Critical Reviews in Biochemistry and Molecular Biology, 39:297–317, 2004

ISSN: 1040-9238 print / 1549-7798 online
DOI: 10.1080/10409230490900658

Taylor & Francis
Taylor & Francis Group

Beyond Transcription—New Mechanisms for the Regulation of Molecular Chaperones

Jeannette Winter and Ursula Jakob

Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI, USA

Molecular chaperones are an essential part of the universal heat shock response that allows organisms to survive stress conditions that cause intracellular protein unfolding. During the past few years, two new mechanisms have been found to control the activity of several chaperones under stress conditions—the regulation of chaperone activity by the redox state and by the temperature of the environment. Hsp33, for example, is redox-regulated. Hsp33 is specifically activated by disulfide bond formation during oxidative stress, where it becomes a highly efficient chaperone holdase that binds tightly to unfolding proteins. Certain small heat shock proteins, such as Hsp26 and Hsp16.9, on the other hand, are temperature regulated. Exposure to heat shock temperatures causes these oligomeric proteins to disassemble, thereby changing them into highly efficient chaperones. The ATP-dependent chaperone folding system DnaK/DnaJ/GrpE also appears to be temperature regulated, switching from a folding to a holding mode during heat stress. Both of these novel post-translational regulatory strategies appear to have one ultimate goal: to significantly increase the substrate binding affinity of the affected chaperones under exactly those stress conditions that require their highest chaperone activity. This ensures that protein folding intermediates remain bound to the chaperones under stress conditions and are released only after the cells return to non-stress conditions.

Keywords redox-regulation, temperature regulation, oxidative stress, heat shock, Hsp33, DnaK/DnaJ/GrpE

INTRODUCTION

Molecular chaperones assist in the folding of newly synthesized proteins and prevent the misfolding and/or irreversible aggregation of proteins under both normal and stress conditions (Ellis & Hartl, 1999; Hartl & Hayer-Hartl, 2002). They accomplish this task by temporarily binding to folding intermediates, thereby preventing improper inter- and intramolecular interactions between transiently exposed hydrophobic surfaces (Bukau & Horwich, 1998; Walter & Buchner, 2002).

Many molecular chaperones are constitutively expressed under normal conditions and become massively upregulated under heat stress conditions (Gething & Sambrook, 1992). These chaperones are classified as heat shock proteins (Hsp) and include Hsp60/Hsp10 (GroEL/GroES in *Escherichia coli*), Hsp90 (HtpG in *E. coli*), Hsp70/Hsp40 (DnaK/DnaJ in *E. coli*), small heat shock proteins (sHsps) (IbpA/IbpB in *E. coli*), Hsp100/Clp (ClpB in *E. coli*), and Hsp33. For an overview of all heat shock proteins related to *E. coli*, see Rosen and Ron, 2002. In addition to molecular chaperones, several proteases (ClpAP, ClpXP, DegP, FtsH, HslVU, Lon in *E. coli*) are also upregulated under heat shock conditions. These proteases degrade misfolded proteins and are, therefore, indirectly involved in the prevention of protein aggregation.

The heat shock response is universally found in all organisms examined (Craig, 1985). It is triggered by the accumulation of aggregation sensitive unfolding intermediates and ensures sufficient amounts of chaperones and proteases to prevent the irreversible precipitation of these proteins during stress conditions, such as elevated temperatures, viral infections, or oxidative stress (Parsell & Sauer, 1989; Lund, 2001).

The function of most chaperones is tightly regulated. One group of chaperones is regulated by ATP binding and hydrolysis. These chaperones, which are often termed "foldases," cycle between an ATP- and ADP-bound state, which confers a significantly different binding affinity for their substrate proteins (Wegele *et al.*, 2004). In the case of the DnaK (Hsp70) chaperone machinery, the ADP-bound state represents the high-affinity binding state. DnaK-ADP complexes bind substrate proteins very tightly and prepare them for their refolding. In contrast, the ATP-bound state of DnaK represents the low-affinity state that undergoes

Editor: Elizabeth A. Craig
Address correspondence to Ursula Jakob, Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA. E-mail: ujakob@umich.edu

weak interactions with the unfolded substrate proteins and has a high on/off rate of substrate binding (Schmid *et al.*, 1994; Mayer *et al.*, 2000). Conversion between the states is mediated by co-chaperones that stimulate ATP-hydrolysis (DnaJ) and nucleotide exchange (GrpE) (Liberek *et al.*, 1991; McCarty *et al.*, 1995; Harrison, 2003). In addition to DnaJ and GrpE, which function as co-chaperones of prokaryotic DnaK, a growing number of other co-factors interact with eukaryotic Hsp70 (e.g., Hip, auxilin, BAG-1) (for review see Fink, 1999; Alberti *et al.*, 2003; Young *et al.*, 2003). The continuous cycle of ADP/ATP-induced binding and release of substrate proteins is essential for the successful refolding of the folding intermediates to the native state.

ATP-independent chaperone "holdases" such as DnaJ, Hsp33, and sHsps appear to bind to unfolded substrate proteins or folding intermediates via hydrophobic interactions (Lee *et al.*, 1997; Rudiger *et al.*, 2001; Graf *et al.*, 2004) but are usually unable to support their refolding to the native state directly (Rudiger *et al.*, 2001; Haslbeck, 2002; Linke *et al.*, 2003; Hoffmann *et al.*, 2004; Stromer *et al.*, 2004). Some holdases have been shown to present and transfer their substrate proteins to corresponding chaperone foldases such as the DnaK-system, which then support the refolding of the respective proteins (Lee & Vierling, 2000; Mogk *et al.*, 2003; Hoffmann *et al.*, 2004). This interplay between chaperone holdases and foldases is tightly regulated, so that unproductive folding efforts are prevented. Under non-permissive folding conditions, the unfolded substrate proteins remain bound to chaperone holdases. Only after return to non-stress conditions are the substrate proteins relayed onto the foldase-system for refolding (Hoffmann *et al.*, 2004).

In analogy to the ATP-regulated activity of chaperone foldases, certain chaperone holdases also cycle between high- and low-affinity substrate binding states—here, however, either the redox-state of the environment (e.g., Hsp33, PDI) or the environmental temperature (e.g., sHsps) appears to play the crucial part in the regulation of their binding affinity. Many excellent published reviews have described the more conventional regulation of chaperone activity by ATP binding and hydrolysis (Richter & Buchner, 2001; Wegele *et al.*, 2004). Here, we will focus on the two recently discovered modes of post-translational regulation of chaperone activity: regulation by the redox-state and regulation by temperature.

REDOX-REGULATION OF MOLECULAR CHAPERONE ACTIVITY

Reactive oxygen species (ROS) are continuously generated during aerobic metabolism as toxic byproducts of respiration (Imlay, 2003; Apel & Hirt, 2004). In response, many organisms have developed several small molecules (e.g., glutathione) and protein systems (e.g., glutaredoxin, thioredoxin, catalase, superoxide dismutase) to counteract this hazard during normal cell growth.

During the past years, several physiological and pathological conditions (e.g., neurodegenerative diseases, cancer, ageing, apoptosis, inflammation, and diabetes) have been seen to accompany or possibly cause the increased accumulation of ROS, a condition called "oxidative stress" (Chung *et al.*, 2001; Kyselova *et al.*, 2002; Ueda *et al.*, 2002; Calabrese *et al.*, 2003). Under these conditions, the cell's regular defense mechanisms become overwhelmed, leading to increased levels of ROS that can cause lethal damage to most cellular macromolecules, including DNA, membranes, and proteins. In proteins, oxidative stress is known to cause carbonylation of amino acids as well as the oxidation of sulfur in methionine and cysteine residues (Cabiscol *et al.*, 2000; Requena *et al.*, 2001; Stadtman *et al.*, 2003). These modifications can cause thermal instability of many proteins, and can ultimately lead to the unfolding and aggregation of cellular proteins (Berlett & Stadtman, 1997). It is therefore not surprising that all these conditions can also induce the expression of some heat shock proteins (Gophna & Ron, 2003).

Investigators have identified several proteins that are not damaged by reactive oxygen species, but rather use ROS as the regulator of their protein function. These redox-regulated proteins include the *E. coli* transcription factor OxyR, which is specifically activated by peroxides and induces the oxidative stress response in *E. coli* (Ding & Demple, 1997; Aslund *et al.*, 1999). Other proteins, such as the protein tyrosine phosphatase 1B (PTB1B) or the tumor suppressor phosphatase PTEN, are transiently inactivated during oxidative stress (Leslie *et al.*, 2003; van Montfort *et al.*, 2003). The oxidative stress transcription factor Yap1p from *Saccharomyces cerevisiae* and the anti-sigma factor, RsrA from *Streptomyces coelicolor*, on the other hand, show a redox-regulated change in the affinity for their regulatory partner proteins (Kang *et al.*, 1999; Delaunay *et al.*, 2000). Oxidative stress leads to the accumulation of Yap1p in the nucleus and to the dissociation of RsrA from its sigma factor, SigR. Both mechanisms lead to the induction of the antioxidant protein expression in the respective organism (reviewed in Linke & Jakob, 2003). All these redox-regulated proteins have in common the presence of oxidation-sensitive thiol groups, which are reversibly oxidized to either form disulfide bonds (e.g., OxyR, Yap1, RsrA) (Aslund *et al.*, 1999; Kang *et al.*, 1999; Azevedo *et al.*, 2003), sulfenic acids (e.g. NADH peroxidase, Yeh *et al.*, 1996), sulfinic acids (e.g., peroxiredoxin, Woo *et al.*, 2003), or sulfenyl-amides (e.g., PTP1B, Salmeen *et al.*, 2003; van Montfort *et al.*, 2003). The oxidation of these cysteine residues causes a reversible change in protein conformation and, more importantly, in their protein activity.

A few years ago, the first redox-regulated heat shock protein, Hsp33, was discovered (Jakob *et al.*, 1999). Hsp33 functions as a molecular chaperone, which is specifically activated by ROS and protects cells against the otherwise lethal consequences of oxidative stress. Oxidative stress leads to the rapid oxidation of Hsp33's four absolutely conserved cysteine residues and induces the activation of its chaperone function (Graumann *et al.*, 2001). Aside from Hsp33, protein disulfide isomerase (PDI) has also been noted to bind certain substrate proteins in a redox-regulated manner (reviewed in Graf and Jakob, 2002; Sitia and Molteni, 2004). Tsai *et al.* (2001) and Molinari *et al.* (2002) described PDI as a redox-driven chaperone, which binds to its substrate proteins in its reduced state and releases the substrate proteins when it is in its oxidized state. This redox-regulated substrate binding seems, however, to be restricted to specific substrate proteins of PDI, because binding of PDI to the C-propeptide of procollagen or the α-subunit of prolyl-4-hydroxylase appears to be independent of its redox state (Lumb & Bulleid, 2002).

The Molecular Chaperone Hsp33: Activation by Oxidation

Hsp33 is Induced Under Heat Stress and Protects Cells Against Oxidative Stress. Hsp33 was originally identified as part of a global analysis to identify new heat shock-regulated proteins in *E. coli* (Chuang & Blattner, 1993). Now, Hsp33 has been found in more than 120 different prokaryotic organisms, where it is located in the reducing environment of the cytoplasm. Recently, Hsp33 has also been identified in two eukaryotic organisms, *Chlamydomonas reinhardtii* and *Dictyostelium discoideum*, where it is believed to be localized to the mitochondria (Graf & Jakob, 2002; Linke & Jakob, 2003).

In *E. coli*, transcription of the Hsp33 encoding gene *hslO* is controlled by σ^{32}, and the expression of Hsp33 is strongly induced upon heat shock treatment of cells (Chuang & Blattner, 1993; Richmond *et al.*, 1999). Although Hsp33 is functionally activated by oxidative stress, no direct ROS-induced expression of Hsp33 has been observed. Under normal conditions, the concentration of Hsp33 is ca. 1.4 μM; by comparison, the concentration of the well studied *E. coli* chaperone GroEL in its active conformation is 3.4 μM (Mogk *et al.*, 1999). After heat treatment, the mRNA-level of Hsp33 increases by as much as 30-fold. This translates into an about twofold increase on the protein level, which increases the cellular Hsp33 concentration to about 3.0 μM (Jakob *et al.*, 1999).

Hsp33 is important for bacterial defense against severe oxidative stress. This becomes especially apparent in thioredoxin reductase (Δ *trxB*) deletion strains, which are permanently oxidatively stressed and are thus unable to efficiently resolve disulfide bonds in the cytoplasm (Derman *et al.*, 1993). These cells show a tenfold greater tolerance toward additional H_2O_2 treatment (Takemoto *et al.*, 1998), which appears to be due in part to the presence of functional Hsp33 (Jakob *et al.*, 1999). Additional deletion of Hsp33 leads to a more than 10^6-fold higher sensitivity towards hydrogen peroxide (H_2O_2) treatment at heat shock temperatures when compared to Δ*trxB* cells expressing functional Hsp33 (Jakob *et al.*, 1999). Moreover, Δ*trxB* Δ*hslO* deletion strains are unable to form single colonies at heat shock temperatures on MacConkey agar, whereas Δ*trxB* strains expressing Hsp33 reveal robust growth. The results suggest that Hsp33 is especially vital for the protection of cells against a combination of oxidative stress at elevated temperatures (Graf *et al.*, 2004).

Reduced Hsp33 is Inactive and Monomeric. Hsp33 is a 32.8 kDa protein localized in the reducing environment of the cytoplasm of *E. coli*. It contains four absolutely conserved cysteines in its C-terminal redox switch domain, arranged in a Cys-X-Cys-X_{27-32}-Cys-X-X-Cys motif. Under non-stress conditions, where the redox potential of the cytoplasm is about -260 to -280 mV (Gilbert, 1990), Hsp33 is predominantly in its reduced state (Jakob *et al.*, 1999). Here, the four cysteine residues are present in their thiolate anion state and coordinate one zinc ion with very high affinity ($K_a > 10^{17}$ M^{-1}, 25°C at pH 7.5) (Jakob *et al.*, 2000). The zinc ion is coordinated in a tetrahedral geometry as shown by the recently solved NMR structure of the zinc-binding domain (residues 227 to 287) of Hsp33 (Won *et al.*, 2004). Binding of zinc to Hsp33 stabilizes the C-terminus in a compact α-helical structure (Graf *et al.*, 2004). Importantly, the compact zinc binding domain has been found to effectively mask the substrate binding site of Hsp33 and to block its dimerization interface, both of which are located in the N-terminus of Hsp33 (Graf *et al.*, 2004). This renders zinc-coordinated, reduced Hsp33 species monomeric, and thus inactive as a molecular chaperone (Graumann *et al.*, 2001).

Hsp33 is Activated by Disulfide Bond Formation and Dimerization. Zinc binding is needed to keep Hsp33 inactive under non-stress conditions and seems equally important to prime Hsp33 for its rapid activation under oxidative stress conditions. Activation studies using zinc-bound and zinc-free Hsp33 showed that the activation of metal free Hsp33 was very slow and incomplete (Jakob *et al.*, 2000). After exposure of zinc coordinated Hsp33 to ROS such as H_2O_2 or hydroxyl radicals, the four conserved thiolate anions quickly oxidize to form two intramolecular disulfide bonds connecting Cys^{232} with Cys^{234} and Cys^{265} with Cys^{268} (Barbirz *et al.*, 2000). This reaction causes the release of zinc and is followed by the dimerization of oxidized Hsp33 monomers (Jakob *et al.*, 1999; Graumann *et al.*, 2001). Dimerization of two oxidized

Hsp33 monomers leads to the full activation of Hsp33 chaperone function (Graumann *et al.*, 2001) (Figure 1).

Recently, CD and NMR studies were performed using constitutively monomeric and zinc-free Hsp33 mutants. These studies resulted in a detailed view of the structural changes that accompany the activation of Hsp33. These studies also showed that most of the oxidation-induced conformational changes are restricted to the C-terminal redox switch domain (Graf *et al.*, 2004). While the N-terminus of Hsp33 remains largely unaltered during the oxidation and activation process of Hsp33, the C-terminus of Hsp33 loses its structure and appears to unfold almost completely upon oxidation-induced disulfide bond formation and zinc release (Graf *et al.*, 2004). Structural analysis of the C-terminal zinc binding domain of Hsp33 allowed investigators to explain these massive conformational rearrangements, which seem to accompany disulfide bond formation and zinc release and to form the signal that triggers Hsp33's switch (Won *et al.*, 2004). The amino acid composition of the redox switch domain is unusually low in hydrophobic side chains, and the distribution of these side chains is not ideal for creation of secondary structure of the folded domain. Therefore, the domain has the ability to build a hydrophobic core in the presence of the stabilizing zinc ion. After oxidation-induced loss of zinc binding, however, the many destabilizing elements that are present in this structure contribute to the rapid unfolding of the redox switch domain (Won *et al.*, 2004).

Oxidation-induced unfolding of the C-terminal domain was found to expose a hydrophobic substrate binding site in Hsp33, a finding in close agreement with earlier studies that showed the activation of Hsp33 to be accompanied by a dramatic increase in exposure of hydrophobic surfaces, a prerequisite for efficient substrate binding by molecular chaperones (Raman *et al.*, 2001). Oxidized Hsp33 monomers were shown to exert partial chaperone activity *in vitro* but were found to be unable to protect cells against severe oxidative stress (Graf *et al.*, 2004).

Although zinc release alone was sufficient to expose the substrate-binding site in Hsp33, correct disulfide bond formation was found to be crucial for the unmasking of the dimerization interface. As a consequence, only oxidized Hsp33 monomers are able to dimerize and thus to become fully active chaperones. Oxidized Hsp33 dimers show extensive surface-exposed hydrophobic patches, which may explain their high affinity for unfolding substrate proteins (Kim *et al.*, 2001; Vijayalakshmi *et al.*, 2001).

In contrast to disulfide bond formation and zinc release, the dimerization of Hsp33 is a highly concentration- and temperature-dependent process (Graumann *et al.*, 2001). As measured by ultracentrifugation at 20°C, the dissociation constant K_D for the oxidized (active) Hsp33 dimer is 0.6 μM (Graumann *et al.*, 2001). Dimerization of Hsp33 is, therefore, expected to be favored at heat shock temperatures, where its cellular concentration increases to at least 3 μM. These findings confirmed the observation that Hsp33 plays its most important role under oxidative heat stress conditions in the cell. Whereas oxidative stress triggers the post-translational disulfide bond formation in Hsp33, heat shock temperatures increase the Hsp33 concentration and stimulate its dimerization.

Active Hsp33 Dimers have Wide Substrate Specificity In Vitro. Oxidized *E. coli* Hsp33, which only crystallized in its truncated form (aa 1 to 235 or aa 1 to 255) revealed a domain swapped dimer in the structure (Kim *et al.*, 2001; Vijayalakshmi *et al.*, 2001) (Figure 2). Based on patterns of conserved residues and surface charges, investigators have proposed two potential substrate binding sites, extending over and involving residues of both subunits (Kim *et al.*, 2001; Vijayalakshmi *et al.*, 2001; Graumann *et al.*, 2001). Although the exact location of the substrate binding site is not known, mutational and functional studies clearly indicate that the substrate binding site is located within the N-terminus of Hsp33. A truncated Hsp33 version that harbors only the first 235 amino acids exhibits chaperone activity (Kim *et al.*, 2001), whereas the C-terminal redox switch domain (aa 218 to 287) lacks chaperone activity *in vitro* (VanHaerents, Graf, Jakob, unpublished observations). Once activated and present as a dimer, Hsp33 interacts with a large variety of *in vitro* substrate proteins, including chemically and thermally unfolding firefly luciferase (Graf *et al.*, 2004; Hoffmann *et al.*, 2004), porcine mitochondrial citrate synthase (Jakob *et al.*, 1999), and rhodanese (Jakob & Graf, unpublished report), oxidatively unfolded *E. coli* RrmJ (FtsJ) (Jakob *et al.*, 1999), and thermally unfolding ζ-crystallin (Raman *et al.*, 2001). These complexes between active Hsp33 dimers and unfolding protein intermediates are apparently very stable. Hsp33-luciferase complexes, for example, were found to be stable for at least 20 hours at heat shock temperatures, with no significant aggregation of luciferase observed under these conditions (Hoffmann *et al.*, 2004). This finding suggested that Hsp33 functions as an efficient chaperone holdase, which prevents the aggregation of a large number of substrate proteins by forming very stable, long-lived chaperone-substrate protein complexes.

Substrate Release and Inactivation of Hsp33: A Multi-Step Process. Active Hsp33 dimers form very stable complexes with substrate proteins and function independently of co-chaperones and ATP (Jakob *et al.*, 1999). This raises a question about the fate of the bound substrate proteins. Because Hsp33 becomes activated by disulfide bond formation and dimerization, the most obvious mechanism for substrate release and inactivation of Hsp33 was thought to be the reduction of Hsp33. A model that was similar to the mechanism described for PDI predicted that reducing

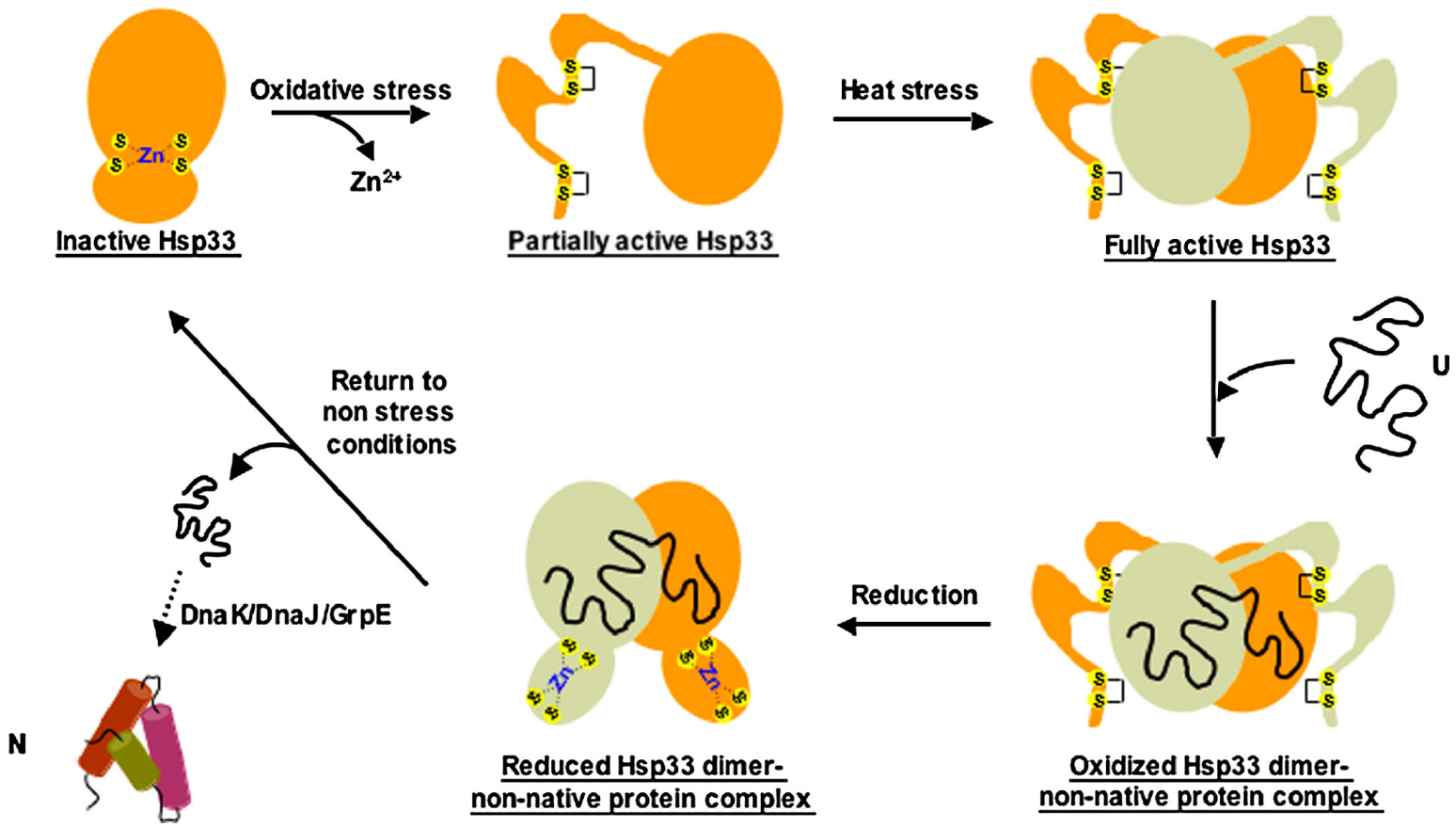

FIG. 1. Redox-Regulation of the Molecular Chaperone Hsp33—a Model. Under non-stress conditions, Hsp33 is reduced and inactive. All four absolutely conserved cysteine residues that are present in the C-terminal redox switch domain are in their thiolate anion state and coordinate one zinc ion with very high affinity. The zinc coordinated C-terminus appears to mask the substrate binding site and dimerization interface of Hsp33 and renders the reduced protein inactive and monomeric. Upon exposure of Hsp33 to severe oxidative stress, two intramolecular disulfide bonds form and zinc is released. This causes the unfolding of the C-terminal redox switch domain and oxidized Hsp33 monomers dimerize to form a highly active chaperone. These Hsp33 dimers have a very high affinity for unfolding proteins and form apparently very stable substrate-complexes during stress conditions. *In vivo*, Hsp33 dimers are quickly reduced by the cellular glutaredoxin and thioredoxin system. This reduction of Hsp33 does not inactivate the chaperone but rather primes Hsp33 for its interaction with the DnaK/DnaJ/GrpE system. Upon return to non-stress conditions, which is reflected by the re-availability of the DnaK/DnaJ/GrpE system, the substrate proteins are released from reduced Hsp33 dimers and can be refolded by the DnaK/DnaJ/GrpE system. At the same time, reduced Hsp33 dimers dissociate into reduced monomers and Hsp33 is inactivated.

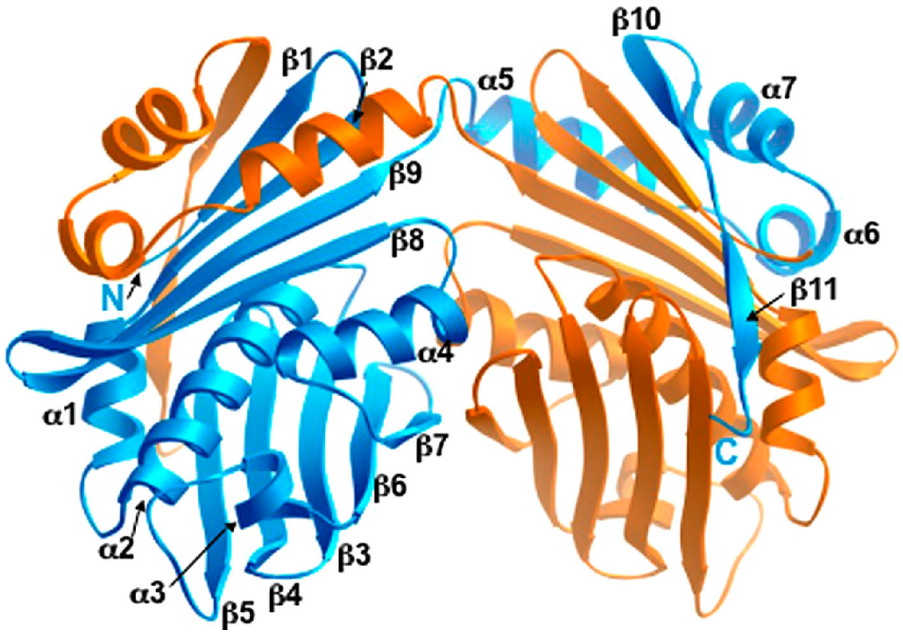

FIG. 2. Crystal Structure of *E. coli* Hsp33 (aa 1 to 255). Ribbon diagram of the active Hsp33(1–255) dimer (side view). Only amino acids 1 to 234 are visible. The two subunits are colored in orange and blue and the α-helices and β-strands of one Hsp33 monomer are numbered. Strands β5, β4, β3, β6, and β7 from both monomers come together and form an intermolecular 10-strand β-sheet. α5, α6, α7, β10, and β11 comprise the C-terminal domain of Hsp33, which is domain-swapped and makes extensive contacts with the other protomer. Figure reproduced with permission from Vijayalakshmi *et al.*, 2001 and Structure.

conditions should cause the dissociation of Hsp33 into the inactive Hsp33 monomers (Tsai *et al.*, 2001). This process should lead to the release of the substrate proteins, which could then refold to their native state. A problem with this simple model is, however, that reducing conditions are usually very quickly restored *in vivo* and often precede the return to permissive folding conditions. The release of any substrate proteins from Hsp33 under these non-permissive folding conditions would, therefore, result in the immediate aggregation of the released substrate proteins—suggesting that the inactivation of Hsp33 might be regulated in a more sophisticated manner than merely by the reduction of the chaperone. This finding was confirmed by a series of *in vitro* experiments that showed that, similar to the activation of Hsp33, the process of substrate release and inactivation of Hsp33 also comprised several highly regulated steps (Hoffmann *et al.*, 2004) (Figure 1). The first step in the inactivation process of Hsp33 was confirmed to be the reduction of oxidized Hsp33 dimers. This reduction is catalyzed *in vivo* by the thioredoxin and glutaredoxin system and appears to happen within minutes after the exposure of cells to H_2O_2-induced oxidative stress (Hoffmann *et al.*, 2004). This process, however, was found to not cause any substrate release but resulted in the formation of kinetically stable, reduced Hsp33 dimers, as shown by anisotropy measurements *in vitro*. This finding was in stark contrast to other redox-regulated proteins such as OxyR, which are also activated under oxidative stress, and whose reduction by the glutaredoxin system exactly parallels their inactivation (Zheng *et al.*, 1998; Aslund *et al.*, 1999). Reduced Hsp33 dimers, however, showed a similar affinity for bound substrate proteins than oxidized Hsp33 dimers and remained in very stable complexes with their substrate proteins (Hoffmann *et al.*, 2004). The release of the bound substrates required a sophisticated interplay with the DnaK/DnaJ/GrpE foldase-system (Hoffmann *et al.*, 2004). Only after incubation of the reduced Hsp33-substrate complexes with the DnaK-system, were the bound substrate proteins released from Hsp33 and refolded by the DnaK-system. The mechanism of substrate transfer and Hsp33 dissociation is not fully understood. Because oxidized and reduced Hsp33 dimers show a very similar affinity to their substrate proteins, simple competition between the DnaK/DnaJ/GrpE system and reduced Hsp33 dimers for substrate binding seems rather unlikely. It is conceivable, however, that the DnaK-system specifically interacts with reduced Hsp33 dimers because of significant structural rearrangements that might accompany the reduction of oxidized Hsp33 dimers and the refolding of the redox switch domain. This model is supported by the recently solved crystal structure of reduced full-length Hsp33 from *Bacillus subtilis*, which

showed a non-domain swapped zinc-coordinated dimer (Janda *et al.*, 2004). This conversion of a domain-swapped oxidized Hsp33 dimer to a non-domain swapped reduced Hsp33 dimer could trigger the interaction of Hsp33 with the DnaK-system. Upon transfer of the substrate proteins to the DnaK-system, reduced non-domain swapped Hsp33 dimers dissociate, and Hsp33 could return into its inactive, monomeric state (Hoffmann *et al.*, 2004) (Figure 1).

Hsp33 is Part of a Redox-Regulated Chaperone Network. Analysis of the chaperone function of Hsp33 suggested that Hsp33 is part of a highly regulated redox-linked chaperone network. Activation of Hsp33 requires oxidizing conditions and heat shock temperatures, which allow for disulfide bond formation and dimerization of Hsp33. Inactivation of active Hsp33 dimers requires the establishment of reducing conditions and the presence of the DnaK/DnaJ/GrpE system. This apparently very stringent regulation of Hsp33 inactivation ensures that substrate proteins remain bound to Hsp33 until permissive folding conditions are restored and inevitably links substrate release to the establishment of reducing conditions and the availability of the DnaK foldase-system (Figure 1).

Escherichia coli cells harbor at least 10 different cytosolic chaperones and folding systems, which are strongly upregulated under stress conditions. This immediately raises the question of why bacterial cells need yet another chaperone, particularly one whose function is as tightly regulated as Hsp33. Hsp33 appears to be the only known molecular chaperone in *E. coli*, which is specifically activated under combined oxidative and heat stress. This finding clearly emphasized the need of *E. coli* to protect its cellular proteins under those extreme stress conditions, which cause massive aggregation of cellular proteins. This aggregation might overwhelm the conventional chaperone systems in *E. coli* and may require additional chaperone systems to combat this stress. Alternatively, one or the other chaperone system might itself fall victim to those extreme stress conditions and become inactivated during oxidative heat stress. Then, Hsp33 as a very powerful chaperone holdase, would ensure that unfolding proteins are protected against aggregation and that release of bound substrate proteins only occurs under permissive folding conditions, in which sufficient amounts of the DnaK-system are available for the refolding of the substrate proteins.

Protein Disulfide Isomerase: Controversy About a Redox-Regulated Chaperone

Protein Disulfide Isomerase (PDI) is a member of the oxidoreductase family and contains two active site sequences Cys–X–X–Cys (reviewed in Noiva, 1999; Freedman *et al.*, 2002). PDI exerts oxidoreductase and chaperone activity and assists proteins in their folding and degradation, both *in vivo* and *in vitro* (Weissman & Kim, 1993; Gillece *et al.*, 1999; Mayer *et al.*, 2000a; Meunier *et al.*, 2002; Molinari *et al.*, 2002; Shin *et al.*, 2002; Winter *et al.*, 2002). PDI is highly abundant in the lumen of the endoplasmic reticulum (ER), and several additional PDI homologues in the ER have been identified over the past few years (for review, see Frand *et al.*, 2000; Goldberger *et al.*, 1963).

The ER provides a very unique and special folding environment; it contains a large number of chaperones, which are associated with protein folding and quality control, and PDI takes an active role in both processes (reviewed in Ellgaard & Helenius, 2003; Sitia and Braakman, 2003; Bottomley *et al.*, 2001). In addition, PDI has also been found to be a subunit of prolyl-4-hydroxylase and microsomal triglyceride transfer protein (Pihlajaniemi *et al.*, 1987; Wetterau *et al.*, 1990). Prolyl-4-hydroxylase, a $\alpha_2\beta_2$-tetramer with PDI being the β-subunit, catalyzes the hydroxylation of proline residues in procollagen during collagen synthesis (Pihlajaniemi *et al.*, 1987). Microsomal triglyceride transfer protein consists of a heterodimer of PDI and a 97 kDa subunit, and catalyzes the transfer of neutral lipids onto nascent lipoprotein particles (Wetterau *et al.*, 1990). The role of PDI in these complexes appears to be primarily structural, because the isomerase activity of PDI is not required in these complexes.

In the yeast ER, PDI is mainly found in its oxidized state and catalyzes oxidative protein folding in concert with Ero1p (Tu *et al.*, 2000), which functions as the physiological oxidase of PDI (Frand and Kaiser, 1998). The transfer of oxidizing equivalents to folding proteins in the ER follows a sequential mechanism. Molecular oxygen first oxidizes the FAD-dependent enzyme Ero1p, which directly oxidizes PDI via a disulfide exchange reaction. This enables PDI to introduce disulfide bonds into folding proteins (Tu *et al.*, 2000). Interestingly, only PDI seems to be oxidized by Ero1p, given that other members of the PDI family do not interact with Ero1p. This discrimination leaves numerous PDI homologues in the ER in their reduced form, and thus allows them to fulfill redox reactions other than protein oxidation (Tu & Weissman, 2004).

The successful introduction of disulfide bonds into folding proteins involves two properties of PDI, the chaperone function and the oxidoreductase function. During the past few years, PDI has been discovered to interact with some proteins in a redox-regulated manner. These proteins include the cholera toxin (Tsai *et al.*, 2001) and a pancreatic isoform of human β-secretase (BACE457) (Molinari *et al.*, 2002).

Interaction of PDI with the A1-Chain of Cholera Toxin is Redox-Regulated. The A and B subunits of the cholera toxin fold and form disulfide bonds in the periplasm of *Vibrio cholerae*. Five B-subunits form a ring around the C-terminus of one A-subunit, and the holotoxin is formed

(Zhang *et al.*, 1995). During secretion of the holotoxin from *Vibrio cholerae*, the A-subunit is clipped at position R192 (Mekalanos *et al.*, 1979). The A1- and A2-fragments stay connected through a disulfide bond and remain associated with the B-subunits via non-covalent interactions. After infection of mammalian cells, the holotoxin travels via retrograde transport into the ER (Lencer *et al.*, 1995). There, the toxin undergoes disulfide bond reduction and disassembly, and the A1-subunit unfolds. Upon unfolding, the A1-subunit is then transported into the cytosol, where it catalyzes the ADP ribosylation of the Gαs protein (Hirst, 2001). Consequently, the intracellular cyclic AMP levels dramatically increase, which in turn causes massive water and salt secretion and finally results in severe diarrhea (Hirst, 2001).

In 2001, Tsai *et al.* investigated the mechanism of toxin disassembly and unfolding in the ER and discovered that PDI plays a leading role in this process. The authors used ER lumenal extracts prepared from canine pancreas. PDI was found to disassemble the toxin and to unfold the A1-subunit in a strictly redox-dependent manner. Only under reducing conditions was PDI able to bind to and unfold the A1-subunit. In contrast, under oxidizing conditions, PDI did not interact with the A1-subunit (Tsai *et al.*, 2001). Binding of PDI to the A1-subunit under reducing conditions could be reversed by the addition of very high concentrations of oxidized glutathione (GSSG), which quickly oxidized PDI (Tsai *et al.*, 2001). The interaction between PDI and the A1-subunit appeared to be determined by redox-dependent conformational changes in PDI, and to occur independently of any thiol-disulfide-exchange reactions between PDI and the A1-subunit. A PDI-variant, whose active site cysteines were irreversibly carbamidomethylated, interacted and unfolded the A1-subunit independently of the presence of oxidizing or reducing conditions (Tsai *et al.*, 2001).

Notably, however, stable complex formation between reduced PDI and the A1-subunit could not be achieved when an ER lumenal extract containing PDI was used (Tsai *et al.*, 2001). One possible explanation for this observation was that an ER component might cause the re-oxidation of PDI, therefore causing repeated cycles of A1-subunit–binding and release from PDI, and thus preventing formation of a stable complex with PDI. Which ER component would cause such a release of the toxin? Because an excess of GSSG caused release of the toxin from purified PDI, it was conceivable that an oxidizing component is responsible for the dissociation. Because Ero1p functions as the physiological oxidase of PDI in the yeast ER (Frand & Kaiser, 1998), Ero1p quickly became a potential candidate. Indeed, in 2002, Tsai and Rapoport identified Ero1p as the "release factor" of PDI in the ER extract from dog microsomes. Ero1p was found to exclusively oxidize the C-terminal active site cysteine pair in PDI, which immediately caused the dissociation of PDI from the toxin (Tsai & Rapoport, 2002).

The data suggested that the interaction of PDI with the A1-subunit of cholera toxin depends on the redox state of the active site cysteines of PDI. The PDI-toxin complex forms under reducing conditions, and PDI dissociates from the complex after oxidation of its active site cysteines either by Ero1p or GSSG (Tsai *et al.*, 2001; Tsai & Rapoport, 2002). After release from PDI, the A1-subunit is ready to be retro-translocated into the cytosol.

Redox-Driven Chaperone Activity of PDI During ER-Associated Degradation of BACE457. Endoplasmic reticulum-associated degradation (ERAD) is a process in which misfolded proteins of the ER are degraded by the cytosolic proteasome (McCracken and Brodsky, 2003). For their effective degradation in the cytosol, misfolded ERAD substrates are first transported in a retrograde fashion from the ER to the cytosol. This process requires a very high degree of substrate selectivity in the ER to discriminate between proteins that are on the folding pathway and those that are on the unfolding pathway and are in need of degradation. The proteins that assist in this discrimination process include chaperones such as the Hsp70-related BiP, PDI, several other co-chaperones, as well as calreticulin and calnexin (Brodsky & McCracken, 1999; Gillece *et al.*, 1999; McCracken & Brodsky, 2003). Molinari *et al.* (2002) identified calnexin and the BiP/PDI chaperone system to be sequentially involved in the ER-associated degradation of the type I membrane glycoprotein BACE457. As shown with the A1-subunit of the cholera toxin, PDI appears to participate in this reaction as an oxidoreductase and as a redox-driven chaperone.

BACE457 folds very inefficiently in the ER, leading to ERAD of a significant portion of the newly synthesized protein (Molinari *et al.*, 2002). Preceding the degradation process, the oxidized, monomeric BACE457 species first accumulates in the ER. It later converts into disulfide-linked complexes of heterogeneous size. Diagonal two-dimensional gels showed that PDI specifically associates with these disulfide-bonded complexes of BACE457. The detected complexes include disulfide-linked BACE457 oligomers as well as mixed disulfide bonds between BACE457 and PDI. This observation clearly shows the involvement of PDI as an oxidoreductase in BACE457 folding and preparation for degradation (Molinari *et al.*, 2002). The release of BACE457 from PDI was found to depend on non-reducing conditions. Similar to the interaction of PDI with A1-subunits of the cholera toxin, under reducing conditions, BACE457 was completely reduced but remained associated with PDI, despite the absence of any disulfide bonds. After removal of DTT, however, BACE457 was released from PDI, and ERAD was induced. This mechanism seems to be unique for PDI and BACE457, because

many other ERAD-substrates are degraded faster under reducing conditions.

These data suggested that PDI acts as a redox-driven chaperone during ERAD of BACE457. The redox state of PDI determined its interaction with BACE457. Similar to the interaction of PDI with the A1-subunit of cholera toxin, only reduced PDI associated with BACE457. Dissociation from the substrate proteins required the oxidation of PDI.

PDI Interacts Independently of its Redox State with the C-Propeptide of Procollagen and the α-Subunit of Prolyl-4-Hydroxylase. ER resident proteins such as prolyl-4-hydroxylase (P4H), BiP, and PDI assist the folding and assembly of a procollagen trimer, which consists of trimerized N- and C-propeptides that are separated by a long triple helix (Chessler & Byers, 1993; Prockop & Kivirikko, 1995; Walmsley *et al.*, 1999; Bottomley *et al.*, 2001). PDI specifically and transiently interacts with the C-propeptide of procollagen and retains the unassembled procollagen chains in the ER until they reach their native state. Analysis of the interaction of PDI and the C-propeptide of procollagen in semi-permeabilized human fibrosarcoma cells showed that this interaction occurs independently of the redox state of PDI (Lumb & Bulleid, 2002). The PDI–C-propeptide complex was incubated under either reducing or oxidizing conditions and remained stable under both conditions. Under these experimental conditions, PDI was shown to be either reduced or oxidized, suggesting that the interaction between PDI and the C-propeptide is not influenced by the redox state of PDI.

A second interaction of PDI, which appears to occur independently of its redox state, is the stable association of two PDI subunits with two α-subunits of P4H to form the $\alpha_2\beta_2$-heterotetramer P4H (John *et al.*, 1993). This interaction between PDI and the α-subunits of PH4 seemed independent of the active site cysteines in PDI (Vuori *et al.*, 1992), and the majority of PDI was found to be present in its oxidized state. This observation was in contrast to the results obtained for the A1-subunit of cholera toxin and BACE457 but could be due to the fact that the α-subunits of P4H are not substrates of PDI, but rather permanent binding partners. Interestingly, when Lumb and Bulleid (2002) incubated oxidized P4H tetramers with excess GSSG or mastoparan, a cysteine-free peptide that binds to PDI (Klappa *et al.*, 1998), they observed dissociation of PDI from the P4H complex. The authors concluded that this oxidation-induced dissociation of PDI from the P4H complex is caused by the competition of PDI binding to GSSG or mastoparan, rather than by a change in the redox state of PDI. Noteworthy, P4H tetramers were found to dissociate under reducing conditions (John and Bulleid, 1994). This finding, however, was attributed to the reduction of stabilizing disulfide bonds within the α-subunits of P4H and not of PDI.

PDI: Redox-Dependent or Independent Substrate Binding and Release? All four proteins, whose interactions with PDI were investigated in detail, are either natural substrate proteins or binding partners of PDI. Although BACE457 and the A1-subunit of cholera toxin were shown to interact with PDI in a redox-regulated manner, the interaction of PDI with the C-propeptide of procollagen and the α-subunits of P4H was found to occur independently of its redox state. What causes these differences?

Tsai *et al.* (2001) were the first investigators to suggest that the binding cycle of PDI is regulated by the redox state. They argued that the redox state directly affects the conformation of PDI, which changes the affinity for its substrate proteins. Although Lumb and Bulleid (2002) also suggested that conformational changes in PDI trigger substrate release, they did not link this process to the redox state of PDI. They argued instead that the release of the A1-subunit of cholera toxin from oxidized PDI (Tsai *et al.*, 2001) is mediated by substrate competition with GSSG, rather than by the oxidation of PDI. Lumb and Bulleid (2002) questioned the general concept of the redox-regulated chaperone activity of PDI and used previously published *in vitro* data obtained using purified proteins to support their arguments. In contrast to the data obtained under ER conditions, no connection has yet been shown between the redox state of the active site cysteines and the chaperone function of PDI in studies using purified components. PDI, with its active site cysteines either carbamidomethylated or mutated, is still able to entirely fulfill its chaperone function via repeated cycles of substrate binding and release (Quan *et al.*, 1995; Winter *et al.*, 2002). When Tsai *et al.* (2001) used carbamidomethylated PDI in their assays with A1-subunit, however, this mimic of constitutively reduced PDI remained associated with the A1-subunit independently of the presence or absence of GSSG. This observation contradicted the suggestions of Lumb and Bulleid (2002) that simple competition between GSSG binding and substrate binding caused the oxidation induced substrate release. In addition, the fact that Ero1p, which oxidizes PDI directly via thiol disulfide exchange (Tu *et al.*, 2000), mediated the release of the A1-subunit from PDI (Tsai & Rapoport, 2002) made such a proposed substrate competition even more unlikely.

With our current knowledge, it is clearly difficult to generalize the influence of the redox state on the chaperone function of PDI. It is probably safe to say, however, that the nature of the substrate protein, together with the experimental conditions, probably define the substrate binding affinity of PDI and the involvement of the redox state of PDI's cysteines. This is somewhat reminiscent of other chaperones, such as GroEL/ES, whose requirement for the co-chaperone GroES and the cofactor ATP was also found to be determined by the nature of the substrate proteins and the folding environment (Schmidt *et al.*,

1994). Under "permissive" folding conditions *in vitro*, malate dehydrogenase, citrate synthase, and ribulose-1,5-bisphosphate carboxylase only required the interaction of GroEL and ATP for their successful refolding (Schmidt *et al.*, 1994). Under non-permissive folding conditions *in vitro*, however, most proteins such as rhodanese (Martin *et al.*, 1991; Mendoza *et al.*, 1991) and citrate synthase (Buchner *et al.*, 1991) required the complete GroEL/GroES and ATP machinery for efficient folding.

REGULATION OF CHAPERONE ACTIVITY BY TEMPERATURE

The expression of heat shock proteins is massively induced upon the shift of cells from normal to heat shock temperatures (Gething & Sambrook, 1992). Although this process has been known for many years, only recently have investigators discovered the fact that the molecular chaperone function of certain heat shock proteins is also tightly regulated by heat shock temperatures. The small heat shock proteins Hsp26 (from *Saccharomyces cerevisiae*) and Hsp16.9 (from wheat) have been found to increase their chaperone holdase activity dramatically under heat shock temperatures (Haslbeck *et al.*, 1999; van Montfort *et al.*, 2001). The protein folding activity of the ATP-dependent chaperone foldase GroEL, on the other hand, has been found to decrease as temperature increases. This process slows the release of GroES from GroEL, and effectively transforms GroEL into a chaperone holdase (Llorca *et al.*, 1998). This is very similar to the DnaK/DnaJ/GrpE folding machinery, in which a temperature-dependent change in GrpE activity effectively switches the DnaK/DnaJ/GrpE machinery from a foldase to a holdase at elevated temperatures (Grimshaw *et al.*, 2001; Groemping & Reinstein, 2001).

Small Heat Shock Proteins

Small heat shock proteins (sHsps) (subunits: 12 to 43 kDa) are a widespread, and by far the most poorly conserved, family of molecular chaperones. All sHsps appear to have in common the presence of a conserved C-terminal sequence, which is homologous to the family of α-crystallins (de Jong *et al.*, 1998). Furthermore, most sHsps have been found to form large oligomeric structures with a highly dynamic quaternary structure, and were shown to be effective chaperone holdases that prevent the aggregation of thermally unfolding proteins both *in vivo* and *in vitro* (reviewed in Haslbeck, 2002; Haslbeck *et al.*, 2004). Upon exposure to heat shock temperatures, certain sHsps show an increase in subunit exchange or in some cases (Hsp26 and Hsp16.9) even dissociation into smaller subunits. This dissociation leads to the additional exposure of hydrophobic surfaces, which may constitute additional substrate binding sites (Lee *et al.*, 1997; Rao *et al.*, 1998; Datta & Rao, 1999; Haslbeck *et al.*, 1999; van Boekel *et al.*, 1999; van Montfort *et al.*, 2001).

Most sHsps interact with various non-native proteins and effectively prevent their aggregation (Jakob *et al.*, 1993; Lee *et al.*, 1997; Haslbeck *et al.*, 1999; Giese & Vierling, 2002; Stromer *et al.*, 2003; Haslbeck *et al.*, 2004). Similar to the function of the redox-regulated chaperones Hsp33 and PDI, the chaperone activity of sHsps is also independent of ATP (Jakob *et al.*, 1993). During heat stress conditions, the main task of sHsps appears to be to provide a large reservoir of surfaces that can bind and sequester aggregation-sensitive folding intermediates. These intermediates are bound in a folding-competent state and are ready to be refolded after return to non-stress conditions (Ehrnsperger *et al.*, 1997; Mogk *et al.*, 2003a). Upon release from sHsps, the substrate proteins either refold spontaneously or their refolding is supported by the ATP-dependent Hsp70 chaperone system, alone or in concert with ClpB (Ehrnsperger *et al.*, 1997; Lee & Vierling, 2000; Mogk *et al.*, 2003a).

Chaperone Activity of Hsp26 from Saccharomyces cerevisiae *is Increased by Temperature-Dependent Disassembly.* *Saccharomyces cerevisiae* cells contain at least two different small heat shock proteins, Hsp26 and Hsp42 (Haslbeck, 2002). Both sHsps are absent from exponentially growing yeast cells at physiological pH, but their expression is rapidly induced during the diauxic shift, as well as upon exposure of cells to heat stress conditions (Haslbeck *et al.*, 2004). Interestingly, both Hsp26 and Hsp42 have very similar chaperone activities *in vitro* and show a 90% overlap in their substrate proteins under heat shock conditions *in vivo* (Haslbeck *et al.*, 2004). Under heat stress conditions, protein aggregation is significantly increased in strains lacking either the Hsp26 gene or the Hsp42 gene, indicating that both proteins play an important part in preventing heat-induced protein aggregation *in vivo* (Haslbeck *et al.*, 2004). It is interesting to note, however, that although Hsp42 is active under both physiological and heat shock conditions, Hsp26 appears to be predominantly functional as a molecular chaperone under heat shock conditions—probably due to the specific activation of Hsp26 by heat shock temperatures (Haslbeck *et al.*, 1999; Haslbeck *et al.*, 2004).

Under non-stress conditions *in vitro*, yeast Hsp26 forms well defined and regular complexes of 24 subunits (Bentley *et al.*, 1992; Haslbeck *et al.*, 1999) (Figure 3A), independent of the protein concentration used. Incubation of Hsp26 with thermally unfolding citrate synthase at heat shock temperatures leads to the formation of even larger Hsp26-citrate synthase complexes (Figure 3B). These complexes, however, do not represent 24-mers of Hsp26 that sequester unfolding substrate protein on their surfaces,

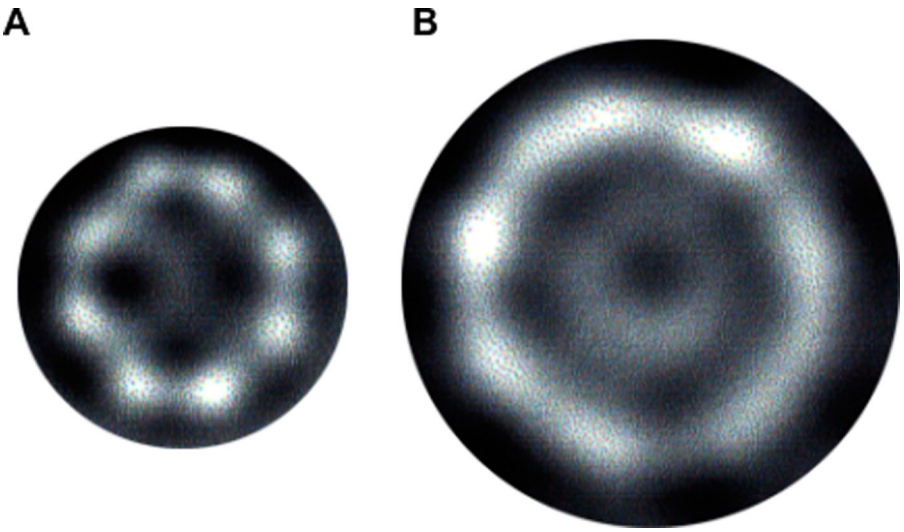

FIG. 3. Cryo-Electron Microscopic Images of the Hsp26 complex and Hsp26-Citrate Synthase Complex. **A.** Hsp26 from *Saccharomyces cerevisiae* forms regular, spherical complexes at 25°C, which consist of 24 subunits. Hsp26 complexes have an apparently uniform structure and size, and a diameter of approximately 150 Å. **B.** Upon incubation with citrate synthase at 43°C, Hsp26 forms large, globular-shaped complexes. These Hsp26-citrate synthase complexes have a diameter of approximately 230 Å. The Hsp26 complex and Hsp26-citrate synthase complex are shown in relative size to one another. Figure reproduced with permission from Haslbeck *et al.*, 1999 and Nature (http://www.nature.com/).

but apparently constitute a completely novel structure (Haslbeck *et al.*, 1999). How and why does Hsp26 form a completely new structure under these conditions? Haslbeck *et al.* (1999) discovered that, at heat shock temperatures, the Hsp26-oligomers disassemble into dimers. These Hsp26 dimers show significantly higher chaperone activity than the 24-mers, bind the substrate proteins, and reassemble into higher oligomeric structures (Figure 4). The observed dissociation of Hsp26 oligomers into the active dimers is again independent of the concentration of Hsp26 used and depends solely on the presence of elevated temperatures. This finding suggests a temperature-dependent shift from the low-affinity Hsp26-oligomers at 25°C to the high-affinity Hsp26-dimers at 43°C. In the absence of substrate proteins, these Hsp26 dimers appear to be stable and do not re-oligomerize for a period of up to 60 minutes at 43°C (Haslbeck *et al.*, 1999). In the presence of unfolding substrate proteins, such as citrate synthase, on the other hand, the Hsp26 dimers associate after the substrate binding in a highly cooperative process into large Hsp26-substrate complexes with a diameter of about 230 Å (Figure 4). It appears that the maximum binding capacity of Hsp26 for citrate synthase is achieved at a molar ratio of one citrate synthase monomer per Hsp26 dimer. The large Hsp26-substrate complexes are highly stable against dissociation (Stromer *et al.*, 2004), confirming that Hsp26 is a very efficient chaperone holdase. Release of the bound substrate proteins after return to normal temperature conditions appears to occur either spontaneously or with the help of other chaperones such as Hsp70, which assist their folding to the native state (Ehrnsperger *et al.*, 1997; Lee & Vierling, 2000). This process presumably allows Hsp26 dimers to assemble back into their low-affinity 24-mer state, which is also called the inactive Hsp26 storage form (Haslbeck *et al.*, 1999) (Figure 4). So far, however, neither the regulation nor the exact order of this process of substrate release, disassembly, and reassembly into the Hsp26 storage form is fully understood.

Hsp26 is an excellent example of a molecular chaperone, whose chaperone function is tightly regulated by the stress conditions under which it is required. Overall, this regulation has intriguing parallels with the redox-regulation of Hsp33. Activation of the two proteins includes significant conformational changes, which lead to a dramatic increase in their hydrophobicity and substrate binding affinity. This process transforms Hsp26 and Hsp33 into very efficient chaperone holdases, which apparently release their bound substrate proteins only after return to non-stress conditions.

Hsp16.9 from Wheat Forms Sub-Oligomeric Species at Heat Shock Temperatures. A second example of a small heat shock protein that changes its oligomerization state at heat shock temperatures is Hsp16.9 (from wheat) (van Montfort *et al.*, 2001; Wintrode *et al.*, 2003). The radiographic structure of the Hsp16.9 oligomer has been solved and shows that Hsp16.9 forms a dodecameric double disc with the dimensions 95 Å × 55 Å and with a central hole 25 Å in width (van Montfort *et al.*, 2001) (Figure 5).

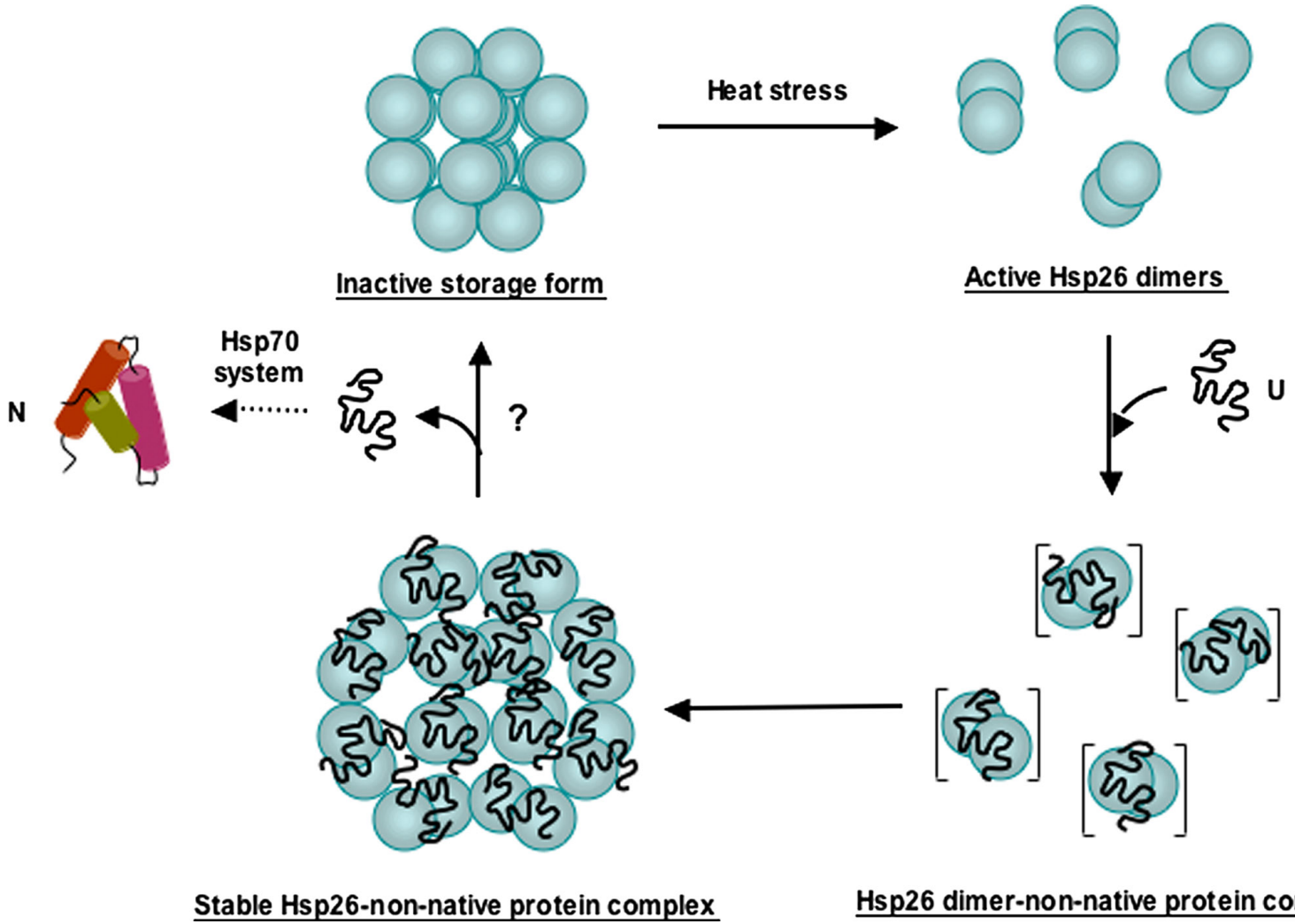

FIG. 4. Model of the Activation of the Temperature Regulated Chaperone Hsp26. Under non-stress conditions, Hsp26 forms large and regular complexes with 24 subunits. This inactive Hsp26-storage form shows low affinity for unfolding protein intermediates. Upon exposure of Hsp26 to heat shock temperatures, the 24-mers disassemble into Hsp26 dimers. This goes along with a significant increase in overall hydrophobicity and chaperone activity of Hsp26. The Hsp26 dimers associate with unfolding substrate proteins and reassemble into large Hsp26-substrate protein complexes with a diameter of approximately 230 Å (Haslbeck *et al.*, 1999). This effectively prevents the aggregation of a number of unfolding proteins both *in vitro* and *in vivo*. Return to non-stress conditions allows the release of substrate proteins and the reassembly of Hsp26 into the low-affinity storage form. In analogy to other small heat shock proteins, Hsp26 presumably transfers its substrate proteins to the Hsp70/Hsp40 system for refolding. Note that the Hsp26 dimer-substrate complexes have not been directly detected and are, therefore, depicted in parenthesis. Note also that the exact structure of the 24-mer Hsp26 and the assembled Hsp26-protein complexes are unknown—the depicted structures represent a model based on the electron microscopic images (see Figure 3).

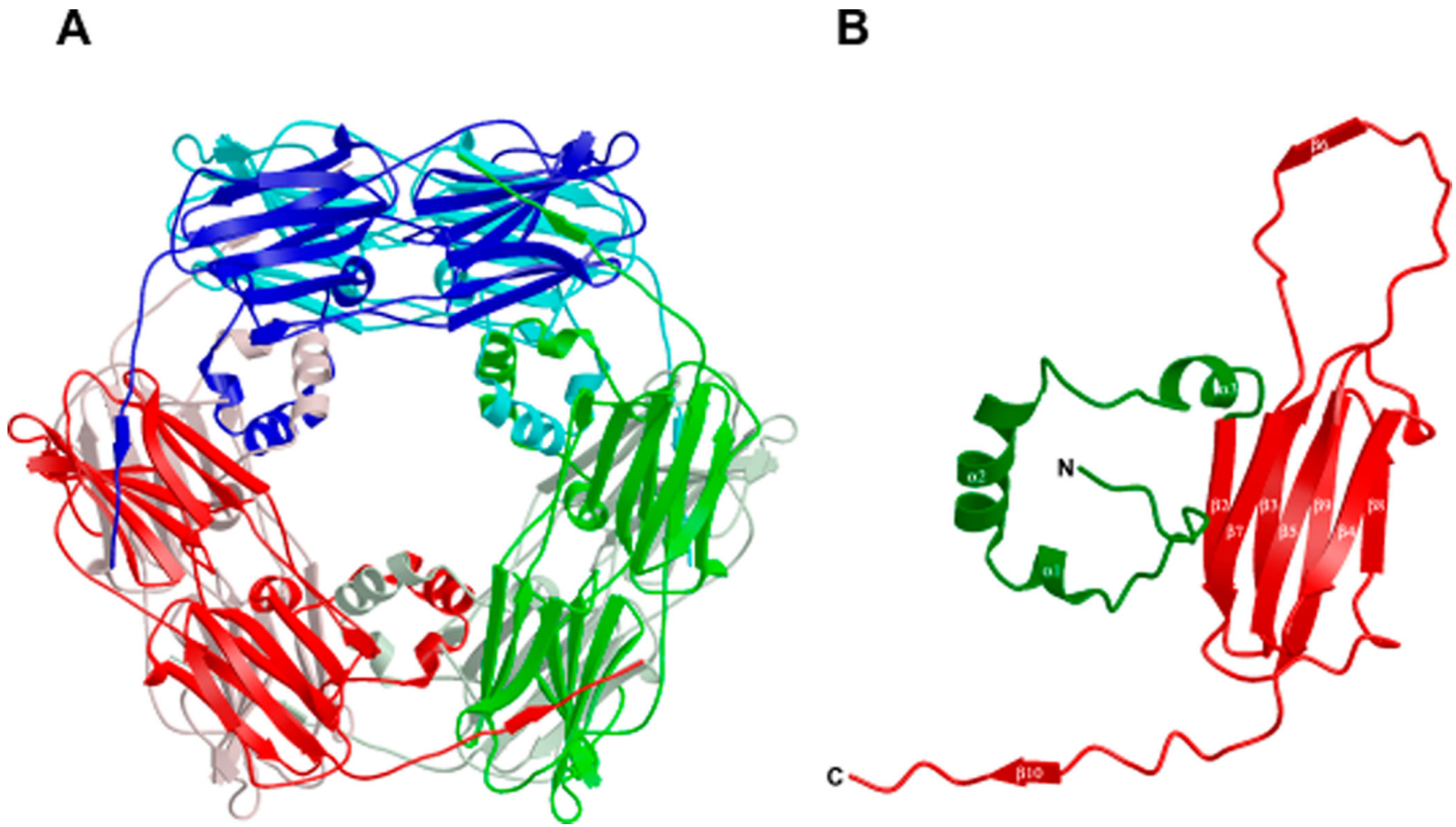

FIG. 5. Crystal Structure of Wheat Hsp16.9. **A.** Wheat Hsp16.9 is a dodecamer, which is arranged as two discs. Each disc consists of six α-crystallin domains, which are organized as trimers of nonsymmetrical dimers. In the dimer, the N-terminus of one monomer is ordered, while the other one is disordered in the crystal structure. This nonsymmetrical alignment allows flanking sequences to form higher-order interactions, which stabilize the assembly. **B.** Ribbon diagram of the monomer. The ordered N-terminal arm (helices α1, α2, α3) is shown in green, and the α-crystallin domain and the C-terminal extension (strand β10) are shown in red. Figure reproduced with kind permission from van Montfort *et al.*, 2001 and Nature (http://www.nature.com/).

Each disc consists of six α-crystallin domains, which are organized as trimers of nonsymmetrical dimers. The N-terminus of one monomer is ordered, whereas the other one is disordered in the crystal structure. This non-symmetrical alignment allows flanking sequences to form higher-order interactions, which stabilize the assembly. Despite these stabilizing inter-domain contacts, the dodecamer is flexible enough to allow for rapid subunit exchange with sub-oligomeric, supposedly dimeric, species. This subunit exchange was also observed with a closely related dodecameric small Hsp from plants, Hsp18.1 (from the pea). Incubation of Hsp16.9 with Hsp18.1 at room temperature resulted in the formation of intermediate dodecameric species, which had a significantly different size than the native dodecamers (van Montfort *et al.*, 2001). At heat shock temperatures, the dodecamer-to-sub-oligomer equilibrium shifts toward the sub-oligomeric species and the Hsp16.9 complexes disassemble into stable dimers. These dimers are the active species, which bind substrate proteins with high affinity and reassemble into large oligomeric structures (van Montfort *et al.*, 2001; Wintrode *et al.*, 2003). This process is very similar to the activation mechanism of yeast Hsp26 (Haslbeck *et al.*, 1999). So far, the extent to which Hsp16.9 is populated under non-stress conditions remains unclear, and it remains also to be elucidated whether the oligomeric state of Hsp16.9 exerts any chaperone activity *in vivo*. If the Hsp16.9 oligomer was populated and indeed inactive as a molecular chaperone under non-stress conditions, the regulation of Hsp16.9 would become even more analogous to the redox-regulated *E. coli* chaperone Hsp33. Here, the chaperone active state is clearly restricted to conditions in which large numbers of aggregation-sensitive folding intermediates accumulate. Based on the high affinity of Hsp33 and Hsp16.9 for unfolding substrate proteins, it is conceivable that the down-regulation of their chaperone activity under non-stress conditions is necessary to prevent their non-productive interactions with newly synthesized protein folding intermediates. High-affinity binding of protein folding intermediates by abundant chaperone holdases under non-stress conditions could conceivably prevent the folding of many cellular proteins.

Hsp26, Hsp16.9, and Hsp16.6 from *Synechocystis* sp. strain PCC6803 are members of the sHsp family, which show activation through heat-induced dissociation (van Montfort *et al.*, 2001; Haslbeck *et al.*, 1999; Giese & Vierling, 2002). Although pea Hsp18.1 has been shown to be in rapid equilibrium with sub-oligomeric species at ambient temperature (van Montfort *et al.*, 2001) and has been found to increase its chaperone activity at heat shock temperatures (Lee *et al.*, 1997), it has not been shown to require disassembly before efficient substrate binding (Lee *et al.*, 1997). Small Hsps such as Hsp42, on the other hand, appear to be constitutively active, and it remains unclear what distinguishes the sHsps that are regulated by heat shock temperature from those that are constitutively active. Certain sHsps of higher eukaryotes (e.g., mouse Hsp25 and human Hsp27), in contrast, appear to be regulated by phosphorylation (Rogalla *et al.*, 1999). Quite similar to the temperature-induced activation of sHsps from lower eukaryotes, phosphorylation seems to modulate the oligomerization state and activity of these mammalian small heat shock proteins.

Temperature-Induced Conformational Changes in GrpE Regulate the Activity of the DnaK-Foldase System

The heat shock induced DnaK/DnaJ/GrpE folding machinery is the major cellular foldase system, which protects cells against stress situations that are accompanied by the accumulation of large quantities of aggregation-sensitive folding intermediates (Mogk *et al.*, 1999). The DnaK system binds to and protects unfolding intermediates against aggregation during heat shock and supports their refolding to the native state upon return to non-stress conditions. The DnaK (Hsp70) system has been shown to interact with chaperone holdases such as Hsp25 (Ehrnsperger *et al.*, 1997) and Hsp33 (Hoffmann *et al.*, 2004). These chaperone holdases bind aggregation-sensitive folding intermediates during stress and transfer them to the DnaK (Hsp70) system for their efficient refolding after the stress conditions have subsided. In cooperation with ClpB, the DnaK system has also been shown to disaggregate smaller protein aggregates successfully, both *in vitro* and *in vivo* (Tomoyasu *et al.*, 2001; Mogk *et al.*, 2003a; Mogk *et al.*, 2003b).

Importantly, the DnaK/DnaJ/GrpE system is also constitutively expressed under non-stress conditions, where it assists in the folding of newly synthesized proteins, in the regulation of the prokaryotic heat shock response, as well as in degradation processes (Gamer *et al.*, 1996; Muffler *et al.*, 1997; Tomoyasu *et al.*, 1998; Deuerling *et al.*, 1999; Teter *et al.*, 1999). The mechanism of DnaK action is very similar in all of these various functions; it is characterized by ATP-dependent cycles of binding, folding, and release of substrate proteins, in a process that is tightly regulated by the joint action of the chaperone holdase DnaJ and the nucleotide exchange factor GrpE (Liberek *et al.*, 1991; McCarty *et al.*, 1995) (Figure 6). DnaJ presents unfolding substrate proteins to DnaK-ATP complexes for binding. Concerted substrate binding and interaction of DnaJ with the N-terminal ATPase domain of DnaK stimulates the ATP hydrolysis of DnaK, and thereby the conversion of DnaK from the ATP-bound low affinity T-state to the ADP-bound high-affinity R-state (T $\rightarrow$ R conversion) (Mayer *et al.*, 2000b; Mayer *et al.*, 2000c). GrpE functions as nucleotide exchange factor in the bacterial and mitochondrial

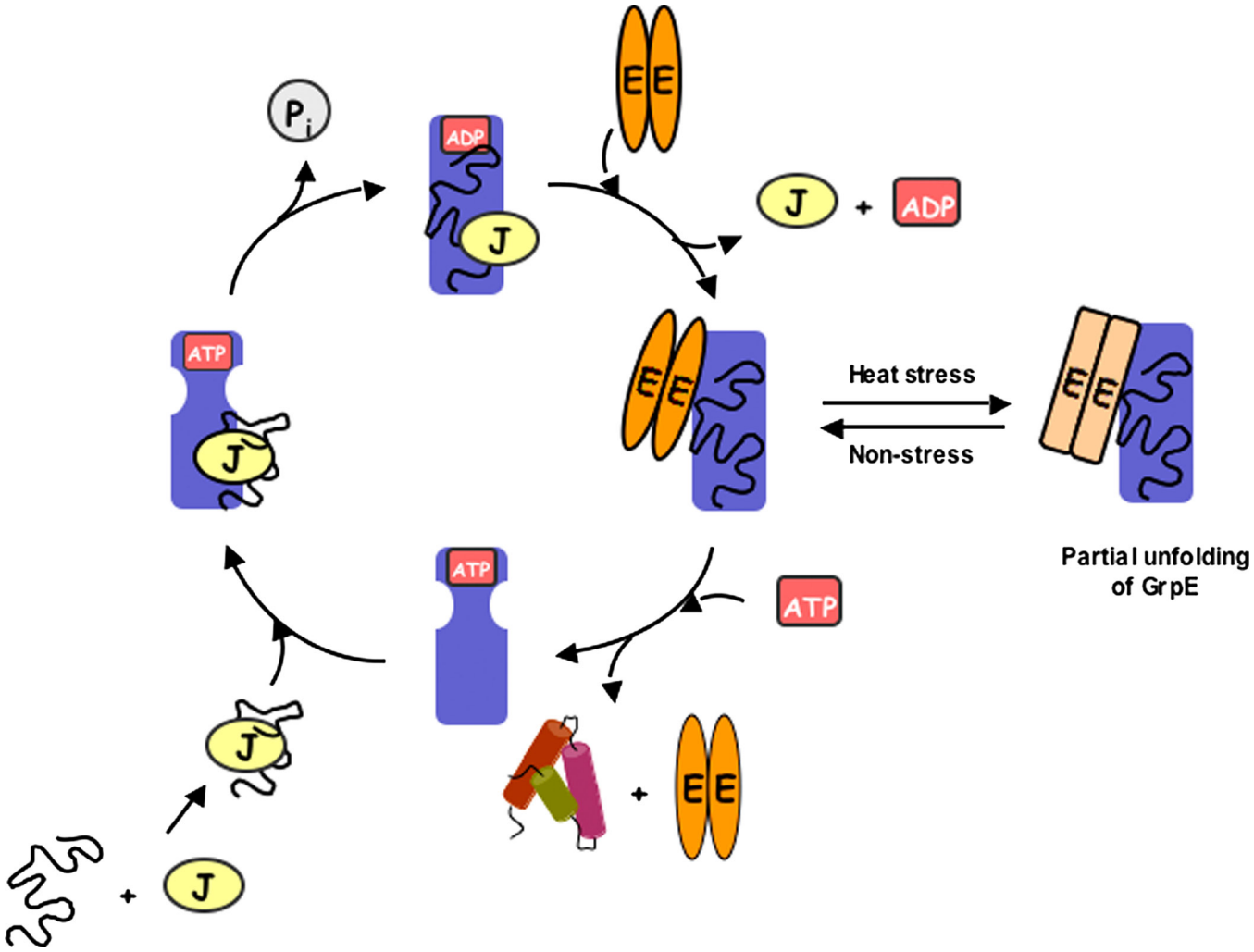

FIG. 6. Model of the Inactivation of GrpE by Heat Shock Temperatures: Turning a Foldase-system into a Holdase-System. The DnaK/DnaJ/GrpE system is an effective protein folding machine. Protein folding intermediates and unfolded proteins are first captured by the chaperone holdase DnaJ and presented to DnaK-ATP, which is in its low affinity T-state. Upon binding of the folding intermediate and interaction with DnaJ, ATP-hydrolysis is stimulated and DnaK turns into its high affinity R-state. DnaJ leaves the complex and the nucleotide exchange factor GrpE binds. ADP is exchanged for ATP, which converts DnaK into its low affinity T-state. This causes the release of the bound substrate proteins. Under heat stress conditions, however, GrpE partly unfolds. This does not seem to affect the affinity of GrpE to DnaK but appears to affect the ADP/ATP exchange activity of GrpE. This dramatically slows down the R→T transition and maintains DnaK in its high affinity holding mode for a longer period of time.

DnaK system. Active GrpE is a homodimer that interacts with the N-terminal ATPase domain of DnaK (Figure 7). It stimulates the ADP/ATP exchange in DnaK by stabilizing the open conformation of the ATPase domain (Harrison *et al.*, 1997; Harrison, 2003), thereby accelerating the R → T conversion of DnaK. This nucleotide exchange then triggers the release of bound substrate (reviewed in Walter & Buchner, 2002) (Figure 6).

A few years ago, the nucleotide exchange factor and co-chaperone GrpE was postulated to function as a thermosensor in *E. coli* (Grimshaw *et al.*, 2001; Groemping and Reinstein, 2001) (Figure 6). The homodimer GrpE was known to stimulate the ADP/ATP exchange in DnaK with a maximum rate of ADP/ATP exchange at 40°C. Above 40°C, however, GrpE was found to undergo two major conformational changes. The first and physiologically more relevant temperature transition is completely reversible, starts at approximately 40°C and shows a midpoint of transition of approximately 48°C. It appears to be due to the melting of the long helix pair of the GrpE dimer (Harrison

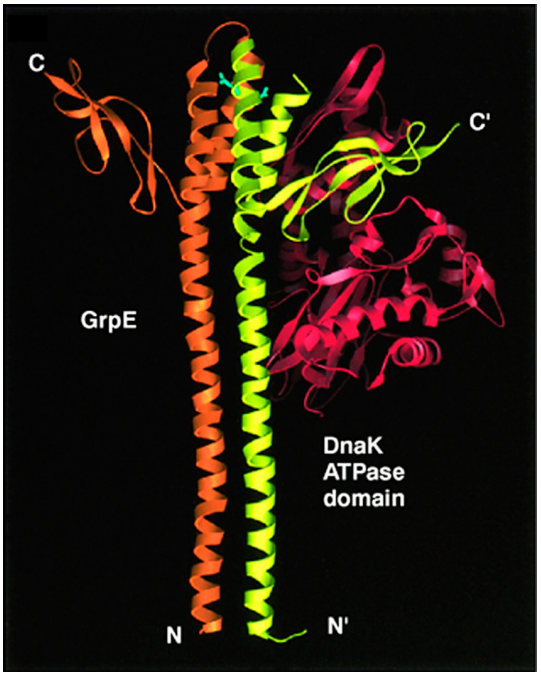

FIG. 7. Crystal structure of the complex of GrpE and the ATPase domain of DnaK. Ribbon diagram of *E. coli* GrpE (amino acids 34 to 197) in complex with the ATPase domain of *E. coli* DnaK (amino acids 1 to 388, shown in red). The N- and C-termini of GrpE are indicated and amino acid 122 (Gly mutated to Asp) is shown in blue. The GrpE-DnaK complex is free of nucleotide. GrpE is a tightly associated homodimer (shown in orange and green) that binds DnaK with 2:1 stoichiometry. The GrpE dimer interface comprises two long, paired α-helices, leading into a small four-helix bundle, where each monomer contributes two α-helices. The loop connecting the long and short α-helices is totally disordered in one of the GrpE monomers and partially disordered in the other. Contacts between GrpE and DnaK are mainly made by the GrpE monomer proximal to DnaK. (Figure reproduced with permission from Harrison *et al.*, 1997. *Science* **276**:431–435. AAAS.)

et al., 1997; Grimshaw *et al.*, 2003) (see Figure 7). The unfolding of GrpE did not appear to cause any significant changes in the affinity of GrpE for DnaK, but led to a dramatically decreased ADP/ATP exchange rate in DnaK, and therefore to a decrease in the rate of R → T conversion (Grimshaw *et al.*, 2001) (Figure 6). The fact that this decrease in the rate of R → T conversion of DnaK was indeed due to the thermal unfolding of GrpE became evident when a disulfide-linked GrpE dimer was tested (Grimshaw *et al.*, 2003). This mutant protein showed wild-type stability and activity under reducing conditions, but was stable against thermal unfolding at 48°C when disulfide-linked. When the ADP/ATP exchange rate of DnaK was investigated using this mutant GrpE protein, the rate of R → T conversion of DnaK showed the expected decrease under reducing conditions, but showed no significant decrease in the rate of conversion when GrpE was stably disulfide-linked. This finding clearly showed that the unfolding of GrpE causes the temperature-induced decrease in R → T conversion of DnaK.

What does the inactivation of GrpE above 40°C mean for the functionality of the whole DnaK system? Slow ADP/ATP exchange rates at heat shock temperatures result in a DnaK system that remains in its high affinity R-state over a prolonged period (Grimshaw *et al.*, 2001). This process effectively reduces the number of substrate binding and release cycles and increases the fraction of substrate proteins sequestered by DnaK (Siegenthaler *et al.*, 2004) under conditions that are non-permissive for protein folding and are, therefore, not expected to lead to the productive refolding of the proteins. This regulation turns the DnaK system from an ATP-consuming folding machine into an effective chaperone holdase that binds tightly to substrate proteins. This finding was in good agreement with earlier observations showing that certain substrate proteins remain bound to DnaK under heat stress conditions in a folding-competent form (Diamant & Goloubinoff, 1998). This arrests the refolding of these substrate proteins, and decreases the futile consumption of ATP until non-stress conditions are restored. Then, GrpE presumably refolds and the subsequent binding of ATP displaces GrpE. This lowers the affinity of DnaK for the folding intermediates and causes substrate release (Figure 6).

This regulation is similar to observations with GroEL/GroES, the second major folding system in *E. coli*, which is responsible for the folding of a large number of newly synthesized proteins (Houry *et al.*, 1999; Walter & Buchner, 2002; Ellis, 2003). At elevated temperatures, the GroEL/ES system has also been proposed to switch from a folding mode into a holding mode (Goloubinoff *et al.*, 1997; Llorca *et al.*, 1998). Both GroEL and GroES molecules have been found to be thermostable proteins, but as yet the underlying mechanism of this temperature regulation is not fully understood. Temperature-induced changes either in the affinity of GroEL for its co-chaperone GroES (Goloubinoff *et al.*, 1997) or in the inter-ring signaling of GroEL (Llorca *et al.*, 1998) seem to be responsible for this functional regulation. At heat shock temperatures, GroES was found to show reduced affinity to GroEL. This reduced affinity regulates the protein folding/protein release activity of GroEL-GroES in a way that slows GroEL/ES-mediated protein folding at heat shock temperatures (Goloubinoff *et al.*, 1997). Llorca et al. (1998) also observed that GroEL slows release of its bound substrates at heat shock temperatures but interpreted this finding to be due to a decreased inter-ring signaling in GroEL

induced by heat shock temperatures. This would result in a slowed release of GroES from GroEL and, in turn, would inhibit release of encapsulated proteins from the GroEL ring. Both models agreed with the finding that GroEL was unable to refold its substrate proteins at heat shock temperatures (Llorca *et al.*, 1998). It maintained them in a folding-competent state, a functional characteristic of chaperone holdases. GroEL/ES supported refolding of these substrate proteins was resumed as soon as non-stress temperatures were restored (Llorca *et al.*, 1998). As discussed with the DnaK/DnaJ/GrpE system, the temperature-induced switch from a foldase-system to a holdase-system at elevated temperatures appears to prevent senseless cycles of binding and release of substrate proteins under conditions that are non-permissive for protein folding. Perhaps more importantly, it would prevent the waste of valuable cellular ATP, which is otherwise hydrolyzed by either folding system in every cycle of their substrate binding and release.

DISCUSSION

This review focuses on recently identified molecular strategies that have been used in molecular chaperones to regulate their functional activities. Until a few years ago, most molecular chaperones were thought to be mainly regulated on the transcriptional level, where they were found to be under heat shock control and massively overexpressed under stress conditions. This was thought to be sufficient to allow cells to cope with the damaging effects of heat stress, which causes the thermal unfolding and aggregation of several proteins *in vivo*. For chaperone foldases, such as the prokaryotic DnaK/DnaJ/GrpE system or the GroEL/ES system, ATP-binding and hydrolysis was shown to be a major functional regulator, attenuating the affinity for the substrate proteins. All other molecular chaperones that are efficient in preventing protein aggregation, but are unable to support the refolding of proteins, were thought to be mainly regulated on the transcriptional level. This view dramatically changed with the finding that certain chaperone holdases, such as Hsp33 or the small heat shock proteins Hsp26 and Hsp16.9, were regulated very tightly on a posttranslational level. In the case of Hsp33, the redox conditions of the environment appear to play the critical role in switching the molecular chaperone activity of Hsp33 on and off. In the case of Hsp26 and Hsp16.9 and the co-chaperone GrpE, on the other hand, temperature appears to be the main regulator of their chaperone activity.

It seems that all these new modes of post-translational regulation of molecular chaperone activity serve one purpose: specifically to generate highly efficient chaperone holdases, which tightly bind to unfolding substrate proteins under stress conditions and do not release them until permissive folding conditions have been restored. This is achieved either by the specific activation of chaperone holdases that are only active under stress conditions, where their protective action is absolutely required (e.g., Hsp33; Hsp26, Hsp16.9), or by turning constitutively active chaperone foldases such as the DnaK system into efficient chaperone holdases under stress conditions. This protects stress-sensitive proteins from aggregation without futile cycles of ATP-consuming protein binding and release. Upon return to non-stress conditions, the ATP-dependent DnaK system returns into a folding machine, which is capable of refolding its own substrate proteins. After this has been accomplished, the DnaK system is able to take over substrate proteins from the activated chaperone holdases. This allows the refolding of the protected proteins and at the same time returns the chaperone holdases to their inactive storage form, ready to jump into action when required again.

REFERENCES

Alberti, S., Esser, C., and Hohfeld, J. 2003. BAG-1–a nucleotide exchange factor of Hsc70 with multiple cellular functions. *Cell Stress Chaperones* **8:**225–231.

Apel, K. and Hirt, H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Physiol Plant Mol Biol* **55:**373–399.

Aslund, F., Zheng, M., Beckwith, J., and Storz, G. 1999. Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. *Proc Natl Acad Sci USA* **96:**6161–6165.

Azevedo, D., Tacnet, F., Delaunay, A., Rodrigues-Pousada, C., and Toledano, M.B. 2003. Two redox centers within Yap1 for H2O2 and thiol-reactive chemicals signaling. *Free Radic Biol Med* **35:**889–900.

Barbirz, S., Jakob, U., and Glocker, M.O. 2000. Mass spectrometry unravels disulfide bond formation as the mechanism that activates a molecular chaperone. *J Biol Chem* **275:**18759–18766.

Bentley, N.J., Fitch, I.T., and Tuite, M.F. 1992. The small heat-shock protein Hsp26 of Saccharomyces cerevisiae assembles into a high molecular weight aggregate. *Yeast* **8:**95–106.

Berlett, B.S. and Stadtman, E.R. 1997. Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* **272:**20313–20316.

Bottomley, M.J., Batten, M.R., Lumb, R.A., and Bulleid, N.J. 2001. Quality control in the endoplasmic reticulum: PDI mediates the ER retention of unassembled procollagen C-propeptides. *Curr Biol* **11:**1114–1118.

Brodsky, J.L. and McCracken, A.A. 1999. ER protein quality control and proteasome-mediated protein degradation. *Semin Cell Dev Biol* **10:**507–513.

Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R., Schmid, F.X., and Kiefhaber, T. 1991. GroE facilitates refolding of citrate synthase by suppressing aggregation. *Biochemistry* **30:**1586–1591.

Bukau, B. and Horwich, A.L. 1998. The Hsp70 and Hsp60 chaperone machines. *Cell* **92:**351–366.

Cabiscol, E., Tamarit, J., and Ros, J. 2000. Oxidative stress in bacteria and protein damage by reactive oxygen species. *Int Microbiol* **3:** 3–8.

Calabrese, V., Scapagnini, G., Colombrita, C., Ravagna, A., Pennisi, G., Giuffrida Stella, A.M., Galli, F., and Butterfield, D.A. 2003. Redox

regulation of heat shock protein expression in aging and neurodegenerative disorders associated with oxidative stress: a nutritional approach. *Amino Acids* **25:**437–444.

Chessler, S.D. and Byers, P.H. 1993. BiP binds type I procollagen pro alpha chains with mutations in the carboxyl-terminal propeptide synthesized by cells from patients with osteogenesis imperfecta. *J Biol Chem* **268:** 18226–18233.

Chuang, S.E. and Blattner, F.R. 1993. Characterization of twenty-six new heat shock genes of Escherichia coli. *J Bacteriol* **175:**5242–5252.

Chung, H.Y., Kim, H.J., Kim, J.W., and Yu, B.P. 2001. The inflammation hypothesis of aging: molecular modulation by calorie restriction. *Ann N Y Acad Sci* **928:**327–335.

Craig, E.A. 1985. The heat shock response. *CRC Crit Rev Biochem* **18:**239–280.

Datta, S.A. and Rao, C.M. 1999. Differential temperature-dependent chaperone-like activity of alphaA- and alphaB-crystallin homoaggregates. *J Biol Chem* **274:**34773–34778.

Jong, W.W., Caspers, G.J., and Leunissen, J.A. 1998. Genealogy of the alpha-crystallin–small heat-shock protein superfamily. *Int J Biol Macromol* **22:**151–162.

Delaunay, A., Isnard, A.D., and Toledano, M.B. 2000. H_2O_2 sensing through oxidation of the Yap1 transcription factor. *Embo J* **19:**5157–5166.

Derman, A.I., Prinz, W.A., Belin, D., and Beckwith, J. 1993. Mutations that allow disulfide bond formation in the cytoplasm of Escherichia coli. *Science* **262:**1744–1747.

Deuerling, E., Schulze-Specking, A., Tomoyasu, T., Mogk, A., and Bukau, B. 1999. Trigger factor and DnaK cooperate in folding of newly synthesized proteins. *Nature* **400:**693–696.

Diamant, S. and Goloubinoff, P. 1998. Temperature-controlled activity of DnaK-DnaJ-GrpE chaperones: protein-folding arrest and recovery during and after heat shock depends on the substrate protein and the GrpE concentration. *Biochemistry* **37:**9688–9694.

Ding, H. and Demple, B. 1997. In vivo kinetics of a redox-regulated transcriptional switch. *Proc Natl Acad Sci USA* **94:**8445–8449.

Ehrnsperger, M., Graber, S., Gaestel, M., and Buchner, J. 1997. Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. *Embo J* **16:**221–229.

Ellgaard, L. and Helenius, A. 2003. Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol* **4:**181–191.

Ellis, R.J. 2003. Protein folding: importance of the Anfinsen cage. *Curr Biol* **13:** R881–R883.

Ellis, R.J. and Hartl, F.U. 1999. Principles of protein folding in the cellular environment. *Curr Opin Struct Biol* **9:**102–110.

Fink, A.L. 1999. Chaperone-mediated protein folding. *Physiol Rev* **79:**425–449.

Frand, A.R., Cuozzo, J.W., and Kaiser, C.A. 2000. Pathways for protein disulphide bond formation. *Trends Cell Biol* **10:**203–210.

Frand, A.R. and Kaiser, C.A. 1998. The ERO1 gene of yeast is required for oxidation of protein dithiols in the endoplasmic reticulum. *Mol Cell* **1:**161–170.

Freedman, R.B., Klappa, P., and Ruddock, L.W. 2002. Protein disulfide isomerases exploit synergy between catalytic and specific binding domains. *EMBO Rep* **3:**136–140.

Gamer, J., Multhaup, G., Tomoyasu, T., McCarty, J.S., Rudiger, S., Schonfeld, H.J., Schirra, C., Bujard, H., and Bukau, B. 1996. A cycle of binding and release of the DnaK, DnaJ and GrpE chaperones regulates activity of the Escherichia coli heat shock transcription factor sigma32. *Embo J* **15:**607–617.

Gething, M.J. and Sambrook, J. 1992. Protein folding in the cell. *Nature* **355:**33–45.

Giese, K.C. and Vierling, E. 2002. Changes in oligomerization are essential for the chaperone activity of a small heat shock protein in vivo and in vitro. *J Biol Chem* **277:**46310–46318.

Gilbert, H.F. 1990. Molecular and cellular aspects of thiol-disulfide exchange. *Adv Enzymol Relat Areas Mol Biol* **63:**69–172.

Gillece, P., Luz, J.M., Lennarz, W.J., de La Cruz, F.J., and Romisch, K. 1999. Export of a cysteine-free misfolded secretory protein from the endoplasmic reticulum for degradation requires interaction with protein disulfide isomerase. *J Cell Biol* **147:**1443–1456.

Goldberger, R.F., Epstein, C.J. and Anfinsen, C.B. 1963. Acceleration of reactivation of reduced bovine pancreatic ribonuclease by a microsomal system from rat liver. *J Biol Chem* **238:**628–635.

Goloubinoff, P., Diamant, S., Weiss, C., and Azem, A. 1997. GroES binding regulates GroEL chaperonin activity under heat shock. *FEBS Lett* **407:**215–219.

Gophna, U. and Ron, E.Z. 2003. Virulence and the heat shock response. *Int J Med Microbiol* **292:**453–461.

Graf, P.C. and Jakob, U. 2002. Redox-regulated molecular chaperones. *Cell Mol Life Sci* **59:**1624–1631.

Graf, P.C., Martinez-Yamout, M., VanHaerents, S., Lilie, H., Dyson, H.J., and Jakob, U. 2004. Activation of the redox-regulated chaperone Hsp33 by domain unfolding. *J Biol Chem* **279:**20529–20538.

Graumann, J., Lilie, H., Tang, X., Tucker, K.A., Hoffmann, J.H., Vijayalakshmi, J., Saper, M., Bardwell, J.C., and Jakob, U. 2001. Activation of the redox-regulated molecular chaperone Hsp33–a two-step mechanism. *Structure (Camb)* **9:**377–387.

Grimshaw, J.P., Jelesarov, I., Schonfeld, H.J., and Christen, P. 2001. Reversible thermal transition in GrpE, the nucleotide exchange factor of the DnaK heat-shock system. *J Biol Chem* **276:**6098–60104.

Grimshaw, J.P., Jelesarov, I., Siegenthaler, R.K., and Christen, P. 2003. Thermosensor action of GrpE. The DnaK chaperone system at heat shock temperatures. *J Biol Chem* **278:**19048–19053.

Groemping, Y. and Reinstein, J. 2001. Folding properties of the nucleotide exchange factor GrpE from Thermus thermophilus: GrpE is a thermosensor that mediates heat shock response. *J Mol Biol* **314:**167–178.

Harrison, C. 2003. GrpE, a nucleotide exchange factor for DnaK. *Cell Stress Chaperones* **8:**218–224.

Harrison, C.J., Hayer-Hartl, M., Di Liberto, M., Hartl, F., and Kuriyan, J. 1997. Crystal structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK. *Science* **276:**431–435.

Hartl, F.U. and Hayer-Hartl, M. 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* **295:**1852–1858.

Haslbeck, M. 2002. sHsps and their role in the chaperone network. *Cell Mol Life Sci* **59:**1649–1657.

Haslbeck, M., Braun, N., Stromer, T., Richter, B., Model, N., Weinkauf, S., and Buchner, J. 2004. Hsp42 is the general small heat shock protein in the cytosol of Saccharomyces cerevisiae. *Embo J* **23:**638–649.

Haslbeck, M., Walke, S., Stromer, T., Ehrnsperger, M., White, H.E., Chen, S., Saibil, H.R., and Buchner, J. 1999. Hsp26: a temperature-regulated chaperone. *Embo J* **18:**6744–6751.

Hirst, T.R. 2001. Cholera. A toxin with emerging therapeutic potential. *Lancet* **358 Suppl:**S7.

Hoffmann, J.H., Linke, K., Graf, P.C., Lilie, H., and Jakob, U. 2004. Identification of a redox-regulated chaperone network. *Embo J* **23:**160–168.

Houry, W.A., Frishman, D., Eckerskorn, C., Lottspeich, F., and Hartl, F.U. 1999. Identification of in vivo substrates of the chaperonin GroEL. *Nature* **402:**147–154.

Imlay, J.A. 2003. Pathways of oxidative damage. *Annu Rev Microbiol* **57:**395–418.

Jakob, U., Eser, M., and Bardwell, J.C. 2000. Redox switch of hsp33 has a novel zinc-binding motif. *J Biol Chem* **275:**38302–38310.

Jakob, U., Gaestel, M., Engel, K., and Buchner, J. 1993. Small heat shock proteins are molecular chaperones. *J Biol Chem* **268:**1517–1520.

Jakob, U., Muse, W., Eser, M., and Bardwell, J.C. 1999. Chaperone activity with a redox switch. *Cell* **96:**341–352.

Janda, I., Devedjiev, Y., Derewenda, U., Dauter, Z., Bielnicki, J., Cooper, D.R., Graf, P.C., Joachimiak, A., Jakob, U., and Derewenda, Z.S. 2004. The crystal structure of the reduced, Zn(2+)-bound form of the B. subtilis Hsp33 Chaperone and Its Implications for the activation mechanism. *Structure (Camb)* **12:**1901–1907.

John, D.C. and Bulleid, N.J. 1994. Prolyl 4-hydroxylase: defective assembly of alpha-subunit mutants indicates that assembled alpha-subunits are intramolecularly disulfide bonded. *Biochemistry* **33:**14018–14025.

John, D.C., Grant, M.E., and Bulleid, N.J. 1993. Cell-free synthesis and assembly of prolyl 4-hydroxylase: the role of the beta-subunit (PDI) in preventing misfolding and aggregation of the alpha-subunit. *Embo J* **12:**1587–1595.

Kang, J.G., Paget, M.S., Seok, Y.J., Hahn, M.Y., Bae, J.B., Hahn, J.S., Kleanthous, C., Buttner, M.J., and Roe, J.H. 1999. RsrA, an anti-sigma factor regulated by redox change. *Embo J* **18:**4292–4298.

Kim, S.J., Jeong, D.G., Chi, S.W., Lee, J.S., and Ryu, S.E. 2001. Crystal structure of proteolytic fragments of the redox-sensitive Hsp33 with constitutive chaperone activity. *Nat Struct Biol* **8:**459–466.

Klappa, P., Ruddock, L.W., Darby, N.J., and Freedman, R.B. 1998. The b' domain provides the principal peptide-binding site of protein disulfide isomerase but all domains contribute to binding of misfolded proteins. *Embo J* **17:**927–935.

Kyselova, P., Zourek, M., Rusavy, Z., Trefil, L., and Racek, J. 2002. Hyperinsulinemia and oxidative stress. *Physiol Res* **51:**591–595.

Lee, G.J., Roseman, A.M., Saibil, H.R., and Vierling, E. 1997. A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. *Embo J* **16:**659–671.

Lee, G.J. and Vierling, E. 2000. A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. *Plant Physiol* **122:**189–198.

Lencer, W.I., Constable, C., Moe, S., Jobling, M.G., Webb, H.M., Ruston, S., Madara, J.L., Hirst, T.R., and Holmes, R.K. 1995. Targeting of cholera toxin and Escherichia coli heat labile toxin in polarized epithelia: role of COOH-terminal KDEL. *J Cell Biol* **131:**951–962.

Leslie, N.R., Bennett, D., Lindsay, Y.E., Stewart, H., Gray, A., and Downes, C.P. 2003. Redox regulation of PI 3-kinase signalling via inactivation of PTEN. *Embo J* **22:**5501–5510.

Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C., and Zylicz, M. 1991. Escherichia coli DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. *Proc Natl Acad Sci USA* **88:**2874-2878.

Linke, K. and Jakob, U. 2003. Not every disulfide lasts forever: disulfide bond formation as a redox switch. *Antioxid Redox Signal* **5:**425–434.

Linke, K., Wolfram, T., Bussemer, J., and Jakob, U. 2003. The roles of the two zinc binding sites in DnaJ. *J Biol Chem* **278:**44457–4466.

Llorca, O., Galan, A., Carrascosa, J.L., Muga, A., and Valpuesta, J.M. 1998. GroEL under heat-shock. Switching from a folding to a storing function. *J Biol Chem* **273:**32587–32594.

Lumb, R.A. and Bulleid, N.J. 2002. Is protein disulfide isomerase a redox-dependent molecular chaperone? *Embo J* **21:**6763–6770.

Lund, P. A. 2001. Microbial molecular chaperones. *Adv Microb Physiol* **44:**93–140.

Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A.L., and Hartl, F.U. 1991. Chaperonin-mediated protein folding at the surface of groEL through a 'molten globule'-like intermediate. *Nature* **352:**36–42.

Mayer, M., Kies, U., Kammermeier, R., and Buchner, J. 2000a. BiP and PDI cooperate in the oxidative folding of antibodies in vitro. *J Biol Chem* **275:**29421-29425.

Mayer, M.P., Rudiger, S., and Bukau, B. 2000b. Molecular basis for interactions of the DnaK chaperone with substrates. *Biol Chem* **381:**877–885.

Mayer, M.P., Schroder, H., Rudiger, S., Paal, K., Laufen, T., and Bukau, B. 2000c. Multistep mechanism of substrate binding determines chaperone activity of Hsp70. *Nat Struct Biol* **7:**586–593.

McCarty, J.S., Buchberger, A., Reinstein, J., and Bukau, B. 1995. The role of ATP in the functional cycle of the DnaK chaperone system. *J Mol Biol* **249:**126–137.

McCracken, A.A. and Brodsky, J.L. 2003. Evolving questions and paradigm shifts in endoplasmic-reticulum-associated degradation (ERAD). *Bioessays* **25:**868–877.

Mekalanos, J.J., Collier, R.J., and Romig, W.R. 1979. Enzymic activity of cholera toxin. II. Relationships to proteolytic processing, disulfide bond reduction, and subunit composition. *J Biol Chem* **254:**5855–5861.

Mendoza, J.A., Rogers, E., Lorimer, G.H., and Horowitz, P.M. 1991. Chaperonins facilitate the in vitro folding of monomeric mitochondrial rhodanese. *J Biol Chem* **266:**13044-13049.

Meunier, L., Usherwood, Y.K., Chung, K.T., and Hendershot, L.M. 2002. A subset of chaperones and folding enzymes form multiprotein complexes in endoplasmic reticulum to bind nascent proteins. *Mol Biol Cell* **13:**4456–4469.

Mogk, A., Deuerling, E., Vorderwulbecke, S., Vierling, E., and Bukau, B. 2003a. Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation. *Mol Microbiol* **50:**585–595.

Mogk, A., Schlieker, C., Friedrich, K.L., Schonfeld, H.J., Vierling, E., and Bukau, B. 2003b. Refolding of substrates bound to small Hsps relies on a disaggregation reaction mediated most efficiently by ClpB/DnaK. *J Biol Chem* **278:**31033–31042.

Mogk, A., Tomoyasu, T., Goloubinoff, P., Rudiger, S., Roder, D., Langen, H., and Bukau, B. 1999. Identification of thermolabile Escherichia coli proteins: prevention and reversion of aggregation by DnaK and ClpB. *Embo J* **18:**6934–6949.

Molinari, M., Galli, C., Piccaluga, V., Pieren, M., and Paganetti, P. 2002. Sequential assistance of molecular chaperones and transient

formation of covalent complexes during protein degradation from the ER. *J Cell Biol* **158:**247–257.

Muffler, A., Barth, M., Marschall, C., and Hengge-Aronis, R. 1997. Heat shock regulation of sigmaS turnover: a role for DnaK and relationship between stress responses mediated by sigmaS and sigma32 in Escherichia coli. *J Bacteriol* **179:**445–452.

Noiva, R. 1999. Protein disulfide isomerase: the multifunctional redox chaperone of the endoplasmic reticulum. *Semin Cell Dev Biol* **10:**481–493.

Parsell, D.A. and R.T. Sauer. 1989. Induction of a heat shock-like response by unfolded protein in Escherichia coli: dependence on protein level not protein degradation. *Genes Dev* **3:**1226–1232.

Pihlajaniemi, T., Helaakoski, T., Tasanen, K., Myllyla, R., Huhtala, M.L., Koivu, J., and Kivirikko, K.I. 1987. Molecular cloning of the beta-subunit of human prolyl 4-hydroxylase. This subunit and protein disulphide isomerase are products of the same gene. *Embo J* **6:**643–649.

Prockop, D.J. and Kivirikko, K.I. 1995. Collagens: molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem* **64:**403–434.

Quan, H., Fan, G., and Wang, C.C. 1995. Independence of the chaperone activity of protein disulfide isomerase from its thioredoxin-like active site. *J Biol Chem* **270:**17078–17080.

Raman, B., Siva Kumar, L.V., Ramakrishna, T., and Mohan Rao, C. 2001. Redox-regulated chaperone function and conformational changes of Escherichia coli Hsp33. *FEBS Lett* **489:**19–24.

Rao, C.M., Raman, B., Ramakrishna, T., Rajaraman, K., Ghosh, D., Datta, S., Trivedi, V.D., and Sukhaswami, M.B. 1998. Structural perturbation of alpha-crystallin and its chaperone-like activity. *Int J Biol Macromol* **22:**271–281.

Requena, J.R., Chao, C.C., Levine, R.L., and Stadtman, E.R. 2001. Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. *Proc Natl Acad Sci USA* **98:**69–74.

Richmond, C.S., Glasner, J.D., Mau, R., Jin, H., and Blattner, F.R. 1999. Genome-wide expression profiling in Escherichia coli K-12. *Nucleic Acids Res* **27:**3821–3835.

Richter, K. and Buchner, J. 2001. Hsp90: chaperoning signal transduction. *J Cell Physiol* **188:**281–290.

Rogalla, T., Ehrnsperger, M., Preville, X., Kotlyarov, A., Lutsch, G., Ducasse, C., Paul, C., Wieske, M., Arrigo, A.P., Buchner, J., and Gaestel, M. 1999. Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor alpha by phosphorylation. *J Biol Chem* **274:**18947–18956.

Rosen, R. and Ron, E.Z. 2002. Proteome analysis in the study of the bacterial heat-shock response. *Mass Spectrometry Reviews* **21:**244–265.

Rudiger, S., Schneider-Mergener, J., and Bukau, B. 2001. Its substrate specificity characterizes the DnaJ co-chaperone as a scanning factor for the DnaK chaperone. *Embo J* **20:**1042–1050.

Salmeen, A., Andersen, J.N., Myers, M.P., Meng, T.C., Hinks, J.A., Tonks, N.K., and Barford, D. 2003. Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. *Nature* **423:**769–773.

Schmid, D., Baici, A., Gehring, H., and Christen, P. 1994. Kinetics of molecular chaperone action. *Science* **263:**971–973.

Schmidt, M., Buchner, J., Todd, M.J., Lorimer, G.H., and Viitanen, P.V. 1994. On the role of groES in the chaperonin-assisted folding reaction. Three case studies. *J Biol Chem* **269:**10304–10311.

Shin, H.C., Song, M.C., and Scheraga, H.A. 2002. Effect of protein disulfide isomerase on the rate-determining steps of the folding of bovine pancreatic ribonuclease A. *FEBS Lett* **521:**77–80.

Siegenthaler, R.K., Grimshaw, J.P., and Christen, P. 2004. Immediate response of the DnaK molecular chaperone system to heat shock. *FEBS Lett* **562:**105–110.

Sitia, R. and Braakman, I. 2003. Quality control in the endoplasmic reticulum protein factory. *Nature* **426:**891–894.

Sitia, R. and Molteni, S.N. 2004. Stress, protein (mis)folding, and signaling: the redox connection. *Sci STKE* **2004:**pe27.

Stadtman, E.R., Moskovitz, J., and Levine, R.L. 2003. Oxidation of methionine residues of proteins: biological consequences. *Antioxid Redox Signal* **5:**577–582.

Stromer, T., Ehrnsperger, M., Gaestel, M., and Buchner, J. 2003. Analysis of the interaction of small heat shock proteins with unfolding proteins. *J Biol Chem* **278:**18015–18021.

Stromer, T., Fischer, E., Richter, K., Haslbeck, M., and Buchner, J. 2004. Analysis of the regulation of the molecular chaperone Hsp26 by temperature-induced dissociation: the N-terminal domail is important for oligomer assembly and the binding of unfolding proteins. *J Biol Chem* **279:**11222–11228.

Takemoto, T., Zhang, Q.M., and S. Yonei. 1998. Different mechanisms of thioredoxin in its reduced and oxidized forms in defense against hydrogen peroxide in Escherichia coli. *Free Radic Biol Med* **24:**556–562.

Teter, S.A., Houry, W.A., Ang, D., Tradler, T., Rockabrand, D., Fischer, G., Blum, P., Georgopoulos, C., and Hartl, F.U. 1999. Polypeptide flux through bacterial Hsp70: DnaK cooperates with trigger factor in chaperoning nascent chains. *Cell* **97:**755–765.

Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P., and Bukau, B. 2001. Genetic dissection of the roles of chaperones and proteases in protein folding and degradation in the Escherichia coli cytosol. *Mol Microbiol* **40:**397–413.

Tomoyasu, T., Ogura, T., Tatsuta, T., and Bukau, B. 1998. Levels of DnaK and DnaJ provide tight control of heat shock gene expression and protein repair in Escherichia coli. *Mol Microbiol* **30:**567–581.

Tsai, B. and Rapoport, T.A. 2002. Unfolded cholera toxin is transferred to the ER membrane and released from protein disulfide isomerase upon oxidation by Ero1. *J Cell Biol* **159:**207–216.

Tsai, B., Rodighiero, C., Lencer, W.I., and Rapoport, T.A. 2001. Protein disulfide isomerase acts as a redox-dependent chaperone to unfold cholera toxin. *Cell* **104:**937–948.

Tu, B.P., Ho-Schleyer, S.C., Travers, K.J., and Weissman, J.S. 2000. Biochemical basis of oxidative protein folding in the endoplasmic reticulum. *Science* **290:**1571–1574.

Tu, B.P. and Weissman, J.S. 2004. Oxidative protein folding in eukaryotes: mechanisms and consequences. *J Cell Biol* **164:**341–346.

Ueda, S., Masutani, H., Nakamura, H., Tanaka, T., Ueno, M., and Yodoi, J. 2002. Redox control of cell death. *Antioxid Redox Signal* **4:**405–414.

van Boekel, M.A., de Lange, F., de Grip, W.J., and de Jong, W.W. 1999. Eye lens alphaA- and alphaB-crystallin: complex stability versus chaperone-like activity. *Biochim Biophys Acta* **1434:**114–123.

van Montfort, R.L., Basha, E., Friedrich, K.L., Slingsby, C., and Vierling, E. 2001. Crystal structure and assembly of a eukaryotic small heat shock protein. *Nat Struct Biol* **8:** 1025–1030.

van Montfort, R.L., Congreve, M., Tisi, D., Carr, R., and Jhoti, H. 2003. Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. *Nature* **423:**773–777.

Vijayalakshmi, J., Mukhergee, M.K., Graumann, J., Jakob, U., and Saper, M.A. 2001. The 2.2 A crystal structure of Hsp33: a heat shock protein with redox-regulated chaperone activity. *Structure (Camb)* **9:**367–375.

Vuori, K., Pihlajaniemi, T., Myllyla, R., and Kivirikko, K.I. 1992. Site-directed mutagenesis of human protein disulphide isomerase: effect on the assembly, activity and endoplasmic reticulum retention of human prolyl 4-hydroxylase in Spodoptera frugiperda insect cells. *Embo J* **11:**4213–4217.

Walmsley, A.R., Batten, M.R., Lad, U., and Bulleid, N.J. 1999. Intracellular retention of procollagen within the endoplasmic reticulum is mediated by prolyl 4-hydroxylase. *J Biol Chem* **274:**14884–14892.

Walter, S. and Buchner, J. 2002. Molecular chaperones—cellular machines for protein folding. *Angew Chem Int Ed Engl* **41:**1098–1113.

Wegele, H., Muller, L., and Buchner, J. 2004. Hsp70 and Hsp90-a relay team for protein folding. *Rev Physiol Biochem Pharmacol* **151:**1–44.

Weissman, J.S. and Kim, P.S. 1993. Efficient catalysis of disulphide bond rearrangements by protein disulphide isomerase. *Nature* **365:**185–188.

Wetterau, J.R., Combs, K.A., Spinner, S.N., and Joiner, B.J. 1990. Protein disulfide isomerase is a component of the microsomal triglyceride transfer protein complex. *J Biol Chem* **265:**9801–9807.

Winter, J., Klappa, P., Freedman, R.B., Lilie, H., and Rudolph, R. 2002. Catalytic activity and chaperone function of human protein-disulfide isomerase are required for the efficient refolding of proinsulin. *J Biol Chem* **277:**310–317.

Wintrode, P.L., Friedrich, K.L., Vierling, E., Smith, J.B., and Smith, D.L. 2003. Solution structure and dynamics of a heat shock protein assembly probed by hydrogen exchange and mass spectrometry. *Biochemistry* **42:**10667–10673.

Won, H.S., Low, L.Y., Guzman, R.D., Martines-Yamout, M., Jakob, U., and Dyson, H.J. 2004. The zinc-dependent redox switch domain of the chaperone Hsp33 has a novel fold. *J Mol Biol* **341:**893–899.

Woo, H.A., Chae, H.Z., Hwang, S.C., Yang, K.S., Kang, S.W., Kim, K., and Rhee, S.G. 2003. Reversing the inactivation of peroxiredoxins caused by cysteine sulfinic acid formation. *Science* **300:**653–656.

Yeh, J.I., Claiborne, A., and Hol, W.G. 1996. Structure of the native cysteine-sulfenic acid redox center of enterococcal NADH peroxidase refined at 2.8 A resolution. *Biochemistry* **35:**9951–9957.

Young, J.C., Barral, J.M., and Hartl, F.U. 2003. More than folding: localized functions of cytosolic chaperones. *Trends Biochem Sci.* **28:**541–547.

Zhang, R.G., Scott, D.L., Westbrook, M.L., Nance, S., Spangler, B.D., Shipley, G.G., and Westbrook, E.M. 1995. The three-dimensional crystal structure of cholera toxin. *J Mol Biol* **251:**563–573.

Zheng, M., Aslund, F., and Storz, G. 1998. Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* **279:**1718–1721.