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Destabilization of the Ca^{2+}-ATPase of Sarcoplasmic Reticulum by Thiol-Specific, Heat Shock Inducers Results in Thermal Denaturation at 37 °C†

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ABSTRACT: A number of protein reactive compounds, including the thiol reagents diamide and arsenite, are known inducers of heat shock protein (HSP) synthesis and thermotolerance. These compounds are thought to damage cellular protein, which has been proposed to serve as the signal for induction. The specific mechanism of protein damage and its relation to thermal denaturation are unknown. The Ca^{2+}-ATPase of sarcoplasmic reticulum, a membrane protein that contains 24 cys residues, was used to determine the effect of diamide, arsenite, *N*-ethylmaleimide (NEM), and the cys-specific probes Br-DMC and IAEDANS, which label one or two specific cys residues, respectively, on protein conformation and stability. The Ca^{2+}-ATPase was chosen because diamide has been shown to affect the thermal properties of a class of membrane proteins of CHO cells (Freeman et al., 1995). The labeling of one or two thiols has no effect on activity or conformation, while more extensive reaction (but with less than approximately five to eight groups titrated) results in destabilization of the Ca^{2+}-ATPase such that it denatures thermally at 37 °C. Higher levels of titration result in greater destabilization such that the protein is no longer stable at room temperature, with the production of a state similar to the thermally denatured state as assayed by activity, differential scanning calorimetry, ANS binding, and light scattering. The fractional denaturation induced by these thiol reagents, determined by the decrease in the heat absorbed during thermal denaturation, is directly proportional to inactivation of ATPase activity. Thus, inactivation of the Ca^{2+}-ATPase by thiol reagents occurs because of denaturation not through oxidation of essential thiols. These results indicate that these thiol-specific heat shock inducers function by two mechanisms: (1) destabilization of proteins such that they thermally denature at 37 °C and (2) direct denaturation, apparently driven by thermal processes at room temperature, following more extensive reaction which results in extreme destabilization. We suggest that these are general mechanisms by which heat shock inducers damage proteins.

In eukaryotes, both thermal and non-thermal stress induce transcription of heat shock genes through multistep activation of heat shock factor-1 (HSF-1)[1] (Zuo et al., 1995; Morimoto et al., 1994; Baler et al., 1996). An initial step involves conversion of monomeric HSF-1 to a trimer with subsequent acquisition of DNA binding and transactivational activities. The work of Hightower et al. (1981, 1985) led to the hypothesis that a common feature of thermal and nonthermal stresses that induce heat shock protein (HSP) synthesis (e.g., arsenite, diamide, amino acid analogs, puromycin, short-chain alcohols, anesthetics, or release from hypoxia) was the production of "abnormal" or denatured protein. Direct experimental support for this hypothesis was obtained by a number of investigators. Goff and Goldberg (1985) demonstrated that the heat shock regulatory gene *htpR* was required for transcription of a lon-lacZ operon fusion in *Escherichia coli* cells synthesizing aberrant polypeptides generated by exposure to canavanine, puromycin, or streptomycin. Ananthan et al. (1986) co-injected native or reduced carboxymethylated bovine β-lactoglobulin or BSA plus a *Drosophila* hsp 70-galactosidase hybrid gene into frog oocytes. Only injection of chemically modified proteins resulted in activation of the hybrid gene. Mifflin and Cohen (1994) extended these results by microinjecting a number of different types of chemically modified proteins in the presence of an hsp 70 reporter gene. They found that induction was independent of the type of protein or the type of chemical modification provided that the modified protein underwent aggregation.

The studies reported here were undertaken in order to delineate the mechanisms occurring during chemical modification that lead to aggregation such that the denatured protein can act as a signal for the induction of the stress response. Protein denaturation can be defined as a first-order transition from the native state of low entropy to a

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[1] Abbreviations: HSE, heat shock element; HSF-1, heat shock transcription factor-1; HSP, heat shock protein; Br-DMC, 4-(bromomethyl)-6,7-dimethoxicoumarin; IAEDANS, 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; NEM, *N*-ethylmaleimide; ANS, 1-anilinonaphthalene-8-sulfonate; SR, sarcoplasmic reticulum; LSR, light sarcoplasmic reticulum; DSC, differential scanning calorimetry.

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more disordered state of higher entropy. In some cases the denatured state can be approximated by a completely unfolded, random-walk chain (Privalov, 1979) and in others by partially ordered, compact intermediates such as the molten globule (Ptitsyn, 1992). Thiol-specific reagents were used to restrict the chemical modifications to cysteine residues. With the exception of NEM (Levinson et al., 1980), each of these compounds and several other thiol reagents (diamide, menadione, arsenite, nephrotoxic cysteine conjugates, and IAA) have been shown to activate HSF-1 and induce transcription of HSP 70 (Levinson et al., 1980; Chen et al., 1992; Freeman et al., 1995; Liu et al., 1996; McDuffee et al., 1997).

The Ca^{2+}-ATPase, a membrane protein of sarcoplasmic reticulum (SR), was chosen to investigate denaturation by sulfhydryl specific reagents because of previous observations of diamide-induced denaturation in whole cells. Differential scanning calorimetry (DSC) of several lines of intact tissue culture cells yields profiles of excess specific heat (C_p) vs temperature that are characterized by five main peaks which have been labeled A−E (Lepock et al., 1993). Each peak is composed of the sum of numerous order−disorder transitions, but due to either strong interactions between components or a fortuitous superposition of transitions there is a unique transition temperature for each peak. Exposure of whole cells to diamide produces a characteristic change in the DSC profile (Freeman et al., 1995): there is a loss of intensity in one specific peak (peak B) that, in the absence of chemical stress, exhibits a denaturation transition temperature (T_m) of 60 °C. In addition, diamide also causes the appearance of a new low-temperature peak, characterized by a T_m of approximately 40 °C, that suggests protein destabilization. DSC analyses of isolated subcellular fractions and whole cells have identified this class of proteins as primarily representative of membrane proteins (Lepock et al., 1993) and demonstrated that they are distinct from the thermolabile proteins denatured by heat shock (Freeman et al., 1995).

The Ca^{2+}-ATPase contains 24 cysteine residues (Brandl et al., 1986). Thus, it satisfies the minimal requirements for the class of proteins preferentially modified by diamide in whole cells. In addition, inhibition of the Ca^{2+}-ATPase of ER activates NF-κB, a stress responsive transcription factor, which induces the transcription of target genes such as cytokines, immunoreceptors, and adhesion molecules (Pahl & Baeuerle, 1996). Thus, inactivation of this protein can activate a stress response. We demonstrate that modification of protein thiols of the Ca^{2+}-ATPase lowers the temperature for thermal denaturation. Exceeding this temperature results in a first-order transition (denaturation) to a disordered state of higher entropy with the exposure of hydrophobic domains resulting in protein aggregation.

MATERIALS AND METHODS

Sarcoplasmic Reticulum Isolation. Sarcoplasmic reticulum (SR) was isolated from the hind leg and back muscles of New Zealand white rabbits as described by Eletr and Inesi (1972). The light (LSR) fraction was further purified by using a sucrose density gradient (Campbell et al., 1980). SDS−PAGE was used to access the purity of the Ca^{2+}-ATPase of each isolation. For each experiment, the LSR sample was washed to remove sucrose and DTT and resuspended in a buffer consisting of 10 mM TES, 100 mM KCl, 1 mM EDTA, at pH = 7.0.

Chemical Modification of the Ca^{2+}-ATPase. SR (0.66 mg of protein/mL) in 10 mM TES, 100 mM KCl, and 1 mM EGTA (pH 7.0) was incubated with the desired concentration of diamide or arsenite for 1 h at 22 °C. A 100 mM stock solution of 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS) in 30 mM MOPS buffer, 5 mM $MgCl_2$, 100 mM KCl at pH = 7.0 was added to SR (3 mg of protein/mL) at 0.25 mM and incubated for 1 h at 22 °C (Birmachu et al., 1989). A 30 mM stock solution of *N*-ethylmaleimide (NEM) was added to SR (3 mg of protein/mL) at the desired concentration in a solution of 40 mM TES, 100 mM KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$ at pH = 7.0 and incubated for 30 min at 30 °C (Kawakita & Yamashita, 1987). A 50 mM stock solution of 4-(bromomethyl)-6,7-dimethoxycoumarin (Br-DMC) in dimethylformamide was added to SR (5 mg of protein/mL) at 0.5 mM in 10 mM TES, 100 mM KCl, and 1 mM EGTA at pH = 7.0 and incubated for 1 h at 22 °C (Stefanova et al., 1992). Similar treatment of the SR in dimethylformamide alone (1%) had no effect on ATPase activity or the DSC profile. After incubation the excess Br-DMC was removed by first pelleting undissolved Br-DMC at 10 000 rpm. Soluble Br-DMC and the other unreacted chemicals were removed by washing twice with 10 mM TES, 100 mM KCl, 1 mM EGTA pH = 7.0 at 4 °C (centrifugation for 20 min at 45 000 rpm, and the pellet was resuspended in the same buffer for analysis).

Enzyme Activity. Calcium dependent ATPase (Ca^{2+}-ATPase) activity was assayed by monitoring the absorbance of NADH (λ = 340 nm) using an ATP-regenerating, coupled enzyme system as done previously (Lepock et al., 1990).

Erythrocyte Preparation. Human erythrocytes were purified from freshly drawn human blood by washing three times in a 10-fold excess of phosphate-buffered saline (137 mM NaCl, 3 mM KCI, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH = 7.4). White blood cells were removed by aspiration after each centrifugation. Erythrocytes were suspended in the same solution for the DSC studies, and the cells were incubated in 0.1−1.0 mM diamide for 1 h at 22 °C before scanning.

Differential Scanning Calorimetry. A Microcal-2 high-resolution differential scanning calorimeter (DSC) was used to obtain all scans at a protein concentration of 2−5 mg/mL for SR and 4−8 mg/mL for erythrocytes at a scan rate of 1 °C/min. The noisier scans were smoothed by fast Fourier transform filtering. In all cases the samples were rescanned and this scan, which showed no transitions, was subtracted from the first scan to correct for intrinsic base line curvature. The change in specific heat (ΔC_p) upon denaturation was corrected as previously described (Lepock et al., 1990). All curves were then fitted for irreversible denaturation assuming two-state, irreversible denaturation of the form $N \xrightarrow{k_i} D$ with the data points of the exothermic transition removed before fitting (Lepock et al., 1990). The parameters T_m (temperature of half-completion), Q (total heat absorbed), A (Arrhenius intercept), and E_a (inactivation energy) were obtained from the best fit curves.

Sulfhydryl Titration. The number of free sulfhydryl groups was determined using 5,5′-dithiobis(2-nitrobenzoic acid)

(DTNB) following the procedure described by Janatova et al. (1968).

Polyacrylamide Gel Electrophoresis (SDS−PAGE). A solution of 0.66 mg/mL SR protein in 10 mM TES, 100 mM KCl, 1 mM EGTA (pH = 7) buffer was incubated for 1 h at room temperature with diamide at concentrations of 0, 2, 4, and 6 mM. After being washed to remove the diamide, the pellets were resuspended in the same buffer at 2 mg of protein/mL. 100 μL samples of these suspensions were added to 40 μL of either reducing buffer (1.25 M sucrose, 0.1 M DTT, 0.25 M Tris/HCl, pH = 6.8, 5% SDS, 0.01% bromophenol) or non-reducing buffer (1.25 M sucrose, 0.25 M Tris/HCl, pH = 6.8, 5% SDS, 0.01% bromophenol) and 60 μL of distilled water. The suspension was then heated for 10 min at 100 °C, and solid DTT added to the sample in reducing buffer to raise the final concentration of DTT to 100 mM. All samples were then sonicated for 10 s in a probe sonicator, and 10 μg of each sample was analyzed on a 10% polyacrylamide SDS gel with a 3.75% stacking gel. The intensity of the bands was determined by scanning densitometry.

1-Anilinonaphthalene-8-sulfonic Acid (ANS) Fluorescence and Light Scattering. All ANS fluorescence measurements were performed in an SLM spectrofluorometer (SLM 4800S) with an excitation wavelength of 380 nm and an emission wavelength of 470 nm. A protein concentration of 0.15 mg/mL in 10 mM TES, 100 mM KCl, 1 mM EGTA, and 100 μM ANS at pH = 7.0 was used.

The light-scattering measurements were performed in the same SLM spectrofluorometer with both the excitation and emission wavelengths set to 450 nm at the same protein concentration in the same solution. The ANS fluorescence and light-scattering curves were fit using the exponential function

$$Y(t) = Y_0 + A(-e^{-k_i t})$$

where $Y(t)$ is the fluorescence or scattering at time t, Y_0 is the fluorescence or scattering at $t = 0$, A is a constant equal to the maximum increase in intensity, and k_i is the rate constant of increase in fluorescence or light scattering.

Gel Mobility Shift Assay. Human HepG2 cells were incubated at 37 °C in phosphate-buffered saline for 15 min in the presence of various concentrations of NEM. After the treatment, cells were extracted with buffer containing 10 mM HEPES, 422 mM NaCl, 0.1 mM EGTA, 5.3% glycerol, 0.5 mM PMSF. Binding reactions were performed by adding 20 μg of cell extract to 0.2 ng of ^{32}P-labeled, double-stranded oligonucleotide containing rat Hsp 70 heat shock element (Liu et al., 1993) in binding buffer [20 mM Tris (pH 7.5), 100 mM NaCl, 1 mM DTT, 10% glycerol, 0.05% NP-40, 5 mM $MgCl_2$, 0.05 μg of poly dI-dC] and incubating at 20 °C for 15 min. Competition binding reactions were performed by addition of a 100-fold excess of unlabeled HSE to the binding reaction. Extracts were pretreated with HSF-1 antisera (Affinity Bioreagents) prior to binding reactions.

Northern Blot Analysis. Total RNA was isolated from cesium chloride gradients following homogenization in guanidinium thiocyanate. The isolated RNA, 10 μg per lane, and a RNA ladder were fractionated by electrophoresis in a 1.1% agarose/2.2 M formaldehyde gel, blotted onto nitrocellulose, and baked. Prehybridization and hybridization with ^{32}P nick-translated 2.3 kb *Hin*dIII/*Bam*HI fragment from human hsp 70 (ATCC) were performed at 42 °C. Blots were washed twice at room temperature in 2× SSC/0.1% SDS and twice at 42 °C in 0.1× SSC/0.1% SDS. Blots were then stripped and reprobed with a ^{32}P nick-translated cDNA to chicken β-actin.

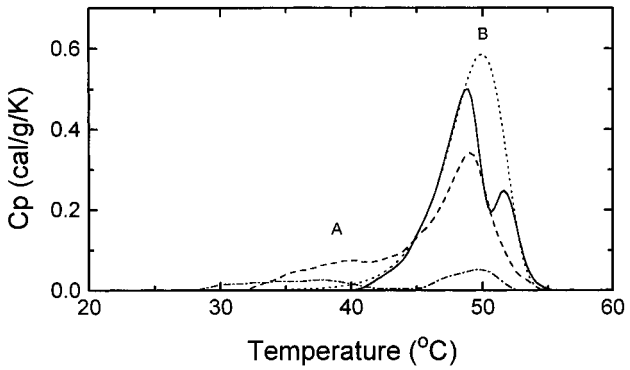

FIGURE 1: DSC scans (apparent excess C_p vs temperature) of the Ca^{2+}-ATPase after 1 h incubation at 0 (—), 1 (- - -), and 5 mM (— · —) diamide. The dotted line (···) is the best fit curve for the control.

RESULTS

Denaturation

(i) Diamide. The effect of chemical modification on the thermal properties of the Ca^{2+}-ATPase of LSR was determined by DSC. The purity of the ATPase was assayed by SDS−PAGE and found to range between 80 and 90% (data not shown). A DSC profile of native Ca^{2+}-ATPase (solid line) is shown in Figure 1. The doublet appearance of the native profile is due to a sharp exothermic transition superimposed on the endothermic transition of denaturation as discussed previously (Lepock et al., 1990). The temperature of the exotherm was variable between 50 and 55 °C, and the heat released, as determined by area, was approximately 0.70 cal/g. The cause of the exotherm is not known with certainty but is presumably due to a step during protein aggregation.

The profile obtained from native ATPase (peak B) was fitted using a irreversible two-state denaturation model (N → D, dotted line). An activation energy (E_a) of 110 kcal/mol was obtained from the irreversible fit, which is consistent with previous results (Lepock et al., 1990). The area under the fitted curve represents the heat absorbed during denaturation (Q) and for the native ATPase was equal to 2.75 cal/g. Since denaturation is not reversible, Q is not necessarily the same as the enthalpy (ΔH) for a reversible process. A transition temperature (T_m), defined as the temperature at which $Q(T)$ is one-half of the total Q, was calculated from the fitted curve and was determined to be 48.7 °C. An excellent correlation exists between the thermodynamic parameters describing irreversible thermal denaturation and thermal inactivation of ATP hydrolysis, demonstrating that thermal inactivation is due to thermal denaturation (Lepock et al., 1990).

The Ca^{2+}-ATPase was incubated with 0.1−5 mM diamide for 1 h at 22 °C and then subjected to DSC analysis. The DSC curves obtained after treatment with 1 and 5 mM diamide are shown in Figure 1. Diamide-induced thiol oxidation (quantitated in Table 1) caused significant alteration

Table 1: Free Sulfhydryl Groups of the Ca^{2+}-ATPase Titratable by 5,5′-Dithiobis(2-nitrobenzoic Acid) (DTNB) after 1 h Incubation with Diamide, Arsenite, or NEM

drug	concentration (mM)	SH/ATPase	minimum number of SH groups reacting
diamide	0	14.5	−
	0.1	10.6	4
	0.5	7.7	7
	1	6.4	8
	2	0	14
arsenite	0	14.5	−
	2	10	4
	4	8.0	6
NEM	0	16.0	−
	0.2	15.2	1
	0.4	11.3	5
	0.8	9.2	7

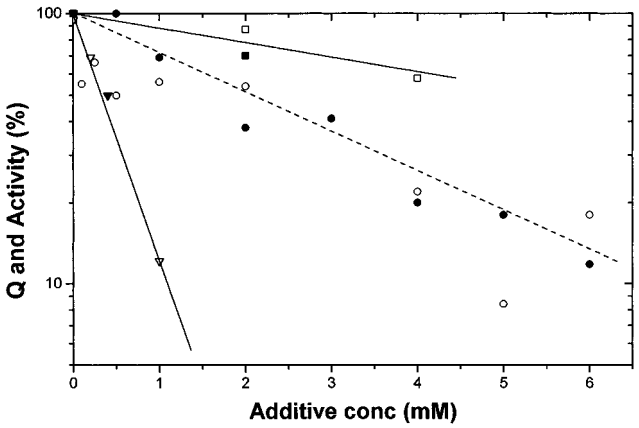

FIGURE 2: Semilog plot of the area (Q, open symbols) under the main peak B (T_m = 48 or 49 °C) of the DSC curves and the ATPase activity (closed symbols) expressed as percent of the control as a function of diamide (circles), arsenite (squares), and NEM (triangles) concentration. The values of Q (open symbols) were calculated from the area under peak B in the fitted DSC curves.

of the DSC profile: (1) The area under peak B was diminished. (2) The T_m for this peak was decreased by approximately 1 °C. (3) A new peak (A) characterized by a T_m of approximately 40 °C appeared. (4) The exotherm disappeared. The normalized value of Q of peak B, determined by deconvolution of the profile, as a function of diamide concentration is shown in Figure 2.

As shown in Table 1, exposure to 1 mM diamide oxidized at least eight of 14 solvent accessible thiol groups. While the ATPase contains 24 cysteines, only 14 are accessible to water soluble probes such as DTNB (Ariki & Shamoo, 1983). We obtained values of 14−16. Thus, only the minimum number of thiols reacting with diamide can be determined.

The reduction of diamide requires the oxidation of two thiols. To determine if diamide oxidation of the Ca^{2+}-ATPase produced inter- and/or intramolecular disulfide cross-links, the protein was incubated with varying diamide concentrations and subjected to non-reducing and reducing SDS−PAGE. Figure 3 shows that after treatment with diamide there is an appearance of higher molecular weight material in the gel and a reduction in the intensity of the Ca^{2+}-ATPase monomer band. The low molecular weight aggregates correspond to the band at the interface between the stacking (3.75%) and resolving (10%) gels, and the high molecular weight aggregates correspond to the band at the top of the stacking gel that was unable to enter this 3.75% gel. No discrete bands corresponding to dimers, trimers, etc.

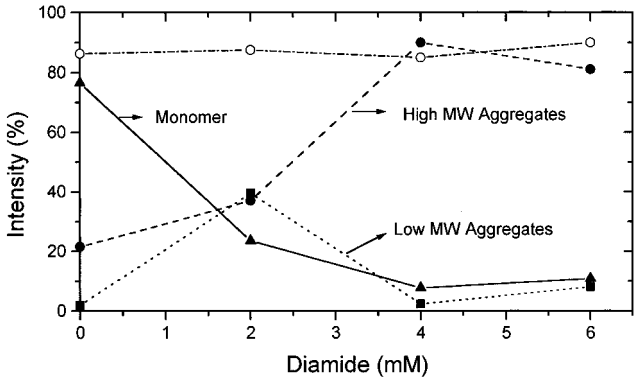

FIGURE 3: Relative intensities of the major bands of non-reducing SDS−PAGE gels of LSR following incubation in diamide for 1 h at room temperature: monomer (▲), low molecular weight aggregates (■), and high molecular weight aggregates (●). Only monomers (○) were present in significant amounts when the same samples were resuspended in reducing buffer with DTT.

were observed. These results confirm that diamide induces intermolecular disulfide bonds with the consequent appearance of high molecular weight material. At 4−6 mM diamide, all of the aggregates were too large to even enter the stacking gel. These aggregates could be solubilized by treatment with the reducing agent DTT at 100 °C and sonication.

The shift of the T_m of peak B from 49 to 48 °C following diamide treatment must be due to destabilization. This low level of destabilization presumably occurs at low levels of thiol modification. The appearance of peak A with $T_m \cong$ 40 °C, determined by deconvolution of the profile (not shown), indicates greater destabilization of the Ca^{2+}-ATPase and was observed for diamide concentrations between 0.1 and 2 mM which modified, on average, four to eight sulfhydryl groups. Denaturation of the Ca^{2+}-ATPase can be approximated by an all-or-none, two-state process (N → D), as is supported by the excellent fit of the two-state, irreversible model. Thus, the transition at T_m = 40 °C indicates either (1) transformation of denaturation from a two-state to a three-state process (N → I → D) where N → I occurs with T_m = 40 °C and I → D with T_m = 49 °C or (2) the formation of a new species (N′) of less stability such that N′→ D occurs with a T_m of 40 °C. Evidence given below supports the second possibility, but either mechanism results in denaturation of the less stable component at 37 °C. Native Ca^{2+}-ATPase with T_m = 49 °C does not denature at 37 °C.

Irreversible curve fitting of peak A gives the parameters E (activation energy) and A in the Arrhenius relation for the rate constant of denaturation (Lepock et al., 1990). The half-life for component A is 8.4 min at 37 °C. Thus, this component is unstable and thermally denatures at 37 °C.

The reason for the disappearance of the exotherm is not known with certainty. However, since aggregation exotherms are commonly observed during DSC studies of denaturation and diamide must cause aggregation (Figure 3), the exothermic event occurring during denaturation presumably occurred during the prior treatment with diamide.

Diamide concentrations in excess of 2 mM resulted in the oxidation of all solvent accessible thiols (Table 1) and a significant decrease in the total area of Peaks A and B in the DSC profile (Figure 1). This relatively flat profile indicates that little native protein was present during the DSC

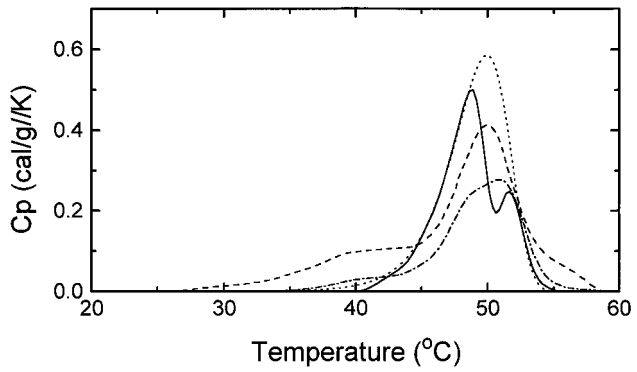

FIGURE 4: DSC scans (apparent excess C_p vs temperature) of the Ca^{2+}-ATPase after 1 h incubation at 0 (—), 2 (- - -), and 4 mM (— · —) arsenite. The dotted line (···) is the best fit curve for the control.

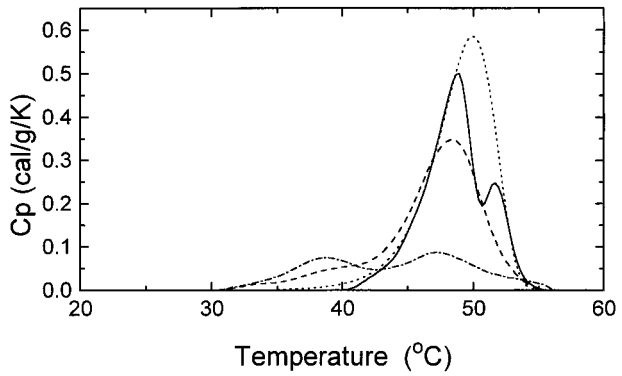

FIGURE 5: DSC scans (apparent excess C_p vs temperature) of the Ca^{2+}-ATPase after 30 min incubation at 30 °C in the presence of 0 (—), 0.4 (- - -), and 1 mM (— · —) NEM. The dotted line (···) is the best fit curve for the control.

scan and signifies that either an irreversible transition to the denatured state occurred during exposure to diamide at 22 °C or that intermolecular cross-linking stabilized the ATPase to such an extent that it could not unfold (denature) over the temperature range assayed to 100 °C. The observation that the T_m did not increase but decreased at low diamide concentrations and the results with arsenite and NEM, which do not cause intermolecular cross-links, argue against the latter hypothesis.

(ii) Arsenite. Compounds of trivalent arsenic react with thiols to form mono- and dithioarsenites (Torchinsky, 1981). Arsenite, unlike diamide, did not induce intermolecular disulfides in the Ca^{2+}-ATPase, as determined by comparison of non-reducing and reducing PAGE (data not shown). However, formation of dithioarsenites would by necessity be intramolecular.

The Ca^{2+}-ATPase was incubated with either 2 or 4 mM arsenite for 1 h at 22 °C and then subjected to DSC analysis (Figure 4). Exposure to 2 mM arsenite altered the DSC profile in a fashion reminiscent of the profile produced by exposure to diamide concentrations less than or equal to 1 mM. There was the appearance of a new peak (A) with a T_m of 40 °C and a decrease in the area of the peak (B) characterized by a T_m of 49 °C. The degree of thiol modification needed to produce this effect is shown in Table 1. Greater thiol modification due to exposure to 4 mM arsenite caused a further reduction in the areas of both peaks A and B, indicative of denaturation occurring during exposure to arsenite at 22 °C.

(iii) N-Ethylmaleimide (NEM). NEM is an alkylating agent that reacts with thiol groups at a stoichiometry of 1:1. Furthermore, it has a more hydrophobic nature than diamide or arsenite and will thus penetrate further into the interior of a folded protein, leading to greater reactivity. Treatment with 0.4 mM NEM labeled approximately five SH groups (Table 1) and yielded a DSC profile similar to that observed after treatment with diamide and arsenite (Figure 5). The T_m of peak B was reduced to 48 °C. This profile appears to be a general characteristic of thiol reactive reagents: a decrease in the area of peak B ($T_m \cong 48$ or 49 °C) and the appearance of a new peak (peak A) with $T_m \cong 40$ °C. The overall area was further reduced after exposure to 1 mM NEM.

Chemical denaturation of the Ca^{2+}-ATPase correlated well with the inhibition of the ATPase activity. A good correlation between the magnitude of peak B and ATPase activity was observed for diamide-, arsenite-, and NEM-induced denaturation (Figure 2). Thus, inactivation of ATPase activity by these thiol reagents is due to the denaturation of the Ca^{2+}-ATPase, not to modification of essential sulfhydryls or steric hindrance by the modifying agent. The order of effectiveness, in terms of inhibiting ATPase activity and reducing Q, is NEM ≥ diamide ≥ arsenite.

The data presented in Figures 1−5 imply that denaturation is a function of the number of thiols modified and possibly the chemical nature of the modifying reagent. An additional parameter most likely includes the spatial location of the modified thiol. Taken together, these data indicate that modification of more than two or three thiols results in a DSC profile exhibiting two peaks (A and B) indicative of destabilization. Further modification results in an increasing reduction in total area indicative of denaturation during incubation at room temperature. The observation that three thiol reactive reagents producing different types of chemical modifications caused the T_m to decrease to the same extent and not increase is not consistent with the hypothesis that the alterations in the DSC profile are a consequence of stabilization produced by cross-linking.

(iv) Probes That Modify a Limited Number of Sulfhydryls. Diamide, arsenite, and NEM are relatively nonspecific in that they will react with all accessible sulfhydryl groups. The effect of limited sulfhydryl modification was examined by reaction of the Ca^{2+}-ATPase with Br-DMC, which specifically labels cys 344 (Stefanova et al., 1992), and IAEDANS, which labels cys 670 and cys 674 (Birmachu et al., 1989). Neither compound affected the DSC profile, ATPase activity, or Ca^{2+} uptake (data not shown). This indicates that labeling of only one or two thiols on the residues cys 344, 670, and 674, even with bulky labels, does not alter conformation in such a way as to affect activity or conformational stability.

Increased Hydrophobicity and Aggregation

The results given above indicate that unfolding of the Ca^{2}–-ATPase, whether native or chemically modified, is thermal in nature and is the consequence of exceeding a specific temperature. Of fundamental importance is the structure of the denatured state. The question of relevance to the stress response is whether the unfolding of native or chemically modified protein exposures hydrophobic residues. The dye ANS can be used as a hydrophobic probe for the study of protein conformational changes because the quantum yield

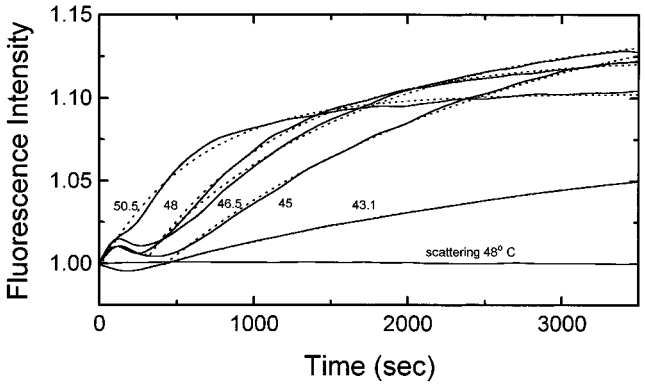

FIGURE 6: ANS fluorescence intensity of SR at temperatures of 43.1−50.5 °C as a function of time (solid lines). The scattering curve was obtained under the same conditions (excitation and emission wavelengths and gain) in the absence of ANS. The dotted lines are the best fit curves to the exponential region.

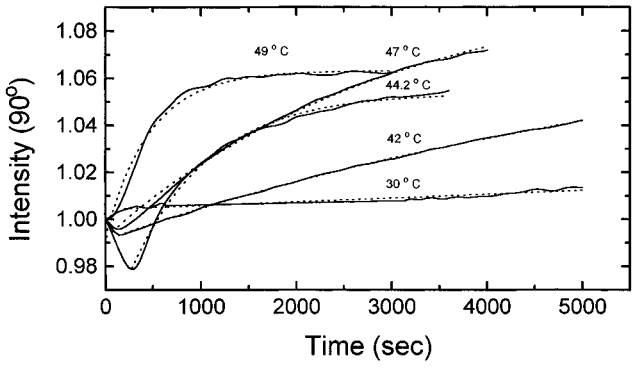

FIGURE 7: Light scattering (90°, λ = 450 nm) of SR heated at temperatures of 30−49 °C as a function of time (solid lines). The dotted lines are the best fit curves to the exponential region.

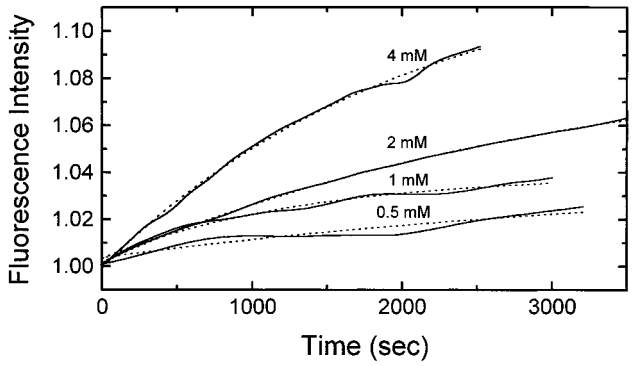

FIGURE 8: ANS fluorescence intensity vs time at 22 °C of SR in the presence of 0, 0.5, 1, 2, and 4 mM diamide (solid lines). The dotted lines are the best fit curves to the exponential region.

of the dye increases significantly after binding to hydrophobic regions of proteins (Cardamone & Puri, 1992).

The data presented in Figure 1 illustrate that the onset for unfolding and denaturation of native Ca^{2+}-ATPase occurs when the temperature exceeds 42−43 °C. The data presented in Figure 6 show that after an initial small increase followed by a dip, the fluorescence intensity of ANS in the presence of SR increased as a function of time at temperatures greater than 43 °C. The initial increase is caused by a 0.5−1 °C drop in temperature when the SR at 0 °C was added to the solution in the cuvette. The fluorescence of ANS bound to membrane lipid is greater at lower temperatures. The original temperature was regained in approximately 5 min, after which the increase in fluorescence was solely due to denaturation of the Ca^{2+}-ATPase. Only points in this exponentially increasing region were used for fitting the curves.

As can be seen, the rate increased with increasing temperature. The curves have been fit to the exponential function described in Materials and Methods. The rate constants obtained from the fits match very closely to the rate of inactivation of ATPase activity and give a predicted transition temperature of 50 °C, calculated as described in Lepock et al. (1990) (results not shown). This value matches the T_m of the Ca^{2+}-ATPase determined from the DSC data (48.7 °C) and confirms that the increase in ANS fluorescence is due to denaturation. Assuming that the plateau in the curves indicates that the conformational change responsible for the increase in ANS fluorescence had gone to completion, at temperatures significantly below the T_m, e.g., 43 °C, unfolding was a rather slow process that did not reach completion over a 3000 (50 min) interval. This is consistent with the predicted rate constants determined from the DSC profile.

When water soluble proteins undergo thermal denaturation, ANS fluorescence increases several fold (Cardamone & Puri, 1992). This was not the case for the Ca^{2+}-ATPase embedded in the SR membrane (Figure 6). Under the conditions used for these experiments, the initial fluorescence intensity, determined at time zero, was the result of extensive ANS binding to membrane lipids. Approximately the same initial level of fluorescence was obtained if ANS was added to liposomes composed of the same amount of lipid (egg phosphatidylcholine) as present in the SR samples (data not shown). The increase in intensity that occurred as the temperature exceeded 43 °C was a consequence of protein unfolding and was not due to a change in lipid ANS binding characteristics since there was no change in ANS fluorescence in the presence of lipid alone (data not shown).

Since the unfolding occurring during thermal denaturation resulted in the exposure of hydrophobic residues, as indicated by the increased ANS fluorescence (Figure 6), one would expect aggregation with an increase in turbidity. Figure 7 illustrates the increase in 90° light scattering that occurred when the Ca^{2+}-ATPase in SR was heated at temperatures in excess of 42 °C. There was little increase in light scattering at or below 30 °C, well below the denaturation temperature. The rate constant for the increase in light scattering at 42−49 °C was similar to that obtained for the increase in ANS fluorescence and is consistent with aggregate formation as a consequence of exposed hydrophobic residues.

The data provided in Figures 8 and 9 illustrate that modification of protein thiols by diamide (Figure 8) and NEM (Figure 9) lowered the temperature to 22 °C at which unfolding and exposure of hydrophobic domains occurred relative to native protein. Neither ANS fluorescence nor 90° light scattering was affected if native protein was incubated at temperaturees below 40 °C. However, treatment with both diamide and NEM also causes an increase in ANS fluorescence (Figures 8 and 9) and a corresponding increase in 90° light scattering. The curves of ANS fluorescence as a function of time during exposure to both diamide and NEM have the same functional dependence as for thermal denaturation. In addition, the rate of increase is dose dependent, increasing as the concentrations of diamide and NEM increase. The maximum increase during exposure to diamide is 10−15%, similar to that for thermal denaturation, sug-

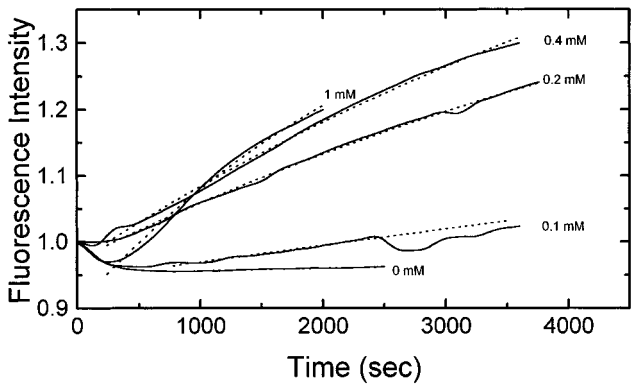

FIGURE 9: ANS fluorescence intensity vs time at 22 °C of SR in the presence of 0, 0.1, 0.2, 0.4, and 1 mM NEM (solid lines). The dotted lines are the best fit curves to the exponential region.

gesting a similar extent of exposure of hydrophobic residues. However, the maximum increase during exposure to NEM is greater than 30%. The curves of 90° light scattering are similar with approximately a 10% and 30% maximum increase during exposure to diamide and NEM, respectively (results not shown). Both the increased ANS fluorescence and light scattering are due to damage to the Ca^{2+}-ATPase since there was no change in ANS fluorescence or light scattering of egg phosphatidylcholine liposomes exposed to 4 mM diamide or 1 mM NEM (results not shown).

Denaturation of Erythrocyte Membrane Proteins by Diamide

Whether or not diamide denatured specific proteins in a whole cell system was investigated with human erythrocytes. Shown in Figure 10 are DSC scans of erythrocytes exposed to diamide (panel A) and an enlargement of the region from 35 to 65 °C (panel B) which contains the denaturation profile of the membrane skeletal proteins. Three weak transitions occurred in the region of 50−65 °C, and a strong transition, due to the denaturation of hemoglobin, was found at T_m = 73.5 °C. The exotherm at 77−78 °C appears to be due to the aggregation of hemoglobin (Lepock et al., 1989). Diamide at 0.5 and 1 mM reduced the area of the hemoglobin peak by 25% and 75%, respectively. In addition, as for the Ca^{2+}-ATPase, the T_m of the hemoglobin transition was reduced 1 °C by 1 mM diamide.

An enlargement of the regions showing transitions A, B, and C is given in Figure 10B. Transition A is due to the denaturation of spectrin, B to the denaturation of several membrane skeletal components including bands 2.1, 4.1, and 4.2 and the cytoplasmic portion of band 3, and C to the denaturation of the transmembrane portion of band 3 (Lysko et al., 1981). Exposure to 0.1 mM diamide had little or no effect on the profile. After exposure to 0.5 mM diamide, the peak representing the transmembrane portion of band 3 was completely lost, the T_m of the denaturation of spectrin (peak A) was decreased by 1 °C, and the combined area of peaks A and B was decreased by approximately 25%. Exposure to 1 mM diamide almost completely eliminated peaks A and B, leaving only very weak peaks at 45 °C, probably a highly destabilized component of spectrin, and at 57 °C, most likely a remnant of the B transition. Thus, the intrinsic membrane protein band 3 is the most sensitive to diamide, followed by the other membrane proteins with hemoglobin being the least sensitive.

Induction of the Heat Shock Response by NEM

The results described above for denaturation of the Ca^{2+}-ATPase, combined with those of Mifflin and Cohen (1994), lead to the hypothesis that any stress that alters conformational stability such that a protein unfolds to expose hydrophobic domains and aggregates must activate HSF-1 and induce synthesis of HSP 70. Such a hypothesis predicts that exposure to NEM should induce a stress response. Induction of HSPs by NEM has not been described to date; rather, it has been reported that NEM does not induce synthesis of HSP 70 (Levinson et al., 1980). However, it is possible to induce the stress response without observable HSP synthesis if the inducing agent is also a general inhibitor of protein synthesis. To test this prediction, human HepG2 cells were exposed to various concentrations of NEM at 37 °C and cell extracts prepared. As shown in Figure 11A, exposure to NEM produced rapid HSF-1 binding activation and increased expression of HSP 70 mRNA (Figure 11B). NEM at 0.05−0.2 mM completely inhibited protein synthesis, which is due to inactivation of eIF 2α (Gross & Rabinovitz, 1973), and there was no detectable synthesis of HSP's (results not shown). These results are consistent with

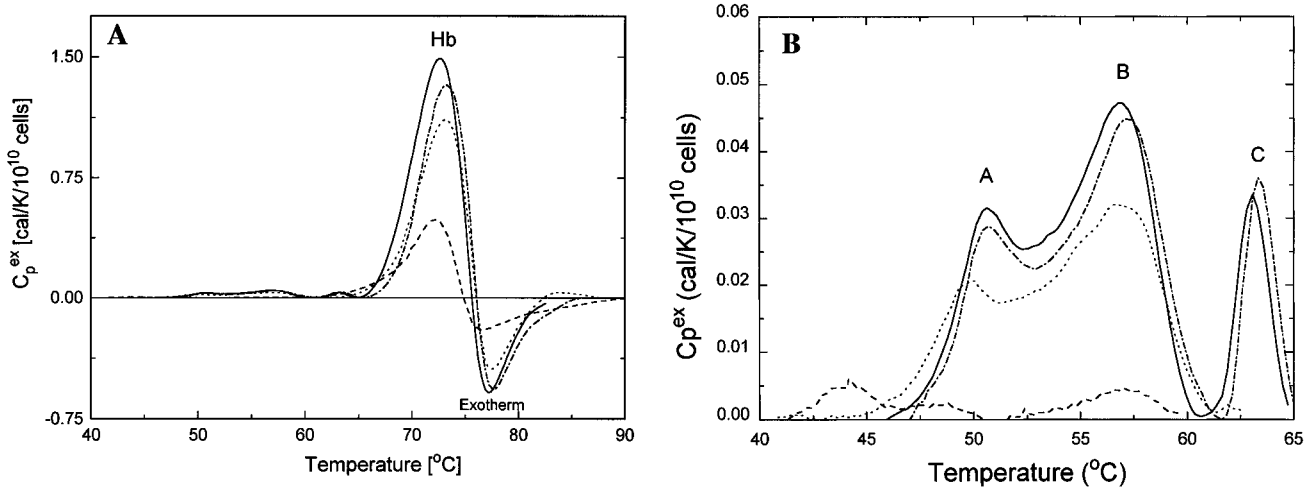

FIGURE 10: DSC scans (apparent excess C_p vs temperature of intact human erythrocytes after 1 h incubation at 0 (—), 0.1 (— · —), 0.5 (···), and 1 mM (- - -) diamide. The temperature ranges plotted are 40−90 °C (A) and 40−65 °C (B) which shows the membrane skeletal transitions only. Hb is the hemoglobin transitions, and A, B, and C are the membrane transitions.

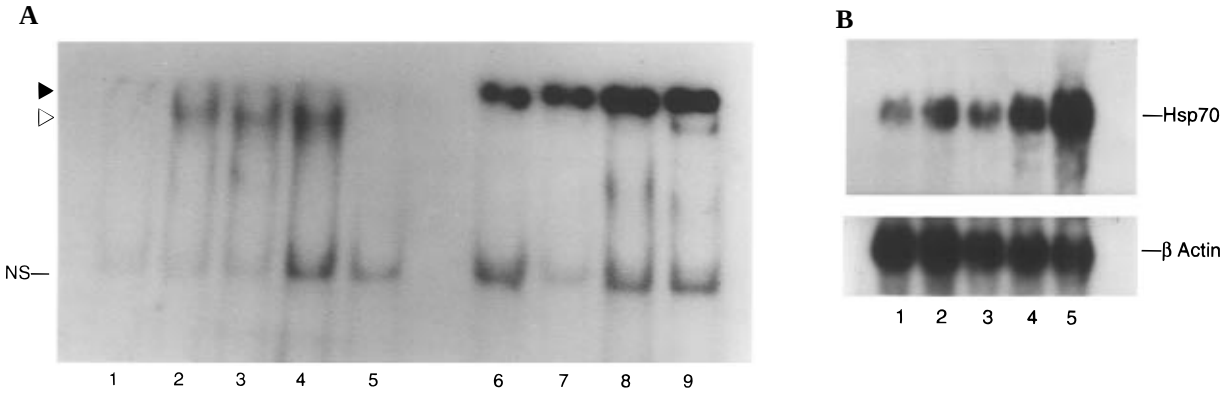

FIGURE 11: (A) Gel mobility shift assay illustrating HSF binding activity following exposure to NEM. Cell incubating at 37 °C in phosphate-buffered saline were exposed to 0 (lane 1), 0.02 (lane 2), 0.05 (lanes 3, 6, and 7), or 0.2 mM NEM (lanes 4, 5, 8, and 9) for 15 min at 37 °C. Specificity of DNA binding was determined by competition with a 100-fold excess of unlabeled HSE (lane 5). The open triangle illustrates HSF−HSE interaction. Addition of polyclonal HSF-1 antisera (diluted 20-fold, lanes 6 and 8, or 100-fold, lanes 7 and 9) to extracts prior to the binding reaction resulted in a super shift, denoted by the filled triangle. NS denotes nonspecific binding. (B) Expression of HSP 70 mRNA following exposure to NEM. Cells were exposed to 0.05 mM NEM for 15 min at 37 °C, washed, incubated at 37 °C in full growth medium for 0 (lane 2), 1 (lane 3), 3 (lane 4), or 5 h (lane 5), after which total mRNA was isolated and subjected to Northern blotting analysis. mRNA obtained from untreated control cells is illustrated in lane 1.

the hypothesis that NEM induces the stress response but inhibits protein synthesis so that there is no HSP synthesis.

DISCUSSION

DSC is a method that directly monitors protein denaturation or unfolding. It can be used to detect and quantitate endothermic transitions in complex structures such as isolated organelles and whole cells (Lepock et al., 1993). The DSC profile obtained from whole cells is characterized by an onset temperature of approximately 40 °C and by five main transitions. The onset temperature is the temperature at which the first endothermic transition can be detected (i.e., the minimum temperature of denaturation). The term denaturation, as used in this discussion, describes a temperature dependent change in conformation from the native state to a more disordered state of higher entropy. A comparison of DSC profiles of denaturation with the minimum temperatures shown to induce HSP 70 in mammalian cells (e.g., 40−41 °C in Hep G2 cells; Freeman et al., 1993) indicates that induction of HSPs by temperatures that exceed physiological (i.e., heat shock) occurs concurrently with the denaturation of thermolabile, cellular proteins with T_m's of approximately 44−48 °C (Lepock et al., 1993). Proteins with these T_m's have an onset of denaturation of approximately 40 °C. Heat shock at 43−45 °C results in a loss of area in the DSC profile in the region from 40−48 °C due to protein denaturation (Lepock et al., 1993). Thus, heat shock denatures a specific set of thermolabile proteins which are characterized by low T_m's corresponding to low conformational stability.

In contrast, thiol-specific reagents that induce HSP 70 at physiological temperatures produce a distinctly different alteration in the DSC profile of whole cells. Modification decreases the area under a peak that is characterized by a T_m of 60 °C (Freeman et al., 1995). In addition, a new peak characterized by a T_m of about 40 °C appears. The $T_m = 60$ °C peak is due predominantly to the denaturation of membrane proteins (Lepock et al., 1993). These results raise the question of how chemically modified proteins, which in the native state exhibit denaturation transitions well above 37 °C, induce a stress response at physiological temperatures.

One possible explanation for the results obtained from whole cells is that modification of protein thiols destabilizes proteins such that there is a decrease in the denaturation temperature T_m to approximately 40 °C, resulting in denaturation at 37 °C. This suggests that unfolding is a function of temperature whether the protein has a native composition or is chemically modified; however, it is not apparent how to demonstrate this in intact cells. This current investigation was undertaken in order to test this concept using the Ca^{2+}-ATPase purified from rabbit sarcoplasmic reticulum. The Ca^{2+}-ATPase consists of 997 amino acid residues and is organized into a cytoplasmic headpiece, a stalk sector, and a transmembrane domain (Brandl et al., 1987). Approximately 200 amino acids are predicted to form 10 transmembrane helices. The majority of the protein is cytoplasmic with 750 amino acids, including 19 of the 24 cys residues, comprising this domain.

The rationale for using Ca^{2+}-ATPase as a model to study protein unfolding by heat shock inducers is based on several points. (1) The Ca^{2+}-ATPase is a membrane protein and membrane proteins in whole V79 cells appear to be the most sensitive category of proteins to diamide. (2) The denatured protein required is nonspecific. The work of Ananthan et al. (1986) and Mifflin and Cohen (1994) indicates that induction of the heat shock response by chemically modified protein did not require that the denatured protein have a direct role in the response or a physiological function in the cell system. Furthermore, neither chemical modification per se nor the ability to form a random coil was a prerequisite for initiation of the stress response; rather, denaturation of any proteins to a state prone to aggregation was sufficient. (3) The Ca^{2+}-ATPase contains 24 cys residues and thus should be sensitive to oxidative damage.

DSC analysis of native, unmodified Ca^{2+}-ATPase yielded a profile that displayed a single transition, characterized by a T_m of 48.7 °C, when the exotherm that is superimposed on the endotherm was removed (Figures 1, 4, and 5). At temperatures that approached T_m (i.e., $T \geq 46$ °C), unfolding was rapid as measured by changes in ANS fluorescence and 90° light scattering, parameters that detect exposure of hydrophobic residues and aggregation (Figures 6 and 7). In

contrast, at temperatures significantly below T_m (i.e., $T \leq$ 43 °C), unfolding was slow and did not approach completion during the 50 min exposure. Unfolding of native protein was not observed at temperatures below 30 °C (data not shown). The interpretation of these results is that as the temperature approached the T_m, native unmodified Ca^{2+}-ATPase unfolded to expose hydrophobic domains, with subsequent aggregation, and the rate at which this occurred was temperature dependent.

Three types of modifications to the DSC profile of chemically modified Ca^{2+}-ATPase were examined: intermolecular disulfides produced by exposure to diamide, intramolecular dithiolarsenites, and alkylation of monothiols by NEM. Qualitatively similar results were obtained using all three reagents. In addition Br-DMC was used to specifically label cys 344 and IAEDANS to label cys 670 and cys 674. For purposes of discussion, let *i* equal the average number of thiol modifications per Ca^{2+}-ATPase. When *i* was 2 or less there was no change in stability [i.e., following Br-DMC and IAEDANS modification only a single transition with a T_m of 49 °C (peak B) was observed]. When *i* was greater than 2 but less than some number *Z*, two transitions were observed: a broad transition with a T_m of about 40 °C (peak A) and the well-defined transition characterized by peak B. Following diamide and NEM modification, the T_m of peak B was decreased by approximately 1 °C indicating a small amount of destabilization, probably due to lesser modification. However, the structure of this species is very similar to the native state since it is still fully active. When $i > Z$, a flat profile was observed, suggesting that denaturation occurred, characterized by a T_m of less than 30 °C, during the time of reaction. In general, one would expect *Z* to be a function of the sulfhydryl reagent. A comparison of the results given in Figure 2 and Table 1 suggests that *Z* is approximately 5−8.

The following model of the DSC profiles was developed on the basis of observations obtained using ANS fluorescence, 90° light scattering, and thiol titration data. The chemical reaction that occurred between the thiol groups of the Ca^{2+}-ATPase and diamide, arsenite, or NEM came to equilibrium rapidly and produced a population of ATPases that contained a specific number of modified thiols. The number of thiols modified per protein most likely exhibits a binomial distribution around a mean. The data presented in Table 1 illustrate the mean values obtained. Modification of the highly reactive, exposed cys residues 344, 670, and 670 had no effect on protein stability. However, treatment with low concentrations of diamide and NEM, presumably resulting in low levels of thiol modification, results in the slight destabilization ($T_m \sim 48$ °C) of the Ca^{2+}-ATPase. A similar degree of destabilization occurs after oxidation with low concentrations of H_2O_2 (results not shown).

Modification of an intermediate number of cys residues results in a species with $T_m \sim 40$ °C. No species with T_m's between 40 and 48 °C were observed. The $T_m \sim 40$ °C species is unstable and denatures with a predicted half-life of 8.4 min at 37 °C.

A rough estimate of the $\Delta\Delta G$ of destabilization can be obtained from the T_m's of the native and modified component and the enthalpy ΔH of the transition. This cannot be done accurately because the irreversibility of denaturation of the Ca^{2+}-ATPase alters the curve shape such that only an approximate value of ΔH can be determined by curve fitting and ΔC_p cannot be determined at all (Lepock et al., 1992). The enthalpy (ΔH) of peak A is approximately 300 kcal/mol, which gives a $\Delta\Delta G$ for this component ($T_m = 40$ °C) of approximately 9 kcal/mol.

The data presented in Figures 7 and 8 indicate that the rate of unfolding could be increased by increasing the concentration of a thiol reagent. This observation combined with the flat DSC profile observed at the highest thiol reagent concentrations suggests that when a larger number of cys residues are modified, the T_m of the protein was shifted below 22 °C, the temperature at which the reaction occurred.

Taken together, the data indicate that the unfolding of both native and chemically modified ATPase resulted in the exposure of hydrophobic domains with the subsequent formation of aggregates. The difference was that modification of protein thiols lowered the transition temperature for destabilization and unfolding. Thus, chemical modification results in thermal denaturation.

The work of Kono et al. (1992) supports the concept that modification of protein thiols can decrease conformation stability. γ-Crystallins were isolated from calf lens and protein thiols modified by reaction with glutathione. Denaturation as a function of temperature was monitored by changes in CD and fluorescence emission. Glutathione modification decreased the $T_{1/2}$ by 2−8 °C. The decrease in stability ($\Delta\Delta G$) was measured using guanidinium hydrochloride denaturation and found to be 0.6−2.4 kcal/mol. This is less than the $\Delta\Delta G$ observed for the Ca^{2+}-ATPase, but γ-crystallins are much smaller with far fewer cys residues.

The erythrocyte results demonstrate that 1 mM and lower concentrations of diamide effectively denature the membrane proteins spectrin, band 3, and other components of the membrane skeleton. In addition, significant denaturation of hemoglobin occurs. Destabilization of spectrin, detected as a decrease in T_m, also occurs as for the Ca^{2+}-ATPase. Previous work has shown that oxidation of spectrin by diamide results in a conformational change in spectrin and cross-linking such that spectrin/band 4.1 binding is reduced (Becker et al., 1986). The erythrocyte has the advantage over other cells (e.g., CHO cells used previously) that the transitions of specific proteins are resolved. The overall sensitivity to diamide was in the order of band 3, membrane skeletal proteins, and hemoglobin. The sensitivity *in vivo* of band 3 and other membrane proteins was greater than the sensitivity of the Ca^{2+}-ATPase *in vitro*. Thus, denaturation occurs not only *in vitro* but *in vivo* in the presence of the normal redox buffering agents.

NEM at 0.02 mM activated HSF-1, while higher concentrations of NEM were used to quantitate denaturation of the Ca^{2+}-ATPase. The curve for NEM given in Figure 2 predicts that approximately 4% denaturation of the Ca^{2+}-ATPase should occur at 0.02 mM NEM. Approximately 1%−2% denaturation of cellular proteins by heat shock is required to induce a heat shock response (Lepock et al., 1993). Thus the amount of protein denaturation of the Ca^{2+}-ATPase by the minimal concentration of NEM required to activate HSF-1 would be expected to be sufficient to induce a heat shock response.

In summary, we have shown that thermal denaturation of the Ca^{2+}-ATPase resulted in unfolding, exposure of hydrophobic residues, and subsequent aggregation. Modification of the ATPase's thiols by a number of different thiol reagents caused a destabilization of protein conformation such that

the temperature for thermal denaturation was lowered significantly compared to the native protein. If such observations can be applied to whole cells, then the activation of HSF-1 and induction of HSP 70 to 27 °C by diamide, menadione, arsenite, IAA, and NEM is a consequence of protein destabilization, resulting in thermal denaturation occurring at physiological temperatures.

REFERENCES

Ananthan, J., Goldberg, A. L., & Voellmy, R. (1986) *Science 232*, 522−524.

Ariki, M., & Shamoo, A. E. (1983) *Biochim. Biophys. Acta 734*, 83−90.

Baler, R., Zou, J., & Voellmy, R. (1996) *Cell Stress and Chaperones 1*, 33−39.

Becker, P. S., Cohen, C. M., & Lux, S. E. (1986) *J. Biol. Chem. 261*, 4620−4628.

Birmachu, W., Nisswandt, F., & Thomas, D. (1989) *Biochemistry 28*, 3940−3947.

Brandl, C. J., Green, N. M., Korczak, B., & MacLennan, D. H. (1986) *Cell 44*, 597-607.

Campbell, K. P., Franzini-Armstrong, C., & Shamoo, A. E. (1980) *Biochim. Biophys. Acta 602*, 97−105.

Cardamone, M., & Puri, N. (1992) *Biochem. J. 282*, 589−593.

Chen, Q., Yu, K., & Stevens, J. L. (1992) *J. Biol. Chem. 267*, 24322−24327.

Eletr, S., & Inesi, B. (1972) *Biochim. Biophys. Acta 282*, 174−179.

Freeman, M. L., Sierra-Rivera, E., Voorhees, G. J., Eisert, D. R., & Meredith, M. J. (1993) *Radiat. Res. 135*, 387−393.

Freeman, M. L., Borrelli, M. J., Syed, K., Senisterra, G. A., Stafford, D. M., & Lepock, J. R. (1995) *J. Cell. Physiol. 164*, 356−366.

Goff, S. A., & Goldberg, A. L. (1985) *Cell 41*, 587−595.

Gross, M., & Rabinovitz, M. (1973) *Biochem. Biophys. Res. Commun. 50*, 832−838.

Haest, C. W. M., Kamp, D., Plaza, G., & Deuticke, B. (1977) *Biochim. Biophys. Acta 469*, 226−230.

Hightower, L. E., & White, F. P. (1981) *J. Cell. Physiol. 108*, 261−275.

Hightower, L. E., Guidon, P. T., Whelan, S. A., & White, C. N. (1985) in *Changes in Eukaryotic Gene Expressions in Response to Environmental Stress* (Atkinson, B. G., & Walden, D. B., Eds.) pp 369−377, Academic Press, Orlando, FL.

Janatova, J., Fuller, J. K., & Hunter, J. (1968) *J. Biol. Chem. 243*, 3612−3622.

Kawakita, M., & Yamashita, T. (1987) *J. Biochem. 102*, 103−109.

Kono, M., Sen, A. C., & Chakrabarti, B. (1990) *Biochemistry 29*, 464−470.

Lepock, J. R., Frey, H. E., Bayne, H., & Markus, J. (1989) *Biochim. Biophys. Acta 980*, 191−201.

Lepock, J. R., Rodahl, M., Zhang, C. H., Heynen, M., Waters, B., & Cheng, K. (1990) *Biochemistry 29*, 681−689.

Lepock, J. R., Ritchie, K. P., Kolios, M. L., Rodahl, A. M., Heinz, K. A., & Kruuv, J. (1992) *Biochemistry 31*, 12706−12712.

Lepock, J. R., Frey, H. E., & Ritchie, K. P. (1993) *J. Cell Biol. 122*, 1267−1276.

Levinson, W., Oppermann, U., & Jackson, J. (1980) *Biochim. Biophys. Acta 606*, 170−180.

Liu, R. Y., Kim, D., Yang, S. H., & Li, G. C. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3078−3082.

Lysko, K., Carlson, R., Taverna, R., Snow, W., & Brandts, J. F. (1981) *Biochemistry 20*, 5570−5576.

McDuffee, A. T., Senisterra, G., Huntley, S., Lepock, J. R., Sekhar, M. J., Meredith, M. J., Borrelli, M. J., Morrow, J. D., & Freeman, M. L. (1997) *J. Cell. Physiol. 171*, 143−151.

Mifflin, L., & Cohen, R. (1994) *J. Biol. Chem. 267*, 15710−15717.

Morimoto, R. I., Jurivich, D. A., Kroeger, P. E., Mathur, S. K., Murphy, S. P., Nakai, A., Sarge, K., Abravaya, K., & Sistonen, L. T. (1994) in *The Biology of Heat Shock Proteins and Molecular Chaperones* ;(Morimoto, R. I., Tissieres, A., & Georgopoulos, C., Eds.) pp 417−455, Cold Spring Harbor Laboratory Press, Plainview, NY.

Nover, L., Scharf, D. K., & Neumann, D. (1989) *Mol. Cell. Biol. 9*, 1298−1308.

Pahl, H. L., & Baeuerle, P. A. (1996) *FEBS Lett. 392*, 129−136.

Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167−241.

Ptitsyn, O. B. (1992) The Molten Globule. In *Protein Folding* (Creighton, T. E., Ed.) pp 243−300, W. H. Freeman and Company, New York.

Stefanova, H. I., East, J. M., Gore, M. G., & Lee, A. G. (1992) *Biochemistry 31*, 6023−6031.

Torchinsky, Y. M. (1981) *Sulfur in Proteins*, pp 46−48, Pergamon Press, Toronto.

Zuo, J. R., Rungger, D., & Voellmy, R. (1995) *Mol. Cell. Biol. 15*, 4319−4430.

BI9711590