The heat shock transcription factor (HSF) mediates the induction of heat shock gene expression. The activation of HSF involves heat shock-induced trimerization, binding to its cognate DNA sites, and the acquisition of transcriptional competence. In this study, the oligomeric properties of Drosophila HSF were analyzed by equilibrium analytical ultracentrifugation and gel filtration chromatography. Previous findings showed that trimerization of purified Drosophila HSF was directly sensitive to heat and oxidation (1). Here we report that low pH, in the physiological range, also directly induces HSF trimerization and DNA binding in vitro. Furthermore, the induction of HSF trimerization by low pH is synergistic with the actions of heat and oxidation. Since heat or chemical stress leads to a moderate decrease of intracellular pH, we suggest that intracellular acidification may contribute to activating the heat shock response in vivo.

All living organisms respond to elevated temperature and a variety of chemical treatments by a rapid increase in the synthesis of heat shock proteins. These protect the structure and activity of proteins from denaturation under environmental stresses (2–5). In eukaryotes, transcriptional regulation of heat shock genes is mediated by a pre-existing transcription activator (heat shock factor, HSF), which binds to an upstream conserved DNA recognition sequence (heat shock element, HSE) and acquires transcriptional potency, leading to an increase in the synthesis of heat shock proteins (6–9). HSF is encoded by a single-copy gene in some species and by several genes in others, but only one HSF, designated HSF1, appears to be sensitive to the heat stress signal. In metazoans, HSF is present in an inactive monomeric form under normal conditions and converts to a trimeric form with high DNA binding affinity after stress induction. The activated HSF trimer also gains transcriptional competence and undergoes hyperphosphorylation. Under certain conditions, the DNA binding activity of HSF can be uncoupled from the acquisition of transcriptional activity (9). All HSFs contain two highly conserved regions: a DNA-binding domain at the amino terminus and an adjacent trimerization domain with clusters of hydrophilic heptad repeats (HR-A/B) separated by a short spacer (9). The DNA-binding domain of HSF forms a compact structure resembling the helix-turn-helix DNA-binding motif (10, 11). The trimerization domain has been proposed to resemble the hemagglutinin trimer of influenza virus, where the long array of heptad repeats self-associates as a parallel triple-stranded, α-helical coiled-coil structure (12–16). The domain responsible for transactivation is generally located in the carboxyl-terminal region of HSF and shows less sequence conservation between organisms when compared with the DNA-binding and trimerization domains (17). The activities of trimerization and transactivation domains are subject to control by other regions of HSF (18–24).

The activation of HSF involves complex signaling pathways, including the loss of feedback repression imposed by the constitutive HSP70 proteins (25–30), serine phosphorylation of HSF (31–37), and direct effects of temperature (38–43). To test these mechanisms, we established an in vitro assay for reversible trimerization and DNA binding of Drosophila HSF purified from nonshocked or heat-shocked Sf9 cells (1). This in vitro system enabled analysis of DNA binding and the equilibrium distributions of monomer and trimer forms of HSF in vitro under a variety of inducing conditions. Using this system, we observed direct and reversible effects of heat and oxidation on HSF trimerization and DNA binding activity, revealing a physico-chemical mechanism for signal transduction of the heat shock response (1). However, the changes in trimerization and DNA binding induced by heat and hydrogen peroxide treatments in vitro were not as pronounced as those observed after heat shock in cells (1). This prompted us to search for additional conditions that could further enhance HSF activation in vitro. Here, we report that moderately low pH in the physiological range directly and reversibly induces trimerization and DNA binding of purified Drosophila HSF. Such a change of pH may play an additional regulatory role in heat shock response in vivo.

EXPERIMENTAL PROCEDURES

Protein Purification—The Drosophila HSFs were expressed in Sf9 (Spodoptera frugiperda) cells infected with baculovirus (multiplicity of infection of 5–10) carrying the Drosophila hsf cDNA inserted with no additional residues in pBlueBac (Invitrogen) (44). Whole cell extracts were prepared from either the nonshocked or heat-shocked (36 °C, 30 min) Sf9 cells, and HSFs were purified to 95% homogeneity by using heparin Sepharose CL-6B, HSE-agarose, and Mono S chromatography (1). The purity of HSF protein was estimated by reverse phase chromatography and SDS-polyacrylamide gel electrophoresis. Absorption spectroscopy was used to determine the concentration of HSF. The extinction coefficient of HSF, 35970 M⁻¹ cm⁻¹ at 276 nm, was calculated according to Pace et al. (45).

Analytical Ultracentrifugation—Equilibrium analytical ultracentrifugation of HSFs was performed using a Beckman XLA analytical ultracentrifuge at a rotor speed of 12,000 rpm at 20 °C. The buffer used was in 20 mM sodium phosphate (pH 6.6 or 7.4), 150 mM KCl, 1 mM EGTA, 0.1 mM PMSF, 1 mg/ml BSA, 0.5 M NaCl, 1 mM DTT, 1 mM MgCl₂, 2% Triton X-100, 0.01% CHAPS, and 0.004% sodium azide. The buffer used was in 20 mM sodium phosphate (pH 6.6 or 7.4), 150 mM KCl, 1 mM EGTA, 0.1 mM PMSF, 1 mg/ml BSA, 0.5 M NaCl, 1 mM DTT, 1 mM MgCl₂, 2% Triton X-100, 0.01% CHAPS, and 0.004% sodium azide.
MgCl₂, 0.2 mM DTT, and 5% glycerol (v/v). Equilibrium was considered to have been attained when the absorbance scans at 230 nm were invariant for 12 h. The models for fitting the absorbance data were monomer only, A_{230}=A_{s}\exp(\sigma + E); dimer only, A_{230}=A_{s}\exp(2\sigma + E); and trimer only, A_{230}=A_{s}\exp(3\sigma + E); where \sigma = M(1 - \exp[-(p - \nu)^2RT]), and K_s is the trimer equilibrium association constant. \sigma is absorbance at 230 nm at the radial position r, and A_s is absorbance at the cell bottom. M refers the molecular mass of the monomer protein, 76,933 daltons, calculated from the predicted amino acid sequence and confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry with an error of \pm 200 daltons. E is the base line; \nu = \nu_0; M, the molar extinction coefficient of the monomer at 230 nm; \nu is the partial specific volume, 0.723 ml/g, calculated according to Perkins (46); p is solvent density (47); \omega is the rotor angular velocity; R is the universal gas constant, and T is absolute temperature. The trimer dissociation constant K_s equals to 1/K_A. 

**Gel Filtration Chromatography—**Precision columns (PC 3.2/30, 2.4 ml) and a SMART system (Amersham Pharmacia Biotech) were used for gel filtration analysis, 0.5 µM purified HSF samples in (30 µl) were fractionated on a Superose 6 column equilibrated in buffer containing 20 mM Tris-HCl (pH 8.0), 1.5 mM MgCl₂, 200 mM KCl, 0.5 mM DTT, and 5% glycerol at a flow rate of 40 µl/min and 8 °C. This low temperature was utilized to minimize trimer dissociation upon sample dilution during the 1-h chromatography, we have shown previously that trimer dissociation is a time- and temperature-dependent (1). Thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa) (Bio-Rad) were used in parallel as standards. The elution profiles at 215 nm were recorded. The trimer equilibrium dissociation constant is defined as K_s = [M]/[T], where for a known polypeptide concentration C, the monomer concentration is [M] = C × [area]_M/[area]_T, and the trimer concentration is [T] = [C - [M]/3].

**Growth Inducibility and Western Blotting Analysis—**Gel mobility shift assays of Drosophila HSF were according to Zhong et al. (44), using 33P-5'-end-labeled double-stranded oligonucleotides corresponding to a HSE. After induction, about 30 ng of HSF was mixed with labeled HSE and held on ice before agarose gel electrophoresis at room temperature. Western blotting was performed as described previously (48), using polyclonal antibodies against Drosophila HSF and enhanced chemiluminescent detection (ECL, Amersham Pharmacia Biotech). Typically, 10–20 ng of HSF was analyzed per sample.

**Intracellular pH Measurements—**SL2 cells were grown at room temperature (22–24 °C) in spinner culture with Schneider’s medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum and 25 µg/ml gentamicin. Cell suspensions (typically 3–5 × 10⁶ cells/ml) were centrifuged, washed, and resuspended in S-medium containing 25 mM KCl, 5 mM NaHCO₃, 15 mM MgSO₄, 5 mM CaCl₂, 20 mM glucose, 100 mM NaCl, and 10 mM Pipes/Tris (pH 6.8–6.9). This solution was designed to approximate the ionic composition of Schneider’s medium (49). Cells were then incubated with 5 µM 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxy methyl ester (BCECF/AM, Molecular Probes) in S-medium (1.5 × 10⁶ cells/ml) for 30 min at 23 °C with constant shaking at 200 rpm. At the end of the incubation, cells were washed twice with S-medium. Aliquots of cells were centrifuged, resuspended at -2 × 10⁶ cells/ml in S-medium in the presence or absence of chemical inducers. At room temperature, cells remained viable with little leakage of BCECF for several hours. The BCECF fluorescence was monitored with a SLM8000C spectrophotometer as the ratio of emission at 530 nm for excitation at 500 nm, to the emission upon excitation at 450 nm (slit width 2 nm). The fluorescence signal was calibrated with the nigericin-high K⁺ technique (50). Briefly, cells were suspended in a series of solutions containing 150 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 2 µM nigericin (Molecular Probes) and 10 mM MES/Tris (pH range 5.5–6.0) or Pipes/Tris (pH range 6.2–7.8). The calibration curves were fitted by polynomial regression of degree 3 [F_{BCECF} = a + b(pH) + c(pH)^2 + d(pH)^3], and pH values were calculated using the fitted parameters.

**RESULTS**

**Low pH Induces HSF Trimerization In Vitro—**Several lines of evidence have previously suggested that there is a change in intracellular pH during heat shock of living cells (51–54). A substantial increase in DNA binding of HSF was also reported when crude extracts were subjected to moderately acidic pH conditions (pH 6.0) (42, 43). To test whether low pH could induce the change in the oligomeric state of HSF directly, or if the pH signal was sensed and transduced by other factors present in the crude extracts, we analyzed the oligomeric states of purified Drosophila HSF as a function of pH. The Drosophila HSF used was overexpressed in either nonshocked or heat-shocked Sf9 cells and purified by chromatography to 95% homogeneity. Fig. 1A shows the pH dependence of the monomer-trimer equilibrium of HSF as measured by gel filtration chromatography. At 0.5 µM HSF concentration and 20 °C, the HSF trimer population showed a sigmoidal increase from ~26% to ~78% over a pH drop from pH 8.3 to pH 5.6, with the greatest increase occurring in the range of pH 7.5 to pH 6.5. Importantly, the induction of HSF trimerization by the pH decrease was reversible. When the HSF sample in pH 6.6 buffer was re-equilibrated in pH 7.4 buffer, the percentage of trimers decreased from ~63% to the original level, ~40% (Fig. 1B). This reversibility indicates that the observed changes in state of HSF are unlikely to be due to irreversible effects of acidic pH on the activity of misfolded protein. We conclude that a moderate decrease of pH is able to make a direct contribution.
Sensitivity of HSF to Low pH

Low pH Activates DNA Binding of HSF in Vitro—The effect of low pH on the DNA binding of HSF at 20 °C was further investigated using the gel mobility shift assay. As shown in Fig. 3, both purified nonshocked and heat-shocked HSF showed enhanced DNA binding activity when the buffer pH was decreased, consistent with increased HSF trimerization. The maximal DNA binding of HSF at 20 °C occurred at pH 7.0 (Fig. 3, lanes c and h). The magnitude of the increase in HSF DNA binding activity caused by lowering the pH from 7.4 to 7.0 was comparable with the increase caused by heat treatment alone (Fig. 3, lanes b and g). Interestingly, a further drop of buffer pH to 6.2 led to a decrease of DNA binding activity (Fig. 3, lanes e and j); this effect is likely to be due to partial denaturation of HSF brought about by increasingly acidic pH.

Synergistic Regulation of Drosophila HSF Trimerization by Low pH, Heat, and Oxidation—Previously, we identified heat and oxidation as two direct inducers of HSF trimerization and DNA binding in vivo. Each individual inducing signal decreases the $K_d$ of Drosophila HSF by 8–11-fold, leading to a 30–40% increase of the HSF trimer population at physiological concentrations (1). To evaluate if low pH could act synergistically with heat and/or oxidation, we measured the $K_d$ values of HSF under multiple inducing conditions using gel filtration chromatography. As shown in Table II, treatments with heat/ $H_2O_2$, heat/low pH (pH 6.6) or $H_2O_2$/low pH (pH 6.6) decreased the $K_d$ values of HSF by about 30.6–25.0–, and 20.4-fold, respectively, somewhat more than the sum of individual changes. In a triple induction by heat, $H_2O_2$, and low pH (pH 6.4), the $K_d$ value of HSF decreased by 45.8-fold (Table II), increasing the HSF trimer population by 50–60%. The synergistic effect of low pH on heat-induced HSF trimerization in vitro may have a role in the heat shock response in vivo.

Intracellular Acidification by Heat and Other Inducers—To investigate the physiological significance of the low pH-induced trimerization and DNA binding of HSF, we measured the intracellular pH ($pH_i$) change in Drosophila SL2 cells during heat or chemical stresses, using the fluorescent indicator BCECF as a marker of the intracellular pH (55). SL2 cells were grown in spinner culture with Schneider’s medium (pH 6.8) at 22–24 °C. The fluorescence signal was calibrated using the nigericin-high K+ technique (50). After exposure of the BCECF-loaded cells to a series of solutions (pH ranging from 5.5 to 7.8) in the presence of nigericin, the ratio of fluorescence intensities (at 530 nm) was measured for the excitation wavelengths of 500 and 450 nm. Fig. 4A shows the BCECF fluorescence ratio as a function of intracellular pH. Data were fitted with a polynomial regression of degree three; the fitted parameters were then used to convert fluorescence ratios to $pH_i$ values.

As shown in Fig. 4B, the resting $pH_i$ of SL2 cells under normal conditions was 7.36 ± 0.02 at 23 °C. After heat shock for 10 min at 37 °C, the $pH_i$ dropped to 7.05 ± 0.02. The magnitude of $pH_i$ change in SL2 cells is consistent with the $pH_i$ decrease reported previously in salivary glands upon heat shock (from 7.38 to 6.91) (51). In addition to heat shock (37 °C, 10 min), chemical inducers of the heat shock response such as ethanol, sodium salicylate, arachidonate, and indomethacin (group I inducers) lowered the $pH_i$ of SL2 cells by 0.15–0.36 units (Table III). Interestingly, sodium arsenite, cadmium sulfate, and canavanine (group II heat shock inducers) had no significant effects on the intracellular pH value (Table III). Among the group I inducers, heat shock and sodium salicylate have been shown to rapidly activate HSF DNA binding, induce chromosomal puffing or HSP synthesis in Drosophila (56, 57), and ethanol, arachidonate, and indomethacin are fast heat shock inducers or potentiators in mammals (58–60). In contrast, it is known that the group II chemicals such as cad-

![Fig. 2. Equilibrium analysis of HSF by analytical ultracentrifugation.](Image)
mium sulfate and sodium arsenite are not very good inducers for HSP synthesis in SL2 cells, and the induction of heat shock proteins by canavanine is a slow process in Drosophila (61, 62). Therefore, the rapid drop in intracellular pH by some heat shock inducers detected in our measurements generally correlates well with the kinetics of induction of HSF DNA binding or HSP synthesis (with the exception of hydrogen peroxide). Taken together, our data suggest that a drop in intracellular pH may contribute to the induction of HSF trimerization in vivo by a subset of heat shock inducers.

**DISCUSSION**

In this study, we demonstrate that low pH in the physiological range of pH 7.5 to 6.5 can directly and reversibly induce trimerization and DNA binding of purified *Drosophila* HSF in vitro. This *in vitro* sensitivity of *Drosophila* HSF to pH is generally consistent with earlier reports showing that the DNA binding of HSF can be induced by low pH in crude human and *Drosophila* cell extracts (42, 43) and also with a recent study showing pH effects on bacterially expressed mouse HSF1 protein (38). We note, however, that the latter effects on mouse HSF1 were not shown to be reversible and that a more acidic

**TABLE I**

Comparison of equilibrium dissociation constants of HSFs determined by analytical ultracentrifugation and gel filtration chromatography

Measurements were performed with 0.5–0.8 μM HSF (in monomer) in 20 mM sodium phosphate, 150 mM KCl, 0.2 mM DTT, and 5% glycerol at 20 °C. The *Kd* values are averages of 3–10 independent measurements and are within ±10% experimental error.

| HSF   | pH  | Analytical ultracentrifugation | Gel filtration |
|-------|-----|-------------------------------|---------------|
|       |     | ln*Kd* | *Kd*    | *Kd* | *Kd* | *Kd* | *
|       |     |        |        |      |      |      | *
| HSF (NS) | 7.4 | 29.3   | 0.19   | 1.0  | 0.55 | 1.0  | *
|        | 6.6 | 31.6   | 0.019  | 10.0 | 0.060 | 9.2  | *
| HSF (HS) | 7.4 | 29.4   | 0.17   | 1.1  | 0.39 | 1.4  | *
|        | 6.6 | 29.5   | 0.15   | 1.0  | 0.52 | 1.0  | *
|        | 6.6 | 29.7   | 0.13   | 1.2  | 0.044 | 11.8 | *

*~8 μM HSF samples in pH 6.6 buffer were diluted more than 10-fold into pH 7.4 buffer and equilibrated at 20 °C overnight before taking the measurements.

**TABLE II**

Synergistic effects of heat, oxidation, and low pH on the equilibrium dissociation constants of Drosophila HSF

0.5 μM nonshocked HSF samples were incubated in 30 μl 20 mM sodium phosphate buffer (pH 7.4 or 6.6) with or without 2 mM H2O2 at 20 °C for 12 h and analyzed by gel filtration chromatography. Buffer contains 150 mM KCl, 1 mM MgCl2, 0.2 mM DTT, and 5% glycerol. The *Kd* values are averages of three to five independent measurements and are within ±10% experimental error.

| Inducer                  | *Kd*  | Fold induction* |
|--------------------------|-------|-----------------|
| None (pH 7.4, control)   | 0.55  | 1.0             |
| Heat (pH 7.4)            | 0.051 | 10.8            |
| H2O2 (pH 7.4)            | 0.067 | 8.2             |
| Heat/H2O2 (pH 7.4)       | 0.018 | 30.6            |
| None (pH 6.6)            | 0.060 | 9.2             |
| Heat (pH 6.6)            | 0.022 | 25.0            |
| H2O2 (pH 6.6)            | 0.027 | 20.4            |
| Heat/H2O2 (pH 6.6)       | 0.012 | 45.8            |

*Fold induction = *Kd*_{pH 7.4, control}/*Kd*_{pH 6.6}.

*Samples were incubated at 36 °C for 30–120 min before gel filtration analysis.
pH was required (maximal effect observed at pH 5.9) (38). We show further that the direct effects of low pH on synergetic with heat and oxidation, leading to a 30–40-fold decrease in the $K_d$ for trimer dissociation of purified HSF. As such, the combined effects of low pH and heat on HSF can account for a substantial portion, though not all, of the induction of trimerization and DNA binding activity of HSF observed in cells. Since both heat stress and a number of chemical inducers of the heat shock response lead to a decrease of the intracellular pH in living cells, our results suggest that moderate intracellular acidification may contribute to the induction of HSF activity during heat shock in vitro by several inducers of the response.

How are the effects of low pH transduced to HSF? Many proteins are known to undergo a conformational change in the process of activation. Influenza virus hemagglutinin, for example, undergoes a low pH-induced conformational change that mediates fusion of the viral and host cell membrane (63). This conformational change of hemagglutinin protein, irreversible by nature, can also be triggered by heat or treatment with urea in vitro (64). Interestingly, the pH effects on HSF activation are reversible, indicating that change in the state of HSF is driven by thermodynamic rather than kinetic considerations. Previous studies have established that trimerization of HSF is subject to negative regulation and requires the integrity of both amino- and carboxyl-terminal hydrophobic heptad repeats, in addition to other regions of the molecule (18–20). These findings have led to a model for trimer regulation whereby the carboxyl-terminal domain of HSF is involved in the suppression of trimerization of the amino-terminal heptad repeats. There are two histidine residues and more than 20 other charged amino acids located in the middle of trimerization domain (between HR-A and HR-B) and in the carboxyl-terminal HR-C, which are conserved in all HSFs or conserved between Drosophila HSF and mammalian HSF1. As was reported for the Fos leucine zipper homodimer and influenza virus hemagglutinin (65–67), low pH-induced changes in salt bridges or hydrogen bonds are likely to impair interactions stabilizing the structure of the HSF monomer and/or strengthen interactions between subunits of the HSF trimer. In either case, the association constant would be accordingly increased. Indeed, an amino acid substitution on the conserved histidine 179 (in the HR-AB regions) of mouse HSF1 led to derepression of trimerization (38). Further mutagenesis and biophysical studies are necessary to elucidate the mechanism by which HSF is responsive to low pH.

Both analytical ultracentrifugation and gel filtration analyses show at most about 10-fold decrease in the equilibrium trimer dissociation constant of Drosophila HSF when buffer pH is decreased from 7.4 to 6.6 at 20 °C. In living cells, heat shock leads to a smaller change of intracellular pH (from pH 7.36 to pH 7.05), which corresponds to ∼3-fold decrease in the $K_d$. However, as heat, oxidation, and low pH show synergistic effects on Drosophila HSF activity in vitro, the effects of high temperature could be amplified by the moderate intracellular acidification associated with heat stress, and possibly by secondary, oxidative effects in vivo (68, 69). In addition, the rapid DNA binding of HSF trimers and the stability of the protein-DNA complex would drive the equilibrium further toward the trimeric state of HSF.

The failure to observe a lowered intracellular pH for certain heat shock inducers indicates that changes in pH are not obligatory for activation of HSF trimerization. Other activating mechanisms have been proposed, including post-translational modification and the loss of feedback repression by heat shock proteins (25–37). Our results show no significant difference in the trimerization constants between nonshocked and heat-shocked Drosophila HSFs overexpressed in Sf9 cells. Similar experiments using endogenous HSFs partially purified from nonshocked and heat-shocked SL2 cells reveal only 2–3-fold differences in the equilibrium trimerization constants for the two forms of HSF. Hence, heat-induced post-translational modifications may not be essential for regulating the trimerization and DNA binding activities of HSF. This conclusion is in concurs with previous studies showing that trimer formation and DNA binding of HSF1 are independent of inducible phosphorylation in mammalian cells (31, 36); similar findings were obtained in a recent study of Drosophila HSF. Some effects of phosphorylation on the transcriptional competence of HSF have been reported (32–34, 36). However, there is increasing evidence showing that heat shock proteins are involved in regulation of HSF transactivation and recovery after heat shock (30, 48, 70). Reversible effects of heat shock proteins on HSF trimerization, DNA binding, or transcriptional activity have not yet been demonstrated with a purified system. It will be of interest to analyze the direct influence of environmental conditions and heat shock proteins on HSF activity using the in vitro system we have developed.

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