Destabilization and denaturation of cellular protein by glutathione depletion

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Abstract This investigation tested the hypothesis that depletion of intracellular glutathione, in contrast to its oxidation, could lead to non-native oxidation of protein thiols, thereby trapping proteins in an unstable conformation. Chinese hamster cells were exposed to the α,β-unsaturated dicarboxylic acid diethylmaleate in order to produce rapid glutathione (GSH) depletion without oxidation. Measurement of the fluorescence of oxidized 2′,7′-dichlorofluorescein diacetate indicated that reactive oxygen species accumulated in GSH depleted cells. Glutathione depletion was found to alter protein thiol/disulfide exchange ratios such that 17 to 23 nmol of protein SH/mg protein underwent oxidation. Differential scanning calorimetry (DSC) of glutathione depleted cells yielded a profile of specific heat capacity versus temperature that was characteristic of cells containing destabilized and denatured protein. In addition, cells depleted of glutathione exhibited a two-fold increase in NP-40 insoluble protein. These results indicate that depletion of intracellular glutathione caused oxidation of protein thiols, protein denaturation and aggregation and provide a mechanism to explain how GSH depletion can initiate stress responses.

INTRODUCTION

Glutathione constitutes the majority of non-protein thiols in cells and participates in many metabolic processes (Meister and Anderson 1983) including reduction and detoxification of hydrogen peroxide generated by endogenous and exogenous oxidative stresses. Reduction of peroxide by GSH results in the formation of GSSG (oxidised GSH) which is then reduced back to GSH by the enzyme glutathione reductase using nicotinamide ademine dinucleotide phosphate, reduced (NADPH) as a co-substrate. During periods of oxidative stress, glutathione depletion can occur if GSSG is generated faster than it can be reduced. Excess GSSG is either transported from the cell or reacts with protein thiols leading to the formation of non-native, intermolecular protein

disulfides (P_1S-SP_2) or GSH-mixed disulfides (Kosower and Kosower 1978). These reactions illustrate two outcomes of glutathione metabolism that should be considered during periods of stress: (i) changes in the GSH/GSSG ratio; and (ii) changes in glutathione concentration.

The total intracellular glutathione concentration can approach 10 mM (Kosower and Kosower 1978). In the cytosol, the ratio of reduced to oxidized glutathione exceeds 10:1 (Meister and Anderson 1983). In contrast, in the endoplasmic reticulum the ratio is approximately 3:1 (Hwang et al 1992). It has been postulated that the specific GSH to GSSG ratios and the high intracellular glutathione concentration function as an intracellular redox buffer. This buffer is thought to contribute to the maintenance of reduced cytosolic protein thiols (Kosower and Kosower 1978; Nishikawa et al 1983; Ziegler 1985; Walters and Gilbert 1986) and the formation of native disulfides in proteins that fold within the endoplasmic reticulum (Hwang et al 1992). A corollary of this hypothesis is that depletion of GSH, in contrast to oxidation, can alter protein thiol redox states in vivo. Recently, Liu et al

(1996) observed that depletion of glutathione was associated with the formation of non-native, intermolecular disulfides. A porcine renal epithelial cell line was exposed to 75 μM ^{14}C iodoacetamide (IDAM) in order to deplete glutathione. Intracellular glutathione was depleted by 80% and 22 nmol protein thiols/mg protein were oxidized. Thiol oxidation was not the result of acetamidylation, suggesting that oxidation of protein thiols was a consequence of GSH depletion.

The significance of the hypothesis that glutathione functions as a redox buffer pertains to the question of whether the conformational stability of a protein can be altered in glutathione depleted cells due to changes in protein thiol redox states. For example, genetically engineered non-native disulfide bond formation increased the conformational stability of phage T4 lysozyme (Matsumura et al 1989). Conversely, random formation of non-native intermolecular disulfide bonds (P_1S-SP_2) and non-native glutathione mixed disulfides (PS-SG) has been shown to destabilize proteins and induce denaturation, which is defined in the context of this investigation as a temperature induced change in conformation from an ordered native state to a partially unfolded intermediate that is prone to aggregate. This can be characterized by the transition temperature T_m, the temperature at which the probability of unfolding is equal to that of folding. Thus, random formation of non-native disulfides can trap certain native proteins, characterized by T_m in excess of 45°C, in conformationally unstable forms that exhibit altered T_m at about 37°C. Such proteins unfold and aggregate at physiological temperatures to become denatured. This has been shown to occur in whole cells following diamide or menadione treatment, using differential scanning calorimetry (DSC) and by measuring the detergent solubility of cellular proteins (Freeman et al 1995; McDuffee et al 1997). Analysis of isolated Ca^{2+} ATPase of sarcoplasmic reticulum by DSC, 1-anilianon-aphthalene-8-sulfonate binding, and light scattering have confirmed and extended the results obtained with whole cells.

This investigation was undertaken to test directly the hypothesis that depletion of glutathione in vivo could alter protein stability. The data presented indicate that rapid depletion of glutathione in CHO cells resulted in a generation of reactive oxygen intermediates, the oxidation of protein thiols, decreased conformational stability of a subset of cellular proteins, and protein aggregation.

MATERIALS AND METHODS

Cell culture

Chinese hamster ovary (CHO) were cultured in McCoy's 5A medium containing 10% fetal bovine serum supplemented with 26 mM sodium bicarbonate, 0.1 g/l streptomycin sulphate, and 0.07 g/l penicillin G potassium.

Diethylmaleate (DEM) (purity ≥ 98%) was dissolved in dimethyl sulfoxide (DMSO). The DEM/DMSO solution was then added to flasks containing growth medium; the final concentration was 100 μM DEM/0.5 mM DMSO. Buthionine sulfoximine was dissolved in Dulbecco's saline and added to flasks so that the final concentration was 50 μM. Control cells not treated with DEM were exposed to DMSO alone.

Glutathione and protein thiol determinations

Cells were lysed in ice-cold 10% perchloric acid (PCA). Glutathione concentrations were measured using high-performance liquid chromatography (Farris and Reed 1983). Acid precipitated protein was washed once in 10% PCA and twice in ice-cold PBS. Thiol oxidation was determined by measuring the change in reduced protein thiols relative to controls, as described by Habeeb (1972).

Measurement of reactive oxygen species

Production of reactive oxygen metabolites was monitored by dichlorofluorescein diacetate fluorescence (DCFA, Ramakrishnan et al 1996). A 30 mM stock solution of DCFA was prepared in absolute ethanol and stored under argon at −20°C until used. The final concentration of DCFA was 30 μM. At the indicated times cells were washed with Hanks buffered saline (HBS) and then scraped into 1 ml of HBS. Samples were quickly homogenized with a Polytron microprobe. Fluorescence was immediately determined in homogenized samples with a Hitachi spectrofluorometer; excitation wavelength 475 nm, emission wavelength 525 nm. Glutathione and fluorescence levels were determined in separate experiments. Protein content was determined using the BCA assay (Smith et al 1985).

Chemical modification of isolated proteins

Light sarcoplasmic reticulum (LSR), consisting of 80–95% Ca^{2+}-ATPase, isolated from rabbit skeletal muscle (Lepock et al 1990) and $β_L$ crystallin (Sigma) were incubated at room temperature for 1 h in TES buffer (10 mM TES, 1 mM EGTA, 100 mM KCl, pH 7.0) containing 0 or 3.3 mM GSH plus 1.65 mM diamide (to induce GSH-mixed disulfide formation as describe by McDuffee et al 1997). Protein was then washed twice in the TES buffer given above. Disulfide formation was assessed by analysis of reducing/non-reducing 1-dimensional SDS-PAGE, protein thiol redox states and concentrations (Habeeb 1972) and corrected for total protein concentration.

Table 1 Oxidation of protein thiols following depletion of glutathione

Experiment	Relative Oxidation of Protein SH[a]
1[b]	0.18 ± 0.01
2	0.15 ± 0.01
3	0.16 ± 0.09
4	0.18 ± 0.09
5	0.27 ± 0.07
6	0.26 ± 0.05
7[c]	0.020 ± 0.003

[a]Relative oxidation of protein SH represents {1 – [the protein SH concentration (nmol/mg) measured in DEM treated cells (n=6 per experiment) divided by the protein SH concentration (nmol/mg) measured in DMSO treated cells (n=6 per experiment)]. The mean protein thiol concentration from the 6 experiments was independent of DMSO treatment (data not shown) and was 113 ± 12 nmol SH/mg protein.

[b]In experiments 1–6, 4 × 106 CHO cells were incubated in full growth medium for 1 h at 37°C in the presence of either 0.5 mM DMSO or 100 μM DEM/0.5 mM DMSO. After exposure, cells were washed in ice-cold PBS, lysed and washed in ice-cold 10% PCA. Protein thiols were measured according to Habeeb (1971).

[c]In experiment 7 cells were incubated in full growth medium for 1 h at 37°C in the presence of either 0.5 mM DMSO or 100 μM DEM/0.5 mM DMSO. Next, cells were incubated for 15 min at 37°C in the presence of 50 mM DTT. After exposure, cells were washed in ice cold PBS, lysed and washed in ice-cold 10% PCA.

in glutathione-depleted cells. Both untreated and cells treated with vehicle alone (DMSO) contained 113 nmol protein SH/mg protein. Approximately 20% of these thiols (17 to 23 nmol protein SH/mg protein) became oxidized in glutathione-depleted cells (Table 1) and this could be reversed by the addition of DTT. This is quantitatively similar to the results obtained when IAA was used (Lui et al 1996).

These glutathione-depletion protocols were found to be non-lethal, as determined by colony forming assays. Furthermore, the rates of protein and DNA synthesis were unaffected by these depletion protocols (Freeman and Meredith 1987; Freeman et al 1988). The profile of polypeptides synthesized was independent of glutathione concentration, with two specific exceptions, increased heme oxygenase and Hsp synthesis (unpubl. data; Saunders et al 1991; Freeman et al 1993; Abe et al 1994).

The relationship between conformational stability of a protein and the temperature of its environment are integrally related. At physiological temperatures, a protein in its native-folded state is only slightly more stable than in an unfolded conformation (Creighton 1990). As temperature is increased, random thermal fluctuations away from the most compact conformation (Butler and Falke 1996) increase the probability of transition to an unfolded state or states. Temperature-induced unfolding will expose hydrophobic domains to the hydrophilic solvent. As the hydrophobic residues seek to limit solvent contact, aggregation occurs, at diffusion controlled rates.
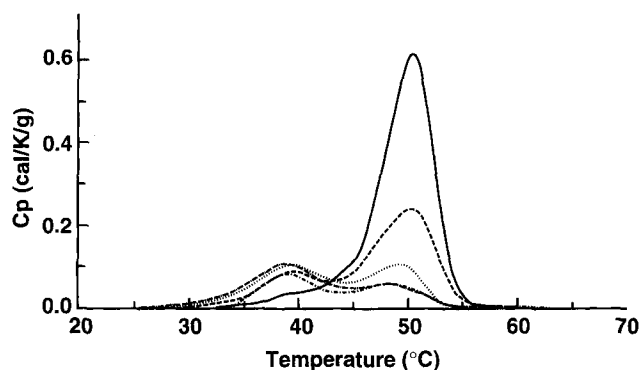
Fig. 2 DSC scans of native (solid line) and H_2O_2 treated Ca^{2+}-ATPase in isolated sarcoplasmic reticulum (SR). The SR was exposed to 0 (——); 10 (- - - -); 20 (•••); 30 (- - • - -); or 40 (- • - • -) mM H_2O_2 in 10 mM TES, 100 mM KCL, 1 mM EGTA, pH 7.0 for 1 h at room temperature, washed and then subjected to DSC analysis.

DSC is a technique that can be used for analysis of both whole cells and isolated proteins to quantify changes in protein stability. As a protein or a domain within a protein undergoes thermal denaturation, there is an increase in heat absorption which is detected as the apparent excess specific heat C_p^{ex}.

Ca^{2+}-ATPase isolated from SR was exposed to hydrogen peroxide treatment and then analyzed by DSC. Peroxide was used because its concentration would be expected to increase in GSH depleted cells, due to diminished glutathione peroxidase activity. The solid curve in Figure 2 illustrates the DSC profile of untreated Ca^{2+}-ATPase. The native enzyme exhibited a T_m of 49°C. Exposure to peroxide decreased the area of the peak representing native protein in a dose-dependent fashion and produced a new broad peak centered at about 38°C. The observation that the peroxide treatment did not shift the T_m to a higher temperature but rather to a lower one is consistent with the concept that peroxide treatment modified amino acid residues such that the protein became destabilized.

Figure 3 illustrates examples of the changes in conformational stability that can occur following non-native oxidation of protein thiols. In this figure, the profile of apparent excess specific heat capacity at constant pressure (C_p^{ex}) was measured for β_L crystallin, purified from bovine lens (Fig. 3A), and Ca^{2+}-ATPase, purified from rabbit sarcoplasmic reticulum (Fig. 3B), as a function of temperature (solid curves). The β_L crystallin profile is distorted by an exothermic peak at approximately 68°C, probably due to aggregation. The profiles can be characterized by two parameters: (i) the onset temperature, the temperature at which the endothermic transition can first be detected; and (ii) the transition temperature, T_m, defined previously. Native β_L crystallin was characterized by an onset temperature of about 45°C and a T_m of about 60°C. The profile

Isolated LSR was also oxidized by exposure to hydrogen peroxide. For these experiments LSR was incubated for 60 min at room temperature in 10 mM TES, 1 mM EGTA, 100 mM KCl, pH 7.0 containing 0, 10, 20, 30 or 40 mM hydrogen peroxide, washed as described above, and then subjected to DSC analysis.

Differential scanning calorimetry (DSC)

DSC profiles of apparent specific heat (C_p) versus temperature were obtained for whole cells in 25 mM HEPES, 5.4 mM KCl, 137 mM NaCl, 5 mM $MgCl_2$ and for the Ca^{2+}-ATPase and β_L crystallin in TES buffer as described by Lepock et al (1993) using a Microcal-2 DSC. The Ca^{2+}-ATPase, β_L crystallin, and CHO cells were scanned from 5°C to 105°C at a rate of 1°C/min. Profiles were corrected for baseline curvature and the shift in specific heat (ΔC_p) upon denaturation as previously described (Lepock et al 1990; 1993). The transition temperature (T_m) is defined as the temperature for half completion of the transition, not the temperature of maximum C_p.

Determination of NP-40 insolubility

Cells were labeled overnight with 0.25 μCi/ml of ^{14}C thymidine and washed twice with phosphate buffered saline (PBS) at 37°C. Cells were then incubated for 30 min at 37°C in leucine free growth medium supplemented with either 0.5 mM DMSO or 100 μM DEM/0.5 mM DMSO. 5 μCi/ml of 3H leucine was then added to the cells and the incubation continued for an additional 10 min. Cells were then trypsinized and washed twice in ice-cold PBS. At this time, 0.1 ml of cell suspension was removed, added to 1 ml of ice-cold 10% TCA, and the insoluble material pelleted (1 000 *g*/4°C/15 min). The remainder of the cells were incubated at 4°C for 5 min in 5 ml of ice-cold buffer (50 mM TRIS, pH 7.4, 50 mM NaCl, 10 mM EDTA, 0.5% NP-40). The lysate was pelleted (1000 *g*/4°C/15 min) and the pellet washed twice in ice-cold 10% TCA. TCA insoluble material was solubilized in 1N NaOH and counted to determine number of ^{14}C and ^{3}H dpm. All samples were processed in triplicate.
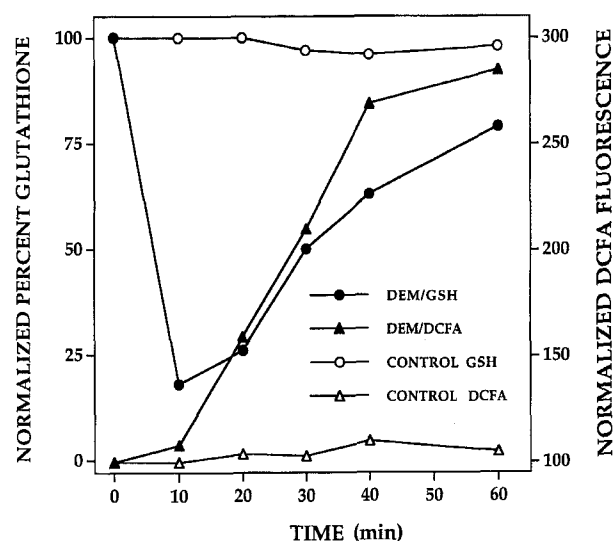
SDS-PAGE

Analysis of polypeptide synthesis was accomplished using SDS-PAGE as described in Freeman et al (1988).

RESULTS AND DISCUSSION

Glutathione depletion and thiol oxidation

The addition of diethylmaleate (DEM) to CHO cells caused rapid and extensive depletion of intracellular glutathione


Fig. 1 The relationship between glutathione concentration and DFCA fluorescence. CHO cells were exposed to 100 μM DEM at time zero. GSH concentrations and DFCA fluorescence measured at the indicated times. (○) GSH concentration in control cells; (●) GSH concentration in cells exposed to DEM; (△) fluorescence in control cells; (▲) fluorescence in cells exposed to DEM.

(Fig. 1). Untreated cells contained 40 ± 2 nmol GSH/mg protein. Ten minutes after the addition of 100 μM DEM, the glutathione concentration was decreased by 80%. It slowly returned to control values over the next 50 min. GSSG concentrations remained below the level of detection (0.1 nmol/mg). In later experiments 50 μM buthionine sulfoximine (BSO) was added simultaneously with 100 μM DEM in order to inhibit glutathione resynthesis. Addition of BSO with the DEM did not affect the rate or degree of glutathione depletion but simply inhibited resynthesis (data not shown).

Production of intracellular oxidizing species can be estimated by measuring the fluorescence produced by accumulation of the oxidized form of 2',7'-dichlorofluorescein diacetate (DFCA; Rothe and Valet 1990). In untreated control cells, fluorescence remained constant over the 1 h experimental time course and was arbitrarily assigned a value of 100% (Fig. 1). In glutathione depleted cells, the intensity of the fluorescence signal increased 250%, relative to control (Fig. 1), suggesting that oxidizing species accumulated during the interval of glutathione depletion. Such results are consistent with the observations of Mehlen et al (1996).

Even in their native state, proteins undergo random thermal fluctuations which can bring cysteine residues into close proximity. In the presence of diffusible oxidizing species, the cysteine residues can form non-native disulfides (Butler and Falke 1996). The data presented in Table 1 illustrate the redox state of cellular protein thiols
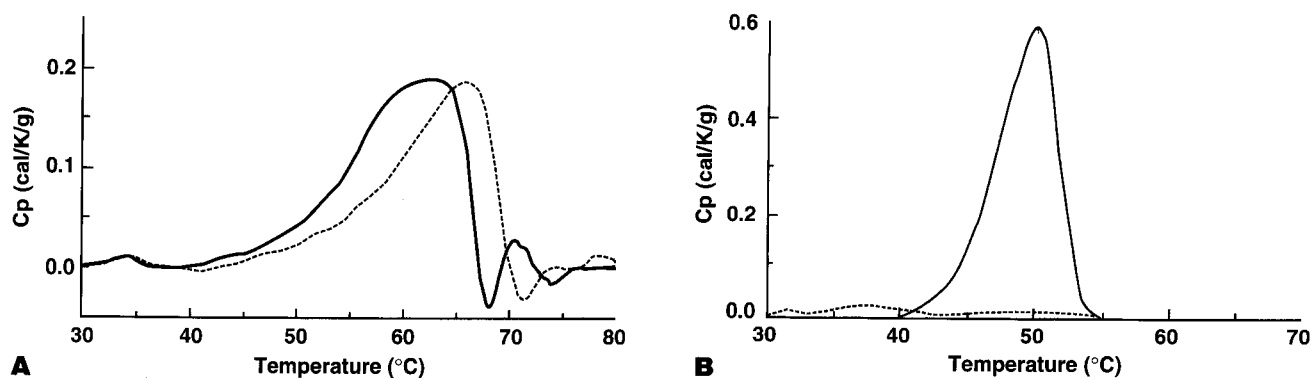
Fig. 3 DSC scans of native (——) and oxidized (- - -) β_L crystallin (A) or Ca^{2+}-ATPase (B). Fig. 3B was redrawn with permission (McDuffee et al 1997). Thiol oxidation was accomplished as described in the Materials and methods section.

produced by native Ca^{2+}-ATPase was characterized by an onset temperature of about 40°C and a T_m of about 49°C.

β_L crystallin contains two cysteine residues while the Ca^{2+}-ATPase contains 24. Incubation of these proteins at 20°C in the presence of GSH plus diamide, as described in the Materials and Methods, oxidized approximately 95% of the thiols of both proteins when calculated on a molar basis. The resulting DSC profiles are shown in Figure 3 (dotted curves). Thiol oxidation was found to increase slightly the thermal stability of β_L crystallin. Both the onset temperature and the T_m increased about 5°C (to ~ 50°C and 65°C, respectively, with no change in peak height). These results are in contrast to those obtained from the Ca^{2+}-ATPase containing non-native disulfides (Fig. 3B, dotted line). There was a large decrease in peak height and almost a complete loss of the profile, indicative of denaturation due to extreme destabilization occurring during the 20°C oxidation reaction.

The differences observed between β_L crystallin and Ca^{2+}-ATPase are not the result of different types of thiol oxidation events. Analysis by reducing and non-reducing SDS-PAGE demonstrated that in both proteins non-native disulfide oxidation under these experimental conditions was the result of GSH-mixed disulfide formation (data not shown). The differences in stability observed for the two proteins may be a consequence of the spacial location and number of thiols oxidized.

Thiol oxidation-induced protein denaturation in CHO cells

In untreated control cells the profile of C_p^{ex} versus temperature consists of at least five broad endothermic peaks (Fig. 4, A–E, solid curve). At about 41°C (onset of denaturation) the profile deviates sharply from base line and the endotherm continues to above 100°C. A number of thermotropic processes contribute to this complex profile (Lepock et al 1993). The profile represents the
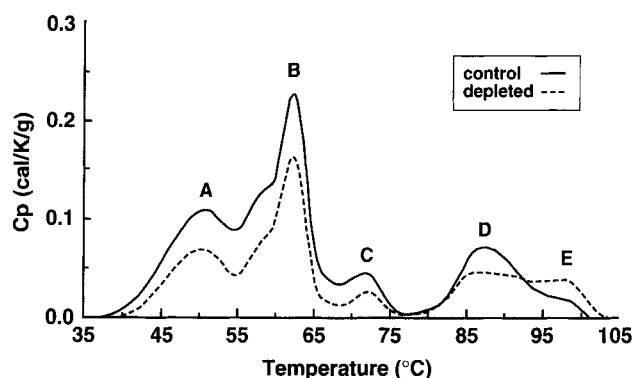
Fig. 4 DSC profile of CHO cells. Cells exposed to either vehicle control (0.5 mM DMSO) or 100 μM DEM/0.5 mM DMSO/50 μM BSO for 1 h at 37°C and then analyzed by DSC. Each scan is the average of three separate scans from four separate experiments.

sum of all cellular transitions including protein denaturation, DNA, and RNA unfolding, molecular aggregation and disaggregation. On the basis of relative specific heats and fractional composition, protein denaturation should predominate over other endothermic process (Lepock et al 1993). In Figure 4, all transitions with the exception of D are irreversible.

The dotted curve shown in Figure 4 represents the DSC profile of glutathione depleted cells. Cells were incubated at 37°C for 1 h in the presence of 100 μM plus 50 μM BSO and then washed of the DEM/BSO. BSO was added back to the cells and DSC analysis performed. The BSO was used to prevent resynthesis of GSH. Under conditions of glutathione depletion, the area under peaks A and B was decreased relative to cells containing control levels of glutathione. There is no evidence that the T_m of peaks A and B have been increased, which would be indicative of protein stablilization due to disulfide bond formation. These results suggest that the oxidizing conditions produced by glutathione depletion cause certain cellular

Table 2 Effect of glutathione depletion on the detergent solubility of proteins[a]

| Exp.	Treatment	^{3}H dpm whole cells	3H dpm NP-40 insoluble	^{14}C dpm whole cells	Relative ^{3}H insolubility
1	DMSO	1.8×10^6	3.6×10^5	2.7×10^6	0.20
	DEM/DMSO	1.7×10^6	4.4×10^5	1.8×10^6	0.39
2	DMSO	1.2×10^6	2.4×10^5	2.0×10^6	0.27
	DEM/DMSO	1.0×10^6	2.7×10^5	1.8×10^6	0.40

[a]CHO cells were labeled overnight with ^{14}C thymidine, washed twice with PBS, then incubated for 30 min at 37°C in leucine free-growth medium supplemented with either 0.5 mM DMSO or 100 µM DEM/0.5 mM DMSO. ^{3}H leucine was then added to the cells and the incubation continued for an additional 10 min. Cells were then trypsinized and washed in PBS. At this time, an aliquot of cell suspension was removed and precipitated with ice-cold 10% TCA. The remaining washed, pelleted cells were resuspended and incubated at 4°C for 5 min in 5 ml of ice-cold buffer (50 mM TRIS, pH 7.4, 50 mM NaCl, 10 mM EDTA, 0.5% NP-40). The lysate was centrifuged at 1 000 *g* and the pellet washed twice in ice-cold 10% TCA. TCA insoluble material was solubilized in 1N NaOH and counted to determine ^{14}C and ^{3}H dpm. All samples were in triplicate.

[b]Relative ^{3}H insolubility represents the amount of NP-40 insoluble ^{3}H obtained divided by the amount of TCA insoluble ^{3}H obtained from whole cells and corrected for the total number of cells in the sample, as determined by ^{14}C dpm. This ratio represents the amount of ^{3}H protein that was insoluble in NP-40 relative to the total amount of ^{3}H protein synthesized per cell.

proteins to lose conformational stability and unfold prior to the DSC analysis. That is, the T_m of such proteins has been decreased. This is analogous to the behavior of the Ca^{2+}-ATPase. The statistical significance of the decrease in area was determined by determining the mean and 95% confidence intervals for the heights of peaks A and B ($n = 4$). Peaks A and B have decreased 38% and 28%, respectively; $P \leq 0.05$.

Stresses that induce protein denaturation will decrease detergent solubility of proteins due to aggregation (Dubois et al 1991; Beckman et al 1992). The data presented in Table 2 indicate that depletion of glutathione increased the amount of cellular protein that exhibited NP-40 insolubility. The interpretation of this data is contingent upon whether or not polypeptide synthesis was altered by the depletion of glutathione. The 1D PAGE shown in Figure 5 indicates that the pattern of polypeptide synthesis was independent of glutathione concentration. Increased expression of heat shock proteins and heme oxygenase were not observed due to the design of the experimental protocol which did not provide sufficient time for expression.

Extrapolation of the results presented above may help to contribute to a molecular rationale for understanding how glutathione depletion can affect stress responses. It has been clearly demonstrated that denatured protein is a key feature for hyperthermic cytotoxicity (Lepock et al 1988), the induction of Hsp 70 (Hightower 1980; Hightower and White 1981; Mifflin and Cohen 1994; Ananthan et al 1986) and heme oxygenase, which contains a functional heat shock element in rodent systems (Muller et al 1989). Thus, the observations that glutathione depletion, specifically depletion of mitochondrial glutathione, significantly enhanced hyperthermic cytotoxicity (Mitchell and Russo 1983; Freeman and Meredith 1988) in V79 and CHO cells, activated HSF-1 and induced
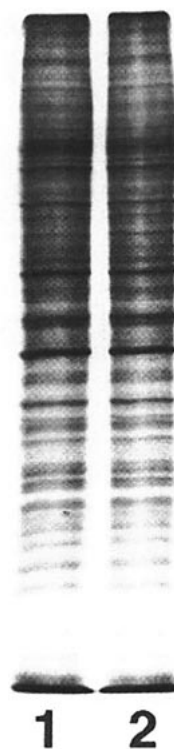
Fig. 5 Autoradiographic analysis of polypeptides synthesized at 37°C. Cells were incubated at 37°C for 30 min at 37°C in 0.5 mM DMSO (lane 1) or in 100 µM DEM/0.5 mM DMSO (lane 2) and then exposed to [^{3}H]leucine for 10 min. Cellular proteins were solubilized and equal amounts of protein analyzed by 1D-PAGE.

Hsp 70 in Hep G2, human amniotic WISH cells, porcine renal epithelial, and porcine aortic endothelial cells (Koizumi et al 1993, Freeman et al 1993; Abe et al 1994; Liu et al 1996), as well as induced heme oxygenase in CHO (Saunders et al 1991) may be attributed to protein

denaturation. In addition, the oxidation of protein thiols upon depletion of intracellular glutathione may impact upon the regulation of the mitochondrial permeability transition pore (Hunter and Haworth 1979) which affects mitochondrial membrane permeability. Voltage dependence of the pore can be regulated at two independent sites. One is in apparent oxidation-reduction equilibrium with pyridine nucleotide pools while the other site is regulated by the oxidation-reduction potential of a critical dithiol (Costantini et al 1996). Depletion or oxidation of glutathione increases the probability of pore deregulation.

In summary, the results presented in this investigation demonstrate that depletion of intracellular glutathione was accompanied by an accumulation of reactive oxidative species, oxidation of protein thiols, protein denaturation and aggregation. Thus, the concentration of glutathione, as well as the GSH/GSSG ratio, alterations of which have been shown to induce Hsp 70 (Freeman et al 1995; McDuffee et al 1997) are important parameters for regulating protein stability in vivo.

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