Glutamic Acid Residues in the C-Terminal Extension of Hsp25 are Critical for Structural and Functional Integrity

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Abstract
Small heat shock proteins (sHsps) are intracellular molecular chaperones that prevent the aggregation and precipitation of partially-folded and destabilized proteins. sHsps are comprised of an evolutionarily conserved region of 80-100 amino acids denoted the α-crystallin domain which is flanked by regions of variable sequence and length: the N-terminal domain and the C-terminal extension. Whilst the two domains are known to be involved in organization of the quaternary structure of sHsps and interaction with their target proteins, the role of the C-terminal extension is enigmatic. Despite the lack of sequence similarity, the C-terminal extension of mammalian sHsps is typically a short, polar segment which is unstructured and highly flexible and protrudes from the oligomeric structure. Both the polarity and flexibility of the C-terminal extension are important for the maintenance of sHsp solubility and the complex it makes with its target protein. In the present study, mutants of murine Hsp25 were prepared in which the glutamic acid residues in the C-terminal extension at positions 190, 199 and 204 were each replaced with alanine. The mutants were found to be structurally altered and functionally impaired. Whilst there were no significant differences in the environment of tryptophan residues in the N-terminal domain or overall secondary structure, an increase in exposed hydrophobicity was observed for the mutants compared with wild type Hsp25. The average molecular masses of the E199A and E204A mutants were comparable to the wild type protein, while the E190A mutant was marginally smaller. All mutants displayed markedly reduced thermostability and chaperone activity compared with wild type. It is concluded that each of the glutamic acid residues in the C-terminal extension is important for Hsp25 to act as an effective molecular chaperone.

Keywords
Molecular chaperone, small heat shock protein, Hsp25, C-terminal extension, protein aggregation

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Glutamic Acid Residues in the C-Terminal Extension of Hsp25 are Critical for Structural and Functional Integrity

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Abbreviations:
ADH, alcohol dehydrogenase; ANS, 1-anilino-8-naphthalene sulphonate, DTT, dithiothreitol; SEC, size-exclusion chromatography; sHsp, small heat shock protein

Keywords:
Molecular chaperone, small heat shock protein, Hsp25, C-terminal extension, protein aggregation
Summary
Small heat shock proteins (sHsps) are intracellular molecular chaperones that prevent the aggregation and precipitation of partially-folded and destabilized proteins. sHsps are comprised of an evolutionarily conserved region of 80-100 amino acids denoted the \( \alpha \)-crystallin domain which is flanked by regions of variable sequence and length: the N-terminal domain and the C-terminal extension. Whilst the two domains are known to be involved in organization of the quaternary structure of sHsps and interaction with their target proteins, the role of the C-terminal extension is enigmatic. Despite the lack of sequence similarity, the C-terminal extension of mammalian sHsps is typically a short, polar segment which is unstructured and highly flexible and protrudes from the oligomeric structure. Both the polarity and flexibility of the C-terminal extension are important for the maintenance of sHsp solubility and the complex it makes with its target protein. In the present study, mutants of murine Hsp25 were prepared in which the glutamic acid residues in the C-terminal extension at positions 190, 199 and 204 were each replaced with alanine. The mutants were found to be structurally altered and functionally impaired. Whilst there were no significant differences in the environment of tryptophan residues in the N-terminal domain or overall secondary structure, an increase in exposed hydrophobicity was observed for the mutants compared with wild type Hsp25. The average molecular masses of the E199A and E204A mutants were comparable to the wild type protein, while the E190A mutant was marginally smaller. All mutants displayed markedly reduced thermostability and chaperone activity compared with wild type. It is concluded that each of the glutamic acid residues in the C-terminal extension is important for Hsp25 to act as an effective molecular chaperone.

Introduction
Small heat shock proteins (sHsps)\(^1\) are a family of intracellular molecular chaperones defined by the presence of an evolutionarily conserved region of 80-100 amino acid residues denoted the \( \alpha \)-crystallin domain [1]. Despite having a relatively small monomeric size (12-43 kDa) [2], sHsps exist under physiological conditions as large oligomers of up to 50 subunits and 1.2 MDa in mass [3, 4]. sHsps are found in most cell types in most organisms and their expression is upregulated under a range of stress conditions such as heat, oxidative conditions, pH changes, infection and in many disease states which are characterized by the formation of insoluble amyloid plaques, e.g. Alzheimer's, Creutzfeldt-Jakob and Parkinson's diseases [5-8]. Increased levels of sHsps, in particular \( \alpha \)B-crystallin and Hsp27, are observed in the brains of sufferers of these diseases [9, 10]. Hsp25 is the murine homologue of Hsp27.

Stress conditions can promote the partial unfolding of proteins, which subsequently leads to the exposure of hydrophobic residues [11]. This increase in surface-exposed hydrophobicity
encourages partially-folded proteins to mutually associate and potentially precipitate [12]. sHsps prevent the aggregation of such proteins by interacting with them and sequestering them into a large complex. Recognition of target proteins by sHsps occurs through exposed hydrophobic regions, and the resultant complex is stabilized through electrostatic interactions [13]. Target proteins are held in a folding-competent conformation until conditions are permissive for their refolding or degradation, with the former requiring the input of another chaperone protein, e.g. Hsp70 [14].

sHsps are comprised of three structural regions: the conserved $\alpha$-crystallin domain is flanked by an N-terminal domain and a C-terminal extension, both of which are of variable length and sequence. Overall homology amongst sHsps is therefore low [15]. Whilst extensive work has been undertaken to elucidate the functions of the N-terminal domain and $\alpha$-crystallin domain, the role of the C-terminal extension is less clear. Despite its variability, the C-terminal extension is a short region which is polar, highly flexible and unstructured and extends freely from the sHsp oligomer [16]. These general properties are essential for the proper functioning of sHsps as molecular chaperones. Removal of the C-terminal extension inhibits the chaperone activity of $\alpha$A-crystallin and *Xenopus* Hsp30C [17, 18] and also leads to a reduction in the solubility of Hsp25, $\alpha$A-crystallin and bacterial Hsp16-2 [17, 19, 20].

The C-terminal extension acts as a solubilizer to counteract the hydrophobicity associated with target sequestration [21]. The flexibility of the C-terminal extensions of Hsp25 and $\alpha$A-crystallin is maintained in the final sHsp-target protein complex, with the extensions remaining solvent-exposed. Under heat stress, the extension of $\alpha$B-crystallin has been shown to have reduced flexibility upon sHsp-target complex formation, implying that the extension may be involved in target protein capture and have functions in addition to acting as a solubilizer [22]. The oligomeric sizes of $\alpha$A-crystallin, bacterial Hsp16.3 and bacterial HspH are affected by C-terminal extension removal [23, 24], indicating that the C-terminal extension is involved in the quaternary structural arrangement of sHsps.

Altering the properties of the C-terminal extension also leads to significant changes to the structure and function of sHsps. The chaperone activity of Hsp30C is impaired when the polarity of the C-terminal extension is reduced [25], and introduction of hydrophobicity into the C-terminal extension of $\alpha$A-crystallin results in immobilization of the C-terminal extension and reduced chaperone activity [21]. Conversely, increasing the charge of the extension of $\alpha$A-crystallin results in no significant changes in chaperone activity compared to wild type $\alpha$A-crystallin [26, 27], highlighting the importance of the polar residues in the C-terminal extension of sHsps.
The thermostability of proteins from thermophilic organisms is related to electrostatic interactions through the presence of polar and charged groups as well as hydrophobic and packing effects [28]. These proteins typically have a higher proportion of polar and charged residues, primarily glutamic acid and lysine, than their mesophilic equivalents [29]. Interactions between αB-crystallin subunits can be inhibited by the replacement of glutamic acid residues in the α-crystallin domain with other residues, possibly through decreased electrostatic interactions and increased electrostatic repulsion [30]. Similarly, it is likely that the glutamic acid residues in the C-terminal extension of Hsp25 are important for electrostatic interactions with the solvent and potentially other regions of the sHsp.

Whilst some studies have examined the role of residues in the C-terminal extensions of other sHsps, notably αA- and αB-crystallin, the flexible regions of these proteins are unique and distinct from Hsp25. The α-crystallins contain negatively-charged residues only near the anchor point of the flexible region to the domain core whilst Hsp25 has glutamic acid residues spaced along its flexible extension. Investigation into the function of these uniquely-positioned residues in Hsp25 has not previously been performed.

Site-directed mutagenesis has been used in this study to produce Hsp25 alanine substitution mutants of the glutamic acid residues in the C-terminal extension, i.e. E190A, E199A and E204A. An additional mutant, Q194A, was also prepared and included as a control. The role of the C-terminal extension and each of the mutated residues was investigated by comparison of the structure and function of these mutants with wild type Hsp25. The glutamic acid residue mutants displayed altered structure and impaired thermostability and chaperone activity compared with the wild type protein, highlighting the importance of the negatively-charged glutamic acid residues in the C-terminal extension of Hsp25.

Results

Sequence Analysis of the C-Terminal Extension of Mammalian sHsps

The C-terminal extensions of mammalian sHsps are highly variable in length and sequence yet they share the characteristics of being polar, flexible and unstructured, suggesting that the types of residues present in the C-terminal extension are important rather than their sequence. This was investigated by analyzing the amino acid residues corresponding to the known flexible regions of αA- and αB-crystallin and Hsp25 [16, 26, 31] (Figure 1). Proline is present in six of the eight human sHsps that contain a flexible region (Figure 1, Table 1) and seven of the corresponding murine sHsps (not shown) and is a predominant residue in the flexible regions of both human and murine sHsps. The majority of residues present in the flexible regions of the extensions (71% and 74% for human and murine, respectively) are those that have been shown to promote disorder
(Table 1) [32]. Whilst the C-terminal extension of human Hsp27 contains an aspartic acid residue, that of murine Hsp25 does not (Figure 1). Thus, apart from the C-terminal carboxyl group, the three glutamic acid residues are the only source of negative charge in the flexible extension of Hsp25.

**Expression and Purification of Wild type and Mutant Hsp25 Proteins**

Hsp25 mutants were designed to investigate the importance of the negatively-charged residues in the C-terminal extension. Wild type Hsp25 and the Q194A and glutamic acid residue mutants were purified successfully, as confirmed by their correct masses being obtained by ESI-MS (not shown).

**Circular Dichroism Spectroscopy of Wild type and Mutant Hsp25**

Far-UV circular dichroism (CD) spectroscopy was performed to determine if substitution of the glutamine or glutamic acid residues resulted in any alteration to the overall secondary structure of Hsp25. A broad minimum at 217 nm was observed for all spectra (Figure 2, Table 2), indicative of the predominance of β-sheet structure [33]. The estimation of secondary structure content obtained by deconvolution of the spectra was consistent with previous measurements [20], with wild type Hsp25 having secondary structure contents of 38% β-sheet and 6% α-helix at 25°C. A slight increase in negative ellipticity and flattening of the spectra were observed for wild type and mutant Hsp25 samples with increasing temperature from 25 to 55°C. The increase in negative ellipticity at around 210 nm implies an increase in α-helical content [33]. However, following deconvolution of the spectra, changes to each of the structural element proportions were less than 5% between all spectra and were deemed to be insignificant [34]. The overall increase in negative ellipticity at higher temperatures is consistent with a slight increase in or stabilization of secondary structure [35]. In comparing the mutants with wild type Hsp25, the consistency of the deconvolution data suggests it is unlikely that secondary structure is altered significantly as a result of the mutations, i.e. there was little difference in overall secondary structure between the wild type and mutant proteins.

**Tryptophan Fluorescence Spectroscopy of Wild type and Mutant Hsp25**

Tryptophan fluorescence depends strongly on the local environment of the amino acid and is a sensitive probe of conformation in the vicinity of tryptophan residues [36]. Fluorescence spectroscopy was performed on wild type and mutant Hsp25 to detect any changes in the environment of the tryptophan residues resulting from the mutations. The tryptophan residues of Hsp25 are located in the N-terminal domain at positions 16, 22, 43, 46 and 52 and in the α-crystallin domain at position 99. A fluorescence maximum (F_{max}) of approximately 4,700 arbitrary units with a wavelength at maximum fluorescence (λ_{max}) of 340.2 nm was observed for
wild type Hsp25 (not shown). No shift in $\lambda_{\text{max}}$ was observed for the mutants. A shift in $\lambda_{\text{max}}$ is indicative of a change in the polarity of the tryptophan environment [37]. Therefore, the overall tryptophan environment was not significantly affected by substitution of the glutamic acid residues in the C-terminal extension, which are all distant in primary structure from the tryptophan residues.

**ANS Binding Fluorescence Spectroscopy of Wild type and Mutant Hsp25**

The binding of 1-anilino-8-naphthalene sulphonate (ANS) and other hydrophobic probes to a protein enables the comparative determination of the exposed clustered hydrophobicity of the protein and, if altered, indicates a perturbation in tertiary structure [38]. Such probes bind non-covalently to regions on proteins that have exposed clusters of hydrophobic aminoacyl residues, resulting in an increase in fluorescence [39]. ANS binding fluorescence of wild type and mutant Hsp25 reached a maximum at a final concentration of 85 $\mu$M ANS and fluorescence values presented (Figure 3, Table 2) are the means of the plateau region of the ANS binding curves (75-95 $\mu$M) (not shown). Wild type Hsp25 resulted in ANS binding fluorescence of approximately 570 arbitrary units. The Q194A, E190A, E199A and E204A mutants exhibited increases in ANS binding fluorescence of approximately 27, 45, 53 and 63%, respectively, compared to the wild type protein, indicating that all of the mutants have greater clustered hydrophobicity exposed to the solvent and thus altered tertiary structure.

**Oligomer Formation by Wild type and Mutant Hsp25**

Size-exclusion chromatography (SEC) was performed in order to determine whether the glutamic acid substitutions affected the oligomeric size of Hsp25. Wild type Hsp25 eluted between the molecular weight markers thyroglobulin (mass of 669 kDa) and apoferritin (mass of 443 kDa) (Figure 4, Table 2) with an average molecular mass of 613±185 kDa, as calculated from the standard curve (not shown), corresponding to an average oligomer of 26-27 subunits. The peak maxima of the Q194A, E199A and E204A mutants eluted at volumes almost identical to that of the wild type, indicating very similar average oligomeric sizes to the wild type. The small extra peak in the elution profile of E199A represents protein less than 250 kDa in mass. The oligomeric species of wild type Hsp25 is in equilibrium with a tetrameric form [40], so the smaller species may be a tetramer. Elution of the E190A mutant was delayed slightly compared with the wild type protein, with the elution peak corresponding to a calculated average molecular mass approximately 53 kDa smaller than wild type Hsp25, corresponding to an average oligomer of 24-25 subunits. Thus, with the exception of the E190A mutant, the oligomeric size of Hsp25 was not affected by the glutamine or glutamic acid residue mutations.
Thermostability Studies of Wild type and Mutant Hsp25
The thermostability of wild type and mutant Hsp25 was investigated by monitoring the increase in light scattering at 360 nm as a result of formation of large aggregates followed by precipitation with increasing temperature. Wild type Hsp25 was very heat stable and remained in solution up to temperatures of 100°C (Figure 5, Table 2). No precipitate was observed and the small increase in light scattering at temperatures above approximately 70°C is consistent with an increase in aggregate size [41]. The Q194A mutant showed a light scattering profile comparable to the wild type protein. In marked contrast, the glutamic acid residue mutants all precipitated out of solution within 2°C of the onset of aggregation, i.e. at approximately 68°C for E190A and 70°C for E199A and E204A. Decreased light scattering after maximum precipitation was reached resulted from the precipitate sinking to the bottom of the cuvette and therefore not obscuring the lightpath [42]. Thus, the Q194A mutant showed thermostability similar to that of wild type Hsp25 whilst the glutamic acid residue mutants exhibited significantly decreased thermostability.

Functional Chaperone Activity Assays of Wild type and Mutant Hsp25
The chaperone activity of wild type and mutant Hsp25 was assessed by determining the ability of these proteins to prevent the amorphous aggregation and precipitation of target proteins under stress conditions. Assays were performed with ADH under heat stress and insulin under reduction stress in the presence of varying concentrations of Hsp25.

Yeast ADH is a tetramer of four equal subunits with a total molecular mass of 141 kDa [43]. Thermal stress assays using this enzyme are commonly performed at temperatures of 48-60°C. The optimal rate of precipitation of yeast ADH for monitoring precipitation was found to be 55°C (not shown) and the inactivation and precipitation of yeast ADH at this temperature has been well characterized [44]. This temperature was also well below the onset of aggregation and precipitation for wild type and mutant Hsp25 proteins, as shown by the thermostability studies. Any precipitation observed was therefore not attributable to Hsp25 instability.

Complete suppression [45] of yeast ADH precipitation was observed for wild type Hsp25 at a molar ratio of 1.4:1.0 Hsp25:ADH (Figure 6, Table 2). A reduction in suppression of ADH precipitation was observed at this ratio for all of the glutamic acid residue mutants. At all other ratios, the E199A mutant showed minor reductions in suppression of ADH precipitation compared to wild type Hsp25, whilst the E190A and E204A mutants showed markedly reduced suppression. The Q194A mutant showed similar levels of suppression of aggregation to the wild type protein at all ratios.
Precipitation of insulin can be initiated by the addition of a reducing agent, such as dithiothreitol (DTT), which cleaves the disulphide bonds between the A and B chain of insulin, resulting in the aggregation and precipitation of the B chain. Reduction stress assays are advantageous over thermal stress assays as they can be performed at physiological temperatures, i.e. 37°C. The precipitation of insulin under reducing stress was completely suppressed by wild type Hsp25 at a molar ratio of 0.5:1.0 Hsp25:insulin (Figure 7, Table 2), with all mutants showing comparable suppression of insulin precipitation at this ratio. All of the glutamic acid residue mutants, in particular the E204A mutant, displayed reduced suppression at the lower ratio (0.05:1.0) and the E190A mutant showed reduced suppression at 0.25:1.0. At all ratios, the Q194A mutant exhibited very similar levels of suppression of insulin B chain precipitation to the wild type protein. Taken together, the thermal and reduction stress assays demonstrate that each of the glutamic acid mutants, in particular E190A and E204A, are significantly less effective chaperones than wild type Hsp25.

**Discussion**

Many proteins contain intrinsically-disordered regions that are necessary for their function [46]. Accordingly, these regions have a higher frequency of disorder-promoting residues [32, 47]. Such is the case for the flexible regions located at the extremity of the C-terminal extensions of human and murine sHsps. Despite low sequence similarity, these regions are abundant in disorder-promoting residues, such as proline [48]. The lack of structure and the associated flexibility is essential for the hydrophilicity responsible for the solubilizing role of the sHsp and complexes it makes with target proteins and for potential interactions. The unstructured and highly dynamic nature of this flexible region also ensures that it does not interfere with or block the preceding conserved IXI motif, which is important in subunit-subunit interactions [49].

Despite low sequence similarity throughout the sHsp family, Hsp25 has a very similar, predominately β-sheet, secondary structure to that of other sHsps, including mammalian αA- and αB-crystallin and bacterial IpBb [50, 51]. The glutamic acid residue substitutions did not affect the secondary structure of Hsp25, indicating that these residues, which are part of an unstructured region, are not important for the determination of this level of structure. In support of this conclusion, mutants of αA- and αB-crystallin in which the C-terminal extensions were swapped have been shown to have secondary structure similar to each other and their wild type counterparts [52]. Similarly, the removal of the C-terminal extension of αA-crystallin, αB-crystallin and bacterial Hsp16.3 produced proteins with secondary structure comparable to the respective wild type proteins [17, 53, 54] The secondary structure of wild type and mutant Hsp25 did not change significantly from 25 to 55°C, which is consistent with previous findings that Hsp25, α-crystallin and IpBb resist changes to secondary structure with increasing temperatures up to approximately
60°C [50, 55, 56]. The secondary structure of Hsp25 has also been shown to be stable under mildly denaturing conditions [40], and temperatures of at least 60°C are required for a loss of β-sheet structure [40].

Whilst the secondary structure of Hsp25 was not altered as a result of the mutations, significant differences in exposed hydrophobicity, as indicated by ANS binding, were observed, suggesting that the same elements of secondary structure were adopted but that the subunits were arranged differently than in the wild type protein. Because five of the six tryptophan residues in Hsp25 are located in the N-terminal domain, the comparable overall tryptophan exposure in the mutants compared with the wild type protein indicates that the structure of the N-terminal domain is maintained, at least in the vicinity of the tryptophan residues. The increase in exposed clustered hydrophobicity observed for the mutants is therefore likely to arise from rearrangements of secondary structural elements in the α-crystallin domain.

The slightly reduced oligomeric size of the E190A mutant compared with the wild type suggests that the E190 residue is important for the correct formation of the quaternary structure of Hsp25. Examination of the crystal structures of Hsp16.9 and Hsp16.5, which do not have flexible C-terminal extensions, shows that the conserved IXI motif (residues 185-187 in Hsp25) forms hydrophobic contacts with a groove between β-strands in the α-crystallin domain of another monomer and that this interaction is essential for the oligomerization of sHsps [57]. Truncation from the C-terminus of αA-crystallin to remove the IXI motif renders the protein unable to form oligomers [58], indicating that interactions involving the IXI motif are also essential for the oligomerization of mammalian sHsps containing a flexible C-terminal extension. Because of the proximity between the E190 residue of Hsp25 and the IXI motif, it is possible that substitution of this residue disrupts the interaction between the IXI motif and the hydrophobic groove, resulting in an altered oligomeric structure. The formation of large shSp oligomers is also dependent on interactions between N-terminal domains [23], which are not affected by mutations in the C-terminal extension. The comparability of oligomeric sizes between the mutants and wild type Hsp25 clearly demonstrates this.

In mammalian sHsps, the presence of a flexible, solvent-exposed C-terminal extension helps to counteract the large amount of hydrophobicity exposed by the remainder of the protein [15, 26]. Removal of the C-terminal extension results in a reduction in thermostability of Hsp25, αA-crystallin, frog Hsp30C and nematode Hsp16-2 [17-20]. The drastically reduced thermostability of the glutamic acid residue mutants demonstrates the importance of each of the negatively charged residues in the C-terminal extension in maintaining the solubility of the Hsp25
oligomer. Similarly, introduction of hydrophobicity into the C-terminal extension of αA-crystallin results in a reduction in the thermostability of this sHsp [21]. These data suggest that relatively modest alterations to the C-terminal extension of sHsps resulting in a reduction in polarity are sufficient to disrupt the ability of the C-terminal extensions to efficiently act as solubilizers.

At temperatures above 60°C, Hsp25 and the α-crystallins undergo changes in their tertiary structure that result in exposure of hydrophobic regions [56, 59]. The onset of precipitation of the glutamic acid residue mutants of Hsp25 corresponds approximately to this temperature. The temperature-induced increase in hydrophobic exposure did not induce the precipitation of wild type Hsp25, although a small increase in light scattering implies the formation of larger aggregates [41]. Thermostable proteins display more effective burial of hydrophobic regions than less thermostable proteins [28]. The increase in hydrophobicity associated with the mutations coupled with the temperature-induced increase in hydrophobicity is consistent with the poor thermostability observed for the glutamic acid residue mutants.

The glutamic acid residue mutants showed variably reduced chaperone activity compared with wild type Hsp25 towards target proteins under different assay conditions, a property that has also recently been observed for wild type and mutant forms of αB-crystallin [60, 61]. The E190A and E204A mutants performed poorly compared with the wild type protein in both assays, most notably at the lower Hsp25:target protein ratios used. The E199A mutant showed somewhat decreased chaperone activity toward ADH under heat stress but performed poorly at lower ratios toward insulin under reduction stress. These functional differences were not a result of insolubilization of the chaperone as both wild type Hsp25 and the glutamic acid residue mutants of Hsp25 were stable in solution at the temperatures at which these assays were performed. Recognition of and interaction with target proteins by sHsps is largely hydrophobic in nature [13]. On this basis, it would be expected that the Hsp25 mutants, with increased surface hydrophobicity, would display enhanced chaperone ability compared to the wild type protein [51]. However, the structural changes associated with the mutations appear to have a greater influence on the chaperone activity than simply the degree of exposed hydrophobicity [62].

Whilst conclusive identification of the chaperone binding sites of sHsps remains elusive, there is evidence that binding of target proteins occurs in the groove between monomers and involves a β-sheet region located at the beginning of the α-crystallin domain corresponding to residues 70-88 in αA-crystallin [63, 64]. The changes in tertiary structure observed for the mutants, as evidenced by alteration in exposed hydrophobicity, could result in the disruption of binding sites and thus hindered recognition and sequestration of target proteins. These changes may also inhibit
stabilization by electrostatic interactions, resulting in less effective target protein sequestration. The reduction in polarity of the C-terminal extension may also facilitate interaction between the extension and hydrophobic chaperone binding sites, resulting in the binding sites being less accessible to the target protein, leading to a reduction in target protein binding [26].

In summary, the three negatively charged glutamic acid residues in the C-terminal extension of Hsp25 (E190, E199 and E204) are essential for the correct structure and function of this sHsp. These residues contribute to the polarity of the extension and promote its disorder, ensuring that the C-terminal extension remains unstructured and solvent-exposed and therefore able to perform its solubilizing role in the sHsp and the complexes formed with target proteins during chaperone action. Indeed, the presence of significant regions of structural disorder is a common characteristic of molecular chaperones and is integral to their effective chaperone action [65]. Despite an alteration in exposed hydrophobicity, the Q194A mutant had comparable oligomeric size and functional properties to wild type Hsp25. Thus, residues in the flexible region of the C-terminal extension are not equally important for Hsp25 to perform its role as a molecular chaperone, emphasizing the importance of the glutamic acid residues.

**Experimental procedures**

**Sequence Analysis of the C-Terminal Extension of Mammalian sHsps**

The C-terminal extensions of the ten human sHsps [66] were aligned according to their IXI motifs, where present. Where absent, the alignments were based on those of Fontaine et al. [67] and Franck et al. [68]. Residues that aligned with the known flexible regions of α-A- and α-B-crystallin [16] were tallied. The C-terminal extensions of the equivalent murine sHsps were similarly analyzed.

**Site-Directed Mutagenesis of pAK3038-Hsp25**

Site-directed mutagenesis was performed using the QuikChange® system (Stratagene, La Jolla, U.S.A.) according to the manufacturer's instructions except that 14 cycles were used (Cooled-Palm 96, Corbett Research, Mortlake, Australia). All primers were synthesized by Sigma Genosys (Castle Hill, Australia). Primers pairs for site-directed mutagenesis were: 5'-TTC GAG GCC CGC GCC GCA ATT GGG GCC CCA GAA-3' and 5'-TTC TGG GCC CCC AAT TGC GGC GCG GGC CTC GAA-3' for E190A, 5'-ATT CGG GTT ACT TTC GCC GCC CGC GCC CAA ATT-3' and 5'-AAT TTG GGC GCG GGC CGC GAA AGT AAC CGG AAT-3' for E190A, 5'-CAA ATT GGG GGC CCA GCA GCT GGG AAG TCT GAA-3' and 5'-TTC AGA CTT CCC AGC TGC TGG GCC CCC AAT TTG-3' for E199A and 5'-GAA GCT GGG AAG TCT GCA CAG TCT GGA GCC AAG-3' and 5'-CTT GGC TCC AGA CTG TGC AGA CTT CCC AGC TTC-3' for E204A. Mutated codons
are underlined. Dimethylsulphoxide was added to a final concentration of 5% (v/v) to reactions where strong secondary interactions were likely as advised by the supplier. Successful mutagenesis was confirmed by DNA sequence analysis of the forward and reverse strands with BigDye™ Terminator Ready Reaction Mix (Applied Biosystems, Foster City, U.S.A.) on a Prism 377 DNA sequencer (Applied Biosystems, Foster City, U.S.A.) using the primers 5'-TCTCGGAGATCCGACAGA-3' and 5'-CTTTCGGGCTTTGTTAGCAG-3', respectively.

Expression and Purification of Wild type and Mutant Hsp25
pAK3038-Hsp25 was a gift from Prof. M. Gaestel (Institute of Biochemistry, Hannover, Germany). DNA was transformed into electrically competent BL21(DE3) E. coli before expression. Expression and purification of murine Hsp25 and mutants were performed according to the method described by Horwitz et al. [69] with minor changes. Transformed cells were grown in Luria-Bertani medium containing 0.4% (w/v) glucose and 100 µg/mL ampicillin to select for pAK3038-Hsp25. Protein expression was induced with 0.4 mM isopropyl-β-D-thiogalactoside. Cells were harvested by centrifugation and lysed as described. After ultracentrifugation, dithiothreitol (DTT), polyethyleneimine and ethylenediaminetetraacetic acid were added to the supernatant to final concentrations of 10 mM, 0.12% (v/v) and 1 mM, respectively, and the lysate was incubated and centrifuged as described. The final supernatant was filtered through a 0.22 µm Minisart filter (Sartorius, Epsom, U.K.) before being loaded onto a DEAE-Sephacel (Sigma-Aldrich, St Louis, U.S.A.) column with volume of ~90 mL. Recombinant Hsp25 was eluted with 100 mM NaCl in 20 mM Tris-HCl buffer (pH 8.5) containing 1 mM EDTA and 0.02% (w/v) NaN₃. Fractions containing Hsp25 were concentrated to ~5 mL and DTT was added to a final concentration of 50 mM. The sample was incubated at room temperature for 30 min before being loaded onto a Sephacryl S-300HR (Pharmacia, Uppsala, Sweden) column with a volume of ~470 mL. Recombinant Hsp25 eluted in the first peak with 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 0.02% (w/v) NaN₃. Fractions containing Hsp25 were concentrated, dialyzed exhaustively against, or exchanged into, MilliQ water and lyophilized. Both chromatography steps were performed at 4°C. The purity of recombinant proteins was confirmed by nanoscale electrospray ionization mass spectrometry (nanoESI-MS).

Far-UV Circular Dichroism (CD) Spectroscopy
CD spectra were acquired on a J-810 spectropolarimeter (Jasco, Tokyo, Japan) with an attached Peltier temperature-controlled water circulator. Samples were prepared in 10 mM phosphate buffer (pH 7.5) to a final concentration of 10-15 µM and filtered through a 0.22 µm Minisart filter. Spectra were recorded at 25, 37 and 55°C and are accumulations of 16 scans recorded from 190 to 250 nm with a pathlength of 1 mm. Sample concentration was determined using a bicinchoninic
acid assay (Sigma-Aldrich, St Louis, Missouri, USA). An estimation of secondary structure composition was performed using the CDSSTR program [70-72] in the DICHROWEB Online Circular Dichroism Analysis suite [73, 74].

**Intrinsic Tryptophan Fluorescence and ANS Binding Fluorescence Spectroscopy**
All fluorescence studies were performed at 25°C using a F-4500 Fluorescence Spectrophotometer (Hitachi High-Technologies, Tokyo, Japan) with a Thermomix temperature-controlled water circulator (B. Braun, Melsungen, Germany). Samples were prepared in 50 mM phosphate buffer (pH 7.3) containing 0.02% (w/v) NaN₃ to a final concentration of 5 µM as calculated from A₂₈₀ values of the samples, an extinction co-efficient of 1.87 for a 1 mg/mL solution of Hsp25 [75] and molecular mass. An excitation wavelength of 295 nm was used for intrinsic fluorescence, with emission spectra recorded from 300 to 450 nm. ANS binding fluorescence was performed with an excitation wavelength of 387 nm. Sequential aliquots of freshly prepared ANS were added to the samples and mixed thoroughly until the emission fluorescence at 479 nm reached a maximum. Emission spectra were then recorded from 400 to 650 nm.

**Size-Exclusion Chromatography**
SEC was performed at room temperature on a BioSep-SEC-S 4000 column (Phenomenex®, Torrance, U.S.A.) connected to an ÄKTA™FPLC™ system (Amersham Biosciences, Buckinghamshire, U.K.). Samples were prepared to a final concentration of 30 µM, as determined by A₂₈₀ values, with 100 µL being loaded onto the column. Protein was eluted at 0.5 mL/min with 50 mM phosphate buffer (pH 7.3) containing 0.02% (w/v) NaN₃ and detected at 280 nm. The column was calibrated with blue dextran (2 MDa), thyroglobulin (669 kDa), apoferritin (443 kDa) and catalase (250 kDa). Standard deviations are given as the oligomeric range at half peak height [69].

**Thermostability Studies**
Hsp25 solutions were prepared at 0.2 mg/mL, as determined by A₂₈₀ values, in 50 mM phosphate buffer (pH 7.3) containing 0.02% (w/v) NaN₃. Samples were heated from 25 to 100°C at 1°C/min in 1 mL quartz cuvettes with a 1 cm pathlength in a Cary-500 Scan UV-Vis-NIR spectrophotometer (Varian, Palo Alto, U.S.A.) with a Cary temperature controller. Protein precipitation was monitored by light scattering at 360 nm.

**Chaperone Activity Assays**
Thermally-induced precipitation of 2 µM yeast alcohol dehydrogenase (ADH) in the presence of increasing concentrations of Hsp25 was performed at 55°C in a Hewlett Packard Diode Array
UV-Vis spectrophotometer (Agilent Technologies, Forest Hill, Australia) connected to a Thermomix temperature controlled water circulator (B. Braun, Melsungen, Germany). Samples were prepared in 50 mM phosphate buffer (pH 7.3) containing 0.02% (w/v) NaN₃. Protein precipitation was monitored by light scattering at 360 nm. Assays were performed in duplicate. The precipitation of 45 µM insulin from bovine pancreas in the presence of increasing concentrations of Hsp25 was performed at 37°C in a 96 well plate in a FLUOstar OPTIMA plate reader (BMG LABTECH GmbH, Offenburg, Germany). Samples were prepared in 50 mM phosphate buffer (pH 7.3) containing 0.02% (w/v) NaN₃. Precipitation was initiated with the addition of DTT to a final concentration of 20 mM and monitored by light scattering at 360 nm. Assays were performed in triplicate.

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References


Table 1. Frequency of amino acid residues in the flexible region of the C-terminal extension of human and murine sHsps.

Residues present in the flexible region of the C-terminal extension of each of the eight human sHsps that contain a flexible region (underlined residues in Figure 1) are tallied. Only the totals are given for murine sHsps. Residues that promote disorder are shaded and those that promote order are underlined [32]. Residues are denoted as charged (+ or −), polar (p) or nonpolar (n).

| Residue: | P | A | E | K | S | T | C | R | L | D | Q | G | V | N | Y | I | M | F | W | H |
| Charge:  | n | n | – | + | p | p | p | + | n | – | p | n | n | p | n | n | n | n | + |
| Hsp27    | 1 | 5 | 3 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 2 |
| HspB2    | 1 | 3 | 6 | 1 | 1 | 1 | 1 | 1 |
| αA-crys. | 2 | 1 | 2 | 1 | 3 | 1 |
| αB-crys. | 2 | 3 | 2 | 3 | 1 |
| Hsp20    | 3 | 4 | 1 | 1 |
| Hsp22    | 1 | 2 | 1 | 1 | 1 |
| HspB9    | 1 | 1 | 1 | 1 |
| ODFP     | 4 | 1 | 2 | 4 | 2 | 1 | 1 | 1 | 2 | 1 | 1 |
| Human total | 17 | 16 | 14 | 8 | 7 | 6 | 5 | 4 | 4 | 3 | 3 | 3 | 3 | 2 | 2 | 2 | 2 | 1 | 1 |
| Murine total | 18 | 13 | 14 | 9 | 10 | 1 | 6 | 4 | 3 | 2 | 4 | 5 | 4 | 3 | 1 | 2 | 1 | 1 | 0 | 0 |
Table 2. Summary of changes in structure and function of C-terminal Hsp25 mutants. Comparisons are made with wild type Hsp25. Qualitative comparisons are given for exposed clustered hydrophobicity, thermostability and chaperone activity. ∆C18, Hsp25 truncation mutant lacking the C-terminal 18 residues; ND, not determined.

<table>
<thead>
<tr>
<th>Hsp25 Mutant</th>
<th>Charge</th>
<th>Secondary structure</th>
<th>Exposed clustered hydrophobicity</th>
<th>Average molecular mass</th>
<th>Thermostability</th>
<th>Chaperone activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q194A</td>
<td>No change</td>
<td>No change</td>
<td>27% increase</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
<td>This study</td>
</tr>
<tr>
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<td>53 kDa smaller</td>
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<td>Decreased</td>
<td>This study</td>
</tr>
<tr>
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<td>54% increase</td>
<td>No change</td>
<td>Poor</td>
<td>Decreased</td>
<td>This study</td>
</tr>
<tr>
<td>E204A</td>
<td>+1</td>
<td>No change</td>
<td>65% increase</td>
<td>No change</td>
<td>Poor</td>
<td>Decreased</td>
<td>This study</td>
</tr>
<tr>
<td>∆C18</td>
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<td>Increased α-helix</td>
<td>31% decrease</td>
<td>No change</td>
<td>ND</td>
<td>Decreased</td>
<td>[20]</td>
</tr>
</tbody>
</table>
Figure legends

Figure 1. C-terminal extension sequences of murine Hsp25 and the ten human sHsps aligned at their IXI motifs [67, 68]. The IXI motifs are shown in italics and the known flexible regions of various sHsps as determined by NMR spectroscopy [26, 31, 76] are in bold. Residues used in the tally for Table 1 are underlined. Accession numbers were: Hsp27 (P04792), MKBP (Q16082) αA-crystallin (P02489), αB-crystallin (P02511), Hsp20 (Q14558), HspB7 (Q9UBY9), Hsp22 (Q9UJY1), HspB9 (Q9BQS6) and ODFP (Q14990).

Figure 2. Far-UV CD spectra of wildtype (------), Q194A (----), E190A (-----), E199A (-----) and E204A (-----) Hsp25 at 25°C, 37°C and 55°C in 10 mM sodium phosphate buffer (pH 7.5). No significant differences in secondary structure between wild type and mutant proteins were observed at any of the three temperatures.

Figure 3. ANS binding fluorescence emission spectra of wildtype (------), Q194A (----), E190A (-----), E199A (-----) and E204A (-----) Hsp25 and buffer (-----). Experiments were performed at 25°C with 85 µM ANS and an excitation wavelength of 387 nm. Samples were prepared to a final concentration of 5 µM in 50 mM sodium phosphate buffer (pH 7.3) containing 0.02% NaN₃. Increases in maximum fluorescence of 27, 45, 53 and 63% were observed for the Q194A, E190A, E199A and E204A mutants, respectively, in comparison to wild type Hsp25.

Figure 4. SEC FPLC of wildtype (------), Q194A (----), E190A (-----), E199A (-----) and E204A (-----) Hsp25. Samples were prepared to a final concentration of 30 µM in 50 mM sodium phosphate buffer (pH 7.3) containing 0.02% NaN₃ with 100 µL being loaded onto the column. The peak positions of the elution of molecular standards are indicated at the top of the graph. The elution of wild type Hsp25 corresponds to an average molecular mass of 613 kDa. No significant differences in mass were observed for the Q194A, E199A or E204A mutants. The E190A mutant eluted at a volume corresponding to an average molecular mass of 560 kDa.

Figure 5. Thermostability profiles of wildtype (------), Q194A (----), E190A (-----), E199A (-----) and E204A (-----) Hsp25. Samples were prepared to a final concentration of 0.2 mg/mL in 50 mM sodium phosphate buffer (pH 7.3). Temperature was increased at a rate of 1°C/min. Wild type Hsp25 and the Q194A remained in solution up to temperatures of 100°C. By contrast, the E190A, E199A and E204A mutants precipitated out of solution within 2°C of the onset of precipitation at 68°C for E190A and 70°C for E199A and E204A.

Figure 6. Chaperone activity of wildtype and mutant Hsp25 as measured by the suppression of precipitation of ADH under thermal stress. Ratios represent the molar concentration of Hsp25.
monomers to ADH subunits. Assays were performed at 55°C in sodium phosphate buffer (pH 7.3) containing 0.02% NaN₃. Traces are the average of duplicates. The precipitation of ADH was completely suppressed at a Hsp25:ADH ratio of 1.4:1.0. The Q194A mutant showed comparable chaperone activity to the wild type protein. Each of the glutamic acid residue mutants displayed a decrease in chaperone activity compared to wild type Hsp25.

**Figure 7.** Chaperone activity of wildtype and mutant Hsp25 as measured by the suppression of precipitation of insulin under reducing stress. Ratios represent the molar concentration of Hsp25 monomers to insulin molecules. Assays were performed at 37°C in sodium phosphate buffer (pH 7.3) containing 0.02% NaN₃. Traces are the average of triplicates. The precipitation of insulin was completely suppressed at a Hsp25:insulin ratio of 0.5:1.0. The Q194A mutant showed comparable chaperone activity to the wild type protein. Each of the glutamic acid residue mutants displayed a decrease in chaperone activity compared to wild type Hsp25.
Figures

Figure 1

Murine Hsp25  IPVTFEARaqigpeagkSEQGAK<sup>209</sup>
Hsp27 (HspB1)  IPVTFSRaqlgpeaaksDetaak
MKBP (HspB2)  VYISLPPAPDPEEEEEEAAIVEP
HspB3  -
αA-crystallin (HspB4)  IPVSR<sub>EEKPTSAPSS</sub>
αB-crystallin (HspB5)  IPITR<sub>EEKPFAVTAAPKK</sub>
Hsp20 (HspB6)  AFA<sub>S</sub>AQAPPPAAAk
HspB7  IKI
Hsp22 (HspB8)  LpqDSqEvTCT
HspB9  GSKASNLTR
ODFP (HspB10)  CNPCSPYDPCNPCyPCGSRFSCRRMIL

Figure 2

![Graphs showing temperature effects on protein structure](image)

Figure 3

![Graph showing fluorescence vs wavelength](image)