Protein Denaturation in Intact Hepatocytes and Isolated Cellular Organelles During Heat Shock

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Abstract. There is circumstantial evidence that protein denaturation occurs in cells during heat shock at hyperthermic temperatures and that denatured or damaged protein is the primary inducer of the heat shock response. However, there is no direct evidence regarding the extent of denaturation of normal cellular proteins during heat shock. Differential scanning calorimetry (DSC) is the most direct method of monitoring protein denaturation or unfolding. Due to the fundamental parameter measured, heat flow, DSC can be used to detect and quantitate endothermic transitions in complex structures such as isolated organelles and even intact cells. DSC profiles with common features are obtained for isolated rat hepatocytes, liver homogenate, and Chinese hamster lung V79 fibroblasts. Five main transitions (*A–E*), several of which are resolvable into subcomponents, are observed with transition temperatures (T_m) of 45–98°C. The onset temperature is ~40°C, but some transitions may extend as low as 37–38°C. In addition to acting as the primary signal for heat shock protein synthesis, the inactivation of critical proteins may lead to cell death. Critical target analysis implies that the rate limiting step of cell killing for V79 cells is the inactivation of a protein with T_m = 46°C within the *A* transition. Isolated microsomal membranes, mitochondria, nuclei, and a cytosolic fraction from rat liver have distinct DSC profiles that contribute to different peaks in the profile for intact hepatocytes. Thus, the DSC profiles for intact cells appears to be the sum of the profiles of all subcellular organelles and components. The presence of endothermic transitions in the isolated organelles is strong evidence that they are due to protein denaturation. Each isolated organelle has an onset for denaturation near 40°C and contains thermolabile proteins denaturing at the predicted T_m (46°C) for the critical target.

The extent of denaturation at any temperature can be approximated by the fractional calorimetric enthalpy. After scanning to 45°C at 1°C/min and immediately cooling, a relatively mild heat shock, an estimated fraction denaturation of 4–7% is found in hepatocytes, V79 cells, and the isolated organelles other than nuclei, which undergo only 1% denaturation because of the high thermostability of chromatin. Thus, thermolabile proteins appear to be present in all cellular organelles and components, and protein denaturation is widespread and extensive after even mild heat shock.

EXPOSURE of mammalian cells to 42–45°C for short periods of time (referred to as heat shock or hyperthermia) is sufficient for killing and induction of the synthesis of heat shock proteins (Hsp's) during subsequent incubation at 37°C. The direct effect of heat initiating both of these responses is unknown; however, circumstantial evidence suggests the involvement of protein denaturation. Thermodynamic arguments (Johnson et al., 1974; Alexandrov, 1977), the high activation energy for cell killing (Westra and Dewey, 1971), sensitization by incorporation of amino acid analogs (Li and Laszlo, 1985), the correlation between cellular sensitization and protection for numerous compounds and their effects on protein denaturation, and other observations (Dewey, 1989) suggest a relationship between protein denaturation and cell killing due to hyperthermia. Similar correlations exist for Hsp induction. Agents and conditions that induce Hsp synthesis are thought to produce abnormal proteins (amino acid analogs, puromycin, et cetera), to denature protein (high temperature, short chain alcohols, anesthetics), or to damage proteins (release from anoxia) (Hightower et al., 1985; Goff and Goldberg, 1985; Ananthan et al., 1986; Parcell and Sauer, 1989). Injection of denatured protein into cells induces Hsp synthesis, demonstrating that abnormal protein is a sufficient condition for induction (Anathan et al., 1986). Because denatured protein is rapidly degraded, the observation of an initial stimulation of proteolysis after mild heat shock is additional circumstantial evidence for protein denaturation during heat shock (Parag et al., 1987; Heynen et al., 1989). In addition, a common

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mechanism appears to be involved in cell killing, induction of acquired thermotolerance, and Hsp synthesis because the Arrhenius plots for each of these responses is indistinguishable >43°C (Hahn and Li, 1990).

These observations suggest that some cellular proteins are thermolabile, i.e., have a low transition temperature (T_m) for denaturation or unfolding, and that cellular responses to heat shock are a consequence of the denaturation of thermolabile proteins. In addition, these proteins are destabilized at 37°C by a number of chemicals and other stresses inducing the heat shock response. The reason that some proteins may have low stability and denature so close to the growth temperature is unknown. Proteins may have a high degree of flexibility and plasticity near T_m that is required for optimum function, resulting in thermolability and a susceptibility to other stresses at 37°C.

None of the above studies describe the direct measurement of protein denaturation or prove that it occurs during heat shock, and few studies have been directed at answering this question, due in part, to the lack of suitable techniques for measuring protein denaturation under conditions existing within living cells. Spectroscopic studies using fluorescent and spin labels demonstrate that protein conformational changes occur in Chinese hamster lung V79 cell plasma and mitochondrial membrane proteins at temperatures > 40°C (Lepock et al., 1983, 1987). Spectroscopic studies of complex structures are difficult to analyze quantitatively, and it was only possible to determine the onset temperature for conformational changes in membrane proteins, presumably due to denaturation, which occurs near the minimum temperature for cell killing. Recently, Burgman and Konings (1992) have detected transitions in HeLa cell membranes at ~41, 50, and > 58°C and have identified the denaturation of three proteins during the 50°C transition.

Differential scanning calorimetry (DSC)[1] is commonly used for measuring thermally-induced, protein denaturation and has advantages when applied to complex systems. A detailed analysis of denaturation in the erythrocyte membrane by DSC has made it possible to determine the denaturation temperature of the major protein components and has shown that denaturation begins at 40–45°C with transition temperatures (T_m) of 51, 58, 63, and 75°C for the denaturation of spectrin, several membrane skeletal proteins, the transmembrane portion of band 3, and the aqueous portion of band 3, respectively (Brandts et al., 1977; Lysko et al., 1981). These results suggest that endothermic transitions in other isolated cellular organelles are due to the denaturation of specific proteins. We have obtained DSC profiles from V79 cells consisting of five major endothermic transitions and have interpreted them as indicating that protein denaturation occurs > 40°C (Lepock et al., 1988).

If denatured protein is responsible for hyperthermic cell killing and induction of the heat shock response, then it is important to determine the level of protein denaturation as a function of temperature of heat shock and the localization of denaturation within the cell. One reason is that the level of free Hsp70, which is determined by the amount of substrate (i.e., denatured protein), may be the sensor for the heat shock response (DiDomenico et al., 1982; Craig and Gross, 1991). This study analyzes the endothermic transitions observed in isolated rat hepatocytes, liver homogenates, and isolated subcellar organelles as a means of quantitating and localizing protein denaturation. The thermostability of proteins in microsomes, mitochondria, nuclei, and cytosol are compared and estimates are made of the extent of protein denaturation during heat shock at 40–50°C in each of these cellular components and in intact hepatocytes.

1. *Abbreviations used in this paper*: DSC, differential scanning calorimetry.

Materials and Methods

Isolation of Organelles

The homogenate was obtained by homogenizing livers from 12–16 wk old, male Wistar rats with 20 strokes of a glass, hand-held Dounce homogenizer (tight pestle) in HBS with Mg^{2+} (25 mM Hepes, 5.4 mM KCl, 137 mM NaCl, 5 mM $MgCl_2$, 1 mM PMSF, pH = 7.4) at ~7 ml buffer to 1 g tissue. All isolations were carried out at 4°C. This gave a suspension of 15–25 mg protein/ml which was then used for the DSC scans of homogenate.

Microsomal membranes were isolated after homogenization in 250 mM sucrose, 3 mM histidine, pH = 7.4 as described by Amar-Costesec (1974). The microsomal fraction was defined as the membranes pelleted at 145,000 *g* for 30 min from the supernatant remaining after centrifugation at 57,000 *g* for 6 min. The membranes were washed and resuspended in HBS with Mg^{2+} at ~25 mg protein/ml for DSC.

Mitochondria were isolated after homogenization in 250 mM sucrose, 0.1 mM EGTA, 5 mM DTT, and 25 mM Hepes at pH 7.2 essentially as described by Schneider (1948). The homogenate was centrifuged at 1,000 *g* for 10 min and the pellet discarded. The supernatant was centrifuged at 8,000 *g* for 10 min and the pellet suspended and centrifuged a further 10 min at 10,000 *g*. The pellet was washed and resuspended in HBS with Mg^{2+} for DSC.

Nuclei were isolated by homogenization in 340 mM sucrose, 2 mM EDTA, 0.5 mM EGTA, 60 mM KCl, 15 mM NaCl, 15 mM β-mercaptoethanolamine, 1 mM PMSF, 15 mM TRIS, pH 7.4 and centrifugation through a 2.3 M sucrose cushion (Touchette et al., 1986). As for the other organelles, nuclei were washed and resuspended in HBS with Mg^{2+} for DSC.

The cytosolic fraction was defined as the supernatant remaining after centrifugation of the homogenate in HBS with Mg^{2+} at 10,000 *g* for 20 min followed by 204,000 *g* for 60 min.

Isolation of Hepatocytes

Hepatocytes were isolated as previously described (Malhotra et al., 1986) and maintained in culture on collagen coated plates. The medium was changed 2 h after seeding to remove unattached cells. The viable cells were removed with collagenase 8 h after seeding, washed twice, and resuspended in a Hepes-buffered saline (25 mM Hepes, 137 mM NaCl, 5.4 mM KCl, pH 7.4) at ~6×10^7 cells/ml for DSC.

Growth of Chinese Hamster Lung V79 Cells

Chinese hamster lung (CHL) V79 cells, adapted for growth in suspension, were grown in suspension in MEM containing 10% FCS, 25 mM Hepes, and 10 mM sodium bicarbonate at pH 7.4 with no added calcium. Cells were harvested by centrifugation when they reached a density of 5×10^5/ml, were washed twice, and were resuspended in Hepes-buffered saline at 6×10^7 cells/ml for DSC (Lepock et al., 1990*a*).

Differential Scanning Calorimetry

A Microcal-2 DSC with 1.21 ml sample cells was used to obtain all DSC profiles. Cells, homogenates, and organelles were suspended in Hepes-buffered saline and degassed on ice for 10 min under mild vacuum. A DSC scan of each suspension was run from 2 to 105°C at a scan rate of 1°C/min, the suspension was cooled to 0°C, and a rescan from 2 to 105°C was immediately run to determine baseline curvature and to check for reversibility. The baseline curvature and shift in specific heat (ΔC_p) upon denaturation were corrected as previously described (Lepock et al., 1989).

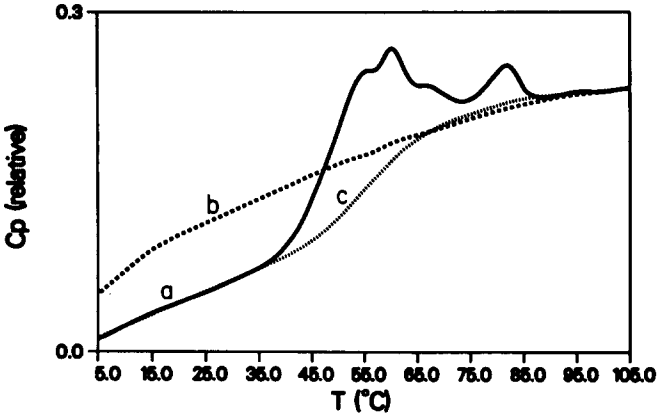

Figure 1. DSC profile of specific heat C_p vs temperature at a scan rate of 1°C/min for (*a*) rat liver homogenate (*solid line*), (*b*) rescan of rat liver homogenate (*dashed lined*), and (*c*) constructed baseline (*dotted line*).

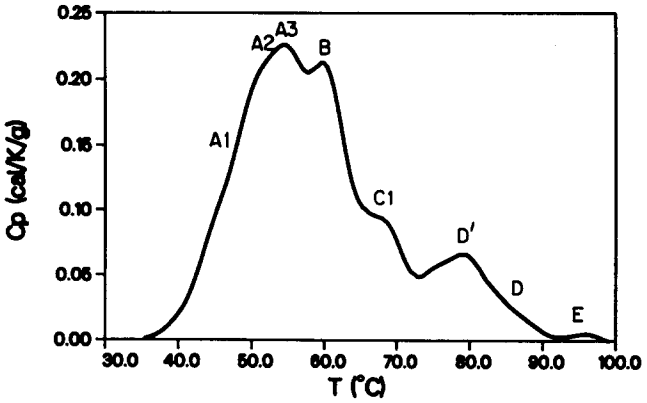

Figure 2. Corrected DSC profile of excess C_p vs temperature for rat liver homogenate.

Results

Thermal Stability of Rat Liver Homogenate

DSC is a powerful technique for detecting and quantifying temperature induced transitions such as protein denaturation (Privalov and Khechinashvilli, 1974; Brandts et al., 1977). Profiles of excess specific heat (C_p) vs temperature are obtained as temperature is increased at a uniform rate. Protein denaturation can be determined directly from such a profile for a simple two state transition of the form $N \rightleftarrows D$ because the temperature integral of C_p (area under the profile) is then proportional to fractional denaturation.

Fig. 1 gives a DSC scan (C_p vs temperature) of rat liver homogenate, the starting material for all organelle isolations. The scan consists of a number of endothermic peaks in the temperature range of 40–95°C. The rescan is almost completely flat, demonstrating that these transitions are irreversible after heating to 105°C. Irreversibility indicates that the reversible model for denaturation is incomplete and that an irreversible model of the form $N \rightarrow I$ or the more realistic model $N \rightleftarrows D \rightarrow I$ where I represents irreversibly denatured protein is needed. The slight curvature visible in the rescan is intrinsic to the calorimeter. The baseline, represented by the rescan, is shifted after scanning through the transitions. This shift in specific heat (ΔC_p) has been attributed to the exposure of buried hydrophobic groups upon denaturation of proteins (Sturtevant, 1977) and was corrected by generating a baseline (Fig. 1, curve *c*) shifted in proportion to the extent of completion of the transitions as a function of temperature as previously described (Lepock et al., 1989).

Subtraction of the baseline gives a profile of excess specific heat (C_p) as a function of temperature (Fig. 2). There are at least four, major, resolvable, endothermic peaks (labeled *A, B, C,* and *D'*) and two minor peaks (labeled *D* and *E*). Peak A consists of three resolvable components A1, A2, and A3 with transition temperatures (T_m) of ~46, 52, and 55°C, respectively. T_m is defined as the temperature of maximum C_p for each peak. Excess C_p is a maximum in the region of peaks *A* and *B*; thus, most cellular transitions occur in this region. The other transition temperatures (T_m) are given in Table I.

A single endothermic peak present in a DSC scan of a pure macromolecule can usually be associated with an order-disorder transition (e.g., protein denaturation or unfolding). Due to the large number of proteins, nuclei acids, and cellular organelles (i.e., membranes, nuclei, cytoskeletal elements, et cetera), the interpretation of the profile in Fig. 2 is not straight-forward. However, it must represent the sum of numerous order-disorder transitions, each represented by a transition temperature (T_m) and a calorimetric enthalpy (ΔH_{cal} defined as the area under the peak). Thus, each major peak is composed of as many as tens or hundreds of specific transitions of varying amplitudes but of similar T_m's, either because of a fortuitous superposition of transitions or a strong interaction between components. In general, this profile can be interpreted as a low resolution separation of all thermotropic transitions based on thermal stability. One would expect protein denaturation to be the principle contribution to the profile since proteins are the major component of cells and protein unfolding has a high ΔH_{cal}. This is supported by the studies on isolated organelles described below.

The profile is also characterized by an onset temperature (T_i), the temperature at which the endothermic transitions can first be detected, which is important for heat shock since this is the temperature above which detectable thermal alterations occur. T_i is defined, as is done for single component transitions, as the intercept temperature of the extrapolation of the linear, low temperature side of peak *A* with the abscissa. The value of T_i is very close to 40°C for the homogenate; however, the first deviation from the baseline appears to occur at a somewhat lower temperature, in the vicinity of 37–39°C.

Thermal Stability of Hepatocytes

Intact hepatocytes were analyzed as a means of determining if significant alternations to the DSC profile occurred during homogenization, which would invalidate any measurements on isolated organelles. The DSC profile for hepatocytes is shown in Fig. 3. It is similar to the profile for homogenate except for some minor differences. An exotherm (release of heat) is centered at 32°C. This can be eliminated with 5–10 mM KCN and is much stronger when the scan is obtained with cells in complete medium or when more oxygen is present (results not shown). Thus, it appears to be due to normal metabolism occurring during the scan until all oxygen is consumed. In nondegassed buffer with a higher oxygen concen-

Table I. Transition Temperatures (T_m) for Each Resolvable Peak in the DSC Profiles of Excess C_p vs Temperature

	T_1	A1	A2	A3	B	C1	C2
Homogenate	39.8 ± 0.9	~46	51.6 ± 0.5	54.7 ± 0.4	59.8 ± 0.1	67.8 ± 0.7	–
Hepatocytes	40.7 ± 0.4	~48	~52	57.2 ± 0.6	59.6 ± 0.4	65.1 ± 0.3	73.4 ± 0.2
Microsomes	39.6 ± 0.3	45.0 ± 0.5	~50	–	58.8 ± 0.2	67.2 ± 0.2	–
Mitochondria	40.6 ± 1.9	~44	50.8 ± 0.3	57.0 ± 0.5		~65	–
Nuclei	39.8 ± 0.8	–	41.7 ± 0.4(I)	–	–	–	71.6 ± 0.2(II)
Cytosol	39.9 ± 0.5	~47	52.4 ± 0.2	~56	58.3 ± 1.3	67.2 ± 0.2	–
V79 Cells	41.6 ± 0.4	–	~52(A)	–	60.7 ± 0.3	–	71.0 ± 0.2

Values given (±SEM) were obtained from at least three scans. The T_m's for the poorly resolved peaks are approximate and are marked with a ~. The nuclear

tration the exotherm is centered at 38–40°C. Oxygen consumption is strongly inhibited > 40°C, implying inactivation of metabolism with a reduction in metabolic heat production above this temperature (Lepock et al., 1987).

The *A* peak is resolvable into three components (*A1, A2,* and *A3*) as in liver homogenate with T_m's of ~48, 52, and 57°C, respectively. The *A3* peak is the major component in both hepatocytes and the homogenate; however, this peak is 2.5°C lower in the homogenate. Thus, there is a slight shift of heat absorption to lower temperatures upon homogenization implying a destabilization of some proteins possibly due to disruption of intermolecular associations. The T_m of the *B* peak is unaltered by homogenization. It will be shown below that the major contribution to the A peak is from cytosolic components while the *B* peak is due to membrane proteins. The *C* peak is resolvable into two peaks *C1* and *C2*. The strong *D'* peak at 81°C is not present in hepatocytes, which were isolated by collagenase treatment; thus, it probably represents the denaturation of extracellular, connective tissue present in liver and differs from the D transition shown in Figs. 3, 6 *C*, and 7.

Other than these modifications there are no changes upon homogenization. The T_m's are similar, and more importantly T_1 differs by only 0.9 ± 1.0°C demonstrating no significant sensitization of the most thermolabile cellular components upon homogenization.

Stimulation of Complex DSC Profiles

The DSC profile of liver homogenate and hepatocytes can be viewed as consisting of the sum of a large number of transitions with random ΔH_{cal} (i.e., area, a measure of the size of each transition) and by a distribution of T_m's defining the general shape of the profile. As can be seen from Figs. 2 and 3 the distribution of T_m's is not random but has a maximum near 55–60°C and a minimum T_m of 40–50°C resulting in a T_1 of ~40°C. In addition, it is slightly skewed towards higher temperatures.

The DSC profiles in Fig. 4 were stimulated by summing a distribution of independent, reversible transitions. For

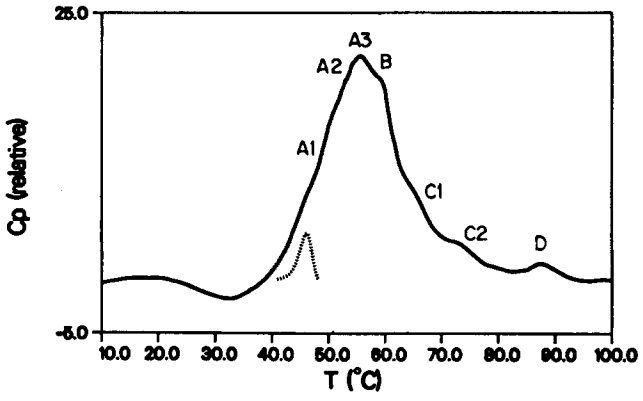

Figure 3. DSC profile of excess C_p vs temperature for rat hepatocytes (*solid line*) maintained in culture for 8 h after isolation. The predicted DSC profile (arbitrary amplitude) for the critical target for the killing of V79 cells is represented by the dotted line.

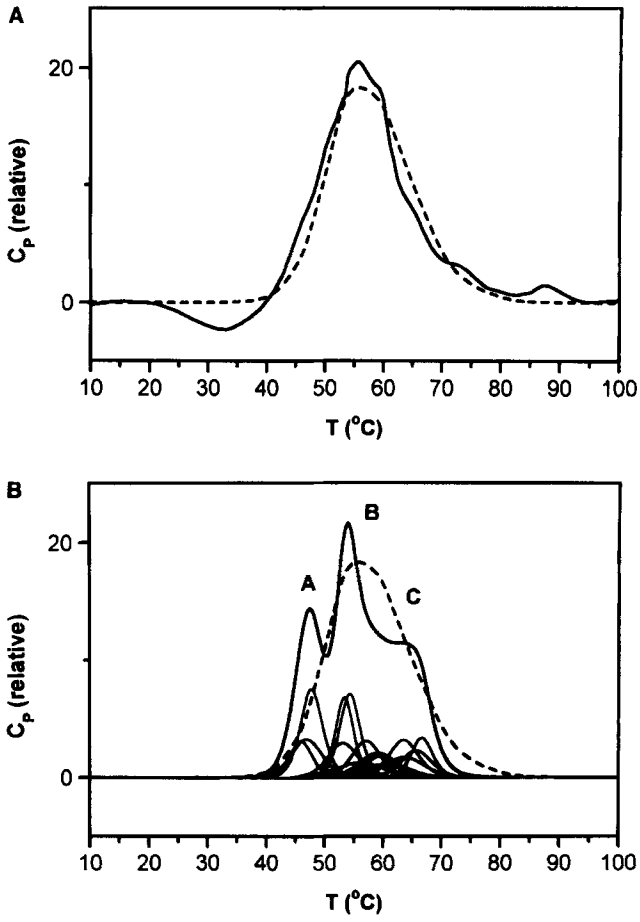

Figure 4. Simulated DSC profiles consisting of a sum of individual transitions with the T_m for each selected from a Poisson distribution with an average T_m of 55°C. Experimental profile of rat hepatocytes (*solid line*) and simulated profile (*dashed line*) consisting of 1,000 transitions (*A*). Simulated profile (*solid line*) consisting of 20 transitions, the individual transition profiles (*thin solid lines*), and simulated profile (*dashed line*) consisting of 1,000 transitions (*B*).

Table I.

D'	D	E
79.2 ± 1.5	85.3 ± 0.6	95.6 ± 0.6
–	85.8 ± 0.2	96 ± 2
–	–	–
–	–	–
–	85.5 ± 0.1(III)	98.1 ± 0.3(IV)
78.3 ± 1.1	–	95.6 ± 0.4
–	84.8 ± 0.4	98.9 ± 0.7

transitions are also labeled I, II, III, and IV.

each transition T_m was randomly chosen from a Poisson distribution shifted to start at 40°C with a mean of 55°C, the van't Hoff enthalpy ΔH was randomly chosen from a normal distribution with a mean of 500 kJ/mol and a standard deviation of 200 kJ/mol, and the ratio of $\Delta H_{cal}/\Delta H$ was allowed to vary randomly from 0 to 1. A Poisson distribution was chosen because it is skewed towards higher T_m's. A large number of transitions (1,000) gives a smooth curve (Fig. 4 *A*) which represents the distribution of T_m's of all cellular proteins of both hepatocytes and liver. Thus, the average T_m, a measure of protein stability, for all cellular proteins of hepatocytes is ~55°C.

When a smaller number of transitions is used the simulated DSC profile contains resolvable peaks which better represents what is found for cells. One simulation using 20 transitions is shown in Fig. 4 *B*. Three resolvable peaks labeled *A*, *B*, and *C* appear in the profile. Other simulations contain between 3 and 5 peaks. Each peak is resolvable because of the superposition of several strong transitions with similar T_m's. Presumably, the peaks in the DSC scans of whole cells and homogenate are also due to the superposition of other, non-resolvable transitions. Peaks *A1*, *A2*, and *A3* (Figs. 2 and 3) are the partial resolution of some of these underlying transitions.

Critical Target Analysis

The onset temperature for the endothermic transitions detectable by DSC is 40°C, roughly the temperature at which the damaging effects of hyperthermia begin. However, what must be determined is the T_m of the transition that is rate limiting for the observed effect induced by hyperthermia. Assuming that a transition, such as protein denaturation, in a critical cellular component during heat shock is responsible for killing, it is possible to calculate the T_m for this rate limiting transition in what can be called the critical target. The T_m for inactivation of the critical target responsible for killing of V79 cells, which was calculated from clonogenic survival curves, is 46.0°C (Lepock et al., 1988; Lepock et al., 1990*a*). This transition can be detected by DSC if it is sufficiently strong.

The T_m for the transition in the critical target is higher than the minimum temperature (~41°C) at which hyperthermic cell killing can be detected because of the procedure for measuring denaturation using DSC. A model assuming irreversible denaturation of the form $N \rightarrow I$ resulting in irreversible inactivation of the critical target was used to predict the T_m of the critical target. Thus, the extent of denaturation is dependent on both temperature and time of exposure. A time on the order of an hour is required to detect killing at 41°C,

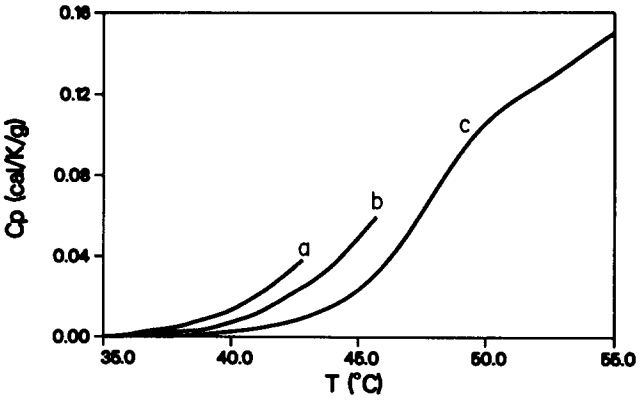

Figure 5. DSC profile of excess C_p vs temperature at a scan rate of 1°C/min for rat liver homogenate scanned to 43°C (*a*), immediately cooled (~2°C/min) to 0°C and rescanned to 46°C (*b*), and immediately cooled again followed by a complete scan (*c*).

whereas a scan rate of 1°C/min was used for DSC. Thus, only 9 min is required to go from 37 to 46°C, effectively increasing the temperature of detectable denaturation during this short time and resulting in a predicted T_m of 46°C for the critical target.

The predicted DSC profile for the critical target is shown in Fig. 3. This was determined from measurements of the killing of V79 cells because it is not possible to measure clonogenic survival of hepatocytes, but most rodent cells have similar heat sensitivity; thus, it is unlikely that the T_m of the critical target for killing in rat hepatocytes differs by more than a few degrees from that shown. Measurements of viability of both V79 cells and hepatocytes by trypan blue exclusion indicate that hepatocytes are slightly more sensitive to heat shock, equivalent to a T_m for the critical target 1–2°C lower than that for V79 cells (results not shown). Thus, those transitions that comprise the *A1* peak, which includes the region of 44–46°C, are the important ones for cell killing. A similar analysis can be applied to the inactivation of any cellular function by heat shock to determine the corresponding T_m of the critical target.

Irreversibility

The transitions are irreversible after scanning to 105°C for both liver homogenate (Fig. 1) and hepatocytes. Irreversible inactivation of the proteins lysozyme (Ahern and Klibanov, 1985) and ribonuclease (Zale and Klibanov, 1986) at 90–100°C at neutral pH is dominated by the formation of incorrect, nonnative conformations. Irreversibility occurs at much lower temperatures for cellular protein denaturation. As shown in Fig. 5, the transitions in the temperature range of 40–50°C are largely irreversible after heating to 43°C and 46°C at 1°C/min and immediately cooling to 0°C. These are relatively mild heat shocks; there is no killing on scanning to 43°C and ~50% killing on scanning to 46°C for V79 cells as determined by clonogenic survival. The onset of the subsequent profiles are shifted upwards in temperature due to the irreversibility of the most thermolabile transitions. For example, T_i is 44°C after heat shock at 46°C. Thus, the transitions under the A1 peak are largely irreversible after exposure to heat shock temperatures.

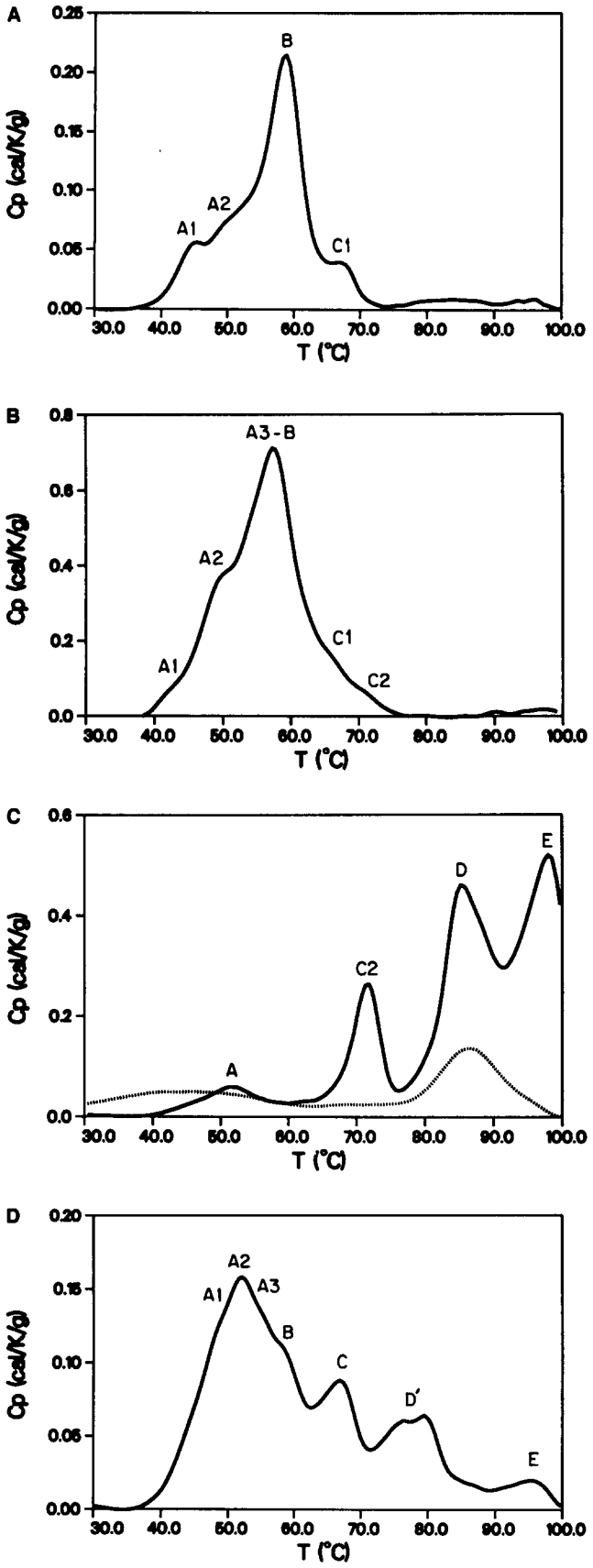

Figure 6. DSC scans of excess C_p vs temperature for isolated rat liver microsomes (*A*), mitochondria (*B*), nuclei (*C*), and a cytosolic fraction (*D*). The rescan of nuclei (*dotted line*) shows the only reversible component in any of the organelles.

Thermal Stability of Cellular Organelles

DSC profiles for isolated rat liver microsomes, mitochondria, nuclei, and a cytosolic fraction are shown in Fig. 6. The T_m's of the resolvable transitions are given in Table I. All the transitions are irreversible except for the *D* transition of nuclei which is partially reversible and is in part associated with the unfolding of DNA. The remaining transitions in each component must represent primarily the denaturation of proteins, as has been definitively shown for the DSC transitions of the erythrocyte membrane (Brandts et al., 1977; Lysko et al., 1981).

The onset for denaturation (T_i) is near 40°C for each component (Table I), demonstrating that each contains some thermolabile proteins denaturating at heat shock temperatures. T_i is no lower than the value in homogenate, implying that little or no thermal sensitization occurs during isolation.

The main transition of microsomes and mitochondria corresponds to the *B* transition in homogenate, suggesting that at least in part this peak in cells is due to the denaturation of membrane proteins. Microsomes isolated by the method used have a very low level of contamination by large cytoplasmic granules (mitochondria, lysosomes, and peroxisomes) of < 8% (Amar-Costesec, 1974). The major transitions of nuclei (*D* and *E*) are barely detectable in hepatocytes and homogenates, probably because the nucleus makes up a relatively small fraction of the total cell volume. The *D* and *E* peaks are much stronger in V79 cells (Fig. 7) which have a larger nucleus to cytoplasm ratio. Other small cells grown in tissue culture (CHO and RIF) also have strong *D* and *E* transitions (results not shown). The *A* peak dominates the cytosolic profile, suggesting that soluble, cytoplasmic components strongly contribute to the *A* peak in cells. All of the components have transitions under the *A1* peak that correlate with the predicted T_m of the critical target for killing. Thus, each organelle undergoes some protein denaturation during heat shock at temperatures of 42–45°C and each organelle could contain the critical target for killing.

Thermal Stability of Chinese Hamster Lung V79 Cells

We have previously analyzed transitions in V79 cells by DSC; however, because of the strong *E* transition it was not possible to determine a good high temperature baseline for a proper correction for ΔC_p (Lepock et al., 1988, 1990*a*). This causes an underestimation of the amount of denaturation in the temperature range of 40–50°C. The scans of homogenate, hepatocytes, and nuclei show that no transitions exist above the *E* transition, allowing a correction for ΔC_p yielding the corrected scan shown in Fig. 7 *A*. The profile for hepatocytes and V79 cells are very similar except for the much weaker *D* and *E* nuclear transitions in hepatocytes and the better resolution of the *A* transition into subcomponents in hepatocytes. The T_m's for each transition match very closely and the bulk of the denaturation occurs between 45 and 65°C for V79 cells as for hepatocytes. Thus, the overall patterns of protein stability, as shown in Fig. 4, are similar for both cell types. In addition, a similar DSC profile is found for CHO cells (Borrelli et al., 1991).

Irreversible protein denaturation also occurs after heat shock of V79 cells in complete medium (Fig. 7 *B*). Cells were heated at 45°C for 10 min. in medium at a density of 5×10^5 cells/ml, which results in ~90% killing, and then

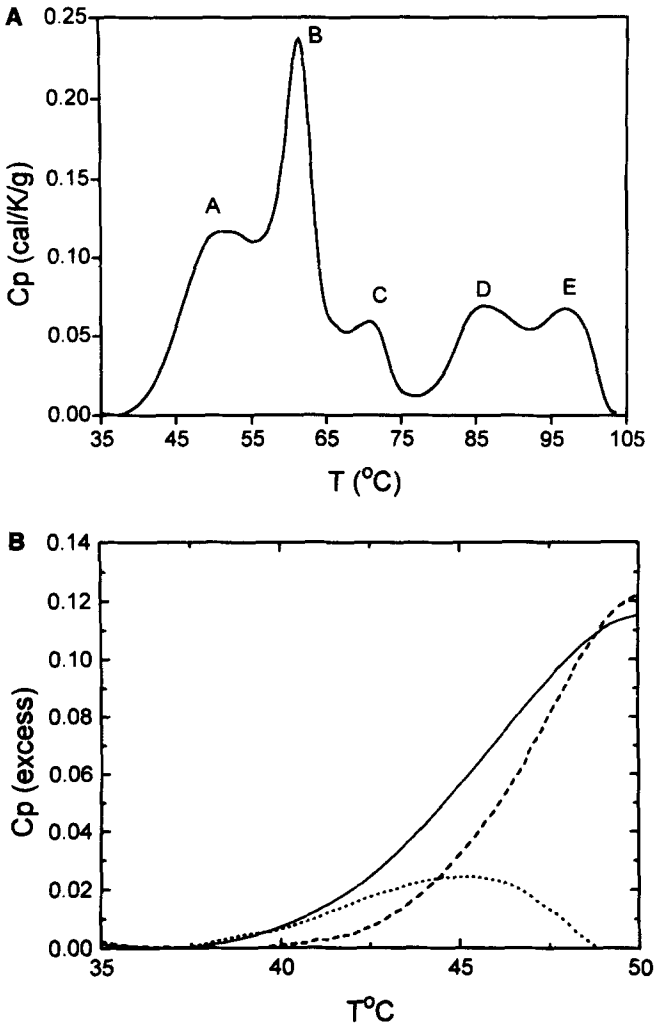

Figure 7. DSC scan of excess C_p vs temperature for intact Chinese hamster lung V79 cells (*solid line*) and V79 cells following a heat shock of 45°C for 10 min (*dashed line*). Region from 35–105°C (*A*) for control, nonheat shocked cells and region from 35–50°C (*B*) including the difference (*dotted line*) between the control and heat shocked cells.

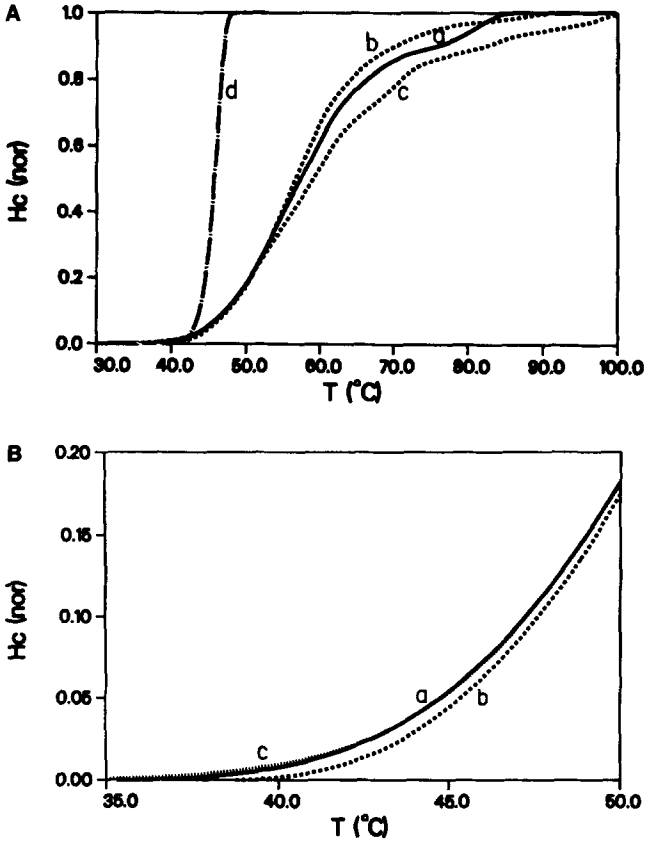

Figure 8. Normalized ΔH_{cal} (approximate fractional denaturation) vs temperature for rat liver homogenate (*a*), hepatocytes (*b*), V79 fibroblasts (*c*), and the predicted critical target for cell killing (*d*) (*A*). Enlargement of the region from 35–50°C (*B*) for homogenate (*a*), hepatocytes (*b*), and V79 fibroblasts (*c*).

washed and prepared for DSC as described in the Materials and Methods. The onset of denaturation is increased from 41.6°C to 43.9°C after heat shock. Also shown in Fig. 7 *B* is the difference from 35 to 50°C between the profiles of control and heat shocked cells. The difference represents the profile of the proteins irreversibly denatured by this heat shock. The cells were maintained at 0–4°C between the heat shock and the DSC scan to block the development of thermotolerance. Thus, irreversible denaturation not only occurs during heating in the calorimeter as shown in Fig. 5, but also during exposure to heat shock in culture medium.

Quantitation of Protein Denaturation

For a pure protein, the area under the profile of excess C_p vs T gives the fractional denaturation on scanning to that temperature. The same is true for the more complex profiles shown here if the specific calorimetric enthalpy for each cellular protein is the same. Although the calorimetric enthalpy is a function of temperature, this appears to hold approximately because the average enthalpy and standard deviation at 60°C is 6.7 ± 1.2 cal/g, with only a factor of two difference between the minimum and maximum enthalpy, for the proteins studied by Privalov and Khechinashvili (1974). Thus, integration and normalization of excess C_p to obtain ΔH_{cal} (normalized) vs T gives curves that should approximate fractional denaturation vs temperature.

These curves are shown for liver homogenate, hepatocytes, and V79 cells (Fig. 8) and the isolated organelles (Fig. 9). The profiles for hepatocytes and liver homogenate (Fig. 8) are very similar but differ slightly for two reasons: (*a*) the low temperature region for hepatocytes is shifted upward in temperature by ~1°C due primarily to superposition of the metabolic exotherm. This shift can be eliminated by scanning in 5 mM KCN. (*b*) The presence of peak *D'* in the homogenate alters the high temperature region. The profile for V79 cells is identical to liver homogenate at lower temperatures (Fig. 8 *B*), but relatively more denaturation is observed at high temperatures due to the greater contribution of the nuclear transitions *D* and *E*. The profiles for microsomes, mitochondria, cytosol, and liver homogenate are very similar in the low temperature region (Fig. 9 *B*). Significantly less denaturation occurs in nuclei at low temperatures due to the relatively greater contribution from the *C2*, *D*, and *E* transitions at high temperatures.

The fractional denaturation on scanning to heat shock temperatures and immediately cooling is given directly by Figs. 8 *B* and 9 *B* because denaturation appears to be nearly

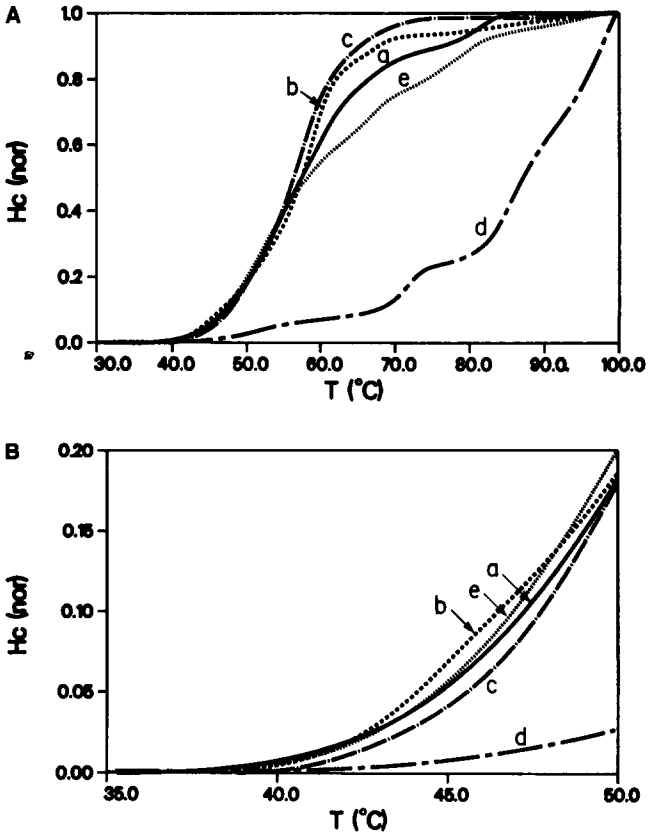

Figure 9. Normalized ΔH_{cal} (approximate fractional denaturation) vs temperature for rat liver homogenate (*a*), microsomes (*b*), mitochondria (*c*), nuclei (*d*), and cytosol (*e*) (*A*). Enlargement of the region from 35–50°C (*B*).

completely irreversible in cells (Fig. 5). About 5.5% denaturation is predicted to occur in homogenate, and the values range from 4 to 7% in microsomes, mitochondria, and cytosol on scanning to 45°C. Significantly less denaturation (1%) occurs in isolated nuclei. The estimated denaturation on scanning to 43, 45, and 47°C for each cell and organelle is given in Table II. Because denaturation at these temperatures is dependent on time of exposure (Lepock et al., 1988), significantly more denaturation will occur during longer exposures to each temperature.

Table II. Estimated Fractional Denaturation on Scanning to 43, 45, and 47°C as Determined from the Curves of ΔH_{cal} vs Temperature (Figs. 8 and 9)

	Fractional denaturation		
	43°C	45°C	47°C
		%	
Homogenate	2.8	5.5	9.5
Hepatocytes	1.8	4.5	8.5
Microsomes	3.2	7.0	11
Mitochondria	1.9	4.3	8.1
Nuclei	0.4	0.8	1.4
Cytosol	2.8	5.8	10
V79 Cells	2.8	5.5	9.5

Discussion

An unanswered question of critical importance for understanding the heat shock response is whether or not protein denaturation actually occurs in cells at relevant heat shock temperatures. In addition, once denaturation is demonstrated, it is necessary to determine how many proteins denature, where they are localized within the cell, and their identity. Protein denaturation is defined as the unfolding of proteins from the native state to a more random state of lower organization. Most proteins appear to retain more structure after thermal denaturation than after denaturation by strong chemical denaturants (e.g., urea), and the thermally denatured state for some proteins is similar to the molten globule state (Ptitsyn, 1992). In cells, the unfolding is almost completely irreversible, due either to the very high protein concentration within cells or to the presence of proteins whose native state is not the state of lowest energy. The high intracellular protein concentration should promote irreversibility by increasing the aggregation of unfolded proteins.

A number of endothermic transitions are observed by DSC, with a first detectable deviation of excess C_p from the baseline at 37–38°C and an onset of the transitions (T_i), determined by extrapolation, of ~40°C in intact hepatocytes, V79 fibroblasts, rat liver homogenate, and isolated microsomes, mitochondria, nuclei, and cytosol. These transitions are irreversible, except for the *D* transition of nuclei which is at least partially due to the reversible unfolding of DNA. The calorimetric enthalpy (ΔH_{cal}) is high (5–6 cal/g), in the range of that found for the denaturation of proteins (Privalov and Khechinashvili, 1974). ΔH_{cal} (per g) for the unfolding of DNA and highly-structured RNA (e.g., tRNA) is similar; thus, these components should contribute to the profiles in proportion to their relative concentration within each organelle. Other possible enthalpic contributions, such as polymerization and depolymerization, should be relatively small (e.g., gelation of deoxyhemoglobin S has a maximum enthalpy of <0.1 cal/g [Ross et al., 1975]). Thus, protein denaturation would be expected to be the major transition of the profile; however, for a system as complex as a cell many other factors must be considered.

The first is the effect of pH changes. The ΔpK_a for Hepes is −0.014 pH U/°C. Thus, the extracellular pH will drop by 1.4 U over the scan range of 100°C, and it must be assumed that the internal pH will change by a similar amount. A decrease in pH can affect the DSC profile in two ways: (*a*) by heats of ionization and (*b*) by altering T_m and ΔH_{cal} for the individual transitions. The lack of detection of significant ionization effects on the rescan suggest that the first factor is not important. Any shifts in T_m should be small and should not influence the final conclusions because the pH change from 40 to 70°C, the region over which nearly all transition are observed, is only 0.4. In addition, heats of ionization are small compared to denaturation (Privalov, 1992) and the shift in T_m between pH 6–8 is small for globular proteins (Privalov and Khechinashvili, 1974). The heat of ionization for lysozyme is linear from pH 1 to 5 and for a pH change of 1 is ~1% of the calorimetric enthalpy of denaturation. Thus, any effects of decreasing pH from 40 to 50°C, the region of interest for heat shock, must be insignificant.

Lipid transitions are endothermic and detectable by DSC

if of sufficient strength. There are no lipid transitions detectable by spin label or fluorescent probes in V79 cell membranes > 40°C (Lepock et al., 1983, 1987). DSC scans of a number of *Bacillus* species clearly show lipid transitions centered 20–30°C below the onset of protein denaturation (Lepock et al., 1990 *b*). The area of the lipid transitions in *Bacillus* is < 10% of the total area of the profile. Thus, it is unlikely that lipid transitions have a significant effect on the DSC profiles of mammalian cells above 40°C.

Most proteins aggregate when denatured and this is one of the major factors causing irreversibility. Aggregation is exothermic and should alter the DSC profiles of cells and organelles. These are no obvious exothermic transitions that can be associated with aggregation in any of the profiles shown here. However, there is an exotherm in erythrocytes that is apparently due to the aggregation of hemoglobin after denaturation (Lepock et al., 1989). The area of this exotherm is 10–15% of the ΔH_{cal} of denaturation. Thus, is seems likely that the total ΔH_{cal} for cells is reduced by at least 10–15% due to aggregation of denatured proteins. This induces an error of similar magnitude in the values of fractional denaturation shown in Table II. However, this error is small compared to that caused by assuming a constant value of ΔH_{cal} for the denaturation of each protein.

Aggregation and irreversibility lead to errors in determining ΔH_{cal}. Thus, this quantity cannot be viewed as the true enthalpy of the system, which is only defined for reversible processes, but is just a measure of the heat flow into the system during denaturation.

Thermally-induced denaturation of proteins at physiological salt and pH can be treated as a transition or series of transitions form the native to an unfolded state thought to be approximated by a random coil (Privalov, 1992) but possibly containing significant secondary structure (Ptitsyn, 1992). Each of the four main peaks of the DSC profile of the erythrocyte membrane has been shown to be due to the denaturation of a single protein or group of proteins with significant unfolding and loss of native structure (Brandts et al., 1977; Lysko et al., 1981).

The DSC profiles shown here can be interpreted in a similar manner. This leads to the question as to whether there has been any selection for a specific degree of thermal stability or if protein stability is completely random as long as proteins are stable enough to remain folded at the growth temperature. This can be determined by analyzing the distribution of T_m's. For cells, homogenate, and most of the organelles, the majority of protein denaturation occurs between 45 and 65°C. The profile for liver homogenate can be fit by a sum of transitions with a distribution of T_m's described by a Poisson distribution starting at 40°C and centered at 55°C. This distribution implies nonrandom selection for proteins with T_m's averaging ~20°C higher than the growth temperature of 37°C. Thus, it is not sufficient for cellular proteins simply to be stable at 37°C, but a specific stability has been selected. A measure of this stability is the difference between the T_m of denaturation and 37°C.

The reason for the relatively low average stability of T_m = 55°C is unknown. It may be that low stability is associated with a high degree of plasticity and flexibility which may be necessary for the proper function of proteins in cells such as hepatocytes and fibroblasts. Nearly all proteins in hepatocytes and fibroblasts adapted to culture denature <65°C except for histones in chromatin. However, this is not true for all tissues. Human hemoglobin, which dominates the DSC scan of erythrocytes, denatures with a T_m of 72°C (Lepock et al., 1989), and the average T_m of bovine lens is ~70°C (results not shown).

Denaturation of proteins in cells is highly irreversible, even during exposure to 43–45°C for short periods of time. Unfolding is usually reversible; irreversibility is then caused by a subsequent step. At high temperature this may be due to covalent modifications, but it usually involves the formation of improper conformations (Arhn and Klibanov, 1985; Zale and Klibanov, 1986) due either to aggregation or disulfide exchange (Lysko et al., 1981; Hahn et al., 1992).

Irreversible denaturation can be modeled by

$$N \rightleftarrows D \rightarrow I$$

where *N*, *D*, and *I* represent the native, reversibly unfolded, and irreversibly denatured forms, respectively. Irreversibility is presumably necessary for lethality because any reversibly unfolded protein should refold on return to 37°C. Any reduction in the rate of $D \rightarrow I$, by Hsp's or other compounds or conditions, should result in increased cellular resistance to heat shock.

Denaturation is observed in each isolated organelle with an onset temperature T_i = 40°C, as found in whole cells. The DSC profile of each organelle is distinct and contributes specifically to different temperature regions of the DSC profile of intact cells. Microsomes and mitochondria have strong transitions in the region of the *B* peak, suggesting that denaturation in membranes is a major contribution to this peak. The *C2*, *D*, and *E* peaks are due primarily, or exclusively, to the denaturation of nuclear components. The cytosolic fraction remaining after centrifugation at 204,000 *g* for 60 min is the major component contributing to the *A* peak, the region of interest for heat shock; however, all isolated organelles contain thermolabile proteins denaturing in this region.

~5% protein denaturation, subject to the errors discussed above, is found in each isolated organelle, except for nuclei, and the intact cells on heating to 45°C at 1°C/min. Because denaturation is irreversible, it must depend on time of exposure. Results from V79 cells show that denaturation is increased by about one half after 10–30 min exposure to 43 or 45°C (Lepock et al., 1988). Thus, 5–10% protein denaturation occurs after even modest heat shocks. This can be viewed as massive levels of denaturation distributed relatively evenly throughout the cell. The lower level of denaturation (1%) detected in isolated nuclei may be underestimate of the level *in vivo* because there is evidence that a considerable amount of loosely associated protein is lost during isolation of nuclei (Cook and Jackson, 1988).

Thus, relatively modest exposures denature a significant amount of cellular protein. All denatured protein may contribute to the induction of Hsp synthesis, but this need not be the case for cell killing. The inactivation of one or a small number of thermolabile proteins, necessary for cell survival, may be sufficient for killing. These are referred to as the critical target, and we predict a T_m of ~46°C for the inactivation of the critical target for cells with a thermal sensitivity similar to CHL V79 cells.

If the protein denaturation observed by DSC relates to the heat shock response, then conditions that sensitize cells to

heat killing and induce Hsp's should lower the onset of denaturation (T_l) whereas conditions that protect cells from killing and inhibit Hsp induction should increase T_l. This is true for short chain alcohols which induce Hsp synthesis and lower T_l and glycerol and D_2O which inhibit induction (Edington et al., 1989) and raise T_l (Lepock et al., 1988; Borrelli et al., 1992). In addition, the DSC profiles of cells made thermotolerant by both a prior heat treatment followed by incubation at 37°C and by exposure to cyclohexamide are shifted to higher temperatures by 1-1.5°C (Lepock et al., 1990*a*; Borrelli et al., 1991). This is the degree of stabilization predicted by a survival assay. Thus, the most thermolabile proteins are stabilized in thermotolerant cells, demonstrating a direct correlation between heat sensitivity and protein denaturation under those conditions.

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