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# DnaK-Sigma 32 Interaction Is Temperature-dependent

## IMPLICATION FOR THE MECHANISM OF HEAT SHOCK RESPONSE*

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**The heat shock response in bacteria is a complex phenomenon in which sigma 32 plays the central role. The DnaK/J chaperone system binds and promotes degradation of sigma 32 at lower temperatures. At heat shock temperatures, the DnaK/J-mediated degradation of sigma 32 is largely abolished by a mechanism, which is not yet fully understood. In this article we have shown that interaction of DnaK with sigma 32 is highly temperature-dependent. This interaction is completely abolished at 42 °C. To investigate the origin of such strong temperature dependence, we have monitored the structural changes that occur in the sigma 32 protein upon upshift of temperature and attempted to elucidate its functional roles. Upon a shift of temperature from 30 to 42 °C, the CD spectrum of sigma 32 becomes significantly more positive without significant change in either tryptophan fluorescence spectra or quenchability to external quenchers. 1,8-Anilinonaphthalene sulfonic acid binding at 42 °C is not significantly affected. The equilibrium guanidine hydrochloride denaturation of sigma 32 is biphasic. The first phase shifts to even lower guanidine hydrochloride concentrations at 42 °C, whereas the major phase remains largely unchanged. The sigma 32-core interaction remains unchanged as a function of temperature. This suggests that increased temperature destabilizes a structural element. We discuss the possible location of this temperature-sensitive structural element.**

Stress response in bacteria and other organisms occurs in response to change in temperature, nutritional deprivation, genotoxic stress, and other kinds of stress (1, 2). The heat shock response in enteric bacteria is one of the most intensively studied stress response systems in bacteria (3, 4). It is clear that upon elevation of temperature, there is increased synthesis of sigma 32, the heat shock sigma factor, caused by lifting of translational control (5, 6). It is also clear now that sigma 32 protein is transiently stabilized upon heat shock. The increased concentration of sigma 32 in heat-shocked cells increases the concentration of sigma 32 holoenzyme leading to transcription of genes encoding heat shock proteins (7). The increased transient stability of sigma 32 after temperature upshift is a result of down-regulation of DnaK/J-mediated degradation (4). Interestingly, at elevated temperatures, increased susceptibility of sigma 32 to DnaK/J independent proteolysis is seen, which ultimately leads to a decrease in sigma 32 levels in the long run and down-regulation of the heat shock response (8). The structural basis of the temperature-dependent regulations of proteolysis is still incompletely understood.

It has been previously thought that one of the mechanisms of down-regulation of DnaK/J-mediated proteolysis is due to increased concentration of denatured proteins. No information is available regarding whether DnaK-sigma 32 interaction itself is temperature-dependent. In this article we demonstrate that DnaK-sigma 32 interaction is highly temperature-dependent and explored the temperature dependence of sigma 32 structure using various spectroscopic tools.

### EXPERIMENTAL PROCEDURES

*Materials*—$Ni^{2+}$-NTA-agarose[1] was purchased from Qiagen. Ampicillin kanamycin, chloramphenicol, spermidine, dithiothreitol, sucrose, ATP-agarose, heparin-agarose, ATP, lysozyme, isopropyl β-D-thiogalactopyranoside, phenylmethylsulfonyl fluoride, and guanidine HCl were purchased from Sigma. Bacto-tryptone, bacto-agar, and yeast extract were purchased from Difco Laboratories (Detroit, MI). β-Mercaptoethanol and glycerol were purchased from Aldrich. ANS and fluorescein maleimide were purchased from Molecular Probes Inc. (Eugene, OR). AG-1-X8 was purchased from Bio-Rad. Core RNA polymerase was obtained from Epicentre Inc. (Madison, WI). All other reagents were of analytical grade.

*Bacterial Strains and Plasmids*—pUHE 211-1 (containing C-terminally His-tagged gene of *Escherichia coli* rpoH and ampicillin resistance marker) was transformed to the bacterial strain NUT-21 containing pDM I,1 a plasmid that harbors *lacI*$^q$ gene and the kanamycin resistance marker (9). BB4708, the Cys mutant of wild type sigma 32 (T128C/N138C), was also transformed similarly in NUT-21 (9).

*Sigma 32 Purification*—The purification was carried out according to Joo *et al.* (9). The NUT-21 strain containing pUHE 211-1 and pDM I,1 plasmids was grown at 30 °C in 1 liter of 2× YT medium with 100 μg/ml ampicillin and 50 μg/ml kanamycin. At $A_{600}$ ~1, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. The cells were grown for another 20 min and poured into tubes of ice. All of the subsequent steps were performed at 4 °C. After centrifugation at 5,000 rpm for 10 min, the cell pellet was resuspended in 18 ml of ice-cold buffer X (50 mM phosphate buffer, pH 7.9, containing 300 mM KCl, 50 mM isoleucine, 50 mM phenylalanine) containing 20 μg/ml of phenylmethylsulfonyl fluoride and disrupted by sonication. The cell lysate was centrifuged for 45 min at 12,000 × *g*. The supernatant was loaded onto a 3-ml $Ni^{2+}$-NTA-agarose column pre-equilibrated with buffer X at a rate 0.4 ml/min. The column was subsequently washed with 40 ml of buffer X and then with 10 ml of buffer X plus 15 mM imidazole. Nickel-bound proteins were eluted with 30 ml of 15–150 mM imidazole gradient in buffer X. Pure fractions of sigma 32 proteins were dialyzed against two changes of 1 liter of 50 mM phosphate buffer, pH 7.9, containing 300 mM KCl and 50% glycerol. The cysteine mutant of sigma 32, BB4708 was also purified following the same protocol. In this case the only alteration that was made was that buffer X additionally contained 1 mM β-ME.

*DnaK Purification*—DnaK purification followed the procedures of Cegielska and Georgopoulos (10) with minor modifications. RLM893

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[1] The abbreviations used are: NTA, nitriloacetic acid; β-ME, β-mercaptoethanol; ANS, 1,8-anilinonaphthalene sulfonic acid; GuHCl, guanidine hydrochloride.

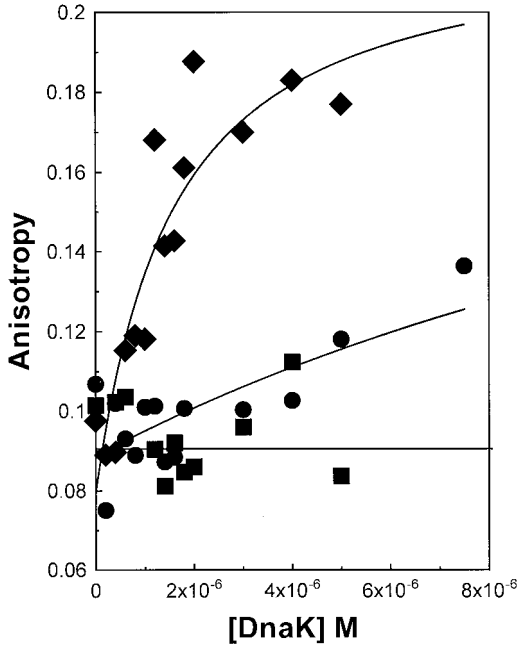


FIG. 1. **Anisotropy increase of fluorescein-labeled sigma 32 as a function of DnaK concentration at three different temperatures, 30 (♦), 35 (●), and 42 °C (■).** Fluorescein maleimide-labeled mutant sigma 32 (T128C/N138C) protein was titrated with increasing concentrations of nucleotide-free DnaK in 0.02 M Hepes, pH 7.6, containing 100 mM NaCl and 25% glycerol at 30, 35, and 42 °C. Anisotropy was determined at each point. Each point is an average of three independent measurements. The excitation and emission were at 480 and 530 nm, respectively. The respective band passes were 10 and 20 nm.

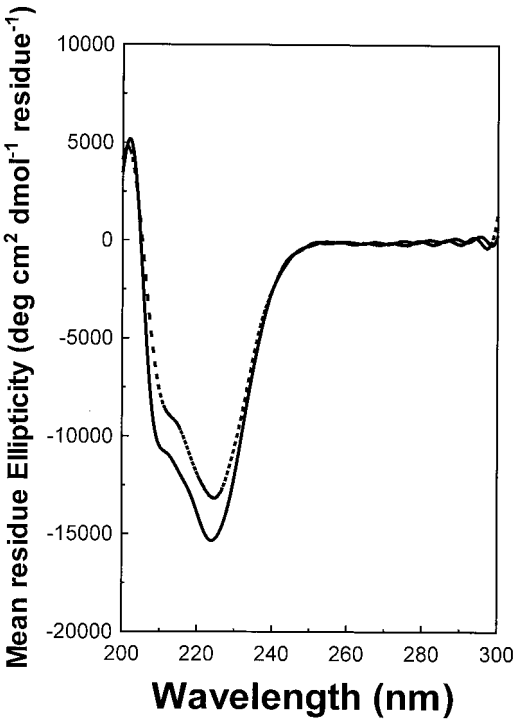


FIG. 2. **Far UV circular dichroism spectra of sigma 32 at 30 °C (*solid line*) and 42 °C (*dotted line*).** The spectra were obtained in a 2-mm-pathlength tightly capped cuvette with a jacket through which water was circulated. The protein concentration was 3.5 $\mu$M in 0.05 M potassium phosphate buffer, pH 7.9, containing 300 mM KCl and 25% glycerol. The protein was equilibrated to 30 °C for 30 min, the spectrum was measured, and then the temperature was shifted to 42 °C. After equilibration for 15 min, the spectra were taken. Two scans were signal-averaged. The bandwidth was 2 nm, and the time constant was 2 s. The actual temperatures were measured using a thermocouple directly placed into the cuvette during an identical run with buffer.

was grown in LB medium containing 25 $\mu$g of chloramphenicol/ml. The cells were first cultured at 30 °C to early log phase and then shifted to 42 °C and incubated until late log phase (4–6 h). The purification steps were all carried out at 4 °C. The cell pellet was washed with buffer B (50 mM Tris-HCl, pH 8, containing10% sucrose (w/v)). The bacterial pellet was then resuspended in buffer K (180 mM spermidine HCl, 50 mM dithiothreitol, 50 mM EDTA, 0.9 M ammonium sulfate) containing 2 mg/ml of freshly prepared lysozyme. After 45 min in ice, the mixture was incubated at 37 °C for 4 min and then returned to ice for another 10 min. It was then subsequently centrifuged in a Beckmann Type 35 rotor at 30,000 rpm for 30 min at 0 °C. After centrifugation to pellet cellular debris, the proteins in the supernatant were precipitated with ammonium sulfate (280 g/1000 ml) followed by another centrifugation for 30 min at 30,000 rpm (70 Ti rotor; Beckmann). The pellet was resuspended in 5–10 ml of buffer A (25 mM imidazole, pH 7.0, 10% sucrose, 25 mM NaCl, 5 mM $MgCl_2$, 5 mM $\beta$-ME) and dialyzed extensively against buffer A (60 volumes) for 12 h at 0 °C. It was then applied onto a 2-ml heparin-agarose column. The flow through was repeatedly applied to a 2.5-ml ATP-agarose column pre-equilibrated in buffer A. The column was washed with 10 column volumes of buffer A containing 500 mM NaCl followed by 2 column volumes of buffer A. The protein was eluted with buffer Q (25 mM Hepes, pH 7.6, containing 50 mM KCl, 1 mM EDTA, 5 mM $\beta$-ME, and 10% glycerol) containing 5 mM ATP. The protein concentrations were determined by the Bio-Rad protein assay.

*Removal of Bound Nucleotide from DnaK*—DnaK is always purified with bound nucleotides. The peak fractions were pooled and loaded onto an AG1-X8 column (Bio-Rad) pre-equilibrated with 20 mM Hepes, pH 7.6, containing 100 mM NaCl, 5% glycerol, and 5 mM dithiothreitol to remove the bound nucleotide (11).

*Chemical Modification*—Prior to labeling, the protein was dialyzed in 1 liter of degassed 0.05 M potassium phosphate buffer, pH 7.9, containing 50% glycerol, 300 mM KCl, and 1 mM EDTA. Labeling of sigma 32 Cys mutant (T128C/N138C) protein with fluorescein maleimide was done by incubating the protein at 0.5 mg/ml with continuous stirring in 0.05 M potassium phosphate buffer, pH 7.9, containing 300 mM KCl and 50% glycerol with 20-fold molar excess of fluorescein maleimide (added as *N*,*N*′-dimethylformamide (DMF) solution in such a way that the final DMF concentration did not exceed 1%). It was then shifted to 25 °C and kept at that temperature for another 15 min. The reaction was then quenched with 1 mM $\beta$-ME and dialyzed extensively against 0.05 M potassium phosphate buffer, pH 7.9, containing 300 mM KCl, 15% glycerol, and 1 mM $\beta$-ME to remove the excess reagent. The incorporation ratio was determined using $\epsilon_{490}$ = 83,000 $M^{-1}$ $cm^{-1}$ (22). The protein concentration was determined from absorbance value at 280 nm after subtraction of the contribution from fluorescein.

*Fluorescence Studies*—All of the fluorescence spectra were measured in Hitachi F 3010 spectrofluorometer having a facility for spectra addition and subtraction. The excitation and emission band passes were 5 nm unless mentioned otherwise. ANS binding to sigma 32 protein was done at both 30 and 42 °C in 0.05 M potassium phosphate buffer, pH 7.9, containing 300 mM KCl and 25% glycerol. The protein (3.3 $\mu$M) was titrated with increasing concentrations of ANS while keeping the volume change to a minimum. The excitation wavelength was 360 nm, and the emission wavelength was 480 nm. The inner filter correction was done as before (12). Tryptophan fluorescence was studied as a function of temperature in 0.05 M potassium phosphate buffer, pH 7.9, containing 300 mM KCl and 25% glycerol. The protein concentration was kept at 2 $\mu$M. The temperature was steadily increased from 30 to 45 °C at intervals of 1 °C by circulating water through the cell holder. Approximately 15 min were required for equilibration and measurement for each time point. Study of protein fluorescence spectra was done in 0.05 M potassium phosphate buffer, pH 7.9, containing 300 mM KCl and 25% glycerol. The temperature was increased at 1 °C intervals. Excitation was at 295 nm, and the band passes were 5 nm each. The protein concentration was 2 $\mu$M.

*Anisotropy Measurements*—Anisotropy measurements were performed using a Hitachi polarizer accessory. The steady state fluorescence anisotropy ($A$) was calculated according to the following equation.

$$A = (I_{\parallel} - G1_{\perp})/(I_{\parallel} + 2GI_{\perp}) \quad \text{(Eq. 1)}$$

where, $I_{\parallel}$ is the intensity when the polarizers were in the same direction, $I_{\perp}$ is the intensity when the polarizers were crossed, and $G$ is the grating factor that corrects for wavelength-dependent distortion of the polarizing system. Fluorescein maleimide-labeled mutant sigma 32 protein was titrated with increasing concentrations of core RNA polymer-

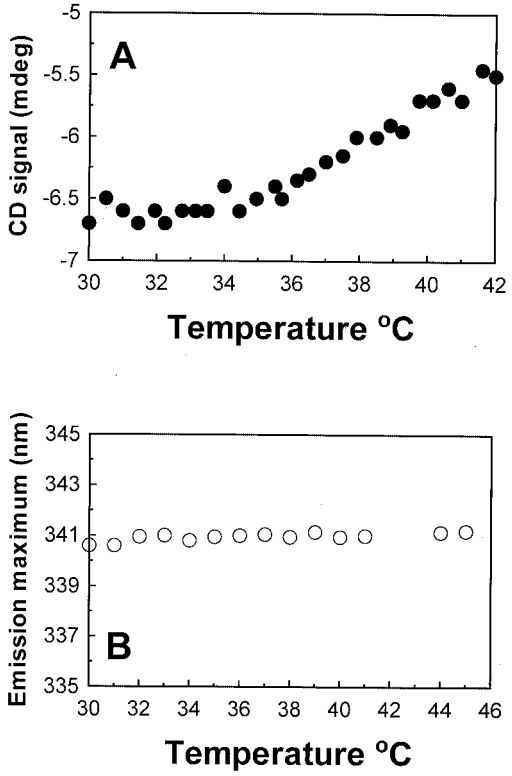


FIG. 3. *A*, temperature dependence of circular dichroism signal at 225 nm of sigma 32. The protein concentration was 3.5 $\mu$M. The bandwidth was 2 nm, and the time constant was 2 s. The temperature was increased from 30 to 45 °C at intervals of ~0.5 °C by circulating water through the jacket. The actual temperature within the cuvette was calibrated with a thermocouple placed in the cell. The spectra was taken in a 2-mm-pathlength cuvette. *B*, temperature dependence of fluorescence emission maximum. The protein concentration was 2 $\mu$M. The excitation was set at 295 nm. The band passes were set at 5 nm. The temperature was increased from 30 to 45 °C at intervals of ~1 °C by circulating water through the jacket. The actual temperature within the cuvette was calibrated with a thermocouple placed in the cell. For both of the experiments, the buffer was 0.05 M potassium phosphate, pH 7.9, containing 300 mM KCl and 25% glycerol.

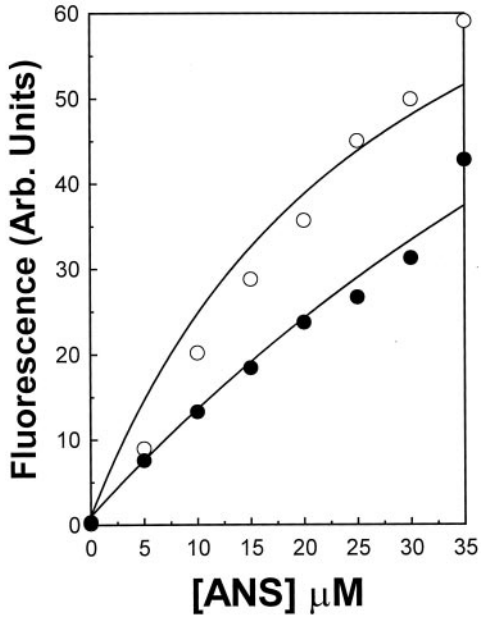


FIG. 4. **ANS binding to sigma 32 at two different temperatures, 30 (●) and 42 °C (○).** The protein concentration was 3.3 $\mu$M in 0.05 M potassium phosphate buffer, pH 7.9, containing 300 mM KCl and 25% glycerol. The excitation and emission wavelengths were 360 and 480 nm, respectively. Each point is the average of four separate experiments. The data were fitted to a straight line.

ase in 0.02 M Tris-HCl, pH 7.5, containing 100 mM NaCl and 25% glycerol at both 30 and 42 °C. The excitation and emission were at 480 and 530 nm, respectively. The respective band passes were 10 and 20 nm. Fluorescein maleimide-labeled mutant sigma 32 protein was titrated with increasing concentrations of nucleotide-free DnaK in 0.02 M Hepes, pH 7.6, containing 100 mM NaCl and 25% glycerol at 30, 35, 37, and 42 °C. The excitation and emission were at 480 and 530 nm, respectively. The respective band passes were 10 and 20 nm.

*Light Scattering*—Light scattering was measured at 400 nm in a Hitachi F3010 spectrofluorometer by setting the excitation and emission wavelengths to 400 nm. The bandpasses were 5 nm each. The experiment was conducted in 50 mM potassium phosphate buffer, pH 7.9, containing 300 mM KCl and 25% glycerol. The protein concentration was 0.131 mg/ml.

*Circular Dichroism*—Circular dichroism measurements were done on a JASCO J600 spectropolarimeter using a water-jacketed 2-mm-pathlength quartz cuvette. The scan speed was 20 nm/min. For equilibrium denaturation measurements, 10 scans were signal-averaged to increase the signal to noise ratio. The spectrum with buffer only was subtracted from the protein spectrum. To study the spectra of protein as a function of increasing temperature, the temperature was increased at intervals of 0.5 °C by circulation of water through the cuvette from 30 to 45 °C, and the spectra were taken. The protein concentration was kept at 3.5 $\mu$M. For measurement of the effect of temperature jump on CD spectra, at first the protein was allowed to equilibrate at 30 °C for about 30 min in a tightly capped cuvette before spectral measurements. Then the temperature was rapidly shifted to 42 °C, and the spectral measurements were made at different times after equilibration. The measurements were done in 0.05 M potassium phosphate, pH 7.9, containing 300 mM KCl and 25% glycerol. A bandwidth of 2 nm was used. Secondary structure prediction was done using web based k2d program (www.embl-heidelberg.de/~andrade/k2d/).

*Equilibrium Denaturation Studies*—GuHCl denaturation studies were performed by diluting the stock protein solution with different volumes of buffer (0.05 M phosphate, pH 7.9, containing 300 mM KCl either in the presence of 25% glycerol or not) and a standard GuHCl solution in the same buffer so as to attain the desired final protein, buffer, and GuHCl concentrations. The solutions were then incubated overnight in a tightly capped tube to attain complete equilibrium at both 30 and 42 °C. The spectral measurements were carried at both 30 and 42 °C by circulating water through the jacket of a jacketed cuvette. The bandwidth was 2 nm, and the time constant was 2 s. 10 scans were signal-averaged.

*Curve Fitting*—The curve fittings were done to various equations such as two-state equilibrium, three-state equilibrium, protein-protein association equilibrium, etc., using Kyplot (Koichi Yoshioka, 1997–1999, version 2.0 beta 4).

*Proteolysis*—Proteolysis was carried out at 44 °C with sigma 32: chymotrypsin ratio (w/w) of 1:50 in 50 mM Tris-HCl buffer, pH 8, containing 100 mM KCl, 1 mM dithiothreitol, 0.02% Triton X-100, and 25 mM $MgCl_2$. Five minutes after the addition of chymotrypsin, the reaction was quenched with phenylmethylsulfonyl fluoride (final concentration, 1 mM). The protein was then loaded on to a 150-$\mu$l $Ni^{2+}$-NTA-agarose column pre-equilibrated with buffer X (50 mM KP, pH 7.9, containing 300 mM KCl, 50 mM isoleucine, and 50 mM phenylalanine). It was washed subsequently with 1.5 ml of buffer X and eluted with buffer X containing 300 mM imidazole. The samples were then analyzed on 16% SDS-PAGE using the Laemmli procedure (13).

## RESULTS AND DISCUSSION

*DnaK-Sigma 32 Interaction*—DnaK/J-mediated proteolysis of sigma 32 plays a crucial role in down-regulating levels of sigma 32 at lower temperatures. Upon heat shock, this proteolytic down-regulation phenomenon is abolished. One of the suggested mechanisms is an increase in the concentration of denatured proteins, which causes titration of DnaK away from its complex with sigma 32. Little is known about how temperature affects interaction of DnaK with sigma 32. One of the better ways of measuring interaction of two proteins is by fluorescence anisotropy. Fig. 1 shows titration of fluorescein labeled sigma 32 with nucleotide-free DnaK at three temperatures, 30, 35, and 42 °C. At 30 °C, the anisotropy increased

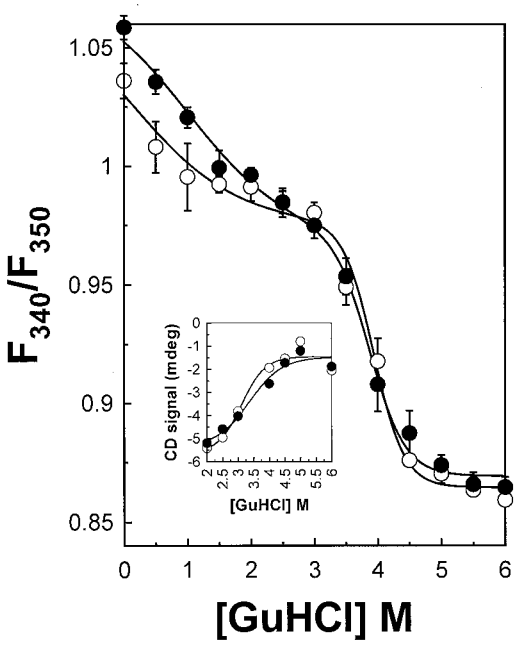


FIG. 5. **Equilibrium GuHCl denaturation profile of sigma 32 at two different temperatures 30 (●) and 42 °C (○), by fluorescence and CD (in the *inset*).** The buffer was 0.05 M potassium phosphate, pH 7.9, containing 300 mM KCl. For fluorescence, the excitation wavelength was 295 nm, and the emission wavelength was 340 nm. Both of the bandpasses were set at 5 nm. The *error bars* are based on the averages of five independent measurements. The CD spectra were obtained in a 2-mm-pathlength cuvette. The protein concentration was 1 $\mu$M.

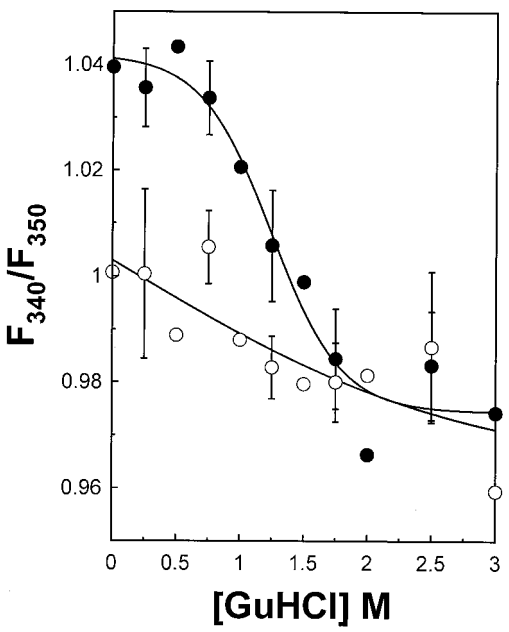


FIG. 6. **Equilibrium denaturation at two different temperatures, 30 (●) and 42 °C (○), at low GuHCl concentrations.** The solutions conditions are same as for Fig. 5 except 25% glycerol was present. Each *error bar* is the result of five independent measurements.

quickly as a function of DnaK concentration but quickly saturated. When fitted to a single-site binding equation, it gives a dissociation constant of $1.4 \times 10^{-6}$ M. At 35 °C, the increase of anisotropy occurred at higher DnaK concentrations. When fitted to a single site equation with same limiting anisotropy, it gave a dissociation constant of $2.1 \times 10^{-5}$ M. At 42 °C, the anisotropy increase is virtually negligible, suggesting a dissociation constant that is much higher. Such a large change in $\Delta G$ of interaction between DnaK and Sigma 32 cannot occur from explicit temperature dependence of $\Delta G$ ($\Delta H - T\Delta S$) and is suggestive of large temperature dependence of $\Delta H$ (heat capacity) or $\Delta S$. One of the very likely mechanisms of such a large temperature dependence of $\Delta H$ or $\Delta S$ may originate from a temperature-dependent conformational change in DnaK or Sigma 32. Because it has been reported that DnaK interaction with its substrates are virtually temperature-independent (14), it is likely that the temperature dependence of sigma 32-DnaK interaction originates from temperature-dependent conformational change of sigma 32. This is also supported by the fact that a peptide (MQQRITLKDYAM) binds to DnaK equally well at 30 and 42 °C (data not shown).

*Temperature-dependent Conformational Change in Sigma 32*—Fig. 2 shows the far UV circular dichroism spectra of sigma 32 at two different temperatures, 30 and 42 °C. The spectra were first taken at 30 °C in a sealed cuvette, and then the temperature was shifted to 42 °C. 15 min after the temperature shift, the spectra were recorded. In a separate experiment, when the kinetics was recorded, it was observed that after 5 min of upshift (first observation point) no further change of spectra occurred, indicating that the change is not due to progressive irreversible aggregation, etc. (data not shown). The protein concentration measured spectrophotometrically after incubation at 30 and 42 °C indicated no change in protein concentration (data not shown). The light scattering measurements were carried out under conditions similar to that of the CD experiment. After attaining 42 °C, the light scattering intensity was measured (from the 16$^{th}$ min). Very little change of scattering intensity is seen (around 5% after 15 min), suggesting that no significant aggregation takes place under these conditions. Also, greater spectral intensity change at 208 nm compared with 220 nm also suggests that it is not merely a loss of protein caused by aggregation or other factors. Such decreased intensity in the far UV range is characteristic of disorder in an organized secondary structural element. Because the loss of intensity does not exceed more than 10–15% of the total, it is likely that it is a relatively small section of the protein that undergoes an order-disorder transition.

If the reduction in CD spectral intensity is related to functional properties of sigma 32, it is likely that a major change would be seen around the temperature where significant heat shock effect is seen, which is above 37 °C. Fig. 3*A* shows far UV circular dichroism spectra of sigma 32 as a function of temperature. Above 35 °C, the far UV circular dichroism spectra showed decreased intensity with some leveling off occurring beyond 43 °C.

Increased disorder in protein structure often leads to protein interiors becoming exposed to solvents. The tryptophans that are situated in the protein interiors may show red shift if the increased disorder leads to increased solvent exposure. Fig. 3*B* shows the response of emission maximum of tryptophan fluorescence as a function of temperature. There are six tryptophans that are distributed throughout the protein sequence. It is clear that there is very little shift of tryptophan emission maximum. Tryptophan emission is generally sensitive to solvent polarity, suggesting that this transition does not involve a significant change in the environment of any of the six tryptophans of sigma 32. This conclusion is also supported by the fact that the acrylamide quenching patterns at the two temperatures are remarkably similar, suggesting no major change in tryptophan accessibility (data not shown). The lack of tryptophan emission maximum change and preservation of most of the CD intensity argues in favor of the conformational change being local in nature.

One possible consequence of order-disorder transition is the

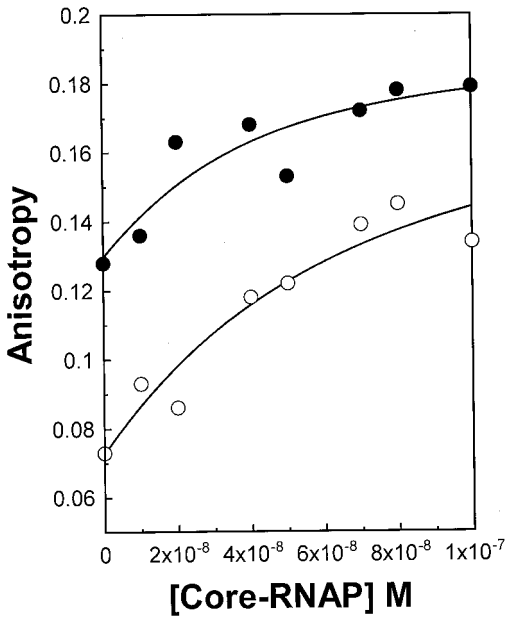


FIG. 7. **Equilibrium binding of sigma 32 to core RNA polymerase at two different temperatures, 30 (●) and 42 °C (○).** Fluorescein maleimide-labeled double mutant sigma 32 (T128C/N138C) was titrated with increasing concentrations of core polymerase, and anisotropy at each point was determined. The excitation and emission wavelengths were 480 and 520 nm, respectively. The excitation and emission bandpasses were 10 and 20 nm, respectively. The solution conditions were 0.02 M Tris-HCl buffer, pH 7.5, containing 100 mM NaCl and 25% glycerol.

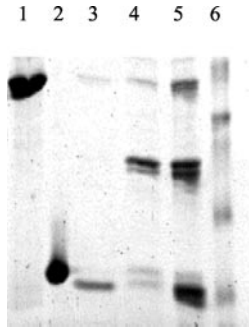


FIG. 8. **SDS-polyacrylamide gel electrophoresis of proteolytic fragments at elevated temperature.** *Lane 1* is purified sigma 32; *lane 2* is chicken egg white lysozyme (molecular mass = ~14 kDa); *lane 3* is high imidazole eluate of $Ni^{+2}$-NTA-agarose column after loading of the C-terminally His-tagged protein proteolyzed at 44 °C; *lane 4* is flow-through from $Ni^{+2}$-NTA-agarose column; *lane 5* is the proteolyzed sigma 32 before loading to the column; and *lane 6* shows the molecular weight markers.

exposure of interior hydrophobic patches. ANS is a hydrophobic probe that has been used widely for the detection of exposed hydrophobic surfaces in proteins. We have used such a probe to detect whether at the elevated temperature sigma 32 shows any excess exposed hydrophobic patches. Fig. 4 shows the ANS binding isotherm at 30 and 42 °C. Their similarity suggests that the disorder-order transition does not cause any increased exposure of interior hydrophobic sites. It has previously been noted that in other proteins, elevated temperatures induce molten globule states (15, 16). One of the common characteristics of the molten globule state is increased binding of hydrophobic probes like ANS and bis-ANS. Clearly such a situation is not evident here, suggesting that the structure of sigma 32 is not a molten globule at 42 °C.

*Equilibrium Denaturation of Sigma 32*—Denaturation of proteins sometimes yields valuable information about domain structure and other structural elements. The stability of domains or structural elements can be assessed based on equilibrium denaturation curves. Given the likely scenario that a small domain/structural element destabilizes at higher temperature, equilibrium denaturation can shed additional light on domain structure and stability of sigma 32. Fig. 5 shows the guanidine hydrochloride-induced denaturation of sigma 32 at 30 °C as monitored by change of $F_{340}/F_{350}$ ratio (a measure of emission maximum shift). In contrast to all other experiments, this experiment was done in the absence of glycerol caused by solubility difficulties of high concentrations of GuHCl in 25% glycerol containing buffers. It is apparent that the denaturation profile is biphasic. The first phase occurs at low guanidine hydrochloride concentration, where significant emission maximum shift occurs along with significant far UV CD change. The second denaturation phase occurs at ~4 M GuHCl, where a large part of the residual CD (shown in the *inset*) disappears along with the complete shift of tryptophan fluorescence emission maximum to greater than 350 nm ($F_{340}/F_{350}$ ratio of about 0.86). We interpret this as denaturation of a relatively small domain at low guanidine hydrochloride followed by unfolding of rest of the protein at a higher GuHCl concentration. The major transition centered around 4 M GuHCl is not significantly different at 30 and 42 °C, suggesting that global stability of the protein is not affected by the temperature upshift. The first phase of denaturation, however, is affected by temperature upshift.

Because most of the experiments were conducted in the presence of 25% glycerol, we have also performed the denaturation experiment at low GuHCl concentration in the presence of glycerol. Fig. 6 shows the denaturation profile of sigma 32 up to 3 M GuHCl at two different temperatures, 30 and 42 °C, in the presence of 25% glycerol. Clearly the two profiles are different, with the one at 42 °C denaturing at significantly lower GuHCl concentrations than the one at 30 °C. When fitted to a two-state unfolding transition, $\Delta G_u$ values of 2.5 and −0.1 kcal/mol values were obtained for 30 and 42 °C, respectively. This suggests that a relatively unstable domain or structural element become destabilized at 42 °C.

We have investigated the functional effects of this conformational change. One of the most important functions of sigma 32 is binding to core-RNA polymerase. We have used fluorescence anisotropy to derive the equilibrium binding isotherm. Fig. 7 shows the titration of fluorescein-labeled sigma 32 with increasing concentrations of core at 30 and 42 °C. The increase in anisotropy shows saturation behavior. When fitted to a single site binding equation, it yields a dissociation constant of 24.1 nM at 30 °C. A similar protocol yields a dissociation constant of 50.6 nM at 42 °C. Clearly, the binding to the core is not significantly affected by the temperature-dependent transition. The core-RNA polymerase binding regions of sigma 32 are now reasonably well documented. The interface is thought to be extensive, including regions 2.1 and 2.2 (17). We conclude that the domain/structural element that undergoes the temperature-dependent transition does not significantly overlap with the regions that are important for core binding.

*Regions Involved in Conformational Change*—We have no direct information on the limits of this domain. Because there is no significant change in tryptophan fluorescence emission in the temperature induced order-disorder transition, it is likely that the part of the domain that undergoes the temperature-induced transition contains no tryptophan residues. There are six tryptophans (26, 107, 120, 147, 216, and 243) distributed throughout the primary structure. It is interesting to note that residues 26–107 and 147–216 are relatively long stretches of amino acids that lack a tryptophan residue. The 147–216 stretch comprises regions 3.1 and 3.2. Based on mutational

**(A)**

```
MTDKMQSLAL APVGNLDSYI RAANAWPMLS ADEERALAEK LHYHGDLEAA KTLILSHLRF    VVHIARNYAG
HHHHHHHEEE CCTTCHHHHH HHHTCCHHCC HHHHHHHHHH HHHCCCHHHH HHHHHHHHHH EEEHHHHHTT

YGLPQADLIQ EGNIGLMKAV RRFNPEVGVRL VSFAVHWIKA EIHEYVLRNW  RIVKVATTK  AQRKLFFNLR
TCCCCCHEEH TTCHHHHHHH HHCCTTCCEE EEEEHHHHHH HHHHHHHHHH HEEEEEECCH HHHHEEEEHH

KTKQRLGWFN QDEVEMVARE LGVTSKDVRE MESRMAAQDM TFDLSSDDDS DSQPMAPVLY LQDKSSNFAD
CCHHHEECCC HHHHHHHHHH HTCCHHHHHH HHHHHHHTTC EEEECCCCCC CCCCCCCEEE ECCCCCCCHT

GIEDDNWEEQ AANRLTDAMQ GLDERSQDII  RARWLDEDNK STLQELADRY  GVSAERVRQL EKNAMKKLRA
CCCCCCHHHH HHHHHHHHHH HHHHTCHHHH HHHHCCTTCH HHHHHHHHHH TCCHHHHHHH HHHHHHHHHH

AIEA
HHHH
```

**(B)**

```
sig70: 375 AKKEMVEANLRLVISIAKKYTNRGLQFLDLIQEGNIGLMKAVDKFEYRRGYKFSTYATWW
           A K ++ ++LR V+ IA+ Y   GL   DLIQEGNIGLMKAV +F    G +  ++A  W
sig32: 49  AAKTLILSHLRFVVHIARNYAGYGLPQADLIQEGNIGLMKAVRRFNPEVGVRLVSFAVHW

sig70: 435 IRQAITRSIADQARTIRIPVHMIETINKLNRISRQMLQEMG--REPTPEELAERMLMPED
           I+  I   +    R +++     +   KL    R+  Q +G   +   E +A  + +
sig32: 109 IKAEIHEYVLRNWRIVKVATTKAQ--RKLFFNLRKTKQRLGWFNQDEVEMVARELGVTSK

sig70: 493 KIRKV-LKIAKEPISMETPIGDDED--------------SHLGDFIEDTTLELPLDSATT
            +R++  ++A + ++ +    DD D              S+  D IED   E
sig32: 167 DVREMESRMAAQDMTFDLSSDDDSDSQPMAPVLYLQDKSSNFADGIEDDNWE--------

sig70: 538 ESLRAATHDVLAGLTAREAKVLRMRFGIDMNTDYTLEEVGKQFDVTRERIRQIEAKALRK
           E       D + GL  R   ++R R+ +D +   TL+E+  ++ V+ ER+RQ+E  A++K
sig32: 219 EQAANRLTDAMQGLDERSQDIIRARW-LDEDNKSTLQELADRYGVSAERVRQLEKNAMKK

sig70: 598 LR
           LR
sig32: 278 LR
```

FIG. 9. *A*, secondary structure prediction for sigma 32. The program used was SOPM (21). *H*, helix; *E*, $\beta$ strands; *C*, coil. *B*, alignment of sigma 70 and sigma 32 sequence by Blast. The *boxes* indicate the putative unstable region in *A* and the insert in *B*.

studies it has been concluded that region 3.2 is not involved in interaction with core (17). It is possible that some structural elements in this region become disordered upon increase in temperature. This hypothesis is also compatible with proteolysis data described below. Yura and co-workers (18) have shown that at elevated temperatures the protein becomes highly susceptible to proteolysis, which initially produces a fragment 12 kDa smaller than the native protein. We have verified this. We have also attempted to locate whether this 12-kDa fragment originates from the N- or the C-terminal side using a His tag as a handle. When the C-terminal His-tagged sigma 32 was proteolyzed and loaded onto the $Ni^{+2}$-NTA-agarose column, the smaller fragment bound tightly to the column, suggesting its C-terminal origin (Fig. 8). It thus suggests that the cleavage takes place ~100 amino acid residues from the C-terminal end. This is very close to the boundary regions 3.1 and 3.2. Fig. 9 shows the alignment of sigma 70 and sigma 32 sequence. Interestingly, residues 190–205 of sigma 32 has no counterpart in the sigma 70 sequence, raising the possibility that this region may be an insertion having sigma 32-specific function. Secondary structure prediction suggests that this region may have a $\beta$ strand and a hairpin loop. Secondary structure content prediction from circular dichroism spectra at two temperatures (30 and 42 °C) suggests a significant loss of $\beta$ sheet structure. One possibility is that this region (the insertion) is in an anti-parallel $\beta$ strand-hairpin turn conformation at lower temperatures that becomes disordered at higher temperatures, thus allowing the protease to gain access. In general, the secondary structure prediction reported here agrees well with the secondary structure of homologous Sigma A of *Thermus aquaticus* (derived from x-ray crystal structure complexed with the core). Insertion region 190–205 aligns with the long connecting loop between the −35 and −10 recognition elements (19). Interestingly, a small deletion in sigma 32 (next to the insertion) also falls in this region, suggesting that this loop may play important role in sigma factor functions.

## CONCLUSION

It has been shown previously that DnaK/J-mediated degradation undergoes temperature-dependent attenuation (4). One possible mechanism is that at higher temperatures, DnaK binds to other misfolded proteins, thus lowering the DnaK-sigma 32 complex concentration and consequently its degradation rate. However, it has been also suggested that this indirect mechanism may not be the only mechanism for lowering degradation rate at higher temperature (20). We have shown that the DnaK-sigma 32 interaction at elevated temperatures is abolished and that a small domain or structural element in sigma 32 undergoes structural transition at elevated temperatures. The abolition may contribute significantly to the lower-

ing of the degradation rate and the initiation of the heat shock response. The structural transition may have a causal relationship with the abolition of interaction between DnaK and sigma 32 reported here, leading to transient stabilization of sigma 32. This same structural transition may also lead to an increased DnaK/J-independent proteolysis rate, leading to adaptation.

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