby inducing polyproline type II helix character and promoting the formation of higher order triple helical structures (14). The in vitro and in vivo interactions of HSP47 with collagen triple helix have recently been demonstrated (15). Finally, the HSP47 trimer was proposed to assist with the propagation of procollagen triple helix generation, once the three procollagen polypeptide chains were associated by disulfide bridge formation (14). More recently, GXR sequences within procollagen have been found to be critical sites of HSP47 interaction (16–18), enhanced by a sequential G sequence in the same poly-peptide strand (19).

HSP47 appears to remain in association with procollagen triple helices until transport from the ER to the cis-Golgi (20), at which point HSP47 is released and recycles back to the ER by a C-terminal RDEL ER retention sequence (21). The pH profile of HSP47-binding affinity substantially (at least 1000-fold) under mildly acidic conditions. These pH-dependent changes in binding affinity were found to correlate with pH-driven trans-conformational changes within HSP47 itself (22), suggesting that the conformational and binding affinity changes are strongly related. This has become known as the “conformational pH switch” or simply “pH switch” mechanism of HSP47 (14). Previously, we have suggested that the pH profile of the switch mechanism would be consistent with an active role for histidine residues in procollagen polypeptide binding and release (22).

Cellular mechanisms and the potential for therapies emanating from HSP47—procollagen/collagen interactions have been extensively researched (23, 24). But still little has become known, including the architecture and inner workings of the pH switch mechanism. The lack of x-ray crystal structural information has been a significant limitation. Fortunately, HSP47 belongs to a protein family that is very well characterized, namely the serine protease inhibitor (or serpin) family. Serpins belong to a protein family that is very well characterized, including the architecture and inner workings of the pH switch mechanism. These histidine-to-alanine (HA) mutants designed to probe for those HSP47 subdomains affected by the pH-dependant conformational changes.

**EXPERIMENTAL PROCEDURES**

**Molecular Modeling**—HSP47 WT shares a high homology with serpin family members (generally ~30% identity) (28). This allows the construction of a homology model of the chaperone based on the known structure of α1-antitrypsin (Protein Data Bank code 1QLP) (29) using Deepview (version 4.0.1) (30). The model constructed has a pairwise alignment score of 97% when analyzed with ClustalW (31, 32) and thus allows the use of the model as a guide for mutagenesis experiment.

**Cloning and Site-directed Mutagenesis**—The mouse HSP47 cDNA was amplified by PCR using 5’ and 3’ primers with HindIII and NdeI restriction sites, respectively. Subsequently, the gene was cloned into the pET-24b(+) vector (Novagen, Germany), which contains the equivalent restriction sites. HA mutations were introduced using a QuikChange site-directed mutagenesis kit (Stratagene) and using methods developed previously (33) with Escherichia coli strain DH5α as host. Oligonucleotides were synthesized and supplied by MWG-Biotech or Invitrogen. The primers used were as follows (with the introduced mutation sites underlined): H191A, 5’-ATG TTC TTT AAG CCA CAC TTT GAT GAG AAG TTT GCA GCA AAG ATG GTG G; H198A, 5’-CAC TGG GAT GAG CCG C; H302A, 5’-CAT GAC CTG CAG

**Preparation of HSP47 WT and Mutant Proteins**—Strain BL21(DE3)pLysS carrying HSP47 WT or HA mutant-containing pET-24b(+) plasmid as appropriate was grown on 2 × YT medium (tryptone 16 g/liter, yeast extract 10 g/liter, NaCl 5 g/liter, deionized water) supplied with kanamycin (25 μg/ml),
chloramphenicol (20 μg/ml), and 0.4% (w/v) of glucose, and incubated at 37 °C overnight. Each preculture was used to inoculate an individual main culture of the same medium (without glucose) at a volume ratio of 1:20. The main culture was incubated in a shaker at 20 °C until the optical density at 600 nm reached 0.3. Isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich) was added (0.1 mM of final concentration) and shaking continued for 12–18 h. The cells were then harvested by centrifugation at 2700 × g for 10 min and resuspended in lysis buffer (50 mM sodium phosphate, pH 8.0, 0.5 M NaCl, 0.02% (v/v) Triton X-100, 3 mM β-mercaptoethanol) supplemented with a Complete mini EDTA-free protease inhibitor tablet (Roche Applied Science). Each bacterial lysis was carried out by sonication on ice (2 s on, 3 s off; 1.5 min per cycle, cycle repeated twice with 5 min of resting period between cycles; 20% intensity) with a VCX400 probe sonicator (Sonic and Materials). The resulting lysate was centrifuged at 30,600 × g for 20 min at 4 °C. Supernatant was filtered through a 0.2-μm syringe filter. Purification of HSP47 WT or HA mutants was carried out using collagen-affinity chromatography comprised of a 5-ml HR16/5 column (GE Healthcare) packed with gelatin-agarose beads (Sigma-Aldrich). Packed columns were stored at 4 °C until used. Before purification, the column was equilibrated with cold 50 mM sodium phosphate, pH 8.0, 100 mM NaCl, for ~5 column volumes. The flow rate was fixed at 1 ml/min for this and all of the subsequent steps, using a Fast Protein Liquid Chromatography system (Amersham Biosciences/GE Healthcare). The column was washed using the same buffer, before being eluted with a 70-ml 0–100% gradient of 50 mM sodium phosphate, pH 4.1, with 100 mM NaCl. Fractions (2 ml) were collected, and those with a high pH were immediately neutralized with 200 μl of 1 M sodium phosphate, pH 8.0. Protein samples were used within 24 h of purification and otherwise stored at 4 °C.

For purification of WF mutants, E. coli BL21(DE3) strain carrying WF mutant-containing pET-21a(+) plasmid as appropriated was cultivated as described above, except that purification was performed with a HisTrap HP column (1 ml) (GE Healthcare) according to the manufacturer’s instructions. The filtered supernatant was applied before being washed with binding buffer for at least 10–15 volumes. Finally, the bound protein was eluted with elution buffer (50 mM sodium phosphate, pH 8.0, 150 mM NaCl, 250 mM imidazole) using a linear gradient of 30× vol. Imidazole was removed using HiPrep 26/10 desalting column (GE Healthcare), pre-equilibrated, and eluted using the buffer required for equilibration and washing of the collagen affinity column above.

**Determination of Protein Concentration**—HSP47 protein concentrations were determined using either bichinchoninic acid (BCA) assay kit (Sigma-Aldrich) or from A280 values using the molar extinction coefficient of 42,400 M⁻¹ cm⁻¹ calculated by means of ProtParam (34).

**Collagen Affinity Pulldown Assay**—Lysis buffer was added for resuspension to pelleted cell cultures (30 ml of buffer per gram of cell pellet). Each cell suspension was then sonicated on ice, and 1.5 ml of lysate was removed and subject to centrifugation at 16,100 × g for 20 min at 4 °C. Supernatant (400 μl) was set aside for immunoblotting analysis. Otherwise, further supernatant (400 μl) was added to collagen affinity (gelatin-agarose) beads (20-μl bed volume) (Sigma-Aldrich). The mixture was then tumbled for 1 h at 4 °C before centrifugation at 3400 × g for 3 min at 4 °C. The resulting pellet was washed with washing buffer (200 μl) and subjected to recentrifugation. This process was repeated twice more before water (100 μl) was added, with 2× SDS sample buffer (100 μl) with added β-mercaptoethanol (10 μl), and heated at 95 °C for 5 min. The sample was subject to centrifugation one more time before loading to SDS-PAGE, followed by visualization by immunoblotting analysis.

**Immunoblotting Analysis**—Protein samples following electrophoresis on 12% Tris-glycine SDS-PAGE gel, were first transferred to ECL Hybond™ nitrocellulose membrane (GE Healthcare) at a constant voltage of 100 V for 1 h in 5 mM sodium tetraborate buffer. The membrane was blocked with 5% skimmed milk in PBS(−) buffer (10 mM Na2HPO4, 2 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl) at 4 °C overnight or at room temperature for 1 h. After discarding the blocking solution, anti-HSP47 primary antibody (Stressgen/Enzo Life Sciences) in skimmed milk (1:1000 dilution) was added and incubated at room temperature for another 1 h. After washing twice with 0.1% Tween 20 in PBS(−) for 10 min, the membrane was probed with goat anti-mouse secondary antibody, alkaline phosphatase conjugate (BIOSOURCE/Invitrogen) (1:2000 dilution) for an additional 1 h before 4 × 5-min washing with 0.1% Tween 20 in PBS(−) and 1× in MilliQ water for 3 min for the final wash. Colorimetric detection was performed using the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium tablet (BCIP/NBT) giving permanent dark blue bands on the membrane (Sigma-Aldrich). Quantification was performed using NIH ImageJ software (35).

**Collagen Fibrillization Assay**—The protocol used for collagen fibril formation was similar to that described previously (36, 37). Pepsin-solubilized type I collagen from porcine skin (Nitta Gelatin) (final concentration of 2 μM) was brought to pH 7.3–7.4 by the addition of 50 mM sodium phosphate containing 100 mM NaCl. The formation of collagen fibrils was then monitored the turbidity of the sample measured at A315 using a Thermo Varioskan microplate reader (Thermo Fisher Scientific). Each experiment was monitored over a period of 120 min at a constant temperature of 34 °C. Measurements were made in triplicate.

**Circular Dichroism Spectroscopy**—Circular dichroism (CD) spectra were recorded on a JASCO J715 spectropolarimeter using a thermostatically controlled 10-mm quartz cuvette. HSP47 WT or mutants (3 μM) were studied in 50 mM sodium phosphate buffer, pH 8.0, containing 100 mM NaCl. Far UV CD spectra were recorded from 300–190 nm at a constant temperature of 25 °C using a 2-mm cuvette. Each scan was set at 100 nm min⁻¹ and a standard sensitivity (100 millidegrees). The response was set at 4 s with 2 nm of bandwidth. Each spectrum was an accumulation of 5–16 scans corrected for buffer contribution. For temperature titration experiments, ΔA222 was monitored, whereas the temperature was increased at a constant rate of 1 °C per minute from 25 to 90 °C. Melting temperatures were estimated from the temperature titration profile with HSP47 samples at concentrations ranging from 2.8–3.0 μM. To
calculate $T_{m}$, the fraction unfolded was obtained using the following equation, modified from (38),

$$F(T) = \frac{\theta(T) - \theta_d(T)}{\theta_p(T) - \theta_d(T)}$$  

(Eq. 1)

where $\theta$ is the observed ellipticity and $\theta_d$ and $\theta_p$ are the ellipticities for the denatured and folded. $T_{m}$ was obtained at the midpoint of transition, where $F(T_{m}) = \frac{1}{2}$. The transitional pH was estimated from the pH titration profile. For pH titrations, these were carried out using an autotitrator connected to the spectropolarimeter. Samples were added to a stirred, thermostatically controlled 1-cm quartz cuvette, and pH was then progressively decreased by the addition of 2.5-μl aliquots of phosphoric acid. HSP47 WT and mutants were studied at a final protein concentration of 3 μM in 50 mM sodium phosphate buffer, pH 8.0, containing 100 mM NaCl at 25 °C. The change in $\Delta A_{222}$ was then measured. The actual pH in the cuvette post equilibration after each acid addition was determined using an ultramini pH electrode (Hamilton, Switzerland). Equation 1 was used to estimate the inflection point of transition by replacing $T$, $\theta_p$, and $\theta_d$ with pH, $\theta_{Ac}$, and $\theta_{Al}$, which are ellipticities measured at pH values appropriate for the quantitative appearance of full acid and alkali states, respectively.

Steady-state Intrinsic Tryptophan Fluorescence—Intrinsic tryptophan fluorescence emission spectra were recorded on a Shimadzu RF-5301PC spectrofluorophotometer at 25 °C using a thermostatically controlled 10-mm quartz cuvette in the same buffer as in the CD experiment. Samples of HSP47 WT or WF mutants were excited at 295 nm, and emission detected from 310–410 nm. The excitation slit width was set at 3 nm, and the emission slit widths were varied between 10–15 nm. The pH titration experiments were performed by the addition of small aliquots of phosphoric acid (1–2 μl each) with constant stirring. HSP47 WT or mutant concentrations were fixed to 1 μM for all analyses. Each spectrum was the average of three repetitions. All experiments were background-corrected and performed within 12 h after protein purification.

Averaged individual spectra recorded at various pH values were then fitted to polynomial model second order for the purpose of data extraction using OriginPro software (version 8.5, OriginLab). Fitting ensures more accurate and smoother data for plotting maximum fluorescence intensity ($I_p$) and wavelength at maximum intensity ($\lambda_{em}$). Values of $\Delta \lambda_{em}$ were plotted against pH, and data were then fitted to single pH ionisation model using GraFit Pro (version 7.0.0) (39) based on Equation 2. The $y$ values vary from one value at low pH to another at high pH, depending upon the ionization of a group with a certain $pK_a$.

$$y = L_1 + \frac{(L_2 - L_1) \times 10^{pH - pK_a}}{10^{pH - pK_a} + 1}$$  

(Eq. 2)

$L_1$ represents limit 1 or the upper limit of the $y$ ($\Delta \lambda_{em}$) values, whereas $L_2$ is the lower limit.

RESULTS

Effect of Mutations on Expression and Purification of Mature Recombinant Murine HSP47—Here, we used site-directed mutagenesis of HSP47 WT to probe the pH switch mechanism in detail. Examination of our homology model indicated that six highly conserved histidine residues (one of middling conservation) could be grouped into three clusters based on their locations, here described according to serpin nomenclature (Fig. 1) (40). Seven HA mutants were constructed aimed at these clusters: H191A (located in the s3A-s4C loop), H197A, H198A, and H197A,H198A (s3A-s4C), and H244A (s2B-s3B) all are in the breach cluster based on the homology model; H255A,H256A (s3B-hG) is in the gate cluster; and H302A (h1) is in the shutter cluster. These residues are also predicted to be located at the surface of HSP47 molecule and solvent-accessible (data not shown).

Following the expression of HSP47 WT or HA mutants in E. coli BL21(DE3)pLysS cells, SDS-PAGE analysis was used to demonstrate that the soluble fraction of recombinant protein still represents a substantial percentage of the whole in all cases.
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(FIG. 2A). Following this, soluble fraction HSP47 WT or HA mutants were analyzed using a semi-quantitative collagen affinity pulldown assay. This pulldown assay system was based on the application of gelatin-agarose microbeads. Gelatin itself is a composite denatured form of collagen, containing a mixture of single-stranded and triple helical collagen molecules. Given the broad specificity of HSP47 WT for procollagen peptides and triple helices, gelatin makes for an excellent, readily available source of collagen for in vitro binding assays. Pulldown assays were introduced to quantify the relative proportion of each soluble fraction HA mutant protein that was also competent to bind collagen under mildly alkaline conditions, with results normalized relative to HSP47 WT. To achieve this, HA mutants were combined with beads for 1 h at 4°C. Next, beads were washed thoroughly to remove unbound components and then extracted with SDS to remove collagen-bound protein prior to SDS-PAGE. An equivalent sample of each soluble fraction HA mutant protein was also analyzed by SDS-PAGE for control comparison. HA mutant bands were then detected on gel by immunoblotting and analyzed using ImageJ software (35); in each case, band intensity was assumed to correlate directly with immunodetection analysis was performed using ImageJ software (35); error bars represent ± S.E. B, solution concentration and collagen affinity activity of the HSP47 HA mutants relative to WT, as measured by Western blot. Immunodetection analysis was performed using ImageJ software (35); error bars represent ± S.D. of 3–6 replicates.

FIGURE 2. A, percentage of soluble HSP47 as estimated by SDS-PAGE (12% Tris-glycine gels, stained with Coomassie Brilliant Blue). Gel analysis was performed using ImageJ software (35), and error bars represent ± S.E. B, solution concentration and collagen-affinity bound HSP47 WT.

Following this pulldown assay, we developed a single-step collagen affinity chromatography procedure for the efficient generation of homogeneous HA mutants and WT. This procedure was found to be rapid, reproducible, and appropriate for routine use. These characteristics were all the more important given the observed propensity of recombinant HSP47 WT and HA mutants to aggregate in buffer solution over time. Variations of this approach have been used for HSP47 purification previously (14), but the technique reported here is the fastest, most reproducible, and reliable.

Using our procedure, HSP47 WT was found to elute in a well defined peak at around pH 5.7 (Fig. 3). HA mutants H191A, H244A, H255A,H256A, and H302A showed similar elution profiles. However, upon closer observation, H191A was found to elute with a peak maximum at a slightly higher pH value. More notably, the double mutant H197A,H198A showed little capacity for controlled cooperative release from the affinity column with decreasing pH gradient elution. Instead, following initial column elution with 5 bed volumes of buffer at mildly alkaline pH, the double mutant protein leached off in the form of an extended elution tail with decreasing pH (Fig. 3). SDS-PAGE analysis of the tail fractions demonstrated that only HSP47 was present in the elution tail to >95% purity (Fig. 3). Moreover, H197A and H198A single mutants also exhibited similar if less profound elution behavior. Combining evidence from purification data and the pulldown assay (Fig. 2) H197A, H198A, and H197A,H198A appeared to be soluble mutants that were also essentially as competent to associate with collagen as HSP47 WT under mildly alkaline conditions. However, the mutagenesis of either or both histidine residues appears to have removed the capacity for pH-triggered controlled, cooperative release. In the case of the H191A mutant, the displacement of the elution peak to higher pH suggested that pH-triggered cooperative release was perturbed to some extent by the mutation compared with HSP47 WT. Other histidine mutations were notably silent in terms of their effects on binding and elution profile compared with HSP47 WT. These mutants (H244A, H255A,H256A, and H302A) plus H191A that were purified with relative ease, were then taken on for further analysis. H197A, H198A, and H197A,H198A were found to be refractory to further study due to a strong propensity toward aggregation post-elution and fraction concentration.

Effects of Mutations on Folding and Stability—The far-UV CD spectra of HSP47 WT and HA mutants H191A, H244A, H255A,H256A, and H302A were all essentially equivalent and completely typical of an α/β-protein with negative maxima at 208 and 220 nm (results not shown). Hence the mutations did not appear to have perturbed protein structure at mildly alkaline pH to any significant extent relative to WT. This was confirmed by CD temperature titration experiments that established that HSP47 WT and these HA mutants were subject to essentially equivalent thermal denaturation processes with T_m values centered at 58–59 °C (see Table 1). Such behavior is indicative of HSP47 WT and the indicated HA mutants adopting a comparable serpin ”metastable” protein fold, wherein the central A β-sheet is composed of only five strands, whereas the sixth strand is free to promote the formation of a reactive center.
loop that is responsible for mediating protease inhibition in inhibitory serpin family members (14).

Effects of Mutations on Collagen Anti-fibrillization—HSP47 WT is known to have the ability to arrest collagen fibril forma-

tion in vitro by binding at neutral pH. However, this capability is abolished at around pH 6.2–6.3, presumably as a consequence of collagen release (37). Collagen fibrillization in vitro involves a lag phase, a propagation phase, and finally an equilibrium phase where fibrils are in equilibrium with monomers (37, 41). Collagen type I fibrillization is typically studied at 34 °C by monitoring sample turbidity measured at $A_{313}$ (36). In our case, the turbidity of a collagen solution alone, plotted as a function of time showed a lag phase of ~10 to 20 min (Fig. 4A), which is slightly longer than that reported previously (37). This may be due to the collagen used in this instance; our pepsin treated collagen type I is known to form shorter fibrils over a longer period of time than untreated collagen (42). When the HSP47 WT to collagen ratio was 1:1 (m/m), fibrillization was completely inhibited. However at a 1:2 (m/m) ratio, inhibition of fibrillization was incomplete, presumably due to insufficient binding of collagen by HSP47 WT. Similarly, all four HA mutants were able to arrest fibrillization fully with a HA mutant to collagen ratio of 1:1 (m/m) (Fig. 4B), consistent with previous results demonstrating that all of these mutants were

![FIGURE 3. Collagen affinity chromatography of soluble HSP47 WT and HA mutants, plus SDS-PAGE analyses of resulting fractions. The dotted line shows the pH gradient. A, WT, H191A, H244A, H255A, H256A, and H302A all show peak elution at about pH 5.7, as opposed to in an injection-peak “tail” as seen for H197A, H198A, and H197A,H198A. Close-up view of chromatograms (right) demonstrates that H191A is eluting at a slightly higher pH than WT and the other mutants. B, SDS-PAGE analysis of the early elution (E) and peak elution (E) fractions for HSP47 WT and H197A,H198A (shown as lines in boldface type on the FPLC trace). Fraction numbers are given in min after injection. Results demonstrate the presence of high H197A,H198A concentrations in the tail (W) fractions.

### TABLE 1

Summary of data and biophysical properties of HSP47 WT and HA mutants

Data shown include thermal stability, inflection points observed under pH titration study, degree of conservation in HSP47 family, and the secondary structural locations of the mutations introduced based on the HSP47 homology model. Locations are from Huber and Carrell nomenclature (51).

| Mutations | $T_m$ ± 0.5°C | pH titration inflection points | Conserved in HSP47 family? | Locations |
|-----------|-------------|-----------------|-----------------|-----------|
| WT        | 59.0        | 5.70 ± 0.10     | 100%            | s3A-s4C   |
| H191A     | 57.0        | 5.90 ± 0.05     | 100%            | s3A-s4C   |
| H197A     | 57.5        | 5.65 ± 0.05     | 92%             | s2B-s3B   |
| H198A     | 58.0        | 5.75 ± 0.10     | 46%             | s3B-hG    |
| H244A     | 58.0        | 5.75 ± 0.05     | 92%             | s3B-hG    |
| H255A     | 58.0        | 5.75 ± 0.05     | 92%             | s3B-hG    |
| H256A     | 58.0        | 5.75 ± 0.05     | 92%             | s3B-hG    |

a The following HSP47 species were analyzed: *Alligator mississippiensis* (American alligator), *Carassius auratus* (goldfish), *Cricetulus griseus* (Chinese hamster), *Danio rerio* (zebrafish), *Gallus gallus* (chicken), *Homo sapiens* (human), *Macaca mulatta* (rhesus monkey), *Mus musculus* (mouse), *Oncorhynchus mykiss* (rainbow trout), *Rattus norvegicus* (Norway rat), *Taeniopygia guttata* (zebra finch), and *Xenopus laevis* (African clawed frog).
capable of binding collagen to an extent equivalent with WT (Figs. 2 and 3).

Effects of Mutations on Structural pH Sensitivity—HSP47 undergoes pH-dependent conformational changes involving a two-step conversion from an alkali state to an acid state via an intermediate state (22). CD spectroscopy allows for the monitoring of secondary structural changes that take place as a function of pH. The spectra of HSP47 WT were recorded at different pH values. The results are in broad agreement with our previous reports (22), including the suggestion of two isodichroic points that characterize the two-step conversion process (Fig. 5A). Curve-fitting of both alkali and acid state CD spectra had been interpreted previously as suggesting that \( \alpha \)-helical character decreases and \( \beta \)-sheet content increases as WT transitions from alkali to acid state with change in pH from mildly alkaline to acidic (43, 44). When mutants H191A, H244A, H255A, H256A, and H302A were examined by CD spectroscopy as a function of pH, spectral changes appeared almost identical.

However, CD pH titration experiments performed by monitoring \( \Delta A_{222} \) as a function of pH at fixed temperature did reveal one important difference between the HA mutants and WT (Fig. 5B). Although WT, H244A, H255A, H256A, and H302A all appeared to undergo trans-conformational changes centered on \( \text{pH} 5.70 \pm 0.10 \) (Table 1), in contrast, H191A exhibited changes centered on a slightly higher pH \( 5.90 \pm 0.05 \). This increase in inflection point pH is consistent with the observed displacement of the elution peak of H191A from collagen affinity chromatography relative to the situation observed with HSP47 WT and the mutants H244A, H255A, H256A, and H302A (Fig. 3). From both sets of data, there emerges a suggestion that the H191A mutation is able to induce a mild perturbation of the trans-conformational pathway between alkali and acid states in comparison with WT (see Fig. 5B), leading to the corresponding shift in the pH controlled release from the affinity column (Fig. 3). In other words, H191A mutation has led to a measurable perturbation in the pH switch mechanism of HSP47.

Tryptophan Residue in the Breach Region Show Extent of Trans-conformational Changes during HSP47 pH Switch Mechanism—HSP47 has five tryptophan residues that may be utilized as intrinsic fluorescence reporters, with which to probe trans-conformational changes as a function of pH. In our homology model of mature recombinant HSP47, Trp-110 is located at the end of sheet 2 of \( \beta \)-sheet A (s2A) close to the loop

FIGURE 4. A, effect of HSP47 WT on collagen fibrillization \((n = 3)\). Collagen concentration was fixed at 2 \( \mu \text{M} \), and the molar ratios of HSP47/collagen are 0:1, 1:2, and 1:1. Light scattering was monitored at 313 nm and 34 °C. B, the ability of HA mutants to arrest collagen fibrillization at 1:1 molar ratio under the same conditions.

FIGURE 5. Effects of pH on the secondary structures of HSP47 WT and HA mutants. A, CD spectra were recorded of WT (3 \( \mu \text{M} \)) at different pH values in 50 mm sodium phosphate buffer at 25 °C. B, pH-dependent conformational changes of HSP47 WT and HA mutants. Plots were generated from \( \Delta A_{222} \) values of WT and HA mutants (3 \( \mu \text{M} \)) as a function of changing pH. Fractional state of 0 represents full alkali state, whereas 1 represents full acid state.
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between s2A and helix D (hD), Trp-158 at the hF, Trp-192 in the loop between s3A and sheet 4 of β-sheet C (s4C) (breach region), Trp-275 at the hH, and finally, Trp-341 is located at the loop after s5A near the “hinge” region (Fig. 1).

Intrinsic tryptophan fluorescence has been previously used to investigate the conformation of serpin family members before and after ligand binding, e.g. antithrombin binding to heparin (45). We elected to make single WF mutations to enable the observation of residual fluorescence attributable to the remaining tryptophan residues in any given mutant. If a mutant was to demonstrate only modest changes in intrinsic tryptophan fluorescence as a function of pH (in comparison with WT), then those residues should pinpoint local regions that were undergoing significant trans-conformational changes as part of the pH switch mechanism.

Two mutants, W158F and W275F, were found to have very low solubility even in the presence of 15% glycerol (data not shown). Hence, they could not be reliably studied further. The other three mutants (W110F, W192F, W341F) possessed good solubility and were purified by affinity chromatography as before. CD spectroscopy and CD temperature titration experiments were then used to demonstrate that all three WF mutants were equivalent in structure to the alkaline state of HSP47 WT (data not shown). Fluorescence pH titration experiments were then performed with WT and mutants during which changes in maximum emission wavelength (λem) and maximum emission intensity (Iem) were monitored as a function of pH change. In the event changes in λem (Δλem) were found easiest to monitor, not the least because intensity data per se were found to be prone to quenching during titration. Values of Δλem were plotted as a function of pH for each WF protein and HSP47 WT, and then resulting plots were then subjected to curve-fitting analysis and normalization relative to WT. The observed red shifts in with decreasing pH are consistent with the transfer of at least one tryptophan residue from a hydrophobic local environment to a more hydrophilic local environment as a consequence of pH-induced changes (Fig. 6).

Overall changes in λem with pH are very similar for W110F and W341F mutants in comparison with WT, taking into account standard fitting errors. By contrast, the observed changes for W192F were obviously reduced. This suggests that although Trp-110 and Trp-341 contribute very little toward values of Δλem as a function of pH, Trp-192 makes some measurable contribution. Accordingly, Trp-192 would appear to be the most active intrinsic fluorescence probe of all three tryptophan residues investigated, suggesting that the breach region should experience significant conformational change as a result of the pH switch.

DISCUSSION

During the transport of triple helical procollagen molecules from ER to the Golgi, a drop in pH causes dissociation of HSP47 and procollagen bundle formation takes place, allowing for the recycling of chaperone back to the ER (21). Unfortunately, molecular details of the pH switch mechanism are far from fully revealed. Because the pH dependence of collagen release is close to the pKα of histidine imidazole, one or more histidine residues have long been considered responsible for the pH sensitivity.

To investigate this, we took a site-directed mutagenesis approach with mutant selection based on a homology model of HSP47 WT, constructed using the known crystal structure of α1-antitrypsin (a serpin present in mammalian blood plasma (Protein Data Bank code 1QLP) (29). Analysis of the model revealed that surface histidine residues appear to group into three clusters: the breach cluster located at the top of the A β-sheet close to the serpin proximal hinge region, the gate region opposite the breach (borrowing from the nomenclature of a flexible loop connecting s3C and s4C, which is critical in the conversion of PAI-1 from native to latent structure) (46), and the shutter cluster found toward the bottom of the A β-sheet adjacent to the serpin shutter region (Fig. 1) (40). The locations of the histidine residues, particularly those in the breach cluster, seem to be in excellent agreement with the structures recently reported (47). Each mutant selected for this study was intended to target a separate cluster, to best evaluate the possible locality of residues important for the pH switch. Of particular importance, mutants H197A, H198A, and H197A,H198A were shown to possess essentially WT-like levels of collagen binding at alkaline pH (Fig. 2B), but were shown to have lost the capacity for pH-dependent, controlled release and elution from an affinity chromatography column (Fig. 3). Moreover, the H191A mutant exhibited the ability to bind collagen under mildly alkaline conditions (Fig. 2B) but also exhibited a measurable perturbation in release from the column (Fig. 3) correlated with perturbations in its pH-driven trans-conformational change (Fig. 5B). Other HA mutants were silent compared with WT. Our results suggest that His-197 and His-198 residues of the breach cluster play a critical role to the pH switch mechanism of HSP47, whereas His-191 plays a role in preventing immature release. Otherwise, clusters of histidine residues found elsewhere in HSP47 do not appear to be significant for correct operation of the pH switch.

HSP47 has been shown to bind to the triple helical portion of procollagen/collagen molecules and not at the N or C terminus (37). Previously, we described a putative procollagen peptide binding interface in terms of the hydrophobic cleft formed at its base by the β-sheet B and helices hA and hG/hH as its sides (Fig.
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1) (28). This sits in close proximity to the breach cluster histidine amino acid residues and the breach region of HSP47 WT. Interestingly, this same breach region subdomain of HSP47 was also shown by WF mutant analyses to experience some significant conformational changes as part of the pH-induced changes in HSP47 (Fig. 6). These changes may impact on the structure and capability of the procollagen peptide interface to sustain binding interactions. This would provide a mechanistic explanation to account for at least some of the reduction in binding affinity that takes place with decreasing pH.

We propose that the pH switch mechanism begins with protonation of breach cluster histidine residues, setting up intraprotein interactions responsible for driving trans-conformational changes with decreasing pH. These in turn distort the procollagen peptide binding site, reducing binding affinity and promoting rapid release at a critical pH. The most profound pH-driven secondary structural changes appear to occur between 6.3 and 5.7 (Fig. 5A), consistent with the main transition between the HSP47 alkali (pH 6.4) and acid states (pH 5.7) (22). We suggest that a significant component of the conformational differences between these states should be located in the breach region.

The breach is also where key amino acid residues are found in serpin family members, and this region is also known to control formational differences between these states should be located in the breach region.

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