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Hsp85 Conformational Change Within the Heat Shock Temperature Range

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One of the major mammalian heat shock proteins, hsp85, aggregates extensively when heated in the presence of non-ionic detergents (J Cell. Physiol. 140: 601-607, 1989). The present study used intrinsic fluorescence and susceptibility to tryptic proteolysis to probe hsp85 conformation within the physiological and heat shock temperature ranges. Fluorescence intensity decreased and the emission spectrum was red-shifted (2.5 nm) as hsp85 was heated from 15° to 50°C. Upon heating in the absence of detergent, the red shift, monitored by the ratio of fluorescence emission at 330 nm to that at 350 nm, began at 38°-45°C with a transition midpoint at 45°-50°C, depending on the rate of temperature increase. This transition was masked by 1% n-octyl-O-glucoside - a detergent previously shown to promote aggregation. The spectral changes were not reversible upon cooling to 15°C. Susceptibility to proteolysis in the absence of detergent, measured by the degradation of characteristic large fragments, increased sharply between 40°C and 45°C. These findings suggest that hsp85 undergoes a major conformational change within the range of temperatures known to induce hsp synthesis. This change is consistent with partial unfolding which exposes additional sites to the aqueous environment and influences detergent binding.

Heat shock proteins (hsp's) are defined as the set of proteins induced by exposure to elevated temperature. Although the assumption that synthesis is induced because cellular requirements for the proteins is increased by heat shock appears reasonable, none of the hsp's has been shown to participate in a process that is strongly perturbed within the heat shock temperature range. For example, hsp85 dissociates from steroid-receptor complexes in a temperature-dependent manner [1], but this process occurs at temperatures (ca. 25°C) far below those generally used to elicit the heat shock response in mammalian cells (42-45°C). Similarly, heat shock activator protein binds to hsp regulatory sequences after heat shock [2], but this process has not been shown to involve hsp's per se.

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The finding that hsp85 aggregates to form oligomers after exposure to heat shock temperatures in the presence of non-ionic detergents (Lanks, 1989) suggested that this hsp might be directly affected by heat shock. The present study examines this possibility by asking whether hsp85 undergoes a detectable conformational change within the temperature range known to induce synthesis. Two approaches are taken. Intrinsic fluorescence is used to monitor the exposure of Trp residues to the aqueous environment of the hsp85 molecule, while sensitivity to proteolytic attack is used to assess conformation of the relatively hydrophilic exterior. Both approaches detect a conformational change occurring within the temperature range that is relevant to the heat shock phenomenon.

MATERIALS AND METHODS

Purification of hsp85. The hsp85 was purified from L929 cells by DEAE-Sephadex [4] and hydroxylapatite [5] chromatography. Coomassie blue stained SDS-polyacrylamide gels showed the preparations to consist of a single 85 kDa protein, but silver staining [6] revealed small quantities of actin and other unidentified polypeptides.

Intrinsic fluorescence. All measurements were made on a Spex 212 Fluorolog spectrofluorimeter using 1-cm path length quartz cuvettes. Fluorescence intensity was measured at 280 nm excitation and at both 330 nm and 350 nm emission. For corrected emission spectra, wavelength was scanned from 300 to 400 nm. Background intensity was subtracted from reported values and spectra. The data are plotted as the ratio of emission intensity at 330 nm to that at 350 nm (F_{330}/F_{350}) to take advantage of the red shift expected from moving protein tryptophan residues to a more polar environment while, at the same time, correcting for conformation-independent changes in quantum yield. Cuvette temperature was increased at an average rate of about 0.5°C/min by increasing the circulating bath temperature in 5°C increments and allowing 10 min for equilibration after each temperature change. Cuvette temperature was increased at an average rate of about 2°C by continuous heating with simultaneous measurement of fluorescence.

Tryptic proteolysis. Purified hsp85 (1 mg/ml) was incubated in 0.15 M potassium phosphate buffer (pH 7.5) with the indicated concentration of trypsin (Grand Island Biologicals, Grand Island, NY) for 30 min at 25°C. Reactions were stopped by addition of diisopropylfluorophosphate (1 mM final concentration) and prepared for SDS-PAGE electrophoresis as usual. Gels were silver stained as described by Oakley et al. [6]. Relative fragment abundances were determined by densitometry (Autoscanner, Helena Laboratories, Beaumont, TX).

RESULTS

Effect of temperature on hsp85 intrinsic fluorescence. Figure 1 shows that ratio of emission intensity at 330 nm to that at 350 nm, F_{330}/F_{350}, decreased markedly as the cuvette temperature was slowly increased. This transition began at about 38°C and was 50 percent complete at 45°C. Additional measurements taken as the temperature of the system was decreased showed that the spectral changes were not completely reversible. Figure 2 shows that

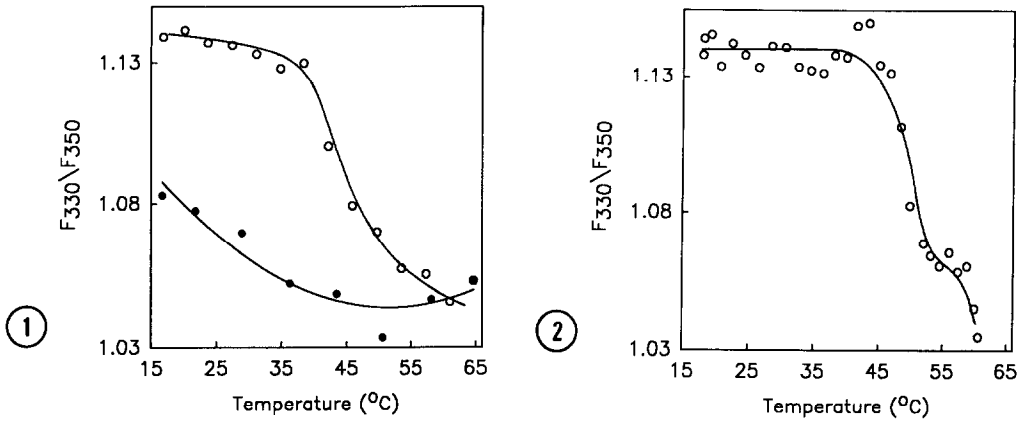

Figure 1. Temperature dependence of hsp85 intrinsic fluorescence. Purified hsp85 (200 µg/ml) in 0.15 M K phosphate buffer (pH 7.4) + 5 mM 2-mercaptoethanol was subjected to increasing temperature at an average rate of about 0.5 °C/min (see Methods) while fluorescence measurements were taken at 330 nm and 350 nm (o—o). Similar measurements were then taken as the temperature was decreased (•—•). Excitation was at 280 nm and a reference cuvette contained buffer alone.

Figure 2. Temperature dependence of hsp85 intrinsic fluorescence. Conditions were exactly the same as in Figure 5 except that cuvette temperature was increased at the rate of about 2°C/min.

with more rapid heating the transition was first detectable at about 45°C and the mid point was shifted to 50°C. Complete emission spectra taken at 18°C before and after exposure to 61°C (as in Figure 2) showed that the emission maximum was red shifted about 2.5 nm (from 335.5 nm to 338 nm) after this in vitro heat shock. Such spectral changes often occur when conformational changes expose buried tryptophan residues to the aqueous solvent environment.

Figure 3 shows that fluorescence did not detect a conformational transition when hsp85 was heated in the presence of n-octyl-O-glucoside. Since this detergent was previously shown to promote aggregation (oligomerization), interaction between protein tryptophans and the hydrophobic hydrocarbon detergent side chain may be occurring as the protein unfolds, thereby masking the transition which can be detected by proteolysis (see below). Interaction with detergent instead of with hydrophobic protein amino acid residues would explain why there is no net change in tryptophan environment at the end of the experiment even though the protein structure is drastically altered.

Proteolytic cleavage of hsp85. Partial tryptic degradation of hsp85 at room temperature in the presence of detergent yielded the fragment pattern shown in Figure 4 (lanes 1, 2, and 3). Apparent molecular weights of the major fragments range from 33 to 70 kDa. Smaller fragments were also observed, but these were not characterized in detail. Yield of all the fragments detected in these experiments was greatly reduced if the protein was heated at 50°C prior to tryptic proteolysis (Figure 4, lanes 4, 5, and 6). This effect was seen even at low protease concentrations which left most of the hsp85 intact (Figure 4, lane 4).

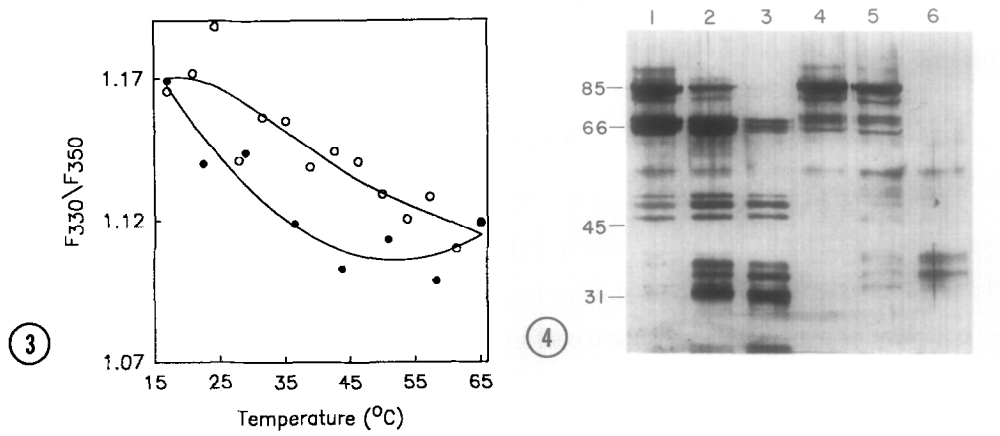

Figure 3. Temperature dependence of hsp85 intrinsic fluorescence in the presence of n-octyl-O-glucoside. The conditions and rates of heating (o—o) and cooling (•—•) were exactly the same as in Figure 1.

Figure 4. Partial tryptic proteolysis of hsp85. Purified hsp85 (1 mg/ml) in 0.15 M potassium phosphate buffer containing 1% Triton X-100 was incubated for 30 min. at 25°C at trypsin concentrations of 0.066 mg/ml **(lanes 1 and 4)**, 0.2 mg/ml **(lanes 2 and 5)**, 0.66 mg/ml **(lanes 3 and 6)**. Fragments were separated by SDS-PAGE and silver stained. **Lanes 1-3**, digestion after preincubation at 25°C; **lanes 4-6**, digestion after preincubation at 50°C for 30 min.

Similar experiments performed in the absence of detergent yielded very similar results except that the yield of 46-50 kDa fragments was reduced. Such an experiment is shown in Figure 5. Yield of all the observed partial proteolytic fragments was highly temperature dependent, but data for only the 66-70 kDa group is shown. Yield of these fragments began to be affected at 40-45°C and had decreased >5-fold at 50°C. This change in protease sensitivity parallels the

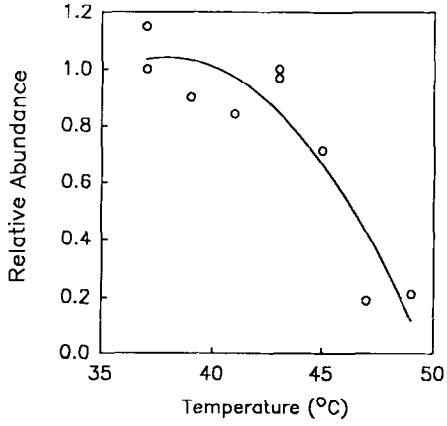

Figure 5. Effect of preincubation at elevated temperatures on partial tryptic proteolysis of hsp85. Purified hsp85 (1 mg/ml) in 0.15 M potassium phosphate buffer was incubated for 30 min. at temperatures ranging from 25°C to 50°C and then subjected to partial tryptic proteolysis (0.2 mg/ml trypsin) for 30 min at 25°C. Fragments were separated by SDS-PAGE and detected by silver staining. Production of 66-70 kDa fragments was determined by densitometry.

change in fluorescence intensity shown in Figure 2 where the temperature of the system was increased rapidly.

DISCUSSION

Cells placed in an incubator equilibrate fairly slowly and are usually heated at 42°-43°C for several hours. Cultures in a water bath equilibrate rapidly and are often heated at 45°C for 20-30 min. Protocols employing both slow and rapid heating were used in the present experiments and both heating regimens demonstrated conformational transitions occurring within the standard heat shock temperature range. Therefore, the observations described in this paper may be relevant to the physiology of intact cells under heat shock conditions.

This conformational change may represent denaturation rather than transition to a specific high temperature state of the protein. Since denaturation is often not readily reversible, the fluorescence measurements showing that the observed conformational change is not reversible within the time frame of the experiments support this conclusion. A specific high temperature state would be expected to exhibit a new set of characteristic partial proteolytic fragments. Whereas preincubation at elevated temperatures did reduce yield of fragments characteristic of the native protein, no new fragment pattern became apparent. This suggests that heating randomized the conformation in some part of the protein instead of promoting a specific conformational transition. Finally, the limited aggregation previously observed when hsp85 is heated in the presence of nonionic detergents is also consistent with denaturation. On the other hand, preliminary differential scanning calorimetry experiments suggest that the main unfolding transitions may occur at slightly higher temperatures than that observed by fluorescence and proteolysis (K.L., E.L. and D.D., unpublished observations). Therefore, it is quite possible that hsp85 undergoes a complex set of unfolding events.

That protein denaturation might play a role in the heat shock response has been suggested several times, but the proposed models invariably involve only the hsp 70 family. For example, bulk microinjecting denatured proteins into cultured cells induces hsp 70 synthesis [7] as does expression of genes for structurally abnormal actins [8]. Hypotheses that hsp 70 induction is triggered by denaturation of cellular proteins are consistent with ATP-dependent binding of hsp 70 to denatured proteins **in vitro** and are reviewed by Pelham [9].

The present study views the role of protein denaturation from a different perspective and suggests that denaturation of the heat shock protein itself, hsp 85 in the present case, may play a pivotal role in the induction process. According to this hypothesis, hsp 85 synthesis is induced when thermal denaturation diminishes the pool of native functional protein. Solvent conditions that stabilize protein folding, e.g., duterium oxide would then inhibit hsp induction [10] by protecting hsp's against thermal denaturation. Validating this hypothesis requires that the

observed conformational change actually affect hsp 85 function and that it occur in cells. Even though the function of hsp 85 is still unknown, the first requirement is probably met **a priori** since virtually any function would be destroyed by extensive irreversible denaturation. Studies designed to detect conformationally altered hsp 85 in cells are now under way.

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