^{1}H NMR spectroscopy reveals that mouse Hsp25 has a flexible C-terminal extension of 18 amino acids

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Abstract The small heat-shock proteins (Hsps) exist as large aggregates and function by interacting and stabilising non-native proteins in a chaperone-like manner. Two-dimensional ^{1}H NMR spectroscopy of mouse Hsp25 reveals that the last 18 amino acids have great flexibility with motion that is essentially independent of the domain core of the protein. The lens protein, α-crystallin, is homologous to Hsp25 and its two subunits also have flexible C-terminal extensions. The flexible region in Hsp25 encompasses exactly that expected from sequence comparison with α-crystallin implying that both proteins have similar structures and that the C-terminal extensions could be of functional importance for both proteins.

Key words: Small heat-shock protein; Chaperone; α-Crystallin; NMR spectroscopy; Flexibility

1. Introduction

The heat-shock proteins (Hsps) are a ubiquitous class of proteins that are involved in the stabilisation of unfolded proteins, and, accordingly, they are expressed in large quantities in response to stress conditions (e.g. increased temperature). Hsps are involved in the processes of folding and unfolding of proteins and, because of this, they are type of chaperone protein. A sub-class of the Hsps is the small Hsps (reviewed in [1] and [2]) which are a diverse group of proteins with masses that range from 15 to over 30 kDa. Amongst this group is αB-crystallin, one of the subunits of α-crystallin, the major lens protein. The chaperone ability of α-crystallin has only recently been described whereby it prevents unfolded proteins from aggregating and precipitating out of solution [3,4]. The two subunits of α-crystallin (A and B) have similar amino acid sequences and masses of approximately 20 kDa, and, like the other small Hsps, the subunits aggregate to form large oligomers with an average mass of around 800 kDa. The structural and functional similarities of α-crystallin and Hsp25 have been noted from a variety of structural studies, including the ability for both proteins to form functional co-aggregates in vivo [5] and in vitro [6], and their very similar chaperone abilities [4,6]. The sequence similarity between the small Hsps and α-crystallin is confined mainly to the C-terminal regions of the molecules corresponding to the putative C-terminal domain [7]. Indeed, removal of the last 42 amino acids of *Drosophila* small Hsp27, which contains the conserved region, leads to a marked reduction in the protective ability of the protein [8]. Likewise, proteolysis of the C-terminal region of α-crystallin to remove the last 16 amino acids of αA-crystallin reduces significantly the chaperone ability of the protein [9]. Thus, the C-terminal region of the small Hsps seems to be important in the action of the molecule.

Despite the large size of the α-crystallin aggregate, a well-resolved ^{1}H NMR spectrum is observed which arises from highly flexible C-terminal extensions of eight (αA) and ten (αB) amino acids [10] that protrude from the domain core of the protein. The amino acid sequences of this region have no homology between the two α-crystallin subunits and with the same region in the mammalian small Hsps (Fig. 1) [11,12]. The purpose of this investigation was to determine, by NMR spectroscopy, whether any region (or regions) in mouse Hsp25 had flexibility, and if so, whether this region corresponded to the comparable flexible regions in α-crystallin. Such information would provide further clues about the structural similarity, or otherwise, of the two proteins.

2. Experimental

Recombinant mouse Hsp25 was expressed and purified as described in [13] and dialysed against buffer 1 containing 20 mM phosphate buffer, 0.02% NaN_3, pH 6.0. The dialysed sample was concentrated by ultrafiltration at $5000 \times g$ using an Ultrafree-MC-Filter (Millipore) with an exclusion molecular weight of 10,000 until it had reached a concentration of 26.8 mg in 0.9 ml of buffer 1. 45.5 μl of D_2O was added to this solution and 0.5 ml of the total solution was withdrawn and placed in an NMR tube. The concentration of this solution was approximately 1.2 mM on a subunit basis.

^{1}H NMR spectra were acquired at 500 MHz and 15°C and 25°C using a Bruker AM-500 spectrometer. Chemical shifts at 25°C were referenced to the residual water resonance at 4.76 ppm. All two-dimensional NMR experiments were acquired in the pure-phase mode using the time-proportional-phase-incrementation method [14]. Through-bond (scalar) connectivities were obtained from double-quantum-filtered correlation spectroscopy (DQF COSY) and total-correlation spectroscopy (TOCSY) experiments. In the TOCSY experiments, a WALTZ-16 pulse train was used for the spin lock which was flanked by z-filter pulses [15]. The duration of the spin lock was 68 ms for the TOCSY experiment at 25°C and 33 ms for the experiment at 15°C. For all correlation experiments, the water resonance was suppressed by continuous, coherent saturation for 1.0 to 1.5 s during the delay between scans. Through-space connectivities were obtained from nuclear Overhauser effect spectroscopy (NOESY) spectra [16] acquired with a mixing time of 100 ms. To reduce the water resonance in these experiments, a DANTE saturation pulse train [17] was used for 1.0 to 1.5 s during the delay between scans and the mixing time. For the TOCSY and NOESY experiments, 512 t_1 increments were acquired over 2048 points and 5814 Hz in t_2 with 32 scans per t_1 increment for the TOCSY

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αA-crystallin	**REEKPSSAPSS**[173]
αB-crystallin	**REEKPAVTAAPKK**[175]
mouse Hsp25	**FEARAQIGGPEAGKSEQSGAK**[205]
human Hsp27	**FESRAQLGGPEAAKSDETAAK**[205]
hamster Hsp27	**FEARAQIGGQEAGKSEQSGAK**[205]

Fig. 1. A comparison of the amino acid sequences of the C-terminal region of bovine α-crystallin subunits and mammalian Hsp25 proteins [11,12]. The flexible C-terminal extensions for the α-crystallin subunits [10] are underlined.

experiments and 96 scans per t_1 increment for the NOESY experiments. The DQF COSY experiment was acquired with the same parameters as for the TOCSY experiments except that 400 t_1 increments were collected with 64 scans per increment. Prior to Fourier transformation, the data for all spectra were zero filled in t_1 and apodized with sine-squared bell window functions shifted by 50° to 70° in both dimensions. All spectra were processed and visualised using Felix software (Biosym).

3. Results

A one-dimensional ^{1}H NMR spectrum of mouse Hsp25 exhibited a variety of well-resolved resonances (not shown), indicating flexibility for a region or regions in Hsp25. A series of two-dimensional NMR experiments were conducted on this sample to assign these resonances using the sequential-assignment procedure of Wüthrich [16]. Fig. 2 compares the NH to α-CH region of DQF COSY and NOESY experiments at 25°C of murine Hsp25 and shows that many cross-peaks were observed via through-bond (scalar) and through-space (dipolar) connectivities, respectively. From the DQF COSY and TOCSY experiments, the various types of amino acids present were determined from one-bond and relayed connectivities and they were linked together from observation of nuclear Overhauser effects (nOes) in the NOESY spectra from the NH proton of one amino acid to the α, β and NH protons of the preceding residue [16]. The NH_i to α-CH_{i-1} nOes are traced out in Fig. 2b. To summarise, a series of such strong nOes were observed from K205 to P194. The connectivity between P194 and G193 was observed via a strong nOe between the δ-CH_2 of P194 and the α-CH_2 of G193 [16]. The strong NH_i to α-CH_{i-1} nOes continued from G193 to I190 from where, due to severe overlap with other cross-peaks and the remaining two cross-peaks being inherently weaker and having almost identical chemical shifts, it was not possible to ascertain conclusively that the connectivity continued onto the cross-peaks of these amino acids, an alanine and an arginine. On the balance of probability, however, it would seem that these two amino acids can be assigned to the two residues that precede I190, i.e. A189 and R188. TOCSY and NOESY spectra were also acquired at 15°C in an attempt to overcome this overlap with no improvement in resolution although the cross-peaks in both spectra were stronger than at 25°C, due to the reduction in NH exchange rate. In addition to the strong NH_i to α-CH_{i-1} connectivities observed in the NOESY spectra, a number of NH to β- and γ-CH_{i-1} and NH to NH nOes were observed. The latter are shown in Fig. 3 and the sequential nOes are summarised in Fig. 4.

Thus, NMR spectroscopy indicates that the flexible region observed in mouse Hsp25 encompasses the region from R188 to K205, the 18 amino acids which, upon sequence alignment [11,12], match exactly the flexible regions observed in the two subunits of α-crystallin (Fig. 1). The assignments for this region in Hsp25 are tabulated in Table 1. The similarity of these values to those for random-coil peptides [16], the observation of strong NH_i to α-CH_{i-1} nOes along with weak, or non-existent NH to NH nOes and the absence of any non-sequential nOes suggests that this region has an extended conformation [16] of little or no preferred structure and a great degree of flexibility. Similar conclusions were drawn concerning the C-terminal extensions in α-crystallin [10].

The C-terminal extension in Hsp25 (and those in α-crystallin) therefore have characteristics of many peptides in an aqueous environment, i.e. flexibility and no defined conformation [16]. The presence of the single proline residue (P194) enabled us to examine this further. The distances between the α-CH and β-CH_2 protons in a proline are fixed at 2.733 Å (β_1, proR) and 2.302 Å (β_2, proS) [18]. The magnitude of the individual nOes between the α-CH and β-CH_2 protons of P194 was measured by integrating the isolated cross-section containing these peaks in the NOESY spectrum acquired at 25°C. Following the notation of Macura and Ernst [19], the ratio of the nOes from the α-CH to the individual β-CH_2 protons of P194 can be related to the ratio of the NOESY mixing coefficients $a_{\alpha\beta1}$ and $a_{\alpha\beta2}$ respectively, i.e.

$$\frac{a_{\alpha\beta1}}{a_{\alpha\beta2}} = \frac{\exp(-R_L^{\alpha\beta1}\cdot t_m)[1-\exp(-R_C^{\alpha\beta1}\cdot t_m)]}{\exp(-R_L^{\alpha\beta2}\cdot t_m)[1-\exp(-R_C^{\alpha\beta2}\cdot t_m)]} \quad (1)$$

where t_m is the mixing time and R_L and R_C are the individual leakage-relaxation and cross-relaxation rates, respectively. In the isolated spin pair approximation, if equal leakage-relaxation rates are assumed for both β protons then, following expansion of the cross-relaxation exponentials and truncation of the power series to the second degree, equation (1) becomes:

$$\frac{a_{\alpha\beta1}}{a_{\alpha\beta2}} = \frac{R_C^{\alpha\beta1}(1-R_C^{\alpha\beta1}\cdot t_m)}{R_C^{\alpha\beta2}(1-R_C^{\alpha\beta2}\cdot t_m)} \quad (2)$$

Following substitution of the appropriate expressions for R_C [19], equation (2) can be solved for the correlation time, τ_c, which can reasonably be assumed to be the same for the reorientation of the α-β_1 and α-β_2 inter-nuclear vectors in a proline residue. From such a calculation, the τ_c of P194 was estimated to be 0.71 ns. The C-terminal extension of mouse Hsp25 has a mass of 1767 Da. From the Stokes–Einstein relationship:

$$\tau_c = \frac{MV\eta}{RT} \quad (3)$$

where M is the molecular weight, V is the partial specific volume (~0.72 cm^3g^{-1} for most proteins), η is the viscosity of the solvent, R is the gas constant and T is the absolute temperature, a free peptide of this mass would have a τ_c of 0.46 ns at 25°C which is comparable, within experimental error, to the value estimated from the NOESY spectrum for P194 in the intact protein. Both values are much less than the τ_c expected for a protein aggregate of mass 800 kDa (207 ns). From such data, it is clear that the last 18 amino acids of Hsp25 have essentially independent motion compared to that of the domain core, protein aggregate.

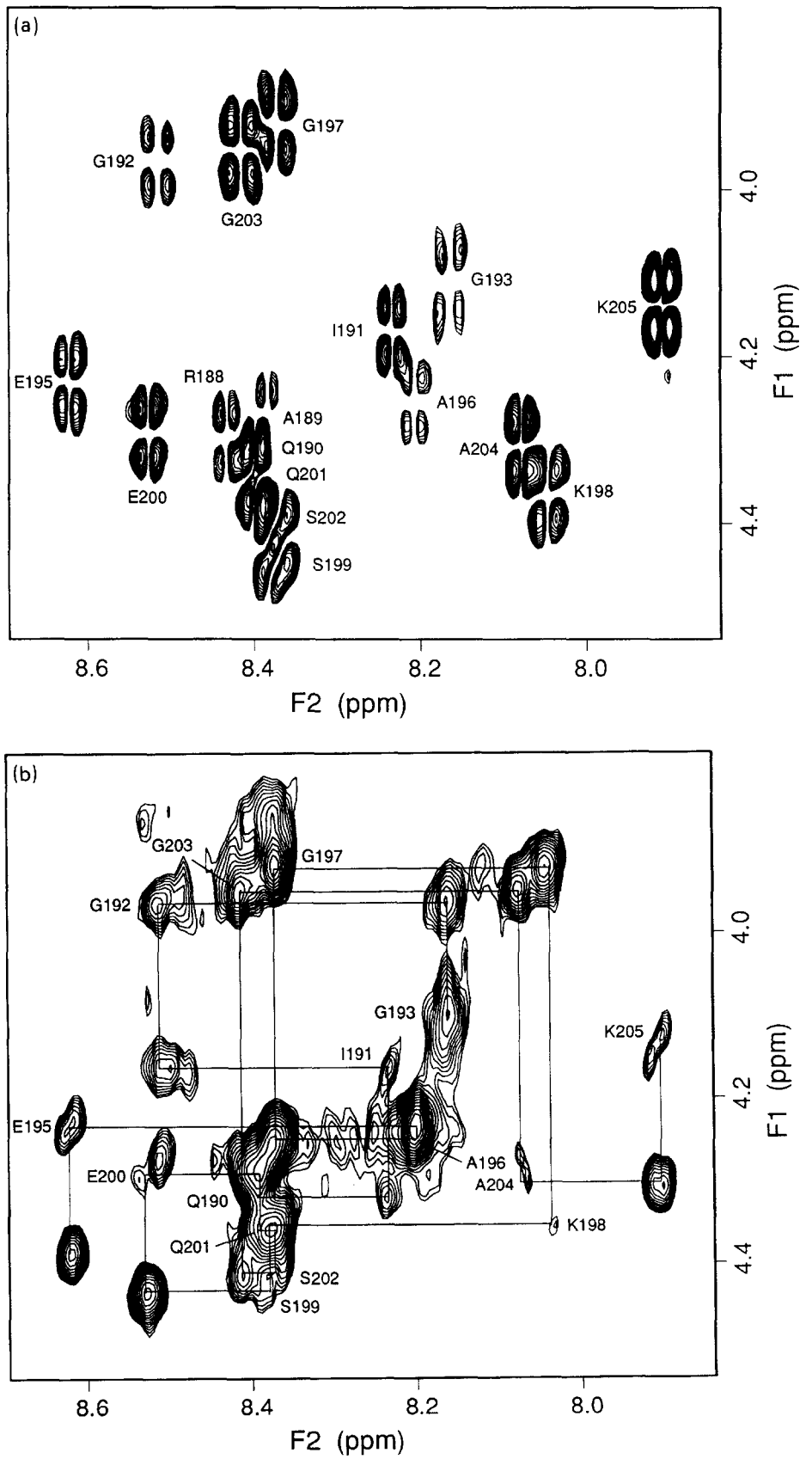

Fig. 2. (a) NH to α-CH region of the DQF COSY spectrum at 25°C of Hsp25. Assignments are indicated. (b) NH to α-CH region of a NOESY spectrum at 25°C of Hsp25. The NH_i to α-CH_{i-1} nOes are traced out. The intra-residue NH_i to α-CH_i nOes are labelled. From this plot it was possible to assign sequentially from K205 to P194 and from G193 to I190 (see text). A number of cross-peaks in this spectrum remain unassigned (e.g. that at 4.28, 8.51 ppm). They probably arise from connections between unassigned protons whose weak cross-peaks were observed in the TOCSY spectra.

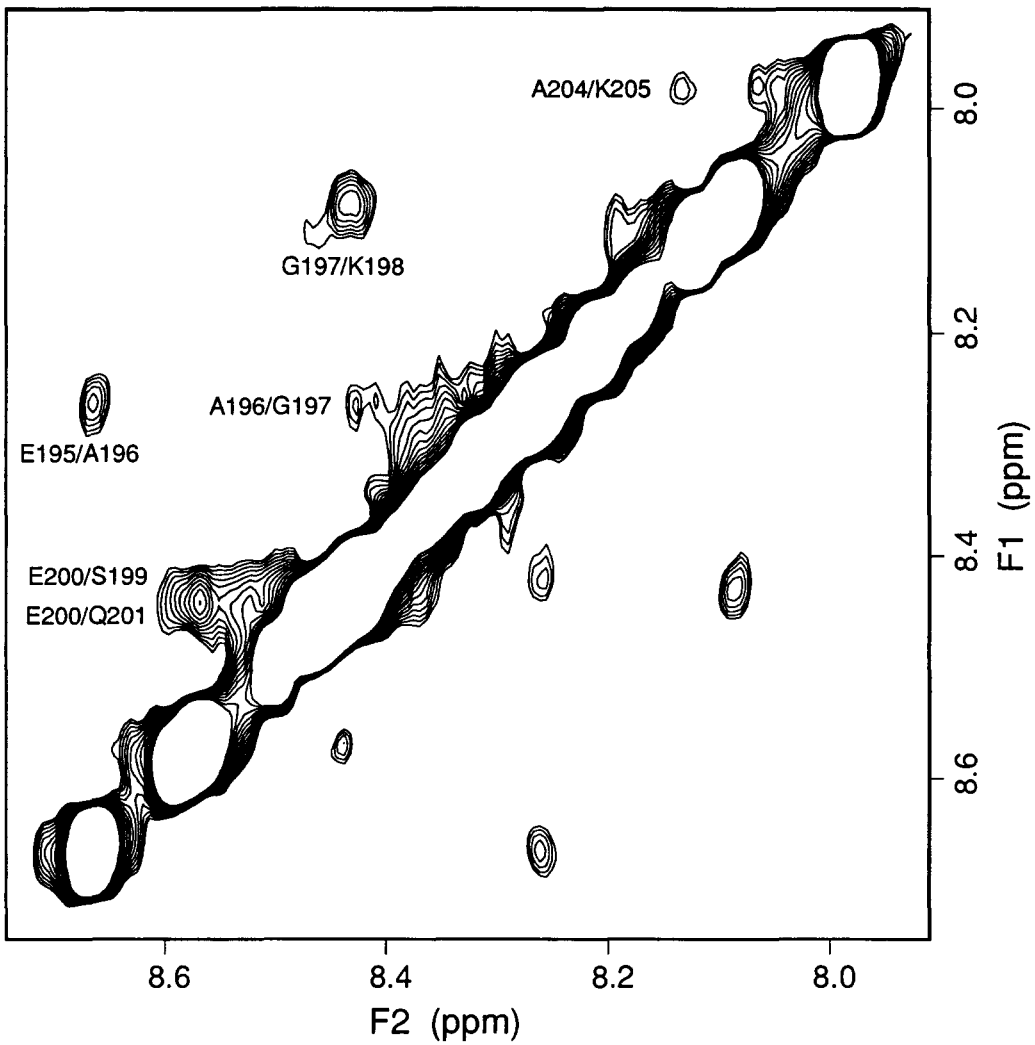

Fig. 3. The NH region of a NOESY spectrum at 15°C of Hsp25. Sequential NH to NH nOes are indicated.

4. Discussion

The observation that Hsp25 has a flexible C-terminal extension like α-crystallin, and that this extension commences exactly at the residue (R188) expected from homology with α-crystallin, implies that both proteins adopt a similar structure. There is other experimental evidence to support such a conclusion. In particular, Merck et al. [6] have shown that both proteins have very similar CD spectra (implying a similar secondary structure), denaturation behaviour (implying similar secondary and tertiary structures), chaperone ability and form co-aggregates (implying similar tertiary and quaternary arrangements). Furthermore, the C-terminal lysine residues in Hsp25 (K205) and αB-crystallin (K175) can act as amine-donor substrates with transglutaminase to form cross-links between polypeptides [6]. The αA-crystallin subunit has a serine residue at its C-terminus (Fig. 1) and cannot therefore be a substrate for transglutaminase. The NMR results presented herein for Hsp25 and previously for αB-crystallin [10] are consistent with the transglutaminase-mediated reactions of both proteins since both C-terminal residues are very exposed to solvent and are therefore accessible to modifying agents. The exposure of the C-terminal region of Hsp25 and α-crystallin to solution is consistent with a number of models for their quaternary structure [20–22].

Both C-terminal extensions in α-crystallin are highly susceptible to modification (reviewed in [23]), e.g. proteolysis, glycation and, as discussed above, cross-linking. Thus, aged α-crystallin frequently contains significant quantities of α-crystallin which has been modified in this region. Presumably, these alterations arise because of the highly exposed nature of the extensions. As the alterations occur to a region that is not part of the domain core of the protein, such alterations should have little effect on the function of the protein. Thus, as there is no protein turnover in the lens, preferential alteration of the extensions may act to ensure that lens α-crystallin remains functionally viable over the lifetime of the individual. One could hypothesise from this work that the longer C-terminal extension in Hsp25 may be also prone to modification.

The C-terminal extension in Hsp25 is much longer than its

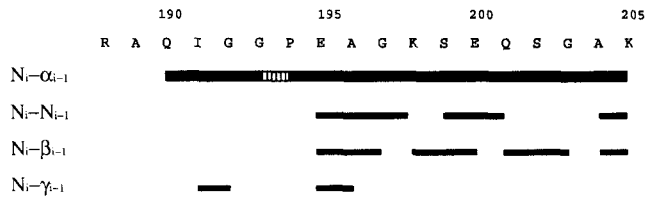

Fig. 4. A summary of the sequential nOes observed for mouse Hsp25 at 25°C. In all cases, the NH_i to α-CH_{i-1} nOes were much stronger than other types of nOes. The nOe connectivity between P194 δ-CH_2 and G193 α-CH_2 protons is indicated by a hatched connectivity.

counterparts in α-crystallin (18 amino acids compared to eight and ten for the αA and αB subunits, respectively). Although this region is relatively well conserved amongst the mammalian Hsp25 proteins [12], it bears little resemblence to those in both crystallin subunits (Fig. 1), which themselves are very different from each other [11]. The question arises as to what is the functional role or roles of these extensions? As discussed above, they may be involved in maintaining the long-term viability of the proteins in preferentially protecting their domain cores from modification. Extensions from both Hsp25 and α-crystallin share the common feature of being polar in nature. They may therefore act as solubilising agents for the hydrophobic protein since it has been demonstrated that α-crystallin possesses a large solvent-exposed hydrophobic area [24] which presumably is also present in Hsp25. Both extensions in α-crystallin do not interact with native proteins (e.g. other crystallins) [25]. The C-terminal extension of αB-crystallin, however, but not that of αA-crystallin, is preferentially involved in interaction with unfolded proteins [26]. The C-terminal extension in Hsp25 may perform a similar task particularly because it, like αB-crystallin, has three positively charged lysine/arginine residues (Fig. 1) which could be involved in electrostatic stabilisation of the unfolded protein.

It is interesting to note that although the small Hsps of mouse, human and hamster show about 80% amino acid sequence homology and share the entire sequence of the most hydrophilic region of the molecule [13], polyclonal antibodies raised in rabbits against human, hamster and mouse small Hsp show only very poor cross-reactivity between these proteins [13,27]. Taking into account that very mobile parts of proteins could be preferred antigenic determinants [28], based on the above NMR data, one may assume that the C-terminal extensions of the small Hsps are the major antigenic determinants. Compared to the most hydrophilic part of the protein, these termini are not as well conserved between species. Therefore, the absence of cross-reactivity of polyclonal antibodies between the human, rabbit and mouse sHsps could be due to the sequence variation in the terminal extensions.

Since the major region of sequence similarity between Hsp25 and α-crystallin arises in the C-terminal region of the molecules [7], the implication is that this region (including the C-terminal extensions) is responsible for the chaperone action. Indeed, removal of regions from the C-terminus into the domain core of both proteins causes a marked reduction in chaperone function [8,9]. The C-terminal region is obviously important for the chaperone action of the both molecules since this region has the greatest homology and, one may argue, from this work and [6], that it adopts a similar structure. The isolated putative C-terminal domains of both proteins, however, cannot function as a chaperone [2], suggesting that the entire protein is required for its function. Mutants of Hsp25 with deletions and alterations in the C-terminal extension are currently being prepared to explore the role of the extension in the chaperone action of the protein.

Flexible regions may be a general feature of chaperone proteins. Thus, in addition to α-crystallin and Hsp25, SecB, a bacterial chaperone involved in protein export, has been proposed to have a highly flexible C-terminal region that is involved in binding to non-native proteins [29]. In GroES, the co-chaperone of GroEL, ^{1}H NMR spectroscopy indicates that a flexible loop of 16 amino acids is present [30]. The flexibility is lost upon complexation with GroEL implying a possible role for this region in recognition between the two proteins [30]. Furthermore, in the X-ray crystal structure of GroEL [31], the last 24 residues of each subunit were disordered suggesting that this region has enhanced flexibility, i.e. like Hsp25 and α-crystallin, a flexible C-terminal extension is present. A flexible but much shorter five-amino-acid N-terminal segment was also observed. The extensions seemed to project into the central cavity of the GroEL aggregate [31]. The significance of these flexible regions in GroEL is not clear but they may regulate transport of unfolded protein within the central cavity. A similar structure to GroEL has been proposed for the α-crystallin aggregate [22] and there is some electron microscopic evidence to support a toroid or annular arrangement for the subunits in Hsp25 [32] and cardiac α-crystallin [33]. As in GroEL, the C-terminal extensions in Hsp25 and α-crystallin may regulate access to the interior of the aggregate.

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Table 1
^{1}H NMR chemical shifts for mouse Hsp25 at 25°C, pH 6.0

	NH	α-CH	β-CH	Others
R188	8.43	4.30	1.74, 1.82	γ-CH_2 1.62; δ-CH_2 3.19
A189	8.38	4.27	1.35	
Q190	8.40	4.34	1.97, 2.12	γ-CH_2 2.35
I191	8.24	4.17	1.85	γ-CH_2 1.17, 1.45; γ-CH_3 0.89; δ-CH_3 0.84
G192	8.51	3.96		
G193	8.16	4.11		
P194	–	4.39	1.93, 2.26	γ-CH_2 2.00; δ-CH_2 3.62
E195	8.62	4.23	1.92, 2.05	γ-CH_2 2.26
A196	8.21	4.25	1.38	
G197	8.37	3.93		
K198	8.04	4.37	1.74, 1.85	γ-CH_2 1.39; δ-CH_2 1.65 ε-CH_2 2.98
S199	8.38	4.44	3.87	
E200	8.53	4.29	1.93, 2.05	γ-CH_2 2.25
Q201	8.40	4.34	1.97, 2.12	γ-CH_2 2.35
S202	8.37	4.42	3.87	
G203	8.42	3.95		
A204	8.08	4.31	1.35	
K205	7.91	4.14	1.66, 1.80	γ-CH_2 1.38; δ-CH_2 1.65 ε-CH_2 2.98

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