Cell, Vol. 96, 751–753, March 19, 1999, Copyright ©1999 by Cell Press

Bridge over Troubled Waters: Sensing Stress by Disulfide Bond Formation

Minireview

Fredrik Åslund† and Jon Beckwith*
Department of Microbiology and
Molecular Genetics
Harvard Medical School
200 Longwood Avenue
Boston, Massachusetts 02115

The regulation of protein activity is a major factor in the cellular response to a changing environment. Well-established mechanisms for such regulation include protein–protein interactions, allosteric changes generated by ligand binding, and chemical modifications such as phosphorylation. It has long been postulated that disulfide bond formation represents a covalent modification that can regulate protein activity. This proposal has come from studies in which enzymes or transcription factors were shown to lose activity after oxidation of cysteine residues in vitro, but to regain activity when exposed to the disulfide reductant thioredoxin. However, such reports may often be based on the fact that the proteins studied usually exist in the reducing environment of the cytosol; the oxidative inactivation observed may simply reflect the unnatural oxidizing conditions of the in vitro system and not reflect the in vivo state of affairs.

Thus, while it is not clear that loss of function by disulfide bond formation has been established as a regulatory mechanism, recent results suggest that the reverse process, gain of function by disulfide bond formation, may be a common way of responding to cellular stress. The utilization of improved techniques for assessing the disulfide-bonded states of proteins in vivo has allowed a reexamination of the role of these bonds in regulating protein activity. These studies provide strong evidence that two bacterial proteins, the transcription factor OxyR and the chaperone heat shock protein 33 (Hsp33), are activated by the oxidation of cysteine residues to disulfide bonds (Zheng et al., 1998; Jakob et al., 1999). These findings and the approaches used should provide impetus to a search for what is likely to be a more widespread occurrence of this mechanism for regulating protein activity.

The two most important means of maintaining the reducing thiol-disulfide status of the cytosol involve the thioredoxin-thioredoxin reductase pathway and the glutathione-glutaredoxin pathway (Prinz et al., 1997). Thioredoxins and glutaredoxins were first detected by their ability to reduce a disulfide bond in the active site of ribonucleotide reductase, as part of the reduction pathway converting ribonucleotides to deoxyribonucleotides. The reduced form of thioredoxin is regenerated by thioredoxin reductase, whereas glutaredoxin is kept reduced by glutathione. Thioredoxin and glutaredoxin are members of the thioredoxin superfamily and exert their action by a disulfide exchange reaction utilizing a Cys-X_1-X_2-Cys active site. Other members of the thioredoxin superfamily are responsible for the introduction and isomerization of disulfide bonds that are often present in secreted proteins. All evidence points to a situation in which cytosolic proteins have evolved to maintain their cysteines reduced in the native form, whereas many secreted proteins have evolved to be more stable when their cysteines are joined in disulfide bonds. Thus, changes in the reducing environment of the cytosol can have profound effects on protein folding and activity.

Perturbations of the cellular redox conditions can be achieved either by mutations that eliminate components of the thioredoxin and glutaredoxin systems or by environmental oxidative stress. Mutant analysis shows that a simultaneous block of both disulfide-reducing pathways is incompatible with growth under aerobic conditions (Prinz et al., 1997). Functional overlap between the two pathways is indicated by the finding that, in the absence of only one of the pathways, strains can survive and grow reasonably well. Nevertheless, the oxidizing conditions in the cytosol of such strains allows for the formation of disulfide bonds in some proteins. This disulfide bond–forming activity can be monitored by expressing in the cytosol a normally exported protein, such as alkaline phosphatase, which requires disulfide bonds for its enzymatic activity. Although it has yet to be directly demonstrated, it seems likely that unwanted disulfide bonds are also generated in the normal resident proteins of the cytosol during oxidative stress—a situation we refer to as "disulfide stress."

Bacteria encounter oxidative stresses in environments with high levels of hydrogen peroxide or other reactive oxygen species. One example of such stress occurs when pathogenic bacteria confront oxidative bursts upon invasion of eukaryotic host cells. These encounters, in addition to damaging other cellular molecules, may also cause the introduction of deleterious disulfide bonds into proteins. Furthermore, studies using cytosolic alkaline phosphatase suggest that *E. coli* in the stationary phase is subject to disulfide stress (Dukan and Nyström, 1998).

How does the bacterial cell respond to the stress that disulfide bond formation in the cytosol poses? Two features common to this response are the restoration of the redox homeostasis in the cytosol and the elimination of the harmful oxidant (Figure 1). Such responses are a requirement for life in an aerobic environment that is accompanied by exposure to reactive oxygen species.

In *E. coli*, the exposure to reactive oxygen species (ROS) such as $O_2^{-}\cdot$ and hydrogen peroxide activates the transcription factors SoxR/S and OxyR (Hidalgo et al., 1997; Zheng et al., 1998). These factors trigger the expression of defense activities including superoxide dismutase and peroxidases. Recently, it was discovered that the response to peroxides and disulfide stress is due to the formation of a disulfide bond within the OxyR protein, thus converting it to a transcriptional activator (Zheng et al., 1998; Åslund et al., 1999). These studies,

*To whom correspondence should be addressed (e-mail: jbeckwith@hms.harvard.edu).
† Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (as of April 1, 1999).

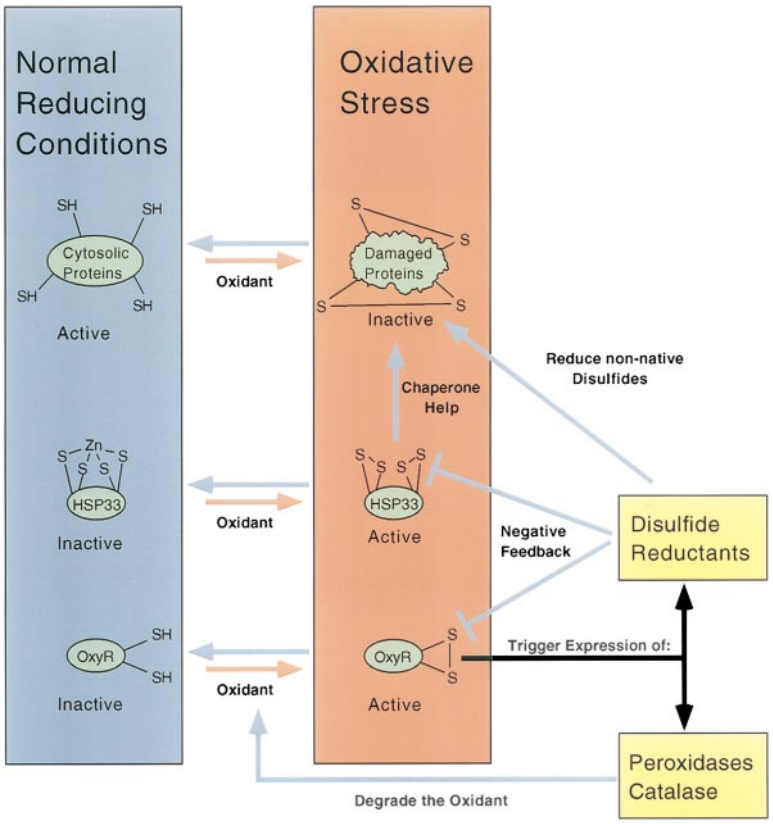

Figure 1. Cellular Redox Homeostasis

Oxidative stress (orange arrows) oxidizes cysteines to disulfide bonds leading to the inactivation of cellular proteins and the activation of defense proteins such as the chaperone Hsp33 and the transcription factor OxyR. Active OxyR triggers the expression of reductive activities (blue arrows) such as enzymes that degrade the oxidant and reduce disulfide bonds. Reduction of the disulfide bonds in Hsp33 and OxyR provides for negative feedback. It is not known which cysteines are ligands for the Zn^{2+} atom, nor have the Hsp33 disulfide bonds been mapped.

relying on both in vivo and in vitro experiments, provided the first well-established example of stress response activation by disulfide bond formation. Hydrogen peroxide can act directly on OxyR, generating a reactive oxidized derivative of a cysteine within the protein, which then can form a disulfide bond. Alternatively, OxyR can be activated by disulfide bond formation resulting from the altered redox state of the cytosol. This is shown by the finding that activation of OxyR takes place in *E. coli* mutants lacking components of the thioredoxin and glutaredoxin pathways. This activation is independent of exposure to hydrogen peroxide.

The active form of OxyR not only triggers the expression of peroxidases that remove the oxidant, but also increases the expression of disulfide reductants such as glutathione reductase and glutaredoxin. Enhanced expression of these cytosolic redox components serves to regenerate the overall reducing conditions of the cell and to deactivate OxyR by reducing its disulfide bond.

Similarly, gram-positive organisms of the actinomycetes group are able to respond to exposure to the disulfide-generating oxidant diamide by elevating the expression of thioredoxin and thioredoxin reductase. Recent results indicate that this transcriptional response is mediated by inactivation of an inhibitor of transcription (Paget et al., 1998). It appears that a protein ("anti-sigma factor"), which normally prevents a particular sigma transcription factor from functioning, is inactivated by disulfide bond formation.

A recent report in *Cell* describes another example of protein activation by disulfide bond formation induced by oxidative stress (Jakob et al., 1999). In this case, the altered protein is the chaperone Hsp33. This activation of a chaperone may have evolved as a response to the effects of hydrogen peroxide, which oxidizes some proteins to inactive, less stable forms that are prone to aggregation. Since these effects are similar to those seen under conditions of heat shock, it is perhaps not surprising that there is considerable overlap in the cellular responses to oxidative stress and heat shock. Hsp33 is one of the proteins induced under both conditions. This chaperone is important for protection against oxidative stress and may be important for keeping oxidized proteins soluble, allowing them to either be reduced and regenerated, or be degraded by cellular proteases.

Under normal, unstressed conditions, the critical cysteines of Hsp33 serve as ligands for an associated zinc atom (Jakob et al., 1999). Upon exposure to oxidative stress, the cysteines are oxidized to disulfide bonds. It is only the oxidized form of Hsp33 that exhibits chaperone activity. As with OxyR (Åslund et al., 1999), demonstration of the physiological significance of this oxidation step was achieved in vivo by the trapping of the active disulfide-bonded form (Jakob et al., 1999). In both cases, the disulfide-bonded forms were detected only after exposure to oxidants such as hydrogen peroxide or in mutants lacking components of the thioredoxin and glutaredoxin systems that exhibit a more oxidizing cytosol. After an oxidative challenge, once the reducing conditions of the cytosol are restored, Hsp33 is returned to its inactive state through reduction of its disulfide bond. This reduction presumably occurs through the activities

of the thioredoxin and glutaredoxin systems, as indicated by the genetic evidence.

Using the formation and reduction of a disulfide bond as an "on-off" switch allows for rapid response to oxidative conditions. Under normal, unstressed conditions, both OxyR and Hsp33 are retained in their reduced, inactive forms, since the disulfide bonds of the active forms of OxyR and Hsp33 are quite unstable (−170 to −185 mV; Zheng et al., 1998; Jakob et al., 1999) and thus easily reduced. Given that the redox potentials of cellular reductants such as thioredoxin and glutathione are strongly reducing (−260 to −280 mV), it is apparent that quite large drops in the cellular redox status are required for activation of OxyR and Hsp33 by thiol-disulfide exchange. While equilibration of Hsp33 and OxyR against the cellular redox buffer is the conceptually most simple mechanism of activation, an additional mode of activation appears to be operative in OxyR. Here, the fast kinetics (close to diffusion rate) of OxyR oxidation by hydrogen peroxide leads to activation of OxyR in an overall highly reducing environment with respect to disulfide bond formation (Åslund et al., 1999). Fast kinetics with hydrogen peroxide enables OxyR to rapidly respond to increments in peroxide levels. After degradation of the oxidant, deactivation of OxyR follows as a thermodynamic consequence of the unstable nature of its disulfide bond.

The overall function of the cellular disulfide reducing systems and the chaperones appear to share a number of properties. These systems both appear to have evolved to help maintain the properly folded state of proteins in the cytosol. A further analogy is that both systems are highly redundant and show little substrate specificity. The requirement for thioredoxin or glutaredoxin for protein activity is obvious for enzymes that are dependent on a disulfide bond reduction for part of their catalytic cycle (e.g., ribonucleotide reductase) or as part of their regulatory cycle (e.g., OxyR and Hsp33). What seems likely, but has yet to be demonstrated for a cytosolic protein, is that part of the damage done to proteins after oxidative stress is the formation of unwanted disulfide bonds, which disrupt protein folding and activity. Although disulfide bonds do form in normally exported proteins (e.g., alkaline phosphatase) when they are retained in the cytosol under altered redox conditions, a similar in vivo finding of aberrant disulfide bond formation has yet to be reported for a normally resident cytosolic protein. Thus, as for the chaperones, it is difficult to assign the actual in vivo substrates for thioredoxin and glutaredoxin, under conditions of oxidative stress.

The difficulty in detecting proteins activated by oxidative stress, on the one hand, and the many in vitro reports that particular proteins can be altered by disulfide reduction, on the other, may be due to artifacts arising during sample preparation. Part of the problem has been the failure to recognize the rapid nature of thiol-disulfide interchange, which makes it difficult to trap proteins with the ratios of thiol-disulfide-bonded species as they exist in vivo. This problem can, to a large degree, be avoided by acid trapping of the proteins of interest (Weissman and Kim, 1991). In addition, the availability of high molecular weight compounds, such as 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate (AMS), that alkylate free cysteines have proved extremely useful in separating reduced forms from disulfide-bonded forms of proteins for assessment of in vivo redox ratios (Kobayashi et al., 1997).

Now that methods to detect evanescent disulfide bonds are readily available, we anticipate the discovery of additional cases where disulfide bond formation regulates the activity of a protein. In eukaryotes, responses to hydrogen peroxide have similarities to those described in *E. coli*. In *Saccharomyces cerevisiae*, the hydrogen peroxide regulon consists of peroxidases, heat shock proteins, and disulfide-reducing enzymes, and an overall shift in metabolism away from glycolysis and toward NADPH production (Godon et al., 1998). This finding is consistent with the role of NADPH as a source of electrons for thioredoxin reductase and glutathione reductase. While transcription factors associated with the yeast response to hydrogen peroxide have been identified, their mechanism of activation has yet to be determined. It seems reasonable to anticipate that disulfide bond formation may be a key signal in this eukaryotic response to oxidative stress also. Such signals may turn out to be significantly more widespread than presently appreciated.

Selected Reading

Åslund, F., Zheng, M., Beckwith, J., and Storz, G. (1999). Proc. Natl. Acad. Sci. USA, in press.

Dukan, S., and Nyström, T. (1998). Genes Dev. *12*, 3431–3441.

Godon, C., Lagniel, G., Lee, J., Buhler, J.-M., Kieffer, S., Perrot, M., Boucherie, H., Toledano, M.B., and Labarre, J. (1998). J. Biol. Chem. *273*, 22480–22489.

Hidalgo, E., Ding, H., and Demple, B. (1997). Cell *88*, 121–129.

Jakob, U., Muse, W., Eser, M., and Bardwell, J.C.A. (1999). Cell *96*, 341–352.

Kobayashi, T., Kishigami, S., Sone, M., Inokuchi, H., Mogi, T., and Ito, K. (1997). Proc. Natl. Acad. Sci. USA *94*, 11857–11862.

Paget, M.S.B., Kang, J.G., Roe, J.H., and Buttner, M.J. (1998). EMBO J. *17*, 5776–5782.

Prinz, W.A., Åslund, F., Holmgren, A., and Beckwith, J. (1997). J. Biol. Chem. *272*, 15661–15667.

Weissman, J.S., and Kim, P.S. (1991). Science *253*, 1386–1393.

Zheng, M., Åslund, F., and Storz, G. (1998). Science *279*, 1718–1721.