Molecular basis for the action of the collagen-specific chaperone Hsp47/SERPINEH1 and its structure-specific client recognition

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Collagen is the most abundant protein in animals and is a major component of the extracellular matrix in tissues such as skin and bone. A distinctive structural feature of all collagen types is a unique triple-helical structure formed by tandem repeats of the consensus sequence Xaa-Yaa-Gly, in which Xaa and Yaa frequently are proline and hydroxyproline, respectively. Hsp47/SERPINEH1 is a procollagen-specific molecular chaperone that, unlike other chaperones, specifically recognizes the folded conformation of its client. Reduced functional levels of Hsp47 were reported in severe recessive forms of osteogenesis imperfecta, and homozygous knockout is lethal in mice. Here we present crystal structures of Hsp47 in its free form and in complex with homotrimeric synthetic collagen model peptides, each comprising one Hsp47-binding site represented by an arginine at the Yaa-position of a Xaa-Yaa-Gly triplet. Two of these three binding sites in the triple helix are occupied by Hsp47 molecules, which bind in a head-to-head fashion, thus making extensive contacts with the leading and trailing strands of the collagen triple helix. The important arginine residue within the Xaa-Arg-Gly triplet is recognized by a conserved aspartic acid. The structures explain the stabilization of the triple helix as well as the inhibition of collagen-bundle formation by Hsp47. In addition, we propose a pH-dependent substrate release mechanism based on a cluster of histidine residues.

Results

Hsp47 binds to two binding sites without conformational change. We determined the crystal structures of Hsp47 alone and in complex with three different collagen model peptides (CMPs), Ac-PGP-GPRGPGPGP-GH2 (named “15-R8”), Ac-PGP-GP-GPRGPGP-GH2 (“18-T8R11”), and Ac-PGP-GPGPGPGP-GP-GPG-PGPGP-GH2 (“18-R11”), each containing the important arginine at the Yaa position of a certain Xaa-Yaa-Gly triplet.
Hsp47 exhibits the typical serpin fold (18, 19), consisting of three β-sheets (A, B, and C) and nine α-helices (Fig. S1). There is no significant change in the Hsp47 conformation upon collagen binding, as can be inferred by comparing the unbound (apo) crystal form with all three complex crystal structures (Fig. S1): The rmsd is only about 0.5 Å for 1,150 main-chain atoms. The intact reactive center loop (RCL, residues 361–379) is flexible, and the segment 368–375 is not visible in the electron density map. There is no partial insertion of the RCL into sheet A. Although it is located close to the collagen-binding site (see below), there are no visible interactions between this stretch and the bound CMPs.

Two chaperones bind to one triple helix. The complex is formed by two Hsp47 molecules that attach in a head-to-head fashion via β-sheet C to two strands of one collagen trimer, i.e., the stoichiometry of (Hsp47)2:(CMP)3 (Fig. 1). The CMPs approximate the canonical 7_3-helical structure that usually is displayed by sequences rich in Pro-Pro/Hyp-Gly triplets (20) and proximate the canonical 75-helical structure that usually is disordered in the electron density map. From inspection of our structure superimposed with Protein Data Bank entry 2DRT (23), 4(R)-hydroxyproline residues at the Yaa positions of the triple helix are compatible with binding to Hsp47, with the notable exception of the Yaa position of the trailing strand, where a clash occurs between the O\(^\gamma\) hydroxyl group and the aromatic ring of Tyr383. This finding is in agreement with previously published biochemical experiments (24).

Each of the two Hsp47 molecules simultaneously forms numerous contacts to either the leading or trailing strand and to the middle strand of the collagen triple helix. Each Hsp47 also makes some contacts with the third strand. A total of 1,000 ± 150 Å\(^2\) of solvent-accessible surface is buried for each Hsp47 monomer/collagen triple helix upon complex formation. These contacts can be made only if collagen is in a proper folded, triple-helical conformation, thus explaining the preferred binding of the chaperone to the folded state of its client (10–13, 15).

In addition to the salt bridge between Asp385 and the arginine at Yaa\(^2\), the same collagen strand hydrogen bonds to the side-chain of Arg222 via its main-chain carbonyl oxygen atoms of Pro5 and Gly6, the former located at the so-called “Yaa\(^3\)” position (i.e., three amino acids before the important arginine residue). The side chain at this position influences the binding affinity, with threonine being the most favored naturally occurring amino acid (24). We can explain this effect from our structure of the Hsp47:18-T8R11 complex, in which the side chain of the threonine makes water-mediated contacts with the Ser305 side chain and the main chain of Ala303.

Furthermore, there are a number of hydrophobic interactions, especially involving Leu381 and Tyr383. We confirmed our crystallographic analysis by performing three different experiments using site-directed mutagenesis and pull-down experiments, solid-phase ELISA (Fig. 3), and surface plasmon resonance (SPR) spectroscopy (Fig. S3).

Mutation of Asp385 to asparagine drastically weakens the interaction, as shown by pull-down experiments and ELISAs (Fig. 3), thus emphasizing the ionic nature of this interaction in agreement with previous studies on the optimal recognition sequence of collagen (9, 13, 24, 25). To verify further the importance of the identified interaction site, two additional mutations were introduced in the vicinity of Asp385, replacing Leu381 and Tyr383 by arginine. Although the wild type binds to collagen with a dissociation constant (K\(_d\)) of about 1 μM, as determined by ELISA and in agreement with IC\(_{50}\) values derived from competitive SPR data (8), all three mutants show drastically reduced binding (Fig. 3 and Fig. S3).

**OI Missense Mutations Occur in the Hydrophobic Core Distant from the Binding Site.** We also mapped the known missense mutation leading to recessive OI, i.e., L78P in humans and L326P in dogs (26, 27), on our experimental structures. As can be seen in Fig. 4, the mutations are located on the distant site of the collagen

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**Fig. 1.** Overall structure of the Hsp47:CMP complex. Hsp47 is shown in cartoon representation, and the CMP chains are depicted as sticks. α-Helices are drawn in gray. β-Strand A is shown in orange, sheet B in magenta, and sheet C in violet. The segment corresponding to the RCL of inhibitory serpins is drawn in red. Asp385 is shown as sticks. The leading strand of the collagen triple helix is drawn in yellow, the middle strand in blue, and the trailing strand in green.
leads to a stabilization of the latter as compared with the unfolded state. The preferred binding of Hsp47 to the folded state of collagen inevitably prevents local unfolding (micro denaturation) region, unlike collagen produced by normal cells. We propose that Hsp47 stabilizes the collagen triple helix.

Discussion
Hsp47 Stabilizes the Collagen Triple Helix and Prevents Lateral Aggregation. Our findings strongly support the notion that Hsp47 stabilizes the folded state of the collagen triple helix. Indeed, the CMPs used for crystallization are not triple-helical at room temperature at low or physiological ionic strength conditions, unlike their conformations observed in the crystal structures of the Hsp47:CMP structures (Fig. S4). Hsp47’s preferred binding to the folded state of collagen sheds light on a possible role in collagen folding and maturation. Both isolated collagen and procollagen are thermodynamically unstable at body temperature (28, 29). Collagen secreted by cells that either completely lack Hsp47 (30–32) or possess only reduced levels, as in OI patients (26), is protease sensitive in the triple-helical region of the RCL.

Another hallmark of Hsp47-deficient cells is the formation of collagen aggregates in the ER (30, 31), although this type of aggregation was not observed in a particular OI patient (26). In part, this agglomeration can be explained by analogy with other proteins that are misfolded and associate via exposed hydrophobic patches. However, even correctly folded procollagen that has passed the ER quality control has an intrinsic propensity for forming lateral aggregates. In normal cells procollagen bundles are formed in the Golgi, where Hsp47 dissociates from its client (33, 34). Thus, Hsp47 also might prevent lateral aggregation of correctly folded procollagen in the ER. A similar effect is used in fibrillation assays, where Hsp47 inhibits collagen aggregation (14). Both effects are easily explainable by our structure, because the collagen triple helix is decorated with one or two chaperones per binding site. The long axes of these chaperones protrude outside each binding position, thereby inhibiting premature lateral multimerization of procollagen.

Chain Register and Number of Binding Sites. The quaternary structure observed in our Hsp47:CMP complexes has implications for the number of chaperones bound to a given site and, in the case of heterotrimeric collagen molecules [e.g., collagen I with composition (α₁)₃(α₂)₁], depends on the register of the three strands. If the register leading:middle:trailing strand is α₁:α₂:α₁, there will be two chaperones on a given binding site on the α₁ chain, and, regardless of the local sequence of the α₂ chain, no Hsp47 will be bound there. On the other hand, if the register is α₁:α₂:α₂, as has been suggested recently (35), one or two Hsp47

Fig. 2. Close-up of the interaction surface. The CMP is color-coded as in Fig. 1. Key residues of Hsp47 with the CMP are numbered; prime numbers refer to the second Hsp47 molecule. Nitrogen atoms are shown in blue, oxygen atoms in red, and sulfur atoms in brown. Hydrogen bonds are indicated as cyan-colored dotted lines. The dotted grey segments indicate the disordered region of the RCL.

Fig. 3. Binding of Hsp47 mutants to collagen I. (A) Pull-down experiment on gelatin agarose. Hsp47 wild type and mutants were bound in equal amounts to gelatin agarose at pH 8. After extensive washing, bound proteins were eluted by shifting the pH to 5. E, eluate; I, total input. (B) ELISA binding assay. Collagen I (rat tail) was coated on 96-well plates and incubated with indicated concentrations of His-tagged Hsp47 constructs. Binding was detected by a murine antibody directed against the His tag and visualized via an HRP-coupled secondary antibody. Every point was measured in triplicate.
molecules will be attached, depending on whether the local sequence of the α2 provides a Hsp47-binding site.

**Does Hsp47 Serve as a Monitor of Quality?** The number of Hsp47-binding sites on any natural collagen has not yet been determined, but multiple sites have been identified in collagen chains α1(I), α2(I), and α1(II) by CNBr mapping (16). Interestingly, the highest-affinity sites were found in the N-terminal region of procollagen. Because triple-helix formation occurs in a zipper-like fashion proceeding from the C to the N terminus (36), binding of Hsp47 to folded segments at the N terminus could signal a successfully terminated folding event and mark this complex for passage to the Golgi. This proposal is in agreement with the finding that Hsp47+ cells secrete collagen much more slowly and are deficient in N-propeptide processing (30).

**Model for pH-Trigged Client Release.** The presumably pH-triggered release of collagen from Hsp47 in the Golgi prompted us to investigate the presence of groups with suitable pK values located in or near the interface. An obvious candidate for pH-triggered release is Asp385, but the pK value of an aspartic acid usually is about 4 and probably is even lower in the complex structure because of the salt bridge to the arginine at the Yaa position of collagen. Thus, we looked for other titratable groups within or near the binding interface with pK values in the range of 6–7. Because the exact locations of binding sites on procollagen are not known, we considered only the Hsp47 partner. Six histidine residues are located in the vicinity of the collagen–Hsp47 interface: His215, His216, His238, His273, His274, and His386. From the pH of our crystallization media (pH 5–6), we deduced that our crystal structures resembled the serine protease catalytic triad, involving Asp220 and replaced by Asn or Tyr in chicken and zebrafish because CMPs are bound, we deduce that our crystal structures discussed above. His238 is the central part of a structure element for interaction with the essential arginine of the Pro-Arg-Gly triplet 

**Materials and Methods**

**Expression and Purification.** A construct encoding amino acids 36–418 of the canine Hsp47 (canine SERPINH1 mRNA, NCBI accession NM_001165888), was cloned into the pET22(b) vector (Novagen) with a C-terminal hexahistidine tag. Expression was induced in BL21(DE3) cells grown to an OD600 of 0.6–0.7 by adding 0.5 mM isopropyl-β-D-thiogalactoside and shaking for 5 h at 37 °C. Cells were resuspended in lysis buffer (300 mM NaCl, 10 mM imidazole, 20 mM Tris, pH 8) and lysed by sonication. Cleared lysate was purified by Ni-NTA affinity chromatography (Ni-NTA superflow; Qiagen). The eluate was reduced with 4 mM DTT, and 1.5 M ammonium sulfate was added to precipitate contaminants. The soluble fraction was concentrated and purified further by gel filtration (Superdex 75; GE Healthcare) in buffer A (20 mM Hepes (pH 7.5), 300 mM NaCl, 4 mM DTT). The purified protein was concentrated to ~15 mg/mL and stored at ~80 °C for further use.

**Crystallization and Structure Solution.** For co crystallization, wild-type Hsp47 and either the 18-residues-long collagen peptide 18-R11 or 18-T8R11 or the shorter 15-residues-long peptide 15-R8 (Ac-PPGPPGPPGPPGP-NH2) (BioMatik) were mixed in a 1:1 molar ratio with respect to trimeric collagen at a concentration of about 0.2 mM. Crystals belonging to space group P1 (co crystallization with 15-R8), P422 (co crystallization with 18-R11), and P2 (co crystallization with 18-T8R11) were grown under the same crystallization conditions containing 8% (vol/vol) Tacsimate (Hampton Research) and 20% (vol/vol) PEG 3500 in 18% (wt/vol) PEG 3500 in space group C2 at similar protein concentrations. Data from optimized crystals were collected at beamline X06DA, Swiss Light Source (Paul Scherer Institute, Villigen, Switzerland). For phasing, selenomethionine-labeled Hsp47 was produced by the pathway inhibition method (37), and data from co crystallization with 18-R11 in space group P422 were collected at the 0.65-Å resolution. The selenium positions of this single-wavelength anomalous dispersion experiment were determined with SHELXD (39), and the initial model was built by SHELXE and phenix.autobuild (40). The P1, P2, and C2 structures were solved by molecular replacement using Phaser (41) with one Hsp47 monomer from the P422 crystal form as search model. All structures were refined using iterative cycles of phenix.refine (42), COOT (43), and Buster (44).

**Site-Directed Mutagenesis and Characterization of Mutants.** The mutations D385N, Y383R, and L381R were introduced according to an optimized mutagenesis protocol (45) using overlapping primers. The mutants were expressed and purified following the protocol used for the wild-type Hsp47. For co crystallization experiments, the Hsp47 monomer (15 mg/mL) was added to 25 μL of gelatin-agarose (Sigma-Aldrich) in loading buffer [5 mM Tris (pH 8), 150 mM NaCl, 1 mM DTT] and incubated for 10 min at room temperature. The agarose beads were washed twice with 1 mL of washing buffer [5 mM Tris (pH 8), 400 mM NaCl, 1 mM DTT] and eluted in 80 μL of elution buffer [100 mM MES (pH 5.5), 150 mM NaCl, 1 mM DTT]. Samples were analyzed by SDS/PAGE and Western blot. Equal volumes from the input and elution fractions were loaded on the gel. For the Western blot, mouse anti-his (Qiagen) was used as primary antibody, and IRDye 800CW goat anti-mouse (LI-COR) was used as the secondary antibody. The green fluorescent signal of the secondary antibody was detected at 800 nm on an Odyssey infrared imaging system. The prestained molecular weight marker (fermentas) was visualized at a wavelength of 700 nm.

For ELISA-style binding assays, rat tail collagen I (BD Bioscience) was diluted in TBS and coated at 10 μg/mL (500 ng per well) overnight at room temperature onto 96-well plates (MaxiSorp, Nunc). After washing with TBS/0.05% (vol/vol) Tween-20, plates were blocked for 1 h at room temperature with 5% (wt/vol) milk powder in TBS. Hs-tagged HSP47 proteins were added at concentrations ranging from 2.4 to 10,000 nM. After washing with TBS/0.05% (vol/vol) Tween-20, bound ligands were detected with a murine antibody directed against the His tag (Penta-His; Qiagen) and visualized with an anti-mouse-phosphatase-coupled secondary antibody (GE Healthcare) and tetramethylbenzidine as substrate. The reaction was stopped by adding 10% (vol/vol) H2SO4, and absorption was measured at 450 nm. The data were analyzed using QTIPlot and fitted using a four-parameter logistic model (46).
All measurements were done at least three times, and representative curves are shown.

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Supporting Information

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SI Materials and Methods

For surface plasmon resonance spectroscopy (SPR) experiments rat tail collagen I (BD Biosciences) was diluted in 10 mM sodium acetate (pH 5.0) to a final concentration of 25 μg/mL and was coated on the activated surface of a CM5 sensor chip (GE Healthcare) at a flow rate of 5 μL/min. The surface was activated with N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and was blocked with 1 M ethanolamine hydrochloride (pH 8.5) after coupling of both ligands. HSP47 proteins were diluted in 20 mM Hepes (pH 7.4), 150 mM NaCl, 0.005% P20, and increasing concentrations ranging from 37.5–10,000 nM were passed over the surface at a constant flow rate of 30 μL/min for 120 s. Dissociation was monitored for 350 s. A Biacore 2000 system (Biacore) was used for all binding experiments, and the data were analyzed using the BIAevaluation software 4.1. Results are presented in Fig. S3.

Fig. S1. Overlay of unbound (apo) HSP47 and collagen-bound HSP47. (A) Cartoon representation of apo-Hsp47. The segment corresponding to the reaction center loop (RCL) in inhibitory active serpins is drawn in red, and the three characteristic serpin β-sheets are indicated. Asp385 is indicated in stick form. (B) Overlay of apo and complex HSP47. Ribbon representation of a superimposition of of the apo-form Hsp47 (red) with the collagen model peptide-bound structure (cyan).

Fig. S2. Experimental solvent-modified electron density map. The solvent-modified experimental electron density map is shown in purple at a contour level of 1.4 σ together with the refined model. The leading strand of the collagen model peptide is shown in yellow, the middle strand in blue, and the trailing strand in green. Hsp47 is drawn in cyan.
Fig. S3. SPR experiments. Interactions of soluble HSP47 proteins with immobilized collagen I (from rat tail; Sigma) was measured using SPR spectroscopy. Each curve presents one concentration, and descending curves correspond to decreasing concentrations of the HSP47 proteins ranging from 10–0.0375 μM (brown, 10 μM; gray, 5 μM; cyan 2.5 μM; magenta, 1.25 μM; blue, 0.6 μM; yellow, 0.3 μM; green, 0.15 μM; red, 0.075 μM; black, 0.0375 μM).

Fig. S4. CD spectra of collagen model peptides at room temperature. CD spectra were recorded using a Jasco J-715 instrument at 20 °C. The concentration of the collagen model peptides was 20 μM calculated with respect to the trimeric triple-helical quaternary structure and the path length of the cuvette was 0.5 mm. The dashed line represents the CD spectrum of peptide 18-R11 that was crystallized in complex with Hsp47, sequence Ac-PPGPPGPPRPGPPGPP-NH₂. This peptide is not folded at room temperature. The solid line represents the CD spectrum of the longer 27-meric peptide 27-T11/R14, sequence Ac-PPGPPGPTGPRPGPPGPPGPP-NH₂. This peptide is triple-helical at room temperature.
Table S1. Data collection and refinement statistics

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a.s.u., asymmetric unit; trimeric CMP, collagen model peptide triple helix.

*Hsp47/collagen trimer.
†Friedel pairs are counted separately.
‡The values in parentheses of resolution range, completeness, Rmerge, and Kα/λ correspond to the outermost resolution shell.
§Rmerge = ΣjIj‖Ij(hkl)‖ − 〈I(hkl)〉/Σj‖Ij(hkl)‖, where Ij(hkl) is the jth measurement of the intensity of the unique reflection (hkl), and 〈I(hkl)〉 is the mean over all symmetry-related measurements.

*Correlation coefficient between random half data sets. A value above 30% indicates a useful signal (ref. 1).