Proteolytic mapping of heat shock transcription factor domains

MIN ZHONG¹ AND CARL WU¹

Laboratory of Biochemistry, National Cancer Institute, Bethesda, Maryland 20892-4255

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Abstract

Heat shock transcription factors (HSFs) of higher eukaryotes respond to physical and cellular stress signals by trimerizing, binding to a specific site on DNA, and transactivating genes encoding the heat shock proteins. In this work, limited proteolysis was used as a biochemical probe of the domain organization of *Drosophila* HSF. Both unshocked monomeric and heat-shocked trimeric HSF possess an internal protease-sensitive region located between the amino-terminal and carboxyl-terminal hydrophobic heptad repeats, suggesting that this is a less structured region compared to those defined for DNA-binding, trimerization, and transactivation. For a few cleavage sites, the heat-shocked form of HSF is more accessible to proteases than the unshocked form, providing an additional diagnostic marker for inducible changes in conformation or modification between the latent and activated forms of HSF.

Keywords: heat shock factor; protease mapping; protein conformation

The regulation and function of heat shock proteins are of interest because of the important roles of these proteins for cell survival under stress conditions and their expression associated with pathophysiological states (for a review, see Morimoto et al., 1994). In eukaryotes, transcriptional regulation of heat shock genes is mediated by a preexisting transcription activator (heat shock factor, HSF), which binds to an upstream conserved DNA recognition sequence (the heat shock element, HSE), leading to an increase in the synthesis of heat shock proteins (For reviews, see Sorger, 1991; Lis & Wu, 1993; Morimoto, 1993; Scharf et al., 1994; Wu, 1995).

HSF genes have been cloned from a number of organisms. The predicted protein sequences vary in size, from 301 residues for tomato HSF24 to 833 residues for *Saccharomyces cerevisiae*. All HSFs contain two highly conserved regions: a DNA binding domain at the amino terminus and an adjacent trimerization domain with clusters of hydrophobic heptad repeats (HR-A/B) separated by a short spacer (Sorger & Nelson, 1989; Wu, 1995). The DNA binding domain of HSF forms a compact structure consisting of a three-helix bundle capped by a four-stranded antiparallel β -sheet, resembling the structure of the helix-turn-helix class of DNA binding proteins (Harrison et al., 1994; Vuister et al., 1994a, 1994b). The trimerization domain has been proposed to form a parallel triple-stranded, α -helical coiled-coil structure (Sorger & Nelson, 1989; Clos et al., 1990; Peteranderl & Nelson, 1992). Another conserved hydrophobic heptad repeat region with the potential to

form a coiled-coil structure is found at the carboxyl terminus of HSF in higher eukaryotic cells (Clos et al., 1990; Rabindran et al., 1991). Deletion mapping and mutagenesis studies suggest that the carboxyl-terminal heptad repeat (HR-C) may be a part of a larger domain involved in negative regulation of oligomerization, possibly by means of an intramolecular coiled-coil interaction between amino-terminal and carboxyl-terminal heptad repeats (Rabindran et al., 1993; Sarge et al., 1993; Zuo et al., 1994). In contrast to the amino-terminal DNA-binding and trimerization domains, the domain responsible for transactivation is generally located in the carboxyl-terminal region of HSF and has not been conserved widely in evolution (Treuter et al., 1993). The transactivation domain is subject to negative control by other parts of HSF under normal environmental conditions (Green et al., 1995; Shi et al., 1995; Wisniewski et al., 1996).

The activation of HSF generally involves at least two steps: DNA binding and transactivation. Stress-inducible high-affinity binding is accomplished by a monomer to trimer transition of the protein (Sorger & Nelson, 1989; Sorger, 1990; Baler et al., 1993; Sarge et al., 1993; Westwood & Wu, 1993). Trimerization increases the binding affinity of HSF for the HSE by more than 1,000-fold (Kim et al., 1994). It is known that HSF is a phosphoprotein and undergoes hyperphosphorylation after heat stress (Sorger et al., 1987; Larson et al., 1988; Sorger & Pelham, 1988; Sarge et al., 1993), although the functional significance of these modifications is not clear and the positions of phosphorylation have not been mapped completely (Cotto et al., 1996). On the other hand, the heat shock proteins themselves have been proposed to function as suppressors of HSF activity via an autoregulatory loop (for a review, see Morimoto, 1993). Further experiments are awaited to support this model, however.

Reprint requests to: Carl Wu, NIH Building 37, Room 4C09, Bethesda, Maryland 20892; e-mail: carlwu@helix.nih.gov.

¹Present address: Laboratory of Molecular Cell Biology, National Cancer Institute, Bethesda, Maryland 20892-4255.

In order to help elucidate the native molecular structure of HSF, we have applied limited protease digestion to map the structural domains of *Drosophila* heat shock transcription factor. Limited proteolysis has been used successfully to identify functional protein domains (Smith et al., 1984; Marzouki et al., 1986; Lieberman et al., 1991; Konigsberg, 1995), to detect conformational transitions upon ligand binding (Shuman et al., 1990; Tan & Richmond, 1990; Lindsley & Wang, 1991; Allan et al., 1992), and to probe protein topography as well as footprint macromolecular interactions (Zhong et al., 1995). Comparison of the cleavage patterns between unshocked latent HSF and heat-shocked active protein provides further information about possible differences in conformation, posttranslational modifications, or both, between these two states of HSF.

Results

Figure 1 outlines the experimental strategy. Partially purified *Drosophila* HSF (dHSF) was subjected to limited proteolysis. The

Fig. 1. Outline of the experimental strategy. Heat-shocked and unshocked *Drosophila* HSFs were partially purified from Schneider line 2 (SL2) cells and subjected to limited digestion by proteases. Cleavage products were analyzed by Tricine-SDS-PAGE and western blotting using antibodies to a HSF C-terminal peptide.

digestion products were resolved on a high-resolution Tricine/SDS polyacrylamide gel (Schägger & von Jagow, 1987), transferred to PVDF membranes, and analyzed by western blotting using polyclonal antibodies specific for a carboxyl-terminal peptide of HSF (676-QMQTQSSVLNTPRHEL-691). By comparing the relative mobility of the visualized cleavage products with those of molecular weight standards, the positions of the cleavage sites can be mapped precisely.

The DNA binding and oligomeric state of the dHSF preparations used for proteolytic mapping were confirmed initially by gel mobility shift assay and gel filtration chromatography (Fig. 2). As expected based on published studies, the specific DNA binding activity of unshocked dHSF is much less (undetectable) than that of dHSF from heat-shocked samples (Fig. 2A). On a Superose 12 column, unshocked HSF had an apparent molecular weight corresponding to the monomeric form, whereas that from the heat-shocked sample migrated as a trimer, consistent with previous results using crude cell extracts (Westwood & Wu, 1993). Together, these biochemical data suggest that the oligomeric states of both unshocked and heat-shocked *Drosophila* HSFs remain intact after partial purification.

Limited proteolysis by endoproteinase Glu-C

Figure 3 shows a limited digestion pattern of unshocked and heat-shocked dHSF by endoproteinase Glu-C as revealed by western blotting. In general, the C-terminal peptide antibody displays high specificity for HSF in the partially purified preparations; however, because of the high sensitivity required for detection of the products of limited proteolysis, some cross-reacting protein species or

Fig. 2. **A:** Gel mobility shift assay of partially purified unshocked and heat-shocked *Drosophila* HSF. In contrast to the heat-shocked sample, the unshocked HSF lacks specific DNA binding. **B:** Gel filtration analysis of unshocked and heat-shocked *Drosophila* HSF. Thyroglobulin (670 kDa) and γ -globulin (158 kDa) were used as molecular weight standards and run on parallel chromatograms. Unshocked HSF has a significantly smaller apparent molecular weight than that of the heat-shocked sample on the Superose 12 column, because HSF forms trimers upon heat shock exposure (Westwood & Wu, 1993).

Fig. 3. Endoproteinase Glu-C digestion of unshocked and heat shocked *Drosophila* HSF. An autoradiogram of a 10% Tricine-SDS-PAGE/western blot is shown. Partially purified unshocked and heat shocked HSFs were exposed to Glu-C, and the cleavage products were analyzed as described in the Materials and methods. Ratios of total substrate proteins to protease (w/w) were 6,000 (lane 2), 3,000 (lane 3, 6), 1,500 (lane 4, 7) and 750 (lane 8), respectively. Lanes 1 and 5 were uncut controls. Hypersensitive sites are indicated by arrows and the apparent molecular weights of the peptide fragments are marked in kDa. Strong cleavage at E499 was confirmed by sequencing the particular band from a parallel digestion of recombinant *Drosophila* HSF. Molecular weight markers were run on the same gel and stained by Ponceau S.

products of endogenous protease are revealed on the western blot. This is especially evident for the unshocked HSF, whose purity is significantly lower than that of the DNA-affinity purified heat-shocked form (Fig. 3, lane 1). Therefore, bands smaller than the full-length HSF in the no-protease samples are discounted as cross-reacting proteins or products of endogenous protease if their intensity does not increase after protease digestion. True proteolytic products are indicated by arrows. High ratios of total substrate protein to protease (w/w) and mild conditions (10–15 min, 25 °C) were used to ensure partial digestion or “single-hit” kinetics. In general, the proteolytic reaction was controlled such that at least 80% of HSF remained intact.

For the Glu-C digests, although there are a total of 50 glutamic acid residues in the full-length HSF, the enzyme cleaves preferentially at a position that maps unambiguously to E499 (corresponding to the 25.12-kDa fragment) for both heat-shocked and unshocked HSF. In addition, one weak band (26.47 kDa) and two very weak, but reproducible, bands (38.93 and 41.03 kDa) appear in the heat-shocked HSF sample only. The positions of cleavage sites are summarized in Tables 1 and 2, and in Figure 7. All sites cleaved by endoproteinase Glu-C are located in the region between amino-terminal heptad repeats (HR-A/B) and the heptad repeats near the C-terminus of HSF (HR-C) (Table 2; Fig. 7). The very strong

Table 1. Summary of cleavage products of *Drosophila* HSFs by limited proteolysis

Protease	MW of peptide (kDa)	No. of residues ^a	Calculated cleavage position ^b	Predicted sites ^c
Glu-C	41.03 ^d	308	383	E384
	38.93 ^d	292	399	E395/E397
	26.47 ^d	199	492	E489
	25.12	189	502	E499 ^e
Lys-C	75.10	564	127	K135
	39.89	300	391	K400
	38.05	286	405	K405/K409
	34.21	257	434	K432
	23.30	175	516	K508/K518
	19.98	150	541	K541/K545
Bromelain	58.59 ^d	440	251	—
	57.31 ^f	430	261	—
	56.57 ^d	425	266	—
	37.81	284	407	—
	34.43	259	432	T436 ^e

^aNo. of residues = number of residues of full-length HSF (691) × MW of peptide/MW of full-length HSF (92 kDa).

^bCalculated cleavage position = 691 – number of residues of peptide.

^cPredicted cleavage sites = possible substrate residues near the calculated cleavage position.

^dHeat-shocked HSF-specific.

^eCleavage sites that were confirmed by sequencing. About 2% experimental error (in terms of the predicted length of the polypeptides) was estimated from the differences between the calculated sites and the sequencing data.

^fUnshocked HSF-specific.

cleavage at E499 suggests that there may be a flexible structural joint around this amino acid residue. We confirmed the location of this strong cleavage site by microsequencing the peptides generated from parallel Glu-C digestion of recombinant dHSF (data not shown). In this case, the predicted cleavage at residue E499 using Tricine-SDS-PAGE matches the sequencing data exactly (Table 1).

Limited proteolysis by endoproteinase Lys-C

Limited digestion of heat-shocked and unshocked HSF by endoproteinase Lys-C generates six bands of apparent molecular weight

Table 2. Comparison of limited digestions of *Drosophila* HSFs by endoproteinase Glu-C and Lys-C

Positions	Protease	No. of sites	No. of cuts ^a	Cleavage (%) ^b
N-terminus	Glu-C	15	0	0
(1–240)	Lys-C	20	1	5
Interheptad	Glu-C	26	4	15.4
Repeats (241–560)	Lys-C	11	5	45.5
C-terminus	Glu-C	9	0	0
(561–691)	Lys-C	3	0	0

^aNumber of cuts include the cleavage products for both heat-shocked and unshocked HSF.

^bCleavage (%) = number of cuts/number of sites × 100.

Fig. 4. Endoproteinase Lys-C digestion of unshocked and heat-shocked *Drosophila* HSF. An autoradiogram of 10% Tricine-SDS-PAGE/western blot is shown. Ratios of total substrate proteins to the protease (w/w) were 55,600, 33,300, 20,000, 12,000 for lanes 2, 3, 4, 5; and 40,000, 20,000, 10,000, 5,000 for lanes 7, 8, 9, and 10, respectively. Lanes 1 and 6 were uncut controls. Hypersensitive sites are indicated by arrows. Note that there are no major differences in Lys-C digestion between unshocked and heat-shocked HSFs.

75.1, 38.89, 38.05, 34.21, 23.3, and 19.98 kDa (Fig. 4). These fragments correspond to the cleavage sites at K135, K400, K405/K409, K432, K508/K518, and K541/K545, respectively (Table 1). Among them, five cleavage sites (K400, K405/K409, K432, K508/K518, and K541/K545) are within the region between the N- and C-terminal heptad repeats, revealing that nearly half of lysine residues in this region are exposed to the protease (Tables 1, 2; Fig. 7). The cleavage at K135 (75.1-kDa fragment) is located at the end of DNA-binding domain. The fact that K135 participates in the formation of a poorly defined, unstructured loop determined by NMR spectroscopy (Vuister et al., 1994a, 1994b) may explain its hypersensitivity to endoproteinase Lys-C. In contrast to the increased number of cleavages in heat-shocked HSF produced by endoproteinase Glu-C digestion, the cleavage patterns produced by Lys-C are rather similar for the heat-shocked and unshocked HSF proteins.

Limited proteolysis by bromelain

Unlike endoproteinase Glu-C and Lys-C that are highly specific for charged amino acid residues, bromelain is a relatively nonspecific protease with weak preferences for Lys-C, Ala-C, and Tyr-C (Carrey, 1989). Figure 5 shows the digestion pattern of unshocked and heat-shocked HSF upon exposure to bromelain. Again, only

Fig. 5. Limited proteolysis of unshocked and heat-shocked HSF by bromelain. An autoradiogram of the Tricine-SDS-PAGE/western blot is shown. Ratios of total substrate proteins to the enzyme (w/w) were 30,000, 20,000, 15,000 for lanes 2, 3, 4; and 240,000, 120,000, 60,000 for lanes 6, 7, 8, respectively. Lanes 1 and 5 were uncut controls. Hypersensitive sites are indicated by arrows. Cleavage at T436 was confirmed by sequencing the digestion products of recombinant *Drosophila* HSF.

several cleavage products (58.59, 57.31, 56.57, 37.81, and 34.43 kDa) can be visualized in the region corresponding to the internal region between the N- and C-terminal heptad repeats (Tables 1, 2; Fig. 7), suggesting that the amino acid residues in this region are more accessible to proteases. Interestingly, around amino acid residues 250–270, two bands (58.59 and 56.57 kDa) appear in heat-shocked sample (Fig. 5, lanes 6–8), whereas only one band (57.31 kDa) can be observed in the unshocked counterpart (Fig. 5, lanes 3 and 4). As shown in Figure 5 and Table 1, the 34.43-kDa peptide produced by bromelain was sequenced to locate the precise cleavage site. In this case, the cleavage site estimated by SDS-PAGE differs by only four amino acid residues from the site, T436, identified by microsequencing (data not shown).

Limited proteolysis by other nonspecific proteases

In addition to bromelain, several other nonspecific proteases (ficin, papain, and chymotrypsin) have been employed for limited proteolysis of *Drosophila* HSF. In general, the limited digestion pattern of unshocked and heat-shocked dHSF using chymotrypsin are similar to those observed using bromelain (data not shown), whereas digestion with ficin and with papain share a common pattern (Fig. 6). As found for the other endoproteases, the cleavage products of dHSF are observed in the internal region between the N- and C-terminal heptad repeats (Fig. 7). Around residues 320–370, one band appears in unshocked dHSF sample after ficin and papain digestion (Fig. 6, lanes 3, 4, 6, 7, 8). In contrast, multiple bands

Fig. 6. Nonspecific endoproteinase papain and ficin digestions of unshocked and heat-shocked *Drosophila* HSFs. The limited cleavage products were separated on a 10% conventional SDS-PAGE (Laemmli, 1970) followed by western blotting. An autoradiogram of the western blot is shown. Ratios of total substrate proteins to protease (w/w) were 300,000, 150,000, 75,000, 48,000, 24,000, 12,000 for lanes 2, 3, 4, 6, 7, 8; and 400,000, 200,000, 100,000, 64,000, 32,000, 16,000 for lanes 10, 11, 12, 14, 15, 16, respectively. Lanes 1, 5, 9, and 13 were uncut controls. Hypersensitive sites are indicated by arrows.

near the same region are detected in the heat-shocked form (Fig. 6, lanes 11, 12, 15, 16).

Discussion and conclusion

Limited proteolysis with endoproteases of various specificities combined with western blotting has been used to probe the native structure of *Drosophila* HSF from both unshocked and heat-

shocked SL2 cells. Using high-resolution SDS-PAGE, confirmed in two instances by microsequence analysis, we were able to locate the positions of the cleavage sites to within several amino acid residues. We have found that: (1) the latent and activated forms of *Drosophila* HSF are relatively protease-resistant with few residues sensitive to proteases; (2) there is a protease-sensitive region located between trimerization domain (HR-A/B) and the C-terminal heptad repeats (HR-C); and (3) for a few sites, significant differences were detected between heat-shocked and unshocked dHSFs, which may reflect conformational changes and/or differential post-translational modifications of HSF upon heat-shock.

The overall domain structure of HSF observed from limited proteolysis is consistent with the previous sequence alignment analyses (Wu, 1995). All conserved functional domains (N-terminal DNA-binding, trimerization, and C-terminal heptad repeats and activation domains) identified by sequence alignment are resistant to proteolysis, whereas the nonconserved internal region is more accessible to proteases (Table 2; Fig. 7). These results are consistent with the idea that the structural flexibility in this region may be necessary for the conformational changes of HSF that are related to the monomer to trimer transition and to the activity of the activation domain.

At present, we are unable to distinguish between differential posttranslational modifications of HSF residues that affect protease activity from true conformational differences in the two forms of HSF. Given the fact that HSF is a phosphoprotein, it is possible that the different protease sensitivities noted in some regions of HSF between unshocked and heat-shocked forms may be due to the differential phosphorylation. For example, around HSF residues 250–270, one band is detected for unshocked HSF, but two bands are observed for heat-shocked HSF upon bromelain digestion (Fig. 5, lanes 3 and 4 versus lanes 6–8). There are seven potential phosphorylation sites (T254, T255, S256, T258, S260, S262, and H269) in this short region. Similarly, around residues 320–370, one band is evident with unshocked HSF and multiple

Fig. 7. Summary of protease accessibility of *Drosophila* HSF. Schematic representation of full-length HSF is shown. Boxes show conserved domains from HSF sequence alignments (Wu, 1995). Cutting sites for proteases used in the study (Glu-C; Lys-C; bromelain; ficin and papain) are indicated by different arrows. Cleavage at sites T436 and E499 were confirmed by peptide microsequencing. The cleavage site specific for unshocked HSF is marked with a star, and those specific for the heat-shocked sample are indicated with dots.

bands are visible with heat-shocked HSF while digested with ficin or papain (Fig. 6). Further studies of HSF modifications induced by heat-shock will be required to address this issue. Nevertheless, the present study provides insight into the domain organization of HSF structure. In addition, those sites that respond to limited proteolysis differently in the heat-shocked and unshocked HSFs may be used as guides for mutagenic studies of HSF regulation, and as an additional diagnostic assay for the two forms of HSF. Because the native structure of the latent form of HSF is currently revealed only from its hydrodynamic properties and the lack of high-affinity DNA binding, the limited proteolysis used in this study provides a useful complement to knowledge of the monomeric state of the protein.

Materials and methods

Antibody production and purification

Polyclonal rabbit antibody 946 was generated against the extreme C-terminal peptide (residues 676–691) of *Drosophila* HSF (Hazleton). The synthetic peptide H₂N-CQMQTQSSVLNTPRHEL-COOH was coupled to bovine serum albumin (BSA) with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) before immunization. The purification of antipeptide antibody was carried out at 4 °C. Briefly, about 30 mL rabbit anti-peptide serum was diluted onefold in phosphate-buffered saline (PBS), pH 7.4, spun to remove insoluble materials, and applied to dHSF C-terminal peptide-Sepharose column (5-mL bed volume, Research Genetics). The column was washed with 10 volumes of 50 mM Tris·HCl, pH 7.5, and 0.5 M NaCl. Then the antibody was sequentially eluted with 2 volumes of 50 mM Tris·HCl, pH 8.8, and 3.5 M MgCl₂ (final pH < 7), and 2 volumes of 0.1 M Glycine·HCl, pH 2.5. To minimize the loss of activity, the former eluate was collected in 500 µL 2% BSA in 1 M Tris·HCl, pH 7.5, directly, whereas the latter was neutralized in 500 µL 2% BSA, 1 M Tris·HCl, pH 8.8. The eluates were combined and vacuum-dialyzed immediately against 10 mM Tris·HCl, pH 7.5, and 50 mM NaCl first, then against PBS, pH 7.4, containing 0.01% Tween-20 (v/v) (PBST) at 4 °C. About 3 mL concentrated antibody solution was recovered and stored in aliquots at –80 °C. More than 90% of dHSF binding activity of the peptide antibody was recovered after purification. The specificity of the purified antibody directed against dHSF was confirmed by competition experiments using its cognate antigenic peptide.

Cell culture and extracts

Drosophila Schneider line 2 (SL2) cells were grown at room temperature (22–24 °C) in spinner culture with serum-free HyQ-CCM3 medium (Hyclone) supplemented with 25 µg/mL gentamicin. Cytoplasmic and nuclear extracts were prepared at 4 °C as described previously (Westwood & Wu, 1993) with minor modification. For heat-shocked extracts, cell suspensions (typically 5 × 10⁶ cells/mL) were concentrated and then incubated in 36 °C water bath with shaking for 30 min. Cells were washed with cold PBS, pH 7.4, and with cold buffer A containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 5% glycerol (v/v), and homogenized in two original pellet volumes of buffer A then centrifuged at 25,000 × *g* for 20 min. The nuclear pellet was

resuspended with two original pellet volumes of buffer B containing 10 mM HEPES, pH 7.9, 400 mM NaCl, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.5 mM DTT, 0.5 mM PMSF, and 5% glycerol (v/v). The extract was then centrifuged at 110,000 × *g* for 30 min. The supernatant was the heat-shocked nuclear extract containing the activated HSF. For unshocked extracts, cell homogenates were prepared as described above without the cell concentration and heat shock steps. After 25,000 × *g* centrifugation, the supernatant was centrifuged again at 110,000 × *g* for 30 min. The 110,000 × *g* supernatant was the unshocked cytoplasmic extract containing the latent HSF. All cell extracts were stored at –80 °C.

Purification of *Drosophila* HSF

All purification procedures were performed in 4 °C. For heat-shocked HSF, the heat-shocked nuclear extract (starting with 15 L spinner culture) was diluted onefold with buffer C containing 10 mM Tris·HCl, pH 8.0, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT, 0.5 mM PMSF, and 5% glycerol (v/v), and applied to a heparin Sepharose CL-6B column (35-mL bed volume) equilibrated in buffer C containing 200 mM NaCl. After washing, heat-shocked HSF protein was eluted with the buffer C containing 500 mM NaCl. The heparin eluate was concentrated and subsequently diluted with buffer C to lower the salt concentration to about 200 mM NaCl, mixed with 50 µg/mL poly(dI-dC) (Pharmacia), and then applied to a heat shock element (HSE)-agarose column (10 mL). HSF was eluted with buffer C containing 1 M NaCl. The 1 M salt eluate was concentrated and dialyzed against 10 mM Tris·HCl, pH 8.0, 100 mM NaCl and 5% glycerol (v/v). The purity of heat-shocked HSF after HSE affinity chromatography was estimated to be about 70–80% by SDS gel electrophoresis (Laemmli, 1970) and Coomassie blue staining. For unshocked HSF, the unshocked cytoplasmic extract was applied directly to a heparin Sepharose CL-6B column. The 200 mM NaCl (in buffer C) flow-through was then fractionated on a Q Sepharose column (15 mL) by elution with 1–2 volumes 250, 300, 350, 400, 450, and 500 mM NaCl in buffer C. The 300-mM NaCl fraction containing most of unshocked HSF was concentrated and dialyzed against 10 mM Tris·HCl, pH 8.0, 100 mM NaCl, and 5% glycerol (v/v). The purity of the unshocked dHSF was estimated about 0.3–0.4% after the Q Sepharose column fractionation. All partially purified HSFs were stored at –80 °C.

Recombinant *Drosophila* HSF was expressed in baculovirus-infected SF9 cells and purified to 95% homogeneity using heparin Sepharose CL-6B, HSE-Dynabeads, and Mono S chromatography. Detailed protocols are described elsewhere (Zhong et al., 1996).

Coomassie blue staining was used to quantitate recombinant HSF, and quantitative western blotting was applied to estimate the endogenous SL2 HSF concentration.

Gel mobility shift assay

DNA binding of unshocked and heat-shocked *Drosophila* HSF was performed as described previously (Zimarino et al., 1990) using ³²P 5'-end-labeled double-stranded oligonucleotides corresponding to a heat shock element (HSE):

For the experiments shown in Figure 2A, 10 ng of HSF were mixed with 0.1 pmol of ³²P-labeled HSE, 0.5 µg of fragmented

Escherichia coli DNA, 20 μ g of BSA in 15 μ L of 10 mM Tris·HCl, pH 7.5, 120 mM NaCl, 0.1 mM EDTA, and 5% glycerol (v/v). Samples were incubated at 4 °C for 30 min and electrophoresed on a 0.8% agarose gel with 0.5 $\times$ TBE buffer (45 mM Tris-borate, 1 mM EDTA) at room temperature. The gel was blotted onto DE81 paper (Whatman), dried, and exposed to X-ray film.

Gel filtration analysis

The partially purified unshocked and heat-shocked *Drosophila* HSFs (in 25 μ L containing 50 ng HSF) were fractionated on a Superose 12 3.2/30 column (Pharmacia) equilibrated in a buffer containing 20 mM Tris·HCl, pH 8.0, 200 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, and 5% glycerol at 8 °C. Fractions (40 μ L each) were collected and analyzed by 8% SDS-PAGE (Laemmli, 1970) and western blotting. Thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa) (Bio-Rad) were used in parallel as standards.

Limited protease digestions

For digestion of endogenous *Drosophila* HSFs, 25 μ L of partially purified unshocked HSF (about 10 μ g/mL HSF and 3 mg/mL total protein) or heat-shocked HSF 10 μ g/mL HSF with 0.5 mg/mL BSA (Boehringer Mannheim) and 0.5 mg/mL β -lactoglobulin A (Sigma) used as carriers were exposed to appropriate concentrations of proteases at 25 °C for 10–15 min. All proteases used were purchased from Boehringer Mannheim, except bromelain (Sigma). For endoproteinase Lys-C and chymotrypsin, the digestion buffers included 20 mM Tris·HCl, pH 8.5, 100 mM NaCl, and 1 mM EDTA. Endoproteinase Glu-C digestions were performed in 50 mM NH_4HCO_3 and 100 mM NaCl, and bromelain digestions were performed in a buffer containing 20 mM Tris·HCl, pH 8.5, and 100 mM NaCl. Papain and ficin digestion buffers consisted of 20 mM Tris·HCl, pH 6.8, and 100 mM NaCl, 0.5 mM EDTA, and 1 mM DTT. L-1-Chloro-3-tosylamido-7-amino-2-heptanone hydrochloride (0.4 mM) and PMSF (0.4 mM) were added for stopping Lys-C, bromelain, chymotrypsin, papain and ficin cleavage. After digestion, SDS sample buffer was added immediately, followed by heat inactivation. All protease cleavage products were separated by SDS-PAGE.

For mapping recombinant *Drosophila* HSF expressed in baculovirus-infected SF9 cells (Zhong et al., 1996), 25–30 μ g purified protein without any carriers was exposed to protease digestion as described above.

Tricine-SDS-PAGE

Sample preparation and Tricine-SDS-PAGE were performed according to Schägger and von Jagow (1987). Briefly, protein samples were incubated for 5 min at 90 °C in 4% SDS, 12% glycerol (w/v), 50 mM Tris·HCl, pH 6.8, 2% mercaptoethanol (v/v) and 0.01% Coomassie brilliant blue G (Sigma), then loaded onto a slab gel, 1.0-mm thick, consisting of a 20-cm separating gel (10% T, 3% C or 16.5% T, 5% C) and a 2–3-cm stacking gel (4% T, 3% C). These discontinuous gels were initially run at 30 V for 1–2 h, then at 140 V for 20–25 h at room temperature. Protein low molecular weight calibration kit (Pharmacia) and combithek (Boehringer Mannheim) were used as standards.

Electrotransfer and protein detection on PVDF membranes

After electrophoresis, the proteins were transferred to either Immobilon P (Millipore, 0.45- μ m, for western blotting) or Immobilon-

P^{8Q} (Millipore, 0.1- μ m, for microsequencing) membranes in buffer containing 39 mM glycine, 48 mM Tris base, 0.01% SDS, and 20% methanol (v/v). Electrotransfer was performed for 2 h at 0.6 mA/cm² in a semidry blotting apparatus (LKB Pharmacia). Protein molecular weight markers on the immobilon P membranes were visualized by staining with 0.2% Ponceau S (w/v) in 3% trichloroacetic acid (w/v) and 3% sulfosalicylic acid (w/v). Following destaining, the immobilon P membranes were blocked with 5% (w/v) BSA or skim milk in PBST and incubated with affinity-purified rabbit anti *Drosophila* HSF C-terminal peptide polyclonal antibodies (1:2,000 dilution in PBST containing 0.5% BSA) for 1–2 h at room temperature. Membranes were washed with PBST, incubated with donkey anti-rabbit IgG antibody-horseradish peroxidase conjugate (Amersham, 1:10,000 dilution) for 1 h at room temperature. The blots were washed and developed with ECL reagents (Amersham) according to the manufacture's instruction.

For protein microsequencing, the Immobilon-P^{8Q} membranes were rinsed in water and then stained in 0.1% Coomassie brilliant blue R-250 (w/v) in 50% methanol (v/v) for 1 min. The background was cleared by destaining in 45% methanol (v/v) and 10% acetic acid (v/v) for 5–10 min. Membranes were then rinsed with water extensively and air dried. The appropriate stained polypeptides were excised for microsequencing.

Assignment of protease cleavage sites

The positions of protease cleavage sites corresponding to bands on a western blot were identified by their apparent molecular weights. The log(Mw) of molecular weight markers versus their relative mobility was plotted to obtain a linear standard curve (data not shown). The typical R^2 for the fitting is 0.99. The apparent molecular weights of the cleavage products were determined from these standard curves and used to calculate the cleavage sites. Because we were able to sequence some typical bands from recombinant HSF digestion products, about 2% experimental error (in terms of the predicted length of the polypeptides) was estimated from the differences between the calculated sites and the sequencing data.

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