BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **242,** 1–9 (1998)
ARTICLE NO. RC977812

# BREAKTHROUGHS AND VIEWS

## Recent Trends in Glutathione Biochemistry—Glutathione–Protein Interactions: A Molecular Link between Oxidative Stress and Cell Proliferation?

Ian A. Cotgreave and Robert G. Gerdes
*Division of Biochemical Toxicology, Institute of Environmental Medicine, Karolinska Institute, Box 210, S-17177 Stockholm, Sweden*

Received November 10, 1997

Since its discovery in the 192Os by Sir Frederick Gowland Hopkins, glutathione (GSH: $\gamma$-glutamyl-cysteinyl-glycine) has stimulated diverse interest from biochemists, physiologists and clinicians alike. Studies of the biochemistry and function of this tripeptide have enjoyed periodic bouts of renaissance, which have closely paralleled general developments in biochemistry. Thus, in parallel with developments in protein biochemistry and enzymology during the 1960s to 1970s, the pioneering work of Meister and colleagues both clarified the enzymatic basis of the rather unusual mode of peptide synthesis employed, and as well provided a metabolic basis for several heritable diseases resulting from dysfunction in some of these enzymes (1). During this period GSH was also demonstrated to be a co-substrate for a number of important enzymatic reactions. For instance, the first GSH-S-transferase was demonstrated in 1961 by Booth and co-workers (2), and it was subsequently proposed that this family of enzymes served as a front-line defence against electrophilic insult (3). Today, this function of GSH is placed centrally in concepts such as drug and foreign compound detoxification (4) and multi-drug resistance (5).

During the 1970s, interest was focussed on cellular redox processes not directly related to respiration. In particular, the natural occurrence of reduced, reactive metabolites of oxygen in the biological milieu, indicated by the discovery of superoxide dismutase in 1969 (6), spawned the concept of the antioxidant function of GSH. This was primarily due to the unique redox chemistry of the cysteinyl thiol of the molecule. Indeed, the discovery, in 1973, of a GSH-dependent selenoprotein peroxidase with specificity for $H_2O_2$ is of particular importance (7). It was during the 1980s, however, that the antioxidant function of GSH attracted particular attention in toxicological research, particularly in relation to the mechanisms of toxicity of both redox-cycling drugs and foreign chemicals (8). Such studies led, by the mid 1980s (9), to a clear biochemical and cytological definition of the concept of "oxidative stress". This concept defines a dynamic situation in which the balance between the occurrence of oxidants and antioxidants in biological systems can indirectly influence cellular phenotype through an effect on other cellular components, particularly redox-sensitive functional groups on proteins. For the purposes of this review it should be stated, however, that the term "stress" often carries rather negative conotations which, becuase of the propensity for association of the situation of redox alteration with toxicity events. As positive roles for "oxidative stress" are proposed here one might better more acurately use a term such as "oxidant-mediated regulation" instead.

At about the same time, one potential link in these events, involving both the redox properties and structure of the GSH molecule itself began to emerge. Pioneering work in the mid 1980s by Thomas and co-workers showed that glutathione may interact in a reversible manner with protein cysteinyl thiols of many cellular proteins during bouts of oxidative stress (10). This, was coupled to accumulating evidence that the activities of many proteins were sensitive to modifications induced on thiols by redox changes. Furthermore, the demonstration of enzymes capable of catalysing protein thiol-disulphide interchange involving GSH, began to suggest that GSH may have an important role to play in the regulation of complex biochemical processes in intact cells. Examples of such catalysts include GSSG reductase (11), the thioredoxin (TRX)-thioredoxin reductase (TRXred) system (12), glutaredoxin (GRX) (13), some times termed thiol transferase and protein-disulphide isomerase (PDI) (14).

Perhaps one conceptual obstacle to progress has been widespread belief that glutathionylation of protein sul-



0006-291X/98 $25.00

phydryls simply reflects the attainment of a rapid chemical equilibrium between protein thiols and GSSG, at levels characteristic of oxidative stress. Indeed, the quantitative importance of GSH in maintaining the redox poise of the cell is established dogma. However, such reactions can be quite slow in a biological time-frame, and there is no reason for assuming *a priori* that chemical equilibrium is the rule.

However, research into the biological significance of these GSH-based processes has been restricted to a very few groups. despite the compelling, albeit indirect evidence of its biological importance.

The 1990s has been characterised by a veritable explosion in information concerning the functional genetics of human cells and particularly the mechanisms by which selective gene expression is achieved. Simultaneously, our knowledge of the cytological and biochemical processes involved in cell cycle regulation have also taken a leap. Interestingly, the concepts of redox biochemistry and oxidant-mediated regulation, are emerging as potentially central players in these processes. Thus, several transcription factors, such as AP-1, Nfkb, p53 and SP-1, have been defined, whose DNA-binding activity and stimulatory effect on gene expression are reliant on the redox status of cysteinyl thiols in their structures (15). Similarly, roles for oxidative stress have been proposed in both the stimulation of cell proliferation (16) and the stimulation of cellular deletion by apoptosis (17). Despite these emerging concepts, there has been a paucity of molecular explanation linking oxidant-mediated regulation to these complex processes.

An indication of sustained interest in this field is given by a Medline analysis which shows that reviews concerning GSH have been published at about 4 weekly intervals over the past years. In addition to several classical reviews of glutathione biochemistry (1, 18), there have been a number of excellent recent reviews of the biochemistry of glutathione (19-21). However, the specific purpose of this review is to specifically examine the possibility that redox modification of critical cellular proteins by reversible interaction(s) of selected cysteines with GSH, particularly during oxidative stress, plays an important role in the mechanisms by which oxidative stress affects the cell cycle, and as a result influence mitogenesis and apoptosis. Towards the end of the review we also suggest some short-comings in our present knowledge which may restrict a full appraisal of these relationships, as well as providing some suggestions for future research trends.

## REVERSIBLE S-GLUTATHIONYLATION OF CELLULAR PROTEIN: WHAT IS KNOWN?

Over the years, a number of lines of evidence have emerged, supporting a fundamental role for redox modification of certain proteins in normal cellular biochemistry and physiology, as well as in pathophysiological processes. Many purified proteins have been shown to be sensitive to thiol disulphide interchange reactions. These include enzymes involved in both central and peripheral metabolism, such as glucose-6-phosphate dehydrogenase and pyruvate kinase, or aldehyde dehydrogenase and carbonic anhydrase, some involved in signal transduction, such as 5′-lipoxygenase and guanylate cyclase and even enzymes of protein catabolism, such collagenase and trypsin (22). These discoveries are continuing and notable recent additions to this list include the homodimeric HIV-1 protease, with suggestions that the action of this protease may be partially regulated by redox changes to its cysteines by S-glutathionylation (23) and a variety of antioxidative enzymes, including Cu-Zn SOD (24) and microsomal glutathione-S-transferase, purported to be involved in the regulation of catalytic activity (25).

The occurrence of GSH-protein mixed disulphides in cells and tissues has long been utilised as strong evidence that these processes have significance in intact biological systems (22, 26, 27). Glutathione also occurs extensively in human plasma as mixed disulphides with serum albumins (22). However, the mechanisms underlying the control of mixed disulphide levels and variations in human circulation are only now being investigated (28). Interestingly, some recent work provides insight for a role for nitric oxide in the formation and regulation of plasma mixed disulphides, via the transient formation of reactive sulfenic acids on serum proteins (29).

Most of the early observations of bulk quantities of GSH bound to cellular protein has been made in association with the development of intracellular oxidative stress (11, 30, 31). These kinds of observations continue to appear in other cell types (32, 33). Bulk measurements of GSH-protein mixed disulphides have been made on a variety of tissues, again mostly during oxidative stress. For instance, mixed disulphide formation has been recently shown to be associated with embryotoxicity elicited by oxidative stress during organogenesis in the rat conceptus (34). Additionally. The accumulation of protein-GSH mixed disulphides in lens crystallins has long been proposed to be a molecular linker between oxidative stress and the development of cataracts, particularly diabetes-related ones. Recent focus has been placed on the compartmentation of bulk mixed disulphides (35), as well as the role of lens senescence (35) and the influence of protein glycosylation on these oxidative modifications (36). Several lines of evidence are also emerging that mixed disulphide formation is of importance in the response of the mitochondrion to oxidative stress. As a molecular link in the development of symptoms of Parkinsonism, monoamine oxidase-derived $H_2O_2$ was shown to inhibit mitochondrial respiration in association with stimulated mixed disulphide formation on mitochondrial proteins

(37). Additionally, the formation of certain protein-GSH mixed disulphides in the mitochondrion may be critically important for mitochondrial membrane permeability changes induced by peroxides and peroxynitrite (38). Mitochondrial mixed disulphide formation has also been proposed to underlie various inhibitory effects of oxidative stress on mitochondrial enzyme activities, particularly those of succinate and isocitrate dehydrogenases (39). Mitochondrial effects of mixed disulphides may be of importance as a mechanism for mitochondrial alteration in triggering apoptosis, particularly by oxidative stress (see below). A recent series of experiments also has suggested the formation of protein-GSH mixed disulphides as a suitable trigger in the response in cells to intracellular redox cycling, by contributing to destabilization of protein structure (40).

In the above observations, one common theme which has limited the implication of mixed disulphide formation in the biological effect is the method of analysis employed. The analytical problems in this area will be addressed below, but the lack of suitably selective techniques has resulted in only a limited number of proteins being identified as undergoing reversible S-glutathionylation reactions in intact cells. Work by Thomas and co-workers, developed analytical approaches (41) and applied these to the identification of patterns of S-glutathionylated proteins formed during oxidative stress in intact cells (10, 42). Such pattern recognition has, in most cases, been extended to identification of the major S-glutathionylated proteins. Thus, carbonic anhydrase III was identified as a substrate in hepatocytes during quinone-, peroxide- and neutrophil-promoted oxidative stress (43, 44). Indeed, the extent of S-glutathionylation of this substrate has been proposed as a quantitative analytical marker for cellular protein thiol modification during oxidative stress (45). Creatine kinase and glycogen phosphorylase b have also been identified as undergoing reversible S-glutathionylation in myocytes and cardiac tissue during cyclic oxidative stress (46, 47). These alterations were postulated to be important in the metabolic response of the muscle cells to this kind of stress. Glyceraldehyde-3-phosphate dehydrogenase has been identified as a major S-glutathionylated protein in endothelial cells in response to hydrogen peroxide (48) and as a major protein species so modified in monocytes during the endogenous oxidative burst (49). The functional significance of this modification in terms of a relevant response to stress at present eludes us. Interestingly however, GAPDH has also been shown to be inactivated in the ischemic myocardium, although the physiological role of S-glutathionylation in this case is also uncertain (50). Another common substrate identified in both endothelial cells (51) and gastric mucosal cells (52) is the structural protein actin. Here, reversible S-glutathionylation of the protein has been associated with alterations to the cytoskeletal organisation of the cells in response to oxidative stress. Actin has also been identified as a major substrate for S-glutathionylation in the neutrophil during stimulation of the respiratory burst (53). The list may extend in the near future, but with the application of the present analytical techniques only those protein substrates in relatively high abundance, and undergoing stimulated S-glutathionylation during oxidative stress will be amenable to identification.

One important set of evidence which supports a central role for S-glutathionylation of cellular protein as a link between oxidative stress and alterations to cellular phenotype is the demonstration that the processes are generally reversible. The reversibility of S-glutathionylation of protein after oxidative stress has clearly been demonstrated in an number of cell types (54-55). Thomas and co-workers and our group have shown that the reductive cleavage of mixed disulphides in intact cells involves both NADH- and NADPH-dependent processes. Work in cardiac myocytes also implicated roles for both "dethiolase" enzymes with properties similar to those of mammalian TRX-TRXred system and that of GRX (47, 56). More recent work has clearly implicated roles for both TRX and GRX in the reductive cleavage of GSH-protein mixed disulphides in intact cells (57, 58). The latter work clearly delineated GRX as the primary catalyst of mixed disulphide cleavage. This is not surprising in view of the affinity of TRX (13) and PDI (14) for inter- and intra-peptide disulphides, and the established preference of GRX for mixed disulphides between GSH and proteins (59), selectivity dictated by the presence of the $\gamma$-L-glutamyl-L-cysteinyl moiety of GSH in the disulphide (60). Here, it is of interest to note that thiol transferase activities are being steadily purified from new biological sources. For instance, proteins catalysing GSH-dependent reductive cleavage of protein GSH-mixed disulphides have been recently purified from the gastrointestinal mucosum (61) and in the ocular lens (62). Despite this interest in thiol transferase as the major player in regulation of the state of cellular GSH-protein mixed disulphides, recent focus has been drawn to the possibility that PDI plays a more considerable role in the reduction of protein-GSH mixed disulphides of biological relevance, with the protein also demonstrating the rather novel ability to relax protein substrates around the area of reductive cleavage (63). The relevance of this may only be limited due to the intracellular localisation of the enzyme, predominantly in the lumen of the endoplasmic reticululum.

Another more discrete line of evidence for a physiological function of reversible S-glutathionylation of proteins arises from the association of S-glutathionylation with the initiation and maintenance of complex, integrated physiological processes. Indeed, the exquisite reversible regulation of mixed disulphide formation in intact cells has provoked suggestions that the reactions

may serve as a quantitatively relevant antioxidant function in cells (64), particularly endothelial cells (54). Increased levels of mixed disulfide formation have also been shown to prime monocytes for the production of reactive oxygen species during the stimulated respiratory burst (65). But perhaps the most important issue which may, in the future, find a place in this list of biological end-points containing links with reversible modification of cellular proteins, extends to processes controlling cell proliferation. In order to give substance to this possibility, we will now briefly review the consensus of evidence coupling the occurence of oxidants and oxidant-mediated regulation in cells with processes controlling cellular mitogenesis and apoptosis.

## OXIDANT-MEDIATED REGULATION IN MITOGENESIS AND APOPTOSIS

During the 1980s, most focus was placed on the detrimental effects of oxidants in biological systems, particularly the role of oxidative stress in necrotic cytotoxicity. However, in 1985, Cerruti postulated that the occurrence of oxidative stress in cells had profound stimulatory effects on cell proliferation in the process of tumour promotion (66). At that time, knowledge of processes controlling mitogenic responses and apoptosis in cells was limited, but the postulate did underscore the potential physiological importance of oxidant-mediated regulation in this fundamental biological process. We now know that oxidants and oxidant-mediated regualtion is involved with mitogenesis and apoptosis in cells. It should be stated immediately that a simple, universal mechanism is not presently apparent. Thus, oxidant-mediated effects are associated with both stimulated proliferation (16, 67) and the onset (68) and effector phase (17) of apoptosis. Similarly, the reactions of cells appear to be cell type-specific, dependent on the source (intracellular versus extracellular), species ($H_2O_2$, $\cdot O_2-$ or NO), and their intracellular concentration. Thus, the overall involvement of oxidant-mediated regualtion in the cell cycle regulation process is likely to be a complex one, dependent on a large number of biochemical and cellular factors.

Let us first consider the positive effects of oxidants on proliferation in cells. Several studies have shown that both $H_2O_2$ and $\cdot O_2-$, added exogenously to a variety of mammalian cells (69, 72), have mitogenic effects. Interestingly, some studies have shown synergistic effects for oxidants with other endogenous mitogens (72). Additionally, some evidence also suggests that the response of tumour cells is particularly amenable to oxidant activation (73). At the same time, a number of studies have concentrated on the proliferative index of cells in response levels of reactive oxygen metabolites generated endogenously in cells, either as a consequence of endogenous metabolism or the metabolism of prooxidant compounds. Thus, addition of antioxidant principles to cells, such as SOD or catalase (74) and $\beta$-carotene and flavanoids (75) have been shown to have anti-proliferative effects in cells, presumably by lowering endogenous levels of oxidants in the cells. Additionally, studies have shown clear correlations between decreases in endogenous levels of oxidants in cells and a decline in their proliferative index (76). Further, a number of redox cycling quinonoid compounds have been shown to induce cell proliferation in association with the induction of intracellular oxidative stress (77, 78). Despite the apparent unity of responses in these cellular systems it should be emphasised that a number of studies describe opposing effects of oxidant-mediated regulation, depending on the absolute level of oxidants applied, or the intracellular oxidant level. Thus, a number of studies emphasise the induction of proliferation at low levels of oxidants, or as a result of transient exposure (72, 77, 78). whereas higher levels actually induce anti-proliferative effects, mainly evident in apoptosis or necrosis (78). This would seem logical in view of the levels of oxidants normally occurring in unstressed cells.

Another situation of cell proliferation tentatively coupled to oxidative stress in the response of cells to peroxisome proliferators, such as hypolipidemic drugs. This has been adequately reviewed elsewhere (79, 80) and will not be dealt with further here except to point out that a direct relationship between peroxisome proliferation, induced oxidative stress and increased hepatocellular proliferation may be questionable (81). Additionally, recent attention has been brought to the effects of these compounds on tissues other than liver, such as the myocardium (82).

In recent years, our understanding of the mechanisms controlling cell proliferation have been greatly enriched by accumulating knowledge of the processes in apoptosis. As a result of accelerating interest in this area, a great deal of evidence is presently accumulating directly or indirectly implicating oxidative stress and the redox status of intracellular thiols, such as GSH, in this processes. Since the literature is rapidly accumulating in this area and a number of recent reviews have dealt amply with the evidence (17, 68, 83, 84), we will concentrate on recent developments which place particular emphasis on the role of thiol redox status in these processes. Some of the earliest concrete ties between apoptosis and oxidative stress were achieved by demonstrating that $H_2O_2$ induces apoptosis in a number of different cell types, particularly of lymphocytic origin. (85). Since these early beginnings it has become apparent that a number of prooxidant and other agents also induce apoptosis in cells by elevating the intracellular concentration of reactive oxygen metabolites. This list now diversly includes arachidonic acid metabolites, particularly some prostaglandins (86, 87), lipid hydroperoxides (88), redox cycling com-

pounds, such as bleomycin (89) and quinones (78), reactive aldehydes, such as methyl glyoxal (90), dithiocarbamates (91), some growth regulatory molecules such as transforming growth factor B (92) and other well established apoptotic stimuli such as methylprednisolone and thapsigargin (93) and tumour necrosis factor-alpha (94).

In addition to a correlation between the occurrence of elevated oxidant levels in cells and the tendency to undergo apoptosis, strong indications of a tie between apoptosis and oxidative stress come from the demonstration that a wide variety of antioxidants function as anti-apoptotic agents against a wide variety of stimuli in different cells types. These include spin trapping antioxidants (95) as well as a variety of thiols including N-acetyl cysteine (96), dihydrolipoate (97), glutathione (98), dithiothreitol (99) and the protein thioredoxin itself (100). Here, it might also be mentioned that extracellular GSH, S-acetylGSH (101) and a number of other glutathione derivatives (102) have been shown to actually selectively induce apoptosis in tumour cells.

Additionally, strong evidence has accumulated that alterations to the intrinsic intracellular antioxidant capacity greatly affects apoptotic responses. Perhaps the most stimulating finding in this area was the initial hypothesis that the product of the BCL-2 gene, the expression of which protects against apoptosis in most cells, functions as an antioxidant (103). Additionally, a number of investigations have demonstrated that the expression of antioxidant enzymes, such as superoxide dismutase, catalase and DT-diaphorase, as well as TRX, are down regulated during early events in apoptosis in lymphoid cells (104). But of particular relevance to this review are the observations that the levels of intracellular GSH are very important for the correct execution of apoptotic programming. Since the early observations that a fall in GSH levels correlates closely to apoptosis in lymphoid cells (105), this inverse correlation has been noted in a wide variety of cells types subjected to pro-apoptotic stimuli including neutrophils (106), thymocytes (107) and monocytes (108). Interestingly, it has also been shown that monocytes from a patient with glucose-6-phosphate dehydrogenase deficiency, thus unable to correctly maintain intracellular GSH in a reduced form, are more susceptible to apoptosis than those from normal individuals (109). Additionally, decreased G-6-PDH activity in human lymphoid cells, as a result of over-expression of the pentose phosphate pathway enzyme transaldolase promoted by transfection, resulted in potentiated sensitivity of T-cells to apoptosis in association with diminished GSH levels (110). It is also of interest to note that, to date, little interest has been paid to the consequences of hereditary defects in enzymes of GSH biosynthesis, or of other GSH-dependent enzymes, on apoptotic processes in general.

Conversely, elevations of intracellular GSH levels by cysteine precursors (see above), other drugs (111), growth regulatory protein (112) and protein synthesis inhibitors, which re-route intracellular cysteine from protein synthesis to GSH synthesis (113) all increase the resistance of cells towards entery into apoptotic processes.

Another important observation centred on GSH and apoptosis stemmed from the observation that CD 95(FAS/APO-1)-mediated apoptosis in lymphoid cells is associated with the rapid loss of reduced GSH from the cells (114). This has since been demonstrated in other cell types, notably with normal human T-lymphocytes (115), and with other pro-apoptotic stimuli (108). This loss of GSH was shown to be tightly coupled to a number of down-stream events in apoptosis including caspase activation and events in chromatin (114). Interestingly, FAS-induced killing of multi-drug resistant tumour cells has also been strongly linked to stimulated efflux of GSH from the cells (116). Similarly, agents which prevent the depletion of GSH (110) delay FAS-directed apoptotic killing. Recent advances in the understanding of the auto-regulation of this process include observations in human neutrophils showing that FAS-directed apoptotic killing could be abrogated in relation to augmented GSH synthesis caused by cross linking of the beta 2 integrin of the cells (117). The exposure of cells to thiol agents have also been shown to result in down regulation of membrane FAS expression in lymphoid cells (118).

## A LINKING ROLE FOR GLUTATHIONE–PROTEIN INTERACTIONS? PROBLEMS AND POTENTIAL SOLUTIONS

An obvious premise to a potential link between oxidative stress and cell cycle regulation is that oxidants have to either directly or indirectly affect the activity of key proteins in these processes through structural alterations. Most amino acids in proteins will react directly with reactive oxygen metabolites, particularly species such as the hydroxyl radical, however such oxidation reactions in the biological setting would likely be indiscriminate. Additionally, in terms of homeostatic regulation, these kinds of oxidation reactions would be largely irreversible. The redox chemistries of the group XVIa elements O and S, facilitate direct oxidation of sulphur containing amino acids occurs readily, resulting in products with an oxidation state higher than +2, i.e. sulphinic acids and sulphonic acids. However, these species are relatively stable in biological systems. On the other hand, redox transition from the −2 (thiol) to −1 (disulphide) state is relatively facile and reversibility is energetically favourable. Thus, based on chemical arguments, this provides a strong possibility for transducing "oxidative information" from intracellular oxidants via the GSH redox buffer to individual proteins containing "regulatory thiols".

Despite these clear chemical arguments and the popularity of study in the areas of research detailed in this review, there is a remarkable lack of direct molecular evidence that a critical protein(s) is(are) redox modified in relation to oxidant-stimulated mitogenesis. Indeed, studies with purified proteins are also lacking. One exception to this is the case of protein kinase C, which has been shown to be activated by oxidative modification of the protein under situations of intracellular oxidative stress (119-121). However, a role for GSH-protein interactions has not been clarified. In the case of apoptosis, several lines of evidence clearly implicate GSH in the process, particularly the demonstration that modulation of cellular GSH levels directly influences apoptotic responses initiated by a number of different agents. Additionally, it is now clear that alterations to mitochondrial function are centrally involved in the apoptotic process (122). Mitochondrial function is known to be particularly sensitive to oxidative stress, in association with mixed disulphide formation on mitochondrial proteins (38, 39). Recent studies have shown that the loss of inner mitochondrial membrane potential and increased membrane permeability are early critical events common to apoptotic events (123). This event has been coupled to alterations to protein redox balance, although again a role for mixed disulphide formation was not investigated (124). Thus, most publications in this area fail to clarify that, where cellular GSH levels are altered, not only is the antioxidative capacity of the cells altered, but all redox equilibria between free GSH and glutathione bound in proteins are also individually altered.

The question which now arises is "what is impeding further developments in this area?" One highly relevant problem is the lack of suitable analytical techniques. At present the analysis of mixed disulphides between GSH and proteins is limited to radiochemical techniques in which intracellular GSH is selectively labelled with ($^{35}$S) during inhibited protein synthesis. This indeed allows individual S-glutathionylated proteins to be identified, however three problems are apparent. Firstly, the necessity for protein synthesis inhibition introduces artefacts into cells. Many proteins have high rates of turnover and, thus, may be present at aberrant levels when actual experiments are performed. Protein synthesis inhibition may also be incomplete, providing high radiochemical back grounds on all proteins. Secondly, the depletion of intracellular GSH prior to radiochemical labelling may place the cell in a situation of acute oxidative stress, inciting compensatory stress-responses in prelude of experimentation. Thirdly, low molecular weight, sulphur-containing species other than GSH can become labelled in cells. Thus, the technique fails to directly identify GSH as the protein-bound species. Finally, the intrinsic limits of sensitivity of the use of radiochemicals reduces their suitability to situations of severe oxidative stress in cells. This specifically precludes application to experiments on cell cycle regulation as it is clear that the mitogenic and pro-apoptotic effects of oxidants occur during relatively "mild" or regulatory oxidative stress. Additionally, the proteins involved in these cell cycle regulatory events are generally present at very low copy number, in comparison to those proteins where S-glutathionylation has been clarified.

Clearly, there is a strong requirement for the development of analytical techniques which allow the study of constitutive S-glutathionylation events on specific, potentially low copy number proteins. We have been engaged in this problem and are at present trying to develop a method based on the selectivity of GRX to recognise GSH bound to proteins, reduce off the GSH and facilitate specific labelling of the revealed thiols coupled to chemiluminescence analysis on 2-dimensional gels. It is hoped that this approach will provide the selectivity and sensitivity required to begin probing for reversible S-glutathionylation of key proteins involved in controlling mitogenesis and apoptosis.

Another major obstacle to progress in this area lies in the primitive nature of our present understanding of the catalysis of these processes in the biological setting. It would be logical to assume that processes as important as mitogenesis and apoptosis, each involving the activities of a multitude of protein components, would demand great selectivity in any regulatory mechanisms operating. This is generally achieved by the action of a wide variety of enzymatic proteins exhibiting selective catalysis. At present less than half a dozen mammalian proteins have been characterised which are capable of catalysing thiol-disulphide interchange reactions. This is a severe restriction to understanding factors governing the selectivity of S-glutathionylation in mammalian cells. Further, in the case of reversible S-glutathionylation of proteins, it is mostly the dethiolation reaction, catalysed by GRX and TRX, which has received most attention. Clearly, there is a lack of evidence supporting the catalysis of S-glutathionylation of proteins. It should not be forgotten, however, that these proteins are theoretically able to catalyse the reactions in both directions (125), perhaps increasing the impact of this small number of proteins in the biological setting. We are at present investigating the ability of GRX to catalyse S-glutathionylation reactions, both by using model protein substrates, and in human cells following transient and stable transfection.

Apart from rather uniquely catalysing the thiol-disulphide interchange reactions, proteins such as GRX and TRX may achieve selectivity by differing forms of physical interactions with protein substrates. For instance, it has been suggested from X-ray crystallographic data that "target" proteins contain a regulatory domain constituting a GSH-binding "footprint", which assists in the selectivity process (64). Thioredoxin itself

is thought to exist in homodimeric form in biological systems (126), as well as in heterodimers with the nuclear reductant protein REF-1 (127). These multimeric forms of redoxin proteins may possess different manners of physical interaction with target proteins from their respective monomers. Finally, a very recent paper has shown that GRX is specifically associated with viral proteins during the assembly of the HIV-1 particle, where it is thought to be necessary for the activation of the HIV-1 protease (128). This elegantly demonstrates that strong physical interactions with protein substrates in complex protein aggregates may constitute an important part of substrate selectivity. Clearly, in the future the use of the yeast "two-hybrid" system might prove useful in defining cellular proteins forming fairly stable interactions with proteins such as GRX and TRX. Additionally, it is envisaged that, as the human genomic organisation program progresses, further proteins will be discovered which are capable of catalysing reversible S-glutathionylation of proteins in a selective manner.

Through the accumulated data and arguments presented above, it must be concluded that GSH, which is strictly conserved throughout all higher forms of aerobic life, indeed plays a role in controlling mammalian cell proliferation, both at the levels of mitogenesis and apoptosis. The relationships to these processes are likely to be complex, involving the interaction of other regulatory processes, and cell-specific. As we pass into a new millennium of scientific endeavour, it is likely that our understanding of the biochemistry of GSH in this area will be required to match the pace of discovery in cell biology in general.

## REFERENCES


1. Meister, A., and Andersson, M. (1984) *Annu. Rev. Biochem.* **52,** 711–760.
2. Booth, J., Boyland, E., and Sims, P. (1961) *Biochem. J.* **79,** 516–524.
3. Boyland, E., and Chasseaud, L. F. (1969) *Biochem. J.* **115,** 985–991.
4. Smith, G. J., Ohl, V. S., and Litwack, G. (1977) *Cancer Res.* **37,** 8–14.
5. Moscow, J. A., and Dixon, K. H. (1993) *Cytotechnology* **12,** 155–170.
6. McCord, J. M., and Fridovich, I. (1969) *J. Biol. Chem.* **244,** 6049–6055.
7. Flohe, L., Gunzler, W. A., and Schock, H. H. (1973) *FEBS. Lett.* **32,** 132–134.
8. Moldeus, P., and Orrenius, S. (1984) *Trends in Pharm. Sci.* **5,** 432–436.
9. Sies, H., and Cadenas, E. (1985) *Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences* **311,** 617–631.
10. Grimm, L. M., Collison, M. W., Fisher, R. A., and Thomas, J. A. (1985) *Biochim. Biophys. Acta.* **844,** 50–54.
11. Bellomo, G., Mirabelli, F., DiMonte, D., Richelmi, P., Thor, H., Orrenius, C., and Orrenius, S. (1987) *Biochem. Pharmacol.* **36,** 1313–1320.
12. Holmgren, A., and Bjornstedt, M. (1995) *Methods in Enzymology* **252,** 199–208.
13. Holmgren, A., and Aslund, F. (1995) *Methods in Enzymology* **252,** 283–292, 1995.
14. Freedman, R. B., Hawkins, H. C., and McLaughlin, S. H. (1995) *Methods in Enzymology* **251,** 397–406.
15. Sun, Y., and Oberley, L. W. (1996) *Free Rad. Biol. Med.* **21,** 335–348.
16. Burdon, R. H. (1995) *Free Rad. Biol. Med.* **18,** 775–794.
17. Buttke, T. M., and Sandstrom, S. (1994) *Immunol. Today* **15,** 7–10.
18. Kosower, N. S., and Kosower, E. M. (1978) *Int. Rev. Cytol.* **54,** 109–160.
19. Bray, T. M., and Taylor, C. G. (1993) *Can. J. Physiol. Pharmacol.* **71,** 746–751.
20. Lomaestro, B. M., and Malone, M. (1995) *Ann. Pharmacother.* **29,** 1263–1273.
21. Meister, A. (1995) *Methods in Enzymology* **252,** 26–30.
22. Cotgreave, I. A., Atzori, L., and Moldéus, P. (1990) *in* Glutathione: Metabolism and Physiological Function (Vina, J., Ed.), pp. 155–179. CRC Press.
23. Davis, D. A., Dorsey, K., Wingfield, P. T., Stahl, S. J., Kaufman, J., Fales, H. M., and Levine, R. L. (1996) *Biochem.* **35,** 2482–2488.
24. Schinina, M. E., Carlini, P., Poltichelli, F., Zappacosta, F., Bossa, F., and Calbreses, L. (1996) *Eur. J. Biochem.* **237,** 433–439.
25. Dafre, A. L., Sies, H., and Akerboom, T. (1996) *Arch. Biochem. Biophys.* **332,** 288–294.
26. Ziegler, D. M. (1985) *Ann. Rev. Biochem.* **54,** 305–329.
27. Cotgreave, I. A., Atzori, L., and Moldeus, P. (1989) *in* Sulfur drugs and Related Chemicals. (Damani, L. A., Ed.), Vol. 2B, pp. 101–119. Ellis-Horwood, England.
28. Di Simplicio, P., Lupis, E., and Rossi, R. (1996) *Biochem. Biophys. Acta.* **1289,** 252–260.
29. DeMaster, E. G., Quast, B. J., Redfern, B., and Nagasawa, H. T. (1995) *Biochemistry* **34,** 11494–11499.
30. Nakagawa, Y., Moldéus, P., and Cotgreave, I. A. (1992) *Biochem. Pharmacol.* **43,** 2519–2525.
31. Schuppe, I., Moldéus, P., and Cotgreave, I. A. (1992) *Biochem. Pharmacol.* **44,** 1757–1764.
32. Rahman, I., Li, X. Y., Donaldson, K., Harrison, D. J., and Macknee, W. (1995) *Am. J. Physiol.* **269,** L285–L292.
33. Toxopeus, C., Van Holsteijn, I., Thuring, J. W., Blaauboer, B. J., and Noordhoek, J. (1993) *Arch. Toxicol.* **67,** 674–679.
34. Hiranruengchok, R., and Harris, C. (1995) *Teratology* **52,** 196–204.
35. Luo, M. F., Xu, G. T., and Cui, X. L. (1995) *Curr. Eye Res.* **14,** 951–958.
36. Dickerson, J. E., Lou, M. F., and Gracey, R. W. (1995) *Curr. Eye Res.* **14,** 109–118.
37. Cohen, G., Farroooqui, R., and Kensler, N. (1997) *Proc. Natl. Acad. Sci.* **94,** 4890–4894.
38. Scarlett, J. L., Packer, M. A., Porteous, C. M., and Murphy, M. P. (1996) *Biochem. Pharmacol.* **52,** 1047–1055.
39. Bernard, O., and Balasubramanian, K. A. (1995) *Int. J. Biochem. Cell Biol.* **27,** 589–595.
40. MacDuffee, A. T., Senisterra, G., Huntly, S., Lepkock, J. R., Sekhar, K. R., Merridith, M. J., Borrelli, M. J., Morrow, J. D., and Freeman, M. L. (1997) *J. Cell. Physiol.* **171,** 143–151.

41. Thomas, J. A., and Beidler, D. (1985) *Analyt. Biochem.* **157,** 32–38.
42. Collison, M. W., Beidler, D., Grimm, L. M., and Thomas, J. A. (1986) *Biochem. Biophys. Acta.* **885,** 58–67.
43. Rokutan, K., Thomas, J. A., and Sies, H. (1989) *Eur. J. Biochem.* **179,** 233–239.
44. Chai, Y. C., Jung, C. H., Lii, C. K., Ashraf, S. S., Hendrich, S., Wolf, B., Sies, H., and Thomas, J. A. (1991) *Arch. Biochem. Biophys.* **284,** 270–278.
45. Lii, C. K., Chai, Y. C., Zhao, W., Thomas, J. A., and Hendrich, S. (1994) *Arch. Biochem. Biophys.* **308,** 231–239.
46. Collison, M. W., and Thomas, J. A. (1987) *Biochim. Biophys. Acta.* **928,** 121–129.
47. Park, D. M., and Thomas, J. A. (1989) *Arch. Biochem. Biophys.* **274,** 47–54.
48. Schuppe-Koistinen, I., Moldéus, P., Bergman, T., and Cotgreave, I. A. (1994) *Eur. J. Biochem.* **221,** 1033–1037.
49. Ravichandran, V., Seres, T., Moriguchi, T., and Thomas, J. A. (1994) *J. Biol. Chem.* **269,** 25010–25015.
50. Knight, R. J., Kofoed, K. F., Schelbert, H. R., and Buxton, D. B. (1996) *Cardiovasc. Res.* **32,** 1016–1023.
51. Schuppe-Koistinen, I., Moldéus, P., Bergman, T., and Cotgreave, I. A., (1995) *Endothelium* **3,** 301–308.
52. Rokutan, K., Johnston, R. B., Jr., and Kawai, K. (1994) *Am. J. Physiol.* **266,** G247–G254.
53. Chai, Y. C., Ashraf, S. S., Rokutan, K., Johnston, R. B., and Thomas, J. A. (1994) *Arch. Biochem. Biophys.* **310,** 273–281.
54. Schuppe-Koistinen, I., Gerdes, R., Moldéus, P., and Cotgreave, I. A. (1994) *Arch. Biochem. Biophys.* **315,** 226–234.
55. Seres, T., Ravichandan, V., Moriguchi, T., Rokutan, K., Thomas, J. A., and Johnston, R. B. (1996) *J. Immunol.* **156,** 1973–1980.
56. Miller, R. M., Park, E. M., and Thomas, J. A. (1991) *Arch. Biochem. Biophys.* **287,** 112–120.
57. Fernando, M. R., Nanri, H., Yoshitake, S., Nagata-Kuno, K., and Minakami, S. (1992) *Eur. J. Biochem.* **209,** 917–922.
58. Jung, C. H., and Thomas, J. A. (1996) *Arch. Biochem. Biophys.* **335,** 61–72.
59. Gravina, S. A., and Mieyal, J. J. (1993) *Biochemistry* **32,** 3368–3376.
60. Rabenstein, D. L., and Millis, K. K. (1995) *Biochem. Biophys Acta.* **1249,** 29–36.
61. Nakamura, S., Matsushima, M., Song, H., and Kikuchi, M. (1996) *J. Biochem.* **120,** 525–530.
62. Bernard, and Balasubramian, K. A. (1996) *Int. J. Biochem. Cell Biol.* **28,** 1051–1059.
63. Raghavachari, N., and Lou, M. F. (1996) *Exp. Eye Res.* **63,** 433–441.
64. Thomas, J. A., Poland, B., and Honzatko, R. (1995) *Arch. Biochem. Biophys.* **319,** 1–9.
65. Moriguchi, T., Seres, T., Ravichandran, V., Sasada, M., and Johnston, R. B. (1996) *J. Leukocyte Biol.* **60,** 191–198.
66. Cerutti, P. (1985) *Science* **227,** 375–381.
67. Burdon, R. H., and Rice-Evans, C. (1989) *Free Rad. Res. Comms.* **6,** 345–358.
68. Slater, A. F. G., Nobel, C. S. I., Van den Dobbelstein, D., and Orrenius, S. (1996) *Cell Death Diff.* **3,** 57–62.
69. Burdon, R. H., Gill, V., and Rice-Evans, C. (1989) *Free Rad. Res Comms.* **7,** 149–159.
70. De Souza, R. J., Phillips, E. M., Jone, P. W., Strange, R. C., and Aber, G. M. (1993) *Clin. Sci.* **86,** 747–751.
71. Los, M., Dröge, W., Stricker, K., and Baeuerle, P. A. (1995) *Eur. J. Immunol.* **25,** 159–165.
72. Fiorani, M., Cantoni, O., Tasinto, A., Boscoboinik, D., and Azzi, A. (1995) *Biochim. Biophys. Acta.* **1269,** 98–104.
73. Burdon, R. H., Gill, V., and Rice-Evans, C. (1990) *Free Rad. Res. Comms.* **11,** 65–76.
74. Burdon, R. H., and Gill, V. (1993) *Free Rad. Res. Comms.* **19,** 203–213.
75. Alliangana, D. M. (1996) *East African Med. J.* **73,** 752–757.
76. Burdon, R. H., Alliangana, D., and Gill, V. (1995) *Free Rad. Res. Comms.* **23,** 471–486.
77. Yang, M., Nazat, N. B., Jiang, X., Kelsey, S. M., Blake, D. R., Newland, A. C., and Morris, C. J. (1996) *Br. J. Haematol.* **95,** 339–344.
78. Dypbukt, J. M., Ankarcrona, M., Burkitt, M., Sjöholm, A., Ström, K., Orrenius, S., and Nicotera, P. (1994) *J. Biol. Chem.* **269,** 30553–30560.
79. Rao, and Reddy, J. K. (1991) *Env. Health Perspect.* **93,** 205–209.
80. Lake, B. G. (1995) *Toxicol. Lett.* **82–83,** 673–681.
81. Rao, M. S., and Subbarao, V. (1997) *Fundam. Appl. Toxicol.* **35,** 78–83.
82. Zipper, J. (1997) *J. Mol. Cell. Cardiol.* **29,** 149–161.
83. Slater, A. F. G., Nobel, C. S. I., and Orrenius, S. (1995) *Biochem. Biophys. Acta* **1271,** 59–62.
84. Buttke, T. M., and Sandstrom, P. A. (1995) *Free Rad. Res.* **22,** 389–397.
85. Sandstom, P. A., Roberts, P. A., Folks, T. M., and Buttke, T. M. (1993) *AIDS Res. Human Retroviruses* **9,** 1107–1113.
86. Sandstrom, P. M., Tebbey, P. W., Van Cleave, S., and Buttke, T. M. (1994) *J. Biol. Chem.* **269,** 798–801.
87. Kim, H. S., Lee, J. H., and Kim, I. K. (1996) *Prostaglandins* **51,** 413–425.
88. Aoshima, H., Satoh, T., Sakai, N., Yamada, M., Enokido, Y., Ekeuchi, T., and Hatanaka, H. (1997) *Biochim. Biophys. Acta.* **1345,** 35–42.
89. Hamilton, R. F., Li, L., Felder, T. B., and Hollihan, A. (1995) *Am. J. Physiol.* **269,** L318–L325.
90. Okado, A., Kawasaki, Y., Hasuiki, Y., Takahashi, M., Teshima, T., Fujii, J., and Taniguchi, N. (1996) *Biochem. Biophys. Res. Comms.* **225,** 219–224.
91. Nobel, C. S. I., Kimland, M., Lind, B., Orrenius, S., and Slater, A. F. G. (1995) *J. Biol. Chem.* **270,** 26202–26208.
92. Sanchez, A., Alvarez, A. M., Benito, M., and Fabregat, I. (1996) *J. Biol. Chem.* **271,** 7416–7422.
93. Bustamante, J., Tovar, B. A., Montero, G., and Boveris, A. (1997) *Arch. Biochem. Biophys.* **337,** 121–128.
94. Cossarizza, A., Francheshi, C., Monti, D., Salvioli, S., Bellesia, E., Rivabene, L., Rainaldi, G., Tinari, A., and Malorni, W. (1995) *Exp. Cell Res.* **220,** 232–240.
95. Slater, A. F. G., Nobel, C. S. I., Maellaro, E., Bustamante, J., Kimland, M., and Orrenius, S. (1995) *Biochem. J.* **306,** 771–778.
96. Sandstrom, P. A., Mannie, M. D., and Buttke, T. M. (1994) *J. Lekocyte Biol.* 221–226.
97. Bustamante, J., Slater, A. F. G., and Orrenius, S. (1995) *Free Rad. Biol. Med.* **19,** 339–347.
98. Gabby, M., Tauber, M., Porat, S., and Simatov, R. (1996) *Neuropharmacology* **35,** 571–578.
99. Abello, P. A., Fidler, S. A., and Buchman, T. G. (1994) *Shock* **2,** 79–83.
100. Iwata, S., Hori, T., Sato, N., Hirota, K., Sasada, T., Mitsui, A.,

Hirakawa, T., and Yodoi, J. (1997) *J. Immunol.* **158,** 3108–3117.

101. Donnerstag, B., Ohlensclager, G., Cinatl, J., Amari, M., Hofmann, D., Flindt, S., Treusch, G., and Trager, L. (1996) *Cancer Lett.* **110,** 63–70.
102. Edwards, S. G., Adesida, A., and Thornally, P. J. (1996) *Leukemia Res.* **20,** 17–26.
103. Hockenbury, D. M., Oltavi, Z. N., Yin, X. M., Milliman, C. L., and Korsmeyer, S. J. (1993) *Cell* **75,** 241–251.
104. Briehl, M. M., Cotgreave, I. A., and Powis, G. (1995) *Cell Death Different* **2,** 41–46.
105. Fernandes, R. S., and Cotter, T. (1994) *Biochem. Pharmacol.* **48,** 675–681.
106. Watson, R. W., Rotstein, O. D., Nathens, A. B., Dackiw, A. P., and Marshall, J. C. (1996) *Surgery* **120,** 150–157.
107. Macho, A., Hirsch, T., Marzo, I., Marchetti, P., Dallaporta, B., Susin, S. A., Zamzami, N., and Kroemer, G. (1997) *J. Immunol.* **158,** 4612–4619.
108. Ghibelli, L., Coppola, S., Rotillio, G., Lafavia, E., Maresca, V., and Ciriolo, M. R. (1995) *Biochem. Biophys. Res. Comms.* **216,** 313–320.
109. Efferth, T., Fabry, U., Glatte, P., and Osieka, R. (1995) *J. Mol. Med.* **73,** 47–49.
110. Banki, K., Hutter, E., Colombo, E., Gonchoroff, N. J., and Perl, A. (1996) *J. Biol. Chem.* **271,** 32994–33001.
111. Perovic, S., Pialoglou, P., Schroeder, H. C., Pergande, G., and Muller, W. E. (1996) *Eur. J. Pharmacol.* **317,** 157–164.
112. Hyde, H., Borthwick, N. J., Janossy, G., Salmon, M., and Akbar, A. N. (1997) *Blood* **89,** 2453–2460.
113. Rattan, R. R., Murphy, T. H., and Baraban, J. M. (1994) *J. Neurosci.* **14,** 4385–4392.
114. Van den Dobbelsteen, D., Nobel, S., Schlegel, J., Cotgreave, I. A., Orrenius, S., and Slater, A. (1996) *J. Biol. Chem.* **271,** 15420–15427.
115. Chiba, T., Takahashi, S., Sato, N., Ishii, S., and Kikuchi, K. (1996) *Eur. J. Immunol.* **26,** 1164–1169.
116. Efferth, T., Fabrey, U., and Osieka, R. (1996) *Blood Cells Mol. Dis.* **22,** 2–9.
117. Watson, R. W., Rotstein, O. D., Jimenez, M., PArodo, J., and Marshall, J. C. (1997) *Blood* **89,** 4175–4181.
118. Delneste, Y., Jeanne, P., Sebille, E., Aubry, J. P., and Bonnefoy, J. Y. (1996) *Eur. J. Immunol.* **26,** 2981–2988.
119. Skoglund, G., Cotgreave, I. A., Rincon, J., Patarroyo, M., and Ingelman-Sundberg, M. (1988) *Biochem. Biophys. Res. Comms.* **157,** 443–449.
120. Kass, G. E., Duddy, S. K., and Orrenius, S. (1989) *Biochem. J.* **260,** 499–507.
121. Palumbo, E. J., Sweatt, J. D., Chen, S. J., and Klann, E. (1992) *Biochem. Biophys. Res. Comms.* **187,** 1439–1445.
122. Ozawa, T. (1997) *Physiol. Rev.* **77,** 425–464.
123. Marchetti, P., Castedo, M., Susin, S. A., Zamani, N., Hirsch, T., Macho, A., Haeffner, A., Hirsch, F., Geuskens, M., and Kroemer, G. (1996) *J. Exp. Med.* **184,** 1155–1160.
124. Stacey, N. H., and Craig, G. K. (1989) *Experimentia* **45,** 180–181.
125. Lawrence, D. A., Song, R., and Weber, P. (1996) *J. Leukocyte Biol.* **60,** 611–618.
126. Marchetti, P., Decaudin, D., Macho, A., Zamani, N., Hirsch, T., Susin, S. A., and Kroemer, G. (1997) *Eur. J. Immunol.* **27,** 289–296.
127. Mannervik, B., and Axelsson, K. (1980) *Biochem. J.* **190,** 125–130.
128. Weichsel, A., Gasdaska, J. R., Powis, G., and Montfort, W. R. (1996) *Structure* **4,** 735–751.
129. Qin, J., Clore, G. M., Kennedy, W. P., Kuszewski, J., and Gronenborn, A. M. (1996) *Structure* **4,** 613–620.
130. Davis, D. A., Newcomb, F. M., Starke, D. W., Ott, D. E., Mieyal, J. F., and Yarchoan, R. (1997) *J. Biol. Chem.* **272,** 25935–25940.