Thiol/disulphide exchange reaction: A key regulatory process in biological systems

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Cysteine residues in proteins serve many important functions such as stabilizing and maintaining the three-dimensional conformation of many proteins[1], in enzyme catalysis, as a residue undergoing post-translational modification[2] and in the formation of DNA-binding domain of a class of transcriptional activators[3]. It is also involved in biological redox coupling[4] and xenobiotic metabolism[5]. Disulphide bonds formed by oxidation of cysteine residues have been used as a probe to study the structure/function relationships of proteins. Introducing novel disulphide bonds in proteins to increase their thermal stability and, therefore, the shelf life is an important goal of protein engineering[6,7]. In addition, the thiol group of cysteine residue participates in a reaction termed as thiol/disulphide exchange reaction, the biological significance of this reaction being the theme of this review.

THIOL/DISULPHIDE exchange reaction is a reversible transfer of reducing equivalents between a thiol/disulphide pair. This process induces covalent modification of proteins by oxidizing cysteine residues in them. Free sulphydryl groups in proteins can get oxidized by reacting with disulphides to form a mixed disulphide. The presence of another reactive sulphydryl group in near proximity can lead to the formation of an intramolecular disulphide bond. By the same mechanism, isomerization of disulphide bonds within a protein or between two proteins can occur. The possible outcomes of thiol/disulphide exchange reaction involving cysteine residues in proteins are shown diagrammatically in Figure 1.

The rate of thiol/disulphide exchange reaction is dependent on various parameters.

pH

Since the initiation of a thiol/disulphide exchange reaction involves a nucleophilic attack by the thiolate anion (S^-), the ionization of the reacting sulphydryl group is important. Also, since thiol groups have a pK_a in the alkaline range, the reaction is favoured in an alkaline pH. Conversely, any factor that decreases the pK_a of a thiol group will alter the rate of the reaction. The sulphydryl groups in proteins show wide variation in their pK_a from the typical value of 8.5. For example, one of the two active site thiols of thioredoxin reductase[8] has a pK_a of 7.0 while that of lipoamide dehydrogenase[9] is < 5.5. Such alteration in the pK_a of protein thiols has been attributed to the presence of a positively charged centre that stabilizes the thiolate anion electrostatically.

Charge and steric factors

Thiol/disulphide reaction is a bimolecular reaction and therefore the reaction between a thiol and a disulphide compound of opposite charge is expected to be faster than a comparable reaction involving neutral molecules. For example, the rate constant for the reaction of thiolate anions of mercaptoethanol (–1 charge, pK_a 9.6) and thioglycolate (–2 charge, pK_a 9.8) with GSSG differ by 1.3-fold[10]. Studies by Szajewski and Whitesides[11] on the

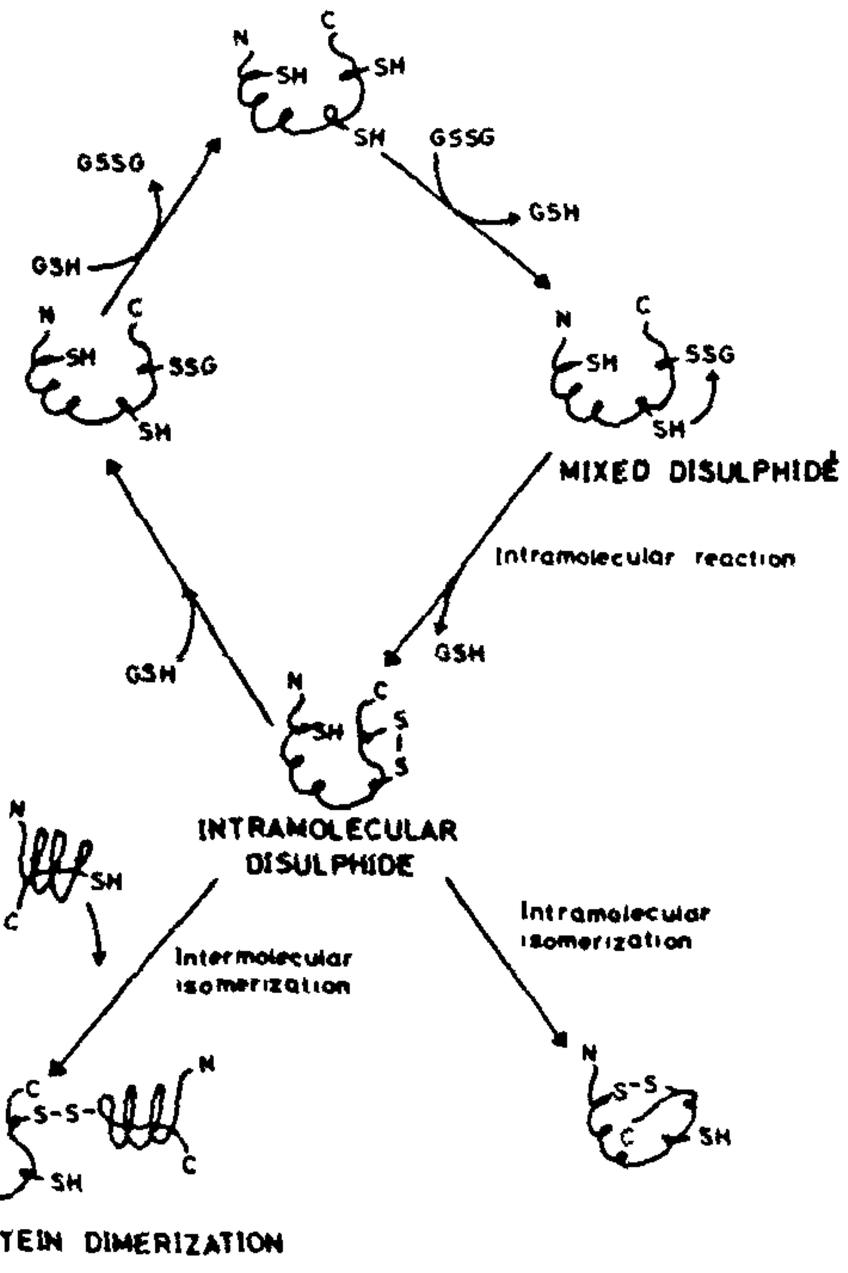

Figure 1. Fate of protein thiols and disulphides in the presence of oxidized (GSSG) and reduced (GSH) glutathione. Conformational change in the protein has been envisaged during intramolecular disulphide bond formation or intramolecular isomerization of disulphide bond.

factors that contribute to the rates of thiol/disulphide exchange reaction indicate deviations between observed rate constants and those predicted from pK_a values alone. Such deviations are relatively small (< 10-fold). However, the effect of charge and binding interactions play a dominant role in determining the rate of thiol/disulphide exchange reactions involving proteins. The following observations support such a conjecture:

(a) the free thiol group in bovine serum albumin reacts with a series of charged or uncharged disulphides at different rates that varied 150,000-fold[11];

(b) the reaction between GSSG and the dithiol at the active site of yeast glutathione reductase is 10^6-fold faster than what is expected from reactions involving small molecules;

(c) the rate of inactivation of HMG-CoA reductase by GSSG at pH 7.0 is 100-fold faster than what is expected for a reaction between small molecules[12]. Such large differences in the observed rates can be attributed to binding, and/or charge interaction between protein thiols and low molecular weight disulphides.

Entropy

Entropy plays an important role in determining the rate of thiol/disulphide exchange reaction in proteins, especially when it leads to the formation of intramolecular disulphide bonds. The formation of intramolecular disulphide bond from a dithiol involves two steps, as shown in Figure 1. The first step is the formation of a mixed disulphide and the second step is an intramolecular reaction between the free thiol and the mixed disulphide to give the intramolecular disulphide bond. The rate of the second reaction is influenced by the translational and rotational entropy barriers around the free thiol group and the mixed disulphide. If the free thiol and the mixed disulphide are in close proximity and are also placed in a rigid region of the protein molecule, the rate of formation of disulphide bond will be enhanced. Conversely, if the thiol groups are present in a flexible region of the protein molecule, the rate of disulphide bond formation will be lowered.

Thiol/disulphide exchange reaction alters the oxidation states of cysteine residues in a protein, thereby keeping them either in the reduced or in the oxidized forms. If these forms of a protein assume different conformations, the alteration in the conformation is likely to be associated with changes in its biological activity. This is the basis of regulation by the thiol/disulphide exchange reaction, as was first suggested by Guzman-Baron[13]. A considerable amount of evidence from various systems have supported this hypothesis. The occurrence of thiol/disulphide exchange reaction in the cell necessitates the presence of active thiols and disulphides, which are the key players in the exchange reaction, as shown in Figure 1. These active thiols and disulphides constitute the cellular redox buffer.

Cellular thiol/disulphide redox buffer

A thiol/disulphide redox buffer constitutes any redox-active thiol or dithiol that is present in both the thiol and the disulphide oxidation states. By virtue of being either in the reduced or in the oxidized states, a thiol/disulphide buffer functions to maintain or modulate the cellular redox status. The redox-active thiol or disulphide can be part of low molecular weight compounds or proteins.

The most abundant thiol/disulphide redox buffer is reduced and oxidized glutathione (GSH and GSSG) respectively, which is present in most cells including mammals, plants and aerobic bacteria[14]. In halobacteria the reduced and oxidized forms of γ-glutamyl–cysteine constitute the cellular-redox buffer[15], whereas in trypanosomes it is trypanothione, a conjugate of glutathione and polyamine[16]. Besides low molecular weight compounds, a class of proteins, thioredoxins and glutaredoxins constitute a thiol/disulphide redox buffer which is important in normal cellular functions. The role of these proteins is discussed later. The importance of thiol/disulphide exchange reaction in biological systems is discussed below.

Biological significance of the thiol/disulphide exchange reaction

Folding of proteins

Proteins render their services by virtue of their unique three-dimensional conformation. The process through which an unfolded polypeptide chain attains a three-dimensional conformation is referred to as protein folding. Since the sequence of amino acids in a protein (its primary structure) dictates the final conformation (the tertiary structure), it means that the pattern of inter-amino acid interactions which hold any protein in its native form is highly conserved. In this regard proteins having disulphide bonds face a unique problem during folding. Since disulphide bonds can be formed between any two cysteine residues, a protein with more than two cysteine residues can form disulphide bonds in many different combinations. Therefore, as the number of cysteine residues in a protein increases, the probability of forming the correct combination of disulphide bonds becomes less. How do proteins circumvent this problem during folding? This question has been clarified from the pioneering work of Creighton[17] on the folding of bovine pancreatic trypsin inhibitor (BPTI) *in vitro*.

BPTI has six cysteine residues which form three disulphide bonds [30–51; 5–55; 14–38]. During the

folding of BPTI, the protein undergoes a series of intramolecular thiol/disulphide exchange reactions till the correct combination of disulphide bonds is produced. Especially interesting was the observation made by Weissman and Kim[18] that the pro-region of BPTI plays a significant role in the folding of the protein *in vitro*. In the folding of BPTI the rate-limiting step is the slow intramolecular rearrangement of a native two-disulphide intermediate [30–51; 14–38] into a more stable native intermediate [30–51; 5–55]. The presence of the pro-region circumvents this rate-limiting rearrangement process, allowing direct formation of three disulphide bonds. Also, during the folding of BPTI, about 50% of the molecules accumulate as a kinetically trapped dead-end intermediate containing two native disulphide bonds [5–55; 14–38]. This intermediate is unable to form the third disulphide bond between Cys-30 and Cys-51 because these residues get buried in the protein molecule[19]. In the presence of the pro-region this intermediate does not accumulate, resulting in an increase in the yield of the mature protein. The important role that the pro-region plays in the folding of BPTI is due to a cysteine residue in it, which facilitates intramolecular thiol/disulphide exchange reaction.

This isomerization of disulphide bonds in a protein is catalysed by protein disulphide isomerase (PDI) *in vivo*[20]. PDI is present in very high concentrations (mM level) in the lumen of the endoplasmic reticulum where the folding of secretory proteins is completed. Studies *in vitro* have demonstrated that PDI has a very high affinity for cysteine-containing peptides. Further, two of the six cysteine residues in PDI which take part in the thiol/disulphide exchange reaction have a pK_a of 6.7, indicating greater reactivity at the physiological pH. The notion that PDI may be essential for the folding of proteins in the endoplasmic reticulum came from the work of Bulleid and Freedman[21]. They observed that dog pancreas microsomes depleted of their luminal contents, including PDI, were able to translate and translocate a plant storage protein Y-gliadin, but were unable to support their proper folding into the native form. More direct evidence regarding the significance of PDI *in vivo* was revealed from the genetic manipulation of PDI expression in *Saccharomyces cerevisiae*. Repression or disruption of PDI gene resulted in the formation of non-native disulphide bonds in carboxypeptidase Y[22]. Conversely, overexpression of PDI in yeast resulted in a 24-fold increase in the secretion of a highly disulphide bonded protein antistasin from Mexican leech saliva[23].

Thermoinactivation of proteins

Proteins subjected to high temperatures lose their native conformation and become largely disordered due to extensive co-operative intramolecular motions, resulting in thermo-inactivation. For an enzyme this process results in loss of catalytic activity. Proteins containing free cysteine residues or disulphide bonds undergo incorrect structure formation due to thiol/disulphide exchange reaction. Such reactions can be prevented by heating the protein in the presence of *N*-ethyl maleimide or other thiol-scavenging chemicals. Zale and Klibanov[24] reported that the irreversible loss of activity of ribonuclease A at 90°C was significantly lower when thiol-scavenging compounds are included in the reaction mixture. As discussed earlier, thiol/disulphide exchange reaction involves a reaction between a thiol and a disulphide. At high temperatures, thiols are generated as a by-product from the destruction of disulphide bonds in proteins by a process known as β-elimination. Thiols generated by this process can initiate thiol/disulphide exchange reaction, resulting in the formation of scrambled structure in proteins[25].

Bacterial metabolism

Regulation of enzyme activity by thiol/disulphide exchange reaction in *Escherichia coli* was first elucidated by Thelander *et al.*[26] in the course of their work on ribonucleotide reductase. This enzyme catalyses the reduction of ribonucleotides to their corresponding deoxyribonucleotides and is essential for the survival of the organism. The enzyme was found to be inactivated in purified preparations. The inactive enzyme could be reactivated by reductants such as dithiothreitol or reduced lipoic acid. The cellular hydrogen donor for ribonucleotide reductase was identified to be a redox-active protein, thioredoxin. Reduced thioredoxin reduces a disulphide bond in inactive ribonucleotide reductase by thiol/disulphide exchange reaction. Oxidized thioredoxin is then reduced by NADPH in the presence of thioredoxin reductase and the next cycle is initiated. Thioredoxin has since been shown to serve different functions in *E. coli*, besides activating ribonucleotide reductase. The finding that thioredoxin-deficient strains of *E. coli* could synthesize deoxyribonucleotides as efficiently as the wild-type bacteria led to the discovery of a second redox-active protein, glutaredoxin. The glutaredoxin system differs from the thioredoxin system in that it utilizes reduced glutathione as the reductant instead of NADPH. The thioredoxin and glutaredoxin systems form an important control mechanism for the regulation of enzyme activities by the thiol/disulphide exchange reaction[27].

Thiol/disulphide exchange reactions also play an important role in regulating the response of *Salmonella typhimurium* and *E. coli* to oxidative stress. The inducible defence mechanism to counter oxidative damage in these bacteria involves the synthesis of catalase, superoxide dismutase and peroxidase which protect the cell from

reactive oxygen species. The genes of these enzymes are positively regulated by a protein oxy-R. Storz *et al.*[28] showed that the oxy-R protein can switch between a reduced and an oxidized form by the oxidation/reduction of essential cysteine residues. The oxidized protein acts as a transcriptional activator inducing the expression of genes involved in the defence mechanism. By this process, the response time for induction of specific genes is reduced since the protein itself acts as a transducer in response to signals generated during oxidative stress.

Plant metabolism

The primary biochemical reaction that generates biomass in our biosphere is photosynthesis. This light-dependent process, occurring in the chloroplasts, generates sucrose from atmospheric CO_2 and NADPH via a series of sequential enzymatic reactions known as the Calvin cycle. Why are chloroplasts unable to fix CO_2 in the dark? One reason is the lack of NADPH which is generated only in light. However, a convincing body of evidence indicates that key enzymes of the Calvin cycle are kept inactive in the dark and are activated only in light. The regulatory role of light in the activation of key enzymes is mediated by thiol/disulphide exchange reaction via the ferredoxin/thioredoxin system[29]. Ferredoxin is a component of the photosynthetic electron transport chain that transduces light energy into chemical energy by accepting electrons generated by light from photosystem I. The reduced ferredoxin, besides channelizing its reducing power for the generation of NADPH, also transfers electrons for the reduction of a disulphide bond in thioredoxin. This reduction is catalysed by an enzyme ferredoxin–thioredoxin reductase. The reduced thioredoxin then reduces essential disulphide bonds in fructose-1,6-bisphosphatase, seduheptulose-1,7-bisphosphatase and phosphoribulokinase, which are the key enzymes of the Calvin cycle. In the dark, the non-availability of reduced thioredoxin keeps these enzymes in an inactive form, thus ensuring the operation of the Calvin cycle only in light.

Animal metabolism

In animal systems, metabolic regulation involving thiol/disulphide exchange reaction is due to the tripeptide glutathione (γ-glutamyl–cysteinyl–glycine), which exists in the cell in reduced (GSH) and oxidized (GSSG) forms. GSH promotes reduction of oxidized cysteine residues in proteins whereas GSSG promotes their oxidation. By their reciprocal effects these compounds maintain protein thiols either in their reduced or in their oxidized states. The reduced and the oxidized states of cysteine residues in protein will be dependent both on the relative amount of GSH and GSSG in the cell and on the kinetic and thermodynamic properties of the thiol groups involved. The role of thiol/disulphide exchange reaction in the regulation of enzyme activities was elucidated following the perturbation of thiol/disulphide balance by chemicals that oxidize glutathione such as butthionine sulfoximine[30] or diamide[31]. Such studies have indicated that concomitant with the change in the cellular environment from reducing to oxidizing, there is a stimulation of glycogen hydrolysis and inhibition in glucose oxidation by the glycolytic pathway[32]. The results of these artificial perturbation experiments supported the observed *in vivo* changes in the level of glutathione in the liver cells after starvation or high diet and the changes in enzyme activities associated with it . These experiments provided circumstantial evidence that alteration in the cellular redox status can, in fact, alter metabolism by changing the activities of the catalytic enzymes. Several enzymes of sugar metabolism such as phosphorylase phosphatase, phosphofructokinase, pyruvate kinase and glucose-6-phosphatase were found to be regulated by thiol/disulphide exchange reactions *in vitro*[33].

Unlike the plant system, wherein thiol/disulphide exchange reaction is primarily involved in the light/dark regulation of the Calvin cycle, the contribution of this reaction in the regulation of animal metabolism is still circumstantial. The main reasons for this are:

(a) in animals the regulatory switching is not an all-or-none phenomenon, unlike in plants;

(b) many of the enzymes in animals that are regulated by thiol/disulphide exchange reaction are also regulated by other mechanisms such as phosphorylation/dephosphorylation, binding of specific ligands, and hormones and therefore their individual contribution in the regulation of enzymes is uncertain. However, it is important to analyse the enzymes that are regulated by the exchange reaction *in vitro* and the effect of activation/inactivation of these enzymes in the regulation of metabolic pathways *in vivo*.

Enzymes regulated by thiol/disulphide exchange reaction fall into two classes, those which are activated by GSH and inactivated by GSSG and others which are activated by GSSG and inactivated by GSH. The enzymes belonging to the first category require free SH group(s) for activity while those of the second category become catalytically active when their SH-groups are oxidized. Therefore, the effect of a high GSH/GSSG ratio on these two groups of enzymes will be opposite. The enzymes of the first group will remain active, while the members of the second group will be inactive. If these two groups of enzymes occupy control points of metabolic pathways, these pathways can be regulated by changing the GSH/GSSG ratio. A few examples are now drawn from the literature to highlight this point:

(1) The activities of phosphofructokinase and fructose-1,6-bisphosphatase switch carbohydrate metabolism between

glycolysis and gluconeogenesis[34]. These two enzymes are reciprocally regulated by thiols and disulphides. Phosphofructokinase is inhibited by GSSG[35], but fructose-1,6-bisphosphatase is activated by GSSG[36]. Therefore, at high GSH/GSSG ratio the flux of glucose through glycolysis will be higher than at low GSH/GSSG ratio. The effect will be reversed in the case of gluconeogenesis.

(2) The regulation of hexokinase and glucose-6-phosphatase prevents uncontrolled hydrolysis of ATP[37]. Thiol/disulphide exchange reaction may play an important role in this regulation since hexokinase is positively regulated by thiols[38] and the activity of glucose-6-phosphatase is stabilized by GSSG, although indirectly[39].

(3) Leukocyte collagenase, which plays an important role in phagocytosis is activated by the dissociation of an inhibiting peptide. The dissociation is initiated by disulphides via thiol/disulphide exchange reaction[40]. This mechanism of activation becomes physiologically significant in view of the fact that during phagocytosis, leukocytes accumulate large amounts of GSSG in their cytoplasm, sufficient to activate latent collagenase[41].

Besides regulation of enzyme activities by thiol/disulphide exchange reaction, a very interesting finding has been the regulation of mammalian gene expression by thiol oxidation. In this process, the transcriptional factors act as transducers in the signal transduction pathway, which means that they respond directly to external stimuli by converting themselves into a DNA-binding form. Abate *et al.*[42] demonstrated that the DNA-binding abilities of the fos and jun proteins are regulated by redox control. A critical cysteine residue located in the basic region of these proteins mediates DNA-binding. Oxidation of this residue abrogates the DNA-binding ability of fos and jun. The exceptional reactivity of the cysteine residue in these proteins[43] makes it readily susceptible to oxidation, and therefore, an attractive candidate for regulation by the nuclear redox system. Further, an ubiquitously present 37 kDa protein in the mammalian nuclear extracts was purified and shown to catalyse the DNA-binding ability of the fos and jun proteins. This nuclear factor, designated as redox factor-1 (Ref-1)[44], responds to the cellular-redox status and, therefore, may act as a primary transducer in this signal transduction pathway. The presence of accessory proteins catalysing the activation of transcriptional factors in a redox-dependent manner argue a case for the redox regulation of transcription in the mammalian system. In parallel with the identification of redox-active transcriptional factors, Rushmore *et al.*[45] identified antioxidant responsive elements (ARE) in the 5′-flanking region of the genes which are induced by phenolic antioxidants. The responsiveness of AREs to reactive oxygen species again represents part of a signal transduction pathway that allows eukaryotic cells to sense and respond to oxidative stress.

Another role of thiol/disulphide exchange reaction in animal systems stems from a study of the interaction between hormones and their cognate receptors. The hormone–receptor complexes transduce their signal indirectly by inducing a biochemical cascade either through G-protein, or through adenylate-cyclase, or directly induce the expression of specific genes by binding to their upstream regulatory sequences. Earlier work by Grippo *et al.*[46] indicated that the binding of glucocorticoid hormone to its receptor was abolished by sulphydryl-modifying compounds and the binding capacity of the receptor was enhanced by thioredoxin/thioredoxin reductase system. This observation suggested that a thiol/disulphide exchange reaction between reduced thioredoxin and the oxidized receptor is essential for steroid binding. In this light, the finding of Boniface and Reichert[47] that gonadotropic hormones LH and FSH have thioredoxin-like catalytic properties is very significant. Their results indicate that following the binding of these hormones to their receptor, LH and FSH induce disulphide isomerization or initiate redox reactions that produce structural changes in the receptor, resulting in signal transduction.

The induction of specific genes through a conformational change in the transcriptional factors brought about by thiol/disulphide exchange reaction raises the question whether this mechanism can control developmental cascades. This aspect has not been seriously considered although there are reports in the literature that point to this possibility. The yeast *Candida albicans* can grow in an unicellular yeast form (Y-form) or in a mycelial group (M-form). This transition from Y-form to M-form was found to be accompanied by a 100-fold decrease in the level of GSH[48]. This morphological transition could be blocked by preventing the decline in GSH levels, suggesting that an oxidizing intracellular environment favours such transitions. In a recent communication by Esposito *et al*[49], it has been reported that perturbation of the redox status of myeloid cells prevents their differentiation possibly through structural changes in the transcriptional factors mediated by the thiol/disulphide exchange reaction. It can be expected that many other key regulatory events in biological systems may be governed by thiol/disulphide exchange reactions, but remain to be discovered.

Fungal metabolism

Besides plants and animals, fungi constitute a separate group among eukaryotes. The literature is sparse with reports on the significance of thiol/disulphide exchange reaction in fungal physiology and metabolism. Fahey *et al.*[50] observed a dramatic increase in GSH/GSSG ratio during germination of *Neurospora* conidia. The authors suggested that metabolic switching from a quiescent to

an active state might parallel with changes in cellular-redox status from an oxidizing to a reducing one. While studying the utilization of sucrose in a thermophilic fungus, *Thermomyces lanuginosus*, Maheshwari *et al.*[51] came across a novel invertase whose activity was unstable, both *in vivo* and *in vitro*. Further studies to elucidate the mechanism of instability of invertase *in vitro* indicated that the enzyme activity was modulated by thiol/disulphide exchange reaction[52]. This finding assumed significance with the observation that the appearance and disappearance of mycelial invertase activity during the growth of the fungus correlated with a decline in the mycelial redox status (manuscript in preparation). These observations suggested that thiol/disulphide exchange reaction may constitute an important regulatory mechanism in determining metabolic heterogeneity across the length of a fungal hypha.

Concluding remarks

Although the significance of thiol/disulphide exchange

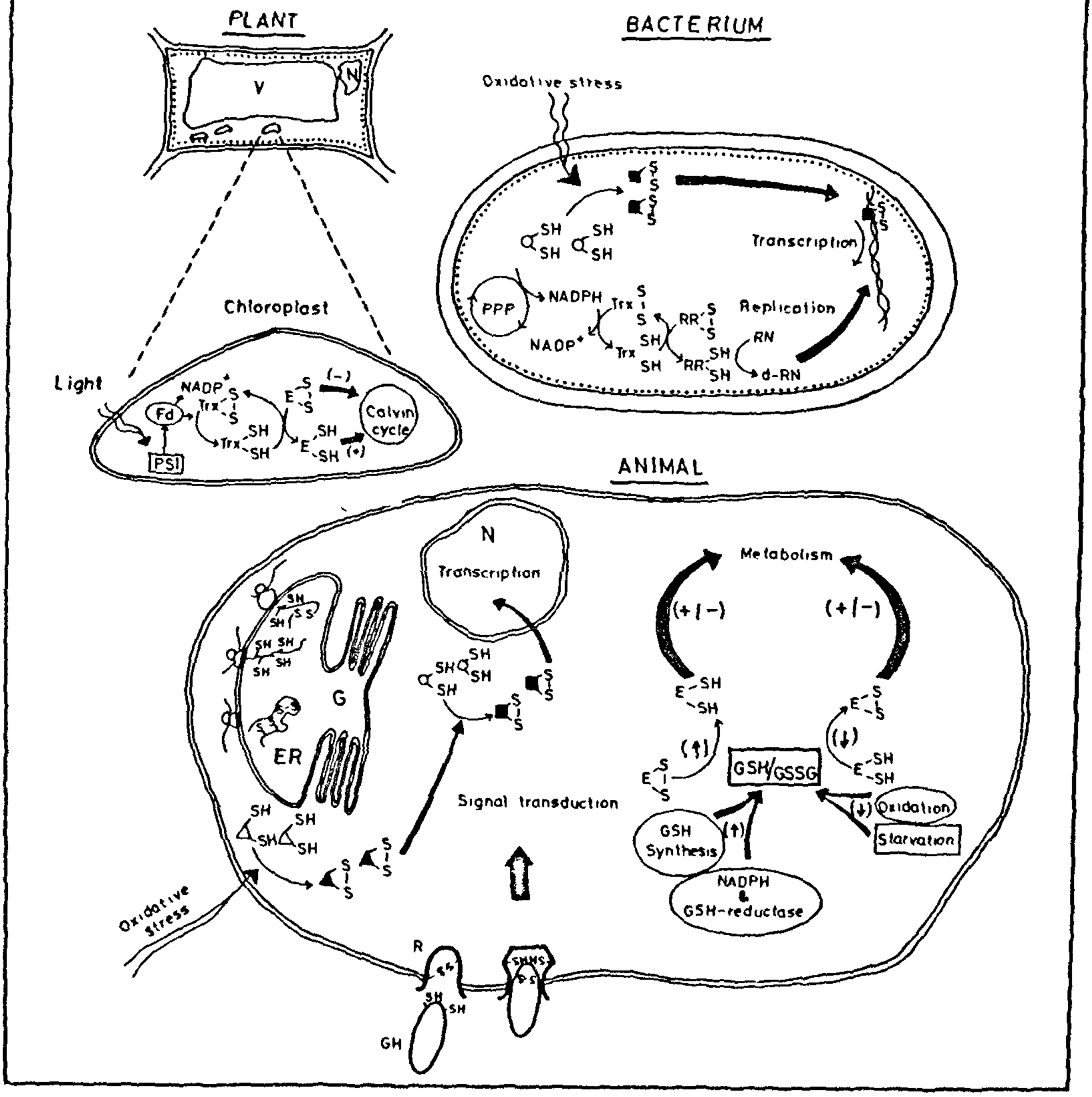

Figure 2. A diagrammatic representation of cellular events controlled by thiol/disulphide exchange reaction. Key: V, vacuole, N, nucleus, ER, endoplasmic reticulum, G, golgi, E, enzyme; GH, gonadotropin hormone; Fd, ferredoxin, PSI, photosystem I, R, receptor, RR, ribonucleotide reductase; RN & d-RN, ribo- and deoxy-ribonucleotides, Trx, thioredoxin, α^{in}_{in}, inactive transcriptional activator, $\alpha^{}_{in}$, active transcriptional activator; $\alpha^{}_{in}$, redox-active transducer protein

reaction in the regulation of cellular metabolism was suggested about 40 years back, the potential of this reaction as a control mechanism has been realized only recently, as shown in the diagrammatic representation in Figure 2. There are many reasons for this lag:

(1) It is difficult to assess *a priori* from experiments conducted *in vitro* whether certain cellular events are controlled by thiol/disulphide exchange reaction. To make a case for such a regulation, two main criteria are to be fulfilled. Firstly, the redox status of the micro-environment in which the event occurs has to be accurately determined. The lability of GSH to oxidation and the presence of GSSG in the cell in very low amounts, however, make this task formidable. Secondly, the redox potential of the reactive dithiol/disulphide pair in a given protein has to be determined. Such measurements have indicated that many enzymes whose activities are regulated reciprocally by thiol compounds and disulphides *in vitro* may not be amenable to modulation by the observed changes in the cellular-redox status[33]. (2) There are again certain limitations associated with this regulation: (a) the mechanism lacks specificity since any thiol group or disulphide in a protein can be oxidized or reduced; (b) the reaction is freely reversible without any energy barrier between the reactants and the products; and (c) the reaction occurs spontaneously if the redox potentials of the reacting groups are compatible.

However, to circumvent these limitations, the cellular machinery has adopted different strategies. A major strategy is to vary the reactivities of cysteine residues so that they respond differently to a change in the cellular-redox status. Another strategy, evident in the hormone–receptor interaction is the specificity of binding of the hormone to its cognate receptor that precedes thiol/disulphide exchange reaction.

Finally, although the amino acid cysteine is present in a wide variety of proteins and enzymes, only a fraction of these enzymes are regulated by thiol/disulphide exchange reaction. This strongly suggests that these enzymes were under a strong selection pressure to evolve a mechanism of regulation by oxidation/reduction of cysteine residues. This aspect assumes importance because no attribute in a cell is selected and maintained unless a fitness component is associated with it.

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