Chemical cross-linking of the chloroplast localized small heat-shock protein, Hsp21, and the model substrate citrate synthase

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Abstract

The molecular mechanism whereby the small heat-shock protein (sHsp) chaperones interact with and prevent aggregation of other proteins is not fully understood. We have characterized the sHsp–substrate protein interaction at normal and increased temperatures utilizing a model substrate protein, citrate synthase (CS), widely used in chaperone assays, and a dodecameric plant sHsp, Hsp21, by chemical cross-linking with 3,3'-Dithiobis[sulfosuccinimidylpropionate] (DTSSP) and mass spectrometric peptide mapping. In the absence of CS, the cross-linker captured Hsp21 in dodecameric form, even at increased temperature (47°C). In the presence of equimolar amounts of CS, no Hsp21 dodecamer was captured, indicating a substrate-induced Hsp21 dodecamer dissociation by equimolar amounts of CS. Cross-linked Hsp21–Hsp21 dipeptides indicated an exposure of the Hsp21 C-terminal tails and substrate-binding sites normally covered by the C terminus. Cross-linked Hsp21–CS dipeptides mapped to several sites on the surface of the CS dimer, indicating that there are numerous weak and short-lived interactions between Hsp21 and CS, even at normal temperatures. The N-terminal arms especially interacted with a motif in the CS dimer, which is absent in thermostable forms of CS. The cross-linking data suggest that the presence of substrate rather than temperature influences the conformation of Hsp21.

Keywords: chemical cross-linking; mass spectrometric peptide mapping; small heat-shock protein; protein–protein interactions; citrate synthase

Small heat-shock proteins (sHsps) exist in essentially all organisms and are especially abundant in plants (Waters et al. 1996; Haslbeck et al. 2005). The sHsps have a particularly important role during heat stress, where they

protect other proteins from aggregation (Horwitz 1992; Narberhaus 2002) and prevent irreversible accumulation of large aggregates within the cell (Sun and MacRae 2005). By maintaining unfolded proteins in a folding-competent state, sHsps work with the ATP-dependent Hsp70 systems, which can then refold the proteins when the stress conditions return to normal (van Montfort et al. 2001b; Haslbeck 2002; Stromer et al. 2003; Haslbeck et al. 2004).

The sHsp monomers range from 12 to 43 kDa and assemble into large multimers (van Montfort et al. 2001b; Haslbeck et al. 2005). The three-dimensional structures have been determined for the plant Hsp16.9 from wheat

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Abbreviations: DTSSP, 3,3'-Dithiobis[sulfosuccinimidylpropionate]; DTT, dithiothreitol; MDH, malate dehydrogenase; CS, citrate synthase; nanoESI-MS, nanoelectrospray-ionization mass spectrometry.

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(*Triticum aestivum*) (van Montfort et al. 2001b), the bacterial Hsp16.5 from the archaeon *Methanococcus jannaschii* (Kim et al. 1998), and the animal Tsp36 from tapeworm *Taenia saginata* (Stamler et al. 2005). The Hsp16.9 structure revealed a double-disc dodecamer, whereas the Hsp16.5 was reported to be a spherical 24 mer. Tsp36, which has duplicated α -crystallin domains, was shown to be a dimer in a reducing environment and a tetramer in a nonreducing environment.

The sHsps are defined by a conserved β -strand rich domain (~90 amino acids, so-called α -crystallin domain), a flexible C-terminal tail, and an N-terminal arm. This N-terminal region is of variable length and highly divergent in sequence between different sHsps, yet conserved between orthologs (Kim et al. 1998; van Montfort et al. 2001a,b; Narberhaus 2002; Haslbeck et al. 2005). Even though the quarternary structure differs between Hsp16.9, Hsp16.5, and Tsp36, the α -crystallin domain shows similar folding, containing two antiparallel β -sheets, one with β -strands 2, 3, 8, and 9, and the other with β -strands 4, 5, and 7. The β -strand 6 is positioned outside the α -crystallin domain, and by strand exchange with the next monomer it contributes to a strong dimer interface (van Montfort et al. 2001b).

Dissociation of the oligomer in response to increased temperature or increased rate of dynamic subunit exchange is supposed to expose hydrophobic substrate-binding sites (Kim et al. 1998; Bova et al. 2000; van Montfort et al. 2001b; Sobott et al. 2002; Wintrode et al. 2003; Lentze and Narberhaus 2004). Therefore, substrate protection by sHsps is not strictly dependent on the oligomeric conformation or the dissociated oligomers (Franzmann et al. 2005; Haslbeck et al. 2005; Basha et al. 2006).

Substrate hydrophobic binding sites have been proposed in the α -crystallin domain, and in the N- and C-terminal regions (Studer et al. 2002; Haslbeck et al. 2004; Stromer et al. 2004; Ghosh et al. 2006; Aquilina and Watt 2007; Biswas et al. 2007). It has also been suggested that different sHsp domains may interact with different substrates (Giese et al. 2005; Haslbeck et al. 2005; Basha et al. 2006), and that different sHsp domains could be responsible for recognition of unfolded substrate and maintaining the sHsp-substrate complex in solution (Stromer et al. 2003; Basha et al. 2004; Ghosh et al. 2006).

To investigate the contact regions between a sHsp and a model substrate, we have performed chemical cross-linking and mass spectrometric peptide mapping with the chloroplast localized sHsp, Hsp21, from *Arabidopsis thaliana* and the model substrate citrate synthase (CS). Hsp21 has an important role for protection of *A. thaliana* plants during stress (Harndahl et al. 1999). The model substrate, CS, is widely used in chaperone assays and is

well-characterized in terms of thermostability and unfolding, with the structure resolved to atomic resolution for both thermosensitive and thermostable forms (Remington et al. 1982; Jakob et al. 1995; Russell et al. 1997).

Here, we have used nanoelectrospray mass spectrometry (nanoESI-MS) to confirm that the Hsp21 oligomer is a dodecamer. The Hsp21 sequence was then modeled onto the structure for dodecameric Hsp16.9 (van Montfort et al. 2001b) to facilitate data interpretation and mapping of cross-linked dipeptides both within the Hsp21 dodecamer and between Hsp21 and CS.

In the absence of CS, the cross-linker captured Hsp21 in dodecameric form, remarkably also at increased temperature, which is assumed to cause sHsp dissociation into dimers (Bova et al. 2000; van Montfort et al. 2001a; Giese and Vierling 2002; Sobott et al. 2002; Lentze et al. 2004; Haslbeck et al. 2005). Mass spectrometric mapping of cross-linked dipeptides confirmed that the proposed substrate-binding sites in sHsps, the flexible N- and C-termini, and the C-terminal hydrophobic binding groove, are involved in interactions with CS. Furthermore, the cross-linking data suggest that there are several different sites on CS for weak and short-lived interactions with Hsp21. The interactions with the N-terminal arm of Hsp21 are specifically mapped onto the CS dimer in an area that is absent in thermostable forms of CS.

Results

Hsp21 is a dodecamer that can prevent CS aggregation

NanoESI-MS was used to determine the oligomer stoichiometry of Hsp21. By the careful manipulation of gas pressures to preserve noncovalent interactions, large macromolecular assemblies like protein oligomers can be transported intact through the mass spectrometer so that the exact oligomeric mass can be determined and, thereby, the oligomer stoichiometry. A series of peaks from 6800 m/z to 8000 m/z arise from the 37+ through 32+ charge states of an oligomer with a mass of 252.7 kDa (Fig. 1, spectrum inset). The theoretical mass of unmodified Hsp21 is 20,968 Da, which gives a stoichiometry of 12.05 subunits (252,700 Da/20,968 Da), showed that the Hsp21 preparation was dodecameric.

Further confirmation was obtained by tandem MS (MS/MS), whereby a highly charged monomer was dissociated from the parent oligomer. Specifically, the peak corresponding to the 35+ charge state was isolated and dissociated into monomers and complementary stripped oligomers. The resulting spectrum (Fig. 1, main spectrum) exhibited two series of peaks; one at low m/z and the other at m/z values higher than the original oligomer. The peaks at low m/z arose from a monomer of mass 21,003 Da, whereas the peaks between 10,000 m/z and

Figure 1. The small-heat shock protein Hsp21 is dodecameric. NanoESI mass spectra were recorded under conditions where noncovalent interactions are conserved. (*Inset*) Mass spectrum with a series of charge states ranging from 37+ to 32+ corresponding to a single oligomeric species with a mass of 252.7 kDa. MS/MS spectrum obtained by isolating the peak arising from the 35+ charge state of Hsp21 and as schematically outlined applying high-energy collisions with argon gas for dissociation of Hsp21 oligomers into highly charged species at low m/z and species of much lower charge at high m/z . The mass of the products giving rise to these charge-state series corresponded well with monomer (low m/z) and undecamer (high m/z), respectively.

15,500 m/z were from an undecamer of mass 231.5 kDa. The experimental mass of the monomer is 35 Da higher than that calculated from the sequence. This is most likely due to the presence of residual water molecules. Thus, both nanoESI-MS and MS/MS clearly showed that Hsp21 is dodecameric.

To ensure that the Hsp21 preparation could protect CS against temperature-induced aggregation *in vitro*, CS aggregation was performed at 47°C, in the absence or presence of Hsp21 (Fig. 2). Soluble protein (supernatant, S) was separated from aggregated protein (pellet, P). In the absence of Hsp21, all of the CS aggregated and was recovered in the pellet. In contrast, CS was prevented from aggregation and maintained in a soluble form in the presence of Hsp21. Similar results were obtained with another frequently used model substrate, MDH. Thus, two different temperature-sensitive enzymes were both effectively protected from aggregation by Hsp21.

Chemical cross-linking of the Hsp21 dodecamer

Cross-linking was first performed with Hsp21 without CS. As seen on nonreducing SDS-PAGE (Fig. 3A), a sample of Hsp21 dodecamers without cross-linker migrated as monomeric Hsp21, as expected. However, after treatment with the cross-linker DTSSP, the whole population migrated as Hsp21 dodecamers with a mass of ~250 kDa (Fig. 3B). A similar result was observed with a 10-fold or 50-fold (data not shown) lower cross-linker concentration, at both 25°C and 47°C. Cross-linked Hsp21 occurred only as dodecamers; neither monomers, dimers, or tetramers were seen. Thus, the cross-linker captured Hsp21 in dodecameric form under the conditions

described showing that in the absence of substrate, Hsp21 does not largely dissociate, but remains dodecameric, even at 47°C.

On the other hand, if Hsp21 was oxidized prior to cross-linking, no dodecamers were seen (Fig. 3C). Oxidation of the conserved methionines in the N-terminal arm of Hsp21 has previously been shown to cause unfolding of the methionine-containing amphipathic α -helices, abolishing chaperone activity and causing oligomer disassembly (Gustavsson et al. 1999, 2001). The addition of DTT to cleave the disulphide bridge in

Figure 2. Exposure of the model substrates to increased temperature causes aggregation that is prevented by Hsp21. Proteins, 1 μ M citrate synthase (CS) or 1 μ M malate dehydrogenase (MDH) were incubated for 20 min at 47°C in the absence or presence of Hsp21 (12 μ M). After centrifugation, soluble protein (supernatant, S) was separated from aggregated protein (pellet, P) and examined by SDS-PAGE; protein corresponding to 2.5, 1.8, and 12.5 μ g loaded per lane of CS, MDH and Hsp21, respectively. The gel was silver stained.

Figure 3. Chemical cross-linking of the Hsp21 dodecamer with DTSSP. Hsp21 (12 μ M) was pre-incubated at 25°C or 47°C for 20 min, after which the chemical cross-linker DTSSP (0.5 or 5 mM) was added for 20 min. Samples were lyophilized and dissolved in nonreducing SDS-PAGE loading buffer (5 μ g/lane). (A) No cross-linker; (B) 0.5 or 5 mM cross-linker; (C) 0.5 mM cross-linker, Hsp21 oxidized with (10 mM H_2O_2 , 20 min) before use; (D) same as in A, but 50 mM DTT added prior to SDS-PAGE. The gel was silver stained.

the cross-linker before SDS-PAGE resulted in the recovery of monomeric Hsp21 (Fig. 3D), which was shown by MS analysis to be modified with halves of the cross-linker (data not shown).

Chemical cross-linking of Hsp21 in the presence of CS

Cross-linking was next performed after a preincubation of Hsp21 with CS. We used either an excess of Hsp21 (molar ratio 12:1) or equimolar amounts of Hsp21 and CS (molar ratio 1:1) since these ratios are often used in sHsp-substrate studies (Mogk et al. 2003; Stromer et al. 2003; Basha et al. 2004; Friedrich et al. 2004).

When Hsp21 was preincubated with CS at a 12:1 ratio before adding the cross-linker, a major band was observed by SDS-PAGE for samples incubated at 25°C and 47°C (Fig. 4A, lanes 4,5). The position of this band corresponded to that of the cross-linked Hsp21 dodecamers (Fig. 3B), and excision of this band from the gel for mass spectrometric analysis showed that it did contain Hsp21 but no CS (data not shown). Some material did not enter the gel, especially after preincubation at 47°C, presumably large cross-linked Hsp21-CS complexes (>1000 kDa).

In the presence of higher amounts of CS, no Hsp21 was captured as cross-linked dodecamers (no dodecameric band is visible, either at 25°C or 47°C) (Fig. 4A, lanes 6,7), in contrast with the situations in the absence of CS (Fig. 3B) and with Hsp21-CS in a 12:1 ratio (Fig. 4A, lanes 4,5). Remarkably, there was a complete absence of Hsp21 dodecamers at both temperatures, indicating a substrate-induced structural rearrangement of Hsp21 that is not dependent on temperature. Instead, Hsp21 appeared

as monomers (Fig. 4A, lanes 6,7) and CS as cross-linked CS dimer and trimer bands (no Hsp21 was detected in these two bands by mass spectrometry; data not shown). Again, some material did not enter the gel, especially after preincubation at 47°C, indicating again the formation of large cross-linked Hsp21-CS complexes (>1000 kDa). Thus, although the presence of higher amounts of CS induced a considerable structural rearrangement of Hsp21 that was independent of temperature, there was also some structural rearrangement of Hsp21 that was dependent on temperature.

Figure 4. Chemical cross-linking between Hsp21 and CS with DTSSP. Hsp21 (12 μ M) and CS (1 or 10 μ M) were preincubated at 25°C or 47°C for 20 min, after which the chemical cross-linker DTSSP (5 mM) was added. (A) Nonreduced SDS-PAGE; (B) SDS-PAGE with 50 mM DTT added to samples prior to loading to reduce the disulphide bridge in the cross-linker. The gel was silver stained.

The addition of DTT to cleave the disulphide bridge in the cross-linker before SDS-PAGE recovered monomeric Hsp21 and CS (Fig. 4B), even the cross-linked Hsp21–CS complexes that did not enter the gel. That DTT amplifies that the staining of weak bands was evaluated separately (data not shown). This partly explains why CS is clearly visible in Figure 4B at a 12:1 ratio, yet barely visible in Figure 4A (very faint dimer and trimer bands are seen in lanes 4 and 5 compared with lanes 6 and 7). That some CS is trapped in the large cross-linked Hsp21–CS complexes (Fig. 4A, lanes 5,7) can also explain why there is less CS (Fig. 4A, less in lane 7 compared with lane 6).

Analysis of cross-linked dipeptides

To obtain more detailed information about where the sites of interaction between Hsp21 and CS are, the samples were analyzed by mass spectrometric peptide mapping for cross-linked dipeptides. Peak lists were retrieved from the mass spectra and cross-linked dipeptides identified by running these peak lists through the software GPMW. In the so-called Search Protein MS X-link function in this software, the theoretical combination of all possible cross-linked peptides between two proteins (or within

one protein) is compared against the peak list of interest, resulting in a set of suggested cross-linked dipeptides. To avoid false positives, the lists were thereafter manually inspected to remove peaks that did not fulfill two other requirements, namely, that the peak should not be detected in a control sample without the cross-linker DTSSP, and that the peak should disappear if DTT was added to reduce the disulphide bond of the cross-linker. For this purpose, three separate mass spectra were recorded for each sample, as illustrated in Figure 5, and used to validate cross-linked dipeptides.

This procedure was applied to all samples as shown in the Venn diagram for the sample in the first row of Table 1. Samples cross-linked with DTSSP contained a total of 223 peaks; of these, 42 peaks were suggested as modified, either as cross-linked or dead-ends, by GPMW. Comparison with the mass spectrum from the corresponding sample where DTT was added showed that 132 peaks of the 223 disappeared with DTT. Comparison with the mass spectrum from the corresponding sample where no DTSSP was added showed that 134 peaks of the 223 appear due to the cross-linker DTSSP. The number of peaks that disappear with DTT and appear with DTSSP are 108; of these, 27 were suggested as modified, either as cross-linked or dead-ends,

Figure 5. MALDI mass spectra used to identify cross-linked dipeptides. Mass spectra recorded for tryptic digests of Hsp21 control (top), Hsp21 with DTSSP (middle), and Hsp21 with DTSSP and DTT (bottom), showing how the peak ($MH^+ 1527.58$), which corresponds to a cross-linked dipeptide, appears upon addition of the cross-linker DTSSP and disappears upon addition of DTT, which reduces the disulphur bond in the cross-linker. A new peak corresponding to a single peptide with half the cross-linker in alkylated form appears ($MH^+ 822.33$) in the bottom spectra.

Table 1. Identification of DTSSP-cross-linked peptides in trypsin digested samples

		Total number of peaks in MS	1= # of peptides recognized as modified by GPMW as crosslinked or dead-ends or internal crosslinks	2= # of peptides disappearing with DTT	3= # of peptides appearing with DTSSP	4= # of peptides fulfilling criteria 2 AND 3	5= # of peptides fulfilling criteria 1 and 4	From 5: # of Hsp21- CS dipeptides	From 5: # of Hsp21- Hsp21 dipeptides	From 5: # of peptides with dead- ends or internal crosslinks
Hsp21:CS	47°C	223	42	132	134	108	27	4	7	11
12:1	25°C	205	36	127	114	90	23	3	10	8
Hsp21:CS	47°C	194	23	153	81	68	14	1	3	9
1.2:1	25°C	226	27	147	192	142	24	8	4	16
Hsp21	47°C	123	17	69	84	60	3	-	3	7
only	25°C	128	28	57	98	46	13	-	5	8

Data analysis of MS spectra was performed to extract and validate peaks representing cross-linked dipeptides and omitting peptides with dead-ends (when only one end of the cross-linker reacts with a lysine residue but not the other end) or internal cross-links (between two lysines within the same peptide).

by GPMW. Thus, of 42 cross-linked dipeptides suggested by GPMW, 15 dipeptides did not fulfill the two other requirements and were removed from the list to yield 27 validated cross-linked dipeptides. Of these 27, four were suggested to be cross-linked Hsp21–CS dipeptides, and seven to be cross-linked Hsp21–Hsp21 dipeptides.

The number of dipeptides detected at 47°C were the same or slightly lower than at 25°C (Table 1), which could be an effect of faster hydrolysis of the cross-linker (DTSSP) at higher temperature (Pierce 2004). Several more Hsp21–Hsp21 dipeptides were detected when Hsp21 was incubated with CS (seven dipeptides at 47°C and 10 at 25°C) than without (three dipeptides at 47°C and five at 25°C). This could be due to some structural rearrangement of Hsp21 in the presence of CS into a more open conformation, providing greater access for the cross-linker.

The Hsp21–CS interactions were investigated by cross-linking under different conditions and with two different proteases to increase sequence coverage (Hjerno and Roepstorff 2005), which yielded the lists of 15 identified Hsp21–Hsp21 dipeptides (Table 2) and 17 Hsp21–CS dipeptides (Table 3). To interpret the cross-linking data, multiple alignments were generated with Hsp21 and

Hsp16.9 (Fig. 6) with the four suggested substrate-binding sites in Hsp16.9 (van Montfort et al. 2001b) indicated in the alignment, i.e., C-terminal tail IXI/V-motif and the C-terminal binding groove that is covered by the C-terminal tail, the N-terminal arm, and a hydrophobic patch in the α -crystallin domain covered by the N-terminal arm. A dodecameric Hsp21 structure was also generated (Fig. 7) by modeling the Hsp21 sequence onto the Hsp16.9 structure (van Montfort et al. 2001b).

Cross-linking within the Hsp21 dodecamer and a CS-induced conformational change

Cross-linked Hsp21–Hsp21 dipeptides (Table 2) could be detected in cross-linked Hsp21 samples whether or not CS was present. For example, all seven dipeptides detected in the absence of CS (MH⁺ 1527, 1747, 2474, 1792, 2521, 1482, and 3125) (boxed in Table 2) were also detected in the presence of CS. These dipeptides included either the Hsp21 N terminus (five) or lysines in the Hsp21 C-terminal tail (two). The cross-links were to either another N terminus or to lysines in the C-terminal tail, or to a number of other lysines. The C-terminal tail was

Table 2. Cross-linked Hsp21–Hsp21 dipeptides

MH ⁺	Cross-linked Hsp21–Hsp21 dipeptides	Hsp21 aa nr	Hsp21 aa nr	Condition	#:
1527,581	M ₁ QDQR ₅ + M ₁ QDQR ₅	N-term	N-term	a,b,c,e,f	7
1747,908	K ₁₇₇ VIDVQIQ ₁₈₄ + V ₁₇₂ ERK ₁₇₆	K177	K173	a,b,c,d,e,f	6
2474,078	M ₁ QDQR ₅ + A ₈₄ PWDIKEEEHEIK ₉₆	N-term	K89	a,b,c,e	4
1792,839	M ₁ QDQR ₅ + K ₁₇₇ VIDVQIQ ₁₈₄	N-term	K177	a,b,c,e,f	4
2521,210	M ₁ QDQR ₅ + I ₁₁₁ SVEDNVLVIKGEQK ₁₂₅	N-term	K121	a,b,e	3
2252,104 ^Δ	K ₁₇₇ VIDVQIQ ₁₈₄ + D ₁₅₄ KIKAELK ₁₆₁	K177	K155/157	c	1
1785,666*	M₁QDQRE₆ + M₁QDQRE₆	N-term	N-term	a	1
3209,426	M ₁ QDQR ₅ + E ₆ NSIDVVQGGQKGNQGSSVEK ₂₇	N-term	K18	c	1
1551,733	M ₁ QDQR ₅ + K ₁₅₆ AELK ₁₆₁	N-term	K157	a	1
1482,650	M ₁ QDQR ₅ + V ₁₇₂ ERK ₁₇₆	N-term	K173	a,b,c,e	4
1353,596	M₁QDQR₅ + D₁₅₄KIK₁₅₇	N-term	K155	a	1
1506,802	V₁₇₂ERK₁₇₆ + K₁₅₆AELK₁₆₁	K173	157	a,b	2
3125,440	K ₁₂₆ EDSDSWSGR ₁₃₆ + I ₁₁₁ SVEDNVLVIKGEQK ₁₂₅	K126	K121	c,d,e,f	4
2462,252	G ₁₂₂ EQKK ₁₂₆ + E ₁₀₇ DVKISVEDNVLVIK ₁₂₁	K125	K110	c	1
2421,082	M ₁ QDQR ₅ + G ₁₉ NQGSSVEKRPQQR ₃₂	N-term	K18	b,c	2

(Gray-shaded) Hsp21–Hsp21 dipeptides involving the N terminus of Hsp21; (boxed) Hsp21–Hsp21 dipeptides detected in absence of CS; (bold) Hsp21–Hsp21 dipeptides detected in presence, but not in absence of CS; (#) number of mass spectra in which dipeptide was detected; (Δ) cross-linked peptide also modified with a dead-end; (*) peptide derived by cleavage with V8 instead of trypsin.

Searching for dipeptides with GPMW was made in eight different samples described in Table 1 and as listed below.

(Molar ratio 1:1) Either 12 μM Hsp21:10 μM CS, or 1.2 μM Hsp21:1 μM CS

(a) Hsp21–CS molar ratio 12:1 at 25°C.

(b) Hsp21–CS molar ratio 12:1 at 47°C.

(c) Hsp21–CS molar ratio 1:1 at 25°C.

(d) Hsp21–CS molar ratio 1:1 at 47°C.

(e) Hsp21 without CS at 25°C.

(f) Hsp21 without CS at 47°C.

cross-linked to the peptide D₁₅₄KDKIKAELK₁₆₁ (MH⁺ 2252), which is located in the putative substrate-binding C-terminal binding groove (see Fig. 6).

In the presence of CS, several more cross-linked Hsp21–Hsp21 dipeptides could be detected (MH⁺ 1785, 1551, 1353, and 1506) (indicated in bold in Table 2). Two of these dipeptides (MH⁺ 1551 and 1353) included cross-linking between the N terminus and lysines in the above-mentioned putative substrate-binding C-terminal binding groove (lysine residues K106, K110, K153, K155, and K157) (see Fig. 6 and below). It is not clear why novel Hsp21–Hsp21 dipeptides could be detected in the presence of CS; however, the presence of CS may act to induce a conformational change resembling cooperative binding, which affects the cross-linking.

Cross-linking within the Hsp21 dodecamer between neighboring tetramers

The Hsp21–Hsp21 cross-linked dipeptides (Table 2) demonstrated that especially the N-terminal arms and the C-terminal tails are involved in cross-linking within the Hsp21 dodecamer. The dipeptide involving the two N-termini (MH⁺ 1527) was easily detected in most mass spectra (all samples except 1:1, 47°C). This cross-linking

between pairs of tetramers through the intertwined N-terminal arms (Fig. 7C) is most likely the underlying explanation for the capture of Hsp21 as a cross-linked dodecamer, as seen by nonreducing SDS-PAGE (Figs. 3, 4).

A surprising result in the cross-linking of the Hsp21 dodecamer was the absence of suboligomeric species like dimers or tetramers seen on SDS-PAGE (Fig. 3A,C, 4A). Dimers or tetramers were expected to be cross-linked, since each monomer has an interface to five other monomers. The fact that this was not seen indicates that the above-mentioned cross-linking between pairs of tetramers predominates.

Cross-linking between Hsp21 and CS at multiple sites on the CS dimer

In total, 17 cross-linked Hsp21–CS dipeptides could be detected (Table 3). Cross-linked dipeptides were not only detected at 47°C, but also at 25°C, indicating that Hsp21 and CS interact not only at increased temperatures, but also at normal temperatures. The total numbers of lysine residues (25) in CS are evenly distributed across the surface of the CS dimer (Fig. 8A). The 17 cross-linked Hsp21–CS dipeptides involved 12 CS lysine residues, which were also fairly evenly distributed (Fig. 8B). An

Table 3. Hsp21–CS dipeptides detected in eight different samples according to Table 1

MH+	Crosslinked Hsp21-CS dipeptides	Hsp21 aa nr	CS aa nr	Condition
1577,726*	M₁QD₃ + I₉LADLIPKE₁₇	N-term	K16	c,d
1998,931	M ₁ QDQR ₅ + E ₁₇ QARIKTFR ₂₅	N-term	K22	a,b
2344,065	M₁QDQR₅ + S₄₂₄MSTDGLIKLVDSK₄₃₇	N-term	K432/437	c,d
3210,432	M ₁ QDQR ₅ + Y ₃₃₀ TCQREFALKHLPDPMFK ₃₄₈	N-term	K339	b
3407,618	E ₉₀ EEHEIKMRFDMPGLSKEDVK ₁₁₀ + M ₇₇ LPKAK ₈₂	K96/106	K80	a,b,c,d
1278,643*	N ₁₁₆ VLVIKGE ₁₂₃ + S ₄₃₆ K ₄₃₇	K121	K437	a,d
1910,958	G₁₂₂EQKK₁₂₆ + E₁₇QARIKTFR₂₅	K125	K22	c,d
2558,215	G₁₂₂EQKK₁₂₆ + F₆₆RGYSIPECQKMLPK₈₀	K125	K76	c,d
3305,578	G ₁₂₂ EQKKEDSDSDSWSGR ₁₃₆ + I ₃₅₆ VPNVLLEQGKAK ₃₆₈	K125/126	K366	a,b
1894,877*	Q ₁₂₄ KKEDSDD ₁₃₁ + G ₄₂₉ LILKLV ₄₃₅	K125/126	K432	b
1894,894*	Q ₁₂₄ KKE ₁₂₇ + G ₁₆₁ IHRTKYWE ₁₆₉	K125/126	K166	b
2118,922	K ₁₂₆ EDSDSDSWSGR ₁₃₆ + I ₂₁ KTFR ₂₅	K126	K22	a,b
1365,665	D ₁₅₄ KIK ₁₅₇ + E ₁₁₃ WAKR ₁₁₇	K155	K116	a,b,c
1733,892	D ₁₅₄ KIKAELK ₁₆₁ + K ₃₂₅ TDPR ₃₂₉	K155/157	K325	a,b
2271,038^	D ₁₅₄ KIKAELK ₁₆₁ + D ₂₉₅ VSDEKLR ₃₀₂	K155/157	K300	a,d
1494,719	V₁₇₂ERK₁₇₆ + E₁₁₃WAKR₁₁₇	K173	K116	c,d
2515,229	V₁₇₂ERK₁₇₆ + E₃₃₅FALKHLPDPMFK₃₄₈	K173	K339	c

(Gray shaded) Hsp21-CS dipeptides involving the N terminus of Hsp21; (=) peptide derived by cleavage with V8 instead of trypsin; (^) cross-linked peptide also modified with a dead-end; (boxed) Hsp21-CS dipeptides involving either the Hsp21 N terminus (I), the suggested substrate-binding sites (II) or (III), see Figure 6 and (van Montfort et al. 2001b; (bold) Hsp21-CS peptides only observed at 1:1 ratio.

(a) Molar ratio 12:1 at 25°C.

(b) Molar ratio 12:1 at 47°C.

(c) Molar ratio 1:1 at 25°C.

(d) Molar ratio 1:1 at 47°C.

interpretation of these data is that Hsp21 interacts with CS at numerous interaction sites, rather than at one specific site.

Different substrate-binding regions have been proposed for sHsps, as outlined in Figure 6. The Hsp21–CS dipeptides in Table 3 were divided into three classes, depending on whether they were involved in interactions with the Hsp21 N terminus (I) the region corresponding to the C-terminal hydrophobic binding groove (van Montfort et al. 2001b) in Hsp16.9 (II) or the Hsp21 C-terminal tail (III) (boxed in Table 3; color coded green, red, and yellow in Fig. 6). Thereafter, the CS lysine residues involved in cross-links with Hsp21 lysine residues in substrate-binding regions I, II, and III were mapped onto the CS structure (Fig. 8C). The different substrate-binding regions in Hsp21 apparently interact with different surfaces in CS.

The Hsp21 N terminus was cross-linked at normal temperatures to only one side of the CS dimer (Table 3) (MH⁺ 1577, 1998, 2344, and 3210), as indicated in green. On this side, the N-terminal α -helices in the CS dimer intertwine with the C terminus in the other subunit and together form a stem-like structure that protrudes from the CS dimer. In contrast, the Hsp21 region corresponding to substrate-binding regions II and III were cross-linked to completely different surfaces on the other side of CS (Table 3 (MH⁺ 1365, 1733, and 2271, and 1494 and 2515, respectively), as indicated in red and yellow. The cross-links with region III, the Hsp21 C-terminal tail (Table 3) (MH⁺ 1494 and 2515) were only seen when the molar ratio

between Hsp21 and CS was 1:1. The C-terminal tail and the IXI/V motif in Hsp21 are indicated in Figures 6 and 7C.

There were also cross-linked Hsp21–CS dipeptides not involving any of the Hsp21 regions I, II, and III. Six of these eight dipeptides (Table 3, not boxed) contained the Hsp21 lysine residues K125 and K126, located at the dimer interface in the loop that acts as a stabilizing element by β -6-strand exchange with the other monomer. This part of Hsp21, to our knowledge, has previously never been suggested to be involved in substrate binding.

Discussion

The Hsp21 dodecamer dissociates in response to substrate

Using chemical cross-linking and mass spectrometric mapping of cross-linked dipeptides, we have shown that the Hsp21 is captured as a dodecamer (Figs. 3A, 4A), which, according to the detected dipeptides (Table 2), is due to cross-linking between the N-termini of adjacent tetramers as illustrated in Figure 7. Changed mobility in size-exclusion chromatography has previously suggested the dissociation of wheat Hsp16.9 dodecamers into dimers at increased temperature (van Montfort et al. 2001b). However, that only one band corresponding to the dodecamer is visible at increased temperature in Figure 3B indicates that Hsp21 essentially does not dissociate into smaller sub-oligomeric species in the absence of CS. Rearrangements

Figure 6. Multiple sequence alignment of Hsp21 and Hsp16.9. Secondary structure elements are marked below the sequences according to the structure determined to atomic resolution for Hsp16.9 (1GME). The four hydrophobic sites suggested to become exposed by dodecamer disassembly to dimers (van Montfort et al. 2001b) are color coded in the following way: The IXI/V-motif in the C-terminal tail, which in Hsp21 and its orthologs is extended to IXVXI, including three hydrophobic residues I179, V181, I183, (yellow); the C-terminal binding groove covered by the IXI/V-motif (red); the N-terminal arm (green); the patch in α -crystallin domain covered by the N-terminal arm (purple). Lysine residues are marked in blue. Sequence alignment was performed with Clustal W (<http://www.ebi.ac.uk/clustalw/>).

within the Hsp21 dodecamer are induced by the presence of substrate rather than by increased temperature, as seen by the absence of a captured Hsp21 dodecamer (Fig. 4A, at a 1:1 molar ratio of CS) and the altered pattern of cross-linked dipeptides in the presence of CS (Table 2, not boxed; Table 3, bold face).

These data suggest that substrate ratio, alone or in combination with temperature, determines the rearrangements of Hsp21. It was recently shown that Hsp26 from yeast *Saccharomyces cerevisiae* undergoes a rearrangement to shift the oligomer to a high-affinity state for binding substrates, without oligomer dissociation (Franzmann et al. 2005). This was also visualized by cryo-EM, implying considerable flexibility between the two forms (White et al. 2006). For Hsp21, we have obtained data by single-particle negative-stain electron microscopy, also suggesting that the conformation of the Hsp21 dodecamer is altered by the presence of substrate (data not shown).

Several weak and short-lived Hsp21-CS interactions

Cross-linked dipeptides were detected not only at increased temperature (47°C) but also at normal temperature (25°C). In total, 17 different cross-linked Hsp21-CS dipeptides were detected, which were equally distributed

around the surface of CS (Fig. 8B). One interpretation of this finding is that CS aggregation, which is preceded by unfolding and monomerization (Russell et al. 1994, 1997; Arnott et al. 2000), can be prevented (Fig. 2) by numerous weak and short-lived sHsp-substrate interactions rather than by strong sHsp binding to one particular substrate region. Weak and short-lived interactions, with $K_d > 10^{-4}$ M and binding surfaces $< 1000 \text{ \AA}^2$, are being recognized more frequently with better tools for measurement and evaluation (Vaynberg and Qin 2006). That all the cross-linked Hsp21-CS dipeptides mapped to the surface indicates that Hsp21 interacts with the intact CS dimer. However, interactions with unfolding intermediates of CS cannot be excluded, since interactions can only be found as DTSSP-cross-linked dipeptides if lysine residues are present at the interaction surfaces and there are no lysine residues in the CS dimer interface.

The N-terminal arm and other substrate-binding sites in Hsp21

The N terminus of Hsp21 was detected in numerous cross-linked dipeptides within the Hsp21 dodecamer (Table 2) and in dipeptides with lysines in CS (K16, K22, K339, and K437) on one side of the CS dimer (Fig. 8C, green). This

Figure 7. Hsp21 modeled onto the Hsp16.9 structure. (A) Top view of the dodecamer showing the double-disc structure with an upper and a lower ring of three dimers and six of 12 N-terminal arms forming intertwined α -helices in the center of the dodecamer. (B) Side view showing one of the three tetramers. In each tetramer the dimers are stabilized through strand exchange between monomers and dimer–dimer stabilization obtained by the C-terminal tail of one of the monomers (the one with disordered N terminus) in each dimer strapping across to the partner dimer in the other disc (encircled, corresponding to cross-linked dipeptides MH⁺ 2252 and 1506) (Table 2). (C) Side view showing the contact region between two adjacent tetramers formed by the N-terminal α -helices of one monomer (light blue) in a dimer in the upper disc and one monomer (pink) in a dimer in the lower disc (eclipsed, corresponding to cross-linked dipeptides MH⁺ 1527, 1785, 3209, and 2421) (Table 2). Stabilization of tetramers is also obtained by the C-terminal tail of one of the monomers (the one with an ordered N terminus) in each dimer strapping across to the partner dimer in the neighboring tetramer (encircled, corresponding to cross-linked dipeptides MH⁺ 2252, 1506) (Table 2). Close-up shows the C-terminal binding groove (red) in one monomer, formed by residues V109, I111, and V113 (β -strand 4), and I156, A158, and L160 (β -strand 8), covered by the C-terminal tail (yellow) with the three hydrophobic residues I179, V181, and I183, belonging to a neighbor tetramer (cyan). The Hsp21 model was generated using the PDB-file 1GME and the chains A and B used as the template structure for a homology model created by 3D-JIGSAW. (<http://www.bmm.icnet.uk/~3djigsaw/> and the program Chimera at <http://www.cgl.ucsf.edu/chimera> were used to generate images.)

region has a stalk-like structure protruding from the CS dimer (Russell et al. 1994, 1997; Arnott et al. 2000). We have noted that this stalk-like structure is missing in thermostable forms of CS when we used peptide array screening, and found that several sHsps interacted strongly with a peptide (L₁₃IPKEQARIKTFRQQ₂₇) in this stalk-like structure (Ahrman et al. 2007).

The stalk-like structure in thermosensitive CS is composed of the most N-terminal helix in each monomer together with the C-terminal of the other monomer. In thermostable forms of CS these helices are absent, and the C-terminal of one monomer folds over and interacts with the other monomer, resulting in a close monomer–monomer

interaction, promoting dimer integrity at higher temperatures (Russell et al. 1997). Also, the N-termini in the two subunits are close to each other, whereas in thermosensitive CS the N-termini are flexible and situated on each side of the dimer. The interactions between the stalk-like structure on this side of CS (Fig. 8C, green) and the six flexible N-terminal arms in Hsp21 (invisible and unordered in the structure determined for Hsp16.9) (van Montfort et al. 2001b) could therefore provide a means of stabilizing the otherwise flexible N-termini and C-termini of CS. Evidence for such stabilizing interactions are provided here by the detected cross-linked Hsp21–CS dipeptides (MH⁺ 1577, 1998, and 2344) (Table 3).

Figure 8. The CS dimer and lysine residues involved in interaction with Hsp21. (A) All lysines in CS marked in blue. (B) All lysines detected in cross-linked CS-Hsp21 dipeptides marked in purple. (C) Lysine residues detected in cross-linked CS-Hsp21 dipeptides involving different suggested substrate-binding regions in Hsp21 color coded according to Figure 6 in the following way: the IXI/V-motif in the C-terminal tail (yellow), the C-terminal binding groove covered by the IXI/V-motif (red), and the N-terminal arm (green). The image was generated using the program Chimera (<http://www.cgl.ucsf.edu/chimera>) and the PDB-file 4CTS.

The other substrate-binding regions in Hsp21 (Fig. 8C, red, yellow) appears not to be called into action until the concentration of CS is higher. For example, cross-linked dipeptides indicative of an interaction between the Hsp21 C-terminal tail and CS were seen (Table 3, MH⁺ 1494 and 2515, marked in bold). These dipeptides were not detected at a 12:1 molar ratio, when the Hsp21 C-terminal tail interacts instead with the putative substrate-binding patch in another Hsp21 subunit, the C-terminal hydrophobic groove, as visualized in Figure 7C. Cross-linked dipeptides corresponding to the Hsp21 C-terminal K173 cross-linked to Hsp21 K155/157 (Fig. 7C, inset) were also detected (Table 2, MH⁺ 2252 and 1506). The cross-linked dipeptide 1506, composed of the Hsp21 C-terminal K173 cross-linked to K157, was only detected in the presence, but not in the absence of CS. This could reflect an important conformational change in the Hsp21 dodecamer, induced by the presence of a low amount of CS. Taken together, these observations provide experimental support to the idea that the C-terminal tail is released from the hydrophobic groove such that both of these surfaces become exposed and available for substrate binding (van Montfort et al. 2001b) when the Hsp21 dodecamer is rearranged in a substrate-induced conformational change.

As outlined schematically in Figure 9, it should be possible to increase the number of substrate-binding sites

gradually with increasing amounts of substrate by gradually pushing the equilibrium between dodecamer and suboligomeric species. Assuming that each monomer has a certain (even) number of potential, hydrophobic substrate-binding sites hidden in pairwise hydrophobic interactions with each other in the dodecamer, the number of substrate binding sites will increase gradually upon dodecamer dissociation.

The methionine-rich α -helix in the N-terminal arm of Hsp21 has been proposed as a substrate-binding site (Harndahl et al. 2001). Unfortunately, this region contains no lysine residues, so how this region interacts with CS could not be investigated with DTSSP, since lysines or other primary amines are required for cross-linking. The primary amine on the Hsp21 N-terminal, amplified by the 12 Å cross-linker, could nevertheless serve as a probe to document the importance of the N-terminal arm in the interactions with CS and within the Hsp21 dodecamer.

It is possible that the N-terminal arm plays an even more prominent role in the chloroplast-localized Hsp21 compared with other plant sHsps like Hsp16.9, since it is longer (33 amino acids more) and very conserved in terms of the methionine-rich amphipathic helix motif (Chen and Vierling 1991; Waters and Vierling 1999). By site-directed mutagenesis of Hsp21, novel amino acids can be introduced to permit chemical cross-linking of this methionine-rich region. We have also initiated a

Figure 9. Increasing numbers of available sHsp substrate-binding sites. The number of substrate-binding sites can increase gradually with an increasing amount of substrate. (A) Low amount of substrate. In the structure determined for Hsp16.9 (van Montfort et al. 2001b), every second N-terminal arm is invisible, since it is not interacting with another N-terminal arm, and therefore not ordered. These six flexible arms, each containing two substrate-binding sites (color-coded green and purple in Fig. 6) may be available for interaction with substrates without dodecamer disassembly. (B) Larger amounts of substrate: Via dynamic subunit exchange, or dodecamer disassembly, more substrate-binding sites are mobilized. The number of available substrate-binding sites increases gradually, from 12 (in dodecamer) to 24 (if tetramers) to 36 (if dimers) to 72 (if monomers) as calculated below, incorporated into large sHsp–substrate complexes (for simplicity only tetramer-based complexes are visualized here). Numbers of substrate-binding sites hidden in the dodecamer in pairwise hydrophobic patch interactions: assuming that each monomer has six potential substrate-binding sites (the color-coded four sites in Fig. 6, plus the loop at the dimer interface indicated in substrate binding by the cross-linked Hsp21–CS dipeptides containing K125/126 [Table 3], plus the surface this loop interacts with in the dimer). In the dodecamer, 12 available substrate-binding sites are offered by the flexible N-terminal arms and their patches. If tetramers 24 (all 12 N-terminal arms and their patches); if dimers 36 (add 12, the six C-terminal tails that cover six patches, within tetramers and between tetramers); if monomers 72 (add 12, the C-terminal tails that cover six patches within dimers, plus 12 more, the released loops and their patches) substrate-binding sites become exposed.

study using ^{15}N -labeled Hsp21 and NMR to determine physical parameters such as on- and off-rates, chemical shifts, affinity, and kinetics and to further investigate the role played by weak and short-lived interactions between various regions in Hsp21 and various substrate proteins.

Material and Methods

Stock solutions of proteins

Porcine citrate synthase (CS) (10 mg/mL) was obtained from Roche. Desalting and buffer exchange into 12.5 mM Tris-HCl (pH 7.0), 75 mM NaCl, 10 mM DTT was performed using disposable protein desalting columns (Pierce) to yield a stock solution of 2.1 mg/mL. Recombinant Hsp21 was obtained as described below. Protein concentrations refer to the monomer mass of Hsp21 (21 kDa) and CS (50 kDa).

Purification of Hsp21

Hsp21 was recombinantly expressed in *Escherichia coli* as described previously (Harndahl et al. 2001) and further purified by urea-induced monomerization and unfolding (Blennow et al. 1995) in order to remove trace amounts of bound bacterial proteins. The preparation of Hsp21 oligomers (1 mL, 3 mg/mL) was incubated in urea-buffer (4 M urea, 25 mM Tris/HCl, 150 mM NaCl, 10 mM DTT at pH 7.0) on ice for 20 min and loaded on to a size-exclusion chromatography column (Pharmacia

HiLoad/Superdex 200 HR 16/60) equilibrated with urea-buffer. The dominant peak contained the Hsp21 monomers. Collected fractions from this peak were dialyzed (4°C, 15–20 h, one buffer change) with urea-free buffer (12.5 mM Tris, 75 mM NaCl, 10 mM DTT at pH 7) using 12–14 kDa cutoff Spectra/Por membranes (Spectrum Medical Industries Inc) to permit re-assembly of Hsp21 oligomers. The dialyzed sample was concentrated with Macrosep (30 K cut off, PALL Life Sciences, SPG Media) for 1 h at 7100g. Any Hsp21 monomers that had not reassembled into oligomers were thus discarded. Finally, protein concentration to 2–5 mg/mL was performed with Microsep (100 K cutoff, PALL Life Sciences, SPG Media), 30 min at 2600g.

Nanoelectrospray-ionization mass spectrometry

To characterize the Hsp21 preparation and to evaluate the oligomer stoichiometry, nanoelectrospray mass spectrometry was conducted as described in Aquilina et al. (2003) by using a modified Q-ToF 2 mass spectrometer (Waters/Micromass). Typically, 2 μL of solution was electrosprayed from gold-coated glass capillaries prepared in-house as described in Nettleton et al. (1998). To preserve noncovalent interactions, the following instrument parameters were used: capillary voltage, 1.5 kV; cone gas, 150 Lh^{-1} ; sample cone, 200 V; extractor cone, 60 V; ion transfer stage pressure, 8.0×10^{-3} mbar (1 mbar = 100 Pa); and ToF analyzer pressure, 3.0×10^{-6} mbar. Tandem experiments were performed using quadrupole resolution settings of 7.0 to isolate the entire peak of interest. A voltage of 128 V was applied to the collision cell, containing 3.5×10^{-2} mbar of argon, to promote dissociation of monomers from the native oligomers. All spectra were calibrated externally by using a

solution of cesium iodide (100 mg/mL) and processed with MassLynx software (Micromass). Spectra are shown here with minimal smoothing and without background subtraction.

Aggregation-protection assay

CS aggregation and Hsp21 protection was performed as in Basha et al. (2004). A 20- μ L sample containing 1 μ M CS (porcine citrate synthase, P00889) or 1 μ M MDH (porcine malate dehydrogenase, P00346) was incubated with Hsp21 (12 μ M), or without Hsp21, for 20 min at 47°C. To separate soluble protein from aggregated, the sample was centrifuged for 10 min at 14,000g. A total of 15 μ L of the supernatant was removed (soluble protein) and mixed with SDS-PAGE loading buffer. The remaining 5 μ L were washed once with 100 μ L buffer (50 mM HEPES, 5 mM MgCl₂ at pH 8.5) and then centrifuged 10 min at 14,000g; thereafter, a 95- μ L sample was removed. The remaining 5 μ L (aggregated protein) was solubilized in SDS-PAGE loading buffer.

Chemical cross-linking

The cross-linker used, DTSSP, contains an amine-reactive *N*-hydroxysulfosuccinimide (sulfo-NHS) ester that reacts with primary amines (the side chain of lysine [K] residues and the N terminus of each polypeptide). Protein samples with only Hsp21 (12 μ M) or Hsp21: CS at a molar ratio (monomer: monomer) of either 12:1 or 1:1 were used. For molar ratio, 1:1 concentrations of 1.2 μ M and 1 μ M, respectively, and 12 μ M and 10 μ M, respectively, were tested and gave essentially the same results. Samples, in a volume of 20 μ L, were preincubated 20 min at 25°C or at 47°C on a thermo-shaker (250 rpm) before adding DTSSP (final concentration 5 mM). The cross-linker DTSSP, 3,3'-Dithiobis (sulfosuccinimidylpropionate) (Pierce) was dissolved in cross-linking buffer (50 mM HEPES at pH 8.0, 5 mM MgCl₂) immediately before use. DTSSP contains an amine-reactive *N*-hydroxysulfosuccinimide (sulfo-NHS) ester that reacts with primary amines at pH 7–9 to form stable amide bonds along with release of the *N*-hydroxysulfosuccinimide leaving group. After 20 min, the cross-linking reaction was quenched by addition of 1 M Tris (final concentration 20 mM). To analyze cross-linked products, the sample was divided into two identical aliquots, one of which was analyzed by SDS-PAGE, and one by mass spectrometric peptide mapping (see below). Prior to SDS-PAGE, samples were concentrated to dryness in a SpeedVac (Savant).

SDS-PAGE

Samples were dissolved or mixed 1:1 (v/v) with SDS-PAGE loading buffer (0.0625 M Tris-HCl, 25% glycerol [v/v], 2% SDS [w/v], 5% β -mercaptoethanol [v/v], 1.25% of 1% bromophenol blue [v/v]), and incubated at 95°C for 5 min. The gels (Ready gels, 12% Tris-HCl, BioRad Laboratories) were run in a Laemmli buffer system at 200 V, 100 mA, and visualized by silver-staining (Sorensen et al. 2002).

MALDI mass spectrometry

Unreacted DTSSP was removed by acetone precipitation of proteins and the pellet was dissolved in 25 mM NH₄HCO₃

(pH 7.8) (20 μ L). Half of the sample (10 μ L) was reduced (50 mM DTT 30 min 37°C) and alkylated (50 mM IAM 30 min in the dark). The samples were digested in-solution with sequencing-grade modified Trypsin (Promega) or Endoprotease Glu-C (Roche, Roche Applied Science). Protease was added 1:50 (w/w protease: protein) for 1 h at 37°C, followed by 1:25 (w/w protease: protein) for 3 h (trypsin) or overnight (Endoprotease Glu-C) at 37°C. Digests were then directly applied onto a MALDI-target plate and allowed to dry before a 0.5 μ L matrix solution (5 mg/mL α -cyano-4-hydroxy cinnamic acid, 70% acetonitrile, 0.5% TFA, and 50 mM citric acid) was added.

MALDI-TOF-MS was recorded on an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOFTM optics (Applied Biosystems) in positive reflectron mode. The obtained MS spectra were internally calibrated using trypsin autolysis peptides (*m/z* values 842.51 and 2211.097 Da) and two Hsp21 peptides (*m/z* values 1269.56 Da, aa 62–72, and 1989.02 Da, aa 33–50) that contained no lysine residues.

Data analysis

Identification of cross-linked dipeptides was performed using the software GPMaw (General Protein/Mass Analysis for Windows, Version 7.02. Lighthouse data; <http://www.gpmaw.com>) with the MS X-link function used at 10 ppm and allowing four missed cleavage sites. Cross-links within the same peptide are referred to as internal cross-links. A dead-end cross-link is formed if only one end of the cross-linker reacts with a lysine residue but not the other end, and an internal cross-link is within one peptide. For samples with reduced DTSSP, a modification file based on Bennett et al. (2000) was created, 174.24 Da (C₆H₆O₂S₂) for cross-links and 192.25 Da (C₆H₈O₃S₂) for dead-ends. Since samples were alkylated after reduction of DTSSP, an extra mass of 145.18 Da was also included in the modification file. Peptides identified as cross-linked dipeptides by GPMaw were manually inspected with the requirements that cross-linked dipeptides should neither be present in the control sample without cross-linker nor in the corresponding reduced sample. Approximately half of the dipeptides listed in Tables 2 and 3 were confirmed by MALDI-MS/MS or LC-ESI-ion trap MS/MS (data not shown).

Molecular modeling of the Hsp21 dodecamer

The structure of Hsp16.9 (van Montfort et al. 2001b) was retrieved from the Protein Data Bank (www.pdb.org, 1GME), and the chains A and B used as template structure for a homology model created by 3D-JIGSAW (<http://www.bmm.icnet.uk/~3djigsaw/>) (Bates et al. 2001).

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